Mixed mechanisms of multi-site phosphorylation

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Multi-site phosphorylation is ubiquitous in cell biology and has been widely studied experimentally and theoretically. The underlying chemical modification mechanisms are typically assumed to be distributive or processive. In this paper, we study the behaviour of mixed mechanisms that can arise either because phosphorylation and dephosphorylation involve different mechanisms or because phosphorylation and/or dephosphorylation can occur through a combination of mechanisms. We examine a hierarchy of models to assess chemical information processing through different mixed mechanisms, using simulations, bifurcation analysis and analytical work. We demonstrate how mixed mechanisms can show important and unintuitive differences from pure distributive and processive mechanisms, in some cases resulting in monostable behaviour with simple dose–response behaviour, while in other cases generating new behaviour-like oscillations. Our results also suggest patterns of information processing that are relevant as the number of modification sites increases. Overall, our work creates a framework to examine information processing arising from complexities of multi-site modification mechanisms and their impact on signal transduction.

1. Introduction

Cells process information, respond to their environments and make decisions through the orchestrated functioning of complex protein and genetic networks [1–3]. A key component of this chemical information processing is cascades and networks of post-translational modification [4–10]. As a result, post-translational modification networks and cascades have been the focus of numerous investigations, and the study of information processing through such networks is one of the central themes in systems biology.

Post-translational modification has been studied at the enzymatic level as well, with a particular focus on phosphorylation [11]. As it is known that phosphorylation (and dephosphorylation) of substrates by enzymes can occur on multiple locations, a natural focal point of interest has been multi-site phosphorylation. Multi-site phosphorylation systems can be regarded as an extension of the basic covalent modification cycle, wherein the same pair of enzymes (kinases and phosphatases) effects multiple modifications. This naturally expands the range of information processing of single-site modifications, by providing a wider array of downstream targets, by providing new capabilities for controlling substrate function and behaviour and also by providing essentially new modes of information processing. Multi-site phosphorylation plays important regulatory roles in many cell signalling contexts in eukaryotic cells of all types [12–15]. It plays a critical role in cell cycle regulation and inflammation pathways, and is implicated in multiple disorders, including Alzheimer disease [16–19]. In fact, multi-site modifications are routinely encountered in signalling, attesting to their broad relevance.

Understanding chemical information processing through multi-site phosphorylation requires a mechanistic description of how enzymatic modifications are effected. In fact, there are different mechanisms by which these multi-site modifications are realized. Thus, multi-site phosphorylation systems may be classified as distributive, wherein each modification needs a new enzyme binding event, and processive, where a single binding can lead to a sequence
of modifications [20]. Each of these mechanisms has been studied in considerable detail. For a given mechanism, there can be different variants depending on what kinds and sequences of modifications are allowed. This leads to variants such as ordered (also referred to as sequential) [21,22] and random [13,23,24] mechanisms. An ordered mechanism allows only a specific order in the modifications, whereas a random mechanism allows any sequence of modifications. Thus, a number of distinct variants of ordering and mechanism are possible.

As enzymatic mechanisms can affect information processing and hence pathway/network behaviour, it is of interest to examine intrinsic information-processing capabilities associated with different mechanisms. A number of studies of distributive and processive mechanisms have revealed many insights. The possibility of exhibiting threshold behaviour under conditions of similar catalytic characteristics of different stages has been studied [25]. It was shown that two-site modifications involving distributive mechanisms are capable of bistability, and that increasing the number of sites can result in unlimited multi-stability [26,27]. We showed how distributive mechanisms can naturally demonstrate biphasic dose–response, based on fairly generic kinetic descriptions of the mechanism, and that this can be combined with ultrasensitive behaviour or multi-stability, providing new modes of information processing [28]. Likewise, studies of processive models focus on dose–response characteristics [21]. Sequestration, an important theme in signalling [29], plays a crucial role here.

While existing studies have resulted in an extensive dissection of distributive and processive mechanisms, there are other so-called mixed mechanisms which exist [30]. Mixed mechanisms can arise for multiple reasons. One possibility is that phosphorylation and dephosphorylation occur through different mechanisms. Another possibility is that phosphorylation and dephosphorylation occur through different mechanisms. Furthermore, it is possible to have phosphorylation and/or dephosphorylation on multiple sites could act through a combination of distributive and processive mechanisms, and this has been implicated in particular contexts [30,31]. It is also possible in certain contexts that both distributive and processive routes to modification are available [32]. In such a case, purely distributive and purely processive mechanisms may be seen as extreme limiting cases, where one mechanism dominates the other. While different mixed mechanisms have been postulated or employed in models in particular contexts, there has been no systematic study of the interplay between distributive and processive mechanisms.

In this paper, we develop a framework to examine the behaviour of mixed mechanisms. Our goal is to study, in detail, the effects of combining processive and distributive mechanisms from the viewpoint of chemical information processing. We approach this using a hierarchy of models. We first consider mechanisms where phosphorylation is distributive and dephosphorylation is processive, and the other way round. We then examine the effects of phosphorylation occurring through a combination of processive and distributive steps, while dephosphorylation is either processive or distributive. Finally, we also discuss the relationship between the results of such models and a more comprehensive model that allows for both processive and distributive mechanisms at each step. In this manner, we create a bridge between purely processive and purely distributive models, isolating clear patterns associated with their interplay, as discussed below.

Our particular focus in this paper is on key qualitative signatures of information processing in mixed systems, such as bistability, biphasic dose–responses and oscillatory behaviour.

Such a framework is useful for multiple reasons. Firstly, mechanisms that are mixed exist in nature [30–32]. Secondly, there are multiple factors that can effectively result in a switching between distributive and processive mechanisms, including crowding agents, presence of scaffolds and applied stress [33–35]. Depending on conditions, this might result in an effectively mixed mechanism. Thirdly, in different cases, both distributive and processive routes to modification may be available, and the mechanism may be regarded as being fully mixed [32]. Finally, tools from protein engineering, nanoengineering and synthetic biology/chemistry can allow for manipulating chemical mechanisms in novel ways, and our study provides a platform for understanding the kinds of kinetics and chemical information-processing behaviour that can emerge from enzyme/substrate engineering.

Models and methods are presented in §2, followed by the results and a summary of analytical results. The paper concludes with a discussion, with analytical work in appendix A.

2. Models

We aim to understand the interplay between distributive and processive mechanisms in multi-site phosphorylation and approach this through a broad hierarchy of three classes of models. All models are described through widely used descriptions of individual modifications involving enzyme binding to substrate to form the complex which irreversibly converts the substrate to yield the product: an abundance of ATP (adenosine triphosphate) is implicitly assumed. In the case of distributive mechanisms, such descriptions of individual modifications are used for all modifications with enzyme binding/unbinding to substrate being involved in each modification. In the case of processive mechanisms, we employ a model wherein the enzyme binds to a substrate, forms a complex, and this leads to the sequence modifications of the substrate while the enzyme is still bound to it (figure 1a). It is possible to have models of processive modification where the intermediate product is released along the way, but we do not study this in detail. This extra feature does not affect any of the qualitative results which we present, and we discuss this subsequently in the context of a comprehensive mixed model (also see appendix A). In all the models, an explicit activation of kinase and/or phosphatase is not employed, for simplicity. The essential results are not dependent on this aspect. All the models are formulated in terms of ordinary differential equations, and can be the basis for subsequent investigations of stochastic effects that build on these results. The conservation of total substrate, kinase and phosphatase implies that these quantities are all parameters. For simplicity, we study ordered mechanisms, which impose a specific order on phosphorylation and dephosphorylation (also called sequential mechanisms: see diagram in electronic supplementary material).

At the outset, we comment on our approach in analysing mixed systems. Mixed systems can, in principle, arise through a combination of sequences of modifications (phosphorylation/dephosphorylation) that are essentially purely distributive/processive, or involve combinations of both components in each modification. A model that incorporates
both distributive and processive routes in each step covers all cases. However, studying such a model in detail parametrically is challenging, becoming rapidly more so as the number of sites increases. While exhaustive parametric analysis is useful, it is not the necessarily the easiest way to isolate key patterns associated with information processing. Therefore, we employ a hierarchy of models where we study a sequence of special cases (e.g. distributive phosphorylation/processive dephosphorylation), alongside the comprehensive model. These special cases can often be analytically studied, revealing features that generalize to different numbers of modification sites. This reveals certain features associated with particular parametric choices/ regimes of the comprehensive model.

Figure 1. Schematic of ordered phosphorylation models. (a) Two types of pure ordered multi-site phosphorylation mechanisms: (i) a distributive and (ii) a processive mechanism. (b) Mixed double- and triple-site models where one direction is distributive and another is processive. (c) Mixed triple-site models where there is a combination of distributive and processive steps in phosphorylation. (d) A mixed composite phosphorylation model where there is a combination of both processive and distributive mechanisms in all steps. (Online version in colour.)
With the above discussion as a backdrop, we now turn to a brief description of the various models. Our models focus on mixed two- and three-site modification systems. The detailed equations for some representative models are included in appendix A. In the description of models, we present a sequence of mixed models where a given step is either purely distributive or purely processive (the first and second class of models below), followed by comprehensive mixed models.

The first class of models (21 and 22) involves double-site modification where either the kinase acts distributively and the phosphatase processively, or the other way around (figure 1b). The essential results of the first case can be mapped onto the second case. The models are obtained as mentioned above.

The second class of models involves situations where the kinase (or phosphatase) can act through a combination of (purely) distributive and processive mechanisms. For this to occur, the minimum number of modification sites is three. We thus consider multiple variants of models of this class (figure 1c). In principle, if one allows for such mixed mechanisms involving one enzyme, one can study (apart from three-site analogues of the distributive/processive models of the first class above, we refer to as models 31 and 32) four other models (models 33, 34, 35, 36). We consider a combination of distributive and processive modification, the latter involving two sites, in phosphorylation. Depending on the location of the processive mechanism in this multi-site modification sequence, we have two possibilities for the mixed phosphorylation. For each of these cases, we consider the dephosphorylation to be either processive or distributive. This leads to a set of four models which we primarily focus on.

The third class of models contains a combination of processive and distributive mechanisms at each step. This model, depicted graphically in figure 1d, combines elements of each of the mechanisms at each step. While for the most part we do not study these mixed models in detail thoroughly (though we do discuss some analysis of such models of two-site mechanisms), we examine these models to see whether their limiting behaviour (when either processive/distributive steps are made weak) approaches that of the previous classes of models. Since the models of the previous two classes are naturally contained within this more comprehensive model, when we study the earlier classes of models, we use them as a bridge to this more comprehensive model. All mixed models are depicted in appendix A.

Taken together, this study allows for a detailed examination of mixed mechanisms. Each of these models has parameters which naturally affects its information processing capabilities. We choose representative parameters for each model (presented in the electronic supplementary material), which encapsulates different behaviour of the mechanisms, and focus on the interaction between processive and distributive steps. Our focus is largely on qualitative signatures. We analyse mixed models in different parametric regimes, where we vary primarily the catalytic constants: these regimes are chosen based on the distinct behaviour seen in pure mechanisms (e.g. bistability), to serve as a contrast. In cases where certain behaviour (e.g. bistability) is ruled out in the mixed model, we demonstrate it analytically. In other cases where we find new behaviour such as oscillations possible we explicitly demonstrate it through simulations and bifurcation analysis (and have checked that such behaviour is possible in plausibly broad parameter ranges). We then discuss the combination of parameters that can give rise to this. While a broad range of parametric analysis has also been performed underpinning this study, we do not discuss it in detail except to illuminate specific points. We perform some parametric analysis of a comprehensive two-site mixed model, where we study the ‘crossover’ between distributive and processive models. Thus, through numerical simulation, bifurcation analysis and analytical work, we consolidate our understanding of the interaction of processive and distributive mechanisms. Our simulations were performed using both ode15s (a stiff ordinary differential equation solver) in MATLAB [36] and COPASI [37] for comparison. The equations are directly programmed in the former, while generated by the software automatically in the latter. This is complemented by bifurcation analysis in MATCONT [38] (which uses the same code for the model as the one used in MATLAB).

At various points, we contrast the behaviour of mixed (ordered) mechanisms to ‘related’ (ordered) distributive and processive mechanisms, and in some cases study the effect of the order of distributive and processive steps, when they occur together in phosphorylation. In situations where we compare a distributive modification step (which has extra binding and unbinding steps) with a processive modification step, we chose the catalytic constants to be the same. The binding and unbinding rates are kept fixed for any sequence of forward or reverse modifications (unless otherwise mentioned). This ensures that sensible comparisons can be made between different sequences of forward and/or reverse modifications, involving distributive and processive steps. Of course, it is possible that binding/unbinding rates can be different for different phosphoforms, but in the absence of specific experimental information regarding this, we adopt the above approach to start with. The effects of these other factors can be studied subsequently. In what follows, we examine only ordered mechanisms and hence omit the qualifier ‘ordered’.

3. Results

At the outset, we discuss the focal point of our results. Our primary goal is identifying and elucidating the presence of key qualitative signatures and capabilities (and constraints) in information processing in mixed systems, such as bistability, oscillations, biphasic dose–responses (as the kinase concentration is varied) or certain combinations thereof. We present a sequence of results for the models above showing ‘representative’ and possible qualitative behaviour in this regard. If certain behaviour is not seen, we demonstrate this analytically (detailed in a subsequent section). Examining these models in sequence also brings to light differences between partial phosphoform behaviour, effects of the order of mechanisms and contrasts with pure models with ‘corresponding’ parameters. We do not focus on a detailed parametric analysis of these models here.

We discuss two-site models with different phosphorylation and dephosphorylation mechanisms followed by three-site models with mixed mechanisms of phosphorylation. We discuss the relationship between these models and mixed models, where each modification can have processive and distributive pathways. In what follows, we employ the following notation (explained further in appendix A):
Figure 2. Steady-state dose–response curves in ordered double phosphorylation models for purely distributive (dis), purely processive (pro) and mixed mechanisms (models 21 and 22). (a–c) show the steady-state input–output response curve of the concentration of maximally phosphorylated substrate, $A_{pp}$ as a function of the total concentration of kinase, $K_{tot}$, while (d–f) shows the corresponding curve for the partially phosphorylated substrate, $A_p$. (Completely phosphorylated substrate: (a) pure mechanism, (b) model 21 and (c) model 22. Singly phosphorylated substrate: (d) pure mechanism, (e) model 21 and (f) model 22.) (Online version in colour.)

$A$ refers to the substrate, and the subscript ‘p’ denotes a phosphorylated substrate. $A_p$ and $A_{pp}$ are thus singly and doubly modified substrates, respectively. $K$ refers to the free kinase, $P$ to the free phosphatase and $K_{tot}$ denotes total kinase concentration. Relevant kinase and phosphatase complexes are denoted by concatenations of relevant substrate symbols with $K$ or $P$: e.g. $A_pK$ denotes the kinase complex where kinase is bound to $A_p$.

3.1. Two-site modification

We start by discussing the two double-site modification models: model 21 refers to the case where the phosphorylation is (purely) distributive and dephosphorylation is (purely) processive, and model 22 describes the opposite scenario. Figure 2 shows the variation of the partial and fully modified substrate as a function of total kinase concentration. We first examine this mixed mechanism in a parameter regime, where the ‘analogous’ purely distributive mechanism is monostable. We find that while the fully modified substrate exhibits a sigmoidal response similar to both distributive and processive models (due to zero-order ultra-sensitivity), we see some differences. In model 21, we find that the partial phosphoform exhibits a decreasing profile asymptoting to zero for large values of $K_{tot}$. This is what is generically expected in a distributive mechanism, with all available substrate (including partial phosphoform) getting converted to the fully phosphorylated form. On the other hand as $K_{tot}$ approaches zero the partial phosphoform $A_p$ does not approach zero (figure 2). In fact, examining the steady state of $A_p + A_pK$ reveals that $[A_p][K]$ is proportional to $[A_{pp}][K]$ (also see appendix A.2). In other words $A$ and $A_p$ are proportional.

We note that introducing a weak distributive component in the dephosphorylation steps (for instance, by considering a fully mixed model as mentioned above) actually shows that, for small values of $K_{tot}$, the concentration of $A_p$ increases very rapidly, reaching a peak for a small value of $K_{tot}$ and then following a decreasing trend as suggested by the above analysis. This can be summarized by saying that the behaviour of $A_p$ as a function of total kinase concentration is essentially monotonically decreasing, except for small values of $K_{tot}$, where small background levels of other mechanisms can result in a sharp increase. Thus, there is a boundary layer-like behaviour (in parametric space) at $K_{tot} = 0$ in the fully mixed model. This can be seen from an analysis of the fully mixed model, which we briefly discuss. In this model, the steady state of $A_p + A_pK$ results in $c_1[A][K] - c_2[A_p][K] + c_3[A_{pp}][P] - c_4[A_p][P] + c_5[A_{pp}][P] = 0$, where all the $c$’s are constants. Neglecting the distributive component to dephosphorylation, we get the result above (obtained by setting $c_3 = c_4 = c_5 = 0$). For simplicity, if we assume a high catalytic conversion of $A_pP$, the concentration of $A_pP$ is negligible. Then $[A_{pp}] = (c_1[A][K] + c_5[A_{pp}][P])/(c_2[K] + c_4[P])$. This indicates that, when $K_{tot}$ approaches 0, $[A_{pp}]$ approaches 0 and $[A_p]$ does indeed approach zero, owing to the presence of the extra term $c_4[P]$ in the denominator. Even if $c_4$ is small in magnitude, the presence of this term results in $[A_p]$ approaching zero as $K_{tot}$ becomes zero. Physically, this can be understood as follows. In the absence of a distributive component in dephosphorylation, a small amount of kinase can move the substrate towards modifications and the cycle is completed through dephosphorylation, though not directly
of $A_p$. With weak distributive dephosphorylation, this is more or less the case except when the kinase levels are low enough for direct dephosphorylation (of $A_p$) to compete.

In the case where the phosphorylation is processive, an analysis of the dephosphorylation (the distributive leg of the pathway) in appendix A.2 shows that the concentration of $A_p$ is proportional to that of $A_{pp}$ (by considering the steady state of $A_p + A_pP$). This explains the sigmoidal increasing variation of $A_p$, which is different from a pure distributive mechanism (figure 2). It also explains the reduced asymptotic amplitude of $A_{pp}$ as $K_{tot}$ becomes high: a fixed fraction of the substrate is in the partially phosphorylated form.

Again we note that if one examines a comprehensive mixed mechanism, introducing a weak distributive step in phosphorylation, then the profile of $A_p$ saturates as above for a broad range of $K_{tot}$ before decreasing to 0 as $K_{tot}$ becomes very high (results not shown). We can summarize this by saying that the comprehensive mixed model follows essentially the same behaviour as model 22, with the possibility of a difference at very high values of $K_{tot}$ (weakening the distributive component above increases the value of $K_{tot}$ at which this happens). This can be understood from analytical work.

We now examine these two mixed mechanisms in parameter regimes, where the purely distributive analogues are bistable. Note that all potential binding/unbinding constants are fixed at the same nominal value, and, by analogous distributive mechanism, we mean the distributive mechanism with these binding/unbinding constants and the same sets of catalytic constants. A choice of sufficiently different catalytic constants in phosphorylation can result in bistability in a purely distributive model, as shown in figure 3a (also see [39]). By contrast, the two mixed models exhibit monostable behaviour. In appendix A.2, we demonstrate analytically how bistability is ruled out in such mixed models, when catalytic conversion steps are irreversible. This analysis also extends, in a straightforward way, to such mixed models with more than two site modifications as discussed there, and thus this analysis of a two-site mixed model reveals a common pattern valid for $n$-site modifications.

Figure 3b shows a dose–response curve of a distributive model in a different regime, indicating a biphasic response for the fully modified phosphoform. The origins of such biphasic responses are discussed in [28], where it is shown that distributive mechanisms are intrinsically capable of generating such biphasic responses in the absence of product inhibition, even when the catalytic conversion step is irreversible. We find that the ‘corresponding’ mixed mechanisms do not exhibit such biphasic dose–response curves (for the fully phosphorylated substrate), and in appendix A.2 we show analytically that biphasic responses under the conditions of irreversible catalytic steps can be ruled out.

Figure 4 presents another aspect of mixed mechanisms. In contrast to the ‘corresponding’ pure distributive mechanism, which reaches a steady state, we find that in a mixed model (for specificity model 22) sustained oscillations can occur (figure 4a). A bifurcation diagram shows that, as the parameter $K_{tot}$ is changed, the steady state does indeed lose stability to a Hopf bifurcation. A Hopf bifurcation denotes a transition in dynamical systems resulting in the ‘birth’ of oscillations [40]. We have not observed oscillations in a two-site ordered distributive mechanism and are not aware.
of such behaviour reported in the literature. However, a random two-site distributive mechanism has been shown to exhibit oscillations, for specific combinations of parameters [41]. Here we find that both ordered as well as random two-site mixed models are able to exhibit oscillations and that the random mechanism is capable of generating oscillations of greater amplitude (results not shown). In a sense, the mixed ordered two-site modification mechanism could be the simplest enzymatic modification scheme that can intrinsically exhibit oscillation, in isolation, without additional explicit feedback regulation. A completely mixed model is also able to exhibit oscillatory behaviour. Naturally, model 21 also exhibits oscillatory behaviour.

3.1.1. Oscillations
It is worth examining the source of these oscillations. We find these oscillations in model 22 in parametric regimes where the binding constant of $A_{pp}$ to the phosphatase is quite high, and when the catalytic constant for the dephosphorylation of $A_p$ is reasonably high, with other parameters all of similar magnitude (order 1). In fact, we also obtain oscillations when the dephosphorylation step of $A_p$ acts via mass action kinetics (i.e. the limit of a very high catalytic constant). We find that, in such a scenario, a significant amount of the phosphatase is sequestered in the complex $A_{pp}P$. However, such a scenario appears not to result in oscillations in two-site ordered distributive models. If we examine the effect of increasing $K_{tot}$, we find that this produces more $A_{pp}$ which largely gets converted to the phosphatase complex $A_{pp}P$ (high binding constant of $A_{pp}$ to $P$), and eventually to $A_p$. However, the paucity of free phosphatase means that this $A_p$ is hardly converted back to $A$. Thus, increasing the total kinase concentration has an effect of depleting/moving away substrate, which contributes a negative feedback effect. In an ordered distributive model, on the other hand, $A_p$ can be directly converted back to $A_{pp}$ by the kinase, something that is not possible/negligible here. This suggests how a combination of enzyme sequestration and modification characteristics combines to readily generate oscillations here.

We discuss some further analysis of a mixed composite two-site modification model in appendix A.4, showing how this bridges the distributive and processive scenarios.

3.2. Three-site modification
We now turn to three-site modification schemes. In this case, it is possible for either phosphorylation and/or dephosphorylation to combine (purely) processive and distributive mechanisms. We examine a selection of mixed mechanisms as follows. We will examine only those cases where the dephosphorylation is either purely processive or purely distributive, and for simplicity we do not consider cases where both phosphorylation and dephosphorylation occur through a combination of mechanisms. We are then left with six mixed mechanisms that can be described as: model 31, dis—pro; model 32, pro—dis; model 33, dis pro pro—pro; model 34, pro dis—pro; model 35, dis pro pro—dis; model 36, pro dis—dis. In these models, the horizontal line separates the phosphorylation mechanism from the dephosphorylation mechanism. Models 31 and 32 have a uniform mechanism for phosphorylation and this is hence referred to by one label. In all other models, the order of modification in phosphorylation is explicitly listed through the sequence of three labels (see figure 1 for a schematic depiction of kinetic steps). Analysing these models allows us to examine the effects of the interplay and ordering of processive and distributive steps in one direction.

Models 31 and 32 are three-site analogues of the two-site models 21 and 22. While the additional modification site enlarges the parameter space, the qualitative behaviour of these models is essentially the same as the two-site models, owing to a similar structure. It can be shown analytically (appendix A) that, if the catalytic step is irreversible, bistability and biphase dose–response curves are ruled out.

We turn to models 33 and 34, involving mixed distributive and processive steps in phosphorylation differing only in order (figure 5), with processive dephosphorylation. Figure 5a shows that the fully modified phosphoform exhibits a sigmoidal dependence on total kinase concentration. Again, we find that the relevant partial phosphoforms $A_p$ for model 33 exhibits monotonic decreasing variation. This is explained by noting that it is proportional to $A$ (see analysis in appendix A.3). In model 33, by considering the steady state of $A_{pp}$, we find that $[A][K]$ is proportional to $[A_{pp}][K]$ ($A_p$ is only produced from $A_{pp}$ in this case, and the conversion of $A_p$ to $A_{pp}$ is proportional to its concentration) and hence the concentration of $A$ is proportional to that of $A_p$. Again, we note that, as $K_{tot}$ becomes very small, this suggests a non-zero asymptote for $A_p$. However by including some weak distributive
component in the dephosphorylation steps, we see that $A_p$ actually increases quite sharply for small $K_{\text{tot}}$ and thereafter the response is essentially the same as that depicted. The reason for this behaviour is the same as described previously. By similar reasoning, we find that, in model 34, $A_{pp}$ exhibits an essentially monotonic decreasing profile and this is because $A_{pp}$ is proportional to $A$. This is seen by considering the steady state of $A_p K + A_{pp} K$.

As both these models have processive dephosphorylation, they are modified variants of the distributive phosphorylation/processive dephosphorylation models discussed above (models 31 and 32). In those models, bistability and biphasic responses for the fully modified phosphoform are ruled out. The current models involve part of the phosphorylation mechanisms being processive, and this further simplifies the model and reduces possible behaviour which may be obtained. An analysis of these scenarios (appendix A.3) shows that both bistability and biphasic behaviour can indeed be ruled out.

Models 35 and 36 involve distributive dephosphorylation. We first examine the system in the monostable parameter regime (figure 6). Figure 6a shows that the fully modified phosphoform exhibits a sigmoidal dependence of the total kinase concentration, with the clear presence of a threshold. This is true for both models 35 and 36, and is in fact seen in the fully distributive analogue as well. We however see a difference between these models at this stage itself. In model 35 (the processive steps are the last two steps in the phosphorylation sequence), $A_{pp}$ exhibits a sigmoidal curve which mirrors $A_{pp}$ (figure 6b). This can be understood in a similar manner to the cases above, since $A_{pp}$ is proportional to $A_{pp}$. We note that, by varying the catalytic constant of the last phosphorylation, it is possible to get a biphasic dose–response curve for both $A_{pp}$ and $A_{pp}$. In contrast to model 36, $A_{pp}$ (but not $A_{pp}$) exhibits a biphasic response. This shows how the order of processive and distributive steps affects phosphoform characteristics.

We then examine these two models in a bistable parameter range. Since the dephosphorylation is distributive and since there are at least two different binding/unbinding pairs of events in the phosphorylation sequence, this system is readily capable of exhibiting bistability (figure 7a). This study shows how the location of the distributive and processive steps can affect the bistable range. We find in this case that having the first two steps in the phosphorylation as processive (model 36) leads to a much greater range of bistability, compared with model 35. This conclusion could of course depend on the choice of enzyme–substrate binding/unbinding constants. Finally, figure 7b also shows how varying the catalytic constant in the last phosphorylation step can result in a biphasic dose–response curve for the maximally phosphorylated substrate. This is much more pronounced when the last step is distributive.

Figure 8 contrasts three mixed models (with same catalytic constants) with distributive dephosphorylation revealing new facets. When the phosphorylation is purely processive (model 32), oscillations are observed over a very broad range of kinase concentrations (figure 8a). We see that model 35 exhibits both bistability and oscillations arising through a Hopf bifurcation of the ‘upper’ branch of steady states. The parameters combine sufficiently different catalytic constants in phosphorylation (giving bistability) and those conducive to oscillations. Model 36 exhibits bistable behaviour with no oscillations, showing how ordering of processive and distributive steps can result in qualitatively different behaviour.

**Figure 5.** Steady-state dose–response curves in triple-site phosphorylation models with mixed phosphorylation and processive dephosphorylation. Panel (a) contrasts the steady-state dose–response curve of the maximally phosphorylated substrate, $A_{pp}$, in mixed triple-site models (model 33 (i) and model 34 (ii)) and that of pure distributive triple-site phosphorylation. (b) The corresponding information for the partial phosphoform. (Online version in colour.)
Figure 6. Steady-state dose–response curves in pure and mixed triple-site phosphorylation models with distributive dephosphorylation (models 35 and 36) under monostable conditions. The steady-state dose–response curves for the maximally phosphorylated substrate, $A_{ppp}$, in these three models are shown in (a) while those for the doubly phosphorylated substrate in these three models are presented in (b). (a) Completely phosphorylated substrate: (i) purely distributive, (ii) model 35 and (iii) model 36. (b) Doubly phosphorylated substrate: (i) purely distributive, (ii) model 35 and (iii) model 36. (Online version in colour.)

We emphasize that all three-site mixed mechanisms exhibit oscillations and that oscillations are readily obtained in the relevant parameter regimes of comprehensive mixed three-site models as well. However, the combination of oscillations and bistability occurs only in models 35 and 36, and does not occur in models 21 and 22, since these other models are not capable of exhibiting bistability as discussed above. This shows how the number of sites, as well as the number of essentially distributive steps in both phosphorylation and dephosphorylation, can introduce new modes of information processing.

We comment about the comprehensive mixed models and models we have analysed. If we examine a sequence of processive modifications (for instance in dephosphorylation in two-site modification), we find that incorporating a distributive pathway introduces extra elements: (i) a pathway converting $A_{pp}P$ to $A_p$ and (ii) a binding of $A_p$ to $P$ to form the complex $A_{pp}P$ and a corresponding unbinding step. It is worth mentioning that, even when the rate constant involved in the reaction converting $A_{pp}P$ to $A_p$ is not small, the qualitative behaviour of the model is essentially similar. Thus, having a modified processive model allowing for the release of intermediate phosphoforms results in essentially similar conclusions. If, however, the binding/unbinding of $A_p$ to $P$ is not small, then the pathway behaves like a distributive pathway.

4. Analytical studies

In this section, we present a summary of some analytical studies performed on these systems. The analytical approach relies on obtaining steady-state concentrations of species through the solution of three coupled equations, involving free kinase concentration $[K]$, free phosphatase concentration $[P]$ and concentration of maximally modified phosphoform $A_{pp}$. This is done, firstly, by eliminating concentrations of all complexes in terms of products of substrate concentrations and those of $K$ or $P$. Then all substrate concentrations are written in terms of that of $A_{pp}$, $P$ and $K$.

In the two-site models 21 and 22, we find that the nature of equations which results is of the form

$$K = K_{tot} - \alpha A_{pp}[P],$$

$$P = \frac{P_{tot}}{1 + \beta A_{pp}},$$

and

$$A_{pp} = \frac{A_{tot}}{\gamma_1 + \gamma_2 [P] + \gamma_3 [P]/[K]},$$

where $\alpha$, $\beta$, $\gamma_1$, $\gamma_2$, $\gamma_3$ are constants. Analysis of this equation (see appendix A.2) shows that bistability and biphasic responses for $A_{pp}$ are ruled out. An $n$-site analogue of these models results in exactly the same form of equation, resulting in the same conclusions.

We then considered three-site modification systems where phosphorylation occurred through a combination of processive and distributive steps. If the dephosphorylation is processive (models 33 and 34), again, the resulting equation is of the form above, and similar conclusions hold good.

We also discuss an extension of such a result: if we have an $n$-site (ordered) modification system of purely processive and purely distributive steps. If distributive steps in phosphorylation and dephosphorylation are disjoint (i.e. no intermediate phosphoform is released in both phosphorylation and dephosphorylation), then the steady-state equations again simplify to a form similar to the above, and bistability is again ruled
out. This indicates that it is necessary to have common distributive steps in phosphorylation and dephosphorylation to produce bistability.

Noting the relationship between these models and comprehensive mixed models, we may say that this analysis reveals various aspects of qualitative behaviour of the latter in different limiting situations.

5. Conclusion

Multi-site phosphorylation is a basic building block of signalling and cellular information processing. It is well known that the nature of enzymatic modification can significantly affect information processing and behaviour of signalling pathways, as seen from studies of distributive and processive systems. Multiple scenarios can result in mixed mechanisms. The natural question that arises is: how does information processing occur through mixed systems and how does this depend on the number of modification sites? In this paper, we developed a framework to elucidate different aspects of the interplay between distributive and processive mechanisms, resulting in mixed models.

5.1. A hierarchy of models

Since increasing the number of modifications increases the number of parameters involved, we analysed mixed mechanisms by considering a hierarchy of models. We studied the scenario where phosphorylation and dephosphorylation occur by different mechanisms (in two-site modification systems), and then considered mixed scenarios for phosphorylation, with dephosphorylation being either processive or distributive (in three-site modification systems). Finally, we also briefly studied a more comprehensive model involving both processive and distributive elements at every stage. This includes the other models (which serve as a bridge to this) as special cases. We were able to check that the behaviour of this model essentially reduces to those of the earlier models when the rates of individual pathways are made small, and also elucidate the source of any differences. Thus, our study simultaneously involves more specific models (that also have the advantage of being more tractable) alongside comprehensive models. We move back and forth between these models, extracting relevant insights therefrom in a consolidated manner. The analysis of the specific cases allows us to identify patterns of information processing applicable to the n-site case.

Figure 7. Steady-state dose–response curves in triple-site phosphorylation models with distributive dephosphorylation: (purely distributive model, models 35 and 36) exhibiting bistability and biphasic responses. (a) The steady-state dose–response curves of fully and doubly phosphorylated substrates, $A_{ppp}$ and $A_{pp}$, in these models under bistable conditions. (b) The dose–response curve of the fully phosphorylated substrate, $A_{pp}$, in these three models demonstrating biphasic behaviour. (a)(i) Purely distributive, (ii) model 35 and (iii) model 36. (b)(i) Purely distributive, (ii) model 35 and (iii) model 36.) (Online version in colour.)
5.2. Two-site mixed systems

When opposing steps follow different mechanisms, we find that behaviour such as bistability and biphasic behaviour in the maximally phosphorylated substrate (as total kinase concentration is increased) is ruled out, when all catalytic conversions are irreversible. This is established analytically and carries through to similar mechanisms involving multiple modification sites. The behaviour of intermediate phosphoforms is quite different from that in pure mechanisms due to the fact that they are bound to the enzyme in the processive leg of the reaction. We found that mixed mechanisms were intrinsically capable of exhibiting oscillatory behaviour. Distributive two-site modifications have been reported to exhibit oscillations in a region of parameter space, when the mechanism is random [41]. However, oscillations in ordered distributive (or processive) double-site phosphorylation have not been reported. Random mechanisms involve more species with additional scope for sequestration effects, and can in general produce behaviour not seen in ordered mechanisms. A strong kinetic asymmetry (in the cycle) in the random mechanism was identified as a key ingredient for oscillations [41]. In contrast we find that, in a mixed model, even ordered mechanisms exhibit oscillations and do so when one of the enzymatic steps acts close to mass action kinetics (high catalytic constant). The presence of a processive leg can combine with the distributive step, in certain parameter regimes (involving enzyme sequestration), and introduce a substrate depletion effect generating oscillations. Thus, two-site ordered mixed mechanisms may be among the simplest multi-site enzymatic systems to intrinsically generate oscillations. Oscillations are also seen with both distributive/processive routes to modification at each step.

5.3. Three-site mixed systems

We studied mixed mechanisms in phosphorylation when dephosphorylation occurred through a uniform mechanism. In certain scenarios, we find that bistable behaviour can be ruled out. Bistability can be observed in these mixed mechanisms, but this requires distributive elements of both phosphorylation and dephosphorylation. The ordering and relative locations of distributive/processive steps can strongly affect system behaviour. Oscillatory behaviour is observed in all variants of three-site mixed mechanisms which we have studied, and, in some cases, is combined with bistability.

5.4. The interplay of distributive and processive steps

Taken together, we see different facets of the interplay between processive and distributive mechanisms (see table 1 and related comments in appendix A). Even when processive and distributive mechanisms exhibit a simple quantitatively similar sigmoidal behaviour, a mixed mechanism can give a quantitatively very different response. While the presence of processive steps can prevent or reduce the capacity of multi-stability, it can also introduce oscillations. Increasing the number of modification sites leads to an increasing number of new information-processing modes. We were able to determine analytically that, if no partial phosphoform was simultaneously associated with distributive steps in phosphorylation and
information-processing capabilities of mixed modification regimes, which bookends a detailed parametric study. Cases allows us to isolate key patterns in specific parametric contexts, both routes are available, in which case distributive and processive steps are just limiting cases. Such models can be 'transmitted' to other signalling entities). Likewise, this suggests under which conditions the mechanism of multi-site phosphorylation may or may not generate bistability or biphasic behaviour, which has a bearing on pathway behaviour in which this is embedded. More generally, certain behaviour in signalling pathways could be made more robust or significantly altered by the behaviour of the enzymatic mechanisms themselves. We emphasize that our study focuses on the intrinsic behaviour of the mechanism in isolation, in well-controlled conditions. It is possible that, in individual cellular contexts, significant deviations from these conditions can occur. In such situations, having a firm basis for understanding the intrinsic behaviour of the mechanisms helps, as it allows us to systematically infer what the effects of the deviations are, rather than misattribute observations to hypothesized mechanisms.

Finally, our analysis provides a framework for suggesting new modes of chemical information processing that arise through the interplay of distributive and processive mechanisms, nonlinearity as well as the number of sites. Using tools of synthetic chemistry and nanoengineering, it is likely that

dephosphorylation, then bistability would not be obtained in the model, irrespective of the number of sites.

This analysis is relevant for multiple reasons. The first reason is that mixed mechanisms are seen in nature. For instance, it is known that the phosphorylation of protein Pho85 occurs through a mixed mechanism (in phosphorylation itself), and this is part of cell cycle regulation. While the modelling of this specific system in its cellular context is not undertaken here, our framework and results provide a basis for understanding what kind of dynamic patterns can result from the interweaving of processive and distributive steps in phosphorylation. Given the widespread occurrence of distributive and processive mechanisms, it is also to be expected that scenarios where the phosphorylation and dephosphorylation occur through different mechanisms and that there will be naturally occurring instances of this.

It is also known that due to various cellular factors (e.g. molecular crowding, scaffold, stress) various distributive mechanisms can behave effectively like processive mechanisms. When we examine this alongside the multitude of scenarios involving multi-site phosphorylation, we see that there are multiple ways for a given multi-site mechanism to behave like a mixed mechanism. Even if some scenarios resulting in such behaviour necessarily result from stochastic effects, having a kinetic framework is useful, as it provides a basis for understanding what arises from the kinetics alone. This can be extended to examine stochastic effects. It is likely that, in some contexts, both routes are available, in which case distributive and processive steps are just limiting cases. Such models have also been invoked in different biological studies [32].

Our analysis provides a framework for analysing the information-processing capabilities of mixed modification systems. Since these modification systems are embedded in signalling networks at different stages, this is directly relevant to information processing in signalling networks as well, indicating what behaviour can arise purely from the modification mechanism. For instance, oscillatory behaviour has been observed in multiple signalling contexts. The combination of factors that generates oscillations has varied from context to context. In cyanobacterium, in vitro studies report oscillation at a covalent modification level via a three-protein network: proteins KaiA, KaiB and KaiC [42]. A model incorporating feedback regulation in this context has been developed in conjunction with experiments [43]. The multi-site phosphorylation of KaiC has been shown to be an important ingredient in this oscillatory circuit. In our study, we show how oscillation can arise from the enzymatic mechanism itself (and hence this can be 'transmitted' to other signalling entities). Likewise, this suggests under which conditions the mechanism of multi-site phosphorylation may or may not generate bistability or biphasic behaviour, which has a bearing on pathway behaviour in which this is embedded. More generally, certain behaviour in signalling pathways could be made more robust or significantly altered by the behaviour of the enzymatic mechanisms themselves. We emphasize that our study focuses on the intrinsic behaviour of the mechanism in isolation, in well-controlled conditions. It is possible that, in individual cellular contexts, significant deviations from these conditions can occur. In such situations, having a firm basis for understanding the intrinsic behaviour of the mechanisms helps, as it allows us to systematically infer what the effects of the deviations are, rather than misattribute observations to hypothesized mechanisms.

Finally, our analysis provides a framework for suggesting new modes of chemical information processing that arise through the interplay of distributive and processive mechanisms, nonlinearity as well as the number of sites. Using tools of synthetic chemistry and nanoengineering, it is likely that

<table>
<thead>
<tr>
<th>Ordered multi-site modification</th>
<th>Phosphorylation</th>
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<tr>
<td>Mechanism</td>
<td>Distributive</td>
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<tr>
<td>Distributive</td>
<td>Bistability</td>
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<tr>
<td>Mixed</td>
<td>Bistability</td>
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<tr>
<td>Processive</td>
<td>No bistability</td>
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Table 1. A summary of qualitative features of mixed mechanisms in two- and three-site modifications. The results, based on the models studied, for various scenarios of mechanisms (distributive, processive and mixed) in phosphorylation and dephosphorylation are discussed. We do not discuss the interplay between mixed phosphorylation and mixed dephosphorylation in detail in the text (see comments in appendix A regarding this entry). (Online version in colour.)
different aspects of enzymatic mechanisms will be engineered in the future. Indeed, engineering chemistry for the purposes of new modes of information processing is not new and is pursued in multiple contexts [44], both biomolecular as well as non-biomolecular. A number of studies aim to harness or control biomolecular information processing, towards the generation of robust information-processing capabilities and applications. These may, in turn, be seen as elementary building blocks for biomolecular computing systems. Our studies point to capabilities and constraints in harnessing mixed mechanisms and multi-site modification mechanisms in general for chemical information processing, through the manipulation of enzymes, substrates, modification sites and protein structures, as well as reaction environments.

Taken together, we find that mixed modification mechanism can exhibit substantially different modes of chemical information processing when compared with pure mechanisms. This is of relevance in a number of scenarios ranging from the elucidation of signal transduction in concrete cellular contexts to the design of new modes of molecular information processing in biomolecular computing.

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Appendix A

The schematic of the models used is depicted in figure 9.

A.1. Models

As mentioned earlier, the models we employ simply describe elementary steps of binding/unbinding of kinase/phosphatase to substrate, as well as catalysis using standard kinetic descriptions. The catalytic reaction is treated as irreversible in the first instance, though we also examine the effects of weak reversibility of this step in selected instances (see §A.2).
The models of distributive mechanisms simply describe the sequence of individual modifications described above, for multiple modifications. Models of purely processive mechanisms involve describing the successive modification of substrate while bound to the enzyme (irreversible, though this does not have much bearing on the results). As mentioned in the text, it is possible to also consider processive mechanisms where the intermediate phosphoforms are released. Many of the qualitative results we discuss analytically hold good even in this case.

Mixed models simply extend distributive and processive models to describe the scenarios we have analysed (a depiction of the kinetic steps is presented in figure 9). For instance, for an ordered two-site modification mechanism where the phosphorylation is distributive and dephosphorylation is processive is described by the following equations:

\[
\begin{align*}
\frac{d[A]}{dt} &= k_2[A]K - k_1[A][K] + k_{10}[A_p]P, \\
\frac{d[K]}{dt} &= (k_2 + k_3)[AK] - k_3[A][K] + (k_5 + k_6)[A_p]K, \\
\frac{d[AK]}{dt} &= k_1[A][K] - (k_2 + k_3)[AK], \\
\frac{d[A_p]}{dt} &= k_3[AK] - k_4[A_p][K] + k_5[A_p]K, \\
\frac{d[A_p]K}{dt} &= k_4[A_p][K] - (k_5 + k_6)[A_p]K, \\
\frac{d[A_{pp}]P}{dt} &= k_5[A_p]K + k_8[A_{pp}]P - k_7[A_{pp}][P], \\
\frac{d[P]}{dt} &= k_8[A_{pp}]P - k_9[A_{pp}][P] + k_{10}[A_p]P, \\
\frac{d[A_{pp}]}{dt} &= k_7[A_{pp}][P] - (k_8 + k_9)[A_{pp}]P, \\
\frac{d[A_p]P}{dt} &= k_9[A_{pp}]P - k_{10}[A_p]P. 
\end{align*}
\]

(A1)

It is evident that this is a simple kinetic description incorporating the single-step modification events. The dephosphorylation involves no enzyme unbinding until the sequence of modifications is achieved. The various k’s are rate constants associated with individual steps.

Such a model can be easily generalized to multiple modifications. A mixed model with phosphorylation being processive and dephosphorylation distributive is described in a similar way, making the switch between the phosphorylation and dephosphorylation descriptions.

We have also considered multiple three-site modification mechanisms, where the dephosphorylation is either purely distributive or purely processive (figure 9). The phosphorylation, on the other hand, involves a combination of distributive and processive mechanisms. This is simply modelled as an appropriate sequence of the elementary processive and distributive steps. For instance, if the first two modifications are processive and the third distributive, then this is modelled simply using a concatenation of two processive and a subsequent distributive step. As the resulting models are a straightforward translation of the kinetic steps presented in figure 9, we do not present them individually in detail.

Finally, we also briefly analyze a comprehensive model that involves both processive and distributive steps at each stage. For instance, when the enzyme K binds to the unmodified substrate it forms the complex AK (this is described exactly as above), from which there are two pathways: one the conversion to A_p with a release of K, or a second pathway where the modification occurs with the enzyme still bound to A_p, which allows subsequent modifications without release of enzymes. Naturally, released substrate can continue to bind with K in subsequent events leading to further modifications. This comprehensive model is simply a kinetic description of the various steps involved (depicted in figure 9), and we do not present the detailed equations here.

Our analysis of this more comprehensive model shows that it approaches the behaviour of other mixed models considered above, when the kinetics of the relevant extra reactions are made weak.

A.2. Analysis of models

To complement some of the results that we presented earlier, we employ analytical approaches in a few cases. In this section, we show that (i) a mixed mechanism with phosphorylation and dephosphorylation processive (or the other way around) will not result in a biphasic dose–response curve for the maximally modified substrate as a function of total kinase concentration. This is true irrespective of the number of modification sites. (ii) A two-site modification equivalent of the scenario considered in (i) is not capable of exhibiting bistability and this is extended to n-site analogues. (iii) A three-site modification mechanism with mixed phosphorylation and processive dephosphorylation does not exhibit either bistable behaviour or a biphasic dose–response curve of the kind considered above. This is extended to n-site modification mechanisms with processive dephosphorylation and mixed mechanisms of phosphorylation.

Our analysis in all cases will rely on eliminating concentrations of most species, and obtaining equations relating the concentration of the maximally phosphorylated substrate, that of the free phosphatase and kinase. Analysis of the resulting equations results in the conclusions which we draw.

We begin by analysing potential biphasic dose–response characteristics in the maximally modified substrate as the total kinase concentration is varied, when the phosphorylation is distributive and dephosphorylation is processive. By examining the steady state of the two-site model (described explicitly in §A.1), we note the following facts:

1. All kinase complex concentrations are proportional to the product of the relevant substrate and the free kinase concentrations. This simply follows by considering the steady state of the complexes.

2. All phosphatase complex concentrations are proportional to that of [A_{pp}P]. This follows by examining the steady state of the relevant phosphatase complexes (noting that dephosphorylation is processive) and relating them to [A_{pp}P]. It also follows that the concentration of A_{pp}P is proportional to the concentration of A_{pp} and that of P. This follows by considering the steady state of A_{pp}P.

3. The concentration of A_pK is proportional to that of A_{pp}P. This follows by considering the steady state of A_{pp} + A_pK. Along with the previous facts, it follows that A_p is proportional to [P][K].

4. By considering the steady state of [A_p] + [A_pK], we see that [AK] ∝ [A_pK], from which it follows that [A] ∝ [A_p] (also see discussion in the text).
Finally, it can be seen that \([A_{pp}p] \approx [AK]\) (by considering the steady state of \([A] + [AK]\)).

Now, we have the conservation conditions

\[
\begin{align*}
[A] + [AK] + [Ap] + [A_{pp}] + [A_{pp}p] + [AP] &= A_{tot}, \\
[K] + [AK] + [Ap] &= K_{tot}, \\
[P] + [A_{pp}p] + [Ap] &= P_{tot}.
\end{align*}
\]

(A2)

By taking account of the above facts and incorporating them in the above equations, we see that \([K] = K_{tot} - a[A_{pp}]p\) for some constant \(a\) (noting that all kinase complexes are proportional to \([A_{pp}]p\)). We also note that \([P] = P_{tot}/(1 + \beta[A_{pp}]p)\) for a suitable constant \(\beta\). This follows by writing concentrations of all phosphatase complexes in terms of \([A_{pp}]p\). Simplifying the conservation equation for the substrate, we find that the concentration of the maximally phosphorylated substrate can be written as

\[
[A_{pp}] = \frac{A_{tot}}{1 + \gamma[P] + \zeta[P]/[K]},
\]

where \(\gamma, \zeta\) are constants.

This is done by eliminating all variables in terms of \([A_{pp}]p\), \([P]\) and \([K]\). Suppose that there is a biphasic response for the maximally phosphorylated substrate as a function of \(K_{tot}\). This means that at some location \(d[A_{pp}]p/dK_{tot} = 0\). We see from the equation for \([P]\) that if \(d[A_{pp}]p/dK_{tot} = 0\) (the requirement for a biphasic response), then it follows that \(d[P]/dK_{tot} = 0\). By differentiating the equation for \([K]\) with respect to \(K_{tot}\) we see that, if such a condition were to hold good, then it immediately follows that \(dK/dK_{tot} = 1\). However, by differentiating the equation for \([K]\) with respect to \(K_{tot}\) we find that if \(d[P]/dK_{tot} = 0\) and \(d[K]/dK_{tot} = 1\), then \(d[A_{pp}]p/dK_{tot}\) can never be zero, contradicting the assumption. Hence biphasic responses for the maximally phosphorylated substrate are ruled out.

While the above analysis was discussed for two-site modification, it follows in an essentially identical way for more than two sites. When we have multiple modifications, this results in more kinase and phosphatase complexes. In fact, all the points mentioned above carry through if we replace \([A_{pp}]p\) by the concentration of the maximally phosphorylated substrate. All additional kinase complex concentrations are still proportional to that of \([AK]\), and hence all additional intermediate phosphorylated forms are proportional to that of \([A]\). All phosphatase complexes are proportional to that of the phosphatase complex of the maximally phosphorylated substrate. Noting this, we see that the argument above carries through in an essentially identical way when the number of sites is increased.

We can consider the case where the phosphorylation is processive and dephosphorylation distributive. Using the same approach and reducing the relevant steady-state equations to those involving \(A_{pp}, p\) and \(K\), we see that a similar functional form of equations from which it follows that a biphasic dose–response curve of the kind above is ruled out.

We return to the case of distributive phosphorylation and processive dephosphorylation. We now turn to the issue of potential multi-stability in this model. Our starting point here will be the expression for \([A_{pp}]p\) in terms of \([P]\), \([K]\) and other parameters derived above. We only need the functional form of this equation. By inverting this equation, we have

\[
1 + \gamma[P] + \zeta[P]/[K] = \frac{A_{tot}}{[A_{pp}]p}.
\]

(A4)

In this equation, we substitute for \([K]\) and \([P]\) in terms of \([A_{pp}]p\) from above. By rearranging terms, we are left with an expression of the functional form

\[
\frac{yP_{tot}}{K_{tot} + (bK_{tot} - aP_{tot})[A_{pp}]p} + \frac{gP_{tot}}{1 + \beta[A_{pp}]p}\frac{A_{tot}}{[A_{pp}]p}.
\]

(A5)

By clearing various constants, this can be reduced to an equation of the functional form

\[
\frac{A_{tot}}{x} = \frac{a}{b + x} + \frac{c}{d + x} + 1,
\]

(A6)

where \(x\) refers to \([A_{pp}]p\), where \(a, b, c, d\) are positive constants. This assumes the constant \(bK_{tot} - aP_{tot}\) is positive. If this constant is negative, the functional form of the equation is

\[
\frac{A_{tot}}{x} = \frac{a}{b - x} + \frac{c}{d + x} + 1.
\]

(A7)

We will examine each of these cases separately to assess the possibility of multi-stability.

Case 1: \(bK_{tot} - aP_{tot} > 0\)

In this case make a change of variable \(y = 1/x\). This results in an equation of the functional form

\[
A_{tot}y = \frac{c_1y}{c_2 + y} + \frac{c_3y}{c_4 + y} + 1.
\]

(A8)

We note that the RHS starts at 1 (when \(y = 0\)) and is an increasing saturating function of \(y\) (\(y\) takes on only positive values). It is straightforward to see that the derivative of the RHS is a decreasing function of \(y\), since it involves linear combinations of \(1/(c_2 + y)^2\) and \(1/(c_4 + y)^2\). Since the LHS increases from zero to infinity as \(y\) increases, we see that there is at least one intersecting point of these functions. Furthermore, we see that the derivative of the RHS has to be less than that of the LHS at this location (since the RHS is larger at \(y = 0\)). But since the derivative of the RHS is a decreasing function of \(y\) and that of the LHS is a constant we see that subsequently the LHS will always be greater than the RHS, and hence only one positive root results. Incidentally, the same argument holds good even when \(aK_{tot} - bP_{tot} = 0\).

Case 2: \(bK_{tot} - aP_{tot} < 0\)

Here we work directly in terms of \(x\). Note that any feasible root has to satisfy \(b - x > 0\) to ensure positive free enzyme concentrations.

By writing out the equation, we get an equation of the form

\[
A_{tot}(b - x)(d + x) = x[a(d + x) + c(b - x) + (b - x)(d + x)].
\]

(A9)

This is a cubic equation which has potentially three roots. By moving the RHS to the LHS, we find that the coefficient of \(x^3\) and that of the constant term have the same sign (all constants are positive here). This means that at least one of the roots is negative since the product of roots is negative. Furthermore, we rewrite the equation in terms of the variable \(u = b - x\). This results in an equation of the form

\[
A_{tot}u(b + d - u) = (b - u)[a(b + d - u) + cu + u(b + d - u)].
\]

(A10)

Taking all terms to one side, we find that by similar reasoning one of the roots for \(u\) is negative. This corresponds to a second unfeasible steady state. This is easily understood from a graphical inspection as well. Thus, we see that there
A.3. Mixed mechanism in phosphorylation

We now discuss two mixed three-site mechanisms where the dephosphorylation is processive. The two mechanisms differ only in whether the first two or the last two phosphorylation steps are processive. We start by consider the latter case. Here we see that

(1) $[AK] \propto [A][K]$ while $[A_pK], [A_{pp}K] \propto [A_p][K]$.
(2) All phosphatase complex concentrations are proportional to that of $[A_{ppp}]$.
(3) The concentration of $A_{ppp}K$ is proportional to that of $A_{ppp}P$. This follows by considering the steady state of $A_{ppp} + A_{pp}P$. Then it follows that $A_p$ is proportional to $[A_{ppp}][P]/[K]$.
(4) By considering the steady state of $[A_p] + [A_pK]$, we see that $[AK] \propto [A_pK]$, from which it follows that $[A] \propto [A_p]$. 
(5) Finally, it can be seen that $[A_{ppp}P] \propto [AK]$.

We therefore see that the free phosphatase satisfies an equation of the form $[P] = P_{tot}/(1 + \beta[A_{ppp}])$ and the free kinase satisfies an equation $[K] = K_{tot} - \alpha[A_{ppp}][P]$. In the conservation condition for substrate, we note that in addition to the complexes, only $[A], [A_p], [A_{ppp}]$ are included.

The procedure for analysing this is exactly as above and again results in an equation

$$[A_{ppp}] = \frac{A_{tot}}{1 + \gamma[P] + \xi[P]/[K]}.$$  \hspace{1cm} (A 11)

The analysis performed in the above case carries through here as well, and we conclude that there is no bistability or biphasic response of $A_{ppp}$ as a function of $K_{tot}$.

We finally examine the situation where the first two steps are processive and the last phosphorylation step is distributive. In the above, the only changes are as follows:

(1) $[AK], [A_pK] \propto [A][K]$ while $[A_{pp}K] \propto [A_p][K]$.
(2) The concentration of $A_{pp}K$ is proportional to that of $A_{pp}P$. Then it follows that $[A_{pp}]$ is proportional to $[A_{ppp}][P]/[K]$.

The equations for free kinase/phosphatase have similar functional forms as before. The only change in the substrate conservation is that, in addition to the complexes, only $[A], [A_p], [A_{pp}], [A_{ppp}]$ are included.

Again this results in an equation for $[A_{ppp}]$ that is of the same form as before and is analysed in exactly the same way, with the same conclusions.

A.3.1. Mechanisms with mixed phosphorylation and processive dephosphorylation

The analysis above examines the combination of processive and distributive steps in three-site phosphorylation with processive dephosphorylation. We now consider an $n$-site analogue of this situation, with an arbitrary combination of distributive and processive steps in phosphorylation. We note that

(1) All phosphatase complex concentrations are proportional to that of $[A_pP]$. This follows by examining the steady state of the relevant phosphatase complexes (noting that dephosphorylation is processive) and relating them to $[A_pP]$. It also follows that the concentration of $A_{pp}P$ is proportional to the product of concentrations of $A_p$ and that of $P$. This follows by considering the steady state of $A_{pp}P$. $A_{pp}$ refers to the maximally phosphorylated phosphoform.
(2) The concentration of $A_{ppp}pK$ is proportional to that of $A_pP$, which in turn is proportional to $[A_p][P]$. This follows by considering the steady state of $A_p + A_pP$.
(3) The steady state of $[A] + [AK]$ implies that $[A_{ppp}P] \propto [AK]$. $[AK] \propto [A][K]$.
(4) By considering the steady states for $A_p + A_{pp}K$, for $m = 1, 2, \ldots, n - 1$ sequentially, we see that all $[A_pK] \propto [AK]$, for $m = 1, 2, \ldots, n - 1$. If the formation of this kinase complex was via a distributive step, then the concentration of this kinase complex is proportional to $[A_{ppp}][K]$ and hence $[A_{ppp}K] \propto [A]$. In this manner, all kinase complexes can be related to one another and all free phosphoforms can be related to $[A_p], [P], [K]$. The relations between these phosphoforms are similar to that observed above.
(5) When we incorporate the conservation of substrate, kinase and phosphatase, we again see that the free phosphatase satisfies an equation of the form $[P] = P_{tot}/(1 + \beta[A_p])$ and the free kinase satisfies an equation $[K] = K_{tot} - \alpha[A_p][P]$. In the conservation condition for substrate, we note that, in addition to the complexes, the relevant individual phosphoforms are incorporated.

The procedure for analysing this is exactly as above and again results in an equation

$$[A_p] = \frac{A_{tot}}{1 + \gamma[P] + \xi[P]/[K]}.$$  \hspace{1cm} (A 12)

The analysis performed in the above case carries through here as well, and we conclude that there is no bistability or biphasic response of $A_p$ as a function of $K_{tot}$.

Therefore, we conclude that, if dephosphorylation is processive, the above conclusions continue to hold good, for any sequence of distributive and processive mechanisms in phosphorylation.

A.4. Comments on mixed mechanisms of phosphorylation and dephosphorylation

In the text, we studied three-site mechanisms where the phosphorylation is mixed while dephosphorylation is either distributive or processive. While we did not examine scenarios where both phosphorylation and dephosphorylation are mixed, we note a couple of points.

(i) If two modifications share processive phosphorylation and processive dephosphorylation (for example,
a pro pro dis—pro pro dis, or a dis pro pro—dis pro dis pro system, in the notation used in the text), then the three-site mechanism behaves essentially like a two-site distributive mechanism. This is the one scenario in all mixed mechanisms we have studied where we have not found oscillations.

(ii) Although bistability needs distributive steps in both phosphorylation and dephosphorylation, if the distributive steps are disjoint as in (dis pro pro—pro pro dis, or pro pro dis—dis pro pro), then bistability is ruled out under the assumptions made.

We demonstrate the following statement in the electronic supplementary material: consider a model of ordered n-site modification mechanism, comprising a combination of purely processive and purely distributive steps. If the mechanism is such that no single partial phosphoform (apart from the completely unmodified and completely modified species) is associated with both distributive phosphorylation and distributive dephosphorylation (i.e. it is not released in both phosphorylation and dephosphorylation), then the resulting model will not exhibit bistability or biphasic behaviour for the maximally phosphorylated substrate.

We note that if there is one phosphoform associated with both distributive phosphorylation and dephosphorylation, then the resulting model (in some parameter regime) has the qualitative capability of an ordered two-site distributive model and the capacity to exhibit bistability and biphasic behaviour.

A.5. Analysis of mixed two-site modification models

Our results in the main text focused on demonstrating particular characteristics exhibited by mixed systems, using simulation (in particular parameter regimes), bifurcation analysis complemented by analytical work. We complement that study by some additional parametric analysis. We consider both a mixed composite two-site model and also model 22 (for specificity). In particular, we discuss (i) the
transition between mixed composite models and distributive and processive models, (ii) the effects of parameters on oscillations in model 22, and (iii) the effect of binding/unbinding constants.

We first discuss the transition between a mixed composite model and distributive and processive models. This can be done by reducing relevant parameters in the mixed composite model, to make one mode of modification dominant, or by augmenting a pure model, with reactions corresponding to the other mode of modification. We employ the latter option, and do this by examining a distributive model in a regime where bistability is obtained. By introducing a processive pathway in phosphorylation, we find that the range of bistability is steadily reduced and eventually bistability is destroyed (figure 10a). As discussed, a model with purely processive phosphorylation and distributive dephosphorylation does not exhibit bistability. Similar transitions between distributive and mixed models are seen in other parameter regimes.

We now analyse the transition between processive and mixed models. Starting with a processive model, we add a distributive component: this involves three additional reactions: release of $A_p$ from $AK$ and the additional binding/unbinding of $A_p$, to the kinase to produce $A_pK$.

In this regard, we note the following. (i) Having small values of the constants associated with release and unbinding of $A_pK$, and moderate values of the rebinding of $A_p$ to $K$, results in behaviour exactly like a processive model. This is seen in the behaviour of both $A_{pp}$ and $A_p$ whose concentration is small. Now if these three constants are of similar magnitude, then a greater amount of $A_p$ may be seen. Thus, results of the processive model are obtained with strong effect of rebinding relative to release of the intermediate phosphoform; as the magnitude of these three constants is reduced together (for example, by keeping the ratio fixed), keeping the rebinding stronger, the behaviour approaches the processive model. Similar trends are found if the extra reactions are added to both phosphorylation and dephosphorylation steps as well (figure 10b).

We now consider module 22, which involves processive phosphorylation, in the regime shown in the text exhibiting oscillations. We add a distributive component to phosphorylation. As this component is increased, the oscillation amplitude decreases and eventually vanishes, and likewise the range of $K_{tot}$ resulting in oscillations shrinks and eventually vanishes (not shown). Sufficiently strong distributive components in both directions can destroy oscillations.

We now examine the effects of other parameters in module 22. As discussed in the text, oscillations occur when the binding constant of $A_{pp}$ to the phosphatase is high. When this constant is gradually reduced, we find the range of $K_{tot}$ over which oscillations occurs is reduced and eventually oscillations are destroyed (figure 10c). This is consistent with the analysis in the text. On the other hand also increasing the binding constant of $A_p$, to $P$ can destroy oscillations as well, as now a significant fraction of the phosphatase may be sequestered in the complex $A_pP$ also. As noted, when the dephosphorylation of $A_p$ acts close to mass action kinetics, oscillations can be seen.

References


36. MATLAB. 2010 v. 7.10.0 (R2010a). Natick, MA: The MathWorks Inc.


