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Modeling the role of covalent enzyme modification in *Escherichia coli* nitrogen metabolism

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Abstract

In the bacterium *Escherichia coli*, the enzyme glutamine synthetase (GS) converts ammonium into the amino acid glutamine. GS is principally active when the cell is experiencing nitrogen limitation, and its activity is regulated by a bicyclic covalent modification cascade. The advantages of this bicyclic-cascade architecture are poorly understood. We analyze a simple model of the GS cascade in comparison to other regulatory schemes and conclude that the bicyclic cascade is suboptimal for maintaining metabolic homeostasis of the free glutamine pool. Instead, we argue that the lag inherent in the covalent modification of GS slows the response to an ammonium shock and thereby allows GS to transiently detoxify the cell, while maintaining homeostasis over longer times.

1. Introduction

The metabolic network of enteric bacteria is heavily regulated to allow cells to grow on a wide variety of nutrients and to adjust the growth rate over a range of more than a factor of 10 in response to the availability of nutrients in the environment [1]. The *Escherichia coli* nitrogen utilization network is a particularly well-studied example of metabolic regulation, with all major inputs and factors characterized. Nevertheless, the purpose of the bicyclic cascade at the heart of nitrogen regulation in *E. coli* (figure 1(a)) remains unclear, though a number of theories have been proposed [2–5]. Here we propose a role for this complex regulatory architecture in detoxifying the cell following ammonium shock.

The nitrogen in all compounds synthesized by *E. coli* originates from one of two central intermediates, the amino acids glutamine and glutamate. In turn, glutamine and glutamate derive their nitrogen from ammonium (*E. coli*'s preferred nitrogen source) via two different pathways (reviewed in [6]). One pathway involves glutamate dehydrogenase (GDH), which converts ammonium $\left(NH_4^+\right)$ and the carbon compound α-ketoglutarate (α-KG) directly into glutamate. The other pathway consists of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT). GS uses ATP to convert one molecule each of ammonium and glutamate into one molecule of glutamine, while GOGAT converts one glutamine molecule and one α-KG molecule into two molecules of glutamate [7]. Unlike the GDH pathway, the GS–GOGAT pathway has a high affinity for ammonium, making it the

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principal pathway in nitrogen-limiting environments [8]. GS–GOGAT can use as much as 15% of the cell's ATP resources when in operation, making it critical that the cell be able to regulate GS activity [6].

Indeed, the activity of the GS–GOGAT pathway is regulated at both the transcriptional and post-transcriptional levels. The transcriptional control of GS–GOGAT is part of the Ntr response, which up-regulates numerous genes involved in metabolism in response to nitrogen limitation [6]. Post-transcriptional regulation of GS–GOGAT involves control of the activity of GS by covalent modification. The GS regulatory cascade (figure 1(a)) consists of two covalent modification cycles [9]. In the first cycle, GS is converted to an inactive form by addition of an adenylyl (AMP) group by the bifunctional enzyme adenylyltransferase/adenylyl-removing enzyme (AT/AR). In the second cycle, the signaling protein PII, which regulates AT/AR activity [10], is modified by addition of a uridylyl (UMP) group by a second bifunctional enzyme, uridylyltransferase/uridylyl-removing enzyme (UT/UR). Glutamine promotes AT and UR activity and inhibits UT and AR activity, thereby implementing negative-feedback regulation of GS [9]. The cascade is also influenced by α-KG, which inhibits both the AT-promoting and AR-inhibiting activities of unmodified PII [11].

Kustu *et al* [12] have shown that post-transcriptional down-regulation of GS prevents depletion of cellular glutamate reserves in high-ammonium environments. Why does the cell employ a complex bicyclic enzyme-modification cascade for this purpose? Previous numerical models of the GS regulatory cascade have revealed some of its properties. Bruggeman *et al* [5] performed a simulation of the combined GS–GOGAT and GDH network (ignoring the effects of α-KG). They found that while the glutamine pool could fluctuate significantly in response to changes in the ammonium concentration, the total flux of nitrogen into the cell (via glutamine and glutamate) remained relatively constant at steady state regardless of the external ammonium concentration. Mutalik *et al* [13] found that the bicyclic cascade with totally unsaturated enzymes responded to changes in the ammonium concentration with a consistent level of sensitivity (as measured by Hill coefficient), regardless of small variations in parameters. However, they did not include the effects of feedback in their model. The cyclic GS regulatory cascade also plays a role in signal integration, since it allows the activity of GS to respond to cellular levels of multiple metabolites, including glutamine, α-KG and ATP [14].

However, none of these considerations explain why GS activity is regulated by a bicyclic cascade, as opposed to a simpler allosteric feedback scheme. Enzymes that sense and respond allosterically to many metabolites at once are common [15], and our modeling suggests that allosteric regulation is both fast and capable of robust feedback control.

In this paper, we attempt to understand the use of a cyclic cascade to regulate GS in *E. coli* by analyzing the qualitative properties of cyclic feedback cascades. We compare a bicyclic cascade (figure 1(a)) to a monocylic cascade (figure 1(b)) as well as direct allosteric feedback (figure 1(c)). We find that the most important property effecting the behavior of a cyclic feedback network is the saturation of the regulatory enzymes (e.g. AT/AR) with respect to their substrates (e.g. GS). We find that saturation of the enzymes that implement covalent modification results in tighter feedback regulation, but reduces the stability of the system. In particular, highly saturated enzymatic cascades can display slowly damped or, in some cases, sustained oscillations. The highly sensitive response of saturated cyclic enzyme cascades has been previously analyzed by Goldbeter and Koshland in the context of zero-order ultrasensitivity [16, 17]. Oscillations in cyclic cascades have also been observed in a number of other contexts [18–21]. Indeed, the mathematical properties of feedback and signaling systems have long been a subject of study [22, 23]. In 1977, Chock and Stadtman

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analyzed the stability and sensitivity properties of various cyclic cascades, including the glutamine synthetase cascade, and found many of the properties later observed by Goldbeter and Koshland for more general models [2, 24]. However, none of these models have considered the properties of a cyclic cascade as part of an integrated feedback system, in which the enzyme of interest is both producing and being regulated by a particular metabolite. Curiously, whether or not the modifying enzymes are saturated, the bicyclic cascade appears to be suboptimal for pure homeostatic feedback regulation. We propose, instead, that the bicyclic cascade is designed to respond relatively slowly following ammonium shock to allow glutamine synthetase (GS) to remain active, so as to detoxify the cell by converting ammonium to amino acids, while over longer times the bicyclic cascade adequately maintains homeostasis of the free glutamine pool.

2. Models

We compare three different schemes for GS regulation shown in figure 1. In all cases, we assume that the concentrations of the various reactants are homogenous, so that the kinetics can be described by ordinary differential equations. We also neglect the effects of dilution by cell growth and assume that the total concentrations of the enzymes (GS, AT/AR and UT/UR) are constant, since both division and gene expression occur on time scales longer than the phenomena we examine here. We assume that the rate of glutamine production by an active GS enzyme depends only on the concentration of ammonium, and treat the ammonium concentration as an exogenous input. Since the pKa value for ammonium $\left(\mathrm{NH}_4^+\right)$ over ammonia (NH_3) is 9.24, ammonium is the dominant species at cellular pH, and we neglect the presence of ammonia.

2.1. Monocyclic cascade

The equations describing the reactions of the monocyclic cascade (figure 1(b)) are

$$\frac{\mathrm{d}[\mathrm{GS}]}{\mathrm{d}t}=\frac{V_{\mathrm{AR}}[\mathrm{GS}-\mathrm{AMP}]}{K_{\mathrm{AR}}+[\mathrm{GS}-\mathrm{AMP}]}-\frac{V_{\mathrm{AT}}[\mathrm{GS}]}{K_{\mathrm{AT}}+[\mathrm{GS}]},$$

$$V_{\mathrm{AR}}=[\mathrm{AR}]v_{\mathrm{AR}}=\frac{[\mathrm{AT/AR}]_{\mathrm{tot}}}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}v_{\mathrm{AR}}; \quad (1)$$

$$V_{\mathrm{AT}}=[\mathrm{AT}]v_{\mathrm{AT}}=\frac{[\mathrm{AT/AR}]_{\mathrm{tot}}[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}v_{\mathrm{AT}};$$

$$\frac{\mathrm{d}[\mathrm{Gln}]}{\mathrm{d}t}=\frac{v_{\mathrm{GS}}[\mathrm{GS}]\left[\mathrm{NH}_4^+\right]}{K_{\mathrm{GS}}+\left[\mathrm{NH}_4^+\right]}-V_g-v_l[\mathrm{Gln}], \quad (2)$$

where V_g is the rate at which the cell consumes glutamine for growth, v_l is the first-order rate constant for leakage of glutamine and its products from the cell, the v_i are the maximal rates of the various enzymes, the K_i are the Michaelis constants and $K_{\mathrm{Gln}}^{\mathrm{AR}}$ is the dissociation constant for the binding of glutamine to AR. The notation $[\mathrm{AT/AR}]_{\mathrm{tot}}$ refers to the total

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concentration of adenylyltransferase/adenylyl-removing enzyme. We have assumed that AR is rapidly interconverted to AT by the binding of glutamine.

To characterize the stability and response to perturbations of the steady state of the monocyclic cascade, we consider a linearized approximation that describes the dynamics of small perturbations about the steady-state concentrations of each species in equations (1) and (2):

$$\frac{\mathrm{d}}{\mathrm{d}t}\delta[\mathrm{GS}] = -k_{\mathrm{GS}}^{\mathrm{GS}}\delta[\mathrm{GS}] - k_{\mathrm{Gln}}^{\mathrm{GS}}\delta[\mathrm{Gln}], \quad (3)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\delta[\mathrm{Gln}] = -k_{\mathrm{GS}}^{\mathrm{Gln}}\delta[\mathrm{GS}] - k_{\mathrm{Gln}}^{\mathrm{Gln}}\delta[\mathrm{Gln}] + k_{\mathrm{NH}_4^+}^{\mathrm{Gln}}\delta[\mathrm{NH}_4^+], \quad (4)$$

where δ[x] is the perturbation from the steady-state concentration of species x, and $k_{\mathrm{y}}^{\mathrm{x}}$ is equal to

$$\frac{\partial(\mathrm{d}[\mathrm{x}]/\mathrm{d}t)}{\partial[\mathrm{y}]}$$

(where we have chosen the signs so that all $k_{\mathrm{y}}^{\mathrm{x}} \geq 0$). The solutions to equations (3)–(4) have a time dependence of the form $e^{\lambda t}$, where λ is a root of the characteristic equation

$$\left(\lambda + k_{\mathrm{GS}}^{\mathrm{GS}}\right)\left(\lambda + k_{\mathrm{Gln}}^{\mathrm{Gln}}\right) + k_{\mathrm{GS}}^{\mathrm{Gln}}k_{\mathrm{Gln}}^{\mathrm{GS}} = 0. \quad (5)$$

The steady state is therefore stable if and only if all the roots λ have negative real parts.

To determine the effectiveness of feedback regulation by the monocyclic cascade, we need to know how the glutamine concentration changes in response to a change in the ammonium concentration. We therefore compute the amplitude of the oscillation of δ[Gln] that occurs in response to a sinusoidal oscillation of $\delta\left[\mathrm{NH}_4^+\right]$ at frequency ω. To do this, we Laplace transform the linearized equations (3)–(4). The Laplace transform of a function $f(t)$ is defined as

$$\mathscr{L}[f(t)] = \tilde{f}(\omega) \equiv \int_0^{\infty} f(t)\mathrm{e}^{-\omega t}\mathrm{d}t,$$

where ω is an imaginary number corresponding to frequency. The Laplace transform converts the differential equations in equations (3) and (4) into a mathematically equivalent description in terms of the response to an oscillatory input with a certain frequency. Because equations (3) and (4) are linear, an oscillation in the input (i.e. the perturbation about the steady-state concentration of ammonium) at a particular frequency will produce an oscillation in output (i.e. the perturbation about the steady-state concentration of glutamine) with the same frequency. The Laplace transform allows us to calculate the magnitude of the oscillations of the output of the cascade for any frequency of the input, including zero, which corresponds to a step-like change. It is mathematically equivalent to inserting a solution of the form $A(\omega)$ exp(iωt) into equations (3) and (4) and solving for $A(\omega)$.

The Laplace transforms of equations (3)–(4) are given by

$$\omega\delta[\tilde{GS}]_\omega = -k^{GS}_{GS}\delta[\tilde{GS}]_\omega - k^{GS}_{Gln}\delta[Gln]_\omega, \quad (6)$$

$$\omega\delta[Gln]_\omega = k^{Gln}_{GS}\delta[\tilde{GS}]_\omega - k^{Gln}_{Gln}\delta[Gln]_\omega + k^{Gln}_{NH_4^+}\delta[\tilde{NH}_4^+]_\omega, \quad (7)$$

which can be solved for the response function

$$\chi_{1cyc}(\omega) \equiv \frac{\delta[Gln]_\omega}{\delta[\tilde{NH}_4^+]_\omega} = \frac{k^{Gln}_{NH_4^+}\left(\omega + k^{GS}_{GS}\right)}{\left(\omega + k^{GS}_{GS}\right) + \left(\omega + k^{Gln}_{Gln}\right) + k^{Gln}_{GS}k^{GS}_{Gln}}. \quad (8)$$

2.2. Bicyclic cascade

Our model for the bicyclic cascade is a simplified version of the real GS regulatory cascade. We ignore the effect of α-KG, as well as the transcriptional regulation of GS, and the effects of the PII homologue GlnK. This is because we are interested only in the immediate effect of a sudden ammonium shock, so the influence of these other effectors can be assumed constant (see section 4). We also simplify the interactions between PII, Gln and AT/AR. In particular, we assume that PII and PII-UMP modify GS directly, rather than by binding to AT/AR. We will still refer to AT and AR when discussing the modification of GS, however; in equations (1)–(3) we simply set [AR] = [PII-UMP] and [AT] = [PII]. This is roughly equivalent to the model of Mutalik *et al* [13] with tight binding of AR to PII-UMP and of AT to PII and with the concentration of AT/AR substantially in excess of PII/PII-UMP. Here we are ignoring the effects of ATP and α-KG [25] on these interactions. In effect we have simplified the interaction between PII, AT/AR and GS, and subsumed the effects of molecules other than glutamine into this effective interaction. We also assume that the AT/PII-UMP complex is not active unless it is also bound to a glutamine, and that the AR/PII complex is not active when bound to glutamine. This simplified bicyclic-cascade model is described by the following equations:

$$\frac{d[PII]}{dt} = \frac{V_{UR}[PII-UMP]}{K_{UR}+[PII-UMP]} - \frac{V_{UT}[PII]}{K_{UT}+[PII]},$$

$$[PII]_{tot} = [PII] + [PII-UMP]$$

$$V_{UR} = [UR]v_{UR} = \frac{[UT/UR]_{tot}}{1+[Gln]/K^{UR}_{Gln}}v_{UR}, \quad (9)$$

$$V_{UT} = [UT]v_{UT} = \frac{[UT/UR]_{tot}[Gln]/K^{UR}_{Gln}}{1+[Gln]/K^{UR}_{Gln}}v_{UT},$$

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$$\frac{\mathrm{d}[\mathrm{GS}]}{\mathrm{d}t}=\frac{V_{\mathrm{AR}}[\mathrm{GS}-\mathrm{AMP}]}{K_{\mathrm{AR}}+[\mathrm{GS}-\mathrm{AMP}]}-\frac{V_{\mathrm{AT}}[\mathrm{GS}]}{K_{\mathrm{AT}}+[\mathrm{GS}]},$$

$$V_{\mathrm{AR}}=\frac{v_{\mathrm{AR}}[\mathrm{PII}]}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}, \quad (10)$$

$$V_{\mathrm{AT}}=\frac{v_{\mathrm{AT}}[\mathrm{PII}-\mathrm{UMP}][\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}},$$

$$\frac{\mathrm{d}[\mathrm{Gln}]}{\mathrm{d}t}=\frac{v_{\mathrm{GS}}[\mathrm{GS}][\mathrm{NH}_4^+]}{K_{\mathrm{GS}}+[\mathrm{NH}_4^+]}-V_g-v_l[\mathrm{Gln}], \quad (11)$$

where V_g is the rate at which the cell consumes glutamine for growth, v_l is the first-order rate constant for leakage of glutamine and its products from the cell, the v_i are the maximal rates of the various enzymes, the K_i are the Michael is constants, and $K_{\mathrm{Gln}}^{\mathrm{AR}}$ and $K_{\mathrm{Gln}}^{\mathrm{UR}}$ are the dissociation constants for the binding of glutamine to AR and UR, respectively. We have assumed that AT and AR are rapidly activated and inactivated, respectively, by the binding of glutamine.

To characterize the stability and response to perturbations of the steady state of the bicyclic cascade, we consider the linearized approximation

$$\frac{\mathrm{d}}{\mathrm{d}t}\delta[\mathrm{PII}]=-k_{\mathrm{PII}}^{\mathrm{PII}}\delta[\mathrm{PII}]-k_{\mathrm{PII}}^{\mathrm{PII}}\delta[\mathrm{Gln}], \quad (12)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\delta[\mathrm{GS}]=-k_{\mathrm{GS}}^{\mathrm{GS}}\delta[\mathrm{GS}]+k_{\mathrm{PII}}^{\mathrm{GS}}\delta[\mathrm{PII}]-k_{\mathrm{Gln}}^{\mathrm{GS}}\delta[\mathrm{Gln}], \quad (13)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\delta[\mathrm{Gln}]=k_{\mathrm{GS}}^{\mathrm{Gln}}\delta[\mathrm{GS}]-k_{\mathrm{Gln}}^{\mathrm{Gln}}\delta[\mathrm{Gln}]+k_{\mathrm{NH}_4^+}^{\mathrm{Gln}}\delta[\mathrm{NH}_4^+], \quad (14)$$

where δ[x] is the perturbation from the steady-state concentration of species x, and $k_{\mathrm{y}}^{\mathrm{x}}$ is equal to

$$\frac{\partial(\mathrm{d}[\mathrm{x}]/\mathrm{d}t)}{\partial[\mathrm{y}]}$$

(where we have chosen the signs so that all $k_{\mathrm{y}}^{\mathrm{x}}\geq 0$). The solutions to equations (12)–(14) have a time dependence of the form $e^{\lambda t}$, where λ is a root of the characteristic equation

$$\left(\lambda+k_{\mathrm{PII}}^{\mathrm{PII}}\right)\left(\lambda+k_{\mathrm{GS}}^{\mathrm{GS}}\right)\left(\lambda+k_{\mathrm{Gln}}^{\mathrm{Gln}}\right)+k_{\mathrm{GS}}^{\mathrm{Gln}}k_{\mathrm{Gln}}^{\mathrm{GS}}\left(\lambda+k_{\mathrm{PII}}^{\mathrm{PII}}\right)+k_{\mathrm{Gln}}^{\mathrm{PII}}k_{\mathrm{PII}}^{\mathrm{GS}}k_{\mathrm{GS}}^{\mathrm{Gln}}=0. \quad (15)$$

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The steady state is therefore stable if and only if all the roots λ have negative real parts. We can gain some intuition for when this will occur by examining the cubic equation

$$\lambda^3+K=0 \quad (16)$$

which has a pair of complex solutions given by $|K|^{1/3}\exp(\pm i\pi/3)$. Since these solutions have a positive real part, the solutions to a similar cubic equation with added quadratic and linear terms should also have positive real part, as long as the coefficients of the linear and quadratic terms are sufficiently small in comparison to K and 1. This means that the characteristic equation for the bicyclic cascade (equation (15)) should also have solutions with positive real part for sufficiently small values of $k_{\mathrm{PII}}^{\mathrm{PII}}$, $k_{\mathrm{GS}}^{\mathrm{GS}}$ and $k_{\mathrm{Gln}}^{\mathrm{Gln}}$.

The linear response function of the bicyclic cascade, calculated by Laplace transform as in equations (6)–(8), is given by

$$\begin{aligned}\chi_{1\mathrm{cyc}}(\omega) &\equiv \frac{\delta[\mathrm{Gln}]_\omega}{\delta[\mathrm{N\tilde{H}}_{+}^{4}]_\omega}\\ &=\left[\left(\omega+k_{\mathrm{PII}}^{\mathrm{PII}}\right)\left(\omega+k_{\mathrm{GS}}^{\mathrm{GS}}\right)\left(\omega+k_{\mathrm{Gln}}^{\mathrm{Gln}}\right)+k_{\mathrm{GS}}^{\mathrm{Gln}}k_{\mathrm{Gln}}^{\mathrm{GS}}\left(\omega+k_{\mathrm{PII}}^{\mathrm{PII}}\right)+k_{\mathrm{Gln}}^{\mathrm{PII}}k_{\mathrm{PII}}^{\mathrm{GS}}k_{\mathrm{GS}}^{\mathrm{Gln}}\right]^{-1}\\ &\times\left(k_{\mathrm{NH}_4^+}^{\mathrm{Gln}}(\omega+k_{\mathrm{PII}}^{\mathrm{PII}})(\omega+k_{\mathrm{GS}}^{\mathrm{GS}})\right).\end{aligned} \quad (17)$$

2.3. Allosteric feedback

The equations describing the allosteric-feedback scheme shown in figure 1(c) are

$$\frac{\mathrm{d}[\mathrm{Gln}]}{\mathrm{d}t}=\frac{v_{\mathrm{GS}}[\mathrm{GS}][\mathrm{NH}_4^+]}{K_{\mathrm{GS}}+[\mathrm{NH}_4^+]}-V_g-v_l[\mathrm{Gln}], \quad (18)$$

$$[\mathrm{GS}]=\frac{[\mathrm{GS}]_{\mathrm{tot}}}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{GS}}}, \quad (19)$$

Where $K_{\mathrm{Gln}}^{\mathrm{GS}}$ is the dissociation constant for the allosteric binding of glutamine to GS, which is assumed to inactivate the enzyme and to be in rapid equilibrium. Equations (18)–(19) lead to the following linearized approximation about the steady state (where $k_{\mathrm{y}}^{\mathrm{x}}$ is defined as below equation (4)):

$$\frac{\mathrm{d}}{\mathrm{d}t}\delta[\mathrm{Gln}]=-k_{\mathrm{Gln}}^{\mathrm{Gln}}\delta[\mathrm{Gln}]+k_{\mathrm{NH}_4^+}^{\mathrm{Gln}}\delta[\mathrm{NH}_4^+]. \quad (20)$$

Equation (20) implies that the glutamine concentration only oscillates if the ammonium concentration oscillates, so the steady state is always stable, and the response to a step change in the ammonium concentration is simply exponential decay to the new steady state. The response function, defined as in equation (8), is

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$$\chi_{\mathrm{allos}}(\omega)=\frac{k_{\mathrm{NH}_4^+}^{\mathrm{Gln}}}{\omega+k_{\mathrm{Gln}}^{\mathrm{Gln}}}. \quad (21)$$

In the zero-frequency limit ($\omega = 0$), which describes the long-time response to a step-like change in the ammonium concentration, the response function reduces to $\chi_{\mathrm{allos}}=k_{\mathrm{NH}_3}^{\mathrm{Gln}}/k_{\mathrm{Gln}}^{\mathrm{Gln}}$. To investigate the effectiveness of allosteric regulation, we assume that $K_{\mathrm{Gln}}^{\mathrm{GS}}$ is very small, corresponding to tight binding of glutamine to GS. The result, after dropping terms in the denominator that contain $K_{\mathrm{Gln}}^{\mathrm{GS}}$, is

$$\chi_{\mathrm{allos}}(0)=\frac{k_{\mathrm{NH}_4^+}^{\mathrm{Gln}}}{k_{\mathrm{Gln}}^{\mathrm{Gln}}}=\frac{K_{\mathrm{Gln}}^{\mathrm{GS}}[\mathrm{GS}]_{\mathrm{tot}}\upsilon_{\mathrm{GS}}}{V_g K_{\mathrm{GS}}(1+[\mathrm{NH}_4^+]/K_{\mathrm{GS}})^2}. \quad (22)$$

This formula indicates that the effectiveness of allosteric feedback regulation is set by the magnitude of the dissociation constant for the binding of glutamine to GS, $K_{\mathrm{Gln}}^{\mathrm{GS}}$. In other words, with a very small value of $K_{\mathrm{Gln}}^{\mathrm{GS}}$, the effect of a change in the ammonium concentration on glutamine levels will be very small.

2.4. Parameters

The following parameters were used to simulate the monocyclic cascade, except where otherwise mentioned, $\upsilon_{\mathrm{AR}} = \upsilon_{\mathrm{AT}} = 1\ \mathrm{min}^{-1}$, $K_{\mathrm{Gln}}^{\mathrm{AR}}=1\,\mu\mathrm{M}$ for the unsaturated cascade and $K_{\mathrm{Gln}}^{\mathrm{AR}}=1\,\mathrm{mM}$ for the saturated cascade, $\upsilon_{GS} = 460$ mM min $^{-1}$, $K_{\mathrm{GS}} = 500\ \mu$M, $V_g = 100\ \mu$M min $^{-1}$, $[\mathrm{AT/AR}]_{\mathrm{tot}} = 0.1\ \mu$M, $[\mathrm{GS}]_{\mathrm{tot}} = 1.3\ \mu$M and $\upsilon_l = 0.1$ min $^{-1}$. We used the same parameters for the bicyclic cascade, with the addition of $\upsilon_{\mathrm{UR}} = \upsilon_{\mathrm{UT}} = 1$ min $^{-1}$, $[\mathrm{PII}]_{\mathrm{tot}} = 0.1\ \mu$M, $[\mathrm{UT/UR}]_{\mathrm{tot}} = 0.5\ \mu$M and $K_{\mathrm{Gln}}^{\mathrm{AR}}=K_{\mathrm{Gln}}^{\mathrm{UR}}=10\,\mu\mathrm{M}$. The corresponding parameters in the allosteric scheme are also the same, except for $K_{\mathrm{Gln}}^{\mathrm{GS}}=0.5\,\mu\mathrm{M}$. The parameters for the bicyclic cascade were chosen to correspond roughly to experimentally measured values [5, 11, 26]. However, since the model is simplified and some interactions are effective in nature (in particular the interaction of glutamine with GS and PII and of PII with GS), the model does not accurately reproduce cellular glutamine concentrations. In order to make a controlled comparison to the monocyclic cascade model, simulations at various saturation levels had the glutamine binding constant $K_{\mathrm{Gln}}^{\mathrm{AR}}$ adjusted so that the steady state glutamine concentration was always around 1 mM. This was also done in the allosteric cascade model. Parameters are discussed further in the supplementary material (available at stacks.iop.org/PhysBio/7/016006/mmedia).

3. Results

3.1. Response to ammonium shock

Figures 2 and 3 show simulations of the response of different feedback schemes to an ammonium shock. The equations describing the monocyclic cascade, equations (1)–(2), and the equations describing allosteric feedback, equations (18)–(19), were numerically integrated from initial conditions corresponding to a steady-state internal ammonium concentration of 100 μM, but with a sudden increase of the ammonium level to 100 mM to simulate ammonium shock. Figure 2(a) shows the intracellular free glutamine level following ammonium shock for a monocyclic cascade in which AT/AR is highly saturated

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with respect to both its substrates, GS and GS–AMP ($K_{AT} = K_{AR} = 1$ pM). This means that the concentrations of the substrates GS and GS–AMP are much higher than the Michaelis constants for AT/AR and therefore that both AT and AR are operating at their maximal rates, and that the activity of AT/AR is independent of the concentration of the substrate molecules. Figure 2(b) shows a corresponding simulation for an unsaturated monocyclic cascade ($K_{AT} = K_{AR} = 1$ μM). In the unsaturated limit, the concentrations of GS and GS–AMP are much lower than the Michaelis constants of AT and AR and therefore the activities of AT and AR are roughly proportional to the concentrations of GS and GS–AMP, respectively. In both simulations, the dissociation constant for the binding of glutamine to either AT/AR (in the monocyclic cascade) or GS (in the allosteric cascade) is adjusted to make the steady state of glutamine roughly 1 mM at 100 mM ammonium. There are two notable features of these plots. Firstly, the glutamine steady state of 1 mM in the saturated cascade is unchanged by the ammonium shock (over a longer time than shown the oscillations in the glutamine concentration relax to 1 mM), whereas the glutamine steady state in the unsaturated cascade rises by more than a factor of 2. In addition, the size of the transient increase in the glutamine concentration is much larger in the unsaturated cascade. This suggests that the highly saturated cascade is more effective at minimizing internal changes in glutamine levels in response to an external change in ammonium. Secondly, in the unsaturated cascade after the initial glutamine spike, the glutamine concentration decays nearly monotonically to a new steady state. By contrast, in the saturated limit the glutamine concentration exhibits slowly decaying oscillations after the ammonium shock. This suggests that while the saturated cascade may be more effective than the unsaturated cascade at minimizing the magnitude of response to ammonium shock, it is also more prone to oscillations. Figure 3 shows simulations of ammonium shock for the bicyclic cascade (equations (9)–(11)), with similar results. The saturated cascade is unstable and displays sustained oscillations, but has a smaller overall response than the unsaturated cascade. In fact, from these examples, one might conclude that the saturated casacades are overall superior for negative feedback regulation, since their oscillations are relatively small and their steady-state response to a change in input is much less than in the unsaturated cascades.

The saturation of the cascade enzymes is the most important variable affecting the qualitative behavior of a cyclic cascade (see the supplementary material available at stacks.iop.org/PhysBio/7/016006/mmedia). These results indicate that in optimizing the saturation level of the regulatory enzymes in a cyclic feedback cascade, one faces a tradeoff between effective feedback and stability. Effective negative feedback requires that the steady-state concentration of a metabolite not change in response to changes in external resources. However, even if the steady state of a particular regulatory cascade is insensitive to changes in external inputs, feedback regulation may in practice be ineffective if steady state is reached via slowly damped oscillations, since the concentration of the regulated metabolite will take a long time to reach its equilibrium value.

3.2. Instability of the saturated cascade

What is the origin of the oscillations in the saturated monocyclic and bicyclic cascades? A tradeoff between tight feedback control and stability is a well-known phenomenon in control theory [27]. This tradeoff can be understood in terms of two general properties of feedback control loops, gain and time delay. The effectiveness of a feedback control loop can be increased by increasing its gain. However, the presence of a time delay in the loop can contribute to oscillations and instability by causing over-corrections to the input signal. A high-gain loop will have a greater tendency to over-correct in the presence of a time delay. In the Supplemental Information we present a calculation of the feedback gain in the monocyclic cascade which demonstrates that the gain approaches infinity as the Michaelis constants of the regulatory enzymes approach zero. However, the phenomenon of tradeoff

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between accurate control and stability can be understood in simpler terms, as presented below.

To find the reason for the reduced response but greater instability of the saturated cascade, we examine equation (1) in the limit of total saturation of AT/AR with respect to both its substrates, GS and GS–AMP:

$$\frac{\mathrm{d}\,[\mathrm{GS}]}{\mathrm{d}t}=V_{\mathrm{AR}}-V_{\mathrm{AT}};$$

$$V_{\mathrm{AR}}=[\mathrm{AR}]v_{\mathrm{AR}}=\frac{[\mathrm{AT/AR}]_{\mathrm{tot}}}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}v_{\mathrm{AR}};\quad (23)$$

$$V_{\mathrm{AT}}=[\mathrm{AT}]v_{\mathrm{AT}}=\frac{[\mathrm{AT/AR}]_{\mathrm{tot}}[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}{1+[\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{AR}}}v_{\mathrm{AT}}.$$

The first equation clearly reaches steady state only for the particular level of [Gln] at which $V_{AR} = V_{AT}$, namely $[\mathrm{Gln}]=(v_{\mathrm{AR}}/v_{\mathrm{AT}})K_{\mathrm{Gln}}^{\mathrm{AR}}$, independent of the ammonium concentration. Therefore, the saturated cascade has the potential to implement perfect negative feedback control, always returning [Gln] to precisely the same level. When the glutamine concentration is below the steady-state level, the first term on the right-hand side of equation (23) dominates, increasing GS activity. When the glutamine concentration is below the steady-state level, the second term in equation (23) dominates, decreasing GS activity. This corresponds to the onset of zero-order ultrasensitivity [16]. This switch-like sensitivity allows the cascade to implement very precise control. However, high sensitivity is also the source of instability. A small perturbation in the glutamine concentration (e.g. caused by a change in the ammonium concentration) can cause an overshoot in GS activity, resulting in oscillations. In fact, since an arbitrarily small change in the glutamine concentration can flip the ‘switch’, driving GS either to full activity or zero activity, the fully saturated monocyclic cascade has effectively infinite feedback gain, which can result in oscillations even if the feedback time delay is very small.

3.3. Glutamine synthetase (GS) is deactivated more quickly in the saturated cascade

Another difference between the saturated and unsaturated cascades is the speed with which GS is deactivated in response to an ammonium shock. As shown in figure 2, GS is deactivated more rapidly by the saturated cascade than by the unsaturated cascade. This is related to the differing values of $K_{\mathrm{Gln}}^{\mathrm{AR}}$ in the two cascades. To achieve a steady state, the intracellular glutamine concentration of 1 mM at an ammonium concentration of 100 mM requires $K_{\mathrm{Gln}}^{\mathrm{AR}}\approx 1\,\mathrm{mM}$ in the saturated cascade, but requires $K_{\mathrm{Gln}}^{\mathrm{AR}}\approx 1\,\mu\mathrm{M}$ in the unsaturated cascade. In both cases, glutamine synthetase activity is low at 100 mM ammonium (i.e. [GS] $\ll$ [GS–AMP]). However, for the saturated cascade at steady state, the enzyme modification rates V_{AT} and V_{AR} remain high (and equal) at $[\mathrm{AT/AR}]_{\mathrm{tot}}/(1/v_{AT} + 1/v_{AR})$, from equation (23). In contrast, for the unsaturated cascade at steady state at 100 mM ammonium, the low value of [GS] implies that the rate of adenylylation of GS to GS–AMP is low, and therefore, necessarily, so is the rate of de-adenylylation of GS–AMP to GS. For this steady-state rate of de-adenylylation to be low even though almost all of the glutamine synthetase is in the

adenylylated form GS–AMP, there must be very little adenylyl-removing enzyme present (i.e. [AR] ≪ [AT]). Recall that AR and AT are two forms of a bifunctional enzyme, with the ratio set by the glutamine concentration at $[\mathrm{AR}]/[\mathrm{AT}]=K_{\mathrm{Gln}}^{\mathrm{AR}}/[\mathrm{Gln}]$. So the requirement [AR] ≪ [AT] for the unsaturated cascade at 100 mM ammonium is the reason that the glutamine dissociation constant $K_{\mathrm{Gln}}^{\mathrm{AR}}=1\,\mu\mathrm{M}$ had to be set much lower than the steady-state glutamine pool [Gln] ≈ 1 mM (i.e. in order to strongly repress the AR concentration and thus reduce the rate of de-adenylylation of GS–AMP).

For the unsaturated cascade, the low rates of adenylylation/de-adenylylation of glutamine synthetase at steady state also imply a slow rate of deactivation of GS following ammonium shock. For a perturbation around steady state at high ammonium,

$$\frac{\mathrm{d}\delta[\mathrm{GS}]}{\mathrm{d}t} \approx -\frac{V_{\mathrm{AT}}}{K_{\mathrm{AT}}}\delta[\mathrm{GS}], \quad (24)$$

Where $V_{\mathrm{AT}} \approx V_{\mathrm{AT}}^{\mathrm{max}}=[\mathrm{AT}/\mathrm{AR}]_{\mathbf{tot}} v_{\mathrm{AT}}$. Therefore, the relaxation time after an ammonium shock will be roughly determined by the ratio $K_{\mathrm{AT}}/V_{\mathrm{AT}}^{\mathrm{max}}=10$ min. This is in contrast to the saturated cascade, where the steady-state rates of adenylylation and deadenylylation are independent of the concentration of GS (and therefore free to be fast), yielding a much faster relaxation time.

3.4. Response to ammonium shock following nitrogen limitation

Figure 4 shows simulations of the responses of the saturated and unsaturated cascades to a change in the ammonium concentration from 0.2 μM to 100 mM. With a low initial ammonium concentration of 0.2 μM instead of 100 μM, the initial glutamine concentration in the unsaturated cascade is much closer to $K_{\mathrm{Gln}}^{\mathrm{AR}}=1\,\mu\mathrm{M}$. As the plots demonstrate, this substantially reduces the difference in the initial responses of the two cascades. Put simply, with GS activity initially high (≳40%) in both cases, the early response to ammonium shock is the deactivation of GS by adenylylation at nearly the maximum rate $V_{\mathrm{AT}}^{\mathrm{max}}$. Only at late times (>10 min) does the slowing of the GS adenylylation rate for the unsaturated cascade become evident.

3.5. Allosteric feedback

Figure 2(c) (red/blue curves) shows the intracellular free glutamine level following ammonium shock for the allosteric-feedback scheme (figure 1(c)). The glutamine concentration monotonically approaches a new steady state without overshoot or oscillations. In fact, it is impossible for the allosteric feedback scheme to ever result in oscillation of the glutamine concentration. The system can be described in the linear approximation by a single first-order linear differential equation, equation (20), which does not admit an oscillatory solution. The steady-state change in the glutamine concentration shown in figure 2(c) is roughly the same as the steady-state change in the unsaturated monocyclic cascade shown in figure 2(b). The change in the steady-state glutamine level in response to a step change in the ammonium concentration is proportional to the dissociation constant, $K_{\mathrm{Gln}}^{\mathrm{GS}}$, for the binding of glutamine to GS, equation (22). In figure 2(c), $K_{\mathrm{Gln}}^{\mathrm{GS}}$ is set to 0.5 μM so that the final value of [Gln] is about 1 mM. Dissociation constants with values as low as 1 pM are possible [15], so in principle allosteric feedback could achieve even tighter control.

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Tighter control can also be achieved by allosteric feedback with cooperative ligand binding. Figure 2(c) (green/black curves) shows the allosteric feedback scheme with cooperativity. In particular, equation (10) is replaced by

$$[\mathrm{GS}]=\frac{[\mathrm{GS}]_{\mathrm{tot}}}{1+\left([\mathrm{Gln}]/K_{\mathrm{Gln}}^{\mathrm{GS}}\right)^{h}}, \quad (25)$$

where h is the Hill coefficient. In figure 2(c) (green/black curves), we set $h = 3$ and changed the value of $K_{\mathrm{Gln}}^{\mathrm{GS}}$ to 70 μM to retain a final glutamine concentration of roughly 1 mM. Note that in the allosteric feedback scheme the total change in the glutamine concentration resulting from the ammonium shock is lower in the case of cooperative binding.

3.6. Instability of the bicyclic cascade

Although the monocyclic cascade may display lengthy oscillations as in figure 2(a), it is never truly unstable. In particular, the root of the characteristic equation, equation (5), will always have a negative real part, meaning that oscillations will decay, unless the saturation constants of both AT and AR are zero *and* the leakage rate for glutamine is zero.

In contrast, permanent oscillations can occur in the bicyclic cascade, as shown in figure 3(a). In particular, the characteristic equation for the bicyclic cascade is a cubic equation which can have roots with positive real parts when the values of the saturation constants of UT/UR and AT/AR are sufficiently low. In general, higher-order feedback systems (see the supplemental information available at stacks.iop.org/PhysBio/7/016006/mmedia) are more prone to instability. Figure 5 shows the boundary at which the bicyclic cascade becomes unstable as a function of the saturation constants (assuming that $K_{\mathrm{AT}} = K_{\mathrm{AR}}$ and $K_{\mathrm{UT}} = K_{\mathrm{UR}}$). The inset shows sustained oscillations in the intracellular free glutamine concentration that occur in response to an ammonium shock in the bicyclic cascade with highly saturated enzymes. The fact that the bicyclic cascade becomes unstable for non-zero values of the saturation constants for UT/UR and AT/AR means that, although both cyclic cascades are prone to oscillation, the problem is worse in the bicyclic cascade.

3.7. Linear response of monocyclic and bicyclic cascades

Figure 5(b) shows the linear-response functions of both monocyclic and bicyclic cascades (equations (7) and (17)). The linear-response function measures the response of the internal glutamine level to an oscillation in the ammonium concentration at frequency ω. The $\omega = 0$ limit of the response function thus gives the response of the steady-state glutamine concentration to a step-like change in the ammonium level. The low frequency regime of the response function also dictates the response of the glutamine pool to noisy fluctuations in the ammonium concentration. We find that for both the monocylic and bicyclic cascades, the magnitude of the response function depends sensitively on the saturation of the regulatory enzymes, with more saturated enzymes exhibiting smaller response, up to the point of onset of sustained oscillations (see the supplementary material available at stacks.iop.org/PhysBio/7/016006/mmedia). In order to make a controlled comparison, the response function of the bicyclic cascade is plotted for values of the saturation constants that are in the stable regime. The saturation constant of the monocyclic cascade is set to a small positive value, such that both cascades will decay to their steady-states at about the same rate. The response of the bicyclic cascade is stronger than the response of the monocyclic cascade for all frequencies. This relation holds over a wide range of saturation constants (up to the unsaturated regime, see the supplementary material available at stacks.iop.org/PhysBio/7/016006/mmedia),

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suggesting that the bicyclic cascade, in addition to being less stable than the monocyclic cascade, is also less effective at feedback regulation.

3.8. Ammonium consumption/detoxification

If adding a second cycle to a monocyclic cascade results in *less* effective feedback regulation, we reason that the bicyclic cascade may serve some other purpose in *E. coli*. This purpose could be ammonium detoxification. While ammonium is the preferred nitrogen source for *E. coli*, ammonium is also toxic at high concentrations [28, 29]. This means that, although down-regulation of GS in response to a high ammonium level maintains glutamine homeostasis and limits ATP consumption by GS, it could be more important in some circumstances for the cell to retain GS activity to convert potentially toxic ammonium to glutamine. The same properties that make cyclic cascades imperfect as feedback regulators, such as the large initial jump in the glutamine concentration following an ammonium shock (figure 2), make them effective as ammonium detoxifiers.

Figure 6 compares ammonium consumption following ammonium shock for two different feedback schemes. The plot shows the cumulative amount of ammonium that would be consumed after a jump in the ammonium concentration from 100 μM to 100 mM in a cell regulating GS with a bicyclic cascade and in a cell regulating GS by allosteric feedback. In this plot we have adjusted the strength of glutamine binding to AT/AR and UT/UR in the bicyclic cascade to make the steady-state glutamine concentration 1.3 mM at 100 mM ammonium, as in the model of allosteric feedback. For the allosteric model we used the same parameters and initial conditions as in figure 2. Once the glutamine concentration has equilibrated, the cell consumes ammonium at the same rate in both models. However, during the first 10 min, GS as regulated by the bicyclic cascade consumes ammonium at roughly triple the rate of GS as regulated by allosteric feedback. Note that 10 min is roughly the time it takes for the cell to effect changes in gene expression that protect it from high ammonium concentrations (see section 4). Figure 6(c) shows schematically our model for ammonium detoxification—ammonium is converted into non-toxic glutamine by GS, and thence into other amino acids which may be leaked from the cell.

The same differences in feedback regulation that make the monocyclic cascade an improvement over allosteric feedback in terms of ammonium detoxification also make the bicyclic cascade an improvement over the monocyclic cascade. In fact, the goal of homeostatic feedback regulation—to minimize changes in the glutamine pool—is precisely the opposite of those of ammonium detoxification—to rapidly consume a large amount of ammonium for a brief period after a sudden shock.

4. Discussion

In *E. coli*, the primary source of cellular nitrogen in low ammonium environments is the conversion of ammonium into glutamine by the enzyme glutamine synthetase (GS). When active, GS can consume as much as 15% of the cell's ATP budget, making regulation of the activity of GS critical to the cell [12]. GS is regulated by a bicyclic covalent-modification cascade (figure 1(a)), in which GS is inactivated by adenylylation, and the adenylylation/ deadenylylation cycle is further regulated by the signaling protein PII, which is itself regulated by covalent modification.

We have presented several pieces of evidence that the bicyclic cascade in *E. coli* is not optimal for homeostatic feedback regulation.

1. Allosteric feedback is fast and, unlike cyclic cascades, suffers from no tradeoff between stability and effective feedback regulation.

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2. Monocyclic cascades offer more effective feedback regulation with less tendency to oscillate than bicyclic cascades.

3. Cyclic cascades with highly saturated enzymes tend to offer better feedback regulation, but the enzymes of the bicyclic cascade that regulate GS in *E. coli* are unsaturated [11, 26].

We used a simple model of a monocyclic regulatory cascade to show that cyclic cascades as a whole suffer from a tradeoff between tight regulation and the stability of their equilibria. We have shown that cyclic cascades with enzymes working at saturation can become unstable, displaying slowly damped or sustained oscillations in response to external perturbations (as has been observed previously [19]). However, a cyclic cascade whose regulatory enzymes work at saturation (tight feedback regulation) deactivates GS more quickly in response to ammonium shock, and has a lower steady-state response function than a cascade whose regulatory enzymes have high Michaelis constants (loose feedback regulation). Experiments [11] have found that the regulatory enzymes of the GS cascade in *E. coli* operate far from saturation, suggesting that the GS cascade is not intended solely for homeostatic regulation of the glutamine pool. We also showed that the bicyclic cascade does not provide tighter feedback control than the monocyclic cascade (figure 1(b)) and suffers from greater instability.

While it is clear that the GS regulatory cascade in *E. coli* is necessary to protect the glutamate pool during ammonium upshift [12], our results suggest that the cascade may have some other purpose as well. Many authors have pointed out that the bicyclic structure of the cascade serves to integrate signals of the nitrogen (via glutamine) and carbon (via α-KG) status of the cell [11, 25, 30]. Westerhoff and colleagues have proposed [3, 31] that the combination of PII and its homologue GlnK enables the cell to precisely adjust its strategy for GS regulation in response to varying external ammonium concentrations. They find that the combined regulation of GS–GOGAT and GDH allows *E. coli* to maintain a roughly constant rate of nitrogen influx regardless of ammonium levels [5]. However, none of these theories are able to directly account for the detailed bicyclic structure of the GS regulatory cascade. In particular, one can ask the following. Given that AT/AR is able to sense glutamine concentration allosterically, why is UT/UR also glutamine sensitive? Why do both UT/UR and AT/AR operate in an unsaturated regime when highly saturated enzymes are more effective regulators?

To answer these questions, we hypothesize that the bicyclic cascade regulating GS plays a role in ammonium detoxification following ammonium shock. High concentrations of ammonium are known to be toxic to many plants and animals, as well as yeast [32–35]. The experimental evidence regarding ammonium toxicity in *E. coli* is less clear. Two experiments have found a toxic effect of ammonium on *E. coli* at submolar concentrations, but only after exposure times on the order of hours [28, 29]. A more recent experiment [36] found that ammonium was not toxic at realistic physiological concentrations. However, ammonium cannot diffuse through the cell membrane and *E. coli* cells are known to deactivate the membrane ammonium transporter AmtB when exposed to a high-ammonium environment [37–39]. This may explain why the experiment in [36] did not see a detrimental effect of ammonium on initial growth rates, while other experiments have seen an effect of ammonium on the viability of *E. coli* after long exposures [28, 29]. Indeed, the authors of [36] speculated that *E. coli* are able to resist ammonium toxicity by preventing ammonium from passing through the cell membrane. It seems likely then that *E. coli* grown in a low ammonium environment, and thus expressing the transporter AmtB, could be vulnerable to sudden ammonium shock. It has been shown that yeast suffer from ammonium toxicity when their ability to block ammonium transport through the membrane is taken away [34].

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We showed that a cyclic cascade converts toxic ammonium into non-toxic glutamine after a sudden ammonium shock more effectively than does an allosteric feedback scheme (figure 6). The qualities that make cyclic cascades more effective at ammonium detoxification than allosteric feedback make them generally less effective as feedback control systems. Thus, the fact that *E. coli* uses an unsaturated bicyclic cascade to regulate GS activity is consistent with the possibility that GS may play a role in ammonium detoxification.

Our model for ammonium detoxification by GS makes a testable experimental prediction. *E. coli* exposed to high internal levels of ammonium should overproduce glutamine. It is likely then that *E. coli* cells with high internal ammonium levels will leak excess glutamine and other amino acids into their environment. However, because *E. coli* cells cease active membrane transport of ammonium when internal glutamine levels are high, a high ammonium environment will not always correspond to a high internal ammonium concentration.

E. coli defend themselves from sudden ammonium shock during nitrogen limitation by shutting down the high-affinity ammonium transport protein AmtB. AmtB activity is regulated by interaction with the PII homolog GlnK. It may be possible to separate the effects of GS regulation on ammonium detoxification from the effects of AmtB regulation by exposing *E. coli* to a low potassium/high ammonium environment. Under potassium limitation, *E. coli* express a high-affinity potassium transporter, Kdp, which is unable to distinguish K^+ and NH_4^+ ions. Experiments have shown that *E. coli* possessing the Kdp transporter increase oxygen consumption when exposed to ammonium ions while under potassium limitation [40]. The authors attribute this to futile cycling of protons from NH_4^+, but it may also be due in part to increased GS activity. It may also be advantageous to achieve high internal ammonium concentrations without exposing *E. coli* to a high potassium environment by constitutively expressing the Kdp channel [41].

Post-transcriptional regulation of GS acts over short time scales, roughly on the order of a minute. At longer time scales, the transcriptional regulation of GS becomes relevant. In particular, for *E. coli* living continuously in a high-ammonium environment, GS expression is low. This means that the role of GS in ammonium detoxification is probably most important when cells are exposed to a sudden ammonium shock after growing under ammonium limitation.

In attempting to understand why GS is regulated by a bicyclic cascade in *E. coli*, it is useful to compare GS regulation in other species. In particular, *Bacillus subtilis* and other Gram-positive bacteria regulate GS activity by allosteric feedback, not by a cyclic cascade [42]. In *Bacillus* there is no GDH activity, so GS–GOGAT is the sole pathway for nitrogen assimilation. GS produces glutamine through two different reactions, one requiring Mg^{2+} and one requiring Mn^{2+}. The Mg^{2+}-dependent reaction is strongly inhibited by glutamine and other amino acids, but the Mn^{2+}-dependent reaction is not [43]. Normally, the Mn^{2+} concentration is much lower than the Mg^{2+} concentration, but during sporulation, the two can be roughly equal. *B. subtilis* lacks a global nitrogen regulatory apparatus, and it has been conjectured that the main survival strategy of *B. subtilis* during nitrogen limitation is sporulation [42]. If sporulation is the primary response to nitrogen limitation, then cells at risk for ammonium shock would be sporulating or already have formed spores. During sporulation, Mn^{2+} levels are high, and GS is less subject to inhibition by glutamine. This would also make GS available to detoxify the cell during sporulation or in germinating spores.

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5. Conclusions and outlook

We have argued from a series of simple models of enzymatic regulatory cascades that the glutamine synthetase regulatory cascade in *E. coli*, in addition to its role in integrating carbon and nitrogen metabolism [14], and in protecting the glutamate pool [12], also protects the cell form sudden ammonium shock by allowing for an extended period of conversion of ammonium to glutamine following ammonium upshift. We have also proposed experimental tests of our predictions, similar to those already carried out in yeast [34]. It is important to keep in mind that the regulation of glutamine synthetase is among the most complex of any known enzyme [44]. PII is known to respond to the concentration of ATP and α-KG in addition to glutamine, and is also known to influence the transcription of GS by modulating the Ntr response [6, 25]. A number of authors have speculated that PII serves to coordinate the response of GS activity to these diverse signals as well as to the concentration of glutamine [11, 25, 30]. Our model is focused on studying the effect of glutamine feedback on GS at short time scales, and does not consider the effects of other modes of GS regulation. It will be important for further modeling work to consider these complexities in order to understand the regulation of GS in detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

GS (glutamine synthetase): ATP-consuming enzyme that catalyzes the conversion of NH_4^+ into glutamine.

AT/AR (adenylyltransferase/ adenylyl-removing enzyme): Bifunctional enzyme responsible for regulating the activity of GS in *E. coli* by covalent modification.

PII: Signaling molecule responsible for modifying the activity of AT/AR in *E. coli*. Can either promote or inhibit glutamine production, depending on the presence of uridylyl groups added by UT/UR.

UT/UR (uridylyltransferase/ uridylyl-removing enzyme): Bifunctional enzyme responsible for regulation the signaling molecule PII, which in turn regulates the activity of AT/AR in *E. coli*.

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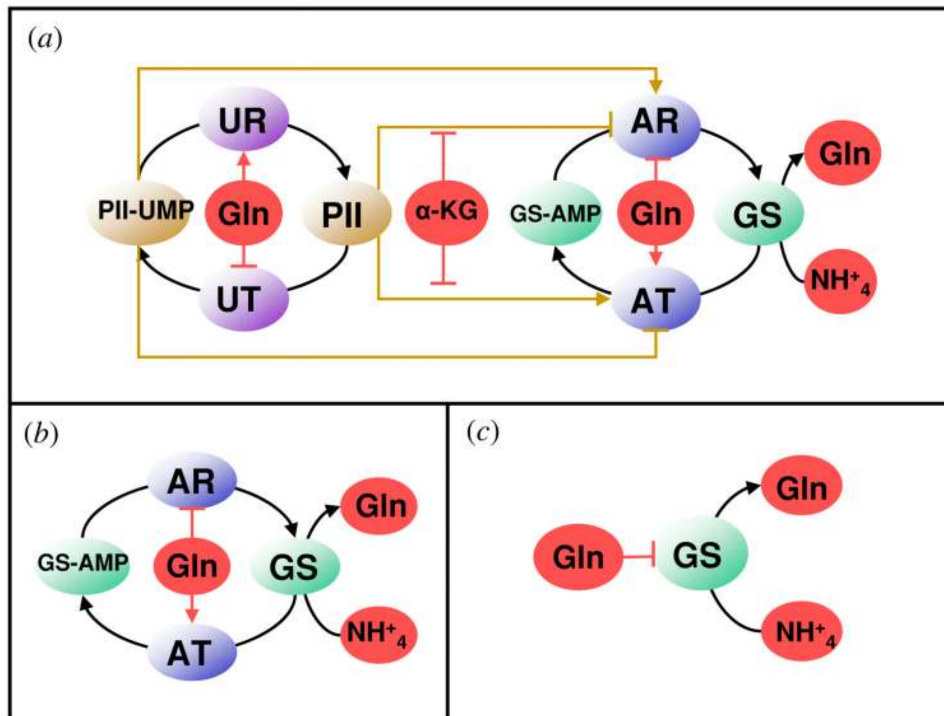

Figure 1.

Schemes for feedback regulation of enzyme activity. (*a*) In *E. coli* the activity of glutamine synthetase (GS) is controlled by a bicyclic cascade. GS activity is suppressed by covalent adenylylation by the bifunctional adenylyltransferase/adenylyl-removing enzyme (AT/AR). AT/AR activity is modulated by the signaling protein PII, whose activity depends on covalent modification by the bifunctional uridylyltransferase/uridylyl-removing enzyme (UT/UR). Glutamine (Gln) affects the activities of both AT/AR and UT/UR so as to implement negative feedback. The activity of unmodified PII is believed to be inhibited by α-ketoglutarate (α-KG). (*b*) Simpler hypothetical scheme in which GS activity is controlled by a single covalent-modification cycle. (*c*) Hypothetical scheme in which GS is allosterically regulated by glutamine.

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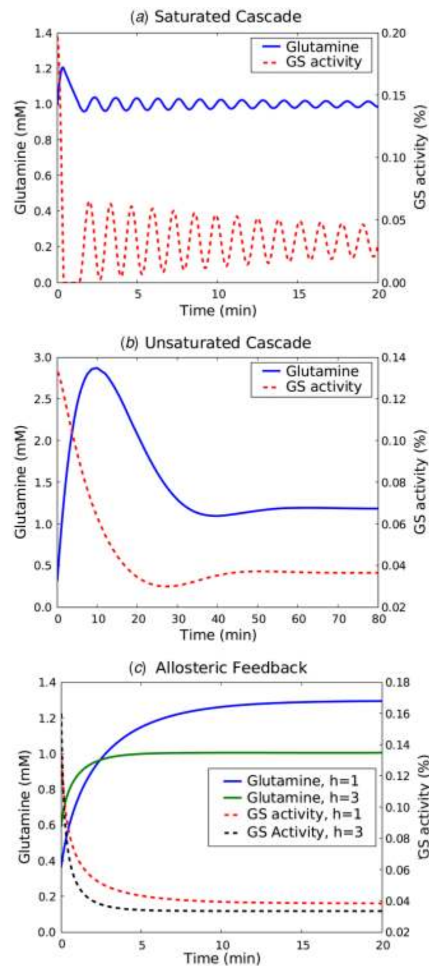

Figure 2.

Comparison of change in intracellular free-glutamine level (solid curves) and the percentage of GS in the active form (dashed curves) following a change in ammonium at time zero from 100 μM to 100 mM for different feedback schemes. (*a*) Highly saturated monocyclic cascade, equations (3)–(4), with $K_{AT} = K_{AR} = 1$ pM and initial condition [Gln] = 1 mM. (*b*) Unsaturated monocyclic cascade, as in (*a*) but with $K_{AT} = K_{AR} = 1$ μM; the initial condition is [Gln] = 0.33 mM and the final condition is [Gln] = 1.2 mM. (*c*) Allosteric regulation, with blue/red curves corresponding to a Hill coefficient $h = 1$ (equation (18)), with $K_{\mathrm{Gln}}^{\mathrm{GS}}=0.5\mu\mathrm{M}$ and green/black curves corresponding to a Hill coefficient $h = 3$ (equation (24)), with $K_{\mathrm{Gln}}^{\mathrm{GS}}=70\mu\mathrm{M}$. The initial and final conditions are, respectively, [Gln] =0.37 mM and [Gln] = 1.3 mM for the $h = 1$ curves and [Gln] =0.60 mM and [Gln] = 1.0 mM for the $h = 3$ curves. All additional parameters are given in section 2

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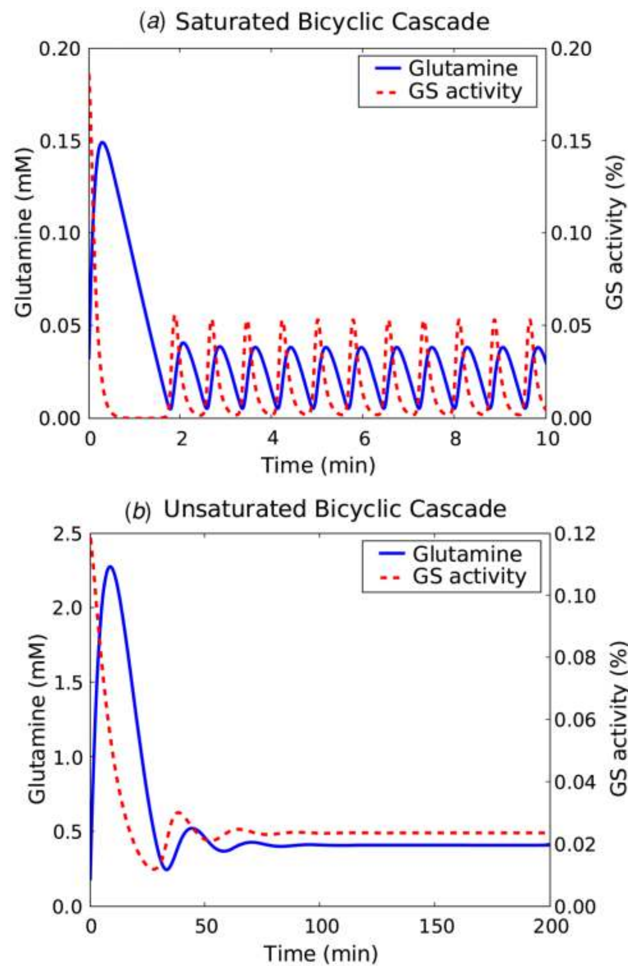

Figure 3.

Comparison of change in the intracellular free-glutamine level (solid blue curves) and the percentage of GS in the active form (dashed red curves) following a change in ammonium from 100 μM to 100 mM for the bicyclic cascade, equations (9)–(11). (*a*) Saturated cascade with $K_{AT} = K_{AR} = 10$ nM, $K_{UT} = K_{UR} = 0.2$ nM and initial [Gln] = 32.8 μM. (*b*) Unsaturated cascade with $K_{AT} = K_{AR} = 1$ μM, $K_{UT} = K_{UR} = 1$ μM and initial [Gln] = 184 μM.

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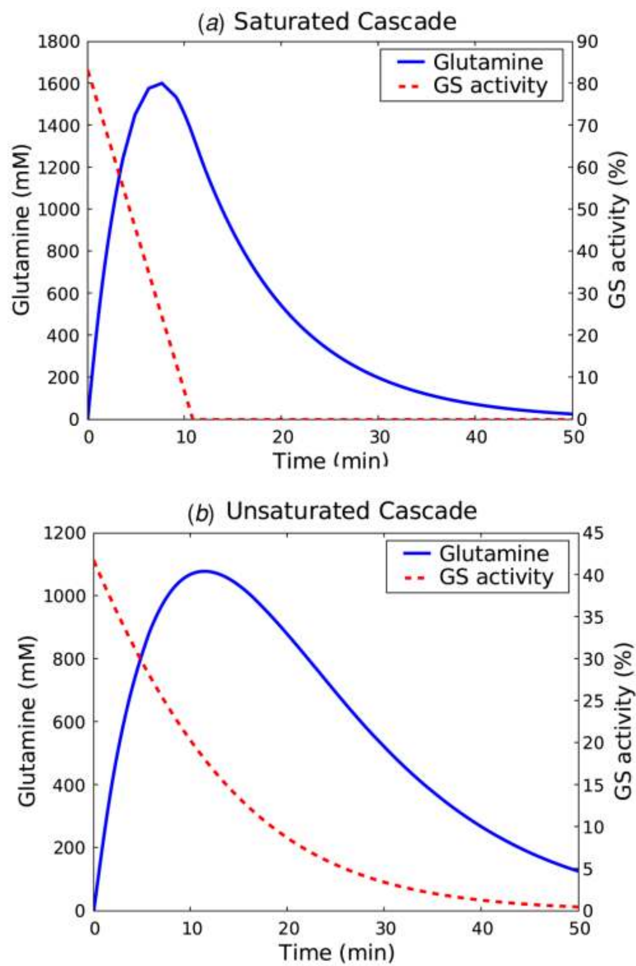

Figure 4.

Comparison of change in the intracellular free-glutamine level (solid blue curves) and the percentage of GS in the active form (dashed red curves) following a change in ammonium from 0.2 μM to 100mM for different feedback schemes. (*a*) Saturated monocyclic cascade, equations (3)–(4), with the same parameters as in figure 2(a), with initial [Gln] = 1 mM. (*b*) Unsaturated monocyclic cascade, as in figure 2(b), with initial [Gln] = 1.23 μM.

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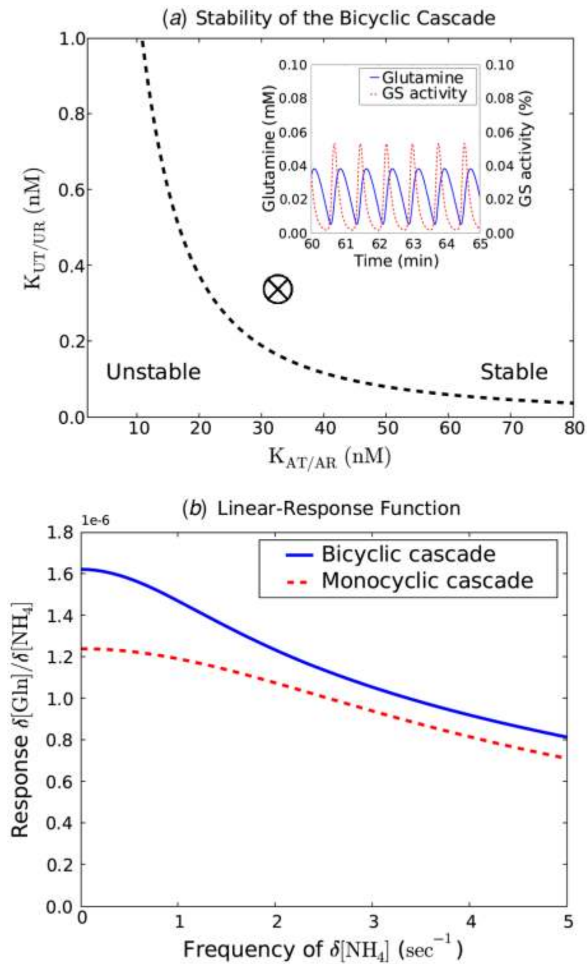

Figure 5.

(*a*) Stability diagram of the bicyclic cascade (figure 1(a)) as a function of the saturation of AT/AR and UT/UR. ⊗ marks the point in the diagram corresponding to the parameters used to calculate the linear-response function. (*b*) Linear-response function of the glutamine level to ammonium variation for the monocyclic cascade (equation (8)) at $K_{AT} = K_{AR} = 5$ nM and for the bicyclic cascade (supplementary equation (9)) at $K_{UT} = K_{ur} = 30$ pM, $K_{AT} = K_{AR} =$ 30 nM. Bifurcation diagrams here and in the supplementary material were generated using the software package PyDSTool (http://pydstool.sourceforge.net).

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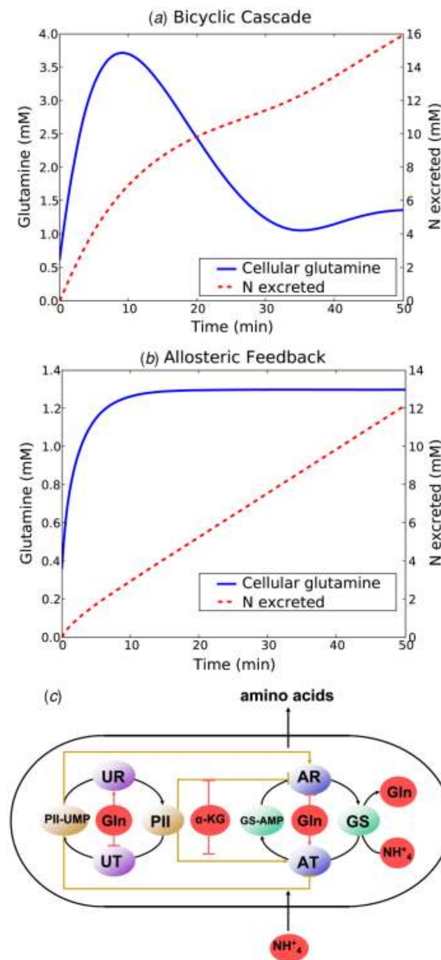

Figure 6.

(*a*) Intracellular free glutamine level and total consumed nitrogen (N) following a 100 mM ammonium shock for a bicyclic enzyme cascade (equations (3)–(4)). Glutamine binding constants have been adjusted to make the steady-state glutamine concentration 1.3 mM, as in the allosteric cascade, so $K_{\mathrm{Gln}}^{\mathrm{UR}}=K_{\mathrm{Gln}}^{\mathrm{AR}}=40\mu\mathrm{M}$ with $K_{\mathrm{AT}} = K_{\mathrm{AR}} = 1$ μM, $K_{\mathrm{UT}}= K_{UR} = 1$ μM and initial [Gln] = 629 μM. (*b*) Intracellular free glutamine and consumed nitrogen for allosteric regulation (equations (18)–(19)). Parameters and initial conditions are the same as in figure 2. (*c*) A model for ammonium detoxification, in which nitrogen enters the cell as ammonium and is metabolized and excreted as amino acids.