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Multisite Mechanisms for Ultrasensitivity in Signal Transduction

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Abstract One of the key aspects in the study of cellular communication is understanding how cells receive a continuous input and transform it into a discrete, all-or-none output. Such so-called ultrasensitive dose responses can also be used in a variety of other contexts, from the efficient transport of oxygen in the blood to the regulation of the cell cycle and gene expression. This chapter provides a self contained mathematical review of the most important molecular models of ultrasensitivity in the literature, with an emphasis on mechanisms involving multisite modifications. The models described include two deeply influential systems based on allosteric behavior, the MWC and the KNF models. Also included is a description of more recent work by the author and colleagues of novel mechanisms using alternative hypotheses to create ultrasensitive behavior.

Keywords Systems Biology · Ultrasensitivity · Allostery · Cooperativity · Signal Transduction

1 Introduction: Ultrasensitive Dose Responses

Chemical reaction networks (CRN) lie at the heart of many biochemical processes inside the cell. They have been extensively modeled to understand the behavior of specific systems, and they have also been systematically studied at the theoretical level [5,14,21]. Although often implicitly assumed to converge globally towards a unique equilibrium in chemical engineering and other applications, CRNs can have exceedingly complex dynamical behavior. Moreover, many biological systems have arguably evolved towards precisely such relatively rare complex examples, driven by a need to exhibit behaviors such as oscillations (e.g. circadian rhythms, cell cycle) and multistability (e.g. cell differentiation).

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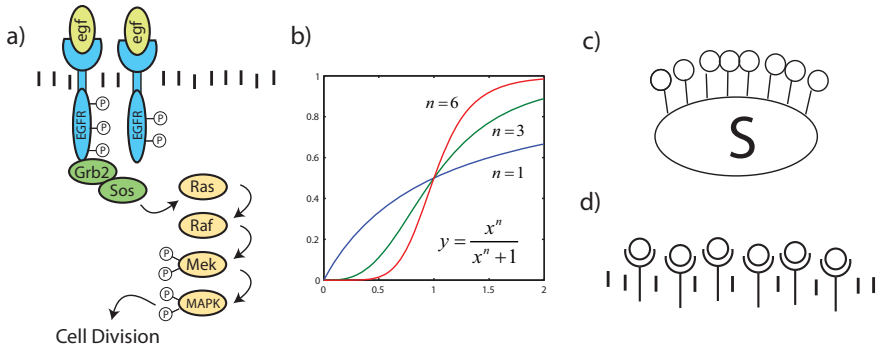

Fig. 1 a) A sample signal transduction pathway involving the signaling protein egf and the output protein MAPK, which can trigger cell division. **b)** The function $y = x^n/(x^n + 1)$ is the canonical example of an ultrasensitive function. The coefficient n quantifies the ultrasensitive behavior. **c)** A protein can be activated through phosphorylation at multiple specific locations. **d)** A receptor complex is activated through the collective binding of ligand to its multiple receptors.

Here we focus on chemical reaction networks in the context of signal transduction, i.e. in the study of cell communication and the processing of information. Such systems have a parameter that is usually regarded as an input, and which corresponds e.g. to a signal molecule binding on the cell or to a component of a larger network. They also have an output, i.e. a molecule in the system that is thought to produce a downstream response and which represents the overall activity level of the network. For instance, a protein known as *epidermal growth factor*, or egf for short, is used as a messenger molecule to induce cells near the skin to divide after a wound (Figure 1a). If a sufficient number of egf molecules bind to the membrane of a cell, a series of internal reactions takes place resulting in the activation of the output protein MAPK. This protein goes on to activate many transcription factors that can cause the cell to divide [2].

There are good reasons to study nonautonomous networks with inputs and outputs in biology, rather than autonomous networks. First, sometimes the full model would take place at a scale much larger than desired. If a hormone is used as a cell ligand input, the cell behavior might ultimately feed back into the tissues that produce the hormone. But modeling the full system would involve including other parts of the body, which is much larger in scope than an intra-cellular model. Another reason is that understanding subsets of a larger network, e.g. the way that Cdc28 affects Wee1 in the cell cycle, is often a fairly difficult and open problem in itself [29,30] and a step towards an understanding of the complete network.

In general one would like to understand the so-called *dose response* of the system, that is, the steady state value of the output as a function of the input (assuming such a steady state is uniquely defined). A special case of high interest to many experimental biologists is that of *ultrasensitive*, or all-or-none behavior (Figure 1b). Imagine that a cell is intended to divide in response to a sufficiently large egf stimulus, and to do nothing for a low egf stimulus. This essentially transforms a *continuous* egf input signal into a *binary*, all-or-none output, which should be reflected in the concentration of the output protein of this system, the protein MAPK. The all-or-none conversion of a continuous input into a binary output is quite common, not only in cell communication but also for the internal components of biochemical pathways such as the cell cycle, and simple networks that achieve it would likely be favored by evolution.

The most well-known mechanisms for ultrasensitivity involve *multisite* systems, in which one of the proteins has many identical modification sites. For instance, a very common protein modification known as phosphorylation involves the covalent attachment of a phosphate group to a specific location in the protein. Many proteins have not only one but multiple specific locations that can be phosphorylated at any given time (Figure 1c). Multisite phosphorylation can cause dramatic changes to the shape and properties of the protein, to the point that it can effectively activate an otherwise inactive protein (or vice versa) [58]. In fact, the egf signal transduction cascade in Figure 1a contains several proteins that are activated through multisite phosphorylation as indicated. Proteins can also be covalently modified in other ways, for instance they can be acetylated or methylated, also in very specific locations. These can act as the modification sites in other mathematical models [62].

An entirely different category of multisite protein modification is multisite ligand binding (Figure 1d). For example, a group of membrane receptors can cluster together and trigger a downstream signal only when sufficiently many of them are bound to some signaling molecule [9,17,72]. A protein may bind to multiple nearby sites on a DNA molecule, in order to promote or prevent the expression of a gene [11,26]. The direction of rotation of the flagellar motor in *E. coli* is controlled by the binding of a protein to one of 34 sites on a ring around the motor [18]. More often than not it is still unknown exactly how the different sites interact with each other and why there are many sites and not just fewer sites or even one.

This manuscript will review the main mechanisms known to create ultrasensitive dose responses in biochemistry, as well as some newer mechanisms (some developed recently by the author) that have not been directly tested experimentally. The focus will be on multisite ultrasensitivity, but other well known mechanisms will also be discussed in a separate section. See also the lively review on cooperativity by Ferrell [23], and the recent more general review on signal transduction, including ultrasensitive responses, by Bluthgen et al. [7].

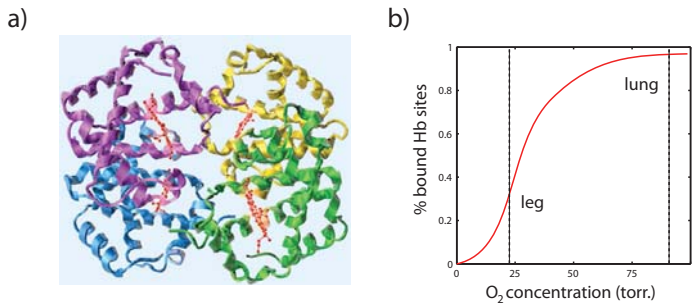

Fig. 2 a) The structure of the hemoglobin protein as it binds to four O_2 molecules. From *Biology* by Brooker, Widmaier, Graham, and Stiling, copyright McGraw-Hill. **b)** Hemoglobin has a high affinity to O_2 under high O_2 concentrations (such as in the lung) and a low affinity in low concentrations (such as in distant tissues), which allows for an efficient O_2 transport in the blood.

2 Hemoglobin and Hill functions

Early work on ultrasensitive behavior in biochemistry appears to have focused on hemoglobin, the molecule that transports much of the oxygen in the bloodstream (Figure 2a). Physiologists were puzzled about the behavior of this protein: when oxygen concentration is low, it has a low binding affinity to oxygen. But when oxygen concentration increases, the affinity to oxygen grows fairly dramatically. This makes physiological sense: when the blood is in the lung, where oxygen abounds, hemoglobin captures as much of it as it can. When it is in the far reaches of the body (e.g. in a leg), the oxygen concentration is low and hemoglobin unloads its cargo (Figure 2b). This leads to a much more efficient transport of oxygen than merely binding and unbinding at random times.

The open question was how hemoglobin can work in this efficient way. In 1910, a 23-year old scientist called A.V. Hill proposed a simple hypothetical reaction that could explain this. Each hemoglobin molecule would have n O_2-binding sites rather than one, and they would bind or unbind at the same time:

$$H + nO_2 \to C, \quad C \to H + nO_2. \tag{1}$$

Using mass action reaction kinetics [20, 21], the differential equation for the complex C is

$$\frac{dC}{dt} = \alpha H O_2^n - \beta C,$$

where α and β are the binding and unbinding reaction rates respectively. One mass conservation law that holds for this system is the preservation of the total amount of hemoglobin, whether bound or unbound to oxygen: $H + C = H_{tot}$.

At steady state, one can set $\alpha H O_2^n = \beta C$ and replace H by $H_{tot} - C$ in order to solve for C as a function of the oxygen concentration:

$$C = H_{tot} \frac{O_2^n}{\frac{\beta}{\alpha} + O_2^n}. \tag{2}$$

As O_2 increases, the amount of oxygen bound to hemoglobin increases in an ultrasensitive way. The function $x^n/(K^n + x^n)$ became known as a Hill function, and it is one of the most important functions in mathematical biology. The exponent n in this function is known as the *Hill coefficient*, and it is a measure of the ultrasensitivity of the function (Figure 1b). Incidentally, A.V. Hill went on to receive a Nobel prize in 1922 for his work on muscle physiology, and he is considered one of the founders of biophysics.

One should actually not take equation (1) too seriously, because a reaction involving such a large number of molecules is highly unlikely to take place. At most, this reaction can be thought of as a shorthand version of a reaction involving multiple steps. Depending on how the different steps are specifically spelled out, the high ultrasensitive behavior may or may not be preserved. Nevertheless this is an illustrative example of how one can derive a Hill function from first principles, involving the collective action of multiple individual sites.

3 Cooperativity and the Adair model

A ubiquitous concept in the study of multisite ultrasensitivity is that of *cooperativity*. A multisite protein is said to be cooperative if the modification of one of its sites (phosphorylation, ligand binding, etc) increases the rate of modification of its neighboring sites. The general idea is that cooperativity leads to ultrasensitive behavior, which is illustrated quantitatively in this section.

Suppose that a protein with n sites can be in states S_0 through S_n, where S_i represents the concentration of protein with exactly i modified sites. S_i turns into S_{i+1} at a linear rate equal to $a_i E S_i$, where E is the input concentration (enzyme, ligand, etc) (Figure 3a). Assume that S_{i+1} turns back into S at a rate of $b_{i+1} S_i$. The differential equation for this system is

$$S_i' = a_i E S_{i-1} - b_i S_i - a_{i+1} E S_i + b_{i+1} S_{i+1}, \tag{3}$$

for $i = 1 \ldots n-1$. For $i = 0$ and $i = n$ simply omit the first and last two terms, respectively. At steady state one can prove that $S_i = \frac{a_i}{b_i} E S_{i-1}$ for $i = 1 \ldots n$ (prove first for $i = 1$, then through induction on i). Therefore

$$S_i = \frac{a_1 \ldots a_i}{b_1 \ldots b_i} E^i S_0, \quad i = 1 \ldots n.$$

Defining the new parameters

$$A_0 := 1, \quad A_i := \frac{a_1 \ldots a_i}{b_1 \ldots b_i}, \quad i = 1 \ldots n,$$

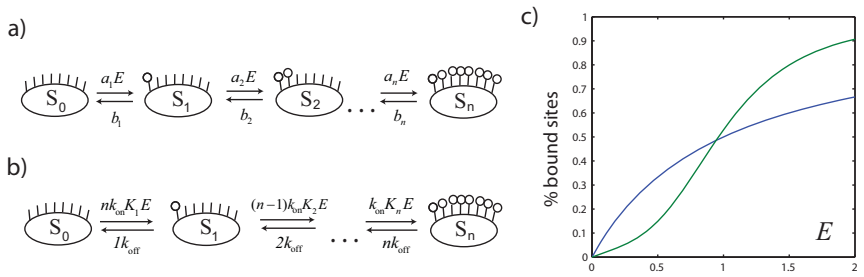

Fig. 3 **a)** General sequential modification model. The corresponding ODE can be derived from the linear flow rates. **b)** Adair model. In the special case $K_i = 1$, this model replicates a nonsequential ligand binding model with n independent sites. **c)** Simulation of the Adair model for $n = 4$. If all $K_i = 1$, the fraction of bound sites is equal to $cE/(cE + 1)$, plotted in blue for $c := k_{on}/k_{off} = 1$. If $K_1 = K_2 = K_3 = 1$, $K_4 = 1000$, and $c = 0.2$, a more ultrasensitive behavior is obtained (green).

the total substrate concentration can be calculated as

$$S_{tot} = S_0 + \dots S_n = S_0 \sum_{i=0}^{n} A_i E^i.$$

Solving for S_0 it follows that

$$S_i = S_{tot} \frac{A_i E^i}{A_0 + A_1 E + A_2 E^2 + \dots + A_n E^n}, \quad i = 0, \dots, n. \tag{4}$$

A simple assumption made often in the literature is that the protein S is only active when it is fully modified. In that case, the dose response function is

$$f(E) = S_{tot} \frac{A_n E^n}{A_0 + A_1 E + A_2 E^2 + \dots + A_n E^n}.$$

By exploring different values for the parameters, one can observe that the ultrasensitive behavior of this dose response increases when the last few net modification constants a_i/b_i are larger than the first. This is because the middle terms in the denominator have a smaller influence and the function becomes similar to a Hill function with Hill coefficient n. For instance, set $n = 3$, $b_1 = b_2 = b_3 = 1$, and $a_1 = \epsilon$, $a_2 = 1$, $a_3 = 1/\epsilon$ for $\epsilon < 1$. Then $A_1 = \epsilon$, $A_2 = \epsilon$, $A_3 = 1$, and

$$f(E) = \frac{E^3}{1 + \epsilon E + \epsilon E^2 + E^3}.$$

The Adair model

The so-called Adair model attempts to replicate the nonsequential behavior of multisite systems within the above framework (Figure 3b). There is usually no order in which to modify or de-modify the sites, and any of the 2^n possible configurations can be found at any time. Suppose that each of the sites binds at a rate of $k_{on}E$ and unbinds at a rate of k_{off}. Then the rate at which S_0 flows into S_1 can be described as $nk_{on}E$, since there are n possible locations at which the modification can happen. Protein S_i can be de-modified at i different locations, so one can set $b_i := ik_{off}$. Similarly, set $a_1 := nk_{on}, a_2 := (n-1)k_{on}, \ldots, a_n := k_{on}$. If $c := k_{on}/k_{off}$, then $A_i = \binom{n}{i}c^i$. Also, at steady state

$$S_i = \binom{n}{i} c^i E^i S_0. \tag{5}$$

Using the binomial formula one also obtains

$$S_n = \frac{c^n E^n}{(1+cE)^n}.$$

In the case of transport molecules such as hemoglobin, one might want to define another output such as the fraction of bound ligand, $Y(E)$. A calculation shows that at steady state

$$Y(E) = \frac{S_1 + 2S_2 + \ldots + nS_n}{nS_{tot}} = \frac{cE}{cE+1}.$$

This is consistent with the idea of n sites independently binding and unbinding to the ligand, and it further confirms the intuition behind this particular choice of parameters.

Notice that in that model the sites are reacting with the ligand independently of each other. In the more general case of the Adair model, the sites are allowed to interact by setting $a_1 := nk_{on}K_1, \ldots, a_n := k_{on}K_n$, and $b_i = ik_{off}$. The abstract parameter $K_i \geq 1$ is intended to represent the cooperative effect of binding i sites beyond what would be expected from independent binding. Once again, it can be observed computationally that when the last few K_i are larger than the rest the ultrasensitive behavior increases. See Figure 3c for a quantitative comparison of the system for $n = 4$ and $K_4 = 1$ as well as $K_4 = 1000$.

4 Allostery and the MWC model

The contemporary theory of multisite ultrasensitivity was founded with an influential 1965 paper by Monod, Wyman and Changeux (MWC) [50]. To this day this paper receives around 200 citations every year according to Google scholar, and it has profoundly affected the way that biologists think of multisite interactions.

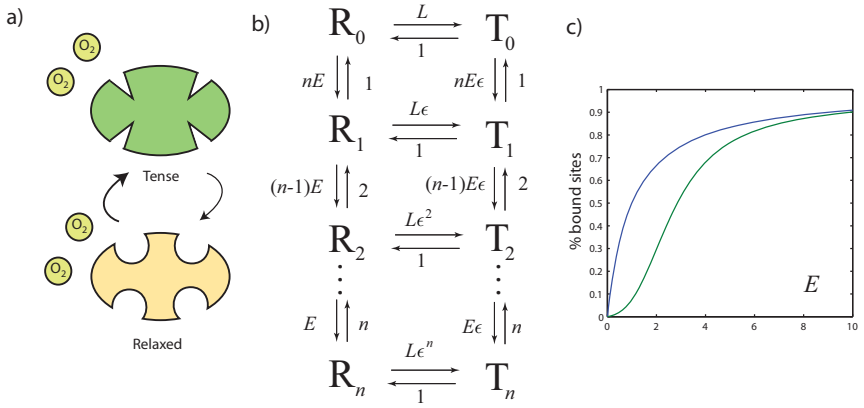

Fig. 4 a) According to the MWC model, all four hemoglobin sites can change simultaneously from a low affinity (tense) state to a high affinity (relaxed) state. Oxygen binding traps the molecule in the relaxed state, allowing other oxygen molecules to bind at a higher rate. **b)** Wire diagram of the MWC model. Here $L \gg 1$ and $\epsilon \ll 1$. **c)** Simulation of the model for $n = 4$ and $L = 100$. For $\epsilon = 1$ the dynamics corresponds to that of the Adair model and the dose response is $E/(E+1)$ (blue). For $\epsilon = 0.01$ the ultrasensitivity increases (green).

The main assumption in the paper is that the hemoglobin protein as a whole can spontaneously jump between a 'tense' state of low O_2 affinity and a 'relaxed' state of high O_2 affinity (Figure 4a). The state is a global property of the protein, i.e. there cannot be both tense and relaxed sites simultaneously in the same protein. This invokes the concept of *allostery*, or the idea that there are strong internal interactions among the sites of a protein, such that changes in one site affect other sites as well.

The model reactions are described in Figure 4b. T_i represents the concentration of the tense hemoglobin protein with exactly i bound oxygen ligands, and similarly R_i represents the relaxed protein with i bound ligands. The oxygen concentration is represented with the variable E. The relaxed molecule R_i has $n - i$ binding sites left and therefore its binding rate is $(n - i)E$. Its rate of unbinding is i, since each of its i sites are equally likely to unbind. This is reminiscent of the Adair model in the previous section, except that $K_i = 1$ in the MWC model. We are also using $k_{on} = k_{off} = 1$ for notational convenience.

A similar argument applies for the tense protein T_i, except that the binding rate is multiplied by a small number ϵ, which is intended to model the fact that the tense protein T_i cannot bind oxygen as well as R_i. R_0 spontaneously turns into T_0 at rate L, and back at rate 1. It is believed that for hemoglobin $L \approx 100$, i.e. in the absence of oxygen most of the protein is in the tense state. The remaining rates of exchange between R_i and T_i are determined by the detailed balance principle [20,21].

At steady state, the detailed balance principle allows us to assume that each of the individual reactions is in equilibrium with its reverse reaction. So for instance,

$$(n-i)ER_i = (i+1)R_{i+1}, \quad (n-i)E\epsilon T_i = (i+1)T_{i+1},$$

for $i = 1 \dots n$. It follows that

$$R_i = \binom{n}{i} E^i R_0, \quad T_i = \binom{n}{i} E^i \epsilon^i T_0.$$

By the binomial formula $R_0 + \ldots + R_n = R_0(E+1)^n$, and $T_0 + \ldots + T_n = T_0(E\epsilon+1)^n$. With some additional work one can also prove $R_1 + 2R_2 + \ldots + nR_n = R_0 nE(E+1)^{n-1}$ by factoring out nE from each of the terms of the left hand side. Similarly for tense variables T_i. The total fraction of occupied sites (i.e. the output of this model) can then be computed as a function of E,

$$Y(E) = \frac{\sum_i iR_i + \sum_i iT_i}{\sum_i nR_i + \sum_i nT_i} = \frac{E(E+1)^{n-1} + LE\epsilon(E\epsilon+1)^{n-1}}{(E+1)^n + L(E\epsilon+1)^n},$$

where $L = T_0/R_0$. This function is fairly ultrasensitive for small ϵ or for large n. In fact one can consider the limit case when the tense protein does not bind oxygen at all, i.e. $\epsilon = 0$, in which case

$$Y(E) = \frac{E(E+1)^{n-1}}{(E+1)^n + L}.$$

This is similar to a Hill function with Hill coefficient n [39]. See Figure 4c for a comparison of the dose response using $\epsilon = 1$ or $\epsilon = 0.01$.

The Monod-Wyman-Changeux model has proved hugely popular in pharmacology and molecular biology ever since its publication, because of its wide applicability in many enzymatic and other biophysical systems. In the mind of many biologists, multisite ultrasensitivity goes hand in hand with allostery and the MWC model, along with the concept of cooperativity.

5 The KNF cooperative model

The model by Koshland, Nemethy, and Filmer [42], also known simply as KNF and the second of the two classical models in the field, is a spiritual heir to the Adair model described in Section 3. It also attempts to describe how cooperative interactions between sites can affect the dose response of a multisite biochemical system. But while the Adair model introduces cooperativity in the form of abstract constants $K_i > 1$, the KNF model has the advantage of increased molecular detail. On the other hand, the geometric detail makes it rather tedious to use, which is why many discussions of cooperativity in the literature still remain at the abstract level of section 3.

In the KNF model the multisite substrate is described as a graph, with nodes representing the different sites, and edges representing which pairs of

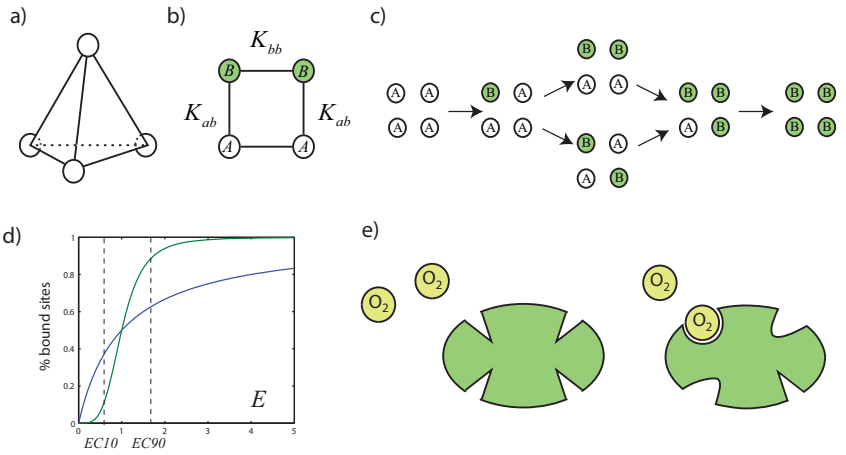

Fig. 5 **a)** In the KNF model, sites are represented as nodes on a graph, and edges represent direct interactions between sites. **b)** Given a configuration of unbound (A) and bound (B) sites, each edge is labeled as AA, BB, or AB. The AB and BB edges lower the overall energy of the molecule by K_{ab} and K_{bb} respectively, increasing its concentration at steady state relative to the fully unbound molecule. **c)** The different conformations of a square molecule. **d)** Simulation of the KNF model for $n = 4$, $K_{ab} = 1$. If $K_{bb} = 1$, the system reduces to the Adair model and the dose response is $cE/(1+cE)$, displayed here for $c = 1$ in blue. For $K_{bb} = 1000$ the dose response becomes ultrasensitive, here shown for $c = 0.001$ in green. For this second graph the inputs EC10 and EC90 are shown, which yield a 10% and 90% response respectively and can be used to quantify ultrasensitive behavior. **e)** According to the induced fit hypothesis, the shape of the binding site changes upon binding, increasing its affinity. This in turn changes the shape of neighboring sites as well.

sites can directly interact with each other. For instance, if $n = 4$ and all pairs of sites are allowed to interact, the corresponding graph is a tetrahedron, as shown in Figure 5a. In the case of the hemoglobin molecule, the graph is rather a square with four nodes and four edges, and each site can only directly interact with two neighbors.

Suppose that in the absence of interactions among sites, the on-rate of binding of the ligand E to a given site is $k_{on}E$, and the off-rate is k_{off}. Say that A and B represent unbound and bound sites respectively. If B_i is the concentration of substrate with i out of n bound sites, then at steady state

$$B_i = \binom{n}{i} c^i E^i B_0.$$

This equation was shown in (5) of the Adair model with independent sites. Here $c = k_{on}/k_{off}$ is the net affinity rate. In fact, $c^i E^i B_0$ is the concentration of each molecule given a *specific* subset of i out of the n ligands bound, and B_i adds together all $\binom{n}{i}$ of them.

Now, in the presence of interactions among neighboring sites, the geometry of the specific molecule matters considerably. Suppose given a square geometry and the molecule B_2 that has two adjacent bound sites. Out of the four edges, two of them are of type AB, one of type AA, and one of type BB (Figure 5b).

The key assumption of the KNF model is that edges involving bound ligands lower the overall chemical energy of a molecule through their interaction, helping to increase the molecule concentration at steady state. Suppose that $K_{ab}, K_{bb} \geq 1$ represent the contribution of AB and BB edges to the lower energy of the molecule, respectively. In the case of the square molecule $B_{2,adj}$ with two adjacent bound sites, the new concentration at steady state is

$$B_{2,adj} = 4c^2E^2K_{ab}^2K_{bb}B_0.$$

The factor 4 represents the number of different specific ligand configurations that have two adjacent bound sites, and the factor $K_{ab}^2K_{bb}$ represents the increased reactivity towards this state due to internal site interactions.

The concentration of a molecule $B_{2,opp}$ with two opposite bound sites is $B_{2,opp} = 2c^2E^2K_{ab}^4B_0$. Using the same logic, one can calculate the concentration of squares with one, three, and four bound sites (Figure 5c) as $B_1 = 4cEK_{ab}^2B_0$, $B_3 = 4c^3E^3K_{ab}^2K_{bb}^2B_0$, $B_4 = c^4E^4K_{bb}^4B_0$. Now the total substrate B_{tot} is calculated as

$$B_{tot} = B_0 + B_1 + B_{2,opp} + B_{2,adj} + B_3 + B_4$$

$$B_{tot} = B_0[1+4cEK_{ab}^2+2c^2E^2(2K_{ab}^2K_{bb}+K_{ab}^4)+4c^3E^3K_{ab}^2K_{bb}^2+c^4E^4K_{bb}^4]. \quad (6)$$

This way one can solve for B_0 as a function of E. Similarly $B_{2,opp}$ and the remaining states can be written in terms of E and the model parameters.

One can also compute the fraction of bound ligands in this model using equation (6) and canceling out B_0 in the numerator and denominator:

$$Y(E) = \frac{B_1 + 2B_{2,opp} + 2B_{2,adj} + 3B_3 + 4B_4}{4B_{tot}}$$

$$= \frac{cEK_{ab}^2 + c^2E^2(2K_{ab}^2K_{bb} + K_{ab}^4) + 3c^3E^3K_{ab}^2K_{bb}^2 + c^4E^4K_{bb}^4}{1 + 4cEK_{ab}^2 + 2c^2E^2(2K_{ab}^2K_{bb} + K_{ab}^4) + 4c^3E^3K_{ab}^2K_{bb}^2 + c^4E^4K_{bb}^4}.$$

Once again, large values of K_{ab} and especially of K_{bb} translate into a higher ultrasensitive behavior in the system. See Figure 5d for graphs of this output in the case $n = 4$ and two different values of K_{bb}.

The induced fit hypothesis

The KNF model is an implementation of the concept of *induced fit*, originally proposed by Koshland in the late 1950's. Binding sites are usually believed to be rigid and have just the right shape for a ligand to bind. Under the induced fit scenario, a binding site is flexible, and when unbound it does not have a very good affinity to the ligand. However, when bound, the binding site adapts

its shape around the ligand, increasing its affinity. When the shape of the site changes, it also allosterically changes the shape of the neighboring sites, so that they also have a higher affinity to the ligand. See Figure 5e.

In this way one can say that ligands have, like the MWC model, two different configurations, one with low affinity and one with high affinity. However the high affinity state is only found when the ligand is bound, so from a mathematical point of view one can simply refer to the bound and unbound states. Also importantly, the KNF model allows for different ligands to be in different states simultaneously, which is ruled out in the MWC model.

6 Generalized Hill coefficients

For Hill functions $x^n/(K^n+x^n)$, the Hill coefficient n is a quantitative measure of the extent to which the function is ultrasensitive: the larger n, the stronger the all-or-none behavior. But if a dose response function $f(x)$ is not a Hill function, this definition cannot be used. Often biologists will measure the approximate Hill coefficient by carrying out a least squares minimization to find the best fitting Hill function, then use the resulting value for h as an estimate of ultrasensitive behavior. However this procedure is ill-posed for carrying out mathematical estimates.

A more useful formula was proposed by Goldbeter and Koshland [25] for use in more general sigmoidal functions:

$$H := \frac{\ln(81)}{\ln(EC90/EC10)},$$

where $EC10$ and $EC90$ are the inputs that produce 10% and 90% of the maximal response, respectively (Figure 5d). The more ultrasensitive the function, the smaller the ratio $EC90/EC10 > 1$, and the larger H becomes.

What is interesting about this particular way of quantifying ultrasensitive behavior is that in the special case of Hill functions $f(x) = x^n/(K^n + x^n)$, it holds that $n = H$. In that sense H is a generalization of the concept of Hill coefficient for arbitrary sigmoidal functions. To see this, set $u = EC10$ and $v = EC90$, so that

$$\frac{u^n}{K^n + u^n} = 0.1, \quad \frac{v^n}{K^n + v^n} = 0.9.$$

Inverting both sides of the first equation,

$$10 = \frac{K^n + u^n}{u^n} = K^n u^{-n} + 1,$$

or $9 = K^h u^{-n}$. For v, the corresponding equation is $1/9 = K^n v^{-n}$. Dividing the two equations one obtains

$$81 = \frac{K^n u^{-n}}{K^n v^{-n}} = \left(\frac{v}{u}\right)^n.$$

Taking natural logarithm on both sides one obtains $n = \ln 81/\ln(\frac{u}{v}) = H$.

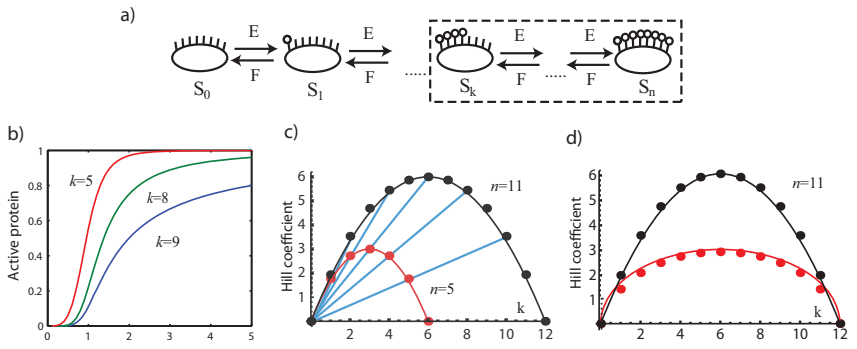

Fig. 6 **a)** The output is redefined by assuming that k modifications or more are sufficient to fully activate the protein. **b)** Dose response $S_k + \ldots + S_n$ for $n = 9$ and different values of k. **c)** Estimated formula for the Hill coefficient (solid lines) along with numerical calculations. **d)** In the nonsequential Adair model (red), the Hill coefficient is proportional to the square root of that in a) (black).

7 Nonessential Modification Sites

After a tour of the classical models, we discuss more recent work carried out in the field by the author and his colleagues. In model MWC as well as KNF, the basis for ultrasensitive behavior is the assumption of internal allosteric interactions among the individual sites, either to force the system to switch globally between two states (MCW), or so that the modification of one site positively affects the rate of modification of its neighbors (KNF). In the new work we have focused on altering instead the definition of the *output* of the system, to encourage ultrasensitive behavior.

In the different models of oxygen transport discussed, the natural output is the fraction of bound ligand at steady state, or $\sum_{i=0}^{n} iS_i/(nS_{tot})$, as a function of the input E. However in signal transduction cascades it is not so much the amount of modification that matters, but the activity level of the multisite substrate. For such models, the standard output in the literature is simply the concentration S_n of the most modified protein, under the assumption that only a fully modified protein is considered active.

Suppose instead that out of n sites, a protein only needs k modifications in order to fully activate the protein [70]. At an intuitive level, this might be considered equivalent to having k sites and requiring all k sites for activation. But as it turns out, having so-called *nonessential sites* in the system can help increase its ultrasensitive behavior.

First we consider the case of a simple sequential system of the type discussed in equation (3) and Figure 3a, setting simply $a_i = b_i = 1$. (In the original work [70] a constant phosphatase concentration F is assumed, which

is equivalent to $b_i = F$ but can also be eliminated through a change of variables.) In that case $A_i = 1$ for all i, and the new output can be calculated from equation (4) as

$$S_k + \ldots + S_n = S_{tot} \frac{E^k + \ldots + E^n}{1 + E + E^2 + \ldots + E^n} = S_{tot} \frac{E^k - E^{n+1}}{1 - E^{n+1}}.$$

Figure 6a describes this dose response for $n = 9$ and several decreasing values of k. By the standards of the previous sections, even the dose response with $k = n = 9$ is ultrasensitive. But as k decreases, the ultrasensitivity clearly becomes stronger.

One can show the ultrasensitive behavior of this dose response analytically for the special case $k = (n+1)/2$. In that case, $n + 1 = 2k$ and

$$\frac{S_k + \ldots + S_n}{S_{tot}} = \frac{E^k - E^{2k}}{1 - E^{2k}} = \frac{E^k(1 - E^k)}{(1 + E^K)(1 - E^k)} = \frac{E^k}{1 + E^k}.$$

This is once again a Hill function, with Hill coefficient k. As n increases, so does k and the ultrasensitivity increases arbitrarily.

In the paper [70] together with L. Wang and Q. Nie, we quantify the ultrasensitive behavior of the dose response for different values of n and k, using the apparent Hill coefficient defined above. We carry out an estimate to conclude that

$$H(n, k) \approx 2k(1 - \frac{k}{n+1}). \tag{7}$$

See Figure 6b for a comparison of the estimated formula and the actual calculated values. This formula shows that in fact the largest Hill coefficient for a given n is found for $k = (n+1)/2$, and that the Hill coefficient grows linearly with n given a fixed ratio $\alpha = k/(n+1)$.

We also carried out a similar analysis in the nonsequential case using an equivalent framework to the Adair model with $K_i = 1$ and $c = 1$ [note: generalize to arbitrary c]. In that case the dose response follows from equation (5) and $S_{tot} = S_0 + \ldots S_n$:

$$S_k + \ldots + S_n = S_{tot} \frac{\sum_{i=k}^{n} \binom{n}{i} E^i}{(E+1)^n}.$$

The Hill coefficient is also estimated in that case, and surprisingly, it is essentially the square root of equation (7):

$$H(n, k) \approx 1.7 \sqrt{k(1 - \frac{k}{n+1})}.$$

In this case the maximum value of H is also reached at $(n + 1)/2$. The Hill coefficient is invariant under horizontal or vertical rescaling of the dose response. In particular the same result will be obtained if, say, $c \neq 1$ in the Adair model.

8 General Activity Gradients

In more recent work by Ryerson and the author, we set out to further generalize the dose response under more natural assumptions. Suppose that S is a multisite substrate with n sites, and that its activity as a signaling molecule increases gradually as its sites are increasingly modified. This relaxes the previous assumption that the protein is either completely active or completely inactive for different number of modifications. To formalize this idea we define the *activity gradient*, a function $h(x) : [0,1] \to [0,1]$ representing the level of activity of a substrate molecule that has a fraction x of its sites modified.

As it turns out, the two different cases treated in the previous section now have a very different behavior. Given a fixed activity gradient $h(x)$, in the sequential case the ultrasensitivity increases arbitrarily for increasing values of n. But in the nonsequential case, the dose response $f(E)$ converges uniformly to a fixed function as $n \to \infty$. In particular, its ultrasensitive behavior is bounded by the ultrasensitivity of this bounded function.

8.1 The sequential case

Consider again the sequential model in (3) and Figure 3a, setting $a_i = b_i = 1$. Given the activity gradient $h(x)$, suppose that activity of S_i is $h(\frac{i}{n+1})$. The dose response at steady state can be written as

$$f(E) = \sum_{i=0}^{n} h\left(\frac{i}{n+1}\right) S_i = S_{tot} \frac{h(\frac{0}{n+1}) + h(\frac{1}{n+1})E + h(\frac{2}{n+1})E^2 + \ldots + h(\frac{n}{n+1})E^n}{1 + E + \ldots + E^n}. \tag{8}$$

Figure 7a displays this dose response for the (non-ultrasensitive) activation gradient $h(x) = x/(1+x)$. Notice that as n increases, this function is increasingly ultrasensitive. In fact, the Hill coefficient of the response appears to increase linearly, as shown in Figure 7b. The following lemma calculates the estimate $H \approx const.(n+1)$ for this system.

Lemma 1 *Suppose that $h(x)$ is a piecewise continuous increasing function, and that $h(0) \leq 0.1h(1)$. Then the Hill coefficient of equation (8) satisfies $H \approx c(n+1)$, where the constant $c > 0$ does not depend on n.*

Proof: The dose response can be approximated by

$$f(E) \approx S_{tot} \frac{\int_0^{n+1} h(\frac{x}{n+1}) E^x dx}{\int_0^{n+1} E^x dx} = S_{tot} \frac{\int_0^1 h(y) E^{(n+1)y} dy}{\int_0^1 E^{(n+1)y} dy},$$

after a change of variables $y = \frac{x}{n+1}$. Defining the function

$$g(\alpha) = \frac{\int_0^1 h(y) e^{\alpha y} dy}{\int_0^1 e^{\alpha y} dy},$$

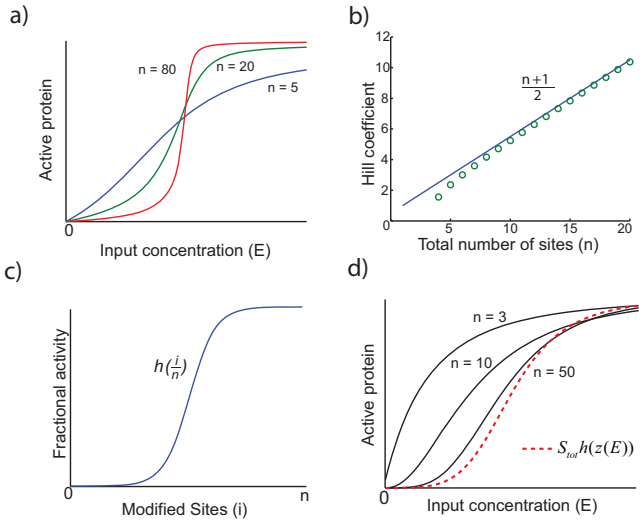

Fig. 7 a) Dose response (8) in the sequential case using the activation gradient $h(x) = x/(1+x)$, for different values of n, illustrating that it becomes arbitrarily ultrasensitive as $n \to \infty$. **b)** Estimated and exact Hill coefficient of the dose responses in a). **c))** Ultrasensitive activation gradient used for the nonsequential model (9). **c))** Dose responses of (9) for increasing n, showing convergence towards $S_{tot}h(z(E))$.

it holds

$$f(E) \approx S_{tot} g((n+1)\ln E).$$

The values $p := EC10_g$ and $q = EC90_g$ are well defined, since $g(\alpha)$ is a continuous, monotone function approaching $h(0)$ for $\alpha \to -\infty$ and $h(1)$ for $\alpha \to \infty$, and $h(0) \leq 0.1h(1)$.

Then $e^{\frac{p}{n+1}}$ is approximately the EC10 of the function $f(E)$ since

$$f(e^{\frac{p}{n+1}}) \approx S_{tot} g((n+1)\ln e^{\frac{p}{n+1}}) = S_{tot} g(p) = 0.1 g_{max} S_{tot} = 0.1 f_{max}$$

Likewise, $e^{\frac{q}{n+1}}$ is the EC90 of f. The Hill Coefficient of f is therefore

$$H_f = \frac{\ln 81}{\ln \frac{EC90_f}{EC10_f}} \approx \frac{\ln 81}{\ln \frac{e^{\frac{q}{n+1}}}{e^{\frac{p}{n+1}}}} = \frac{\ln 81}{q-p}(n+1).$$

8.2 The nonsequential case

Under the Adair model with $K_i = 1$, suppose that the activity of a substrate S_i is $h(i/n)$. Then the dose response is defined as the total protein activity at steady state, or

$$f(E) = \sum_{i=0}^{n} h(i/n) S_i.$$

This expression generalizes both the output used for the transport system used in the classical hemoglobin models, and the output $S_k + \ldots + S_n$ used in the previous section. In the former case this follows from setting $h(x) = x$, and in the latter case this corresponds to a Heaviside function $h(x)$.

Now, recall that the Adair model replicates the behavior of a nonsequential system with n individual sites that are modified at a rate of $k_{on}E$, and demodified at a rate of k_{off}. One can define the function $z(E)$ as the overall fraction of modified sites at steady state. It is easy to calculate that

$$z(E) = \frac{cE}{cE + 1},$$

where $c = k_{on}/k_{off}$. One can then write the steady state protein S_i in terms of $z = z(E)$:

$$S_i = S_{tot} \frac{\binom{n}{i} c^i E^i}{(cE+1)^n} = S_{tot} \binom{n}{i} z^i (1-z)^{n-i}.$$

This leads to an expression for $f(E)$ that can be approximated in surprisingly simple terms:

$$\frac{f(E)}{S_{tot}} = \sum_{i=0}^{n} h(i/n) \binom{n}{i} z^i (1-z)^{n-i} \approx h(z) \tag{9}$$

The middle term above is the so-called Bernstein polynomial of the function $h(x)$. For continuous $h(x)$, the left hand side has been shown to converge towards $h(z)$ uniformly on z as $n \to \infty$ [33]. Even for piecewise continuous functions $h(x)$, the left hand side converges pointwise towards $h(z)$, which is useful in the case of Heaviside functions. Either way we have the following interesting formula,

$$f(E) \approx S_{tot} h(z(E)).$$

If the function $h(x)$ is itself ultrasensitive, then $f(E)$ can also be an ultrasensitive function. In the independent Adair case $h(x) = x$, we have already shown that $f(E)/S_{tot} = z(E)$, which is consistent with this equation. In this way, one can think of the use of ultrasensitive activation functions as an alternative to the use of $K_i > 1$ in the Adair model, or to the corrections proposed in the MWC and KNF models.

See Figure 7d for the dose response in this model for increasing values of n, using the activation function in Figure 7c.

9 Other forms of ultrasensitivity

Although the focus of this review is on multisite mechanisms for ultrasensitivity, other mechanisms have been discussed in the literature that can create ultrasensitive responses. In some cases they are similar enough that the same system can be interpreted by more than one such mechanism, however at the conceptual level they are different enough to be distinguished.

9.1 Signaling Cascades

The reader may have noticed that the reaction described in Figure 1a has multiple steps, i.e. the receptor complex activates Ras, which activates Raf, which in turn activates Mek, and Mek activates MAPK. Why not have the receptor activate MAPK directly and eliminate the other proteins? Such long cascades are actually the rule rather than the exception in signal transduction. For example, the last three steps here are known as a MAP kinase cascade, and almost identical cascades can be found regulating widely diverse signals in mammals, plants, yeast, etc. [56]. There are actually many reasons to have multiple cascades rather than a single regulatory step. For instance, they can help amplify an originally weak signal, and they can increase opportunities for feedback and cross-talk from other pathways [8].

Signal cascades can also increase the ultrasensitive behavior of a response. If the output of an initial step is the input of a second step, then at steady state in the simplest case the overall dose response is the composition of both responses. If two mildly ultrasensitive functions are composed, the result can be a more strongly ultrasensitive function. Notice that the two last proteins of the reaction in Figure 1a have two phosphorylation sites each. Each of them constitutes a multisite system on its own, and the composition of several such systems can lead to a much stronger ultrasensitive response.

In actual signaling cascades, one cannot simply compose the dose responses of the individual steps to obtain the overall dose response. This is due to a retroactivity effect, in which the output molecules of an upstream component are also affected by the downstream molecules that interact with it. Huang and Ferrell investigated the behavior of MAP kinase cascades as a whole and discovered that they can be strongly ultrasensitive, even when the individual steps would not predict such behavior [36]. See also more recent work by Sarkar and colleagues on the properties of synthetically engineered cascades [53] and by Del Vecchio and colleagues on the phenomenon of retroactivity [54].

9.2 Zero-order ultrasensitivity

In 1981, Goldbeter and Koshland proposed an influential framework for ultrasensitive dose responses using a single protein modification site [25] under saturating conditions. In order to understand it, it is useful to describe some basic enzyme biochemistry.

The process by which an enzyme modifies a substrate and converts it into a product is usually modeled using the so-called Michaelis-Menten reactions [49]

$$S + E \leftrightarrow C \rightarrow P + E,$$

where S, E, P represent substrate, enzyme, and product respectively. The molecule C represents a transient complex formed by the substrate and the enzyme. The overall rate of conversion of substrate into product can be estimated as

$$\frac{dP}{dt} \approx kE_{tot}\frac{S}{K_m + S}. \tag{10}$$

Here k is the rate parameter of the reaction $C \rightarrow P + E$, E_{tot} is the total enzyme concentration, and K_m is determined solely from the reaction parameters in the system. The derivation generally uses the assumption $E_{tot} \ll S_{tot}$, although other assumptions can lead to the same result [38]. This is one of the most basic formulas in biochemistry, and it is the origin for many of the terms found in mathematical models of protein interactions.

Suppose that the substrate concentration S in the environment is much larger than K_m. Then the rate of flow of S into P can be approximated as $kE_{tot}\frac{S}{S} = kE_{tot}$, which is independent of S. In the enzyme biochemistry community this reaction is described as *zero-order*, since in general an n-th order reaction $nS \rightarrow P$ would have a rate proportional to S^n, and this reaction seems to fit that description only with $n = 0$.

Goldbeter and Koshland [25] proposed a situation in which one enzyme E modifies a substrate S, and another enzyme F eliminates this modification, assuming a very large substrate concentration of S relative to the K_m of both reactions. Then the rates of modification and de-modification are roughly independent of S, P and the net flow of S into P is approximately $k_1E_{tot} - k_2F_{tot}$. If F_{tot} is left constant and E_{tot} is used as an input, then very minor differences in E_{tot} can result in either a net positive or a net negative flow, resulting in a very large or very small steady state concentration of P. In this way the dose respose may become highly ultrasensitive with respect to E_{tot}. (Of course, the flow is not exactly constant, otherwise S or P would become negative. Rather when one of them becomes very small the assumptions break down and the system settles into steady state.)

Because of its simplicity, this mechanism has many potential applications. It has been used in experimental studies to investigate glycogen metabolism [47] and morphogen gradients in embryonic development [48], among others. From a theoretical point of view, recent work has updated this system by taking into account e.g. stochastic effects [6] and the reversibility of the product-formation reaction in (10) [71].

9.3 Protein Relocalization

Another series of papers points to ultrasensitive behavior though mechanisms using multiple compartments and the sequestration or relocalization of pro-

teins. For instance, Liu et al. consider a multisite protein system similar to those described in previous sections, together with an additional protein that acts as a scaffold [45]. The scaffold protein passively binds and unbinds the substrate, effectively relocalizing it to a different position inside the cell. The relocalization of the substrate affects the rates in which the enzymes can interact with it. Liu et al. show that under certain conditions the presence of the scaffold can significantly improve the ultrasensitive behavior of the dose response.

Perhaps the earliest sequestration mechanism was proposed by Ferrell in 1996 [22], in a system involving the competition of multiple different substrates for the attention of the same enzyme E. The substrate S can be activated e.g. through single-site modification by E. But other substrates are deployed as decoys to bind to the enzyme more tightly than S itself, thus relocalizing it and making it inaccessible to S. The result is that the concentration of E needs to be high enough to bind to the decoy substrates as well as S, leading to ultrasensitive behavior. This mechanism was experimentally tested in 2007 in the context of regulatory proteins in the cell cycle of frog eggs [40]. The enzyme Cdk1 alters the activity of the substrate Wee1 through phosphorylation in specific sites. But other Wee1 phosphorylation sites, as well as other proteins in the cytoplasm, appear to bind Cdk1 and prevent it from activating Wee1.

In 2009, Buchler and Cross built a synthetic circuit inside a cell, also involving protein sequestration by an inhibitor protein [10]. They were able to measure Hill coefficients as high as 12 in this synthetic system and to replicate model predictions on this experimental system.

9.4 Positive feedback and bistability

A more general way of approaching ultrasensitive behavior is through the use of positive feedback interactions (although positive feedback underlies many of the above examples). Such systems could for instance be bistable for a certain range of the input values, leading to hysteresis [4]. A hysteretic dose response can be considered highly ultrasensitive, in the sense that a very small increase in the input beyond the bifurcation point can bring about a very large increase in the output.

Using bistability to create ultrasensitive responses presents a type of chicken and egg problem. Positive feedback interactions are by themselves not sufficient to create bistability, and usually some kind of ultrasensitive nonlinearity is also required in at least one of the feedback interactions. The mechanisms described in this chapter can precisely be used to create the nonlinear interactions necessary for bistability.

That said, positive feedback interactions can be used to enhance dose responses even when they do not lead to bistability. For instance, in a model of DNA packaging regulation, Sneppen et al. assume a positive feedback interaction between histone proteins and their respective enzymes, in order to obtain ultrasensitive dose responses [62]. When a concrete biological system does not

fit the framework of any of the mechanisms above, one can also describe the dose resposes in this way.

9.5 Allovalency and entropic multisite models

Another mechanism involving the spatial distribution of proteins is known as allovalency and was proposed by Tyers and colleagues in 2003 [41,44]. It describes the binding of a multisite, disordered protein to a single binding site. An important aspect of this model is that it distinguishes between three different locations of the protein with respect to its binding site: it can be bound, proximal, and free (i.e. far apart). A thermodynamic argument is used to calculate the resulting linear transition rates between these three states depending on the number n of sites, which leads to a fair degree of ultrasensitivity for large n.

Disordered multisite proteins of course were also highly relevant in the discussion of independent ultrasensitive behavior in previous sections, and they are predicted to be found commonly in nature [37]. Another thermodynamic derivation of ultrasensitive behavior was developed by Lenz and Swain [43], using nonlinear effects in the entropy configurations of disordered multisite systems. This mechanism results in a highly ultrasensitive function $h(x)$ (using our notation) and is therefore complementary to the results described for independent multisite systems.

10 Discussion

There has been a lively debate in the quantitative biology community on possible ways to construct switch-like responses at the molecular level. This question is clearly of interest to experimental biologists trying to understand design principles in cellular physiology. Contributions have been made for several decades, and they have been fueled more recently by advances in quantitative measurements that can potentially distinguish between different models. Clearly, the models that will have the strongest impact are the ones that actually take place in biological systems and contribute to their conceptual understanding.

As described in the introduction, these systems are intrinsically nonautonomous since their behavior depends entirely on the concentration of the outside input. Traditionally the outside concentration is held constant, but recent experimental techniques such as microfluidics are allowing to carry out time-dependent input experiments. Recent work by Sontag, Alon, and colleagues is already revealing some exciting properties of time-dependent signals such as fold-change detection [61]. At the same time, the stochastic properties of ultrasensitive networks have also been explored, showing for instance that signaling cascades have the ability to attenuate noise [65].

While the MCW and KNF models are extremely influential in theoretical biology, biochemistry textbooks tend to provide only an intuitive description

since the mathematical details are beyond the scope of the text. A good description of these models is the book by Cornish-Bowden [13], but it still falls short of sufficient mathematical precision. I tried to strike a balance here between maintaining an informal tone for readability, and sufficient mathematical detail and generality for use as a reference.

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