Integrating Multiple Signals into Cell Decisions by Networks of Protein Modification Cycles

Luca Cerone,† Javier Muñoz Garcia,‡ and Zoltán Neufeld*†
†School of Mathematical Sciences, Complex Adaptive Systems Laboratory, University College Dublin, Dublin, Ireland; and ‡Departamento de Matemáticas, Grupo Interdisciplinar de Sistemas Complejos, Universidad Carlos III de Madrid, Madrid, Spain

ABSTRACT Posttranslational protein modifications play a key role in regulating cellular processes. We present a general model of reversible protein modification networks and demonstrate that a single protein modified by several enzymes is capable of integrating multiple signals into robust digital decisions by switching between multiple forms that can activate distinct cellular processes. First we consider two competing protein modification cycles and show that in the saturated regime, the protein is concentrated into a single form determined by the enzyme activities. We generalize this to protein modification networks with tree structure controlled by multiple enzymes that can be characterized by their phase diagram, which is a partition of the space of enzyme activities into regions corresponding to different dominant forms. We show that the phase diagram can be obtained analytically from the wiring diagram of the modification network by recursively solving a set of balance equations for the steady-state distributions and then applying a positivity condition to determine the regions corresponding to different responses. We also implement this method in a computer algebra system that automatically generates the phase diagram as a set of inequalities. Based on this theoretical framework, we determine some general properties of protein modification systems.

INTRODUCTION

Protein activity is modulated by reversible posttranslational covalent modifications such as phosphorylation, acetylation, methylation, ubiquitination, and SUMOylation (1). A basic model of reversible protein-modification is the Goldbeter-Koshland (GK) cycle (2), which describes the cyclic conversion of a protein between two forms (e.g., active or inactive) catalyzed by a pair of enzymes (e.g., a kinase and a phosphatase). When the reactions are in the saturated regime, the system produces a sharp switch-like response as the enzyme concentration is varied. This all-or-nothing response is highly sensitive to the input signal in a narrow range, and filters out the fluctuations outside this range. This is important for robust cellular responses in the presence of stochastic fluctuations due to cell-to-cell variability and inherent molecular noise.

Reversible protein modification cycles are generic components of pathway modules that produce biochemical switches and oscillators (3). Cascades of protein modification cycles can convert a graded input into a binary on or off output even without saturation of the enzymes (4). A classical example of such switching behavior is the cascade of dual phosphorylations in the MAP kinase pathway involved in cell-cycle progression (5), neuronal differentiation (6), and T-cell selection (7,8). Additional feedback loops lead to further nonlinear behavior, such as bistability and oscillations (9–11). Investigators have studied models of multisite phosphorylation, represented as a chain of GK cycles, considering different molecular mechanisms and theoretical approximations (12–16). When two protein modifications are catalyzed by the same enzyme, competition for that enzyme can produce bistability (17). This was shown to be even more prevalent in modification systems with multiple protein forms, when all reactions are catalyzed by the same enzyme pair and the amount of substrate exceeds that of the concentration of the enzymes (18). Such a system with a large number of coexisting steady states can act as a dynamic multibit memory with a potentially important role in processing information in the cell (19).

Previous work on protein modification systems focused on modifications by a single pair of enzymes. However, intracellular signals are not processed by independent linear pathways. Typically, a significant amount of cross talk creates a network of protein interactions that regulates gene expression and cellular processes in a context-dependent manner (20). This requires proteins whose activity is modulated by multiple enzymes, in similarity to signal processing by neurons (21). A protein modification system controlled by multiple enzymes can involve the same modification on different sites (e.g., glycogen synthase is phosphorylated on multiple sites by six kinases and several phosphatases) or different covalent modifications, sometimes competing for the same residue, e.g., Ser and Thr can be phosphorylated or glycosylated, whereas Tyr can be either phosphorylated or sulfated (22). SMAD7, which regulates response to TGFβ signaling, can be modified on the same residue by acetylation, which stabilizes the protein, or ubiquitination, which promotes its degradation (23,24). PCNA (25), which regulates DNA replication, repair, and silencing, can be acetylated by enzymes activated by DNA damage on the same residue where it is SUMOylated during S phase, switching between different functions of the protein.
Our aim in this study was to develop and analyze a mathematical model for reversible modifications of a protein by multiple enzymes to gain insight into the capabilities and possible biological function of such modification systems. In particular, we focus on the steady-state response of such systems, describing the relationship between the enzyme activities and the resulting distribution of the protein among its multiple forms. This mathematical framework can then be applied to particular proteins for quantitative characterization of the switches and cross talk in signaling pathways to understand how the response and switching threshold of one enzyme activity can be modulated by the activities of other enzymes. To facilitate analysis of the model, we make certain simplifying assumptions, and later we discuss the consequences of relaxing some of these assumptions and possible further extensions of our approach.

**MODEL**

Here we analyze a general model of reversible protein modifications catalyzed by a set of different enzymes, as a generalization of the GK cycle (2) for multiple input signals, extending the signal processing capabilities of the single-input system studied by Thomson and Gunawardena (18). We consider a protein with \( N \) forms \( \{P_i, i=1,\ldots,N\} \) that may activate different downstream pathways and processes, such as proliferation, differentiation, motility, and apoptosis. The conversions of the protein between different forms are catalyzed by separate enzymes whose concentrations are the input signals. This system can be represented as a network (Fig. 1 a) in which the nodes are the protein forms and the edges represent pairs of catalytic reactions controlled by enzymes, \( E_i \), and \( E_j \), that convert \( P_i \) to \( P_j \) and vice versa. On short timescales, the synthesis and degradation of the protein can be neglected and the total protein concentration is conserved:

\[
\sum_i [P_i] = \text{const}.
\]

The system is characterized by the input-output relationship that connects the distribution of the protein forms \( \{P_1, \ldots, P_N\} \) to the enzyme activities \( \{E_i\} \).

We assume that as a consequence of competing, mutually exclusive modifications, the conversion of the protein follows a strictly ordered sequence, i.e., the modification network has a tree structure. Because there are no closed loops, the net flux along each edge of the network has to be zero in the steady state. Using Michaelis-Menten (MM) kinetics for the reaction rates, for each pair of connected nodes, \( i \) and \( j \), the balance between the modification and demodification fluxes can be written as

\[
V_{ij} \frac{p_i}{p_i + k_{ij}} = V_{ji} \frac{p_j}{p_j + k_{ji}},
\]

where \( p_i = [P_i]/[P_T] \) is the proportion of protein in form \( P_i \), \( k_{ij} \) are the Michaelis constants normalized by the total protein concentration, and \( V_{ij} \) are the maximum fluxes that are proportional to the enzyme concentrations \( \{E_i\} \). From Eq. 1 it follows that the steady-state solution depends only on the enzyme activity ratios, defined as \( u_{ij} = V_{ij}/V_{ji} \), which represent the input signals (we use the convention \( i<j \) to select a set of independent parameters). Because each enzyme catalyzes only one reaction, there is no competition between the protein forms for the enzyme. Therefore, a description based on mass action kinetics, including the elementary reaction steps, leads to the same expressions for the steady-state fluxes as the MM description (see Supporting Material). However, in the case of mass-action kinetics, the enzyme-bound forms of the protein also need to be included in the conservation law. Nevertheless, when the amount of substrate protein exceeds that of the enzymes, such sequestration effects (26) are negligible and the MM approximation remains valid. (See Supporting Material for further details and comparison with the mass action kinetics based description.)

The flux balance equations (Eq. 1) together with the conservation of the total protein represent a system of \( N \) quadratic equations for which an explicit analytical solution cannot be obtained in general. To obtain insight into the behavior of such a system, it is instructive to first look at the simplest case with only two modification cycles.

**RESULTS**

**Two competing protein modification cycles**

Consider the modification network with three protein forms in Fig. 1 b, where \( P_1 \) can be reversibly converted into either \( P_2 \) or \( P_3 \). This type of modification system can arise from the competitive reversible glycosylation, with O-GlcNac, and phosphorylation of serine and threonine residues, which has a role in diabetes and neurodegeneration (27). A specific example of such protein is the transcription factor c-Myc, which is glycosylated or phosphorylated at Thr-58 (28), a frequently mutated site in various types of cancer.

The steady-state solution of the modification system with three protein forms (Fig. 1 b) satisfies the flux balance equations:

Please cite this article in press as: Cerone et al., Integrating Multiple Signals into Cell Decisions by Networks of Protein Modification Cycles, Biophysical Journal (2011), doi:10.1016/j.bpj.2011.08.046
\[
\frac{u_{12}}{p_1 + k_{12}} = \frac{p_2}{p_2 + k_{21}}, \tag{2}
\]

Using Eq. 2 and the conservation of the total protein, we obtain the response functions \(p_i(u_{12}, u_{13}), i = 1, 2, 3\) numerically, as shown in Fig. 2 for different values of the Michaelis constants. (For simplicity, we assume that the order of magnitude of the Michaelis constants are the same.) When the total protein concentration is large relative to the Michaelis constants (saturated regime), there are sharp transitions between distinct states in narrow ranges of enzyme activities (right column in Fig. 2). Outside these transition regions, the protein concentrations are almost constant. This generalizes the switch-like response of the single GK cycle to a system with two inputs, which we can characterize by identifying the phase boundaries where the transitions take place. The behavior is qualitatively similar even in the weakly saturated regime (middle column in Fig. 2), except that the sharp switching is replaced by broader transition regions. The response of this system can be understood by considering the two complementary limiting cases that are both solvable analytically.

When the total protein concentration is much smaller than the Michaelis constants \((k_{ij} \gg 1)\), all reactions are far from saturation and the fluxes can be approximated by linear rates. The steady-state solution can be obtained explicitly as

\[
\begin{align*}
\frac{u_{13}}{p_3} & = \frac{k_{21}}{k_{13}} u_{13} + \left(\frac{k_{31}}{k_{13}} u_{12} + \frac{k_{31}}{k_{13}} u_{13}\right), \\
\frac{u_{12}}{p_2} & = \left(\frac{k_{31}}{k_{13}} u_{12} + \frac{k_{31}}{k_{13}} u_{13}\right).
\end{align*}
\]

These are hyperbolic functions of the rescaled activity ratios. Varying the input signals leads to gradual

FIGURE 2  Response surfaces as a function of the input signals. Numerically calculated normalized protein concentrations \(p_0, p_1, p_2\) (responses) as a function of the enzyme activity ratios \(u_{12}\) and \(u_{13}\) (input signals) are shown for two competing protein modification cycles (Fig. 1b) with the parameter values: \(k_{12} = 2, k_{31} = 1, k_{13} = 1, k_{31} = 2\) (left column); \(k_{12} = 0.2, k_{31} = 0.1, k_{13} = 0.1, k_{31} = 0.2\) (middle column); and \(k_{12} = 0.02, k_{31} = 0.01, k_{13} = 0.01, k_{31} = 0.02\) (right column).
changes in the protein form distribution according to $p_1 : p_2 : p_3 = 1 : (k_{21}/k_{12})u_{12} : (k_{31}/k_{13})u_{13}$ (see left column in Fig. 2). However, fluctuations of the input signals are transferred to the protein concentrations, and the system cannot produce a robust switch-like activation of the downstream pathways.

In the opposite saturated limit ($k_{ij} \ll 1$), the concentration of at least one protein form must be much larger than the Michaelis constants. We will call this the dominant form. To determine the response of the system, we use the following strategy: 1) First we consider separately the cases of each node being dominant. 2) We then solve the simplified flux balance equations for the concentration of the protein forms, taking into account the saturation of the fluxes corresponding to the dominant nodes. 3) Because the solution is only valid when the protein concentrations are positive, we determine which conditions the enzyme activities need to satisfy for each case separately. Thus, instead of calculating the steady states corresponding to a given input directly, we first make assumptions about the response and then determine which input conditions are consistent with each of the possible responses.

When the dominant form is $P_1$, the reactions controlled by $E_{12}$ and $E_{13}$ are saturated and the balance between the fluxes leads to the solutions

$$p_2 = \frac{k_{21}u_{12}}{1 - u_{12}},$$

$$p_3 = \frac{k_{31}u_{13}}{1 - u_{13}},$$

and $p_1$ is obtained from the conservation law: $p_1 = 1 - p_2 - p_3$. Having positive values for $p_2$ and $p_3$ requires that $u_{12} < 1$ and $u_{13} < 1$, i.e., the reactions that convert $P_2$ and $P_3$ to $P_1$ should dominate against the opposite modifications in both cycles. When the dominant form is $P_2$, the flux converting it to $P_1$ is saturated, and we obtain

$$p_1 = \frac{k_{12}u_{12}}{u_{12} - 1},$$

$$p_3 = \frac{k_{31}k_{12}u_{13}}{k_{31}(u_{12} - 1) + k_{12}(1 - u_{13})},$$

$$p_2 = 1 - p_1 - p_3.$$

The conditions for a positive solution now are $u_{12} > 1$ and $(u_{13} - 1)/k_{13} < (u_{12} - 1)/k_{12}$. Due to the symmetry of the system, the conditions when $P_3$ is dominant are obtained by permutation of indices 2 and 3. These conditions partition the input parameter space $(u_{12}, u_{13})$ into three regions with different dominant forms (Fig. 3 a) in which the steady states are essentially independent of the input signals. The boundaries in this phase diagram coincide with the switching regions of the numerical solution in Fig. 2, providing a compact analytical description of the robust response with three alternative states.

**General protein modification network**

The above method can be generalized to any network with tree structure to obtain the steady states analytically in both limits. In the strongly saturated case, $Nk_{ij} \ll 1$, the concentration of at least one form is much larger than the Michaelis constants. This dominant form $P_1$ produces saturated fluxes balanced by the corresponding reverse fluxes, leading to linear equations for the concentrations of its first neighbors with solution:

$$p_j = \frac{k_{j}u_{ij}}{1 - u_{ij}}.$$  

Using Eq. 6, we can determine the outgoing fluxes from the first neighbors, producing a new set of balance equations that can be solved for the second neighbors on the network. The same procedure can be carried out expressing the concentrations of all nodes recursively as
Integrating Signals into Cell Decisions

until the whole network is covered. The concentration of the starting node is obtained from the conservation of the total protein. Similarly, for the cases when other forms are dominant, the steady states can be obtained by repeating the same calculation, starting from each node separately. This generates analytical expressions for the steady-state concentrations as a function of the input signals \( \{ u_j \} \) and the normalized Michaelis constants \( \{ k_{ij} \} \). Because only the solutions with positive concentrations are acceptable, the expressions for the concentrations of the depleted nodes can be used to determine which conditions the enzyme activities need to satisfy for each dominant node. Applying the positivity condition to these concentrations, we produce a set of inequalities that determine the regions in the input space consistent with each steady-state solution, representing a partition of the space spanned by the enzyme activity ratios. Thus, we can determine the qualitative structure of the phase diagram from the topology of the reaction network without specifying the values of the reaction parameters (see Supporting Material for a Maple code that implements this procedure, determining the phase diagram from the adjacency matrix of the modification network). Note that this method is different from the standard approach of determining the steady states directly as a function of parameters, which is only applicable to very simple reaction schemes.

**Star network**

To illustrate the above method, we consider a protein modification network with a star structure (Fig. 1 c). In this case, \( N - 1 \) enzymes \( E_{ij}, j = 1, \ldots, N, \) compete for mutually exclusive reversible modification of the same protein form \( P_1 \). The response of this system can be described by considering two separate regimes: one linear and one saturated.

**Linear regime—hyperbolic cross talk**

When the total protein concentration is much smaller than the saturation constants \( \langle k_{ij} \rangle \gg 1 \), the Michaelis-Menten fluxes can be approximated by linear reaction rates. In steady states each modification flux is balanced by their opposite fluxes, from which we obtain the protein concentrations:

\[
p_j = \frac{p_1 u_{ij} k_{ij}}{k_j + p_1 (1 - u_{ij})},
\]

which can be substituted back into the expression for the steady solution for \( p_j \) to give

\[
p_j = \frac{u_{ij} k_{ij}}{k_j (1 - u_{ij}) + k_i (u_{ij} - 1)},
\]

Thus the protein is distributed among the possible forms in proportion of the rescaled enzyme activities:

\[
p_1 : p_2 : \ldots : p_N = 1 : u_{12} k_{21} / k_{12} : \ldots : u_{1N} k_{N1} / k_{1N}.
\]

**Saturated regime—digital cross talk**

In the strongly saturated regime, when \( k_{ij}, k_{ji} \ll 1 \), at least one protein form must have a concentration much larger than the saturation constants, producing a saturated reaction flux that is independent of the substrate concentration. We can consider two different cases:

When most of the protein is in the form \( P_1, p_1 \gg k_{1j} \), from the balance between the forward and reverse reaction fluxes we have \( u_{ij} = p_j / (p_j + k_{1j}), j = 2, \ldots, N \), which can be solved as:

\[
p_j = k_{ij} u_{ij} / (1 - u_{ij}).
\]

These are valid solutions only if \( u_{ij} < 1 \) for all reactions \( j = 2, \ldots, N \). In this case, the reverse modification reactions \( E_{ji} \) dominate over the direct modifications \( E_{ij} \) and hence all forms \( P_j, j = 2, \ldots, N \) are switched off.

When the dominant protein form is \( P_j, j \leq 2 \), we have \( p_j \gg k_{ij} \), and by equating the saturated reverse flux from the node \( P_j \) with the corresponding forward flux, we obtain:

\[
p_1 = k_{ij} / (u_{ij} - 1),\]

which can be solved to obtain the concentrations of all other protein forms:

\[
p_i = \frac{u_{ij} k_{ij} k_{ii}}{k_j (1 - u_{ij}) + k_i (u_{ij} - 1)}.
\]

These solutions are valid if \( u_{ii} - 1 / k_{ij} < u_{ij} - 1 / k_{ij} \) for all \( i = 2, \ldots, N, \) with \( i \neq j \).

The existence conditions for the cases above represent a partition of the space of input signals \( \{ u_{i2}, u_{i3}, \ldots, u_{iN-1} \} \in R^{N-1} \). Thus the input-output response of the module can be summarized as follows: When \( u_{ij} < 1 \) for all \( j = 2, \ldots, N \), all active states are switched off, i.e., \( p_i = 1 \) and \( p_j \ll 1, j = 2, \ldots, N \). When \( u_{ij} > 1 \) for one or more protein forms, these reactions compete for the same substrate, \( P_1 \). This results in the protein being concentrated in a single form, i.e., the one for which \( (u_{ij} - 1) / k_{ij} \) has the largest value. Thus, when the enzyme activities are changed, switching between different protein forms \( P_i \) and \( P_j \) takes place along the hyperplanes defined by \( (u_{ii} - 1) / k_{ii} = (u_{ij} - 1) / k_{ij} \).

**General structure of the switching diagram**

Certain general properties of the phase diagram can be deduced independently of the network topology. It is easy...
to see that varying only one activity ratio while keeping the others constant produces a single switching between the dominant protein forms of the two subnetworks obtained by removing the edge representing the controlled reaction. The dominant nodes and flux distributions of both subnetworks can be obtained separately, and then matching the forward and reverse fluxes of the reaction connecting them determines a unique switching threshold. This typically depends on the other activity ratios within the subnetworks. Therefore, changing the activity of one enzyme can modify the switching thresholds of other enzymes even without producing any visible effect on the protein form distribution. The dominant nodes within subnetworks are determined by their own activity ratios. Thus, varying a selected activity ratio can trigger switching between any pair of nodes that are on the opposite sides of this reaction.

In Fig. 3 b the dominant protein forms for the network shown in Fig. 1 a are depicted as a function of two selected activity ratios, \( u_{3,5} \) and \( u_{3,8} \), for a set of constant values of the other parameters (see Supporting Material). The phase diagram obtained from the proposed method determines the possible dominant forms and partitions the \( (u_{3,5}, u_{3,8}) \) plane into three regions corresponding to the dominant nodes of the subnetworks obtained by removing the reactions represented by \( u_{3,5} \) and \( u_{3,8} \). For this particular case, the subnetworks composed of nodes \( \{P_1, P_2, P_5, P_3\} \), \( \{P_5, P_6, P_7, P_8\} \), and \( \{P_9, P_{10}, P_{11}\} \) have the dominant nodes \( P_2, P_6 \), and \( P_{11} \), respectively. Fig. 3 b shows that, as described above, changing one activity ratio induces a single switch between two of these dominant forms, and the threshold may depend on the other activity ratio. Of interest, the reduced phase diagram obtained by varying two activity ratios is qualitatively the same for the system with only two cycles. This is valid for any tree network because the connections between three subnetworks obtained by removing two edges are always topologically equivalent to the only possible type of tree network with three nodes. However, the location of the phase boundaries depends on the activity ratios and normalized Michaelis constants within the subnetworks.

**DISCUSSION**

In summary, we have presented a model that generalizes the GK protein modification cycle, and a method for analyzing sequential protein modification systems controlled by multiple enzymes. The tree structure of the modification network implies a pairwise balance between the modification fluxes in the steady state, in contrast to general reaction networks in which reaction fluxes are balanced only at the level of network nodes (29). Far from saturation, there is a gradual hyperbolic response and the protein is distributed in proportion to the relative strengths of the input signals. In the more interesting saturated limit, the equations for the steady state can be solved recursively, and the positivity conditions yield a phase diagram composed of regions of input parameters belonging to distinct steady states. This response behavior remains qualitatively correct for weakly saturated systems. Applying the presented theoretical framework to proteins with known modification networks generates experimentally testable predictions regarding their switching behavior, providing information about the cross talk between multiple pathways, and can be used to control or modify important cellular decisions. For example, in the case of the competitive glycosylation and phosphorylation of c-Myc, knowing the qualitative structure of the phase diagram, even without the exact parameters, could be helpful for designing perturbations that might compensate for or restore the normal activity of mutated cells.

The condition for a sharp switching response is that the total amount of the substrate protein should be larger than the enzyme concentrations (\( P_T \ll E_0 \)). When this is not satisfied, the switch-like response is replaced by a gradual redistribution of the protein forms as a function of the enzyme activities. Because in signaling networks the same protein can act as both enzyme and substrate in different modification reactions, the condition of sharp switching clearly cannot be valid for all proteins; however, it is likely to be satisfied for certain components that gain a key regulatory role in the cross talk between multiple pathways.

Our method for constructing the phase diagram is limited to modification networks without closed loops. This may not be satisfied for many real systems, and therefore it would be important to extend the proposed approach to include such systems. Although such an extension does not appear to be straightforward, analyzing some simple cases numerically may provide a starting point for future development.

We neglected the synthesis and degradation of the protein. When the degradation rate is the same for each protein form, the turnover of the protein does not affect the described behavior of the model. However, differential degradation of the protein forms may lead to interesting side-effects because the switching from one form to another induced by an external signal can be accompanied by a change in the total amount of protein that may move the system in or out of the saturated regime.

Here we focused on the steady-state response of protein modification systems. However, in the presence of time-dependent enzyme activities, the transient behavior may also be important. This can be particularly relevant in the strongly saturated regime when the saturation of the enzyme limits the reaction flux, consequently increasing the duration of switching when the enzyme activities are modified. Preliminary simulations for the simple case of only two competing modification cycles indicate that the transition of the protein from one dominant node to another (corresponding to the initial and final steady states) may take place, depending on the parameters, either with or without transient accumulation of the protein in intermediate forms. Thus, in the case of time-dependent (e.g., oscillatory)
Integrating Signals into Cell Decisions

...enzyme activities, the protein may switch between different forms depending on the frequency of input signals.

The systems considered here have unique steady states for a given set of input signals and therefore do not have a memory that requires the coexistence of multiple stable steady states (30). Such multistability arises in protein modification systems when multiple reactions are catalyzed by the same enzyme (18), or when one protein form autocatalytically enhances its own production (9). Clearly, the information storage and signal processing functions can be combined within the same protein modification system, such as when some of the enzymes catalyze multiple reactions. This expands the computational capabilities of proteins that are likely to be employed in cellular signaling pathways (21). By analogy to networks of neurons, in principle, the complex switching controlled by multiple signals in combination with plasticity (18) raises the possibility of cellular learning using signal transduction networks composed of multiply modified proteins (31), complementing the more-rigid and slower regulation based on genetic information. The mechanism of information processing by switching between protein forms (19,32) also provides a possible template for designing synthetic computational systems based on chemically interacting complex molecules.

**SUPPORTING MATERIAL**


L.C. was supported by a postgraduate scholarship from the Irish Research Council for Science, Engineering and Technology. J.M.-G. was supported by the Science Foundation Ireland under grant No. 06/CE/B1129 and the Spanish Ministry of Science and Innovation under the Juan de la Cierva program and grant No. FIS2009-12964-C05-01.

**REFERENCES**


