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Protein-Level Control of Metabolism: Design Principles and Prospects from a Representative System

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Abstract: Significant evidence suggests protein-level or metabolic control is widespread and important in metabolic networks. However, the biophysical interactions responsible for flux control at the metabolic level are not nearly as well-characterized as those which are responsible for control at other biological levels, such as transcriptional regulation. This knowledge gap is a limiting factor in the application of engineered protein-level regulation in Metabolic Engineering for the rational and sensitive control of pathway flux. Here we apply an *in silico* dynamic numerical optimization approach to a representative branched pathway to understand how engineered allosteric regulation could be used to control flux. We consider inhibition sensitivity as a hypothetical tunable parameter to demonstrate that integration of allosteric and transcriptional regulation is necessary to stably achieve arbitrary targets for both downstream metabolite concentrations. We further show that the steady-state ratio of these metabolites can be controlled by tuning the sensitivity of allostery at the branch point. Finally, we demonstrate that system dynamics dictate which type of engineered control is optimal. This work has implications for the co-optimization of transcriptional and allosteric regulatory systems in metabolic networks and provides a framework for the design of allosteric regulation in engineered metabolisms.

Keywords: metabolic engineering, protein engineering, regulation, metabolism, allosteric regulation, control

1. INTRODUCTION

Metabolic Engineering seeks to replace traditional means of chemical production with microbial chemical factories through genome modelling and manipulation. Typically, this is achieved through the deletion of metabolic pathways and/or insertion of heterologous pathway genes to direct metabolic flux toward valuable native and non-native products (Woolston, *et al* 2013). A wide range of optimization techniques at various scales have been explored by metabolic engineers. This study is concerned with enabling tools dynamic engineering of flux. Typically dynamic control involves engineered regulation of flux through the creation of sensor-reporter gene networks which modulate expression of a target enzyme as a function of the intracellular concentration of a downstream metabolite or exogenous chemical (Venayak, *et al* 2015). The widespread use of such gene networks has been facilitated by the rapid growth of Synthetic Biology and the concomitant explosion in the tools available for the efficient engineering of genetic systems.

The field of Protein Engineering has seen similar, though necessarily slower advances in recent years. Protein design, unlike gene network design, is limited largely by the computational power available to engineers (Marcheschi, *et al* 2013). Despite this, *de novo* engineered proteins have been applied in at least two significant Metabolic Engineering projects (Leonard, *et al* 2010) (Siegel, *et al* 2015). Additionally, several methods for the introduction of allosteric control of activity into unregulated proteins have been established and validated (Ostermeier, 2005) (Taylor, *et al* 2016). These techniques have primarily focused on furthering the structural and evolutionary understanding of the phenomenon of allostery (Lindsley and Rutter, 2007), but we suggest that they could be applied in the dynamic control of metabolism analogously to gene networks. In this scenario, an engineered enzyme would act as both sensor and regulator to control metabolic flux.

A significant and growing body of work has characterized the role of metabolic-level control of flux for various network topologies. Metabolic control analysis (MCA) in particular has been useful to demonstrate the significance of metabolic control in regulating pathway flux (Wang, *et al* 2004) (Fell and Sauro, 1985). Other modelling techniques have been used to demonstrate both the theoretical interactions of metabolic and transcriptional control (Oyarzun, *et al* 2007) and to recapitulate these effects as observed in a model biological system (Zaslaver, *et al* 2004). From this work, it is clear that metabolic changes are significant drivers of flux changes (Gerosa, *et al* 2015) and therefore that manipulation at the metabolic-level could be used to optimize engineered metabolisms.

However, the application of these conclusions to the rational design of metabolic control is relatively difficult within these

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modelling frameworks, as they describe potentially tunable biophysical interactions in abstract mathematical terms only. For example, feedback inhibition of enzyme activity is characterized via elasticities in MCA (Fell and Sauro 1985), which are related to, but have no concrete connection to effector binding strength and/or mode of action – i.e. enzyme parameters which are rapidly becoming tunable as Protein Engineering matures. Additionally, many of these approaches rely on linearization for convenience, thereby ignoring the inherently and potentially useful non-linear nature of enzyme inhibition (Wang, *et al* 2004).

We therefore build from this body of work by numerically simulating a branched pathway with explicitly-defined, non-linear, effector-enzyme relationships. Our intention with this approach is to directly link a tunable, biophysical interaction with the flux effects of such an interaction so that it can be optimized. We selected a branching pathway because this is highly relevant for Metabolic Engineering, which is concerned with diverting flux at target nodes toward economically productive pathways. In this topology, metabolic control interactions are most efficient at the entry points to either downstream metabolic branch (Savageau, 1974), so we place the target engineered inhibition at the entry point to one of the branches. We begin by optimizing the global transcriptional control of our system under different sensitivities of allosteric regulation to show that allostery is required for optimal partitioning of flux among the downstream branches to reach relatively arbitrary targets for both terminal metabolites. Then we simulate the system under three different control regimes—transcriptional only, allosteric only, and integrated—to demonstrate that allosteric control of enzyme activity alone can effectively partition flux among the downstream branches, but that the optimal control regime depends strongly on system dynamics and the target steady-state ratio of terminal metabolites in the system.

2. SYSTEM DESCRIPTION

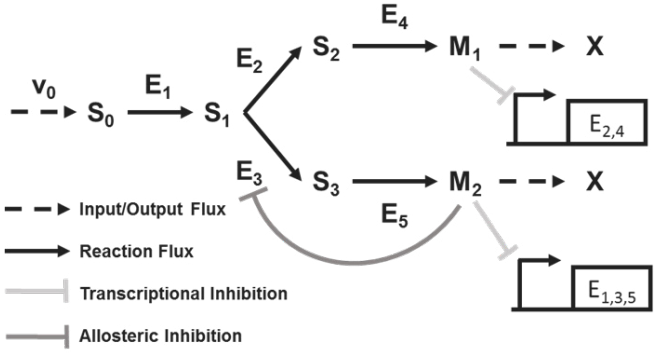

Fig 1. Model system. S_0 enters the system at constant flux v_0 and is converted into either terminal metabolite M_1 or M_2 in a step-wise fashion via the action of enzymes E_i. Terminal metabolites leave the system at rates proportional to their concentrations. Regulatory interactions include transcriptional feedback inhibition of enzymes 1, 3, and 5 by metabolite M_2, and enzymes 2 and 4 by metabolite M_1. In scenarios in which allosteric inhibition is considered, M_2 additionally inhibits the activity of E_3 directly.

In our system (Fig. 1), substrate S_0 enters at a constant flux v_0. It is converted to metabolite S_1 which can enter either of two branching pathways with terminal metabolites M_1 and M_2. Five enzymes (E_i) catalyse the conversion of S_0 to either terminal metabolite. With the exception of E_3, their activity was modelled using standard Michaelis-Menten kinetics. E_3 activity was modelled with Michaelis-Menten kinetics with uncompetitive inhibition. The terminal metabolites leave the system at a rate proportional to their concentrations and enzyme expression is controlled by the concentration of either terminal metabolite in a dose-responsive way.

The kinetic equations describing this system are as follows:

$$\frac{dS_0}{dt} = v_0 - \frac{k_{cat}E_1S_0}{K_m + S_0} \tag{1}$$

$$\frac{dS_1}{dt} = \frac{k_{cat}E_1S_0}{K_m + S_0} - \frac{k_{cat}E_2S_1}{K_m + S_1} - \frac{1}{\alpha}\frac{k_{cat}E_3S_1}{K_m + S_1} \tag{2}$$

$$\frac{dS_2}{dt} = \frac{k_{cat}E_2S_1}{K_m + S_1} - \frac{k_{cat}E_4S_2}{K_m + S_2} \tag{3}$$

$$\frac{dS_3}{dt} = \frac{1}{\alpha}\frac{k_{cat}E_3S_1}{K_m + S_1} - \frac{k_{cat}E_5S_3}{K_m + S_3} \tag{4}$$

$$\frac{dM_1}{dt} = \frac{k_{cat}E_4S_2}{K_m + S_2} - \gamma M_1 \tag{5}$$

$$\frac{dM_2}{dt} = \frac{k_{cat}E_5S_3}{K_m + S_3} - \gamma M_2 \tag{6}$$

$$\frac{dE_i}{dt} = \frac{\beta_i}{1 + \frac{R}{k_i}} - \gamma E_i \tag{7}$$

Where R is the fraction of repressor protein (total concentration assumed constant) bound to the control signal:

$$R = \frac{M_j}{1 \cdot 10^{-4}\, mM + M_j} \tag{8}$$

In (8) j = 1 for i = 2, 4 and j = 2 for i = 1, 3, 5.

Here γ is the rate constant for enzyme degradation and terminal metabolite flux out of the system. We describe it as the "turnover rate constant". The use of first-order kinetics for both processes assumes that they are associated with growth and therefore that cell division is the dominant process by which both enzymes and the terminal metabolites leave the system. This allows for the examination of control dynamics relative to growth rate, as in Fig 5.

Uncompetitive allosteric inhibition ("allostery") in (2) and (4) is described by

$$\alpha = 1 + \frac{M_2}{K_I} \tag{9}$$

This assumes that the fraction of inhibited enzyme reaches equilibrium instantaneously relative to the timescale of transcriptional repression. In (9), K_I is the inhibition constant and it is inversely proportional to the sensitivity of inhibition.

In the limit of large K_I, (9) reduces to unity; this is the case in which allostery has no effect on enzyme activity. We therefore introduce IS, the inhibition sensitivity:

$$IS = \frac{1}{K_I} \tag{10}$$

As IS decreases, α decreases to 1 and the influence of inhibition on enzyme activity is minimized. Therefore, IS is directly proportional to the sensitivity of inhibition of enzyme activity.

In the native case, this control regime is similar to aromatic amino acid biosynthesis which has both global transcriptional control and allosteric control at entry points and branch points in the pathway (Maeda and Dudareva 2012). In engineered cases, it allows for the optimization of the design parameter K_I. In scenarios where engineered transcriptional control of E_3 expression was simulated, the influence of M_2 was maximized by assuming that it binds directly to the gene operator sequence to repress expression:

$$\frac{dE_3}{dt} = \frac{\beta_3}{1 + \frac{M_2}{k_3}} - \gamma E_3 \tag{11}$$

This represents the ideal scenario for engineered transcriptional control in which the transcriptional sensor is also the regulator. In all cases the system has 4 extrinsic, user-defined parameters which determine its time and concentration scales. These parameters were kept constant for all simulations, unless otherwise indicated. They are outlined Table 1.

Table 1. Extrinsic system parameters. Sources for reasonable parameter estimates are provided except for input flux, which was set at a value which allowed for stable system operation.

Parameter	Value	Units	Source
k_{cat}	100	min^{-1}	Bar-Even, *et al,* 2011
K_m	0.1	mM	Bar-Even, *et al,* 2011
v_0	0.005	$mM \cdot min^{-1}$	N/A
γ	0.07	min^{-1}	Anesiadis, *et al,* 2008

3. METHODS

3.1 Native Optimization

To understand the interaction between allosteric control and transcriptional optimization the metabolic cost of the system, C, was minimized to find optimal transcription kinetic parameters (β_i, k_i) over a wide range of inhibition sensitivities (1 – 10000 mM^{-1}).

$$C = a\int_0^T \sum_{i=1}^{5} E_i\, dT + \int_0^T |M_1 - M_{1,goal}|dT + \int_0^T |M_2 - M_{2,goal}|dT \tag{12}$$

We modified the cost equation used by Zaslaver *et al* with the addition of a second metabolite cost to produce (12). This is necessary because of the branching in our system. The first term accounts for the cost of producing enzymes ("enzyme cost"), while the sum of the second and third terms characterize the cost of deviating from the target value of either terminal metabolite ("metabolite cost"). M_{1goal} and M_{2goal} were set to 0.025 mM for each optimization based on measure steady-state concentrations of aromatic amino acids in *E coli* (Bennet, *et al,* 2009). The parameter a in (12) provides the fractional cost of enzymes in the system. For example, we chose a = 0.001 to make the enzyme cost one thousandth of the metabolite cost. Sensitivity analysis on a is not provided here due to space constraints, but for our system this parameter can be increased 100x from the given value with no significant effects on results.

3.2 Engineered Systems Optimization

The goal of engineering this system would not be maintenance of a specific intracellular level of either terminal metabolite, as it might be for normal cellular functioning. Rather, metabolic engineers are concerned with maximizing the amount of one terminal metabolite—M_1 in this case. For this reason, the cost function was redefined as follows for simulations of engineered systems:

$$C = a\sum_{i=1}^{5}\int_0^T E_i dT + \int_0^T \left|\frac{M_1}{M_2} - \frac{M_1}{M_{2_{goal}}}\right| dT \tag{13}$$

This allows for maximal flux through the pathway with maintenance of a target terminal metabolite ratio.

Furthermore, since an engineered system exists in a native transcriptional background, one set of transcriptional parameters was selected for all optimizations of the engineered systems. The transcriptional context used for optimization was selected from optimization results for the native system. Simulation of this transcriptional background was performed to ensure that the system reached a steady-state terminal metabolite ratio of one without any changes to control parameters. The cost function was minimized to find K_I in the allostery-only regime, β_3 in the transcriptional control regime, or both in the integrated regime for a wide range of target M_1/M_2 ratios. In all optimizations, T was set to the time required to reach steady state defined by γ.

4. RESULTS

4.1 Native System

Optimization results for the native system are presented in Fig 2. We present the ratio of terminal metabolites for brevity in this figure. M_1 reached the target value of 0.025 mM in each optimization over the given timeframe, so a ratio below one indicates that the M_2 target was undershot, while a ratio of one indicates that both targets were stably reached.

The addition of allosteric control to the native system changed the transcriptional optimization landscape in two significant ways. First, it allowed the system to reach independent concentration targets for both terminal

metabolites. Without significant protein-level flux control at the entry into the M_2 branch, transcription alone was not able to perfectly balance the terminal metabolite ratios according to the goal values. Fig. 2 demonstrates this with three distinct regions of control influence. For 1 $mM^{-1} \leq IS < 10$ mM^{-1}, transcription dominates and the terminal ratio is significantly undershot. On 10 $mM^{-1} \leq IS \leq 200$ mM^{-1}, control is mixed and the ratio approaches the target value of 1 as the influence of allostery increases. The target ratio is stably reached for IS > 200 mM^{-1} and therefore it is only for this level of inhibition sensitivity that the system can manage two independent optimal metabolite concentrations.

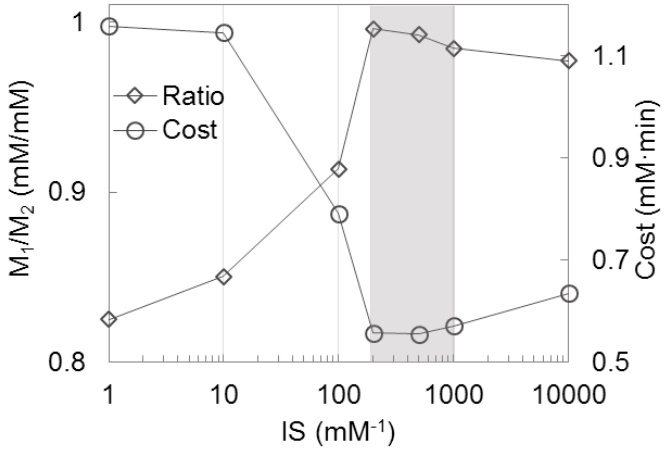

Fig. 2. System performance as a function of inhibition sensitivity. Total system costs, calculated with (12) and the ratio of terminal metabolites for a large range of sensitivity values are presented. When IS ≤ 10, sensitivity is too low to have significant system effects. Conversely, IS ≥ 1000 represents ultrasensitive control in which control effects begin to saturate. The optimal control region is shaded.

The second major effect that the addition of allosteric control had on the system was a significant reduction in the metabolic cost over the same range of sensitivity. This is intuitive, since the majority of the total cost is defined by the metabolite cost. Thus, if the system can reach the target metabolite concentrations quickly, the cost will generally be minimized. In fact, the enzyme cost remained relatively constant over nearly the full range of inhibition sensitivity. However, at extreme sensitivities it began to play a larger role in the total cost; it is responsible for the increasing total cost for IS > 1000 mM^{-1} in Fig. 2. Since the activity of the regulated enzyme has only an indirect effect on its own expression, and since high sensitivities cause a large decrease in activity even for low concentrations of the inhibitor, the system must express the regulated enzyme at much higher levels in order to reach the target terminal metabolite values when allosteric regulation is highly sensitive.

The combined effects of allostery on the terminal metabolite ratio and system cost define an optimum region of control wherein the cost is minimized. This optimal region occurs when IS is on the range of 200 mM^{-1} – 1000 mM^{-1} (shaded region in Fig. 2), and corresponds to an 83%-96% reduction in E_3 activity at steady-state.

4.2 Engineered Systems

Control parameters were optimized for three engineered regulatory regimes – allostery-only, transcriptional-only, and integrated – at the branch point of our system for a range of target M_1/M_2 ratios (Fig. 3). All three control regimes could be feasibly optimized to produce target ratios of 1-10. However, the allostery-only regime could not robustly produce ratios lower than 1 whereas the transcriptional control regime has a minimal feasible ratio of 0.4, beyond which system cost increased. Integration of allostery with transcription further expanded this feasible range by allowing for ratios as low as 0.06.

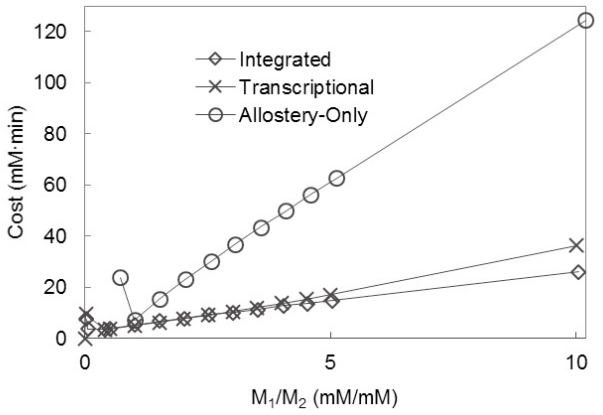

Fig. 3. Metabolic costs of engineered control for three control regimes over a range of target terminal metabolite ratios. The terminal metabolite ratio of the system is the design objective and cost characterizes both how quickly the system reaches the target and the enzyme burden required to do so. Parameters for each regime were optimized to produce a given target ratio and the metabolic cost of control was calculated using (13). Background transcriptional parameters were constant across regimes to simulate the case in which an engineered control system is implemented in a native transcriptional background.

In addition to its limited operational range, the allostery-only regime has the largest cost for all target ratio, i.e. this control regime takes the longest time to reach the target steady-state ratio. Our initial hypothesis was that allostery-only would be the fastest control tu[e and therefore that it would have the lowest associated cost in this optimization. However, the demand for terminal metabolite ratios beyond one is only achievable with highly sensitive allostery in this scenario. Since the control signal (M_2) has a relatively long half-life here, the system takes a proportionately long time to reach the steady-state target when allostery is the only control system. However, this changes as a function of the turnover rate constant (see Fig. 5).

The transcriptional and integrated regimes have similar costs for ratios up to ~2.5. Beyond this point, the cost associated with the integrated system is significantly smaller than that of the transcriptionally-regulated system. The gap in cost between the two systems widens as the ratio increases, suggesting that integration is essential to minimize the cost of large terminal metabolite ratios. Interestingly, the optimal transcription parameter, β, is similar for each target ratio in

the transcriptional and integrated regimes. As a result, the enzyme costs of each system are approximately the same. The cost reduction in the integrated system relative to the transcriptionally-regulated system therefore comes from the metabolite cost alone. This is intuitive, as allostery is expected to reduce time to steady-state in the integrated system relative to the transcriptionally-regulated regime so long as the required sensitivity for allostery is not too high (as in the allostery-only case). The effect of this reduction is most significant for very large or very small target ratios. This would suggest that integration of allosteric and transcriptional regulation is the optimal approach for fine-tuning branched flux distribution.

The increase in operational range in the integrated system relative to transcription-only can be explained by the sharing of control burden across regulatory systems. This is demonstrated in Fig 4: when transcription is paired with allostery, the sensitivity of allosteric regulation required to achieve a given terminal metabolite ratio is reduced by up to two orders of magnitude. For example, in the allostery-only regime, a sensitivity of 526 mM^{-1} is required to reach a target ratio of five. For the same ratio, the integrated system only requires a sensitivity of 8 mM^{-1}. Therefore, a significant portion of the flux distribution control is provided by transcriptional regulation and as a result, less sensitive allosteric regulation is required for fine-tuning. A broader range of target values is thus possible within the limits of sensitivity for allostery in the integrated system. The high sensitivity required for optimal allostery-only control also explains why it has the largest cost for all target ratios: highly sensitive allosteric control is also highly damped, so while it effectively partitions flux, it also takes longer to reach a target M_2 concentration and therefore overshoots the target ratio for a significant portion of the time to steady state.

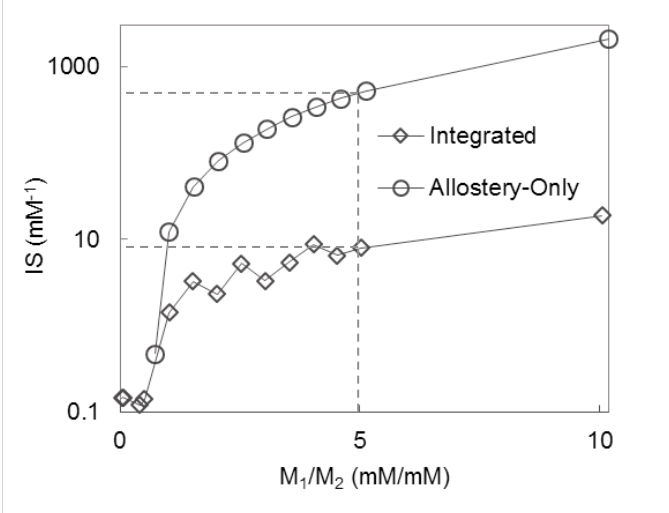

Fig. 4. Inhibition sensitivity required to reach a range of target terminal metabolite ratios in each of the two regimes which use allostery. As in Fig. 3, the ratio of metabolite concentrations is the design target. The sensitivity required to reach the given target can be predicted for either control system; dashed lines indicate the sensitivities required to reach a target ratio of 5 for each regime, for example.

Integration allows the system to perform with the best of both control regimes – it expands the operational range to at least that of a transcriptional controller, but reduces cost relative to transcription by allowing the system to more rapidly achieve the target terminal metabolite ratio.

In each of these optimizations the overall timeframe of the system, defined by the output flux, is constant. We hypothesized that the system timeframe could influence the operation of the proposed control regimes since there is a significant difference in the timeframe over which they respond to control signals. A faster system should favour faster control and vice versa. We therefore expected allostery to play a more significant role for large output fluxes of the control signals – the terminal metabolites. To test this, we optimized the control regimes for a target ratio of 1 for a range of turnover rate constants (γ) for the terminal metabolites.

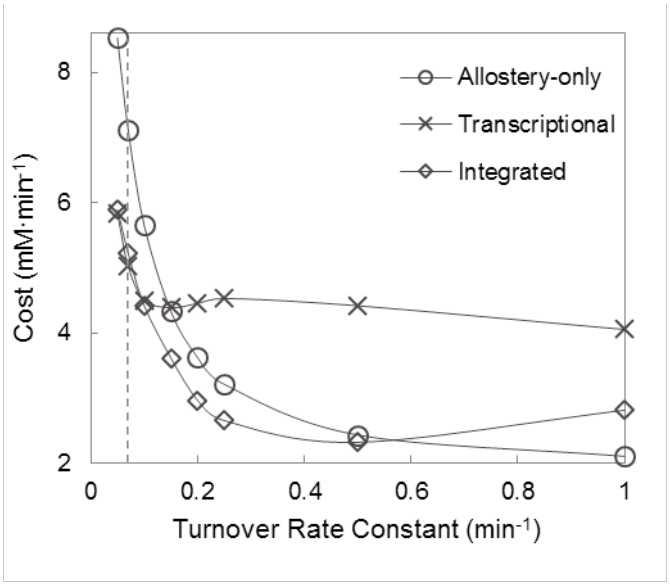

Fig. 5. System costs for each control regime as a function of terminal metabolite turnover rates for a target terminal metabolite ratio of 1. As the turnover rate constant increases, the terminal metabolites leave the system faster and it reaches steady state more rapidly as a result. The dashed line indicates the rate constant used in optimizations of the engineered control regimes.

There are three distinct regions in Fig 5. For small turnover constants (< 0.1 min^{-1}), the effect of transcriptional control dominates as both the transcriptional and integrated regimes minimize cost in this region. For moderate turnovers (0.1 – 5.9 min^{-1}), allostery begins to play a more significant role, but integration minimizes cost. For very large turnover constants (≥ 5.9 min^{-1}), allostery plays a significant role, and transcriptional regulation appears to cause increased costs. As predicted, faster systems require faster control regimes. However, the optimal performance of the integrated regime for moderate timescales is somewhat unexpected and suggests that there is a range of system speeds for which both types of control are necessary for optimal maintenance of a metabolic target.

5. DISCUSSION

We have recapitulated two significant findings here. First, that integration of transcriptional and allosteric control in an engineered system allows for optimal performance in terms of operational range and maintenance of a downstream metabolite concentration (Oyarzun and Chaves, 2011). Second, that system speed dictates which type of metabolic control is optimal to achieve a given target (Chubukov, *et al* 2014). Broadly-speaking, these results are significant for the current understanding of metabolic regulation. They support the notion that our models of metabolism are incomplete without quantification of the interaction between regulatory systems and/or consideration of system dynamics. We have demonstrated here that both of these elements can play significant roles in the direction of flux in an irreversible, branched pathway. In addition, our results suggest that metabolic control – allostery in particular – may be necessary to balance metabolite pools. We suggest that this is especially true in fast systems and that this hypothesis deserves further treatment in future work.

To this body of knowledge, we have added a new design approach to applying engineered proteins in metabolism by treating a potentially tunable biophysical interaction – inhibitor binding – as a design parameter for the rational, metabolic control of flux partitioning. While previous analyses have demonstrated that flux partitioning is significantly controlled at the metabolic level in similar branching pathways, we have shown here that it is possible to rationally control this partitioning by engineering just two system parameters and that for a wide range of system speeds integration of control types is likely optimal.

However, two major caveats to our work exist. First, all of the results we report here are topology-dependent, which is to say that they are only relevant to control of flux partitioning across a branching pathway. Second, our analyses are dependent on strictly irreversible kinetics. MCA has been previously used to demonstrate that the addition of reversibility in one of the arms of a branching pathway can significantly change the metabolic control of flux partitioning through the system (Wang, *et al* 2004). Future work will characterize the effect of reversibility on the results we present here to make this approach more generalizable.

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