The interplay of spatial organization and biochemistry in building blocks of cellular signalling pathways : Supporting Information

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1 Organization

The main text presents results on the effects of spatial organization on building blocks of cellular signalling networks. We analyzed both monofunctional and bifunctional enzymes, and as part of our study have examined both (reversible) covalent modification biochemistry as well as two component systems (TCS). The results are computational, backed up by analytical studies. In the Supporting Information, we present additional material in the following order: (i) We first present a more detailed description of the models, along with model equations. (ii) We subsequently proceed to discuss analytical results to support conclusions made in the text. This is presented in the same order as the relevant sections in the main text (iii) We then present additional plots to supplement those discussed in the text (in somecases referred to in the text) with a brief commentary (iv) We conclude by presenting parameter values for the computational results shown.

2 Models and methods

Our studies are underpinned by a detailed analysis of the effects of spatial organization on both mono and bifunctional enzymes in both TCS and covalent modification cycles (CMC). As part of the analysis, a number of specific augmentations of these basic models were also considered (eg. feedback, dead-end complexes, active localization). Noting this, we first present the basic building block models, and then proceed to describe these augmentations.

We see that there are naturally a number of variants of models, arising from the assumptions of the

kinetics. Our study focusses on the effect of spatial organization. This is implemented in the models by establishing specific patterns of localization or diffusion of species. Noting this, we present the entire suite of models as follows. We present the primary classes of models based on the classification of the kinetics as discussed above. Each model includes (in principle) the diffusion of all species. If the diffusivities of species are set to zero, we obtain the ODE model describing the kinetics. We subsequently discuss how we use these models to spatially dissect their behaviour, by having select species diffuse or having species localized. Finally, we present some kinetic variants of the main classes of models, discussing the significance of these variants. All models presented below are in dimensionless form. θ represents a spatial co-ordinate.

The advantage of examining the basic range of building blocks as well as certain variants is that it allows us to trace the origins of observed behaviour transparently, and analyze whether it arises due to a specific feature of the underlying kinetics, or whether it is seen more broadly.

2.1 Part 1: Models of the biochemical building blocks.

We present, in turn, models of both mono and bifunctional enzymes with both TCS and covalent modification biochemistry. We then comment on the models and possible augmentations.

2.1.1 Mono-functional covalent modification cycle

The model equations for a standard covalent modification cycle, catalyzed by a pair of enzymes (kinase, phosphatase) incorporates a widely employed description of the chemical modification mechanisms involved. The model equations are:

$$\begin{aligned}
\frac{\partial[X]}{\partial t} &= -k_1[X][K] + k_{-1}[XK] + k_4[X^*P] + D_X\frac{\partial^2 X}{\partial\theta^2} \\
\frac{\partial[X^*]}{\partial t} &= -k_3[X^*][P] + k_{-3}[X^*P] + k_2[XK] + D_{X^*}\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[K]}{\partial t} &= -k_1[X][K] + k_{-1}[XK] + k_2[XK] + D_K\frac{\partial^2 K}{\partial\theta^2} \\
\frac{\partial[P]}{\partial t} &= -k_3[X^*][P] + k_{-3}[X^*P] + k_4[X^*P] + D_P\frac{\partial^2 P}{\partial\theta^2} \\
\frac{\partial[XK]}{\partial t} &= k_1[X][K] - k_{-1}[XK] - k_2[XK] + D_{XK}\frac{\partial^2 XK}{\partial\theta^2} \\
\frac{\partial[X^*P]}{\partial t} &= k_3[X^*][P] - k_{-3}[X^*P] - k_4[X^*P] + D_{X^*P}\frac{\partial^2 X^*P}{\partial\theta^2}
\end{aligned} \tag{1}$$

The model describes a reversible modification between X and X^*, where the two directions are catalyzed by independent K (Kinase) and P (Phosphatase) respectively. Each basic modification reaction comprises of binding of enzyme to the substrate, formation of the corresponding complex and irreversible generation of the relevant product, where k_1 and k_3 are the forward rate constants for the binding of K to X and P to X^*, respectively, k_{-1} and k_{-3} are the rate constants for the dissociation of enzyme substrate complexes XK and X^*P, respectively, while k_2 and k_4 are the rate constants for catalysis. θ represents the spatial coordinate and D_j is the diffusion coefficient for any species "j" in the cycle. This is a description of a simple covalent modification cycle where two independent mono-functional enzymes performs a pair of modifications.

2.1.2 Mono-functional two-component system

The model of a monofunctional two component systems, involves a pair of enzymes, with the forward reaction (modification of substrate) involving a phosphotransfer mechanism and the reverse modificationm a covalent modification.The model equations are:

$$\begin{aligned}
\frac{\partial[X]}{\partial t} &= -k_1[X][K^*] + k_{-1}[XK^*] + k_p[X^*P] + D_X\frac{\partial^2 X}{\partial\theta^2} \\
\frac{\partial[X^*]}{\partial t} &= -k_2[X^*][P] + k_{-2}[X^*P] + k_t[XK^*] + D_{X^*}\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[K]}{\partial t} &= -k_a[K] + k_d[K^*] + k_t[XK^*] + D_K\frac{\partial^2 K}{\partial\theta^2} \\
\frac{\partial[K^*]}{\partial t} &= k_a[K] - k_d[K^*] - k_1[X][K^*] + k_{-1}[XK^*] + D_{K^*}\frac{\partial^2 K^*}{\partial\theta^2} \\
\frac{\partial[P]}{\partial t} &= -k_2[X^*][P] + k_{-2}[X^*P] + k_p[X^*P] + D_P\frac{\partial^2 P}{\partial\theta^2} \\
\frac{\partial[XK^*]}{\partial t} &= k_1[X][K^*] - k_{-1}[XK^*] - k_t[XK^*] + D_{XK^*}\frac{\partial^2 XK^*}{\partial\theta^2} \\
\frac{\partial[X^*P]}{\partial t} &= k_2[X^*][P] - k_{-2}[X^*P] - k_p[X^*P] + D_{X^*P}\frac{\partial^2 X^*P}{\partial\theta^2}
\end{aligned} \tag{2}$$

The main difference from the mono-functional CMC model, is seen in the equations depicting the conversion of X to X^*, where the phosphorylation of substrate is resulted from phosphotransfer from K^* (phosphorylated kinase) to X through enzyme substrate complex XK^* (reflected in the K^* equation). This model incorporates a basal interconversion between K and K^*.

In the above k_1 and k_2 are the forward rate constants for the binding of phosphorylated kinase to un-phosphorylated substrate and phosphatase to phosphorylated substrate, respectively, while k_{-1} and k_{-2}

are the respective dissociation rate constants of the complexes. k_t and k_p are the catalytic rate constants for forward and reverse modification reactions. The key difference here in comparison to the previous model is that the substrate affects the enzyme (kinase) not just by sequestration, but by altering the balance of enzyme forms (active, inactive) and this arises as a consequence of phosphotransfer.

2.1.3 Bifunctional two-component system (Batchelor-Goulian model)

This model, studied by Batchelor and Goulian is based on the EnvZ/OmpR system in *Escherichia coli*. It involves a bifunctional enzyme incorporated through a TCS. The model equations are:

$$\begin{aligned}
\frac{\partial[X]}{\partial t} &= -k_1[X][E^*] + k_{-1}[XE^*] + k_p[X^*E] + D_X\frac{\partial^2 X}{\partial\theta^2} \\
\frac{\partial[X^*]}{\partial t} &= k_t[XE^*] - k_2[X^*][E] + k_{-2}[X^*E] + D_{X^*}\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[E]}{\partial t} &= -k_k[E] + k_{-k}[E^*] + (k_{-2}+k_p)[X^*E] + k_t[XE^*] - k_2[X^*][E] + D_E\frac{\partial^2 E}{\partial\theta^2} \\
\frac{\partial[E^*]}{\partial t} &= k_k[E] - k_{-k}[E^*] + k_{-1}[XE^*] - k_1[X][E^*] + D_{E^*}\frac{\partial^2 E^*}{\partial\theta^2} \\
\frac{\partial[XE^*]}{\partial t} &= k_1[X][E^*] - (k_{-1}+k_t)[XE^*] + D_{XE^*}\frac{\partial^2 XE^*}{\partial\theta^2} \\
\frac{\partial[X^*E]}{\partial t} &= k_2[X^*][E] - (k_{-2}+k_p)[X^*E] + D_{X^*E}\frac{\partial^2 X^*E}{\partial\theta^2}
\end{aligned} \tag{3}$$

In the original Batchelor-Goulian model, X, X^*, E, E^*, XE^* and X^*E represent $OmpR$, $OmpRP$, $EnvZ$, $EnvZP$, $(EnvZP)OmpR$ and $(EnvZ)OmpRP$, respectively. This model has an interconversion between two forms of the bifunctional enzyme, E and E^*, highlighting the bifunctional nature of the enzyme. This model also incorporates phosphotransfer in the forward modification and covalent modification in the reverse modification. In relation to the monofunctional TCS model just presented above, we see that the main difference is that K in that model acts here as a phosphatase, and there is no additional phosphatase needed. There is a basal interconversion between the enzyme forms, and owing to phosphotransfer, the balance between enzyme forms is affected by the substrate. k_k and k_{-k} are the forward and backward rate constants for the conversion between E and E^* respectively. The remaining parameter denotations are the same as in mono-functional TCS model.

2.1.4 Bifunctional enzyme with a CMC

We now present an analogue of the previous model with a CMC. Just as before the balance of two forms of the enzyme, E and E^*, are also regulated by external signals Sa and Sb, in addition to basal interconversion (in the most general case: these can be set to 0 if needed). Sa regulates the conversion of E to E^* and Sb that of E^* to E. These reactions were assumed to be in the unsaturated regime, for simplicity. The substrate modification is described as is standard for a covalent modification cycle (describing reversible enzyme substrate binding to form a complex, which gets irreversibly converted to the product). The only additional aspect is the regulation of the enzyme forms. The model equations are:

$$\begin{aligned}
\frac{\partial[X]}{\partial t} &= -k_1[X][E^*] + k_{-1}[XE^*] + k_4[X^*E] + D_X\frac{\partial^2 X}{\partial\theta^2} \\
\frac{\partial[X^*]}{\partial t} &= -k_3[X^*][E] + k_{-3}[X^*E] + k_2[XE^*] + D_{X^*}\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[E^*]}{\partial t} &= k_{sa}[Sa][E] - k_{sb}[Sb][E^*] + k_{fb}[E] - k_{bb}[E^*] - k_1[X][E^*] + k_{-1}[XE^*] + k_2[XE^*] \\
&\quad + D_{E^*}\frac{\partial^2 E^*}{\partial\theta^2} \\
\frac{\partial[E]}{\partial t} &= -k_{sa}[Sa][E] + k_{sb}[Sb][E^*] - k_{fb}[E] + k_{bb}[E^*] - k_3[X^*][E] + k_{-3}[X^*E] + k_4[X^*E] \\
&\quad + D_E\frac{\partial^2 E}{\partial\theta^2} \\
\frac{\partial[XE^*]}{\partial t} &= k_1[X][E^*] - k_{-1}[XE^*] - k_2[XE^*] + D_{XE^*}\frac{\partial^2 XE^*}{\partial\theta^2} \\
\frac{\partial[X^*E]}{\partial t} &= k_3[X^*][E] - k_{-3}[X^*E] - k_4[X^*E] + D_{X^*E}\frac{\partial^2 X^*E}{\partial\theta^2}
\end{aligned} \tag{4}$$

where k_{sa} and k_{sb} are the rate constants associated with the signals Sa and Sb modulating the interconversion between E and E^*, respectively, and k_{fb} and k_{bb} are the basal forward and backward rate constants for the interconversion between the two forms of the enzyme, E and E^*, respectively. The remaining parameter denotations are the same that of a monofunctional CMC.

2.2 Augmentations to the basic models.

We now examine specific augmentations to the basic models to illustrate specific points. These involve ultrasensitivity, feedback and bistability.

Ultrasensitive Response: A small modification of the bifunctional CMC model can be used to demonstrate a particular behaviour, which is the ultrasensitive response. To be concrete let us focus on one

signal (say Sb). The modification of the model above involves explicitly depicting the binding of the signal Sb to the relevant enzyme, to form a relevant ligand-related complex. The modification was based on the model by [Straube, 2014]. It takes the conservation of the ligand or signal (which in this case regulates the conversion of E^* to E) into explicit consideration. A conservation of the following form, results from this model:

$$[Sb_{total}] = [Sb] + [SbE] + [X^*SbE] \tag{5}$$

where $[Sb_{total}]$ is the total concentration of the signal Sb (and the input signal).

This same alteration can be done for both signals Sa and Sb (and this will be discussed later). The sensitivity curve was obtained by plotting X^* against the total concentration of the signal.

In a parameter regime where the two forms of the enzyme are operating at saturation and if the signal binds with high affinity, this modified model is capable of exhibiting an "ultrasensitive" response (the underlying analytical reasoning is shown in [Straube, 2014]). Very briefly, for a very small change in the concentration of the input, the change in the concentration of the output is relatively large- thus the response concentration curve is sharply sigmoidal with respect to the output.

2.2.1 Feedback control

Information in enzymatic modification typically flows from enzyme to substrate. We have briefly examined cases where there is feedback from substrate to enzyme. We explore this in a basic way in the bifunctional CMC model, where the modified substrate can alter the balance between enzyme forms. A feedback model was obtained by incorporating a positive feedback interaction between X^* and E^* in the earlier model.. This may be achieved in two ways- by increasing the conversion of E^* (from E) or inhibiting the conversion of E^* to E. The model does not incorporate any uptake of substrate in mediating this feedback, and we further note that the feedback could incorporate additional entities as well. We note that allosteric feedback has been observed in bifunctional enzymes. We chose the former case for specificity (the interaction is assumed to be in the unsaturated regime for simplicity to focus on the dominant effect).

We note that the way the feedback is incorporated is consistent with the chemistry of modification (in particular all modifications are covalent modifications and there is no phosphotransfer). The substrate modification equations are exactly the same as before and will not be repeated. The equations for the different forms of the enzymes are

$$
\begin{aligned}
\frac{\partial[E^*]}{\partial t} &= k_{sa}[Sa][E] - k_{sb}[Sb][E^*] + k_{fb}[E] - k_{bb}[E^*] - k_1[X][E^*] + k_{-1}[XE^*] + k_2[XE^*] \\
&\quad + k_{pf}[X^*][E] + D_{E^*}\frac{\partial^2 E^*}{\partial \theta^2} \\
\frac{\partial[E]}{\partial t} &= -k_{sa}[Sa][E] + k_{sb}[Sb][E^*] - k_{fb}[E] + k_{bb}[E^*] - k_3[X^*][E] + k_{-3}[X^*E] + k_4[X^*E] \\
&\quad - k_{pf}[X^*][E] + D_E\frac{\partial^2 E}{\partial \theta^2}
\end{aligned}
\tag{6}
$$

where k_{pf} is the positive feedback rate constant. The remaining equations and associated parameters are the same as in bifunctional CMC model. The feedback rate constant k_{pf} is varied in the analysis of the model.

Transcritical Bifurcation: In a certain parameter regime (where the basal rate constants k_{fb} and S_a are zero), this model shows a particular kind of thresholding effect which arises through a transcritical bifurcation. To be specific, suppose k_{fb} is zero and S_a is zero as well. The model has two steady states- one is non-zero and if the signal (Sb) exceeds a threshold (or transcritical bifurcation point) the only stable steady state is one of zero concentration for X^*. This is an example of non-trivial qualitative behaviour (whose essential threshold effect is felt for small values of k_{fb} and S_a). The spatial perturbation of this effect is studied in the main text.

2.2.2 Bistability.

A particular response of interest in cellular systems is bistability. Bistability can be achieved in both monofunctional and bifunctional enzyme-based building blocks. There are two broad ways in which bistability can be realized: one via an explicit positive feedback, and the other via augmentations of a model giving rise to a dead-end complex. We discuss each of these briefly.

Bistability through co-operative feedback : We now consider a variation of the feedback model presented above, which arises in the way feedback occurs. If the feedback between X^* and E^* involves a degree of co-operativity, then this model is capable of exhibiting a bistable response. There are multiple sources to this co-operative effect, but this will not be modelled explicitly. Instead we use this model as a depiction of how a variation in the way feedback is implemented results in bistability. Later in this section, we show how bistability can also arise without co-operative effects. This can arise through simple feedback (as described above) with additional enzyme substrate interactions.

The model equations with co-operative feedback, involve only one specific modification to the earlier model. The new model equations are

$$\begin{aligned}
\frac{\partial[X^*]}{\partial t} &= k_1[X][E^*] - k_2[X^*][E] + D_{X^*}\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[E^*]}{\partial t} &= k_3[Sa][E] - k_4[E^*] + k_{pf}[X^*]^2[E] + D_{E^*}\frac{\partial^2 E^*}{\partial\theta^2} \\
\frac{\partial[X]}{\partial t} &= -k_1[X][E^*] + k_2[X^*][E] + D_X\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[E]}{\partial t} &= -k_3[Sa][E] + k_4[E^*] - k_{pf}[X^*]^2[E] + D_E\frac{\partial^2 E^*}{\partial\theta^2}
\end{aligned} \tag{7}$$

where k_1 and k_2 are the forward and backward rate constants associated with themodification of X to X^* (mediated by enzymes), k_3 and k_4 are the rate constants for the binding of the signal to E and the basal conversion of E^* to E. k_{pf} is the feedback rate constant from X^* to E^*. The effects of the second signal Sb, are implicit in the constant k_4, and for simplicity, basal inteconversion rate constant from E to E^* independent of Sa is set to zero.

Other symbols are exactly as used earlier.

Taken together the bistability can arise through either co-operative feedback, or basic feedback with additional enzyme substrate interactions.

We now turn to a different way of achieving bistability, which is consistent with the biochemistry of TCS. This has been studied for both monofunctional and bifunctional enzymes.

Dead-end complex model (for bistability of TCS). Bistability can be achieved by basic augmentations in the Batchelor-Goulian model (bifunctional enzyme with TCS), which involve additional enzyme substrate interactions to form a "dead-end complex" as well as an additional phosphatase for substrate dephosphorylation (developed and discussed by Igoshin et al (2008)). This has been studied in detail in the literature. The model employed is given by:

$$
\begin{aligned}
\frac{\partial[X]}{\partial t} &= -k_{b1}[X][E^*] + k_{d1}[XE^*] + k_{d3}[XE] - k_{b3}[X][E] + k_{cat}[X^*F] + D_X\frac{\partial^2 X}{\partial\theta^2} \\
\frac{\partial[X^*]}{\partial t} &= -k_{b2}[X^*][E] + k_{d2}[X^*E] + k_{d4}[X^*F] - k_{b4}[X^*][F] + D_{X^*}\frac{\partial^2 X^*}{\partial\theta^2} \\
\frac{\partial[E]}{\partial t} &= -k_{b2}[X^*][E] + k_{d2}[X^*E] - k_{ap}[E] + k_{ad}[E^*] + k_{d3}[XE] - k_{b3}[X][E] + D_E\frac{\partial^2 E}{\partial\theta^2} \\
\frac{\partial[E^*]}{\partial t} &= -k_{b1}[X][E^*] + k_{d1}[XE^*] + k_{ap}[E] - k_{ad}[E^*] + D_{E^*}\frac{\partial^2 E^*}{\partial\theta^2} \\
\frac{\partial[XE]}{\partial t} &= k_{ph}[X^*E] - k_{d3}[XE] + k_{b3}[X][E] + D_{XE}\frac{\partial^2 XE}{\partial\theta^2} \\
\frac{\partial[XE^*]}{\partial t} &= k_{b1}[X][E^*] - k_{d1}[XE^*] - k_{pt}[XE^*] + D_{XE^*}\frac{\partial^2 XE^*}{\partial\theta^2} \\
\frac{\partial[X^*E]}{\partial t} &= k_{b2}[X^*][E] - k_{d2}[X^*E] + k_{pt}[XE^*] - k_{ph}[X^*E] + D_{X^*E}\frac{\partial^2 X^*E}{\partial\theta^2} \\
\frac{\partial[F]}{\partial t} &= k_{d4}[X^*F] - k_{b4}[X^*][F] + k_{cat}[X^*F] + D_F\frac{\partial^2 F}{\partial\theta^2} \\
\frac{\partial[X^*F]}{\partial t} &= -k_{d4}[X^*F] + k_{b4}[X^*][F] - k_{cat}[X^*F] + D_{X^*F}\frac{\partial^2 X^*F}{\partial\theta^2}
\end{aligned}
\tag{8}
$$

Building on the Batchelor-Goulian model, two ingredients are added in this model for the generation of bistability: the formation of dead-end complex, XE, and the existence of an extra and major source, exogenous phosphatase F, for the dephosphorylation of phosphorylated substrate, X^*, which is independent of the bifunctional enzyme, E and E^*. X, E, E^*, X^*E, XE^*, X^*F represents unphosphorylated substrate, unphosphorylated form of bifunctional enzyme (phosphatase activity), phosphorylated form of bifunctional enzyme (kinase activity), the intermediate complex produced by phosphatase form E acting on phosphorylated substrate X^*, the intermediate complex produced by kinase form E^* phosphorylating unmodified substrate X and the intermediate complex produced by exogenous phosphatase F dephosphorylating phosphorylated substrate X^*, respectively. Parameter denotations k_{b1}, k_{d1}, k_{b2}, k_{d2}, k_{ap} and k_{ad} are the same as k_1, k_{-1}, k_2, k_{-2}, k_k and k_{-k} in Batchelor-Goulian model, respectively. However, phosphotransfer occurs between two complexes here, from XE^* to X^*E, with rate constant, k_{pt} following which which there is an irreversible conversion to XE by rate constant, k_{ph}. The exogenous enzyme-phosphorylated substrate complex, X^*F, can dissociate to give F and X with catalytic rate constant k_{cat}. k_{b3} and k_{b4} are the forward rate constants for binding of E to X and F to X^*, respectively, while k_{d3} and k_{d4} are the rate constants for dissociation of XE and X^*F, respectively.

2.3 Spatial organization and localization

Thus far we have discussed various kinetic aspects of the models which we employ. We now describe how we can use the same models, along with some augmentations, to describe the spatial and spatio-temporal regulation of the biochemical modification modules. We note that at the outset, all species are, in principle, diffusible. If no species diffuses, and conditions are uniform (with no localization of species), the modification module is described by the ODE model depicting its kinetics. This incidentally also corresponds to all substrate and enzyme localized in one compartment. Here the effect of compartmentalization/localization is simply incorporated by starting with substrate and enzyme in that compartment and having no species diffuse. With this as a vantage point one can examine various spatial perturbations: (i) one or more substrates exiting this compartment: this is simply described by allowing these substrates to diffuse, while keeping enzymes and complexes non-diffusible (ii) enzymes exiting the compartment: this is described by having the enzymes diffusible. Naturally it is possible to have both enzymes and substrates diffusible and this is incorporated easily. Furthermore it is possible for substrates to be diffusible while enzymes are localized in more than one location (such as two poles of a cell). This is incorporated by simply starting with enzymes at two locations, and having them non-diffusible, while the substrates diffuse.

We have also examined explicit models for regulation of localization of a diffusing species. This is necessary to depict ways in which a cell may impose a localization pattern, which could change dynamically. We focus on depicting control of localization of enzymes (referred to as active localization). This is described in the following way. If an enzyme is diffusing, it can bind to an immobilized species in a given location and become localized. Models which describe the localization of enzymes in such a manner need to account for both forms of the enzyme, when the enzyme is bifunctional. A general model which accounts for the localization of both enzyme forms (of a bifunctional enzyme) at two locations (denoted by L_1 and L_2)is given by

$$
\begin{aligned}
\frac{\partial[E^*]}{\partial t} &= k_{sa}[Sa][E] + k_{fb}[E] - k_{bb}[E^*] \\
&\quad -k_{b1e^*}[S_{L1}][E^*] + k_{u1e^*}[S_{L1} - E^*] \\
&\quad -k_{b2e^*}[S_{L2}][E^*] + k_{u2e^*}[S_{L2} - E^*] \\
&\quad -k_1[X][E^*] + (k_{-1} + k_2)[XE^*] + k_f b_e^*[E][X^*] \\
&\quad +D_{E^*}\frac{\partial^2 E^*}{\partial \theta^2} \\
\frac{\partial[E]}{\partial t} &= -k_{sa}[Sa][E] - k_{fb}[E] + k_{bb}[E^*] \\
&\quad -k_{b1e}[S_{L1}][E] + k_{u1e}[S_{L1} - E] - k_{b2e}[S_{L2}][E] + k_{u2e}[S_{L2} - E] \\
&\quad -k_3[X^*][E] + (k_{-3} + k_4)[X^*E] - k_f b_e^*[E][X^*] \\
&\quad +D_E\frac{\partial^2 E^*}{\partial \theta^2}
\end{aligned}
\tag{9}
$$

where $[Sa]$ is the concentration of the activity regulating signal, converting the enzyme from the phosphatase to its kinase form and k_{sa} is the associated rate constant. The basal forward (E to E^*) and backward (E^* to E) rate constants are k_{fb} and k_{bb}, respectively. This is similar to the models earlier. For simplicity the presence of a signal Sb in mediating the conversion from E^* to E is not included. The new aspects of the model are the incorporation of the binding of E and E^* to localization signals S_{L_1} and S_{L_2} (which are present at different spatial zones). E and E^* bind (and unbind) to the localization signals S_{L1} in location L1 and S_{L2} in location L2 respectively; the related binding and unbinding rate constants are denoted by $k_{b''i''enzymeform}$" and $k_{u''i''enzymeform}$" where i is either 1 or 2- indicating location L1 or L2, respectively, and the enzyme form may be either E or E^* (denoting binding/unbinding of E or E^*, respectively). The localization signals and enzyme bind to give the following localized enzyme forms: $S_{L1} - E^*$ and $S_{L1} - E$ in location L1 and $S_{L2} - E^*$ and $S_{L2} - E$ in location L2. Thus this model incorporates a direct augmentation of the earlier model for the enzyme incorporating localization. Equations for the localized forms of enzymes can be written in a simple way, describing the free enzyme reversibly binding to the localization signal giving the localized enzyme. In the text, when we consider localization, we consider situations where E and E^* bind with equal affinity to the localization signal and also situations where there is an asymmetry of localization. We also consider two broad situations: (i) the localized enzymes dont interconvert (thus for the localized enzymes, the only factors which affect it are reversible binding and unbinding of enzymes to localization signals) and (ii)) the localized enzymes

interact/interconvert exactly as the free enzymes. These are straightforward model descriptions of the network depicted. We do not repeat the models here.

With regard to substrate modification, we assume that (unless otherwise explicitly indicated) localized enzymes modify substrate in the relevant location in exactly the same way as free enzymes. In general all pairs of free enzymes and the localized enzymes modify the substrate species. The modification parameters related to the substrate are similar to those of the models without this localization step (which includes the possibility of both feedforward and feedback regulation). In the case of potential feedback, $k_f b_{e^*}$ is the feedback rate constant associated with the feedback effect of X^* on the conversion between E and E^*. Now if the localized forms are capable of interconverting, then it is possible to incorporate the feedback effect of X^* in regulating the conversion of the localized forms as well: this results in similar terms, with associated feedback parameters - $k_f b_{s1e^*}$ and $k_f b_{s2e^*}$ denoting, respectively feedback acting on interconversion between localized forms at location 1 and 2.

It is worth mentioning that in our model, for simplicity we treat S_{L_1} and S_{L_2} as constant. Our model incorporates localization of both enzymes forms at two locations. It considers both the case where there is no interconversion of localized forms, and that where there is interconversion of localized forms. It also, in specific instances, examines the effect of feedback from substrate regulating the balance of the enzyme forms, and in the latter case, incorporates the possibility of feedback in regulating the balance of localized forms.

In addition to the possibility/absence of interconversion of localized forms, we also use our model to examine the special case where localized forms are catalytically inactive. This is simply incorporated by making the appropriate change in the substrate modification kinetics.

The same localization model is used to describe the localization of bifunctional enzymes, whether they act via CMC or TCS. Likewise, exploring the presence/absence of interconversion of localized forms, or potential lack of activity of localized forms is also implemented in a similar way. We also point that when the enzyme is monofunctional, we have only one enzyme form to focus on and the localization is described analogously.

A comment on localization and compartmentalization. Note that our models focus on the interplay of localization of species in the spatial domain and its interplay with modification chemistry. Our models of localization are representative of compartmentalization of reactions. We comment here that while our models deal with regions of localization and not explicitly with compartments with bounding boundaries (say organelle), a number of insights are also relevant to that instance.For example if we

consider the situation where all species are localized in such a compartment with one species exiting the compartment (as considered at multiple points in the paper). An incorporation of transport across bounding membranes (in an associated model in 1-D) using the Kedem-Katchalsky type equation will still result in a dilution effect due to the species exiting the compartment, and consequently, the key qualitative insights from our model hold good here as well.

2.4 Additional models

We have presented the basic models which we employ for the modification modules, as well as their spatial regulation. We conclude our presentation of the models by presenting a variant of the models presented. This model has also been analyzed alongside the models presented above.

2.4.1 Feedback regulation with additional enzyme substrate interactions.

Earlier we had shown how co-operative feedback can give bistability. Here, for completeness, we briefly examine another model of feedback between enzyme and substrate which can give bistability without co-operative feedback. This model depicts linear feedback between substrate and enzyme, but incorporates an extra interaction between enzyme and substrate. Multispecific interactions between enzyme and substrate have already been studied [Seaton & Krishnan, 2012] and the interplay between simple feedback and conservation of species resulting in bistability has also been studied [Krishnan *et al.*, 2014]. In this model an extra interaction between enzymes and substrates is incorporated by describing the binding of X and E which sequesters E in a complex XE. Note that no catalytic conversion occurs from this reaction, and this is an example of an extraneous interaction between an enzyme and a substrate. This is an example of a multispecific interaction between E and X binding to reversibly form a complex and this additional interaction along with linear feedback can give rise to bistability. The reason we present this model is to simply indicate that there are other models with simpler feedback which can also give bistability. The model equations for the model are

$$
\begin{aligned}
\frac{\partial[X]}{\partial t} &= -k_1[X][E^*] + k_{-1}[XE^*] + k_4[X^*E] - k_5[X][E] + k_6[XE] \\
&\quad + D_X \frac{\partial^2 X}{\partial \theta^2} \\
\frac{\partial[E]}{\partial t} &= k_s[S][E^*] - k_{fb}[E] + k_{bb}[E^*] - k_3[X^*][E] + k_{-3}[X^*E] + k_4[X^*E] \\
&\quad - k_{pf}[X^*][E] - k_5[X][E] + k_6[XE] + D_E \frac{\partial^2 E}{\partial \theta^2} \\
\frac{\partial[XE]}{\partial t} &= k_5[X][E] - k_6[XE] + D_{XE} \frac{\partial^2 XE}{\partial \theta^2}
\end{aligned}
\tag{10}
$$

where k_5 and k_6 are forward and backward rate constants associated with binding of E by X to form XE(the new aspect). All other equations are the same and will not be repeated. All other rate constants are exactly as in the basic model.

Bistable Response: The extra augmentation allows this model to exhibit a bistable response (in a particular parameter regime) even with linear feedback, without requiring co-operativity.

Numerical Method: The partial differential equations were discretized using finite difference equations (centred difference) and the results were checked by doubling the discretization. Note that the only terms which need to be spatially discretized are the diffusion terms, and the centre-difference scheme involves approximating the second derivative of variable u with respect to spatial co-ordinate x, at gridpoint i by $(u(i+1) + u(i-1) - 2u(i))/(dx)^2$, where $u(i)$ represents the value of variable u at gridpoint i and dx denotes the spacing between gridpoints (difference in spatial coordinate between adjacent gridpoints). Implementing this reduces the PDE to a large system of ODEs, which are initial value problems, and can be simulated using ode solvers. All simulations were performed in MATLAB using ode15s. The bifurcation analysis was performed using MATCONT for the basic kinetic models. In the case of spatial designs, in some cases, the steady state in the PDE corresponds to the steady state of the ODEs representing the kinetics, with a modified conservation condition (this is detailed below in the relevant instances). In such cases MATCONT can also be easily used for bifurcation analysis of steady states of the PDE. This is because, for bifurcation analysis of ODEs with conservation conditions (as seen in the kinetic models), the conservation condition is incorporated into the ODE (vector field) by eliminating suitable variables (this introduces new parameters such as total amounts of species) from which bifurcation analysis is easily done. Exactly the same approach applies to these instances of PDEs.

3 Analytical Results

In this section, we provide analytical results to support the conclusions obtained in the main text through computational simulation of the models. This is presented in as follows (i) Analytical results related to monofunctional enzymes (ii) Results on bifunctional enzymes (iii) Results pertaining to active localization and the switching of dominant activity of the bifunctional enzyme (iv) Other miscallaneous results. The order in which the results are presented mirrors the order in which they are presented in the main text.

With regard to the presentation of the analytical results, our focus is on qualitative insights. In some places we adopt a simpler notation (explained in a self contained way) for purposes of the analysis.

Comments on the relationship between different modification systems. We first discuss the relationship between different modification modules. By examining monofunctional and bifunctional enzymes, we identify aspects common to both (eg. effect of feedback) and also important differences. Examples of the latter include absolute concentration robustness as well as qualitative differences in behaviour of different spatial designs. In each of the latter cases, it is easy to trace such behaviour to the bifunctional nature of enzymes.

We have also studied TCS and CMC for both monofunctional and bifunctional enzymes. We discuss similarities and differences between the two, and focus on the bifunctional case. The crucial difference is that in the CMC case, the steady state balance of enzymes is not affected by substrate amounts. While substrates may affect sequestration of enzymes, they do not affect the E^*/E ratio. In the case of the TCS, owing to the asymmetric nature of enzyme substrate modification (phosphotransfer in one direction, hydrolysis/covalent modification in the other), the steady state balance between enzymes forms is affected not only by basal interconversion rates, but also by the substrate. Indeed, we see that $E^*/E = (k_k + k_c X^*)/k_{-k}$, where k_k, k_{-k} represent basal interconversion rates between the enzyme forms, and k_c incorporates the effect of phosphotransfer. Now if substrate levels are low, then the substrate effects become negligible and the behaviour is similar to that of the analogous bifunctional CMC. This is no longer the case substrate levels are high.

Incidentally this substrate dependence also explains the spatial retroactivity for TCS compared to CMC even in monofunctional enzymes as discussed below.

3.1 Monofunctional enzymes and spatial designs.

We present further analysis here to support results presented in the first subsection of the results. The focus was on different aspects of spatial designs of monofunctional enzymes.

We first start with some brief discussions on the kinetics of monofunctional enzymes. We examined two cases: the standard covalent modification cycle, and a monofunctional two component system. The standard covalent modification system has been studied in various contexts in the literature. At steady state, we note that $[XK] \propto [X][K]$, $[X^*P] \propto [X^*][P]$ and further that $k_{c1}[XK] = k_{c2}[X^*P]$, where k_{c1}, k_{c2} are the catalytic constants for phosphorylation and dephosphorylation. The steady states are obtained by solving for these equations, along with the three conservation conditions $[K] + [XK] = K_{tot}$ $[P] + [X^*P] = P_{tot}$, $[X] + [X^*] + [XK] + [X^*P] = X_{tot}$, where $K_{tot}, P_{tot}, X_{tot}$ represent the total amounts of kinase, phosphatase and substrate respectively.

The small modification in the case of a monofunctional TCS involves an inactive and active kinase form (denoted K_0 and K respectively) with the modification mediated by kinase occurring via phosphotransfer. While the first three equations above remain the same, and the conservation conditions for the phosphatase and the substrate remain the same, there are differences with the kinase conservation equation. The kinase conservation equation now reads $[K_0] + [K] + [XK] = K_{tot}$, and further, at steady state $K_0/K = (k_{fa} + k_c X)/k_{ba}$. We see straightaway that in this equation, if the term $k_c X$ in the numerator is negligible, then the ratio of K to K_0 is a constant, and many qualitative aspects of this system are similar to the monofunctional CMC studied earlier.

Spatial designs. We now focus on the results associated with spatial designs: these involve patterns of localization of enzymes and/or substrates. It is instructive to examine the different substrate localization patterns, while the enzymes are co-localized in one location. We focus on the CMC case for specificity, but the main insights we draw are common to the CMC and TCS cases. Thus we examine three cases (i) X^* diffusing (non-localized) (ii) X diffusing (non-localized) (iii) both X and X^* diffusing.

In all cases for specificity, we consider a region where the enzymes are localized in an ambient spatial domain, which are subject to no-flux boundary conditions at both ends. We note that all the main insights are also relevant for other boundary conditions such as periodic boundary conditions. In fact the periodic boundary conditions, for the cases studied, could be reduced to cases involving no-flux boundary conditions in half the domain size.

We first consider case (i). Now adding the equations for X, X^*, XK andX^*P results in all the

kinetic terms cancelling out (this is because the kinetics involve only the interconversion between species): incidentally the kinetics are present only in part of the spatial domain. This is results in $\partial^2 X^*/\partial\theta^2 = 0$. Now given the no-flux boundary conditions at the boundaries, this implies that X^* is uniform throughout the spatial domain, and in particular, at steady state, the flux of X^* out of the reaction compartment is zero. This means that all species satisfy the underlying kinetic equations, with the only change being in the modification to the conservation condition: $L_1(X + XK + X^*P) + (L + L_1)X^* = L_1X_{tot}$ where it is assumed that all species are initially present in the reaction compartment, with a total (local) concentration X_{tot}. Here L_1 is the size of the compartment and L the size of the surrounding domain. Note that when $L = 0$ this reduces to the same conservation condition as the kinetic ODE model. Overall, we see in this case, we see that the only effect of non-localization of X^* is a dilution effect, though we emphasize that the extent of dilution is dependent on the kinetic parameters and the enzyme concentrations (it is not a fixed proportion which is diluted).

Case (ii) where X is diffusing can be analyzed in an identical way: at steady state X is uniform, and consequently the steady state of the system is that of the ODE model with a nmodified conservation condition: $L_1(X^* + XK + X^*P) + (L + L_1)X = L_1X_{tot}$. Again a dilution effect results, though the quantitative effect can be significantly different.

Case (iii) involves both X and X^* diffusing. Now if we analyze each of these species outside the reactive domain we find that they satisfy the diffusion equation, implying a steady state linear profile. Furthermore since one of the boundaries involves a no-flux boundary condition, this implies the slope is zero and the profile is flat. Thus both species have a spatially uniform profile, which also means that at steady state there is no flux outside the reaction compartment. This means again that the variables satisfy the steady state kinetic equations with a modified conservation condition
$L_1(XK + X^*P) + (L + L_1)(X + X^*) = L_1X_{tot}$. Thus again, the only effect is a dilution effect.

We make an additional point in this regard. If both phosphorylation and dephosphorylation are in the unsaturated regime, then the complex concentrations are small. In this case, we see that the dilution effect (which is seen only through the conservation condition) implies that $X + X^*$ (and hence each of these species is diluted by a factor $L_1/(L + L_1)$). As a consequence, in this regime, it is possible to simply correct for the dilution effect by having proportionally more substrate to start with.

Zero output. We now substantiate another comment made in the text of designs which result in zero output (or complete conversion): this is exemplified by the case where kinase and phosphatase are in two different locations and only X^* is diffusing. Now in such a case at steady state, a steady state for species X

must hold good at every spatial location. In particular, it must hold good in the region where the phosphatase is localized. Now X is produced from the X^*P complex and at that location there is no source of consumption. This means that at steady state $X^*P = 0$, from which it follows (by considering the conservation of phosphatase, that $X^* = 0$. We have already seen that X^* has a uniform profile in the domain, and so $X^* = 0$ everywhere. This illustrates the point that spatial designs which prevent local reversibility of the reaction (while also having only some species diffusible while others are not) will result in some species attaining a zero output.

Retroactivity. We also establish a point about retroactivity. To do this we consider kinase and phosphatase diffusible while substrate (and complex) remain localized in one region. Now if the system is at steady state, steady state for complexes implies that there is a kinetic balance between production and dissociation/conversion of the complex (both kinase and phosphatasem complex. In other words $[XK] \propto [X][K]$, $[X^*P] \propto [X^*][P]$ where in each case the proportionaility factor is the ratio $k_b/(k_u + k_c)$ where k_b, k_u, k_c are respectively the corresponding binding inbinding and catalytic constants of the particular modification.

Now examining the free enzyme equations at steady state for a CMC, we find that they satisfy the diffusion equation. This is seen by simply adding the equations for the free enzyme and the complex: the interconversion reactions cancel out and the only remaining term is the diffusion of the free enzyme.

$$\begin{aligned} d/dt(K + XK) &= D_K \partial^2 K/\partial\theta^2 \\ d/dt(P + X^*P) &= D_P \partial^2 P/\partial\theta^2 \end{aligned} \tag{11}$$

This implies that at steady state, the enzyme profiles are uniform, which implies the spatial information about the substrates is not backpropagated into the (free,active) enzyme profiles. This is however not the case for TCS. This was demonstrated computationally in the text and can be explained in similar terms here. Indeed, looking at similar equations, we have (assuming for simplicity, complexes are non-diffusible)

$$\begin{aligned} d/dt(K + XK) &= k_{fa}K_0 - k_{ba}K - k_c XK + & D_K \partial^2 K/\partial\theta^2 \\ d/dt(P + X^*P) & = & D_P \partial^2 P/\partial\theta^2 \end{aligned} \tag{12}$$

Here k_{fa}, k_{ba} represent the basal interconversion between inactive and active kinase, and k_c denotes the

contribution of the phosphotransfer. Notice how the first equation includes only the diffusion of K as well the effects of the interconversion between K_0 and K. From this and noting that at steady state $XK \propto X.K$, we find that information about the substrate is backpropagated to the active kinase at steady state, while the phosphatase profile remains uniform. Note that the phosphatase is constitutively active.

Localization of enzymes. Following this, we examine another point associated with localized enzymes, discussed in the text. We pointed out that with X highly diffusible, localizing enzymes can result in an elevated X^* level relative to (a) the case where X is non-diffusible or equivalently (b) the case where the enzymes are present uniformly. Furthermore, we show how this elevation can even compensate for a product inhibition. We consider the case of the CMC. To focus on the essential point, we examine the case where both kinase and phosphatase act in an unsaturated regime. In this case, the steady state for the substrates is determined by the equilibrium condition $X^*/X = k_{f1}K_{tot}/k_{f2}P_{tot}$ where k_{f1}, k_{f2} represent the forward and backward rate constants (the enzymes are essentially entirely in the free form) along with the conservation condition $L_1X^* + (L + L_1)X = (L + L_1)X_{tot}$. Note an important point implicit in this conservation is the fact that substrate is present everywhere initially at a total local concentration X_{tot}, in the form of unmodified substrate. This results in a steady state for X^* given by

$$X^* \quad = \quad X_{tot}/[k_{f2}P_{tot}/k_{f1}K_{tot} + L_1/(L + L_1)]$$

This expression already highlights the local elevation introduced by the fact that diffusible substrate everywhere from the domain: this is seen in the factor $L_1/(L + L_1)$ in the denominator. Relative to the case $L = 0$ where this term equals 1, this term introduces a reduction in the denominator, and corresponding elevation in output concentration. This can result in a substantial elevation depending on kinetic parameters.

We now show how this effect can even trump a product inhibition. Assuming X^* deactivates the kinase enzyme, the active kinase concentration $K = K_{tot}/(1 + \alpha X^*)$. The steady state of X^* is obtained by solving the quadratic equation which results from

$$X^* \quad = \quad X_{tot}/[k_{f2}P_{tot}(1 + \alpha X^*)/k_{f1}K_{tot} + L_1/(L + L_1)]$$

However it is easy to see that for any value of α, the increase in denominator caused by the product inhibition is due to the term $\alpha X^*.k_{f2}P_{tot}/k_{f1}K_{tot}$ which is always bounded by $\alpha X_{tot}.k_{f2}P_{tot}/k_{f1}K_{tot}$ and that in a range of parameters, this can be offset by the reduction in the denominator owing to species diffusing from outside the diffusion region $1 - L_1/(L + L_1) = L(L + L_1)$.

Writing the resulting quadratic equation for X^* as

$$X^*[k_{f2}P_{tot}/k_{f1}K_{tot}+1]+X^*[\alpha X^* k_{f2}P_{tot}/k_{f1}K_{tot}-L/(L+L_1)] \quad = \quad X_{tot} \tag{13}$$

we see that the last two terms arise from a combination of product inhibition and the X contribution from outside the domain. In the absence of these terms we obtain the steady state of the co-localized system. Now for that value of X^*, if the sign of the second term is negative, then this signifies that this level will be less than the eventual steady state, meaning that there will be a net elevation of steady state. The requirement for that is

$$\alpha k_{f2}P_{tot}/k_{f1}K_{tot}.X_{tot}/(1+k_{f2}P_{tot}/k_{f1}K_{tot}) \quad < \quad L/L+L_1 \tag{14}$$

This provides an explicit criterion connecting product inhibition and the role of the spatial design, in determining what the net outcome is.

3.2 Bifunctional enzymes and spatial designs.

We present basic aspects of kinetics of substrate modification by bifunctional enzymes, followed by discussions on concentration robustness, bistability, feedback and thresholds. We begin by discussing a few aspects of the kinetics of the substrate modification by bifunctional enzymes. We first focus on the case where both substrate modifictaion reactions proceed by hydrolysis, with no phosphotransfer involved.

Kinetics of bifunctional CMC. In this model of bifunctional enzyme based substrate modification, the relative balance of two forms E^* and E is determined by an external signal or basal interconversion reactions (in general). The key feature to note here is that at steady state there is no net consumption of enzymes in the substrate modification cycle. This means that the interconversion reactions of the enzymes have to be balanced at steady state and the ratio $E^*/E = k_f/k_r$, which is the interconversion equilibrium constant (k_f, k_r denote forward and backward interconversion rate constants between E, E^*). This is completely independent of the total enzyme amount.

First, we consider the case where substrate modification reactions all proceed in the unsaturated regime. Based on an analysis of the modification cycle similar to the analysis of the monofunctional CMC, we have X^*/X is proportional to E^*/E which is independent of the total enzyme concentration. The

conservation of the substrate implies that $X + X^* = X_{tot}$ and so X^* is independent of the total enzyme concentration as well.

We now examine the case where substrate modifications may not be in the unsaturated regime. Now, just like in the covalent modification cycle with separate kinase and phosphatase, at steady state, we have $k_2 E^* X = k_4 X^* E$. Therefore the ratio X^*/X is proportional to the ratio of free enzyme concentrations E^*/E. Now considering the dynamics of the bifunctional module, and the interconversion of E and E^*, we find that at steady state $E^*/E = k_f/k_r$ where k_f and k_r are respectively the forward and backward rate constants for the interconversion between E and E^*. Notably, this is independent of the total enzyme concentration. Therefore, we conclude here too, that X^*/X is independent of the total enzyme concentration. This has been seen computationally.

Now the conservation of substrate implies that $X + X^* + XE^* + X^*E = X_{tot}$. This is complemented by the enzyme conservation $E^* + E + E^*X + EX^* = E_{tot}$. Since E^*/E at steady state is a constant independent of E_{tot} and the concentration of the complexes is proportional to the product of that of the enzyme and that substrate, we find that just as in the simpler model, we have $X^*(\beta + kE) = X_{tot}$ and $E = E_{tot}/(1 + \alpha X^*)$ where k , β and α are constants independent of the total enzyme concentration. Thus we see the same trend: with X^* being essentially constant at lower levels of total enzyme concentration and gradually becoming lower. Overall this explains how in this model X^* is relatively constant over a range of total enzyme concentration, while X^*/X is always independent of the total enzyme concentration.

Feedback regulation. We examine a basic case of feedback where the modified substrate concentration affects the balance of enzyme forms. We will assume that the feedback does not involve any sequestration of substrate. In considering feedback the only difference is that the ratio of kinase to phosphatase concentrations depends on the modified substrate. Assuming that the feedback is linear in the concentration of the modified substrate, we have $E^*/E = (k_f + k_b X^*)/k_r$. Here k_f, k_r are basal rate constants for the interconversion between E and E^*, k_b is the feedback constant.

The effect of feedback can be examined in detail by coupling this with the dynamics of the substrate modification. Now if the substrate modification occurred in the unsaturated regime, then we have $X^*/X = k_1 E^*/k_3 E$, where k_1 and k_3 are respectively the rate constants for the substrate modification and demodification reactions. This along with the above equation results in $X^*/X = (k_1/k_3)(k_f + k_b X^*)/k_r$. This coupled with the conservation condition $X + X^* = X_{tot}$ gives the expression for X^*. We see again that here X^* and X^*/X are independent of E_{tot}. In this regard we also note that in the special case

$k_f = 0$, then we have $X = k_3 k_r / k_1 k_b$, $X^* = X_{tot} - X$, which results in a transcritical bifurcation at $k_3 k_r / k_1 k_b = X_{tot}$. This is discussed in Fig. 7(A), where it is shown that for high enough reverse rate constant k_r (Sb in that figure), the output $X^* = 0$. Thus there is a clear threshold behaviour associated with the transcritical bifurcation. Note that the transcritical bifurcation is not seen when $k_f > 0$ although echoes of this behaviour are seen for small values of this constant. Now suppose substrate modification does not occur in the unsaturated regime. We note that $k_2 X E^* = k_4 X^* E$. Since the concentration of the substrate complexes is proportional to that of the enzyme and substrate, we again have a qualitatively similar situation as above: $E^*/E = (k_f + k_b X^*)/k_r = \alpha/\beta(X^*/X)$ where α and β are the constants relating the concentrations of the complexes to the products of concentrations of the respective enzymes and substrates. These constants are easily obtained in terms of the relevant enzyme substrate binding, unbinding and catalytic constants. Now the concentration of modified substrate is obtained from the above equation along with the conservation of substrates, which is identical to what was considered in the previous case (feedforward regulation) without feedback. In this regard, we make the following conclusions: (i) If $k_f = 0$ we again get a transcritical bifurcation as k_r is varied. Basically if k_r exceeds a certain threshold then $X^* = 0$ (ii) Now when k_f is non-zero, we find that when the total enzyme concentration is increased, then neither X^* nor X^*/X remains a constant, and this is directly a consequence of feedback (this result can be compared with the previous cases).

Variations in feedback. We now expand this study to examine a co-operative effect in feedback: here we simply replace the feedback term by $k_b(X^*)^2$. When this is done only one equation changes: $E^*/E = (k_f + k_b(X^*)^2)/k_r$. When combined with the substrate modification kinetics we note the following: (i) Even if the substrate modification in the unsaturated regime the resulting steady state is capable of exhibiting bistability. This confirms the result shown in Fig. 6(A). (ii) Naturally bistability is also possible even when the substrate modification kinetics is far from the unsaturated regime as well. In the latter case, analysis of the model of enzymes as well as substrate modification at steady state, incorporating substrate conservation readily shows that bistability can be obtained.

From the above we see that feedback can give rise to two kinds of qualitative behaviour. When the feedback is linear in the substrate, it is possible (when $k_f = 0$) to obtain a threshold effect as a consequence of a transcritical bifurcation. When the feedback is nonlinear, one can obtain bistability, which is not surprising, given the co-operativity in the feedback. This then suggests the question: is it possible to obtain bistability with just linear feedback? The detailed analysis presented in the study of an autocatalytic module [Krishnan *et al.*, 2014] indicates that linear feedback, coupled with enzyme and

substrate conservation are sufficient to give bistability. In fact in that case the sufficient additional criterion to get bistability is significant sequestration of the phosphatase (i.e. dephosphorylation of substrate far from the unsaturated regime). In the current context, we see two ways in which bistability can be obtained: (i) Substrate dephosphorylation far from unsaturated kinetics (ii) Adding an extra interaction between enzyme and substrate such as X binding to E to give a complex XE which has the effect of sequestering the phosphatase. A simple analysis shows that this is capable of inducing bistability. Note that this interaction is an example of an extraneous interaction between enzyme and substrate, which has been studied earlier [Seaton & Krishnan, 2012].

Substrate modification through the bifunctional TCS. Some analysis of the bifunctional TCS has been performed by Batchelor and Goulian. We note a few basic points relevant to our study. (i) As mentioned earlier E^*/E now depends on the substrate and so is no longer independent of the substrate cycle. This is true even if the substrate modification is in the unsaturated regime. (ii) When the substrate modification is in the unsaturated regime and the "phosphotransfer contribution " to the balance of enzyme forms is negligible, then E^*/E is essentially constant and then X^* and X^*/X are essentially constant independent of E_{tot}. Note that an increase of total substrate amount will eventually introduce a nontrivial substrate dependence on E^*/E. When the substrate modification moves out of the unsaturated regime, the E^*/E and hence X^*/X is essentially constant, but the X^* can exhibit a dependence on parameters. However if the enzyme amounts are much smaller than substrate amounts, then the substrate conservation is dominated by $X + X^* = X_{tot}$, and consequently X^* is essentially constant, independent of total enzyme concentrations. (iii) Finally even if the phosphotransfer contribution to the balance of enzyme forms is not negligible, if the enzyme amounts are much less than the substrate total amounts, then $E^*/E = (k_f + k_1 X^*)/k_r$, $X^*/X \propto E^*/E$ and $X^* + X = X_{tot}$. Solving these indicates that X^* and X^*/X are essentially independent of E_{tot}.

Spatial designs. The first basic point to note regarding spatial designs of bifunctional enzymes is that irrespective of the relative localization of enzymes and substrates a situation of zero concentration of X or X^* will not arise, and this simply stems from the fact that at any location there is always a pair of enzymes functioning.

Concentration robustness. We now turn to a basic aspect of bifunctional enzymes: absolute concentration robustness. We have already seen that absolute concentration robustness arises from a situation where enzyme amounts are much less than substrate amounts: this is true in both the CMC and the TCS, and explained above. Now if we examine spatial designs where the enzyme is in one location and

say X^* is able to exit the compartment, we find that this results in an erosion of the aboslute concentration robustness effect and the associated margin (see Fig. 5 (B) of the text). This can be simply explained by the fact that the amount of substrate in the enzyme compartment is reduced, so that the main conditions for absolute concentration robustness no longer hold good. This is seen in both the CMC and the TCS.

Another aspect of absolute concentration robustness emerges, however. The text (Fig. 5) shows that in the case of the CMC, for the case of X and X^* diffusing, the output is insensitive to the enzyme concentration over a broad range (Fig. 5(C) and Fig. S3(A)). A similar behaviour is seen when X^* diffuses. Interestingly, the ODE does not show this behaviour over this range of enzyme concentations.

This is surprising, since this is a case where the substrate amount should be far from dominating the enzyme amounts locally. This can be explained as follows. Consider the CMC. At steady state X and X^* are uniform and the conservation condition is given by $L_1(XE^* + X^*E) + (L + L_1)(X + X^*) = L_1X_{tot}$. Now for a fixed amount of enzyme, when $L >> L_1$, the contribution of the complexes becomes negligible because they are present in a small region of the domain. Note in particular that when L becomes progressively larger, X and X^* eventually dwarf the respective complex terms XE^* and X^*E in the conservation condition, noting that the complex concentrations are proportional to the product of the substrate and enzyme concentrations, and that the enzyme concentration is bounded by E_{tot}. This means that the complex terms in the above equation can be neglected relative to the free substrate terms, noting that each of them is proportional to a free substrate.

Thus what we find is that $X + X^* \approx X_{tot}L_1/(L + L_1)$ and this is independent of total enzyme amounts. Furthermore, as noted earlier, X and X^* satisfy the kinetic equations and $X^*/X \propto E^*/E$ which is independent of total enzyme amounts. From both of these we find that X^* is independent of total enzyme amounts. Note that our analysis relies on a fixed range of total enzyme amount, and increasing L, the latter effect eventually dominating.

In the case of a TCS, we found that for a lower total substrate concentration to start with (and associated total enzyme range), an exactly analogous behaviour could be seen, for instance when X and X^* were diffusing (see Fig.5 (D)). Again, there was no ACR to start with. This can be explained in analogous terms X^*/X is still independent of total enzyme amount, it being equal to the ratio E^*/E which in turn depends on only rate constants and X and the same argument holds good. In fact when the total substrate concentration is relatively low X^*/X is determined primarily by the ratio of basal interconversion rate constants between E and E^*, and consequently, in multiple cases the results obtained here even quantitatively come close to the CMC.

The question to ask is why the behaviour in a TCS manifests only in a lower level of substrate. For a higher level of substrate, in fact a relative insensitivity to total enzyme amounts exists, but if the enzyme amount are varied across a range of concentration comparable to substrate concentrations, then the enzyme contribution to the substrate conservation becomes substantial, for this fixed length of domain, and so we do not see any robustness over this range.

In summary, if we vary enzyme total amounts over a range comparable to substrate amounts, then a robustness is seen when the domain size is large enough and for reasonably large domains, when the substrate total amounts are moderate (here and below, when we discuss the domain size being large enough, unless otherwise mention, we assume this is done by increasing the size of the ambient domain, L).

We now discuss the fact that when X^* diffuses, then again we can see a situation where X^* is essentially independent of the total enzyme concentration over a range. To understand this, we first note that X^* becomes uniform at steady state. Now in the conservation condition, we have $L_1(XE^* + X^*E + X) + (L + L_1)X^* = L_1X_{tot}$. Now when L becomes large, if the $(L + L_1)X^*$ dominates the LHS, then we find that this establishes a level for X^* independent of E_{tot}, and in fact independent of kinetic parameters. Note however that depends on all other terms being neglected with respect to X^*. This is valid in certain kinetic regimes but not others. For instance, as shown in Fig. S4 and S5, depending on the balance of calytic constants invovled in the modification reactions between X and X^*, if the reverse modification catalytic constant is much higher, then for this length of the domain, the X^* alone diffusing does not give rise to X^* being independent of E_{tot}: the reason is simply that the other substrate has a significant higher concentration which partially offsets the fact that it is present in a smaller domain. In this case it is when X is diffusing that X^* is independent of E_{tot}: the reasoning is that X is constant due to dominating all terms in the conservation condition. Furthermore by examining the ratio X^*/X we see that it is independent of E_{tot} and consequently X^* is independent of E_{tot}. Parallel insights are obtained in the CMC and the TCS.

Effect on feedback: thresholds and bistability.

We start by considering the bifunctional enzyme localized in one location (compartment size L_1 in a surrounding domain of size L). If all substrates and enzymes are localized in this location and are non-diffusible, and X^* diffuses out then, at steady state as seen previously, the variables satisfy the kinetic equations with the modified conservation condition: $L_1(X + XE^* + X^*E) + (L + L_1)X^* = L_1X_{tot}$.

The text has computationally shown that having X^* exiting the compartment and spreading in the ambient domain can destroy bistability. This has been shown for both the CMC model (which has an

explicit feedback) and the TCS model with a deadend complex. The results in the text are established both through simulations in the PDE as well as calculation of the steady state directly with the modified conservation equation.

First consider the CMC. The loss of bistability can be seen in the limit when L becomes very large. From the conservation condition, we see that $X^* \leq X_{tot}L_1/(L+L_1)$, and clearly as L becomes large, X^* becomes small. This automatically precludes two stable steady states with sufficiently separated levels, which is the hallmark of bistability in such contexts. Further analysis also shows why bistability is destroyed by the dilution due to X^* diffusing. The fact that as L becomes larger, we have an upper bound for X^* which gets smaller indicates that the feedback from substrate to enzyme is progressively weakened. Since this feedback is needed for bistability in the CMC model studied, we see that increasing the domain size will eventually lead to a loss of bistability.

Now suppose both X and X^* are diffusing (with equal diffusivity) and exiting the compartment. We can analyze the consequences of this at steady state. For simplicity we start with the case where the substrate modification occurs in the unsaturated regime (i.e. complexes are negligible or absent). Now, adding all the substrate equations, we find that

$$\frac{\partial^2(X+X^*)}{\partial\theta^2} = 0 \tag{15}$$

This implies $X + X^*$ is a constant, which then equals $X_{tot}L_1/(L+L_1)$. In other words, the steady state satisfies the ODE model, with a modified conservation condition $X + X^* = X_{tot}L_1/(L+L_1)$. This reduction of total amount in the compartment is sufficient for a loss of bistability as computational results show. This can be simply seen as follows. For a sufficiently large L, the total substrate concentration at any location can be made sufficiently small. Since bistability in this model depends on feedback from substrate to enzyme, reduction in substrate levels sufficiently can eliminate the possibility of bistability.

Following on from there it is worth asking whether bistability can be maintained even when these species exit the compartment. In fact there is a simple way to counteract the dilution effect: simply start with a proportionally higher amount initially (in fact $X_{tot}(L+L_1)/L_1$). This will guarantee that with diffusion there is a sufficient amount left in the domain to maintain bistability (this in turn boils down to having a sufficient amount of substrate everywhere). Computational results in the main text demonstrate that by starting with this higher amount allows for bistability, which is then retained even following diffusion of the species.

This basic insight which is seen most transparently in this simple case is also relevant in the case

where substrate modification does not occur in the unsaturated regime. Here again, at steady state $X + X^*$ is a constant independent of spatial location(say X_0) and further from the conservation condition $L_1(X^*E + XE^*) + (L + L_1)(X + X^*) = L_1X_{tot}$. Here the complexes do enter the conservation condition. Here again, we find that when the complex concentrations are not large that the same essential conclusions as seen earlier are obtained.

We also briefly discuss the TCS model with dead-end complex model, to show that the dilution effect of X^* diffusing also has the effect of abrogating bistability here. Let us consider the case of X^* exiting the compartment. As L becomes large X^* becomes progressively small, being bounded by $X_{tot}L_1/(L + L_1)$. We sketch out how this dilution effect dismantles the key ingredient for bistability in the TCS dead-end complex model. We sketch the essential steps involved here (refer to Fig. 1(D)): (i) There are three types of complexes involving the enzyme$E$ or E^*. By focussing on the steady state of XE^* we find that $[XE^*] \propto [X][E^*]$. This stems from the fact that the only term producing this complex is the relevant enzyme substrate binding, while the removal is linear in the complex concentration. (ii) Consider the steady state for X^*: the contributions to this involve binding to enxymes and unbinding from complexes. This results in an equation of the form $[X^*](\alpha_{11}[E] + \alpha_{21}[F]) = \alpha_{31}[X^*E] + \alpha_{41}[X^*F]$, where all the α terms are constants. Since $[X^*] \to 0$ noting the positivity of concentrations, it follows that both $[X^*E]$ and $[X^*F]$ approach zero as well. (iii) In the case of X^*E, examining the network and imposing steady state implies that $\alpha_1[X^*][E] + \alpha_2[XE^*] = \alpha_3[X^*E]$, where $\alpha_1, \alpha_2, \alpha_3$ are all constants. Now by using point (ii) $[XE^*] \to 0$, and from point (i) it follows that $[X] \to 0$ (the other posibility will lead to a contradiction violating enzyme conservation) (iv) Now examining the complex XE, we have an equation of the form $\alpha_{21}[X][E] + \alpha_{22}[X^*E] = \alpha_{23}[XE]$. From points (iii) and (ii) it follows that $[XE] \to 0$. Taking all these points together, we find that the concentration of all complexes approaches zero which then means that most of the enzyme is in the free form, a condition which precludes bistability

Perturbation of thresholds. So far we have discussed how the diffusion of one or two species out of the compartment can result in a loss of bistability, as discussed in the text. We now concern ourselves with the situation of simple linear feedback in the CMC model from modified substrate X^* affecting the transition from E to E^*. If the basal conversion constant from E to E^* were exactly zero, this would represent a transcritical bifurcation. This case is also worth examining as it provides insights into the case of small basal conversion. In the text, we asserted that depending on which species was exiting the compartment, it was possible to obtain a non-zero steady state (reduced) or a zero steady state, for the modified substrate concentration.

We establish this point here. We will assume enzymatic modification of substrate in the unsaturated regime (forward and backward modification rate constants k_1, k_3). We start by noting that at steady state $E^*/E = k_b X^*/k_r$ and that at steady state, the diffusing species attain a spatially uniform concentration profile. If only one species is diffusing, this is established exactly as above. In the case of two species diffusing we assume, both substrates diffuse equally. The analysis is again exactly as done above. Now, from this, it follows that the substrate concentrations satisfies the ODEs of the kinetics and so $X^*/X = k_1 E^*/k_3 E$ which from the equation on the balance of enzyme forms results in $X^*/X == (k_1 k_b/k_3 k_r)X^*$. This is supplemented by the appropriate conservation condition.

Case (i) Only X^* diffuses. In this case, the conservation condition is of the form $L_1(X) + (L + L_1)X^* = L_1 X_{tot}$. From the above equation, the steady state is either $X^* = 0$ or $X = k_3 k_r/k_1 k_b$. The latter steady state is obtained if this value is less than X_{tot}. Now in the system localized to one compartment, we did start with a non-zero steady state, which meant that $k_3 k_r/k_1 k_b < X_{tot}$. This being the case we see that this continues to be the steady state, with X^* obtained from the conservation condition $L_1(X) + (L + L_1)X^* = L_1 X_{tot}$. Clearly we obtain a positive concentration for X^* which is reduced (relative to the case of the completely co-localized system) due to dilution. This was shown in the main text. Note that the steady state of X is unaffected by dilution.

Case (ii) Only X diffuses. In this case, the only change is the conservation condition, which is now $(L + L_1)X + L_1 X^* = L_1 X_{tot}$. A non-zero level of X^* is obtained only if $(L + L_1)(k_3 k_r/k_1 k_b) < L_1 X_{tot}$, but this is no longer guaranteed from the non-zero steady state of the co-localized system. In fact it is easy to see that if L becomes large enough, this condition will be violated. In such a case $X^* = 0$ is the only steady state. In other words diffusion of X (in contrast to diffusion of X^*) results in a zero steady state output for the modified substrate concentration.

Case (iii). Both X and X^* diffuse. Here the conservation condition becomes $(L + L_1)(X + X^*) = L_1 X_{tot}$. Again, for the same reason as the previous case, for a sufficiently large domain, the steady state $X = k_3 k_r/k_1 k_b$ is precluded as this would have to be less that $X_{tot} L_1/(L + L_1)$. As a consequence, the only steady state is $X^* = 0$. This demonstrates the point made in the text.

3.2.1 Enzyme localized in two locations.

Dose Response curves. We now briefly examine some aspects of the (local) dose-response curve when the (bifunctional) enzyme (CMC model) is localized in two locations , with a particular focus on the case where the balance of enzyme forms is different at the two locations. To do this we will start with the

following assumptions: (i) Purely feedforward regulation affecting the balance of kinase and phosphatase forms (i.e. no feedback). (ii) Kinetics in the unsaturated limit for the substrate modification.

Suppose the substrate is present everywhere and is non-diffusible. Then at each location, the steady state of the substrate is determined by the kinetics. In particular, the modified substrate concentration only depends weakly on the total enzyme concentration. This has already been discussed in the context of the kinetics of the bifunctional module. Now suppose the substrates are weakly diffusible. The only difference here is that at steady state, the substrate concentration profile between the two poles is linear, and the concentration at the poles is a minor perturbation of the non-diffusible case. Thus in this case, we can make the following conclusions: (i) Varying the total enzyme concentrations at the two locations results in a minor change (ii) Varying the total enzyme concentration even in one location also leads to a minor change. Thus the feature of weak dependence on total enzyme concentration, seen in the kinetics, is seen here as well.

We build on this to examine the opposite limit, one where the substrates are highly diffusible. In this case the substrate concentration profiles are practically uniform. In this case, the substrate at steady state reaches a level, determined by the equation

$$X^*/X = \frac{k_{f1}E_1^*L_1 + k_{f2}E_2^*L_2}{k_{r1}E_1L_1 + k_{r2}E_2L_2} \tag{16}$$

where k_{f1}, k_{f2} are the forward substrate modification rate constants and k_{r1}, k_{r2} are the reverse modification constants.

Now $E_1^* = \alpha E_{1,tot}$, $E_1 = (1-\alpha)E_{1,tot}$,$E_2^* = \beta E_{2,tot}$, $E_2 = (1-\beta)E_{2,tot}$. Here α and β are constants which determine the balance between then kinase and phosphatase forms at location 1 and location 2 respectively. Thus the above equation simplifies to

$$X^*/X = \frac{k_{f1}\alpha E_{1,tot}L_1 + k_{f2}\beta E_{2,tot}L_2}{k_{r1}(1-\alpha)E_{1,tot}L_1 + k_{r2}(1-\beta)E_{2,tot}L_2} \tag{17}$$

We also note that in this kinetic regime there is hardly any substrate present in complexes. Consequently the substrate conservation condition contains terms which are (essentially) independent of total enzyme amounts.

From these points, we can obtain X^* and we note the following points: (i) If $E_{1,tot} = E_{2,tot}$, the output is independent of the total local concentration of enzyme (locally) at each of the locations, i.e. $E_{1,tot}, E_{2,tot}$. Thus any increase of total amounts of enzymes locally (equally at both locations), leaves the output invariant. Note however that if $L_1 \neq L_2$, an equal change of total amount of enzyme at each

location $E_{1,tot}L_1, E_{2,tot}L_2$, will also leave the output unchanged. (ii) Even if this restriction is not met, a proportional increase in total enzyme amount leaves the output unchanged (iii) Now suppose the modification rate constants are the same at each location, and $\alpha = \beta$. Then, it is easy to see that the output does not depend on either enzyme amount. In this case it is possible to vary either enzyme amount, or both, and the output is independent of the total enzyme amount. This is also true if $k_{f1}\alpha = k_{f2}\beta$ and $k_{r1}(1-\alpha) = k_{r2}(1-\beta)$, though that is a special condition combining the relative enzymatic balances as well as substrate modification.

Substrate modification not in the unsaturated regime. In the above if we relax the assumption of substrate modification being in the unsaturated regime, but have the substrates being highly diffusible then we note that (i) Substrate profiles are essentially uniform (ii) The overall mass balance for substrates involve a balance between modification in the forward and reverse directions, each of which can be described by Michaelis Menten like terms, after carefully incorporating enzyme conservation (two terms for each direction of modification). The total amount of enzyme (assumed equal, at both locations) appears in every one of these terms, and is consequently factored out. (iii) Now in the substrate conservation, for large enough domains, this is dominated by the substrates present everywhere in the domain (which can trump the effect of substrate present in enzyme complees (assumed non-diffusible) as already seen previously). In such a case we see that substrate concentrations do not show a dependence on variation of total enzyme amount (equally at each location), even if the balance between enzymes is different at the two loocations.

Taken together, this demonstrates how echoes of the basic features seen previously are present even when there is localization of enzyme at two poles.

3.3 Switching enzymatic activity.

In the main text, we focussed on how the dominant activity of the enzyme could be switched, temporally and spatially. To that end, we focussed on three central "levers" a cell may have: imposing direct activity regulation, controlling localization and feedback. We explore these aspects, foucussing primarily on the CMC system. Our analysis below largely focusses on "upstream" factors which affect the balance of enzyme forms (i.e. factors independent of substrate, such as activity regulation and localization). This "upstream" analysis is sufficient for the CMC, since (apart from feedback) the effect of substrate being modified is to sequester enzymes, without affecting the relative balance of enzyme forms. In the case of the TCS, the role of the substrate on affecting the balance of enzyme forms has been explicitly probed

computationally in the main text: there, the reasons for the observed behaviour can be understood through a combination of basic "upstream" analysis, along with the distinct effect of phosphotransfer, which is discussed in the main text.

3.3.1 Activity regulation.

One of the direct ways of altering activity is by direct activity regulation, by which we mean that a cell can directly alter the balance between kinase and phosphatase forms. Here we briefly explore how activity regulation, along with spatial organization can result in an altered balance of kinase/phosphatase activity. In order to do this, we consider the following situation. The default state of the enzyme is assumed to be phosphatase dominant.

We are concerned with the locations of three sets of species: the enzymes, the substrates and the activating signal. It is assumed that the diffusivity, where relevant, of both substrates is the same, likewise for the two enzyme forms. We considered 3 possibilities for the location of species: (i) At one (designated) pole/location (ii) At both poles and (iii) Everywhere. In the last case, we examine the scenario where the species is essentially non-diffusible as well as that where the species is highly diffusible. This results in a set of 27 different cases, with additional variations, which have all been enumerated and analyzed.

A number of cases can be analyzed very simply. If the substrate is localized to one pole, in order to have the enzymatic activity switched at that pole, it is necessary for the enzyme to be at that pole (it can be present at both poles, or everywhere). Furthermore the activity regulating signal must also be present at that pole. If the enzyme is localized at that pole (or even both poles) the fact that the activity regulating signal is present where the substrate is present is sufficient. If however the enzyme is present everywhere, and is diffusible, then this (localized activating signal) will not result in switching, if the pole is a small fraction of the domain. This simply follows from the fact that a narrow region of localized switching cannot alter the global balance, given the basal state of the enzyme being much more phosphatase dominant. The steady state of enzyme forms emerges as a balance between global regulation converting it from phosphatase to kinase, and the other way around. Since the latter is dominant for most of the domain, it follows that in such a case a switching in activity will not result.

If the substrate is localized in two poles (assumed equally), then to get a clear cut switching in activity, the above conclusions apply, with the enzymes needing to be in both the locations along with the activity regulating signal. If the enzyme or activity regulating signal is present in only one location, then at best the activity will be switched in that location (this will correspond to a spatial switching). In such a case

the overall (average) activity of the enzyme (determined by the extent of substrate modification) will be balanced between kinase and phosphatase, as it is switched in one pole but not the other. Furthermore if the enzyme is present everywhere (and non-diffusible), then the presence of activation signal at both poles will have the desired effect. However even with an activating signal at both poles, if the enzyme is present everywhere and diffusible, then this will not result in the switching for an identical reason to what was considered previously.

Now we consider the substrate present everywhere. We consider the case where the substrate is diffusible. Basically in this case it is necessary and sufficient that the enzyme be present, wherever it is, is present with the activating signal. If the enzyme is present in either one or both poles (or even everywhere) with the activation signals, this is sufficient. If the enzyme is present in both poles, but the activation signal only in one, there will be a balance between kinase and phosphatase activity. If the enzyme is present everywhere and activation signal present in one or two poles this will not result in switching.

We see a difference between the enzyme diffusible case and the substrate diffusible case relative to its upstream regulator. If the enzyme is highly diffusible, a localized presence of activation signal cannot alter its dominant activity (as it is defeated by the preponderance of the basal balance over the domain). In the case of the substrate being diffusible, such a basal balance does not exist (it is determined entirely by the enzyme), and so a localized enzyme (of course with activity signal) can result in the switching of activity.

Taken together we see how the spatial organization/localization of activation signal, enzyme and substrate may result in switching of the activity of the enzyme temporally and spatially.

3.3.2 Localization.

In this subsection, we focus on analytical results pertaining to how the dominant activity of an enzyme can be switched using localization (along with possible feedback (assumed linear)). We start with the case where enzyme and substrate are localized in the same location. We first briefly discuss the effect of feedback.

Feedback. In the most basic form we have from above $E^*/E = (k_f + k_b X^*)/k_r$, which comes out of the steady state of the ODE model. This is coupled to the dynamics of the substrate modification. The first, most basic point to make is that feedback can cause a switching in (dominant) activity. This is most simply seen when the substrate modification occurs close in the unsaturated regime. In this simpler case, we have $X + X^* = X_{tot}$ and $X^*/X = \alpha E^*/E$. From this, it follows that
$X^* = X_{tot}\alpha(E^*/E)/(1 + \alpha E^*/E)$. Substituting this into the equation above and eliminating substrate

concentrations we have $E^*/E = k_f/k_r + k_b/k_r X_{tot}(E^*/E)/(1 + \alpha E^*/E)$. A simple analysis of this equation shows that E^*/E increases as a consequence of feedback, and consequently the dominant activity can be switched.

Analysis of Localization. We now turn to another mode of regulation which can be invoked by a cell: localization. We present a slightly simpler and less cluttered terminology for the purposes of analytical work. We focus on using the ODE model including localization: this corresponds to the enzyme in one location, which may be subject to some active mechanism for localization (the localization network depicted in Fig. 1(F)). We study the effect of localization in two parallel ways. The first is by examining a selection of special cases of this localization network to make specific points ("Analysis of simplified cases.") Following this, we analyze the localization network in full, and make inferences therefrom ("Localization through a direct analysis"). In both cases we examine both symmetric (equal) and asymmetric (biased) localization. In both cases we assume an abundance of localization sites.

Analysis of simplified cases. Much of the basic understanding comes from this simpler cases and is the basis for understanding more complex cases. In the simplest case, we consider a scenario where both E and E^* can become bound, the bound forms being E_b and E_b^* respectively. The binding and unbinding constants of E are denoted by k_{be}, k_{ue} and those of E^* are k_{bes}, k_{ues}. We first consider the absence of feedback and assume that the dominant form of the enzyme is phosphatase. We assume that there is no feedback and thus $k_f < k_r$. Furthermore, as a simplification, we assume that the localized forms do not interconvert. In such a case, the localization reaction network, has an open chain topology. In such a case the steady state corresponds to equilibrium between every pair of reactions. Now at steady state $E^*/E = k_f/k_r$, $E/E_b = k_{ue}/k_{be}$, $E^*/E_b^* = k_{ues}/k_{bes}$. This is supplemented by the conservation condition for enzymes. From this, we find that

$$\begin{aligned} E_b^* &= E_{tot}/(1 + k_{ues}/k_{bes} + k_{ues}k_r/k_{bes}k_f + k_{ues}k_r k_{be}/k_{bes}k_f k_{ue}) \\ E_b^* + E^* &= E_{tot}(1 + k_{ues}/k_{bes})/(1 + k_{ues}/k_{bes} + k_{ues}k_r/k_{bes}k_f + k_{ues}k_r k_{be}/k_{bes}k_f k_{ue}) \end{aligned} \quad (18)$$

Now if we consider the case of symmetric localization (here $k_{bes}/k_{ues} = k_{be}/k_{ue}$), then simplifies

$$E_b^* + E^* = E_{tot}/(1 + k_r/k_f)$$

Now if under basal conditions $k_r >> k_f$, we find that the total enzyme in the kinase form is a negligible fraction of the total enzyme concentration. In other words, symmetric localization cannot alter the dominant activity of the enzyme.

Building on this, we consider the case of asymmetric localization. Now if we choose k_{ues}/k_{bes} small (this corresponds to strong binding of the kinase) so that the product of this and k_r/k_f is small , but at the same time k_{be}/k_{ue} also small, then it is easy to see from the above general expression that $E^* + E_b^*$ can become close to the total enzyme concentration: in other words the kinase form dominates. This demonstrates that the asymmetry in localization can switch the dominant activity of the enzyme.

Interconversion of localized forms. In the model we have considered above, the localized forms do not interconvert. We now examine the possibility that the localized forms interconvert, in exactly the same manner (with the same rate constants) as the free enzyme forms. We now investigate the effect of this interconversion. We start with the case of symmetric localization. Thus here we will assume that $k_{be} = k_{bes}$, $k_{ue} = k_{ues}$ and thus $k_{ue}/k_{be} = k_{ues}/k_{bes} = \gamma$. Now when the binding is strong, γ is small. Now by adding the equations for E and E^* and examining this at steady state, we have $(E + E^*) = \gamma(E_b + E_b^*)$. This then means, from the conservation of enzyme that $E_b + E_b^* = E_{tot}/(1 + \gamma)$ thus indicating that a significant fraction of the enzyme is in the bound form and a negligible fraction is unbound.

We then examine the equation for the bound kinase E_b^*. At steady state $k_f E_b + k_{bes} E^* = (k_r + k_{ues})E_b^*$. Now suppose $k_{bes} = O(1)$ and $k_{ues} << 1$. This is consistent with strong binding. Now, we already know from the strong binding assumption that when γ becomes small that $E + E^* = E_{tot}\gamma/(1 + \gamma)$ and this is small. Therefore the term $k_{bes}E^*$ can be made small. Furthermore on the RHS, the term involving k_{ues} can be neglected. This being the case, we have $E_b^*/E_b = k_f/k_r$ and this shows that most of the bound enzyme is phosphatase. Furthermore since most of the enzyme is bound, this tells us that the dominant activity of the enzyme remains a phosphatase. In essence in such a scenario, most of the enzyme is localized, and they maintain a balance similar to their free forms.

Interestingly, even if there is a feedback between substrate and the interconversion between E and E^*, as we have considered above, but no feedback acting on the bound forms, this exact same argument carries through, since the argument is based on the magnitude of γ, the binding and unbinding constants and the bound species only. This indicates that even if feedback is capable of making the dominant form of enzyme that of a kinase, symmetric localization along with interconversion of localized forms can counteract and compromise this effect, unless the feedback can act on the localized forms as well.

We now examine the effect of asymmetric localization. Here, due to the asymmetry of localization, the analysis is not as simple (however see more general analysis below). We wish to demonstrate the effect as seen in simulations that an asymmetry in localization can also be compromised by interconversion of

localized forms. To do this, we look at two extreme cases. In the first case, we examine the most extreme scenario that only the kinase form localizes (through the binding of the free form of the enzyme) and can do so very efficiently. This is the most extreme form of asymmetric localization. Interconversion of bound forms can occur, but bound E does not unbind (and neither does E bind). By performing an analysis of this system at steady state, noting that steady state in this system corresponds to equilibrium between every pair of reactions we have

$$\begin{aligned} E_b &= E_{tot}/(1 + k_f/k_r + (k_f/k_r)k_{ues}/k_{bes} + (k_f/k_r)k_{ues}/k_{bes}(k_r/k_f)) \\ E_b &= E_{tot}/(1 + k_f/k_r + (k_f/k_r)k_{ues}/k_{bes} + k_{ues}/k_{bes}) \end{aligned} \tag{19}$$

It is clear that when $k_{bes} >> k_{ues}$ and $k_r > k_f$ that a large fraction of the enzyme is in the bound phosphatase form. Intuitively strongly binding of kinase makes most of the enzyme bound, whereas the interconversion of bound forms with a balance strongly tilted towards the phosphatase ensures that most of the enzyme ends up in the bound phosphatase form. This clearly shows how even in one of the most extreme forms of asymmetric localization, that interconversion can compromise the effects of asymmetric localization in switching enzyme activity.

We now examine another tractable extreme case: we assume for simplicity in the full model that $k_{ues} = k_{be} = 0$. In other words, there is no unbinding of the kinase and there is no binding of the phosphatase. Without interconversion of localized forms, this would result in a situation where all the enzyme ends up as bound kinase. Here at steady state we have $k_{bes}E^* = k_{ue}E_b$. Using this and the fact that at steady state $E^* = (k_f/(k_r + k_{bes}))E$ and $E_b = ((k_r/(k_f + k_{ue})E_b^*$, we can obtain an expression for the total concentration of kinase

$$E^* + E_b^* = E_{tot}[1 + (k_{bes}/k_{ue})(k_f + k_{ue})/k_r]/[1 + (k_{bes} + k_r)/k_f + k_{bes}/k_{ue} + (k_{bes}/k_{ue})(k_f + k_{ue})/k_r] \tag{20}$$

When $k_f << k_r$, the dominant term in the denominator is the second term which is large and ensures that the total kinase concentration becomes small. In other words, even with an extreme form of asymmetric localization (while still allowing conversion between bound and unbound forms), interconversion can compromise the effect of asymmetric localization in switching the dominant enzyme activity. This is consistent with computational results.

Taken together, the preceding discussion shows that generally speaking symmetric localization does

not switch the dominant enzyme activity while asymmetric localization can do. However when localized forms interconvert, this works against the effect of localization in altering the dominant activity. As we have seen earlier, a sufficiently strong feedback from substrate to enzyme interconversion can switch the dominant activity of the enzyme. If feedback acts only on nonlocalized forms, then the situation is as follows: (i) In the case of symmetric localization, if there is no interconversion reaction of localized forms, then this feedback can alter the dominant activity of the enzyme. This is simply because we have seen that feedback is capable of this, and having symmetric localization simply makes most of the enzyme localized subsequently, without altering the balance. On the other hand if interconversion of localized forms occurs, then this can reverse the effect of feedback and bring the dominant activity of the enzyme to that of a phosphatase. In essence, once the enzymes are localized, since most of the enzyme is localized (which is the case for strong localization), the balance of enzyme is determined by the localized enzymes. This can also be seen in the analysis of symmetric localization above. However strong feedback may be, as a basis to altering the balance of the free enzyme forms, it creates a minor perturbation to the localized enzyme forms and their balance. Therefore, unless feedback acts also on the interconversion reaction of the localized form, the dominant enzyme activity will not be switched. (ii) In the case of asymmetric localization, feedback is not, of course, needed to switch dominant enzyme activity. As we have seen above, if interconversion of localized forms occurs, this can counteract the effect of asymmetric localization. In such a case feedback occurring at the level of both localized and non-localized enzymes can result in the switching of activity. Taken together, the main conclusion is that if interconversion of localized forms occurs, and if strong localization is present, then to alter the dominant activity of the enzyme, it is important to impact the localized forms directly, and this can be established by having feedback here.

All the above insights were obtained through ODEs, and correspond to situations where enzymes and substrates are non-diffusible. If the enzymes alone are diffusible, then the primary effect is that of reducing the total amount of enzyme at a given location and this does not cause any important qualitative change. This in turn is due to the bifunctional nature of the enzyme, and the fact that the total amount of enzyme does not affect substrate concentrations over fairly broad ranges.

On the other hand if the substrate diffuses while the enzymes are localized (in one location, say), the main qualitative effect caused in the context of the conclusions above, is that this results in dilution and weakening the effect of feedback. Thus this substrate diffusing can by itself compromise one of the main levers responsible for switching activity. The case of enzyme and substrate diffusing simultaneously can be studied as simple extensions of the cases above.

Localization through a direct analysis. We complement our above analysis of localization by a direct analysis of the localization of both enzyme forms as well as their possible interconversion. The steady state of this reaction network can be obtain directly, and this is facillitated by using computer algebra such as Maple. This allows us to draw a number of direct conclusions without considering limiting cases. The steady state of the system, allows one to estimate a number of ratios shown below. For compactness we have used the following shorthand notations: the numbers 1 and 2 in the subscripts (for binding and unbinding constants) refer to E and E^* respectively, k_1, k_{-1} denotes the basal conversion rate constants between E and E^*, while k_2, k_{-2} denote the interconversion rate constants between the localized forms. LE and LE^* denote the concentrations of localized forms of E and E^*. We assume an abundance of localization sites, whose presence is subsumed within the relevant kinetic binding constants.

$$\begin{aligned}
\frac{LE^*}{E^*} &= \frac{k_{-1}k_2k_{b1} + k_1k_2k_{b2} + k_1k_{b2}k_{ub1} + k_2k_{b1}k_{b2}}{k_{-2}k_1k_{ub1} + k_1k_2k_{ub2} + k_1k_{ub1}k_{ub2} + k_2k_{b1}k_{ub2}} \\
\frac{E^*}{E} &= \frac{k_{-2}k_1k_{ub1} + k_1k_2k_{ub2} + k_1k_{ub1}k_{ub2} + k_2k_{b1}k_{ub2}}{k_{-2}k_{-1}k_{ub1} + k_{-2}k_{b2}k_{ub1} + k_{-1}k_2k_{ub2} + k_{-1}k_{ub1}k_{ub2}} \\
\frac{LE^*}{E} &= \frac{k_{-1}k_2k_{b1} + k_1k_2k_{b2} + k_1k_{b2}k_{ub1} + k_2k_{b1}k_{b2}}{k_{-2}k_{-1}k_{ub1} + k_{-2}k_{b2}k_{ub1} + k_{-1}k_2k_{ub2} + k_{-1}k_{ub1}k_{ub2}} \\
\frac{E}{LE} &= \frac{k_{-2}k_{-1}k_{ub1} + k_{-2}k_{b2}k_{ub1} + k_{-1}k_2k_{ub2} + k_{-1}k_{ub1}k_{ub2}}{k_{-2}k_{-1}k_{b1} + k_{-2}k_1k_{b2} + k_{-2}k_{b1}k_{b2} + k_{-1}k_{b1}k_{ub2}} \\
\frac{LE^* + LE}{E^* + E} &= \frac{k_1k_{b2}k_{ub1} + k_{-1}k_{b1}k_{ub2} + \left(\left(k_{-1} + k_{b2}\right)k_{b1} + k_1k_{b2}\right)\left(k_{-2} + k_2\right)}{\left(\left(k_{-1} + k_1\right)k_{ub2} + k_{-2}\left(k_{-1} + k_1 + k_{b2}\right)\right)k_{ub1} + k_2k_{ub2}\left(k_{-1} + k_1 + k_{b1}\right)}
\end{aligned} \tag{21}$$

$$\frac{E^* + LE^*}{E + LE} = \frac{k_{-2}k_1k_{ub1} + k_{-1}k_2k_{b1} + k_1k_2k_{b2} + k_1k_2k_{ub2} + k_1k_{b2}k_{ub1} + k_1k_{ub1}k_{ub2} + k_2k_{b1}k_{b2} + k_2k_{b1}k_{ub2}}{k_{-2}k_{-1}k_{b1} + k_{-2}k_{-1}k_{ub1} + k_{-2}k_1k_{b2} + k_{-2}k_{b1}k_{b2} + k_{-2}k_{b2}k_{ub1} + k_{-1}k_2k_{ub2} + k_{-1}k_{b1}k_{ub2} + k_{-1}k_{ub1}k_{ub2}} \tag{22}$$

This direct analysis allows us to make a few conclusions by examining the expressions above. We focus on a few specific points of interest.

(i). Consider the case of symmetric localization. Here $k_{b1} = k_{b2}, k_{ub1} = k_{ub2}$. Now carefully examining the expressions for the ratios of $(E^* + LE^*)/(E + LE)$ (the ratio of total concentrations of E^* and E, including both localized and non-localized forms), we find that if the interconversion equilibrium constants are the same $k_1/k_{-1} = k_2/k_{-2}$, then this ratio of $(E^* + LE^*)/(E + LE) = k_1/k_{-1}$. This means that with symmetric localization it is not possible in these cases to alter the balance of the enzyme forms. Note that the only requirement is for the equilibrium constants of interconversion to be the same. This can be arrived at via a detailed examination of the expression above. It can also be arrived at more

directly by noting that in such a special scenario, the detailed balance for every pair of reactions holds good (which in turn arises from the fact that the product of rate constants along one arc of the cricle traversing the reaction network $k_b k_2 k_{ub} k_{-1}$ equals the product of rate constants along the other arc of the circle traversing the reaction network$k_{ub} k_1 k_b k_{-2}$.

(ii) However the advantage of the detailed expression is that it can be used to understand asymmetric localization as well. In the previous analysis, we had examined some special cases. Here we examine asymmetric localization through the lens of the expressions above, but by examining some special parameter regimes. (a) First consider the case that the interconversion reaction constants are all large relative to the binding/unbinding constants. While examining the ratio $(E^* + LE^*)/(E + LE)$, we note that this is a ratio of sums of products of three rate constants. Consequently, subject to the assumption made, both numerator and denominator and dominated by terms which contain two interconversion rate constants and one binding/unbinding constant. This expression is given by

$$(E^* + LE^*)/(E + LE) \quad = \quad \frac{[k_1 k_2 k_{b2} + k_1 k_2 k_{ub2} + k_1 k_{-2} k_{ub1} + k_2 k_{-1} k_{b1}]}{[k_1 k_{-2} k_{b2} + k_{-1} k_2 k_{ub2} + k_{-1} k_{-2} k_{ub1} + k_{-1} k_{-1} k_{b1}]} \tag{23}$$

It is clear that if the interconversion reactions have the same equilibrium constant, then the above ratio exactly equals this equilibrium constant. This means that in this case, whatever the asymmetry in localization, this is defeated by the interconversion (of course assuming that there are some non-zero terms in the numerator and denominator respectively).

(b) Another case to examine is the case where the interconversion reaction constants are small relative to the binding and unbinding constants. In this case, the numerator and denominator are dominated by terms contaning two binding/unding constants. The expression is

$$(E^* + LE^*)/(E + LE) \quad = \quad \frac{[k_1 k_{ub2} k_{ub1} + k_2 k_{b1} k_{b2} + k_1 k_{ub1} k_{b2} + k_2 k_{b1} k_{ub2}]}{[k_{-1} k_{ub2} k_{ub1} + k_{-2} k_{b1} k_{b2} + k_{-2} k_{ub1} k_{b2} + + k_{-1} k_{b1} k_{ub2}]} \tag{24}$$

Looking at this expression and looking at like terms in the numerator and denominator (for eg. terms which involve the product of unbinding constants), we see that if $k_1 = k_2$ and $k_{-1} = k_{-2}$ then the ratio of numerator to denominator is $k_1/k_{-1} = k_2/k_{-2}$. This is independent of binding and unbinding constants. This means that notwithstanding any asymmetries or biased localization, the ratio is determined by the interconversion equilibrium constant, and interconversion of localized forms (occurring at similar rates to free forms) can undo the effect of biased localization.

More generally, it is easy to see that when $k_1 = k_2, k_{-1} = k_{-2}$ that the ratio above will always be the interconversion equilibrium constant. This can be seen directly from the ODE equations governing the dynamics. The above expression of course does not require such restrictions and allows for detailed interplay of interconversion and asymmetric binding.

(iii) The expressions above also allow us to evaluate other cases discussed in the text. One is the case where localized forms are not active (computational results were shown for the TCS, but as discussed there, this did not depend on TCS biochemistry in any essential way and that such behaviour could be seen in a CMC). In such a case, we examine the expression for E^*/E. From this expression one can make the following inferences: (a) With an asymmetry in localization it is possible to achieve a switch in activity even when the interconversion of localized forms occurs at the same rate as non-localized forms (b) Unbiased localization does not result in such a switching in this case (the reasoning being similar to that for the unbiased localization considered above).

4 Additional computational results

In this section, we concisely discuss and present additional computational results, related to those in the main text. For the most part, the computational results are variations or extensions of the results shown in the main text. These additional variations are either explicitly referenced in the main text, or serve to complement the results in the main text.

A focal point in our analysis of bifunctional enzyme systems, was in different types of robustness behaviour, some of which have their origins in the kinetics. Fig. S1 shows multiple aspects of a bifunctional CMC model, demonstrating how the ratio of X^*/X is constant in the basic model as total amount of enzyme is varied showing in particular that this ratio is practically constant (i) even in the saturated limit of substrate modification (ii) in the unsaturated limit, even with feedback from the substrate affecting the balance of enzyme forms. A variation of X^*/X is seen when substrate modification is in the saturated limit with feedback also present. Also shown here is an example of linear substrate feedback, but with an extra enzyme-substrate interaction, demonstrating the capacity for bistability (see Supplementary text for details).

Fig. 4 of the main text focussed on the effects of different spatial designs on modification mediated by a bifunctional enzyme (shown for a bifunctional CMC model). Fig. S2 shows an analogous plot for a bifunctional TCS model reveal essentially similar insights.

The main text discussed basic aspects of absolute concentration robustness and how they may be affected by spatial organization. Fig. S3-S6 present multiple associated results. Fig. S3 shows the effect of spatial organization on ACR in the CMC model for a smaller substrate amount. For this smaller total substrate amount the TCS also exhibits the ACR (see main text), while the TCS does not do so for a higher substrate amount. Other basic aspects of the TCS kinetics are also shown. Fig. S4 and S5 complement the results in the main text which shows that different spatial designs may enable absolute concentration robustness even if not seen in the kinetics, and that this arises from an interplay of bifunctionality and the spatial design. Fig. S4 and S5 focus on the effect of varying the balance of modification/demodification catalytic constants of the substrate, shown for both high and low total substrate concentrations, respectively. In each figure we see that as the forward modification catalytic constant is progressively weakened, the X alone diffusing case enables ACR, in the CMC. Furthermore, while for higher substrate amounts there is a noticeable different between the CMC and TCS, this is not the case for lower substrate amounts. Fig. S6 focusses on cases where the enzymes are localized in two locations (say two poles of a cell), and the total enzme amount is varied in both locations simultaneously, building on results in the text. Different measures of the substrate concentration are evaluated: the substrate concentration at either pole, or the average substrate concentration. This complements Fig. 5(E,F). Also worth noting here is Fig. S6 (G,H), where the enzyme balance at one location has the same balance as in Fig. 5(D). By contrasting with Fig. 5(D) for the case of X and X^* diffusing, we find that the presence of enzyme localization at a new pole, with a different balance, introduces significant dependence on total enzyme concentration (here too, enzyme concentrations are varied simultaneously and equally).

The main text examined the perturbation of bistability (realized either by a dead-end complex in the TCS model or by feedback in the CMC model) by spatial organization. Fig. S7 presents additional supplementary plots regarding this.Fig. S7(F) also presents a plot complementing plots in the main text (Fig. 9) regarding how activity of a TCS in the presence of localization of enzymes, may be switched by variation of substrate. Here we present an example of how the balance of enzyme activities remains towards the phosphatase, for a broad range of substrate concentration.

The main text examined multiple aspects of the effects of spatial organization of evolution from monofunctional to bifunctional enzymes. It was shown there that with prior spatial organization, the bifunctional system may exhibit a very different localization pattern in contrast to the parent monofunctional enzymes (for instance even when the bifunctional enzyme localizes where either monofunctional enzyme localizes). Fig. S8 and S9 deal with additional aspects. Fig. S8 explores the effect

of the basal interconversion balance between the enzyme forms, in the context of results shown in the main text. The results demonstrate (Fig. S8(A,B)) that when the bifunctional enzyme localizes where either parent enzyme localizes, the effect of the basal interconversion ratio determines the relative balance of enzymes within a given localization pattern which ensues. A further result (Fig. S8(C)) shows that even under conditions (Fig. 11), where localization is correlated with the enzyme activity, a two pole localization pattern reminiscent of monofunctional enzymes is realized, but with the concentrations of enzymes at the two poles determined by basal interconversion rate constants.

For completeness, Fig. S9 explores an alternate "logic" of localization of thebifunctional enzyme: one where the activity of the enzyme is anti-correlated with the location in the parent monofunctional enzyme—meaning that the phosphatase form localizes where the parent kinase localized (and likewise the kinase form is present/localized where the parent phosphatase was present). Again stark differences are introduced by this localization pattern, in contrast to the monofunctional system.

5 Parameters

Our analysis invovles a combination of computational analysis, along with analytical work. In most places, analytical work complements the computations to reveal the origins of the behaviour. For the purposes of computational analysis, first we use the existing suite of models (CMC and TCS, monofunctional and bifunctional) along with models with some specific augmentations (eg. dead-end complex). We then examine the effect of active localization. As emphasized elsewhere, we employ representative parameters for these models to reveal the presence of certain behaviour, but unless otherwise noted (and where appropriate), similar behaviour is seen in other parameter regimes of the substrate modification as well.

We list the parameters employed as follows: first we define the notations concisely, and certain settings, which are common to multiple setting. We then define various parameter sets we employ in our study, for the various models and their variant. Following this, we proceed figure by figure, making reference to these various parameter sets, as well as figure specific details. This allows us to avoid repetition of parameter sets in multiple figures. The parameters are defined in the model descriptions in this document.

Definitions of localized coordinate:

Generally, L represents the region that $\theta = 4\pi/5$ to $59\pi/50$, $L1$ corresponds to $\theta = \pi/5$ to $29\pi/50$, $L2$ is $\theta = 7\pi/5$ to $89\pi/50$ and $L3$ is $\theta = 0$ to $9\pi/50$ and $9\pi/5$ to 2π (there are certain differences in some

plots, which are noted specifically in those plots). In spatial ODE models (used in the part of Bistable TCS and to be done the bifurcation analysis in MATCONT), l_1 is described by the size of reaction compartment and l represents the width of remaining size or length (total width with excluding l_1). l_{total}, the size of domain, is the sum of l_1 and l. The width l_1 and l are calculated on the basis of x.

Annotations for active localization:

Spatial organizations. Z and Y are immobilized species for active localization. If Z is the only one immobilized species in the model, it is always localized only in $L2$ with absent diffusing ability; if Z and Y exist at the same time, Z is localized only in $L1$ while Y is present only in $L2$ (reversed in Figure S9), both with absent diffusing ability. All the species bound with Z or Y (called bound forms) are also prohibited to diffuse.

Kinetics. k_{bj} and k_{uj} represent the binding rate constant of the species j to Z or Y and unbinding rate from Z or Y, respectively. The kinetic parameters with subscript z and y (based on basic (unbound forms) kinetic parameters) correspond to the kinetics of related bound forms.

5.1 Sets summary

Kinetics.

(CMC.M: a kinetic parameter regime for mono-functional covalent modification cycle; TCS.M: a kinetic parameter regime for mono-functional two-component system; CMC.A(X) and CMC.B(X): four different kinetic parameter regimes for bifunctional covalent modification cycle; TCS.B(X): two different kinetic parameter regimes for bifunctional two-component system; DEC: a kinetic parameter regime for dead-end complex model (the bistable TCS model).)

Set CMC.M: k_1=1.0, k_{-1}=1.0, k_2=1.0, k_3=1.0, k_{-3}=1.0, k_4=0.1.

Set TCS.M: k_1=1.0, k_{-1}=1.0, k_t=1.0, k_2=1.0, k_{-2}=1.0, k_p=0.1, k_a=0.1, k_d=0.1.

Set CMC.B: k_1=1.0, k_{-1}=1.0, k_2=1.0, k_3=1.0, k_{-3}=1.0, k_4=0.1 and $[Sa]$=$[Sb]$=0.0.

Set CMC.BX: k_{fb}=0.1, k_{bb}=0.1, k_1=1.0, k_{-1}=1.0, k_3=1.0, k_{-3}=1.0 and $[Sa]$=$[Sb]$=0.0.

Set CMC.A: k_1= 0.1, k_{-1}= 1.0, k_2= 10.0, k_3= 0.1, k_{-3}= 1.0, k_4= 20.0, k_{sa}=1.0, k_{sb}= 1.0, k_{f1}=1.0, k_{b1}= 1.0.

Set CMC.AX: k_1= 0.1, k_{-1}= 1.0, k_2= 0.5, k_3= 0.1, k_{-3}= 1.0, k_4= 0.5.

Set TCS.B (Rowland & Deeds, 2014): k_1=1.0, k_{-1}=1.0, k_t=1.0, k_2=1.0, k_{-2}=1.0, k_p=0.1.

Set TCS.BX (Rowland & Deeds, 2014): k_k=0.1, k_{-k}=0.1, k_1=1.0, k_{-1}=1.0, k_2=1.0, k_{-2}=1.0.

Set DEC (Igoshin *et al.*, 2008): k_{b1}=0.5, k_{d1}=0.5, k_{b2}=0.05, k_{d2}=0.5, k_{ap}=0.1, k_{ad}=0.001, k_{pt}=1.5,

k_{b3}=0.5, k_{d3}=0.5, k_{b4}=0.5, k_{d4}=0.5 and k_{cat}=0.025. Associated references are [Rowland & Deeds, 2014, Igoshin *et al.*, 2008].

Inactive localization.

(DCS.A-D: four different parameter regimes for the diffusion coefficients of substrate.)

Set DCS.A: D_X=0.1, D_{X^*}=0.0, remaining diffusion coefficients are 0, which allows only X diffusing.

Set DCS.B: D_X=0.0, D_{X^*}=0.1, remaining diffusion coefficients are 0, which allows only X^* diffusing.

Set DCS.C: D_X=0.1, D_{X^*}=0.1, remaining diffusion coefficients are 0, which allows only X and X^* diffusing.

Set DCS.D: D_X=0.0, D_{X^*}=0.0, remaining diffusion coefficients are 0, which prohibits all species including X and X^* diffusing.

Active localization.

(BUR.A-H: eight different regimes for the binding and unbinding rates (k_{bj} and k_{uj}) of species j with immobilized species; I.A-B: two different parameter regimes for the interconversion between two bound forms of bifunctional enzyme; ABZ.A-C: three different parameter regimes for the activity of bound forms which are localized with immobilized species Z (rate constants of the reactions where bound forms are involved) in CMC and TCS systems; ABY.A: a parameter regime for the activity of bound forms which are localized with immobilized species Y (rate constants of the reactions where bound forms are involved) in CMC system.)

(Localization of unbound forms.)

Set BUR.A: only Z, k_{bx}=10.0, k_{ux}=0.1, k_{bx^*}=10.0, k_{ux^*}=0.1.

Set BUR.B: only Z, k_{be}=0.0, k_{ue}=0.0, k_{be^*}=0.0, k_{ue^*}=0.0.

Set BUR.C: only Z, k_{be}=1.0, k_{ue}=0.1, k_{be^*}=1.0, k_{ue^*}=0.1.

Set BUR.D: only Z, k_{be}=1.0, k_{ue}=0.1, k_{be^*}=10.0, k_{ue^*}=0.1.

Set BUR.E: only Z, k_{be}=10.0, k_{ue}=0.1, k_{be^*}=10.0, k_{ue^*}=0.1.

Set BUR.F: Z and Y, k_{be}=10.0, k_{ue}=0.1, k_{be^*}=10.0, k_{ue^*}=0.1 for both.

Set BUR.G: Z and Y, k_{be}=10.0, k_{ue}=0.1 for Y, k_{be^*}=10.0, k_{ue^*}=0.1 for Z.

Set BUR.H: only Z, k_{be}=10.0, k_{ue}=0.1, k_{be^*}=1.0, k_{ue^*}=0.1.

(Activity regulation of bound forms.)

Set I.A: k_{kz}=0.0, k_{-kz}=0.0.

Set I.B: k_{kz}=0.1, k_{-kz}=0.1.

Set ABZ.A: k_{1z}=1.0, k_{-1z}=1.0, k_{tz}=1.0, k_{2z}=1.0, k_{-2z}=1.0, k_{pz}=0.1.

Set ABZ.B: k_{1z}=1.0, k_{-1z}=1.0, k_{2z}=1.0, k_{3z}=1.0, k_{-3z}=1.0, k_{4z}=0.1.

Set ABZ.C: k_{1z}=0.0, k_{-1z}=0.0, k_{tz}=0.0, k_{2z}=0.0, k_{-2z}=0.0, k_{pz}=0.0.

Set ABY.A: k_{1y}=1.0, k_{-1y}=1.0, k_{2y}=1.0, k_{3y}=1.0, k_{-3y}=1.0, k_{4y}=0.1.

Sets for the specific cases in Figure 9 and S7(F).

(ITC.A-E: five different regimes for the variation of initial total concentration of substrate involved in Figure 9 and S7(F).)

Set ITC.A: $[X]_{total}$=0.001, 0.01, 0.1, 1, 5, 10.

Set ITC.B: $[X]_{total}$=0.001, 0.01, 0.1, 0.5, 1.

Set ITC.C: $[X]_{total}$=0.001, 0.01, 0.1, 1, 5, 10, 30, 60.

Set ITC.D: $[X]_{total}$=0.001, 0.01, 0.1, 1, 5.

Set ITC.E: $[X]_{total}$=0.001, 0.01, 0.1, 1, 5, 10, 30, 60, 90.

5.2 Figure 2

Kinetics and spatial organizations (with inactive localization):

A. Set CMC.M, K and P are localized in L.

B. Set CMC.M, K is localized in L but P spreads everywhere.

C. Set CMC.M, K and P are localized in L and $L3$ separately.

Colours of curves in each plot. Blue, set DCS.A; red, set DCS.B; green, set DCS.C; orange, set DCS.D.

Initial conditions:

X, X^* and K are all localized in L with $[X]_{total}$=1.0 and $[K]_{total}$=0.1.

A. P is present only in L with $[P]_{total}$=0.1.

B. P spreads everywhere with $[P]_{total}$=0.02.

C. P exists only in $L3$ with $[P]_{total}$=0.1.

5.3 Figure 3

Kinetics and spatial organizations (with active localization of substrate):

A (blue). Sets TCS.M, BUR.A, ABZ.A, $D_X = D_{X^*} = D_K = D_{K^*} = D_P = D_{XK^*} = D_{X^*P}$=1.0, remaining diffusion coefficients are 0.

A (red). Sets CMC.M, BUR.A, ABZ.B, $D_X = D_{X^*} = D_K = D_P = D_{XK} = D_{X^*P}$=1.0, remaining diffusion coefficients are 0.

B. Set TCS.M, $[K]_{total} = [P]_{total}$=0.1, $[X]_{total}$=1.0 but it becomes varied for bifurcation analysis in MATCONT.

C. Based on Equation (13) in Supplementary, k_{f1}=1.0, k_{f2}=0.1, l_1=20.0, $[P]_{total}$=0.1, $[K]_{total}$=0.1, $[X]_{total}$=1.0. For curves with different colours: α=0.0, l=0.0 (blue); α=5.0, l=0.0 (red); α=5.0, l=80.0 (green).

Initial conditions:

A. X, X^*, XZ and X^*Z are all localized in L with $[X]_{total}$=1.0, K and P are both present only in $L1$ with $[K]_{total} = [P]_{total}$=0.1, Z is localized only in $L2$ with $[Z]_{total}$=10.0.

5.4 Figure 4

Kinetics and spatial organizations (with inactive localization):

A. Set CMC.B, k_{fb}=0.1, k_{bb}=0.1; E and E^* are present only in L.

B. Set CMC.B, k_{fb}=0.1, k_{bb}=0.1; E and E^* are present both in L and $L3$.

C. Set CMC.B, k_{fb}=1.0 and k_{bb}=0.1 in L, k_{fb}=0.1 and k_{bb}=1.0 in $L3$, $k_{fb} = k_{bb}$=0.1 in other locations; E and E^* are present both in L and $L3$.

Colours of curves in each plot. Same as Figure 2.

Initial conditions:

Initially, X and X^* are localized in L with $[X]_{total}$=1.0, $[E]_{total}$=0.1.

A. E and E^* are both localized in L with $[E] = [E^*]$=0.05.

B-C. E and E^* are localized in both L and $L3$ with $[E] = [E^*]$=0.025 in both L and $L3$.

5.5 Figure 5

Kinetics and spatial organizations (with inactive localization):

$[E]_{total}$ is varied.

A. Set CMC. AX, k_{sa}=1.0, k_{sb}= 1.0, k_{fb}=0.1, k_{bb}=0.1.

B. Set TCS.B, k_k=0.1, k_{-k}=0.1; E and E^* are both present only in L; $[X]_{total}$=10.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue).

C. Set CMC.B, k_{fb}=0.1, k_{bb}=0.1; E and E^* are both present only in L; $[X]_{total}$=10.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue).

D. Set TCS.B, k_k=0.1, k_{-k}=0.1, E and E^* are both present only in L; $[X]_{total}$=1.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue).

E-F. Set TCS.B, k_k=10.0, k_{-k}=1.0, in $L1$, k_k=1.0, k_{-k}=10.0 in $L2$. Location $L1$ has a preponderance of E^* and location $L2$ has a preponderance of E. In **E**, set DCS.C; in **F**, $D_X = D_{X^*}$=0.001 (blue), 0.01 (red), 0.1(grey), 1 (orange), 10 (green) with remaining diffusion coefficients equal to 0.

Initial conditions:

X and X^* are initially localized only in L.

B. Start with $[X]_{total}$=10.0; E and E^* are both only present in L with $[E]$ varied and $[E^*]$=0.0 (to vary total enzyme concentration).

C. Start with $[X]_{total}$=10.0; E and E^* are both only present in L with $[E]$=0.1 and $[E^*]$ varied (to vary E_{total}).

D. Start with $[X]_{total}$=1.0; E and E^* are both only present in L with $[E]$ varied and $[E^*]$=0.0 (to vary total enzyme concentration).

E-F. Start with $[X]_{total}$=1.0; E and E^* are localized in $L1$ and $L2$ respectively and these amounts are varied to vary the total enzyme concenetration.

5.6 Figure 6

For Figure 6 (A, E), specifically, $L1$ represents $\theta = 2\pi/25$ to $12\pi/25$ and $L2$ corresponds to $\theta = 27\pi/25$ to $37\pi/25$.

Kinetics and spatial organizations:

A. Bistable model: k_1=0.05, k_2=1.0, k_{pf}=72.0, k_3=2.0, k_4=1.0; $[E]_{total}$=1.0, $[Sa]$ value is varied. Compartment width 20% of domain length. Total substrate amount, $[X]_{total}$=1.0 (blue and red), $[X]_{total}$=3.0 (green). D_{X^*}=0.0 (blue) and D_{X^*}=0.01 (red and green) (remaining diffusion coefficients are 0).

Blue curves in B-D. Set DEC, $[X]_{total}$=6, $[E]_{total} = [F]_{total}$=0.17, k_{ph}=0.05 but it is varied for bifurcation analysis in MATCONT; l_1/l=20/80.

Red curves in C-D. Set DEC, $[E]_{total} = [F]_{total}$=0.17, k_{ph}=0.05 but it is varied for bifurcation analysis in MATCONT; in **C**, $[X]_{total}$=6 and l_1/l=5/95; in **D**, $[X]_{total}$=30 and l_1/l=20/80.

E. Same as **A**. Enzyme is present in equal concentrations in locations $L1$ and $L2$ and $[Sa]$=1.058 at both locations. Both $X*$ and X^* diffuse at $D_{X^*} = D_X$ = 0.00001 (green), =0.0001 (red), = 0.001 (blue), =0.002 (magenta).

F. Set DEC, k_{ph} is varied; X and X^* spread everywhere, other species are localized in both $L1$ and $L2$; $D_X = D_{X^*}$=0.0000001 (blue and red), 0.000001 (green) and 0.001, 0.1, 10 (pink), remaining diffusion coefficients are 0.

Initial conditions:

A, E. $[X]_{total}$ and $[E]_{total}$ are initially present in $L1$; X and X^* or only X^* are allowed to diffuse.

(E). In $L1$, X=1.0, X^*=0.0, E=1.0 and E^*=0.0; in $L2$, X=0.0, X^*=1.0, E=1.0 and E^*=0.0.

B-D. For bifurcation analysis in MATCONT, the spatial ODE models are reduced, and the initial concentrations are set as $[X^*]$=0, $[E]$=0.17, $[XE^*]$=0, $[X^*E]$=0, $[XE]$=0 and $[F]$=0.17 with all species initially present in a certain location.

F. All species are initially localized in both $L1$ and $L2$, with $[X]$=0.5, $[X^*]$=5.5, $[E] = [F]$=0.17 in $L1$ and $[X]$=4.8, $[X^*]$=1.2, $[E] = [F]$=0.17 in $L2$.

5.7 Figure 7

Kinetics and spatial organizations:

A. Transcritical model: k_{sb}=1.0, k_{fb}=0.0, k_{bb}=0.0, k_1=1.0, k_{-1}=1.0, $k2$=10.0, $k3$=1.0, k_{-3}=1.0, $k4$=10.0; $[X]_{total}$=2.0, $[E]_{total}$=1.0, $[Sb]$ value is varied with $[Sa]$=0.0.

B. Same as **A**. D_X=0.01 (red), $D_X = D_{X^*}$ =0.01 (green) (remaining diffusion coefficients are 0). Larger compartment cases: width 6 times the width in the LHS plot.

C. Ultrasensitivity: k_{sb}= 1.0, k_{fb}=0.001, k_{bb}=0.0, k_1=20.0, k_{-1}=1.0, $k2$=1.0, $k3$=20.0, k_{-3}=1.0, $k4$=1.0; $[X]_{total}$=10.0, $[E]_{total}$=1.0, $[Sb]$ value is varied, $[Sa]$=0.0.

D. Same as **C**. Compartment widths shown are 2%, 20% and 90% of the domain length; D_X =0.01 (remaining diffusion coefficients are 0).

5.8 Figure 8

In Figure 8, specifically, $L1$ corresponds to x=1 to 10 and $L2$ is x=91 to 100. The localization is enforced in Matlab through specifying which grid points the entities are non-zero and zero. Subscripts 1 and 2 denote the locations $L1$ and $L2$, respectively, in the rate constant notations.

Kinetics and spatial organizations:

B-D. Set CMC.AX, localization signal concentration $[S_{L1}]$=1.0 at $L1$ and $[S_{L2}]$=0 at $L2$.

(B). Equal or non-selective localization of both enzyme forms in $L1$: k_{b1e}= 10.0, k_{u1e}= 0.1, k_{b1e*}= 10.0, k_{u1e*}= 0.1, k_{b2e}= 0.0, k_{u2e}= 0.0, forward basal =10.0 and backward basal = 1.0 (associated with both,

free and localized enzymes).

(C). Same as **(B)**. Size of compartment is x=1 to 40 and domain length is increased by four fold (from l_{total}=100 to l_{total}=400); the feedback rate constant is 2.0 (blue), 20.0 (red); D_X and D_{X^*}=10.0 (remaining diffusion coefficients are 0).

(D). Kinase form E^* binds strongly in $L1$; k_{b1e*}= 1000.0, k_{u1e*}= 0.01, k_{b1e}= 0.0001, k_{u1e}= 100.0, k_{b2e}= 0.0, k_{u2e}= 0.0, k_{b2e*}= 0.0, k_{u2e*}= 0.0; if Feedback on, the feedback rate constant is 20.0.

F-H. Set CMC.AX, $D_E = D_{E^*}$=0.01, $D_X = D_{X^*}$=0.01 (remaining diffusion coefficients are 0); localization signal concentration $[S_{L1}]$=1.0 at location $L1$.

(F). $[S_{L2}]$ in $L2$ is 0.0. Phosphatase form E binds strongly in $L1$; k_{b1e}= 1000.0, k_{u1e}= 0.01, k_{b1e*}= 0.0001, k_{u1e*}= 100.0, k_{b2e}= 0.0, k_{u2e}= 0.0, k_{b2e*}= 0.0, k_{u2e*}= 0.0. Interconversion is off. Feedback rate constant values are 70,000 (blue), 80,000 (red) and 200,000 (green).

(G). $[S_{L2}]$ in $L2$ is 0.0. Phosphatase form E binds strongly in $L1$ and $L2$; k_{b1e}= 1000.0, k_{u1e}= 0.01, k_{b1e*}= 0.0001, k_{u1e*}= 100.0, k_{b2e}= 1000.0, k_{u2e}= 0.01, k_{b2e*}= 0.0001, k_{u2e*}= 100.0. Interconversion is off. Feedback is turned off and the activity regulation signal is on at location $L2$ (=10.0). Blue: X, X^*, E and E^* are allowed to diffuse; red: only E and E^* are allowed to diffuse.

(H). $[S_{L2}]$ in $L2$ is 1.0. Feedback is turned off and the activity regulation signal $[S_{A2}]$ =10 (location $L2$) and $[S_{A1}]$ =0.0 (location $L1$). For the Activity Regulating Signals: in $L2$ is on, with k_{sa2}=10.0, k_{ba2}=0.1; in $L1$ is off, with k_{sa1}=0.0, k_{ba1}=0.0. In $L1$, the binding rate constant k_{b1e} is varied, k_{b1e}= 1000.0 (blue), 500.0 (red) and 100.0 (green), k_{u1e}= 0.01, k_{b1e*}= 0.0001, k_{u1e*}= 100.0; in $L2$, k_{b2e*}= 100.0, k_{u2e*}= 0.1, k_{b2e}= 100.0, k_{u2e}= 0.1. Interconversion is off.

Initial conditions:

B-D, F and H. All species are initially present in $L1$, $[X]_{total}$=20.0 and $[E]_{total}$=10.0.

G. $[X]_{total}$=20.0 and $[E]_{total}$=10.0. Blue: all species are present in $L1$ initially. Red: all species are present in $L1$ initially, except X, X^* which are present everywhere in the domain at a uniform concentration of 2.0.

5.9 Figure 9

Kinetics and spatial organizations (with active localization of the two forms of bifunctional enzyme):

For kinetics and localization.

Set TCS.B, k_k=0.1, k_{-k}=0.1, $[X]_{total}$ is varied; $D_X = D_{X^*} = D_E = D_{E^*} = D_{XE^*} = D_{X^*E}$=1.0, remaining diffusion coefficients are 0.

A-D. In **A** (blank triangle-dash), set BUR.B; in **A** (circle-line), **B**, **C** and **D**, set BUR.C.

E-H. Set BUR.D.

For interconversion and activity of localized forms.

In **A** (circle-line) and **E**, set I.B and ABZ.A; in **A** (blank triangle-dash), **B** and **F**, set I.A and ABZ.C; in **C** and **G**, set I.A and ABZ.A; in **D** and **H**, set I.B and ABZ.C.

Initial conditions:

Initially, substrate is localized in L with changed $[X]_{total}$ (independent variable) as below, bifunctional enzyme is present only in $L1$ with $[E]_{total}$=0.1, and Z is localized in $L2$ with $[Z]_{total}$=10.0.

A-D. In **A** and **B**, set ITC.A; in **C**, set ITC.B; in **D**, set ITC.C.

E-H. In **E** and **F**, set ITC.A; in **G**, set ITC.B; in **H**, set ITC.D.

5.10 Figure 10

Kinetics and spatial organizations (with active localization of the two forms of bifunctional enzyme):

Sets CMC.B and ABZ.B, k_{fb}=0.1, k_{bb}=0.1, k_{fbz}=0.1, k_{bbz}=0.1; X and X^* are present everywhere; $D_X = D_{X^*} = D_E = D_{E^*}$=0.1, remaining diffusion coefficients are 0. (The results are totally same for: $D_E = D_{E^*}$=0.001, 0.01, 0.1, 1, 10 with $D_X = D_{X^*}$=0.1, and $D_X = D_{X^*}$=0.001, 0.01, 0.1, 1, 10 with $D_E = D_{E^*}$=0.1)

B. Set BUR.E, E and E^* are localized in the forms of EZ and E^*Z respectively in $L1$.

C. Sets ABY.A, BUR.F, k_{fby}=0.1, k_{bby}=0.1; E and E^* are localized in the forms of EZ and E^*Z respectively in $L1$, EY and E^*Y respectively in $L2$.

Initial conditions:

Initially, X and X^* are present only in L with $[X]_{total}$=1.0, E and E^* are everywhere with $[E] = [E^*]$=0.01, Z is localized only in $L1$ with $[Z]_{total}$=20.0.

C. Y is initially localized only in $L2$ with $[Y]_{total}$=20.0.

5.11 Figure 11

k_{cj} represents the release rate constant of the bound form of species j, which is one unbound form of bifunctional enzyme, to the other unbound form of bifunctional enzyme and the immobilized species, where the interconversion between two bound forms of bifunctional enzyme is so quick that it can be negligible due to ensure the unique localization of the objective species j; k_{cj}=0.0 means there is no interconversion occurring.

Kinetics and spatial organizations (selectively active localization of the two forms of bifunctional enzyme):

Set CMC.B, k_{fb}=0.1, k_{bb}=0.1; k_{1z}=1.0, k_{-1z}=1.0, k_{2z}=1.0; form E^* is localized in the form of E^*Z in $L1$; $D_X = D_{X^*}$=0.1, except for D_E and D_{E^*}, remaining diffusion coefficients are 0.

A. Only Z, k_{be^*}=10.0 and k_{ue^*}=0.1; k_{ce^*}=0.1 (red) and 0.0 (blue) for E^*Z converting to E and Z; form E spreading everywhere, $D_E = D_{E^*}$=10.0.

B. Set BUR.G, k_{3y}=1.0, k_{-3y}=1.0, k_{4y}=0.1; k_{ce^*}=0.1 (red) and 0.0 (blue) for E^*Z converting to E and Z, k_{ce}=0.1 (red) and 0.0 (blue) for EY converting to E^* and Y; form E is localized in the form of EY in $L2$. Blue, $D_E = D_{E^*}$=0.001 (the results are essentially the same for: $D_E = D_{E^*}$=0.001, 0.01, 0.1, 1, 10 with $D_X = D_{X^*}$=0.1, and $D_X = D_{X^*}$=0.001, 0.01, 0.1, 1, 10 with $D_E = D_{E^*}$=0.1); red, $D_E = D_{E^*}$=10.0.

Initial conditions:

Initially, X and X^* are present only in L with $[X]_{total}$=1.0, E exists everywhere with $[E]$=0.01, and Z is localized only in $L1$ with $[Z]_{total}$=10.0.

A. E^* is initially localized in $L1$ with $[E^*]$=0.05.

B. E^* is initially present everywhere with $[E^*]$=0.01, Y is localized only in $L2$ with $[Y]_{total}$=10.0.

5.12 Figure S1

Kinetics and spatial organizations (with inactive localization):

A. k_{sa}=1.0, k_{sb}=1.0, k_{fb}=0.1, k_{bb}=0.1, k_1=0.1, k_{-1}=0.001, k_2=10.0, k_3=0.005, k_{-3}=0.001, k_4=0.01.

B. k_{sa}=1.0, k_{sb}=1.0, k_{fb}=0.1, k_{bb}=0.1, k_1=0.1, k_{-1}=1.0, k_2=10.0, k_3=0.1, k_{-3}=1.0, k_4=20.0; feedback rate constant k_{pf}=0.5.

C. $[Sb]$=1.0, $[Sa]$=0.0, same as **A**; feedback rate constant k_{pf}=0.5; $[X]_{total}$=2.0, $[E]_{total}$ is varied.

D. $[Sa]$ is varied, $[X]_{total}$=1.0, $[E]_{total}$=10.0; k_s=1.0, k_{ff}=0.0, k_{bb}=0.0, k_1=1.0, k_{-1}=1.0, k_2=10.0, k_3=1.0, k_{-3}=1.0, k_4=10.0, k_{pf}=8.0, k_5=2.0, k_6=1.0, k_i=0.001.

5.13 Figure S2

Kinetics and spatial organizations (with inactive localization):

A. Set TCS.B, k_k=0.1, k_{-k}=0.1; E and E^* are present only in L.

B. Set TCS.B, k_k=0.1, k_{-k}=0.1; E and E^* are present both in L and $L3$.

C. Set TCS.B, k_k=1.0 and k_{-k}=0.1 in L, k_k=0.1 and k_{-k}=1.0 in $L3$, $k_k = k_{-k}$=0.1 in other

locations; E and E^* are present both in L and $L3$.

Colours of curves in each plot. Same as Figure 2.

Initial conditions: Same as Figure 4.

5.14 Figure S3

Kinetics and spatial organizations (with inactive localization):

A. Set CMC.B, k_{fb}=0.1, k_{bb}=0.1, $[E]_{total}$ is varied; E and E^* are both present only in L; $[X]_{total}$=1.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue).

B. Set TCS.B, k_k=0.1, k_{-k}=0.1, $[E]_{total}$=0.1, $[X]_{total}$=1.0, but $[X]_{total}$ becomes varied for bifurcation analysis in MATCONT.

Initial conditions:

A. X and X^* are initially localized only in L, with $[X]_{total}$=1.0; E and E^* are both only present in L with $[E]$=0.1 and $[E^*]$ varied.

5.15 Figure S4

Kinetics and spatial organizations (with inactive localization):

A-D. Set CMC.BX, $[E]_{total}$ is varied; E and E^* are both present only in L; $[X]_{total}$=10.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue); in **A**, k_2=0.7, k_4=0.3; in **B**, k_2=0.5, k_4=0.5; in **C**, k_2=0.3, k_4=0.7; in **D**, k_2=0.1, k_4=1.0.

E-H. Set TCS.BX, $[E]_{total}$ is varied; E and E^* are both present only in L; $[X]_{total}$=10.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue); in **E**, k_t=0.7, k_p=0.3; in **F**, k_t=0.5, k_p=0.5; in **G**, k_t=0.3, k_p=0.7; in **H**, k_t=0.1, k_p=1.0.

Initial conditions:

X and X^* are initially localized only in L.

A-D. Start with $[X]_{total}$=10.0; E and E^* are both only present in L with $[E]$=0.1 and $[E^*]$ varied.

E-H. Start with $[X]_{total}$=10.0; E and E^* are both only present in L with $[E]$ varied and $[E^*]$=0.0.

5.16 Figure S5

Kinetics and spatial organizations (with inactive localization):

A-D. Set CMC.BX, $[E]_{total}$ is varied; E and E^* are both present only in L; $[X]_{total}$=1.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue); in **A**, k_2=0.7, k_4=0.3; in **B**, k_2=0.5, k_4=0.5; in **C**, k_2=0.3,

k_4=0.7; in **D**, k_2=0.1, k_4=1.0.

E-H. Set TCS.BX, $[E]_{total}$ is varied; E and E^* are both present only in L; $[X]_{total}$=1.0 (orange), sets DCS.A (red), DCS.B (green), DCS.C (blue); in **E**, k_t=0.7, k_p=0.3; in **F**, k_t=0.5, k_p=0.5; in **G**, k_t=0.3, k_p=0.7; in **H**, k_t=0.1, k_p=1.0.

Initial conditions:

X and X^* are initially localized only in L.

A-D. Start with $[X]_{total}$=1.0; E and E^* are both only present in L with $[E]$=0.1 and $[E^*]$ varied.

E-H. Start with $[X]_{total}$=1.0; E and E^* are both only present in L with $[E]$ varied and $[E^*]$=0.0.

5.17 Figure S6

Kinetics and spatial organizations (with inactive localization):

Set TCS.B, $[E]_{total}$ is varied.

A-F. k_k=10.0, k_{-k}=1.0, in $L1$, k_k=1.0, k_{-k}=10.0 in $L2$ and k_k=k_{-k}=0.1 in the remaining locations; E^* and E are present mainly in $L1$ and $L2$ respectively.

(A-D). $D_X = D_{X^*}$=0.001 (**A**), 0.01 (**B**), 1 (**C**), 10 (**D**); remaining diffusion coefficients are 0, which allows only X and X^* diffusing.

(E-F). $D_X = D_{X^*}$=0.001 (blue), 0.01 (red), 0.1(grey), 1 (orange), 10 (green) with remaining diffusion coefficients equal to 0.

G. Set DCS.C, k_k=1.0, k_{-k}=0.1 in $L2$ and k_k=k_{-k}=0.1 in location $L1$. There is a preponderance of E^* in $L2$.

H. Set DCS.C, k_k=0.1, k_{-k}=1.0 in $L2$ and k_k=k_{-k}=0.1 in location $L1$. There is a preponderance of E in $L2$.

Initial conditions:

A-H. X and X^* are initially localized only in L, and start with $[X]_{total}$=1.0; E and E^* are localized in $L1$ and $L2$ respectively with both $[E]$ and $[E^*]$ varied and holding equal during changes.

5.18 Figure S7

Kinetics and spatial organizations:

A-C. Set DEC, k_{ph}=0.05, $[E]_{total} = [F]_{total}$=0.17, but $[E]_{total}$ is varied for bifurcation analysis in MATCONT; in **A, B (blue) and C (blue)**, $[X]_{total}$=6 and l_1/l=20/80; in **B (red)**, $[X]_{total}$=6 and l_1/l=5/95; in **C (red)**, $[X]_{total}$=30 and l_1/l=20/80.

D. Set DEC, $[X]_{total}$=6, $[E]_{total} = [F]_{total}$=0.17, k_{ph}=0.05 but it is varied for bifurcation analysis in MATCONT; l_1/l=5/95.

E. k_1=0.05, k_2=1.0, k_{pf}=72.0, k_3=2.0, k_4=1.0; $[X]_{total}$=1.0, $[E]_{total}$=1.0; $[S]$ is varied. ODE case (blue); PDE case (magenta): D_{X^*}=0.01 (remaining diffusion coefficients are 0), compartment width is 98% of the domain.

F. Sets TCS.B, BUR.H, I.A, ABZ.A, k_k=0.1, k_{-k}=0.1, $[X]_{total}$ is varied; $D_X = D_{X^*} = D_E = D_{E^*} = D_{XE^*} = D_{X^*E}$=1.0, remaining diffusion coefficients are 0.

Initial conditions:

A-D. For bifurcation analysis in MATCONT, the spatial ODE models are reduced, and the initial concentrations are set as $[X^*]$=0, $[E]$=0.17, $[XE^*]$=0, $[X^*E]$=0, $[XE]$=0 and $[F]$=0.17 with all species initially present in a certain location.

F. set ITC.E, bifunctional enzyme is present only in $L1$ with $[E]_{total}$=0.1, and Z is localized in $L2$ with $[Z]_{total}$=10.0; substrate is localized in L with changed $[X]_{total}$.

5.19 Figure S8

Kinetics and spatial organizations (with active localization (A, B) or selectively active localization (C) of the two forms of bifunctional enzyme):

A-B. Sets CMC.B and ABZ.B, k_{fb}=0.1, k_{bb}=1.0, k_{fbz}=0.1, k_{bbz}=1.0; X and X^* are present everywhere; $D_X = D_{X^*} = D_E = D_{E^*}$=0.1, remaining diffusion coefficients are 0. (The results are totally same for: $D_E = D_{E^*}$=0.001, 0.01, 0.1, 1, 10 with $D_X = D_{X^*}$=0.1, and $D_X = D_{X^*}$=0.001, 0.01, 0.1, 1, 10 with $D_E = D_{E^*}$=0.1)

(A). Set BUR.E, E and E^* are localized in the forms of EZ and E^*Z respectively in $L1$.

(B). Sets ABY.A, BUR.F, k_{fby}=0.1, k_{bby}=1.0; E and E^* are localized in the forms of EZ and E^*Z respectively in $L1$, EY and E^*Y respectively in $L2$.

C. Sets CMC.B, BUR.G, k_{fb}=0.1, k_{bb}=1.0; k_{1z}=1.0, k_{-1z}=1.0, k_{2z}=1.0; k_{3y}=1.0, k_{-3y}=1.0, k_{4y}=0.1; k_{ce^*}=0.0 for E^*Z converting to E and Z, k_{ce}=0.0 for EY converting to E^* and Y; form E^* is localized in the form of E^*Z in $L1$, forms E is localized in the form of EY in $L2$; $D_X = D_{X^*}$=0.1, $D_E = D_{E^*}$=0.001 (the results are totally same for: $D_E = D_{E^*}$=0.001, 0.01, 0.1, 1, 10 with $D_X = D_{X^*}$=0.1, and $D_X = D_{X^*}$=0.001, 0.01, 0.1, 1, 10 with $D_E = D_{E^*}$=0.1), remaining diffusion coefficients are 0.

Initial conditions:

Initially, X and X^* are present only in L with $[X]_{total}$=1.0, E and E^* are everywhere with $[E] = [E^*]$=0.01.

A-B. Z is localized only in $L1$ with $[Z]_{total}$=20.0.

(B). Y is initially localized only in $L2$ with $[Y]_{total}$=20.0.

C. Z is localized only in $L1$ with $[Z]_{total}$=10.0, Y is localized only in $L2$ with $[Y]_{total}$=10.0.

5.20 Figure S9

Kinetics and spatial organizations (selectively active localization of the two forms of bifunctional enzyme):

Set CMC.B, k_{fb}=0.1, k_{bb}=0.1; k_{3y}=1.0, k_{-3y}=1.0, k_{4y}=0.1; forms E is localized in the form of EY in $L1$; $D_X = D_{X^*}$=0.1, except for D_E and D_{E^*}, remaining diffusion coefficients are 0.

A. Only Y, k_{be}=10.0 and k_{ue}=0.1; k_{ce}=0.0 (blue) and 0.1 (red) for EY converting to E^* and Y; form E^* spreading everywhere, $D_E = D_{E^*}$=10.

B. Set BUR.G, k_{1z}=1.0, k_{-1z}=1.0, k_{2z}=1.0; k_{ce}=0.0 (blue) and 0.1 (red) for EY converting to E^* and Y, k_{ce^*}=0.0 (blue) and 0.1 (red) for E^*Z converting to E and Z; form E^* is localized in the form of E^*Z in $L2$; $D_E = D_{E^*}$=0.001 (blue) (the results are totally same for: $D_E = D_{E^*}$=0.001, 0.01, 0.1, 1, 10 with $D_X = D_{X^*}$=0.1, and $D_X = D_{X^*}$=0.001, 0.01, 0.1, 1, 10 with $D_E = D_{E^*}$=0.1); $D_E = D_{E^*}$=10 (red).

Initial conditions:

Initially, X and X^* are present only in L with $[X]_{total}$=1.0, E^* exists everywhere with $[E^*]$=0.01, and Y is localized only in $L1$ with $[Y]_{total}$=10.0.

A. E is initially localized in $L1$ with $[E]$=0.05.

B. E is initially present everywhere with $[E]$=0.01, Z is localized only in $L2$ with $[Z]_{total}$=10.0.

6 Figure Captions

See attached file.

Acknowledgment. We thank Vaidhiswaran Ramesh for assistance with Maple computations.

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Figure S1. **Temporal dose responses in different parameter regimes for the bifunctional CMC model**. *X**/*X* (red) and *X** (blue) vs total enzyme concentration *[E]total*. (A) Basic model, substrate modification in the saturated regime. (B) Model with feedback, unsaturated regime for substrate modification (C) Model with feedback and substrate modification in the saturated regime, showing a dependence of X*/X . As discussed in the text, a combination of being far from the unsaturated limit, and having feedback allows for a dependence of *X**/*X* on total enzyme concentration. (D) Alternative model for bistability (linear feedback with extra interaction: see Supplementary Text). Bifurcation diagram showing a bistable response (with linear feedback), S1 and S2 correspond to the signal values bookending the region of bistability.

Figure S2. Spatial designs of bifunctional enzymes (TCS case). An analogous figure to Fig. 4 in the main text, this figure depicts a TCS. The essential insights are similar to those of a CMC. (A), (B), (C) represent different combinations of the enzyme localization and the balance between *E* and *E**: (A) *E* and *E** are localized in one location, *L* (same as in Fig. 2 (A), Main Text), (B) *E* and *E** are localized in two locations, (same as in Fig. 2 (C)), with same balance and (C) different balance between *E* and *E** in two locations. A spatially varying balance of enzyme forms is a necessary factor for producing a graded response. Moreover, due to the local reversibility of the biochemical cycle, resulting from the nature of bifunctional enzymes, there is no scenario of zero outputs of substrate.

Figure S3. Absolute concentration robustness in bifunctional TCS/CMC. (A) In contrast to Fig. 5 (C), we consider a case of reduced total substrate concentration for a bifunctional CMC. While the completely co-localized case does not exhibit ACR (orange curve), specific spatial designs do demonstrate this (green and blue curves). For this reduced total substrate concentration, the TCS also reveals a similar behaviour, whereas for higher substrate concentrations there may be significant differences between the CMC and TCS (see Fig. 5 (B-D)). (B) The effect of variation of total substrate concentration in bifunctional TCS, ODE model. The figure depicts that as total substrate concentration increases in the TCS model, *X** approaches a constant. This can be explained analytically**.**

Figure S4. The effect of conversion balance between *X* and *X on "spatial ACR" in bifunctional CMC and TCS.** This figure investigates the effect of relative balance of kinase and phosphatase activity on the ACR behaviour observed in different spatial (substrate) designs for the CMC (A-D) and the TCS (E-H). In each case, the balance between the catalytic constants of the forward and reverse substrate modification is progressively weakened (by changing the catalytic rate constants). This alteration of balance impacts the effect of single species diffusing (see the relative positions of the red and green curves in (A) and (D)). Note also, that there is a difference between the CMC and TCS results (the latter not exhibiting the robustness results), which has its origins in the relatively high substrate amounts (see Fig. S5 for a contrast).

Figure S5. The effect of conversion balance between *X* and *X on "spatial ACR" in bifunctional CMC and TCS for lower total substrate amounts.** This figure investigates the effect of relative balance of kinase and phosphatase activity on the ACR behaviour observed

in different spatial (substrate) designs for the CMC (A-D) and the TCS (E-H), similar to Fig. S4 but for a lower total substrate concentration. In each case, the balance between the catalytic constants of the forward and reverse substrate modification is progressively weakened (by changing the catalytic rate constants). We see similar trends in the CMC and the TCS here. We also see that as the balance of modification is changed in favour of the reverse substrate modification, the case of only *X* diffusing exhibits a robustness behaviour (a behaviour seen in the CMC in Fig. S4).

Figure S6. Variation of total enzyme concentrations equally and simultaneously at two locations. We see that the destruction of ACR is independent of diffusion coefficients of substrate (A-D), contrasting dose response for average concentrations at location *L1*, *L2* and the overall domain spatial average for different diffusion coefficients. (E) and (F) show location *L2* average and the spatial average respectively, for a range of substrate diffusion coefficients. In all cases there is a dependence on total enzyme concentrations. (G) and (H) consider enzymes localized at two locations, where the enzyme balance at location *L1* is the same as Fig. 5 (D). The presence of enzyme at a second location *L2* with a different balance (kinase dominated in (G) and phosphatase dominated in (H)) contributes to a stronger dependence on total enzyme concentration (contrast with Fig. 5 (D) where both substrates are diffusing). This figure complements Fig. 5 (E,F) in the main text.

Figure S7. The effect of spatial organization on bistability in bifunctional CMC and TCS. Complementing Fig. 6 (B-D) in the main text, (A-C) here examine the variation of total concentration of enzyme on bistability, for the cases of (A) *X* diffusing, (B) *X** diffusing and (C) both *X* and *X** diffusing: spatial organization destroys ((A) and blue curve in (C)) the bistability when only *X* is diffusing as well as both *X* and *X** diffusing respectively, but bistability remains (blue curve in (B)) when only *X** diffusing in bifunctional TCS for the specific parameters (kinetics, spatial) employed (this reinforces the point in the main text, that bistability can be destroyed, and that this can depend on the spatial design). The loss of bistability can depend on the size of compartment where the substrate is localized (see red curve in (B) showing a decrease of size destroying bistability) and also on the total amount of substrate (e.g. it can be regained with greater initial substrate concentration: see red curve in (C)): this is a basic feature seen in all cases of substrate designs. Moreover, (D) demonstrates that a sufficiently small localized domain of enzymes (and non-diffusible substrate) can destroy bistability for all diffusing designs of substrate complementing Fig. 6 (B,D) of the main text. kph denotes the irreversible conversion rate to the dead-end complex. (E) depicts the perturbation of bistability in the CMC model with feedback showing how a non-trivial spatial organization can shrink the range of bistability (red curve), relative to the co-localized case (blue curve). (F) demonstrates a case (in the context of potential switching of activity by localization) of bifunctional enzyme (TCS) where no switching occurs: with a combination of factors of *E* localizing more strongly and no interconversion between localized forms, the phosphatase activity predominates over the entire range of total substrate concentration.

Figure S8. The role of basal interconversion between enzyme forms *E* and *E in the transition from monofunctional to bifunctional enzyme with pre-existing spatial organization.** Building on Figs. 10 and 11 (B), we investigate the effect of different balances of interconversion between *E* and *E**, the (free) forms of the bifunctional enzyme. Similar to Fig. 10 (B,C), (A) shows the species concentration profiles when the monofunctional enzyme *K* is localized in one place (pole) and *P* is everywhere, (B) represents the case when *K* and *P* are localized in two places in the monofunctional CMC. (A) shows how the bifunctional system ends up in a situation where a predominantly phosphatase form localizes at the given pole. (B) A two-pole localization is observed similar to the monofunctional case, though the balance of kinase and phosphatase at the two poles is the same (balance more towards phosphatase), and consequently a non-graded substrate concentration profile is observed (in contrast to the monofunctional case). (C) demonstrates an analogue of Fig. 11 (B) where no interconversion of localized forms occurs. We notice that while a double pole localization does occur, mimicking the monofunctional case, there is a significant difference in concentration of enzyme forms at the two poles. In all cases, it is assumed that the bifunctional enzyme localizes where either parent enzyme is capable of localizing.

Figure S9. Exploration of cases where bifunctional CMC enzyme forms have a completely different localization "logic" to monofunctional CMC. For the two reference cases of the monofunctional enzymes considered previously and in the main text (localization of *K* at one pole, and localization of *K* and *P* at two different poles), (A) and (B) show the species concentration profiles when only *E* can bind to the pole (first case) and *E* and *E** forms of the bifunctional enzymes are localized in opposite locations to the parent monofunctional enzymes (second case). Compared with Fig. 11 (A), in (A) here, a clear quantitative and qualitative change of substrate concentration profiles occurs with the bifunctional enzyme forms ending up essentially fully as phosphatase at one pole (blue curve); in addition, a totally reversed result compared with monofunctional CMC is generated, where *E* is primarily at a pole and *E** is everywhere with graded substrate concentration profiles when the interconversion of localized forms with a strong unbinding of *E** is allowed, (red curve). Moreover, in (B) here, the bifunctional CMC also shows a totally reversed logic with monofunctional CMC, so that *E* and *E** are localized in two different, but exchanged (compared with Fig. 11 (B)), poles whether the interconversion of localized forms does (red curve) or does not (blue curve) exist (if interconversion exists, a strong unbinding of the other form is incorporated to impose the hypothesis of exclusive localization of one form at this pole).

Figure S1

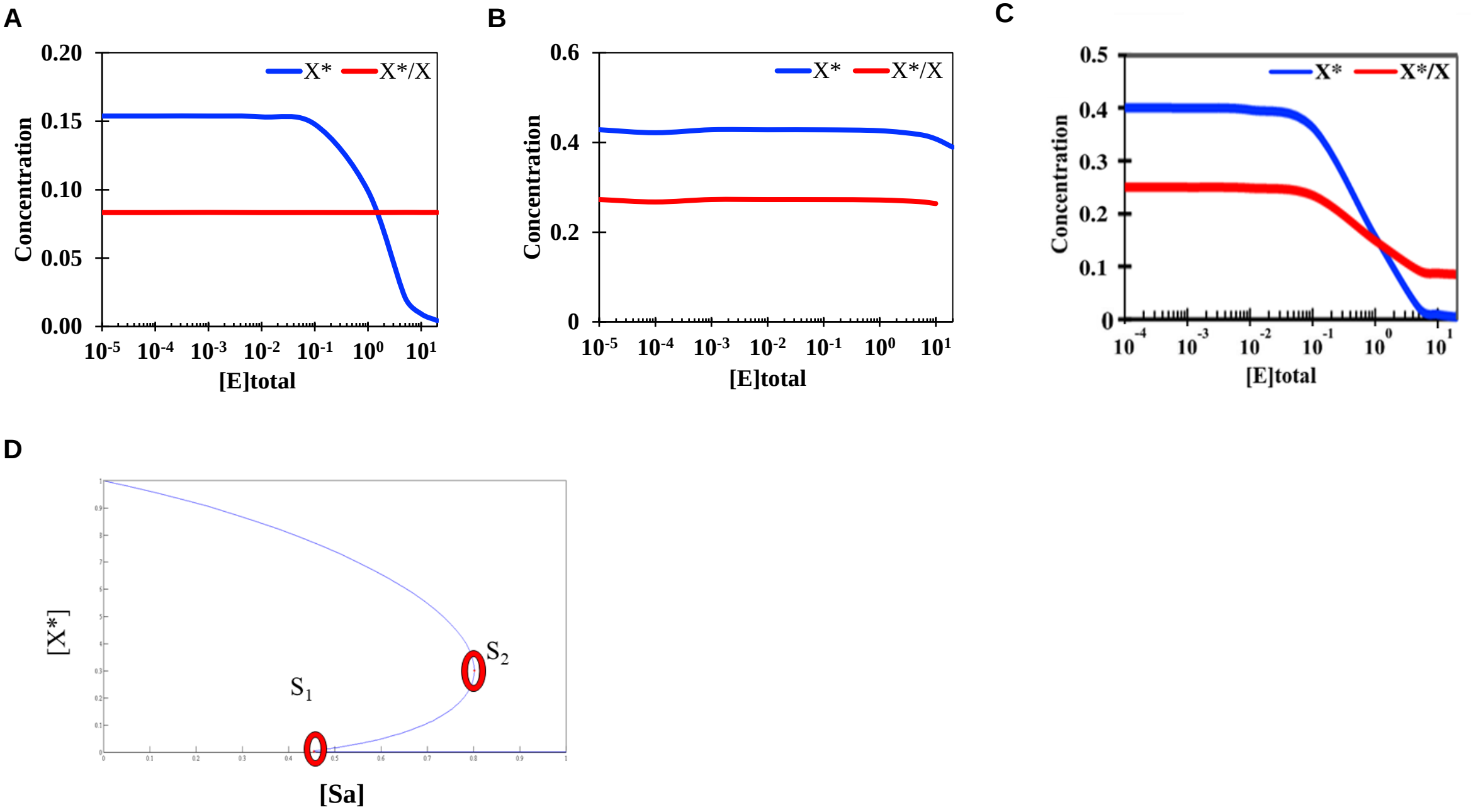


Figure S2

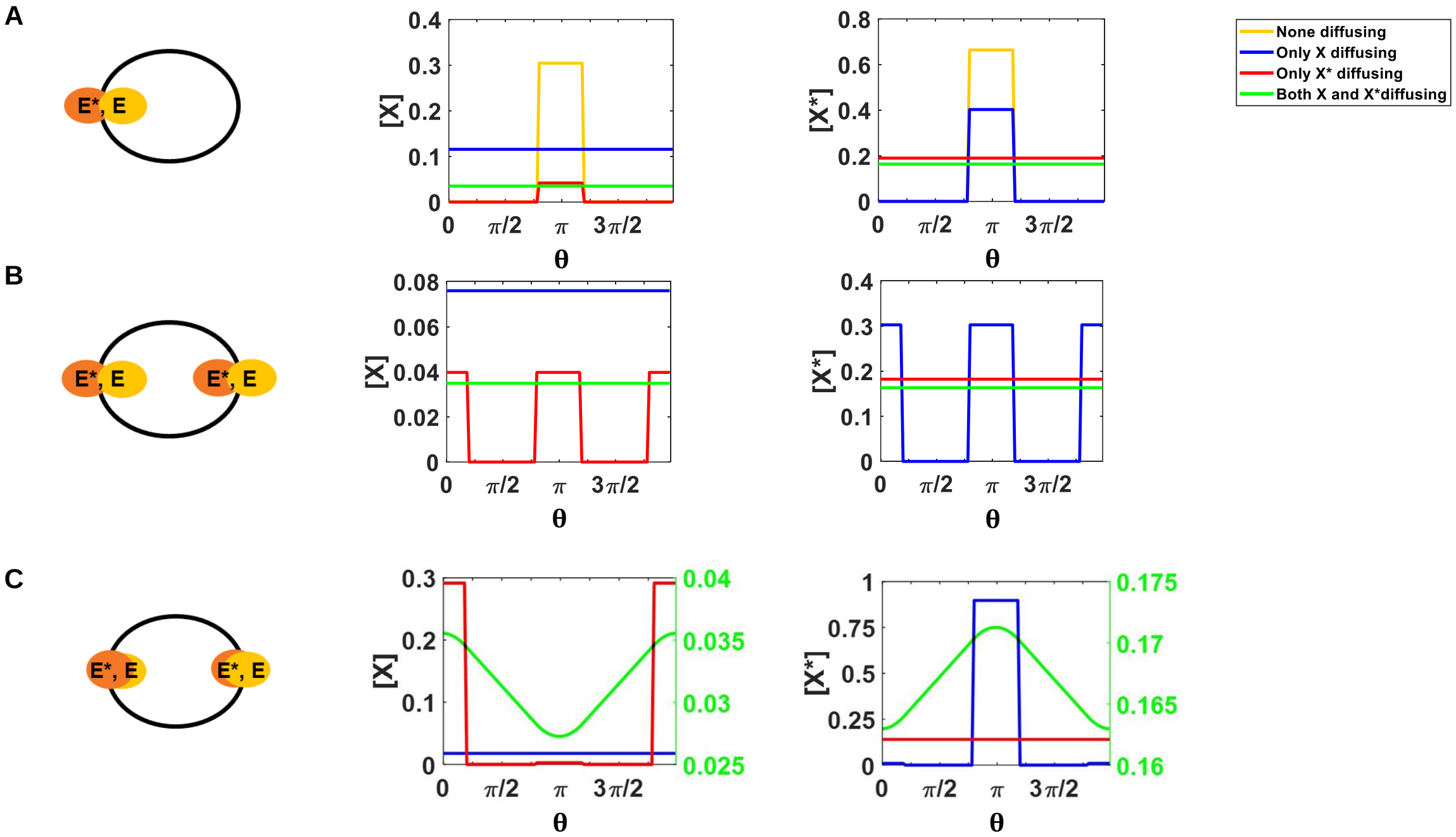

A
B
C
E*, E
E*, E
E*, E
E*, E
E*, E
[X]
[X*]
θ
0
π/2
π
3π/2
None diffusing
Only X diffusing
Only X* diffusing
Both X and X*diffusing

Figure S3

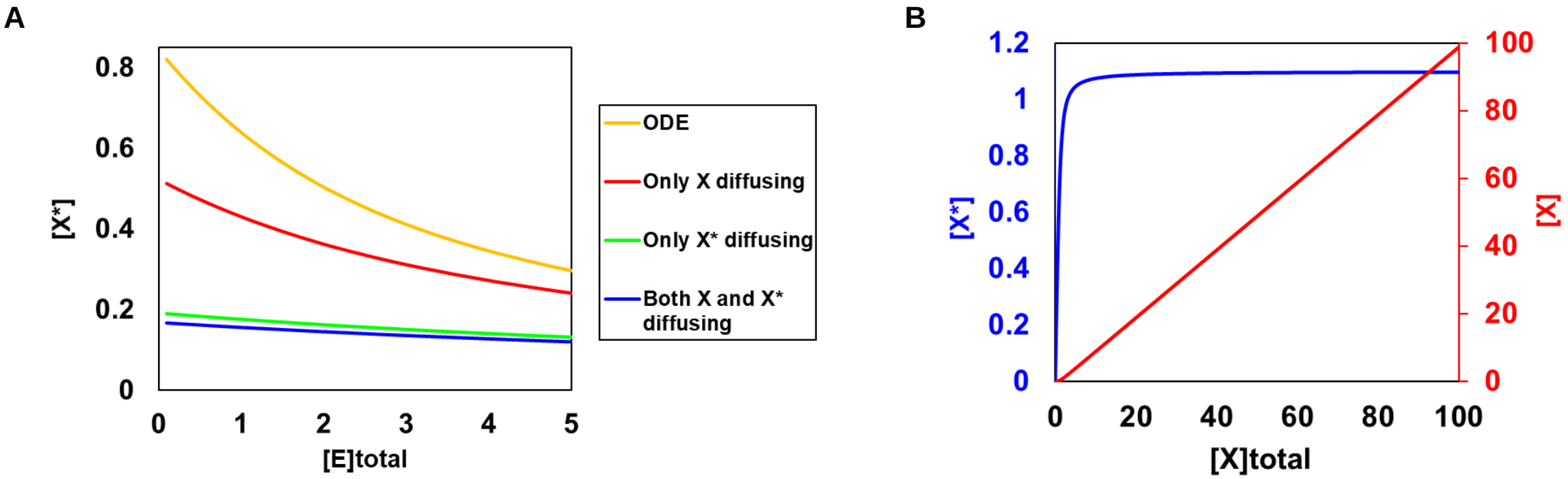

A
0.8
0.6
0.4
0.2
0
[X*]
0
1
2
3
4
5
[E]total
ODE
Only X diffusing
Only X* diffusing
Both X and X* diffusing
B
1.2
1
0.8
0.6
0.4
0.2
0
[X*]
100
80
60
40
20
0
[X]
0
20
40
60
80
100
[X]total

Figure S4

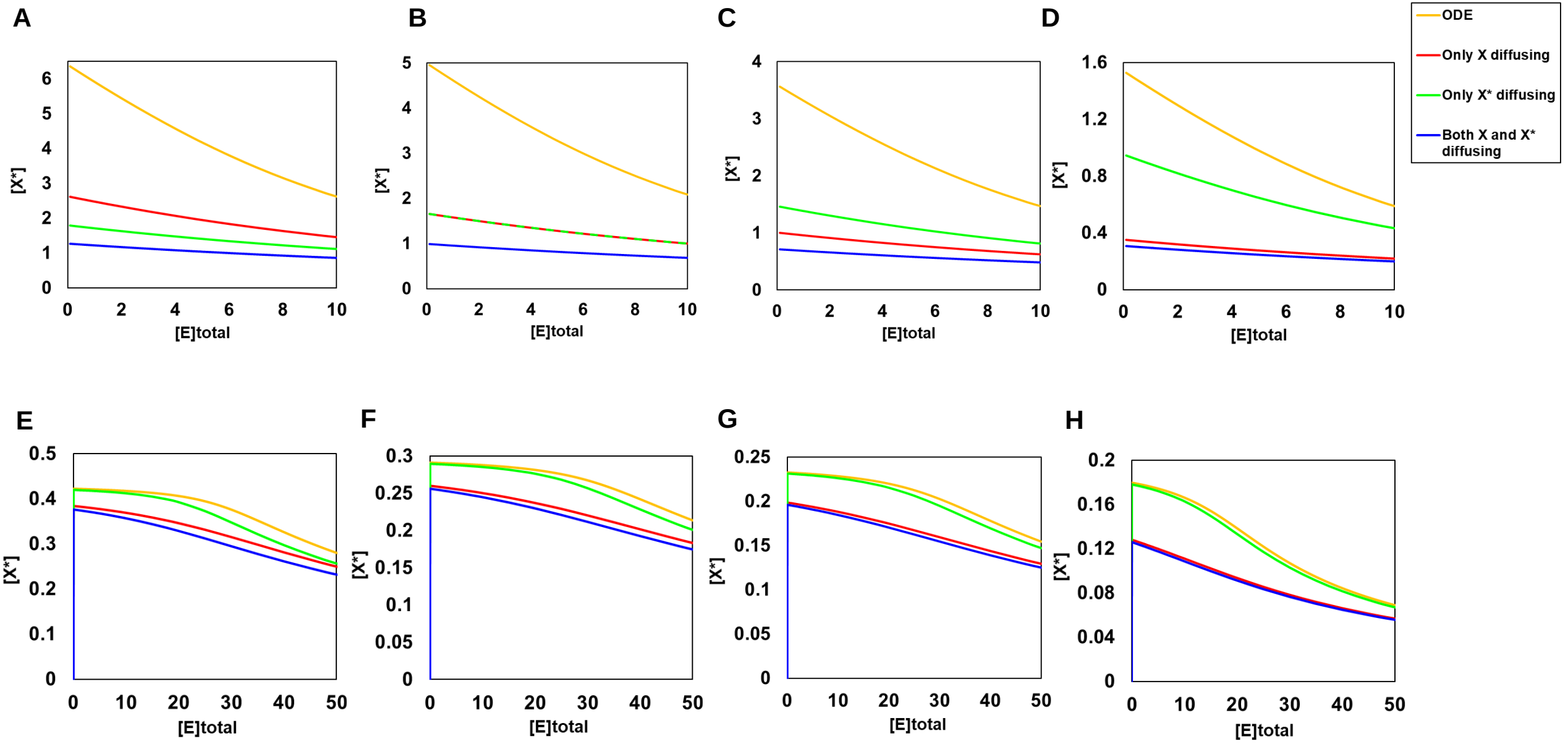

A
B
C
D
E
F
G
H
[X*]
[E]total
ODE
Only X diffusing
Only X* diffusing
Both X and X* diffusing

Figure S5

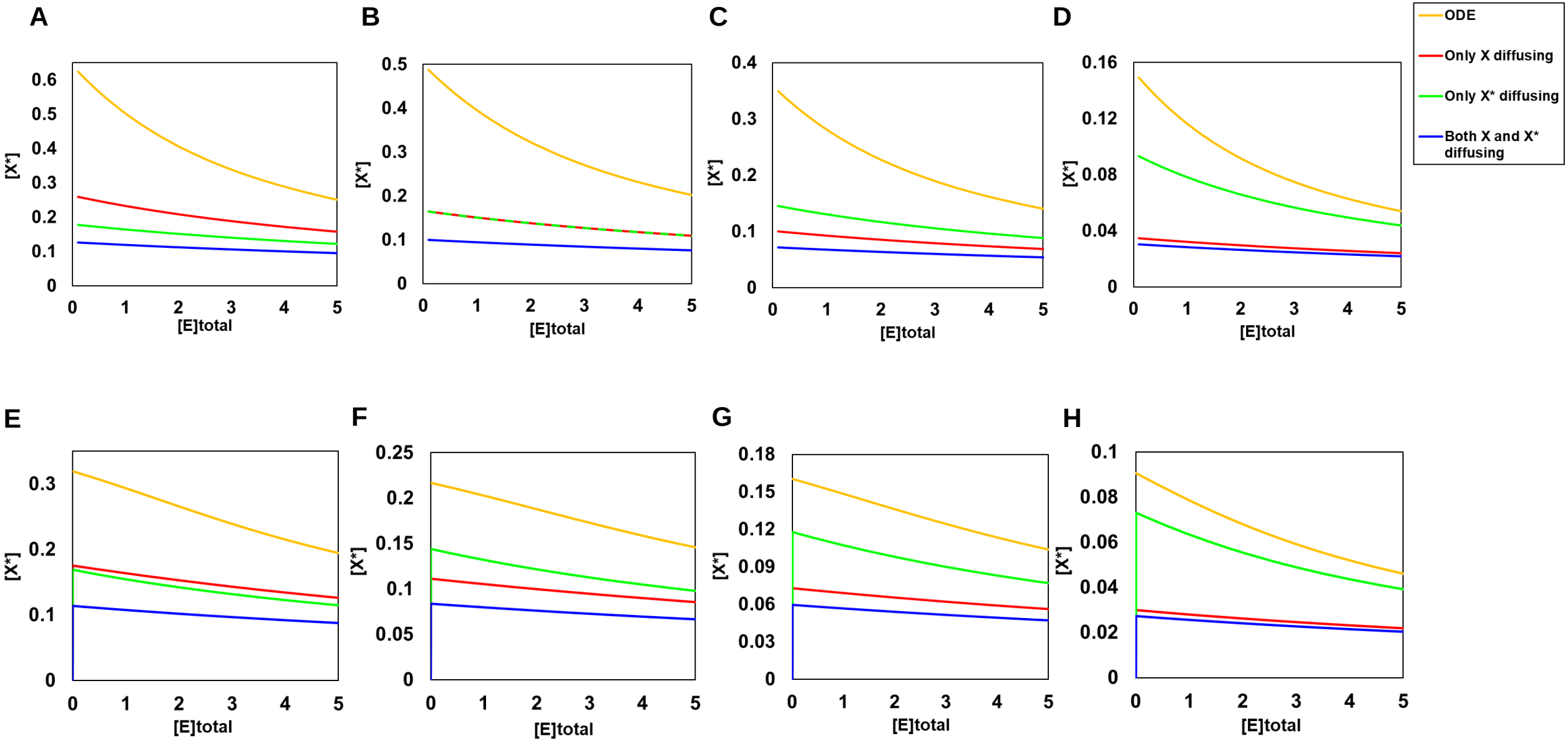

A
B
C
D
E
F
G
H
[X*]
[E]total
ODE
Only X diffusing
Only X* diffusing
Both X and X* diffusing

Figure S6

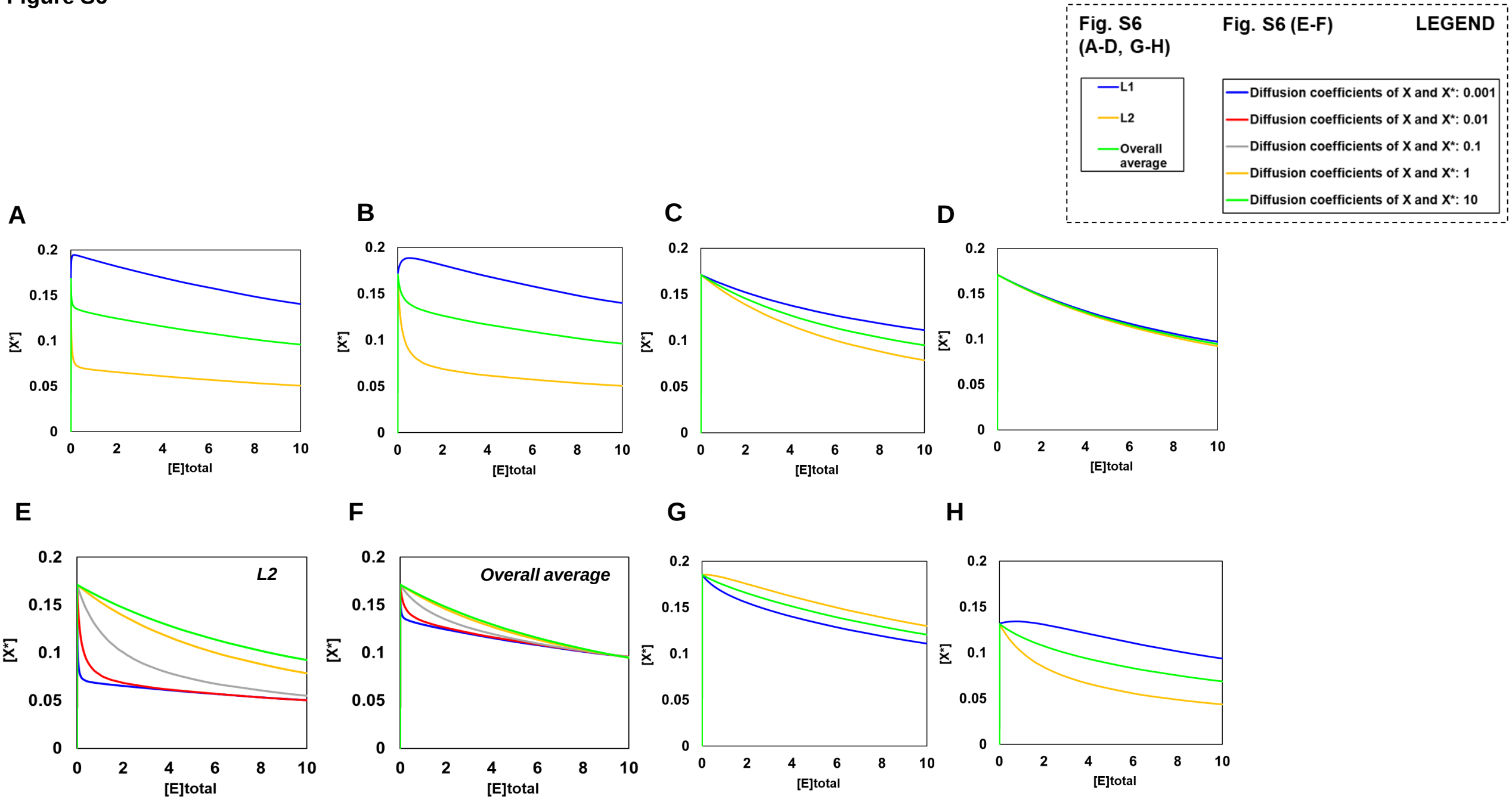


Fig. S6 (A-D, G-H)
Fig. S6 (E-F)
LEGEND
L1
L2
Overall average
Diffusion coefficients of X and X*: 0.001
Diffusion coefficients of X and X*: 0.01
Diffusion coefficients of X and X*: 0.1
Diffusion coefficients of X and X*: 1
Diffusion coefficients of X and X*: 10
A
B
C
D
E
F
G
H
L2
Overall average
[X*]
[E]total
0
0.05
0.1
0.15
0.2
0
2
4
6
8
10

Figure S7

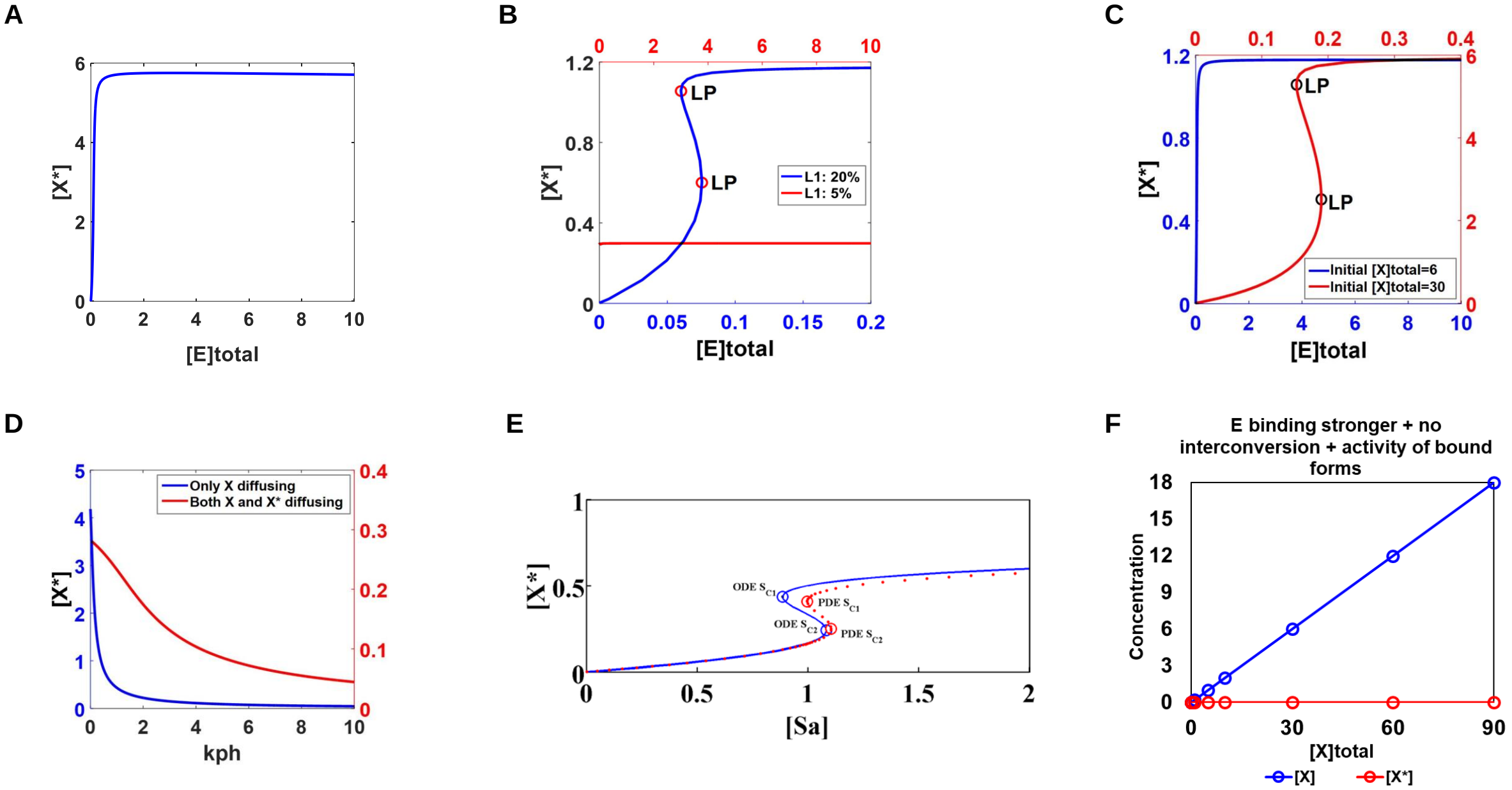

A
[X*]
[E]total
B
LP
LP
L1: 20%
L1: 5%
[X*]
[E]total
C
LP
LP
Initial [X]total=6
Initial [X]total=30
[X*]
[E]total
D
Only X diffusing
Both X and X* diffusing
[X*]
kph
E
ODE S_C1
PDE S_C1
ODE S_C2
PDE S_C2
[X*]
[Sa]
F
E binding stronger + no interconversion + activity of bound forms
Concentration
[X]total
[X]
[X*]

Figure S8

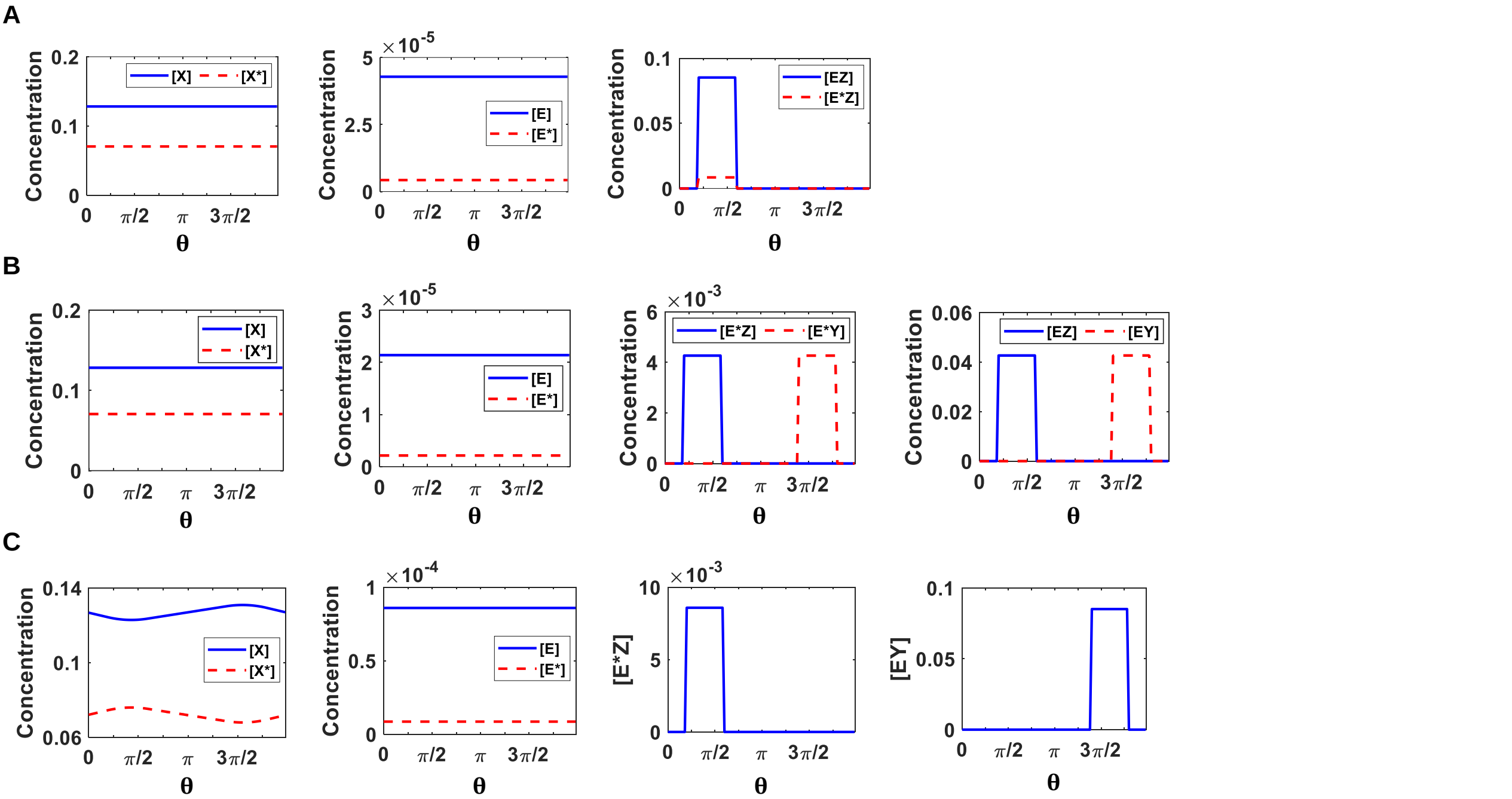
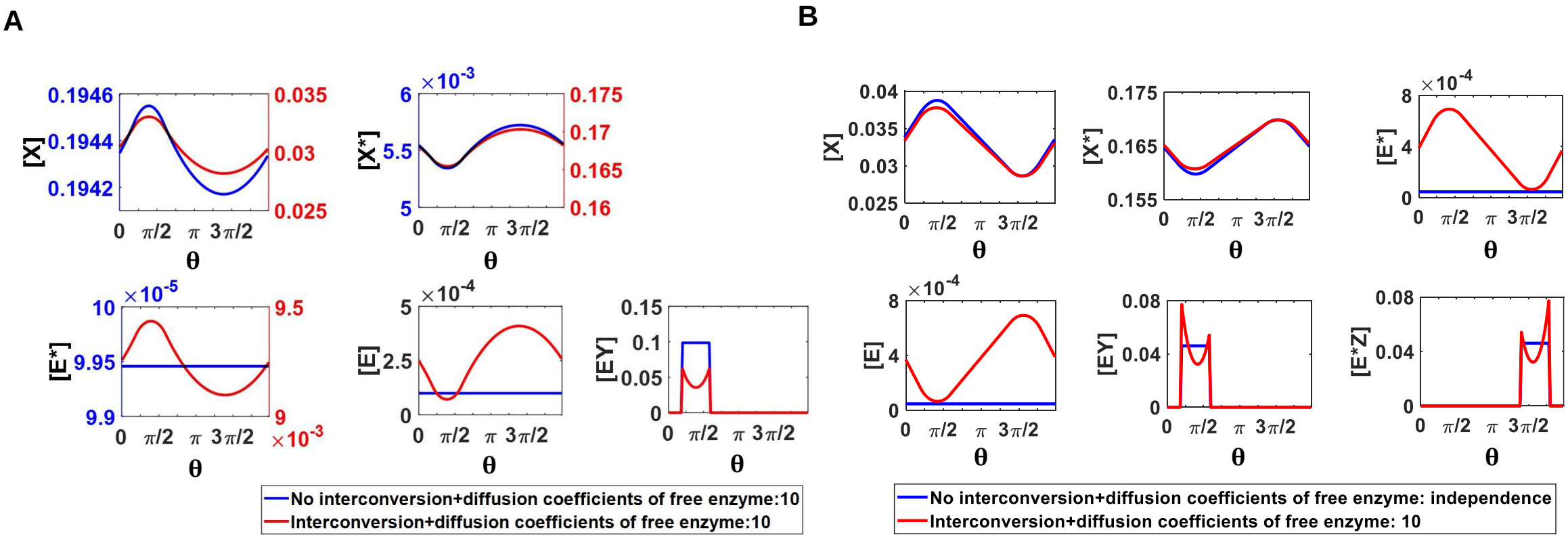


Figure S9


A
B
[X]
[X*]
[E*]
[E]
[EY]
[E*Z]
θ
0
π/2
π
3π/2
No interconversion+diffusion coefficients of free enzyme:10
Interconversion+diffusion coefficients of free enzyme:10
No interconversion+diffusion coefficients of free enzyme: independence
Interconversion+diffusion coefficients of free enzyme: 10