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Multiple feedback loops are key to a robust dynamic performance of tryptophan regulation in *Escherichia coli*

K.V. Venkatesh*, Sharad Bhartiya*, Anurag Ruhela

Department of Chemical Engineering and School of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India

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Abstract Living systems must adapt quickly and stably to uncertain environments. A common theme in cellular regulation is the presence of multiple feedback loops in the network. An example of such a feedback structure is regulation of tryptophan concentration in *Escherichia coli*. Here, three distinct feedback mechanisms, namely genetic regulation, mRNA attenuation and enzyme inhibition, regulate tryptophan synthesis. A pertinent question is whether such multiple feedback loops are "a case of regulatory overkill, or do these different feedback regulators have distinct functions?" [Freeman (2000) Nature 295, 313–319]. Another moot question is how robustness to uncertainties can be achieved structurally through biological interactions. Correlation between the feedback structure and robustness can be systematically studied by tools commonly employed in feedback theory. An analysis of feedback strategies in the tryptophan system in *E. coli* reveals that the network complexity arising due to the distributed feedback structure is responsible for the rapid and stable response observed even in the presence of system uncertainties.

Key words: Systems biology; Multiple feedback loop; Tryptophan system; Robustness; Dynamic model

1. Introduction

Feedback control mechanisms are ubiquitous in nature. In cellular systems, these are employed in the regulation of genetic, signaling, and metabolic networks. Quantification of molecular interactions, leading to a control structure for systems such as above, has been identified through molecular biology [1–4]. A common theme in modeling of biological systems is elucidation of properties implying robust behavior in the presence of uncertainties. A key question that needs to be answered is how the structural elements of the regulatory machinery, evolved by nature, are related to robustness. Indeed, in cellular regulation, a large number of structural motifs including cascades, feedforward control and multiple feedback loops have been observed and these invariably yield complex networks [5–7]. Among these, we focus our attention on multiple feedback loops, whose occurrence has been noted in many regulatory pathways such as those used in cell development [8], genetic regulation [9,10] and signaling pathways [6,11]. This motif is characterized by distribution of feedback to various points of the network leading to a distributed feedback structure (DFS). Such distributed feedback interactions are observed in many other regulatory systems such as in regulation of arabinose uptake in *Escherichia coli* [9], regulation of galactose uptake [10] and osmotic effect [6] in *Saccharomyces cerevisiae* and insulin signaling pathways [11].

In *E. coli*, regulation of tryptophan is achieved by a DFS consisting of three distinct feedback loops, namely genetic regulation [12], mRNA attenuation [12], and enzyme inhibition [13]. Tryptophan is fed back to the transcriptional and anabolic processes in order to regulate its own synthesis. In the *trp* regulon, the activated aporepressor, which is bound by two molecules of tryptophan, interacts with the operator site to repress transcription. Further, tryptophan binds to specific mRNA and protein sites to attenuate and inhibit transcription and tryptophan synthesis, respectively. Such a distributed mechanism of regulation, in contrast to a conventional feedback structure (CFS), is commonly used in engineering control implementations, which typically consist of only a single loop to regulate the network. Thus, an engineering design of the tryptophan system would consist of only genetic regulation, or a single feedback loop.

What, then, is the role of multiple feedback loops found in regulation of tryptophan synthesis in the overall dynamics of the system? Alternatively, how would the system respond in the absence of attenuation and enzyme inhibition, that is, by using a CFS strategy? We apply elements of feedback theory [14] to elucidate the advantages of the DFS over the CFS. Our analysis indicates that the distribution of feedback is necessary for a fast and stable dynamic response even in the presence of uncertainties. Extensive simulations of a dynamic model of the tryptophan system corroborate the results. We conclude from the analysis that DFS is essential for the regulation of the tryptophan system and mere genetic regulation is incapable of providing both a fast and a stable dynamic response.

2. Methods: model description of the tryptophan system

As shown in Fig. 1a, tryptophan is fed back to regulate the processes of transcription, by genetic regulation and mRNA attenuation and anabolism (tryptophan synthesis) by enzyme inhibition. In a recent model that has been validated with experimental data [15], repression, attenuation and inhibition have been quantified by a Hill-type equation given below,

$$C_i(T) = \frac{K_i^{\eta_H}}{K_i^{\eta_H} + T^{\eta_H}} \quad (1)$$

*Corresponding author. Fax: (91)-22-25726895.
E-mail addresses: venks@che.iitb.ac.in (K.V. Venkatesh), bhartiya@che.iitb.ac.in (S. Bhartiya).

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where $C_i(T)$ represents the above three regulatory mechanisms, T denotes the tryptophan concentration, K_i and η_H represent the half-saturation constant and the Hill coefficient, respectively. The tryptophan model can be decomposed into two parts: (a) the 'biological' regulation captured by the Hill equation (explicit dependence on tryptophan concentration), and (b) the 'process' representing transcription, translation and tryptophan synthesis. Thus, we conceptually separate genetic regulation and mRNA attenuation from the transcriptional 'process', and enzyme inhibition from tryptophan synthesis 'process' (see Fig. 1a). Note that the transcriptional process is regulated at two levels. Regulator $C_1(T)$, quantifying genetic regulation, determines the free operator concentration (O_R) available for binding by RNA polymerase. After initiation of transcription, attenuation by $C_2(T)$ is responsible for the premature termination of the 140-nucleotide segment corresponding to the 5′ end of *trp*. At the end of the transcription process, the entire 6720-nucleotide polycistronic *trp* mRNA translates to protein [16].

A description and values of model parameters are summarized in Fig. 1a. The free operator concentration (O_R) is quantified by $k_1 O_t C_1(T)$. Further, $(k_{d,1}+\mu)O_R$ represents the first-order degradation and dilution due to cellular growth. Synthesis of mRNA is represented by $k_2 O_R C_2(T)$, while the first-order degradation and dilution due to growth is given by $(k_{d,2}+\mu)mRNA$. $k_3 mRNA$ and μE represent the synthesis and dilution of the enzyme, respectively. Here, enzyme degradation has been neglected. Tryptophan synthesis has been modeled by $k_4 EC_3(T)$, while $gT/(T+K_g)$ and μT represent tryptophan uptake and its dilution due to growth, respectively. It can be noted that the values of the Hill coefficient for repression, attenuation and inhibition are in a descending order of 1.92, 1.72, and 1.2, respectively. This indicates that repression is more sensitive than attenuation and further, attenuation is more sensitive than inhibition, which is due to stoichiometric binding of two molecules of tryptophan to the aporepressor. Additional details of the model are available in [15]. In this work, the tryptophan system model has been studied for the case of absence of external tryptophan in the medium and this condition has been maintained in the rest of the current study.

3. Results

The dynamic response of the *trp* system, in the absence of external tryptophan, obtained by simulation of the model, is displayed in Fig. 1b. The *trp* system shows a rapid synthesis of enzymes to an in vivo steady-state level of 0.4 μM in less than 5 min, which is in agreement with experimental data [12,13,15] (Fig. 1b, curve E). Tryptophan synthesis also exhibits a rapid surge to its steady-state level in about 5 min, followed by an overshoot, which decays by a factor of a quarter and finally settles to its steady-state value of 4.2 μM (Fig. 1b, curve T).

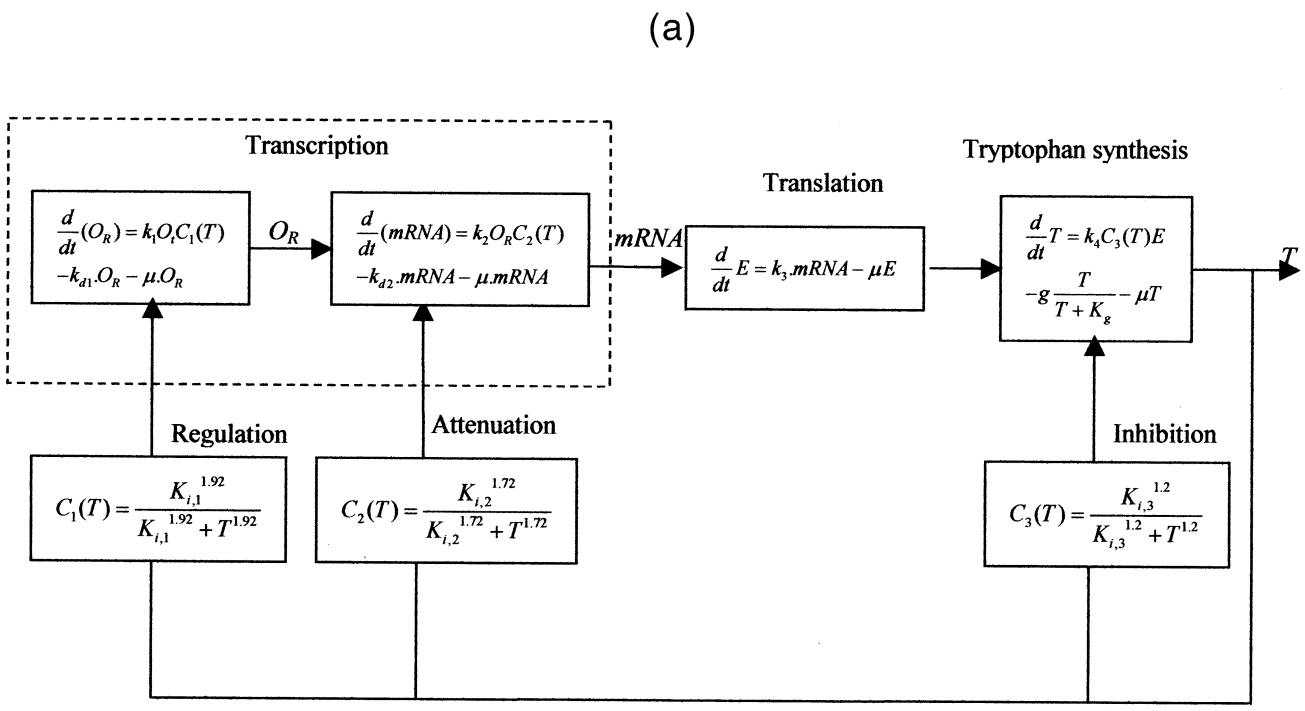
The simulation was rerun by eliminating attenuation and inhibition (C_2 and C_3 in Fig. 1a) in order to investigate their roles in providing robustness. The resulting tryptophan (T) and enzyme (E) responses are shown in Fig. 1c. The results indicate that genetic regulation has evolved to provide a very rapid response which, if acting alone, leads to a poor dynamic performance (see Fig. 1c). Thus, attenuation and enzyme inhibition not only prevent the massive build-up of enzyme and tryptophan levels in the cell, but also ensure a faster settling time to their respective steady-state values.

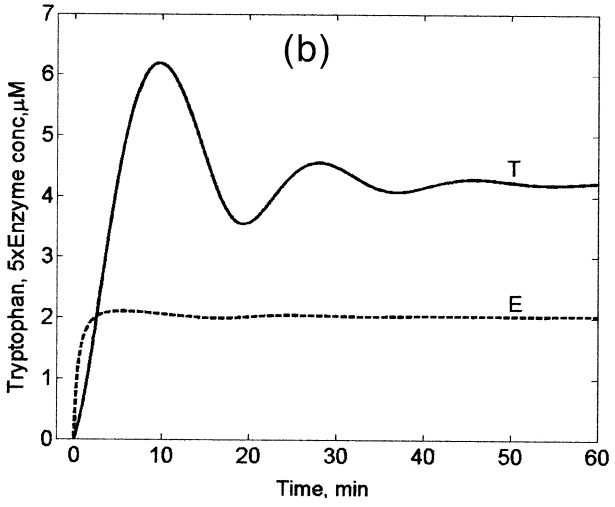
At this juncture, it is pertinent to ask how such a DFS, evolved by nature, accomplishes robust regulation. We make use of tools employed in linear feedback theory that enable delineation of the exclusive role of the feedback structure [17,18]. For this analysis, the tryptophan system was linearized around steady-state mRNA, enzyme and tryptophan concentrations of 0.13 μM, 0.4 μM, and 4.2 μM, respectively. The linear model equations may be represented by transfer functions depicted in the block diagram shown in Fig. 2a. Here, controllers $C_1(s)$, $C_2(s)$ and $C_3(s)$ represent traditional proportional+integral (PI) controllers, commonly used in engineering systems, and were implemented to regulate tryptophan synthesis, $T(s)$, in place of the non-linear biological controllers. Methods to design PI controllers are well documented as in [19]. Implementation of PI controllers with the linearized *trp* system serves to delineate the role of multiple feedback loops observed in the tryptophan system, rather than obtaining insights into the controller per se.

In the conventional control design approach (that is, CFS), where attenuation ($C_2(s)$) and inhibition ($C_3(s)$) are absent, a single genetic regulator ($C_1(s)$) may be designed for control of tryptophan synthesis, $T(s)$, by manipulating initiation of the transcription process $P_{11}(s)$ as shown in Fig. 2a. Constants of regulator $C_1(s)$, representing genetic regulation, can assume different values to achieve three broad qualitative behaviors shown by curves a, b, and c in Fig. 2b. Curve a represents a sluggish tryptophan response due to sub-sensitive genetic regulation, in which case the tryptophan concentration rises slowly to its steady-state value without any overshoot. Alternatively, the regulator can be designed to yield a very rapid synthesis of tryptophan (as shown by curve b). However, this leads to an oscillatory response tending towards instability. Thus, there exists a compromise where sensitivity of the genetic regulation may be designed to trade off between a stable, sluggish response and a fast oscillatory response as shown by curve c. This represents an optimal option with only genetic regulation.

To contrast the role of multiple feedback loops, we now implement the DFS on the linearized *trp* system of the block diagram depicted in Fig. 2a by considering all three feedback loops used for regulation of tryptophan. Sensitivity of the genetic regulator, $C_1(s)$, is retained as in the case corresponding to curve c in Fig. 2b, while regulators $C_2(s)$ and $C_3(s)$ were designed to provide a tryptophan response similar to that in *E. coli*. Fig. 2c shows the comparison between the performances of the tryptophan system of Fig. 1a and the linearized tryptophan system of Fig. 2a. The DFS system for the linearized *trp* system (solid line in Fig. 2c) exhibits an instantaneous rise to its final steady-state value without any significant oscillations. Thus, the DFS eliminates the operating constraint boundaries of a fast oscillatory and a slow stable response as observed in the CFS discussed above. It therefore appears that irrespective of the type of controller employed ('biological' in the *trp* system, or PI in the linearized *trp* system), the DFS itself is responsible for this enhanced performance.

Use of PID controllers and the linearized *trp* system enables a frequency response analysis [20] that can provide insights for the fast and stable response observed in a DFS. Fig. 3a,b shows the comparison between the CFS and DFS on the magnitude and phase plots for the regulation of tryptophan synthesis. The gain margins for the CFS and DFS are noted as 9.7 dB and ∞, respectively. This implies that an increase in the rate constants of transcription, translation and tryptophan synthesis by a factor of 3.2 (equivalent to the gain margin of 10 dB) would completely destabilize the tryptophan system with genetic regulation alone. However, no such limit exists when the DFS strategy is used (equivalent to a gain margin of ∞). A companion measure of system robustness is the phase margin. It is noted that the phase margins for the two cases are 13.8° for the CFS and 27° for the DFS. The increased phase margin in the DFS is an indicator of the ability of the





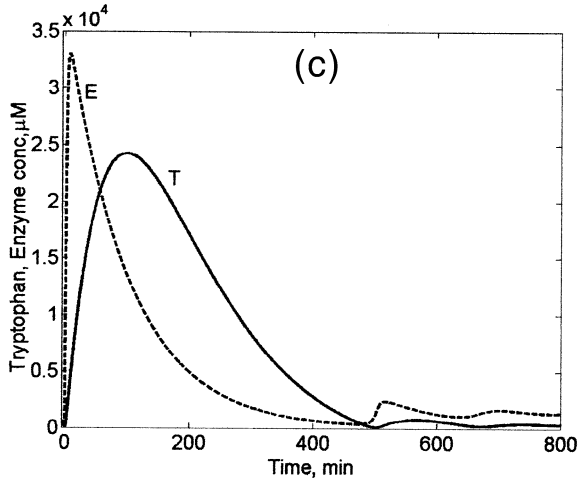


Fig. 1. a: Block diagram of the model used for simulation of the DFS in the *trp* system. Tryptophan concentration is independently distributed to three processes in series. In a CFS, typically used in engineering systems, tryptophan concentration would have been used for genetic regulation alone (that is, controllers $C_2(T)$ and $C_3(T)$ would be absent). k_1, k_2, k_3, and k_4 represent kinetic rate constants for synthesis of free operator, mRNA transcription, translation and tryptophan synthesis, respectively. $K_{i,1}$, $K_{i,2}$, and $K_{i,3}$ represent the half-saturation constants of repression, attenuation, and inhibition, respectively. O_t, μ, k_d1 and k_d2 refer to total operator site concentration, specific growth rate of *E. coli*, degradation of O_R, and mRNA degradation constant respectively. K_g and g are the half-saturation constant and kinetic constant for the uptake of tryptophan for protein synthesis in the cell. Model parameter values are as follows [13,15]: $k_1 = 50$ min^{-1}; $k_2 = 15$ min^{-1}; $k_3 = 90$ min^{-1}; $k_4 = 59$ min^{-1}; $O_t = 3.32$ nM; $k_d1 = 0.5$ min^{-1}; $k_{d,2} = 15$ min^{-1}; $\mu = 0.01$ min^{-1}; $g = 25$ μM. min^{-1}; $K_g = 0.2$ μM; $K_{i,1} = 3.53$ μM; $K_{i,2} = 0.04$ μM; $K_{i,3} = 810$ μM. All simulations were performed in MATLAB environment. b: Dynamic simulation of the *trp* system in the absence of tryptophan in the medium. Beginning from the initial conditions of complete absence of enzyme (E, dashed) and tryptophan (T, solid) in the cell, rapid synthesis of enzyme to its steady-state level is observed. Tryptophan concentration also rises rapidly exhibiting an overshoot and subsequently decays by a factor of a quarter to attain its steady-state value. Enzyme levels have been magnified by a factor of five. c: Dynamic simulation of the *trp* system in the absence of attenuation and inhibition mechanisms. In the absence of attenuation and inhibition, tryptophan (T, solid) and enzyme (E, dashed) levels exhibit an unregulated synthesis with their concentrations increasing by four and five orders of magnitude, respectively. Also, the times required for reaching the steady state are roughly 15- and 80-fold for tryptophan and enzyme, respectively.

tryptophan system to handle transcriptional and translational time delays. It can also be noted that the additional regulation due to transcriptional attenuation and enzyme inhibition doubles the bandwidth from 0.13 rad/s (for the CFS) to 0.26 rad/s. Here too, the increased bandwidth using DFS directly translates to the increased speed of response using the DFS strategy over the CFS as is also evident by comparing Fig. 2b with Fig. 2c. The magnitude plot of feedback sensitivity function measures efficacy of feedback in the *trp* system. The DFS in the *trp* system exhibits efficient control over a larger bandwidth and a smaller peak value (see Fig. 3b) as compared to the CFS. Feedback sensitivity quantifies the effect of feedback on system regulation, whose magnitude in excess of 0 dB implies that the feedback may amplify uncertainties in the system rather than attenuating them [14,20]. In summary, the above frequency response indicators provide analytical insights into the enhanced robust dynamic performance of the DFS over the CFS, thus elucidating the inherent design principles of multiple feedback loops in nature.

It was noted previously in Fig. 1c, that in the absence of mRNA attenuation and enzyme inhibition, a massive build-up of enzyme and tryptophan levels occurs in the cell, far beyond the physiological steady-state levels. Is it possible to redesign the genetic regulator in *E. coli* (that is, C_1 in Fig. 1a) in the

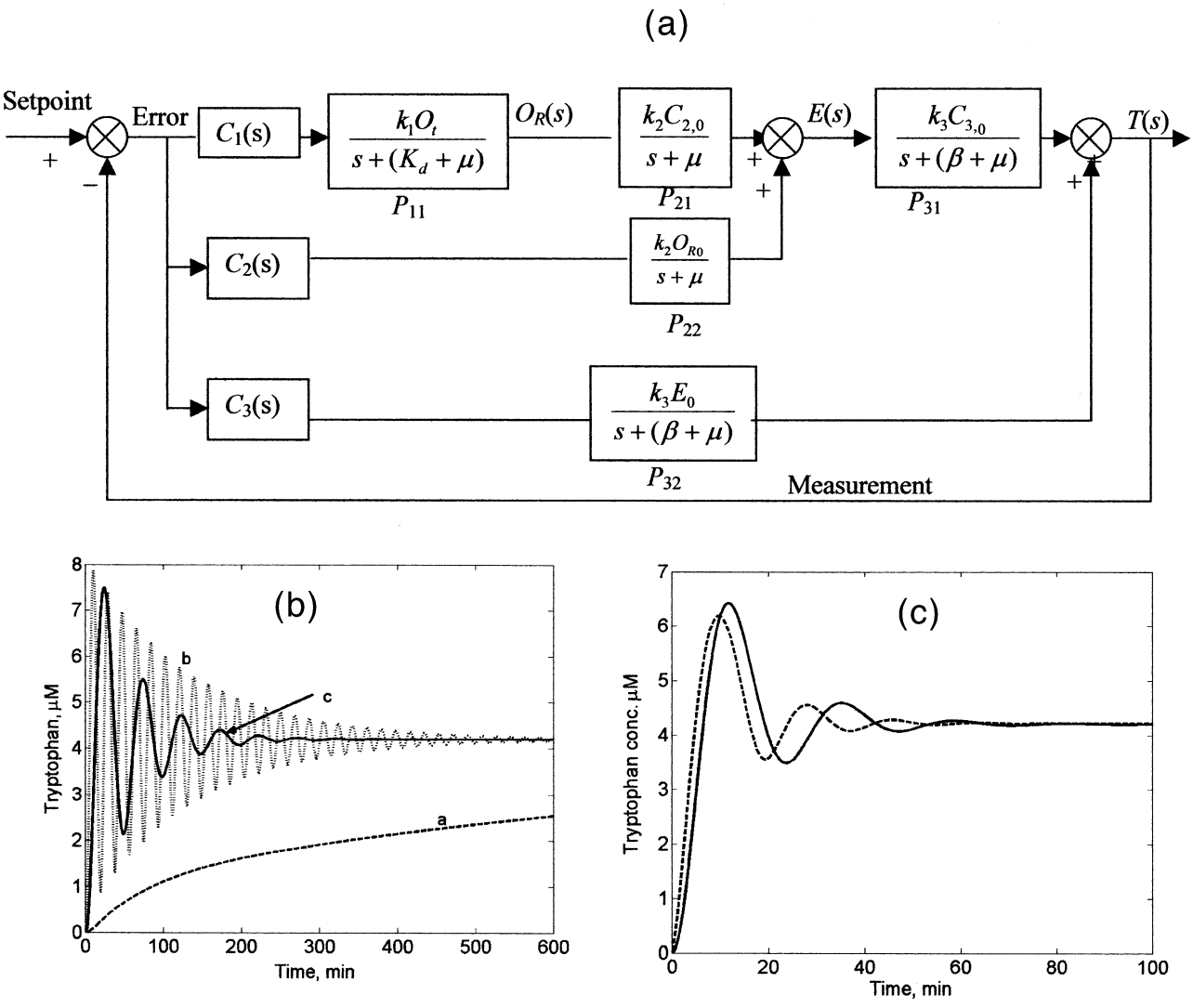


Fig. 2. a: Block representation of linearized *trp* system. Differential equations representing the dynamic model of the *trp* system as shown in Fig. 1a were linearized using Taylor series expansion around the steady-state values (mRNA = 0.13 μM, E = 0.4 μM, and T = 4.2 μM) and further the mRNA was assumed to be at quasi-steady state (that is, d(mRNA)/dt = 0). The resulting linear equations were then transformed into the Laplace domain. Transfer function P_{11} represents transcription, P_{21} and P_{22} represent translation, and P_{31} and P_{32} represent tryptophan synthesis. Here, controllers C_1, C_2, and C_3 represent PI controllers, which replace the biological controllers as shown in Fig. 1a. The three controllers are of the proportional-integral type with the following form of the transfer function, $C_i(s) = K_i + I_i/s$. The controller parameters are as follows: $K_i = \{5\times10^4, 100, 100\}$ and $I_i = \{5600, 5, 5\}$. b: Dynamic simulation of tryptophan synthesis in the absence of controllers C_2 and C_3. Three distinct qualitative responses, namely (a) sluggish, (b) fast, oscillatory, and (c) fast, damped, can be obtained based on different designs of the controller C_1. c: Response of the linearized tryptophan system of the block diagram in panel a with all three PI controllers in the DFS (shown as solid line). This response obtained by the DFS is more rapid and stable when compared with the best response obtained using C_1 alone. The dashed line represents the DFS of the biological tryptophan system of Fig. 1a and matches the response of the linearized tryptophan system.

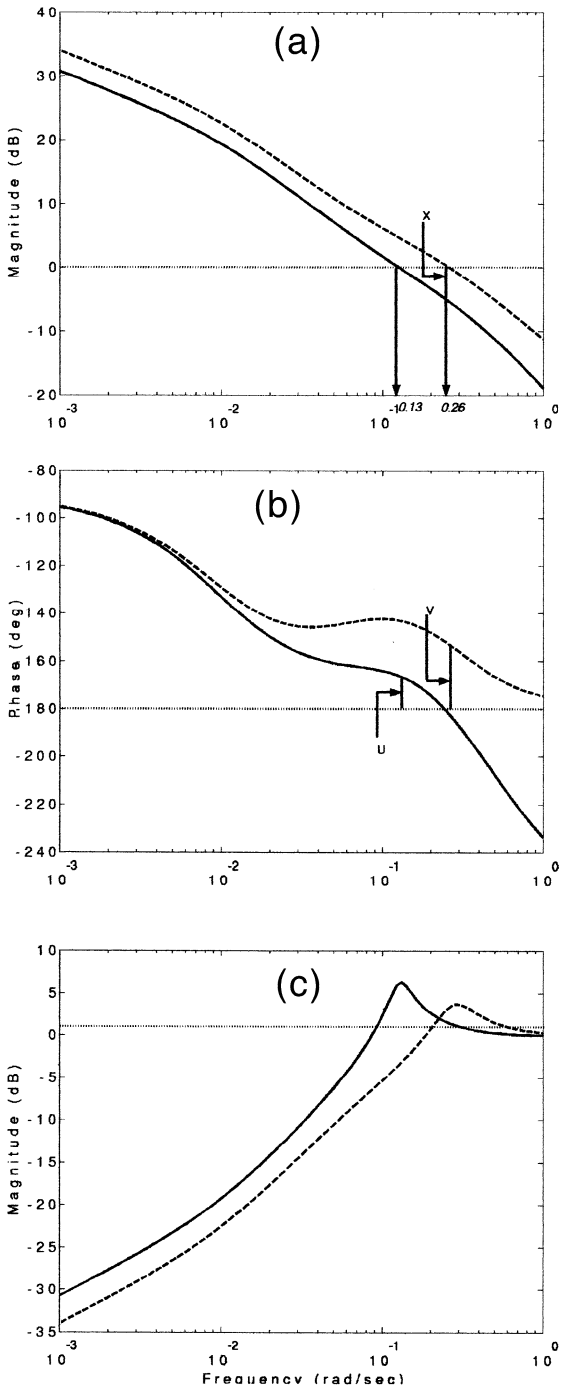


Fig. 3. (a) Magnitude, (b) phase, and (c) sensitivity plots for the tryptophan system in *E. coli*, whose block diagram is shown in Fig. 2a. The solid line represents the CFS controlled by C_1 alone while the dashed line corresponds to the DFS with all three controllers working in unison. $X = 9.7$ dB represents the gain margin of the CFS, while the gain margin of the DFS is infinity (since the phase plot does not exhibit a crossover region). $U = 13.8°$ and $V = 27°$ represent the phase margins of the CFS and DFS, respectively. The increased bandwidth (indicated by arrows at 0.13 rad/s for the CFS and 0.26 rad/s for the DFS) associated with the DFS enables rapid synthesis of tryptophan. The peak value for sensitivity of the DFS (3.6 dB) is smaller in size and occurs at a higher frequency (0.28 rad/s) relative to the CFS (peak value of 6.25 dB occurs at 0.13 rad/s). Frequency response analysis was performed using Control Systems Toolbox in MATLAB.

absence of multiple feedback loops, while retaining the fast and stable response observed in the DFS? Such an alternative strategy would involve changing the parameters of the genetic regulator, C_1. Reducing the Hill coefficient from 1.92 to 0.5 and the half-saturation constant from 3.53 μM to 8×10^{-8} μM leads to an extremely slow synthesis of tryptophan as shown by the dotted line in Fig. 4. Thus, here the speed of tryptophan synthesis is sacrificed for a stable behavior. Further, the small value of the half-saturation constant would make the feedback system extremely sensitive to noise at small values of tryptophan concentration. A stable performance of the CFS can also be achieved if the processes of transcription, translation and anabolism were sufficiently slowed down (a 10-fold decrease in the rate constants). In this case, the tryptophan response corresponds to the solid line in Fig. 4, where physiological steady-state levels of tryptophan are accompanied by a long delay and a poor speed of response. Thus, regardless of the choice of parameters used, the CFS shows a slow rise to the steady-state level, which may starve the cell for tryptophan during the initial transient period. Thus, the additional feedback loops utilizing regulators C_2 and C_3, representing attenuation and enzyme inhibition, respectively, not only prevent the massive build-up of enzyme and tryptophan levels in the cell but also ensure a faster settling time to their respective steady-state values. The aggressive behavior of genetic regulation in *E. coli* may be essential for synthesis of key enzymes, but must be coordinated with attenuation and inhibition to orchestrate a stable dynamic response.

4. Discussion

Cellular functions require regulation to respond rapidly and stably to changes in the environment of the cell. Since this regulation occurs through interacting chemical species, stochasticity in molecular interaction, varying environment, and mutation make the system uncertain. Thus, there is a strong need for a robust design of the regulating mechanism to coun-

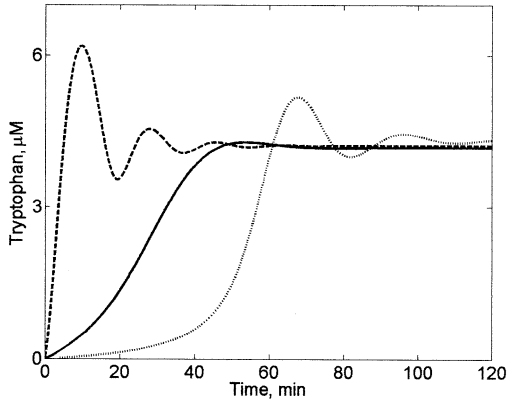


Fig. 4. Comparison between CFS and DFS strategies in the tryptophan system in *E. coli*. The dashed curve shows the physiological response of the tryptophan (that is, DFS). The solid line represents the response without attenuation and inhibition (that is, CFS) and with parameters of genetic regulation altered ($\eta_{H1} = 0.5$, $K_{i,1} = 8 \times 10^{-8}$ μM) to yield a sub-sensitive regulator. The dotted line represents the response for the CFS with the rates of transcription, translation, and synthesis reduced to $k_1 = 2.6$ min^{-1}; $k_2 = 7$ min^{-1}; $k_3 = 5$ min^{-1}.

ter the uncertainties while ensuring a rapid and stable response for adaptation. Further, the regulating mechanism must use simple analog means of information transmission through the interacting species, thereby defining a feedback structure. As seen in the *trp* system, a recurring theme in cellular regulation is the presence of multiple feedback loops. In order to cater to the minimum physiological demand of tryptophan, in its absence in the medium, *E. coli* must quickly synthesize appropriate tryptophan levels (about 4 μM in 5 min) for its survival. Thus, in the tryptophan system, the optimal dynamic response (a) should reach 4 μM in about 5 min, (b) should settle to its steady-state value of 4 μM in about 40 min, and (c) the response between 5 and 40 min should be well-behaved (no excessive overshoot). Further, these objectives must be satisfied in the presence of uncertainty due to mutations and environmental changes. For example, if only one tryptophan molecule were to bind to the aporepressor, then the Hill coefficient for genetic regulation would be unity (see Eq. 1) indicating that the 'controller' parameter has altered [15]. Further, changes in environmental conditions or mutations may alter the binding of RNA polymerase to the specific DNA site, thereby changing the transcriptional rate. In this case, the 'process' parameter k_1 would take on a different value. Uncertainty is also enhanced by the stochastic nature of genetic regulation [21] due to the discrete characteristic of the operator binding site (either the operator is bound with the activator protein or it is in an unbound state). Further studies are necessary to evaluate the performance of DFS in the presence of such intrinsic noise. Extensive simulations with the tryptophan model confirm that the performance of the DFS is robust with respect to changes in transcriptional, translational, and tryptophan synthesis rate constants (results not shown). Yet another parametric uncertainty is in the specific growth rate of *E. coli*, μ, which is dependent on the growth medium. It is interesting to note that the performance of the DFS is superior to CFS even at different growth rates.

The efficacy of feedback motifs in a system in the presence of uncertainties due to rate processes and time delays (such as in transcription and translation) can be analytically quantified using elements of feedback theory. In particular, gain margin indicates the ability of the system to tolerate uncertainty in the rate constants k_1, k_2, k_3, and k_4 and the phase margin deals with robustness against increased delays in transcription and translation. An analysis of the DFS indicates an increase in both the gain and phase margins relative to the CFS (only genetic regulation) implying robust performance in an uncertain environment.

In the CFS, translation and tryptophan synthesis would depend on the dynamic level of mRNA and protein, respectively, and therefore they respond to tryptophan only indirectly resulting in a sluggish response (see Fig. 4). On the other hand, in case of multiple feedback loops, the current tryptophan level is directly transmitted to translation and tryptophan synthesis, in addition to the sluggish response through genetic regulation. Translation and tryptophan synthesis, therefore, respond to tryptophan directly before the indirect response of the genetic regulation manifests completely. Thus, the tryptophan synthesis shows a more rapid response using the DFS strategy. The DFS may have evolved as a strategy to solve the trade-off between a fast and a stable output response.

Although feedback analysis presented in this study can be used to characterize the individual effects of the three controllers, it is very difficult to demonstrate the same through experiments since obtaining mutants in which the attenuation and inhibition by tryptophan is eliminated is difficult. Simulation of the *trp* model shows that in the absence of external tryptophan in the medium, genetic regulation and transcriptional attenuation are mainly responsible for the robust behavior implying redundancy of inhibition. However, when enzyme-rich cells are exposed to external tryptophan in the medium, regulation by enzyme inhibition plays an important role in the dynamic behavior of the *trp* system. It is pertinent to evaluate whether the robust behavior is a result of the distribution of feedback or whether the 'processes' of transcription and tryptophan synthesis have been 'designed' to result in robustness. We have verified that a similar increased speed of response without sacrificing the stability character of the system is obtained upon implementation of the DFS on other processes of engineering interest. This prompts reverse engineering of biologically inspired feedback strategies to industrially relevant systems.

While advanced engineering control strategies involve intense computational algorithms, nature relies on numerous chemical interactions resulting in a structure-based complex network to achieve robustness. Although multiple feedback loops in cellular regulation yield complex networks, they endow the cellular systems with robust regulation in the face of uncertainties. Specific to the *trp* system, multiple feedback loops are essential for robust dynamic performance and do not represent a redundancy in the design. Multiple feedback loops are present in numerous other biological systems encompassing genetic regulation to cellular development. The feedback analysis indicates that it is this strategy of utilizing multiple feedback loops, which endows the system with robustness and will serve similar benefits, irrespective of the biological system. In addition to multiple feedback loops, nature has devised various other strategies in cellular regulation. For example, regulation of cell cycle and DNA repair has been represented by a comprehensive molecular interaction map [22] involving DFSs (for example by p53) and cascade structures within multiple modules. The methodology presented in this work may be extended for study of the underlying design principles of other regulatory motifs and analysis of system-wide molecular interaction maps.

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