Robust global sensitivity in multiple enzyme cascade system explains how the downstream cascade structure may remain unaffected by cross-talk

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Abstract A steady-state framework was applied to the ubiquitous tricyclic enzyme cascade structure, as seen in the mitogen-activated protein (MAP) kinase system, to analyze the effect of upstream kinase concentrations on final output response. The results suggest that signal amplification achieved by the cascade structure ensured that the modifying enzymes at various steps of the cascade were nearly saturated. Thus, there was no change in the response sensitivity with increasing upstream kinase concentration. Analysis was also extended to branching of a signaling pathway as an example of cross-talk. It was observed that the cascade structure confers a larger share of the signal transduction properties to its last kinase. This phenomenon in enzyme cascades may explain how the response of the terminal MAP kinase is unaffected by cross-talk of upstream kinases.

Key words: Signal amplification; Ultrasensitivity; Robustness; Mitogen-activated protein kinase; Enzyme cascade; Cross-talk; Signal transduction

I. Introduction

Living cells continuously sense extracellular cues, transduce signals and respond appropriately with the help of intricate signaling pathways, to survive in an ever changing environment \cite{1,2}. Signaling pathways are made up of a complex web of enzyme cascades, some of which are known to be highly conserved across living systems \cite{3}. Depending on their regulatory design, enzyme cascades have been shown to exhibit signal amplification \cite{1,2,4-7}, flexibility \cite{2,4}, bistability \cite{8}, oscillations \cite{9}, robustness \cite{10,11}, and ultrasensitive responses \cite{1,4-7,12-16}. Multicyclic enzyme cascades are known to integrate multiple signals, thereby permitting coordinated physiological responses \cite{2,4}. The physiological responses are usually ultrasensitive, exhibiting sigmoidal dose-response curves \cite{1}. The functional significance of ultrasensitivity \cite{1,6,12,17} and conditions for generating such a sensitive response have been discussed \cite{18,19}. In enzyme cascades, ultrasensitivity arises when: (a) the converter enzymes (e.g. kinases and phosphatases) operate under saturating conditions (i.e. zero order effect) \cite{5-7}, (b) a signaling component acts on multiple steps of a cascade system (i.e. multi-step effects \cite{5,7} and its manifestations, like dual phosphorylation \cite{12}), and (c) a stoichiometric inhibitor of the activating enzyme is present \cite{12}. Ferrell and coworkers \cite{12-15} have used the Hill coefficient as a sensitivity parameter to quantify the steepness of sigmoidal dose-response curves. A value of the Hill coefficient greater than 1 indicates ultrasensitive response, while values less than 1 indicate subsensitive response.

In eukaryotic cells, mitogen-activated protein kinase (MAPK) modules are highly conserved enzyme cascade systems \cite{3}. They are activated by a wide array of stimuli and are known to regulate numerous cellular processes \cite{20}. The MAPK cascade system typically consists of three sequentially activating enzyme cascades, such that an active MAPK kinase kinase (MAPKKK) activates MAPK kinase (MAPKK), which further activates a specific MAPK. The terminal MAPK then acts as an effector of a unique pathway, by regulating the gene expression to elicit an appropriate physiological response \cite{20}. Experimental and theoretical studies have reasoned that the major significance of having such a cascade structure may be to amplify and integrate various signals arriving from different pathways and also to filter out noise \cite{3,8,12-15,20}. The term cross-talk in the signaling field refers to interconnections between different signaling pathways. The signal can be integrated from different inputs (through components known as ‘junctions’) which can branch out to multiple outputs (through components known as ‘nodes’) \cite{21}. Many components of the MAPK cascades are shared by different pathways and still demonstrate specificity by responding differently to different signals \cite{3,20,22}. If the same protein kinase is being shared between two or more different signaling pathways, then the question arises as to how the same protein determines different responses in different pathways and how its concentration affects the ultimate output response (say, phosphorylation of a specific MAPK).

In the present work, to study the effect of upstream kinase concentration on a final output response, a linear tricyclic enzyme cascade and branching of a signaling pathway, as a case of cross-talk, was analyzed. By steady-state analysis, we show that the cascade structure confers the larger share of the signal transduction properties to its last kinase and responds with an invariant global sensitivity (quantified as apparent Hill coefficient) at various concentrations of upstream kinases.
2. Materials and methods

Fig. 1A shows the schematic of a linear tricyclic cascade wherein a protein kinase (interconvertible enzyme) A gets phosphorylated to A-P, which further catalyzes the phosphorylation of B. In the next step, the phosphorylated form of B (i.e. B-P) further catalyzes the phosphorylation of C. Enzymes E3 and E4 are the phosphatases, which dephosphorylate the modified enzymes A-P, B-P and C-P, respectively. Enzyme E5 is a signal input to the system and is commonly an active kinase (converter enzyme). The signal output of this pathway is the phosphorylation of the terminal kinase C, which can further elicit a cellular response. The steady-state operating equation for individual covalent modification cycles were sequentially connected to evaluate the output response (of the terminal kinase) of the cascade structure to the input stimulus (E_{total}). The concentrations of ATP and PP were considered to be constant in the current analysis. The steady-state operating equation for a simple covalent modification cycle has been taken from the classic work of Goldbeter and Koshland [5-7].

For the first cycle (modification of A) in a linear cascade (Fig. 1A), the steady-state relationship in the form of a cubic equation for fractional modification of protein kinase is given in the Appendix. Similar cubic equations were written for each step of the reversible phosphorylation cycle in a linear cascade to estimate the corresponding fractions of modified and unmodified protein kinases. As the output of one cascade becomes the input to the subsequent downstream cascade (at a fixed phosphatase concentration), the fractional modification of the terminal kinase C can be estimated at various input stimulus concentrations (E_{total}). Such an analysis gives stimulus dose–response curves for fractional modification of all three protein kinases at a specific input stimulus concentration.

A similar analysis was also extended to the cascade structure shown in Fig. 1B. This is a case of cross-talk, wherein a common kinase participates in two different pathways (or routes a signal to different proteins, C and D). The description of the cascade structure is the same as that of Fig. 1A, except that the activated protein kinase B (i.e. B-P) catalyzes activation of two different kinases C and D. Here, it is assumed that the activation of both downstream kinases C and D occurs independently. As explained above, we obtain the stimulus dose–response curves for fractional modification of C and D to a given input E_{total}. In the present analysis, we have considered all enzyme-substrate complexes in the conservation relationship for the corresponding target protein (in an individual cascade). It should be noted that we have neglected the cascade connecting complexes BAP and CBP (of Fig. 1A) in the A_{total} and B_{total} balances respectively, and complexes BAP in the A_{total} balance, CBP and DBP (of Fig. 1B) in the B_{total} balances. This assumption is valid for sets of parameter values that yield greater than 90% modification of the kinases, implying that the complexes are negligible. The parameters used for the above analysis are listed in the Appendix. The parameters were referred from the work of Huang and Ferrell [13], and Bhalla and Iyengar [23], wherein the parameter values were experimentally validated.

The steepness of the dose–response curves can be approximated using the Hill equation. The global output response (fractional modification of C) can then be quantified in terms of apparent Hill coefficient and half-saturation constant, with respect to the input stimulus concentration. Here, the half-saturation constant is the amount of input stimulus required for 50% fractional modification of the corresponding protein kinase. Thus, the half-saturation constant indicates a mid-point on the unmodified to modified kinase transition curve. The parameters of the Hill equation thus also quantify the threshold input concentration required to switch on the response. The apparent Hill coefficient can also be calculated by estimating the primary input concentration required for 10% to 90% modification of terminal kinase C, by using the following equation:

$$n_{HI} = \frac{\log \frac{81}{9}}{\log \frac{I_{90}}{I_{10}}}$$

where $I_{10}$ and $I_{90}$ are input concentrations required for 10% and 90% modification of terminal kinase C, and $n_{HI}$ is the apparent Hill coefficient.

3. Results

The cubic equation (given in the Appendix) was used to obtain the fractional modification of kinases A, B and C at various input concentrations (E_{total}) to yield the dose–response curves. Fig. 2A shows the dose–response curves for three kinases at different concentrations of primary kinase A. For a given primary kinase concentration, the steepness of dose–response curves increases and the half-saturation constant decreases as the signal propagates down the cascade. This indicates amplification of the signal and an increase in sensitivity. The steepness of the fractional modification curve of the first cycle increased as the concentration of primary kinase A increased (curves a1–a3 in Fig. 2A). The increase in steepness (also indicated by the apparent Hill coefficient) is due to zero order effects and this does not affect the half-saturation constant of the first enzyme cycle. The variation in the concentration of primary kinase A did not affect the steepness of the dose–response curves of downstream kinases (i.e. B and C). However, the half-saturation constant of the dose–response curves (of B and C) decreased with an increase in the concentration of primary kinase A, indicating signal amplification. It should also be noted that this increase in signal amplification is limited and saturates beyond a particular concentration of A. Thus, an increase in the primary kinase concentration resulted in an increase in signal amplification to some extent, but did not influence the sensitivity of the dose–response curves of downstream kinases. A similar analysis by changing the second kinase B concentration indicated that the sensitivity of the dose–response curves of the terminal kinase C was not affected. Also, for obvious reasons, the response of the primary kinase was not influenced by the changes in the concentration of downstream kinase C.
can be visualized by plotting the apparent Hill coefficient with respect to the input concentration at various concentrations of upstream kinase as shown in Fig. 3. The apparent Hill coefficient was constant with respect to changes in upstream kinase concentrations (Fig. 3A), as shown in Fig. 2, whereas the apparent Hill coefficient increased with an increase in the terminal kinase concentration C due to zero order effects (Fig. 3B). Therefore, for a given concentration of terminal kinase C, a specific sensitivity was obtained for the overall response (see ‘a’ and ‘b’ in Fig. 3B).

The above results indicate that in enzyme cascades the response sensitivity of the terminal kinase depends mainly on its concentration and thus may remain unaffected by the cross-talk of the upstream kinases. The cascade structure shown in

Fig. 2. Predicted dose–response curves of tricyclic enzyme cascades. Fractional modification of the first kinase (curves a), the second kinase (curves b) and the third kinase (curves c) in a tricyclic enzyme cascade at various concentrations of the input $E_{\text{total}}$ on semi-logarithmic plots. Parameters used for the analysis are listed in the Appendix. A: Predicted dose–response curves at different concentrations of kinase A represented by curve a1, $A_t = 0.003 \, \mu$M, curve a2, $A_t = 0.3 \, \mu$M and curve a3, $A_t = 5 \, \mu$M. Total concentrations of second kinase B and third kinase C were kept constant at 1.2 \, \muM. Curves b1 to b3 ($\eta_1^{\text{App}} \approx 1.34$) and c1 to c3 ($\eta_2^{\text{App}} \approx 1.42$) represent response curves obtained for changes in the first kinase concentration from curve a1 to a3 ($\eta_1^{\text{App}} \approx 1-1.7$) respectively. B: Predicted dose–response curves at different concentrations of second kinase B represented by curve b1, $B_t = 1.2 \, \mu$M, curve b2, $B_t = 4 \, \mu$M and curve b3, $B_t = 10 \, \mu$M. Total concentrations of first kinase A and third kinase C were kept constant at 0.003 \, \muM and 1.2 \, \muM respectively. Curves a1 to a3 ($\eta_1^{\text{App}} \approx 1$) and c1 to c3 ($\eta_2^{\text{App}} \approx 1.42$) represent response curves obtained for changes in second kinase B concentration from curve b1 to b3 ($\eta_1^{\text{App}} \approx 1.34-1.65$) respectively.

Fig. 3. Operating curves for a tricyclic enzyme cascade. A: Plot of the apparent Hill coefficient at various concentrations of upstream protein kinase A (of Fig. 1B). Total concentration of kinase B, kept constant at 1.2 \, \muM, and that of the terminal kinase, C, is given as: curve a1, $C_t = 1.2 \, \mu$M (with $\eta_2^{\text{App}} \approx 1.42$) and curve a2, $C_t = 8 \, \mu$M (with $\eta_2^{\text{App}} \approx 3.25$). B: Plot of the apparent Hill coefficient with increase in the concentration of terminal kinase C showing zero order effects. The total concentrations of upstream kinases were kept constant at $A_t = 0.003 \, \mu$M and $B_t = 1.2 \, \mu$M; dotted lines a1 and a2 indicate the results shown in A, for a particular concentration of target kinase C.

Fig. 1B was analyzed to obtain insight into this phenomenon. The dose–response curves of fractional modification of terminal kinases C and D at various concentrations of input stimulus are shown in Fig. 4. As the concentration of upstream kinase B was increased, the half-saturation constant of the fractional modification curve of both terminal kinases C and D decreased, while the sensitivity of the response remained invariant (with apparent Hill coefficients 1.42 and 1 respectively). We have arbitrarily chosen the parameters for the phosphorylation cycle of terminal kinase D, such that the responses of covalent modification cycles C and D can be differentiated. As the covalent modification cycle of D operates in the first order region, the corresponding response is Michaelian with an apparent Hill coefficient of 1; however, the response sensitivity of cycle C remains unaffected due to cross-talk of upstream kinase B.

In the present work, the main criterion for choosing the parameters used for the analysis was that the cascade system should respond with greater than 90% modification of the terminal kinase. We have used the parameters from the work of Huang and Ferrell [13], while keeping this criterion in mind. To determine whether sensitivity of the response is
constant for a range of parameters, the Michaelis constants and reaction rates were also varied individually by ±10-fold, keeping the rest of the parameters constant. The steepness of the output response of cascade structures (Fig. 1A,B) at different concentrations of upstream kinases was invariant to changes in the parameters. However, the half-saturation constant was again sensitive to these changes. The analysis was also done using the parameters reported by Bhalla and Iyengar [23] for the MAPK system, and similar results were obtained (results not shown here). As listed in the Appendix, these parameters are considerably different from those reported by Huang and Ferrell [13].

In the current analysis, the cascade connecting complexes such as BAP in the A_{total} balance, and CBP in the B_{total} balance were neglected. We have varied the extent of formation of these complexes and they were found to be negligible as compared to the corresponding total kinases. This assumption is valid when the terminal kinase response curve reaches beyond 90% phosphorylation. As can be seen from Fig. 2, greater than 90% phosphorylation of the terminal kinase (kinase C) is reached at a low input concentration of \(10^{-10} \mu M\), which is lower than that required for 5% phosphorylation of the upstream kinases (i.e. A and B). At this point, irrespective of their total concentration, the upstream kinases (A and B) exist mainly in their free form, since the input stimulus is very low for their activation. Also, if the total concentrations of upstream kinases A or B are increased, the overall signal amplification increases and thus leads to further reduction in the threshold input concentration required to switch on the response.

4. Discussion

The analysis of a tricyclic cascade system and signal branching pathway demonstrated an invariant global sensitivity with respect to changes in the upstream kinase concentration, while exhibiting ultrasensitivity with an increase in terminal kinase concentration due to zero order effects. The upstream kinases operate under saturating conditions resulting in the observed invariant response sensitivity. Effectively, the cascade structure imparts robustness to the sensitivity of the output response. In a tricyclic cascade, such as that observed in MAPK, the sensitivity of the overall response depends on the amount of input stimulus to the cascade and on the terminal kinase concentration. Thus, zero order effects observed due to saturating levels of upstream kinases are not entirely transmitted down the cascade.

In such instances, contrary to common notions, the global output responses do not always correspond to a multiplicative function of the Hill coefficient of each cascade response. Though the global sensitivity primarily depends on the terminal kinase concentration, the half-saturation constant and threshold input concentration to switch on the response are influenced by the upstream kinase concentration. The cascade structure decreases the threshold input concentration required to switch on the response due to amplification and this phenomenon is equally important as the invariant sensitivity in signal transduction.

The analysis of a branched signaling pathway indicates that different downstream kinases may respond differently to the same input signal and hence may explain the varied responses obtained through the same signaling pathway at different input threshold concentrations. The response of the terminal kinases (C and D of Fig. 1B) depends on their respective concentrations, Michaelis–Menten constants and rate constants of corresponding modification–demodification reactions. These parameters can thus be responsible to yield distinct responses with respect to the same input stimulus.

In the present work, the response sensitivity of covalent modification cycle C was not affected by changes in the upstream kinase concentration and its cross-talk with the downstream kinase of a different signaling pathway. Our simulation results are consistent with a recent report on evolvability of the MAPK cascade system [24]. Nijhout et al. [24] observed that genetic variation in the regulatory region of MAPK will have a more profound effect on the output response and will be under stronger selection than that of upstream kinases. This emphasizes the importance of the terminal MAPK concentration on the final response of the pathway.

What may be the significance of this robust sensitivity of global output response towards changes in upstream kinase concentration? Ferrell and coworkers have shown that, in Xenopus oocytes, the net amplification is about 30-fold as the signal passes down the Mos-Mek1-Erk2 (MAPK) cascade system [12–15]. However, the signal appears to be attenuated rather than amplified at the Mek1-Erk2 activation step. Since Mek1 (a MAPKK) is more abundant than its downstream Erk2 (a MAPK), the results imply that something other than amplification is accomplished by interposing Mek1. Now the question arises as to what may be the function of this observed zero order ultrasensitivity at the Mek1 activation step (see curves b2 and b3, Fig. 2B), when the net amplification is primarily dependent on the terminal kinase Erk2.

![Figure 4: Predicted dose-response curves of an enzyme cascade system](image-url)
concentration. We postulate that such an effect may be essential for Mek1 to activate other signaling components or to participate in other pathways without affecting the output response of the Erk2 pathway. This result seems to be obvious as it appears that MAPK essentially acts as an effector of each pathway it participates in and has other diverse functions [3,20]. The primary role of upstream kinases on the other hand may be to activate specific downstream kinases by integrating different signals. Thus, according to our analysis, the higher concentration of Mek1 may not affect the output response of Erk2.

Our analysis may also provide insight into the phenomenon of cross-talk in budding yeast. In Saccharomyces cerevisiae, an upstream kinase Ste20p (a MAPKKK kinase) is known to have multiple functions, along with the activation of the MAPK cascade of the pheromone response pathway and filamentation invasion pathway [3,20,22]. Other examples of similar cross-talk include Ste11p (a MAPKKK) and Ste7p (a MAPKK), which are known to be part of different pathways in budding yeast [3,20,22]. It appears that having such common kinases does not affect the overall response of a particular pathway [22]. We postulate that this phenomenon may be due to the robust cascade structure and saturating effects of downstream kinases. Depending on the total concentration of the target kinases the sensitivity of the individual pathway response is fixed, based on the zero order effect. A small percentage of phosphorylated MAPKKK is known to be enough to activate a specific downstream kinase [3,20], which then may allow the utilization of the remaining MAPKKK for other functions. Therefore, having a common MAPKKK or MAPKK for many different pathways may be economical for the performance of the cellular functions.

It is known that the over-expression of component kinases leads to loss of specificity, thus exhibiting a non-specific cross-talk [3,20]. In such a case, can we obtain an invariant global output response of the pathway in question? From the analysis presented here, it can be postulated that the sensitivity of the global output response may remain unaffected due to non-specific cross-talk. However, these arguments have to be studied further.

Recent studies indicate that the enzyme cascades can show a robust output response towards changes in the concentrations of the various cascade components [10,11]. It has been proposed that, to study the signal transduction mechanism, the extent of protein modification may be more important than their total concentrations [25]. Here, we argue that the cascade component concentration may also be vital in obtaining insight into the overall performance of the signaling pathway. Therefore, to ascribe functional significance to individual proteins and to determine the behavior of biological systems, component quantifications (e.g. proteomes, transcriptomes) at different conditions are essential.

Appendix. Cubic equation for covalent modification cycle of A (Fig. 1A)

By considering the enzyme–substrate complexes AE1 and APE2, and neglecting the cascade connecting complex BAP in the conservation relationship of target kinase A (first cycle in Fig. 1A), the cubic equation for fractional unmodified kinase A is given as:

$$af^3 + bf^2 + cf + d = 0 \quad \text{(A1)}$$

where

$$f = \frac{A}{A_1}; \quad a = \left(1 - \frac{k_1 E_{I1}}{k_2 E_{21}}\right)$$

$$b = \left(\frac{K_{m1}}{A_1} + \frac{K_{m2} k_1 E_{I1}}{k_2 E_{21}}\right) + \left(1 - \frac{k_1 E_{I1}}{k_2 E_{21}}\right)$$

$$c = \frac{K_{m1}}{A_1} \left(\frac{K_{m1}}{A_1} + \frac{K_{m2} k_1 E_{I1}}{k_2 E_{21}}\right) + \left(\frac{k_1 E_{I1}}{k_2 E_{21}} - 2\right) + \left(\frac{E_{I1}}{A_1} + \frac{k_1 E_{I1}}{k_2 E_{21}}\right)$$

$$d = - \left(\frac{K_{m1}}{A_1}\right)^2$$

Here, subscript t denotes total concentration of the corresponding species and other nomenclature is as given in Fig. 1. From the constraint $0 < f < 1$, a valid root was obtained as a fractional unmodified kinase A using Eq. A1. The fractional modified kinase A (i.e. AP/A_1) can then be obtained by using the following relationship:

$$AP = 1 - f \left[1 + \frac{E_{I1}}{A_1 + k_1 E_{I1}}\right]$$

$$\text{(A2)}$$

The parameters used for the analysis

Analysis was done using the following parameters on MATLAB (The MathWorks Inc.) software.

1. Parameter set taken from Huang and Ferrell [13]. The total enzyme concentrations: $A_1 = 0.003 \ \mu$M, $B_1 = 1.2 \ \mu$M, $C_1 = 1.2 \ \mu$M, $D_1 = 0.003 \ \mu$M; phosphatase concentration: $E_{21} = E_{31} = 0.0003 \ \mu$M, $E_{41} = 0.12 \ \mu$M and $E_{51} = 0.0003 \ \mu$M; reaction rates: $k_1$ to $k_6 = 150 \ \text{min}^{-1}$, $k_{51} = 10 \ \text{min}^{-1}$, $k_{61} = 20 \ \text{min}^{-1}$; Michaelis constants: $K_{m1}$ to $K_{m6} = 0.3 \ \mu$M, $K_{m61} = K_{m62} = 1 \ \mu$M.

2. Parameter set taken from Bhalla and Iyengar [23]. The total enzyme concentrations: $A_1 = 0.2 \ \mu$M, $B_1 = 0.18 \ \mu$M, $C_1 = 0.36 \ \mu$M; phosphatase concentration: $E_{21} = E_{31} = 0.224 \ \mu$M, $E_{41} = 0.0032 \ \mu$M; reaction rates: $k_1 = 240 \ \text{min}^{-1}$, $k_2 = 360 \ \text{min}^{-1}$, $k_3 = 6.3 \ \text{min}^{-1}$, $k_4 = 360 \ \text{min}^{-1}$, $k_5 = 9 \ \text{min}^{-1}$, $k_6 = 60 \ \text{min}^{-1}$; Michaelis constants: $K_{m1} = 66.6 \ \mu$M, $K_{m2} = 15.65 \ \mu$M, $K_{m3} = 0.159 \ \mu$M, $K_{m4} = 15.65 \ \mu$M, $K_{m5} = 0.046 \ \mu$M, $K_{m6} = 0.066 \ \mu$M. Results using these parameters are not shown in the present work.

References