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Characterization of the Reconstituted UTase/UR-PII-NRII-NRI Bicyclic Signal Transduction System that Controls the Transcription of Nitrogen-Regulated (Ntr) Genes in *Escherichia coli*

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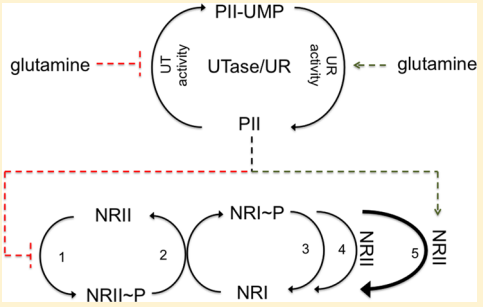
ABSTRACT: A reconstituted UTase/UR-PII-NRII-NRI bicyclic cascade regulated PII uridylylation and NRI phosphorylation in response to glutamine. We examined the sensitivity and robustness of the responses of the individual cycles and of the bicyclic system. The sensitivity of the glutamine response of the upstream UTase/UR-PII monocycle depended upon the PII concentration, and we show that PII exerted substrate inhibition of the UTase activity of UTase/UR, potentially contributing to this dependence of sensitivity on PII. In the downstream NRII-NRI monocycle, PII controlled NRI phosphorylation state, and the response to PII was hyperbolic at both saturating and unsaturating NRI concentration. As expected from theory, the level of NRI~P produced by the NRII-NRI monocycle was robust to changes in the NRII or NRI concentrations when NRI was in excess over NRII, as long as the NRII concentration was above a threshold value, an example of absolute concentration robustness (ACR). Because of the parameters of the system, at physiological protein levels and ratios of NRI to NRII, the level of NRI~P depended upon both protein concentrations. In bicyclic UTase/UR-PII-NRII-NRI systems, the NRI phosphorylation state response to glutamine was always hyperbolic, regardless of the PII concentration or sensitivity of the upstream UTase/UR-PII cycle. In these bicyclic systems, NRI phosphorylation state was only robust to variation in the PII/NRII ratio within a narrow range; when PII was in excess NRI~P was low, and when NRII was in excess NRI phosphorylation was elevated, throughout the physiological range of glutamine concentrations. Our results show that the bicyclic system produced a graded response of NRI phosphorylation to glutamine under a range of conditions, and that under most conditions the response of NRI phosphorylation state to glutamine levels depended on the concentrations of NRI, NRII, and PII.



Nitrogen assimilation in *Escherichia coli* is coordinated with other aspects of metabolism to maintain balanced metabolism under a wide variety of conditions. Part of this control is provided by a signal transduction cascade comprised of two linked cycles of reversible protein covalent modification, the PII-UTase/UR-NRI-NRII system (Figure 1, reviewed in ref 1). This bicyclic system has the capacity to sense three signals of metabolic status (the glutamine concentration, the α-ketoglutarate concentration, and the ratio of ATP to ADP) and to produce as an output the phosphorylated form of the enhancer binding transcription factor NRI (NtrC). NRI~P binds to enhancer elements in the vicinity of nitrogen regulated genes and activates transcription by σ^{54}-RNA polymerase, which it contacts by means of a DNA loop.[2,3] In some cases this transcriptional activation is additionally controlled by other factors that control communication between the enhancer-bound NRI~P and σ^{54}-RNA polymerase, such as by controlling the curvature or flexibility of the DNA.[4] At other promoters, the binding of NRI~P represses transcription by blocking the binding of σ^{70}-RNA polymerase;[5] at yet other promoters the combination of enhancers, a promoter utilized by σ^{54}-RNA polymerase, and "governor" sites results in strong transcriptional activation only when NRI~P is within upper and lower limits ("band-limiter" function).[6] These complexities notwithstanding, a unifying theme is that NRI function in cells is controlled by its reversible phosphorylation,[7] and that transcriptional regulation by NRI enables the cell to respond to nitrogen limitation by activating nitrogen-scavenging pathways and bringing about other adaptive responses.[8] Phosphorylation of the N-terminal domain of NRI results in its oligomerization, which is required for the central AAA+ domain to be able to hydrolyze ATP and activate transcription.[9,10] Phosphorylation of NRI also increases its affinity for DNA and changes its mode of binding DNA (as an oligomer as opposed to as a dimer).[2]

The phosphoryl groups of NRI~P are unstable, with a half-life of ~5 min at physiological pH, and thus it is difficult to measure the level of NRI phosphorylation in cells.[11] Indirect evidence suggests that the cellular concentration of NRI~P slowly rises as cells become nitrogen starved. Transcription of the structural gene encoding NRI is activated by NRI~P, forming a positive feedback loop;[12] the level of NRI in cells ranges from ~10 nM in nitrogen-rich cells to ~100 nM in

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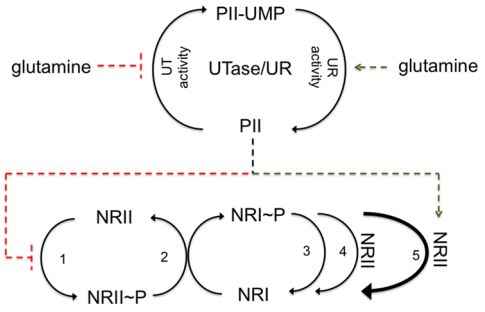

Figure 1. Activities of the UTase/UR-PII-NRII-NRI bicyclic signal transduction system. The UTase/UR-PII cycle brings about the interconversion of PII and PII-UMP. The UT activity of UTase/UR catalyzes the uridylylation of PII and is inhibited by glutamine. The UR activity of the UTase/UR catalyzed the deuridylylation of PII-UMP and is stimulated by glutamine. PII regulates the phosphorylation state of NRI indirectly, by interacting with NRII: 1, autophosphorylation activity of NRII, which is inhibited by PII; 2, phosphotransfer activity in which phosphoryl groups are transferred from NRII~P to NRI; 3, autophosphatase activity of NRI~P, in which it catalyzes its own dephosphorylation; 4, basal "phosphatase activity" of NRII, in which it brings about dephosphorylation of NRI~P; 5, PII-stimulated "phosphatase" activity of NRII. Note that steps 4 and 5 may reflect stimulation of the NRI~P autophosphatase activity by NRII acting alone (step 4) or by NRII in complex with PII (step 5).

nitrogen-limited cells.[13] The NRI~P responsive enhancers elements found upstream of nitrogen regulated genes have variable affinity for NRI~P, and the temporal order in which genes are activated as cells become nitrogen starved appears to correspond in part to the strength of the enhancer.[14] Under certain circumstances only genes under the control of the most powerful enhancer are expressed at a significant level, suggesting that the intracellular concentration of NRI~P is held low under those conditions (reviewed in ref 13). Furthermore, the temporal order of gene expression could be altered by changing the identity of the enhancer element.[15] Together, these data suggest that the level of NRI~P in cells is not regulated in an all-or-none (switch-like) fashion, but rather can be held stably at a low but nonzero level under conditions of moderate nitrogen deficiency, and smoothly increase from that level in a graded way that allows sequential activation of genes as nitrogen deficiency becomes more severe.

The PII-UTase/UR-NRI-NRII bicyclic cascade is comprised of the upstream PII-uridylyltransferase/uridylyl-removing enzyme (UTase/UR) cycle, which has a sensory function and a downstream NRI-NRII cycle, which responds to the concentration of the unmodified form of PII (Figure 1). PII is the product of the *glnB* gene in *E. coli*, while the UTase/UR is the product of *glnD*. NRI and NRII (NtrB) are the "receiver" and "transmitter" proteins, respectively, of a two-component signal transduction system (reviewed in refs 1 and 13). NRII brings about the phosphorylation of NRI indirectly, by binding to ATP and phosphorylating itself on a conserved His residue (autophosphorylation activity, Figure 1,[11]); NRI binds to NRII~P and transfers the phosphoryl group to itself on a conserved Asp residue (phosphotransfer activity, Figure 1). NRI also brings about its own dephosphorylation, at a slow rate (autophosphatase activity,[11,16] Figure 1). In addition to passing phosphoryl groups to NRI, NRII can also stimulate the dephosphorylation of NRI~P.[17] For example, an altered form of NRII with the H139N substitution cannot be autophosphorylated because the H139 autophosphorylation site has been altered, but this form of NRII can still stimulate the dephosphorylation of NRI~P. We refer to this activity as the "basal phosphatase activity" of NRII (Figure 1), although it may be the case that NRII (weakly) stimulates the slow NRI~P autophosphatase activity. PII (GlnB) binds to the C-terminal (ATP-binding) domain of NRII and acts in two ways to bring about the dephosphorylation of NRI~P. First, PII inhibits NRII autophosphorylation and thus slows the flux of phosphoryl groups to NRI.[18] Second, the complex of PII and NRII brings about the very rapid dephosphorylation of NRI~P (regulated phosphatase activity, Figure 1,[7,19]). There is some indirect evidence that the NRII-PII complex may act to stimulate the NRI~P autophosphatase activity,[20] a view that is consistent with studies of the phosphatase mechanisms in the *E. coli* chemotaxis system.[21]

The PII-UTase/UR cycle controls the downstream NRI-NRII cycle by controlling the availability of unmodified PII. PII is one of the most widely distributed signal transduction proteins in nature, and it seems to universally serve as a sensor of α-ketoglutarate and adenylylate energy charge and to be involved in nitrogen assimilation control.[22] PII is a homotrimer, and in *E. coli* and related bacteria each subunit can be uridylylated once (on Tyr51) by the uridylyltransferase (UT) activity of UTase/UR. Thus, the uridylylation state of PII can vary between zero and three uridylyl groups per trimer. The UT reaction occurs by a distributive (nonprocessive) mechanism, and PII trimers bearing only one or two modifications are readily evident in the expected proportions as populations of PII trimers become uridylylated.[23] Only unmodified PII subunits bind to and regulate NRII, and they seem to do so independently of the uridylylation status of the other subunits of the PII trimer.[23,24] Other studies suggested that each PII trimer was only able to bind to a single NRII dimer at any moment, but each NRII dimer was able to bind to and sequester two PII trimers.[25]

The cellular function of the PII-UTase/UR cycle is to integrate three distinct signals (glutamine concentration, α-ketoglutarate concentration, and ATP/ADP ratio) and regulate the downstream PII targets appropriately. In enteric bacteria, glutamine is a key signal of cellular nitrogen status under many conditions;[26] its concentration varies from very low in nitrogen-starved cells to about 4 mM in nitrogen-replete cells.[27] Glutamine acts by binding to the bifunctional UTase/UR and controlling the antagonistic UT and UR activities (ref 28, Figure 1). Glutamine binding to a pair of ACT domains at the C-terminus of the UTase/UR inhibits the UT activity of the N-terminal NT domain and stimulates the uridylyl-removing (UR) activity of the central HD domain.[29] The UR activity of the UTase/UR is relatively weak; it has a discernible basal level and is only activated about 2- or 3-fold by glutamine.[28] Conversely, the UT activity is very powerful, but glutamine can bring about its complete inhibition.[28] The other two signals (α-ketoglutarate, ATP/ADP ratio) are sensed directly by the PII protein, and control the ability of PII to regulate the activities of its downstream targets, such as NRII.[30,31] These signals are unquestionably important in intact cells, and in certain organisms the sensation of α-ketoglutarate levels by PII appears to be the main mechanism for sensation of nitrogen status.[32] [In *E. coli*, α-ketoglutarate is thought to vary from ~0.4 mM in nitrogen-replete cells[27] to ~1 mM in nitrogen-limited cells.[33]] Nevertheless, to begin to investigate the signal-processing properties of the system, we will here study the sensation and signaling of glutamine at fixed α-ketoglutarate and at high ratio

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of ATP/ADP. These studies are relevant to many systems where signals control the activity of converter enzymes of a covalent modification cycle.

Here, we investigate the signal processing properties of reconstituted PII-UTase/UR-NRI-NRII bicyclic systems and of the individual PII-UTase/UR and NRI-NRII monocycles. In particular, we examined the sensitivity of responses and robustness of these responses to variations in protein components. Sensitivity (apparent kinetic order) refers to the steepness of the response characteristic, or more specifically, the range of stimulatory effector concentrations over which the response occurs. A measure of sensitivity is the response coefficient,[34,35] which is the range of stimulus values required to move the system from 10% to 90% of the full response. The response coefficient may be restated in terms of the familiar Hill coefficient (n_H = log 81/log response coefficient), as we shall do here. Ultrasensitivity refers to systems where the response occurs over a very narrow range of stimulation, as in highly cooperative processes, with the apparent $n_H > 1$. Hyperbolic responses ($n_H = 1$) require an 81-fold increase in the stimulatory effector, and subsensitive responses ($n_H < 1$) require an even greater increase in the stimulatory effector, to move the system from 10% to 90% of the full response. Each type of response has application in cellular physiology. Ultrasensitive responses can provide switch-like responses as the stimulus increases beyond a threshold and are thought to play a key role in the morphogenesis and development of metazoans, and the functioning of cellular oscillators and genetic toggle switches (e.g., refs 36−39). Hyperbolic and subsensitive responses are useful for permitting intermediate levels of the system output over a wide range of stimulus amplitude and thus are appropriate for producing graded responses. We show that the sensitivity of the glutamine response of the UTase/UR-PII-NRII-NRI bicyclic system was low and that the downstream NRII-NRI cycle determined (limited) the system sensitivity.

Robustness refers to the ability of a system to function in the face of external perturbations (such as a change in temperature) and internal perturbations (such as fluctuations in the concentration or activity of one of the system components).[40,41] Robustness is a highly desirable property for a signal transduction system, particularly if the system must maintain constant signaling properties in the face of stochastic fluctuations in system components and/or operate in a wide variety of conditions. A few robust signal transduction systems have been identified,[42−45] and it has been hypothesized that robustness to variations in system components plays an important role in the evolution of signaling systems.[46,47] Conversely, with a fine-tuned system the output in response to stimulation depends critically on the parameters of the system such that fluctuations in these parameters result in altered responses. In *E. coli* and related bacteria, the NRI and NRII concentrations are increased as part of a positive feedback loop that becomes activated in nitrogen-starved cells.[12] As already noted, the NRI concentration is increased approximately 10-fold when cells are subjected to nitrogen limitation; NRII, which is encoded by the same operon, is thought to be similarly increased as cells become nitrogen limited. While the PII concentration is constant at approximately 1 μM, *E. coli* produces a second version of PII, GlnK, only when nitrogen starved, due to expression from the NRI~P-dependent *glnK* promoter.[48] It is thought that the level of GlnK can become several fold higher than the level of PII in nitrogen-starved cells.[13] Since GlnK can interact with NRII and act like PII, the level of "PII activity" increases as part of the response to nitrogen limitation.[48,49] Because NRI, NRII, and "PII activity" levels are varied as part of the cellular response to nitrogen limitation, a fine-tuned system is expected. Otherwise, there would be no point to this elaborate regulation of the protein levels. The reconstituted signaling system was decidedly not robust to changes in the relative concentrations of PII and NRII; both the amplitude of responses and the ability to maintain a low concentration of NRI~P at high glutamine concentrations were dramatically affected by the PII/NRII ratio. In the Discussion section, we will correlate this lack of robustness to the biological function of the system.

MATERIALS AND METHODS

Purified Proteins and General Assay Methods. The preparations of PII, NRII, NRII-H139N, NRII-S227R, NRI-N, and UTase/UR were described previously.[17,19,20,28,31] Measurement of steady-state levels of protein uridylylation and phosphorylation were as described[31] and employed the appropriate radiolabeled substrates (γ-[^{32}P]-ATP, or α-[^{32}P]-UTP). Levels of protein modification were determined by absorption and precipitation of aliquots of reaction mixtures onto Whatman p81 cellulose phosphate filters, which were washed extensively to remove unincorporated label and counted by liquid scintillation, as before.[31] Since the specific activity of the radioactive label was known, this permitted calculation of the number of moles of label incorporated; the protein concentrations in the samples were also known, allowing calculation of the extent of protein modification.

UTase/UR-PII Monocycle Experiments. Reaction mixtures contained 100 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, and 100 mM KCl, the concentration of PII (homotrimers) and UTase/UR (monomers) as indicated, α-[^{32}P]-UTP at 0.5 mM or as indicated, ATP at 0.5 mM, α-ketoglutarate at 0.2 mM, DTT at 1 mM, BSA (bovine serum albumin) at 0.3 mg/mL and glutamine as indicated. The system was preincubated at 30 °C in the absence of the ATP and UTP, and reactions were initiated by addition of prewarmed nucleotides. Samples were removed at various times, spotted onto filters, and processed to determine the level of incorporated label. Steady state levels were obtained by simple averaging of the values for later samples in the time-course where the reactions visually were assessed as having reached the steady state. The sensitivities were estimated by determining the dynamic range of stimulus that provided 90% and 10% of the response, and stated in terms of the Hill coefficient by calculating the ratio log81/log($S_{0.1}$/$S_{0.9}$), as described.[25] To allow reader assessment of the reproducibility, data for a number of experiments, including the date stamp for the experiments, are listed in Table 1. Similarly, in Figure 4B, several repititions of the same experiments, performed on different days, are shown. Finally, there is some overlap between the experiments shown in Figures 5 and 6, allowing in three cases direct comparison of similar experiments that were conducted on different days. For the three cases that overlap in Figures 5 and 6, calculated response sensitivities of similar experiments differed by 11%, 13%, and 21%.

NRII-NRI Monocycle Experiments. Reaction mixtures contained 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL BSA, the concentrations of PII, NRII (dimer) and/or mutant form of NRII, and NRI-N (monomer) as indicated, 0.03 mM α-ketoglutarate or as indicated, and α-

Table 1. Sensitivity and Midpoint of Glutamine Responses of the Reconsitituted UTase/UR-PII-NRII-NRI Cascade

experiment	[PII] μM	[UTase/UR] μM	n_H	glutamine $S_{0.5}$
050510	100	2	1.73	0.15
050710	100	2	2.04	0.14
050410	72	1.6	2.20	0.23
022609	36	0.8	2.24	0.30
090309	36	1.2	2.45	0.42
082409	36	1.2	2.28	0.36
050410	36	0.8	2.37	0.47
032712	36	1.0	2.43	0.38
090309	3	0.1	2.32	1.60
082509	3	0.1	2.18	1.40
090909	3	0.1	2.36	1.30
090909	0.5	0.02	1.46	0.90
082009	0.5	0.02	1.47	1.10
091709	0.5	0.02	1.99	0.83
080609	0.5	0.05	1.83	1.20

[^{32}P]-ATP at 1 mM or 2 mM, as indicated. The system was preincubated at 25 °C in the absence of ATP, and reactions were started by addition of the ATP. Time-courses, determination of steady states, and estimation of sensitivities were as for the UTase/UR-PII monocycle. Results are stated as the concentration of phosphorylated NRI-N, and thus the fraction phosphorylated can be determined simply by division by the total concentration of NRI-N used in each experiment.

Experiments with Bicyclic Systems. For the UTase/UR-PII-NRII-NRI bicycle, reaction mixtures contained 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL BSA, proteins as indicated (PII, UTase/UR, NRII and NRI-N), 1 mM γ-[^{32}P]-ATP, 0.5 mM UTP, and α-ketoglutarate at 0.1 mM. To allow detection of the PII uridylylation state, the ATP was unlabeled and α-[^{32}P]-UTP was included, as noted.

RESULTS

Glutamine Sensitivity and Set-Point of the Reconstituted UTase/UR-PII Monocycle Were Dependent on the PII Concentration. Under conditions of fixed α-ketoglutarate and in the presence of ATP as the sole adenylylate nucleotide, the reconstituted UTase/UR-PII monocycle functioned to signal the glutamine concentration (Figure 2A). The sensitivity (apparent kinetic order) of the response to glutamine and the midpoint (concentration of glutamine at which 50% of the response was obtained, $S_{0.5}$) were dependent on the PII concentration (Figure 2A,B, Table 1). At low PII concentration (0.5 μM PII, Figure 2A,B), sensitivity corresponded to a $n_H \sim 1.6$, whereas the maximal sensitivity, corresponding to $n_H \sim 2.35$, was observed when PII was at 36 μM (Figure 2A,B). The glutamine $S_{0.5}$ also changed in a biphasic fashion as PII was varied and was maximal when PII was at 3 μM (Figure 2B).

The UT Activity Was Subject to Substrate Inhibition. Both the ultrasensitivity of the UTase/UR-PII monocycle and the change in sensitivity of the system as PII concentration was varied were not expected. Zero-order ultrasensitivity is a consequence of having the enzymatic activities of a covalent modification cycle saturated by their substrates[35,49−53] and could provide an explanation for an increase in sensitivity as the cycle substrate concentration is increased. Zero-order ultrasensitivity would be possible for a bifunctional enzyme if the two functions were simply tethered together and not coordinately regulated. But, prior theoretical studies have indicated that bifunctional enzymes with tightly coupled activities cannot display zero order ultrasensitivity.[35,54,55] Ultrasensitivity is still possible for a bifunctional enzyme if the number of molecules of stimulatory effector, such as glutamine, required to effect the interconversion between forms of the enzyme is greater than 1.[55] However, in that case the sensitivity of the system will be fixed and will not vary as substrate (PII) concentration is varied. Other sources of ultrasensitivity, such as multistep effects of glutamine acting to control multiple activities[52] can also contribute to the sensitivity of the system, but these contributions to sensitivity would be independent of the PII concentration. [Furthermore, theoretical work has suggested that multistep effects are relatively minor when only two sites of action are involved [52]]. In our case, sensitivity was dependent upon the PII concentration; we therefore investigated factors that could affect sensitivity of the glutamine response. Poor coupling of the regulation of the UT and UR activities of the enzyme by glutamine has already been demonstrated and may provide the potential for zero order ultrasensitivity.[28] Guidi and Goldbeter demonstrated that substrate inhibition of one of the activities of a covalent modification cycle could result in increased sensitivity, and indeed, when the substrate inhibition was severe, the presence of elevated substrate concentration could result in a bistable system.[56] We investigated whether the UT activity of the UTase/UR-PII monocycle was subject to substrate inhibition by PII, and we observed reasonably strong substrate inhibition of the initial rate of PII uridylylation, both in the presence and the absence of glutamine (Figure 2C,D). In Figure 2C, the initial rate of PII uridylylation was examined for a broad range of PII concentrations, using a fairly low concentration of UTase/UR. This experiment demonstrated the presence of substrate inhibition of the UT activity. In Figure 2D, the effect of PII concentration on the initial rate of its uridylylation was examined for higher concentrations of PII, using an elevated UTase/UR concentration to give higher levels of activity, so as to more accurately examine the extent of inhibition by PII. As shown, in both the presence and absence of glutamine, substrate inhibition could reduce the initial rate of uridylylation by more than 50% (Figure 2D).

Since PII exerted substrate inhibition of the UT activity, and Guidi and Goldbeter showed that such systems can display bistability,[56] we also investigated whether the UTase/UR-PII monocycle displayed bistability. For this, we examined whether the same steady state of PII modification was obtained when the system had been allowed to evolve in the presence of glutamine from the outset vs when the system was allowed to evolve in the absence of glutamine until PII modification reached its steady state (~90% of the subunits are modified under these conditions) and then adding glutamine and allowing sufficient time to allow a new steady state to be obtained. Bistability was not evident when PII was at the high concentration of 100 μM (the highest concentration we could examine) and glutamine was 0.6 mM, as the same final steady state seemed to be obtained regardless of the route to that steady state (Figure 2E). We also investigated a number of other glutamine concentrations in similar experiments and did not obtain any evidence for bistability (data not shown).

Level of NRI~P Produced by the NRII-NRI Monocycle Was Not Robust to Changes in the Concentrations of NRII and NRI at Physiological Protein Concentrations. Theoretical studies of two-component signal transduction

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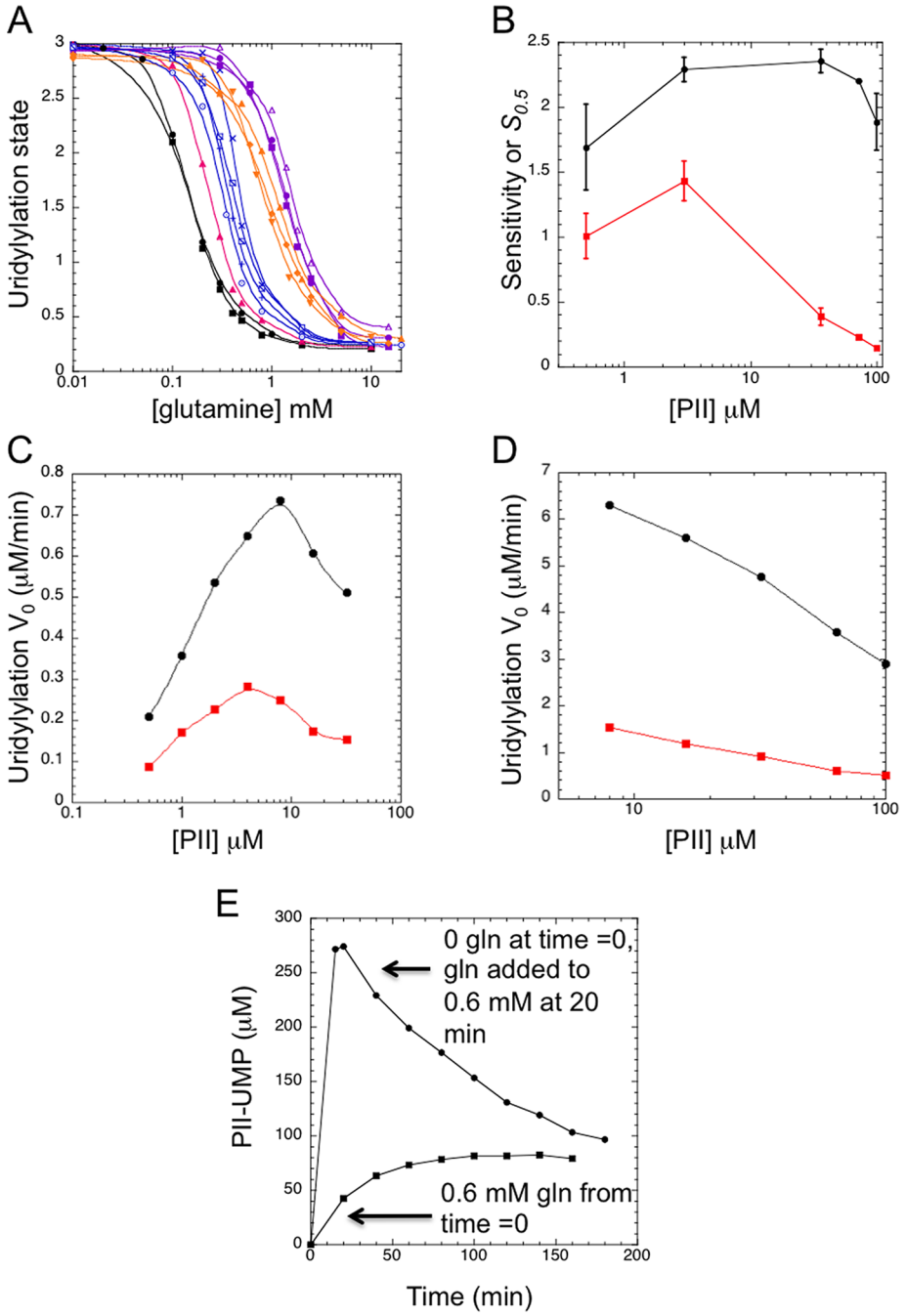

Figure 2. Characterization of the UTase/UR-PII monocycle. (A) Glutamine responses of the UTase/UR-PII monocycle at different PII concentrations. Reactions were conducted as in Materials and Methods, the PII concentrations were 100 μM (black lines); 72 μM (red line); 36 μM (blue lines); 3 μM (purple lines); 0.5 μM (gold lines). Values plotted on the Y axis as 0.01 mM glutamine were actually at zero glutamine, this was done to allow use of the log scale. (B) Replot of sensitivities and $S_{0.5}$ as a function of the PII concentration. Data were from panel A and Table 1. sensitivity, ● and black line; $S_{0.5}$, ■ and red line. The error bars indicate standard deviation. (C) Substrate inhibition of the UT activity of UTase/UR. Initial rate measurements were conducted as described in Materials and Methods, with UTase/UR at 0.01 μM. ● and black line, reactions lacking glutamine; ■ and red line, reactions contained 0.01 mM glutamine. (D) Substrate inhibition of the UT activity studied at high enzyme concentration. Initial rate measurements were conducted as in Materials and Methods, and utilized 0.05 μM UTase/UR. ● and black line, reactions lacking glutamine; ■ and red line, reactions contained 0.1 mM glutamine. (E) Lack of bistability in the UTase/UR-PII monocycle at high PII concentration. The reaction conditions were as in Materials and Methods, with PII at 100 mM, UTase/UR at 1 mM, UTP at 5 mM, and ATP at 0.5 mM. In one reaction mixture (■), glutamine was present at 0.6 mM from the beginning of the experiment. In the other reaction mixture (●), glutamine was not present at the beginning of the experiment, but was added to 0.6 mM after 20 min incubation.

systems by Batchelor and Goulian[44,45] showed that, for systems where the transmitter protein is both a kinase and phosphatase of the receiver, the steady state level of the phosphorylated receiver protein can under certain circumstances become independent of the concentrations of both the receiver and the transmitter proteins. When the concentration of the receiver protein is much higher than the concentration of the transmitter protein, and is saturating for phosphorylation and

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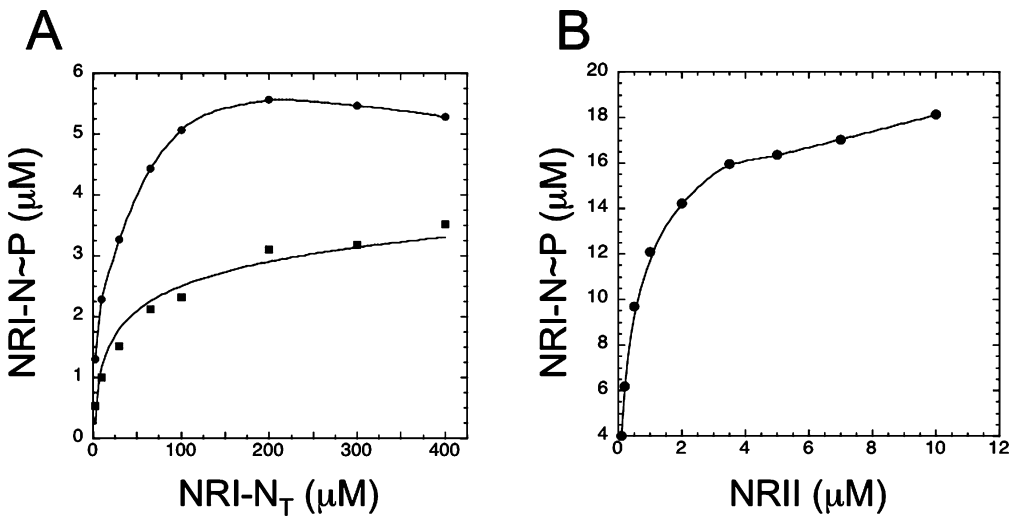

Figure 3. Robustness of the NRII-NRI monocycle to changes in NRII and NRI-N concentrations. (A) Effect of variation of the total concentration of NRI-N on the steady state phosphorylation level of NRI-N. NRII was present at 0.2 μM, and α-ketoglutarate was at 0.1 mM. ●, PII was absent; ■, PII was present at 0.02 μM. (B) Effect of variation of the NRII concentration on the steady state phosphorylation level of NRI-N. NRI-N was present at 200 μM. PII was not present, and α-ketoglutarate was 0.1 mM.

dephosphorylation by the transmitter protein, the level of the phosphorylated receiver is predicted to become insensitive to the total concentrations of the receiver protein.[44,45] Under these conditions, when the rates of autodephosphorylation of the phosphorylated receiver are negligible relative to the transmitter-mediated dephosphorylation rate, the concentration of the phosphorylated response regulator is predicted to also be insensitive to the concentration of the transmitter protein.[44,45] Conversely, when the autodephosphorylation rate of the receiver protein is not negligible (as in our system, Figure 1), the level of receiver phosphorylation is sensitive to the concentration of the transmitter until a threshold is reached, after which it is independent of the transmitter concentration. When the concentration of the phosphorylated receiver protein is independent of the concentrations of receiver and transmitter protein, it is an example of the phenomenon of absolute concentration robustness (ACR[44]). Experiments with intact cells, in which the level of transcription of the osmotically regulated *ompF* and *ompC* were examined, suggested that level of the phosphorylated form of the receiver protein OmpR was indeed insensitive to changes in the cellular level of its cognate transmitter protein, EnvZ.[44] This is consistent with the model,[45] since that system is known to lack significant OmpR~P autodephosphorylation rates. Similarly, the level of OmpR~P was insensitive to the level of its transmitter protein *in vivo* when a mutant form of CpxA was used as the transmitter protein.[45] Here, we examined whether the level of NRI~P produced by a reconstituted NRII-NRI monocycle was dependent on the concentrations of NRII and NRI. To our knowledge, this is the first experimental test of the ACR hypothesis[44] using purified components, although the hypothesis has been discussed in theoretical work and review articles.[43,57,58] To simplify our measurement of NRI phosphorylation state, we utilized the N-terminal domain of NRI (NRI-N) in place of intact NRI in our experiments, as before.[16,59]

The steady state level of NRI-N~P in reconstituted NRII-NRI monocycles depended upon the levels of both NRII and NRI (Figure 3). When NRII was present at 0.2 μM, the level of NRI-N~P increased along with the total concentration of NRI-N (NRI-N$_T$) until this became saturating at about 125 μM (Figure 3A). Above this total concentration of NRI-N, the concentration of NRI-N~P was approximately constant, demonstrating ACR as hypothesized.[45] When PII was present at 0.02 μM, one-tenth of the NRII concentration, the level of NRI-N~P was significantly reduced (Figure 3A). Under these conditions, the level of NRI-N~P rose very slowly when the total concentration of NRI-N was increased above ~125 μM (Figure 3A).

To examine robustness to the NRII concentration, NRI-N was held constant at the high concentration of 200 μM and NRII was varied (Figure 3B). The level of NRI-P became relatively insensitive to NRII concentration when NRII was present at ~3 μM. The slight upward drift in the apparent level of NRI-N~P in Figure 3B may be due to a contribution to the labeled species counted in the experiment by NRII~P; such contribution is negligible in our measurements under typical conditions, but may become detectable at high NRII (and when NRI~P has achieved ACR). Thus, it seems that the system provided absolute concentration robustness of NRI-N~P despite variation in NRII, under the appropriate conditions, as hypothesized.[45]

While it was possible to demonstrate (approximate) robustness of the NRI-N~P level to the concentrations of NRI-N and NRII in reconstituted cycles, robustness to NRI-N$_T$ was only obtained at high NRI-N/NRII ratio, and robustness to NRII only occurred at high concentrations of NRII. But these conditions are not met under physiological conditions, since NRI is thought to vary from several nanomolar concentration to a maximum of several hundred nanomolar, and the NRII concentration is thought to be within a few fold of the NRI concentration.[13] We thus expect that *in vivo* the NRI phosphorylation state will be sensitive to the concentrations of NRI and NRII, even though the capacity for ACR is present in the system.

PII Sensitivity of the Reconstituted NRII-NRI Monocycle. The NRII-NRI monocycle is regulated by PII, which converts NRII from a form that brings about the phosphorylation of NRI to a form that brings about the dephosphorylation of NRI~P.[7] As noted in the introduction, these two activities are not typical kinase and phosphatase activities. We

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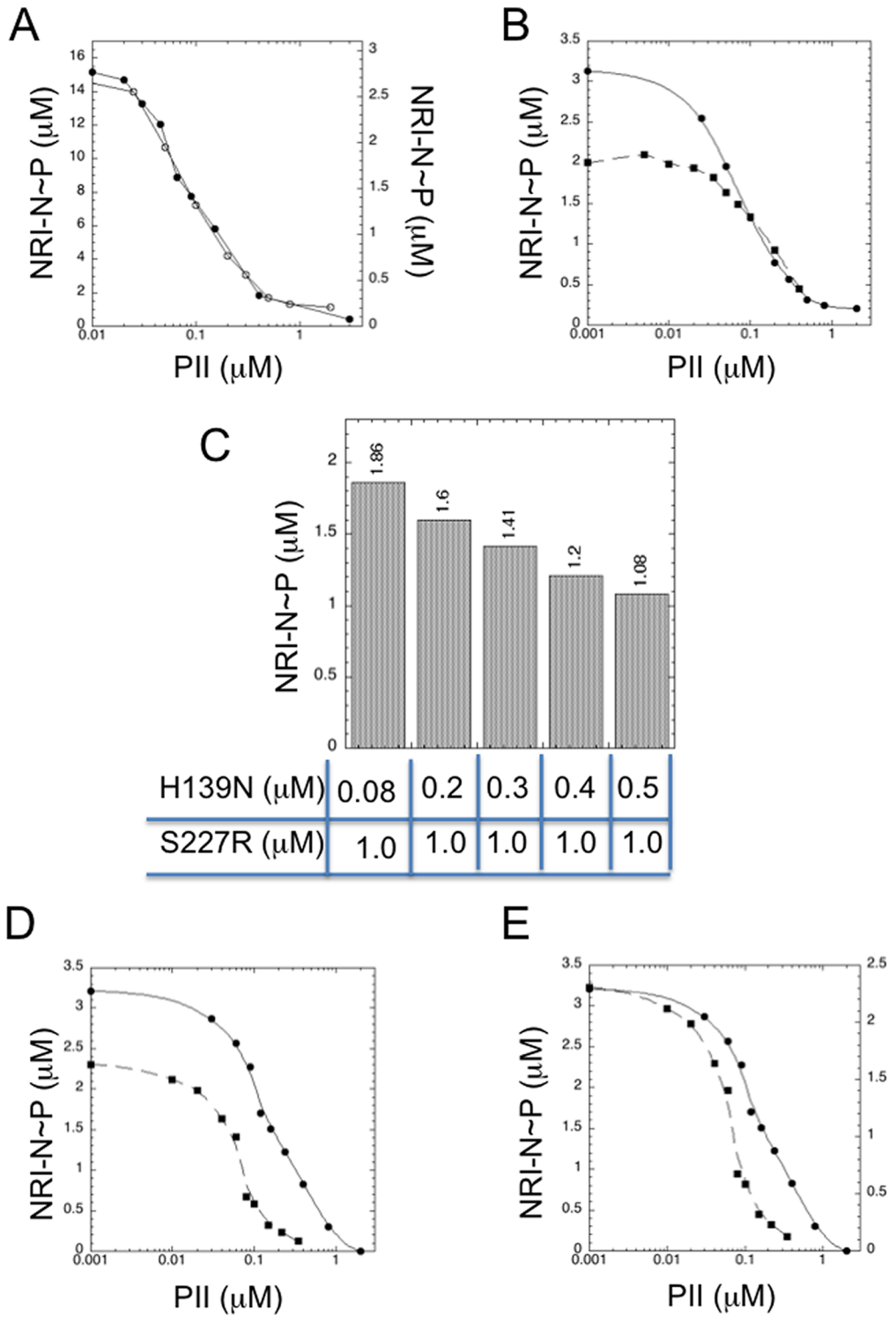

Figure 4. Characterization of the NRII-NRI monocycle. (A) PII response of the reconstituted NRII-NRI monocycle at saturating and nonsaturating NRI-N concentration. NRII was at 1 μM, and NRI was either 3 μM (○) or 300 μM (●). The sensitivity of the responses to PII corresponded to n_H ~ 1.19 and 1.27, respectively. (B) PII response of reconstituted NRII-NRI monocycles containing a combination of wild-type NRII and the altered NRII-H139N protein. ● and solid line, NRII 1 μM and no NRII-H139N; ■ and dashed line, 1 μM NRII + 0.5 μM NRII-H139N. (C) Phosphorylation of NRI-N in reconstituted monocycles lacking PII and containing a combination of the altered proteins NRII-H139N and NRII-S227R in place of wild-type NRII. NRI-N was present at 3 μM. (D) PII response of monocycles that contained a combination of NRII-H139N and NRII-S227R in place of wild-type NRII. NRI-N was present at 3 μM. ● and solid line, NRII-S227R was 1 μM and NRII-H139N was 0.08 μM; ■ and dashed line, NRII-S227R was 1 μM and NRII-H139N was 0.2 μM. (E) Normalized responses of systems with different ratios of monofunctional NRII proteins. Data and symbols are the same as those shown in panel D. For panels A, B, D, and E, values plotted on the Y axis were obtained at zero PII.

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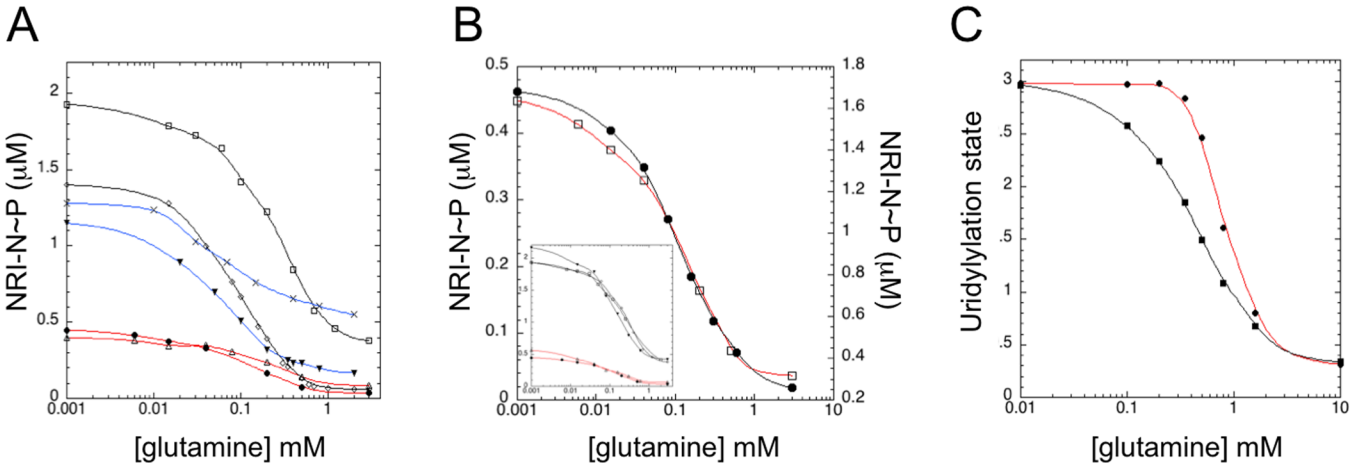

Figure 5. Characterization of reconstituted UTase/UR-PII-NRII-NRI-N bicycles. (A) Glutamine responses of reconstituted bicyclic systems containing various concentrations of NRII and PII. In all experiments, NRI-N was 3 μM. □, PII was 0.5 μM and NRII was 1 μM. The sensitivity of the glutamine response corresponded to $n_H \sim 1.16$. ◊, PII was 0.5 μM, NRII was 0.1 μM, and the sensitivity of the glutamine response corresponded to $n_H \sim 1.23$. X and blue line, PII was 0.1 μM, NRII was 0.5 μM and the sensitivity of the glutamine response corresponded to $n_H \sim 1.25$. ▼ and blue line, PII was 0.1 μM, NRII was 0.1 μM, and the sensitivity of the glutamine response corresponded to $n_H \sim 1.17$. △ and red line, PII was 5 μM, NRII was 1 μM, and the sensitivity of the glutamine response corresponded to $n_H \sim 1.05$. ● and red line, PII was 5 μM, NRII was 0.1 μM, and the sensitivity of the glutamine response corresponded to $n_H \sim 1.06$. (B) Normalized comparison of reconstituted bicyclic systems containing PII at 0.5 μM and NRII at 1 μM (□), and PII at 5 μM and NRII at 0.1 μM (●). The inset shows non-normalized results for these experiments; two repeats of the experiment with high PII and three repeats of the experiment with low PII are shown. (C) PII uridylylation state in bicyclic systems. ● and red line, PII uridylylation data from an experiment where PII was at 5 μM and NRII was at 0.1 μM. ■ and black line, PII uridylylation data from an experiment where PII was at 0.5 μM and NRII was at 1 μM. For all three panels, values plotted on the Y axis were obtained in the absence of glutamine.

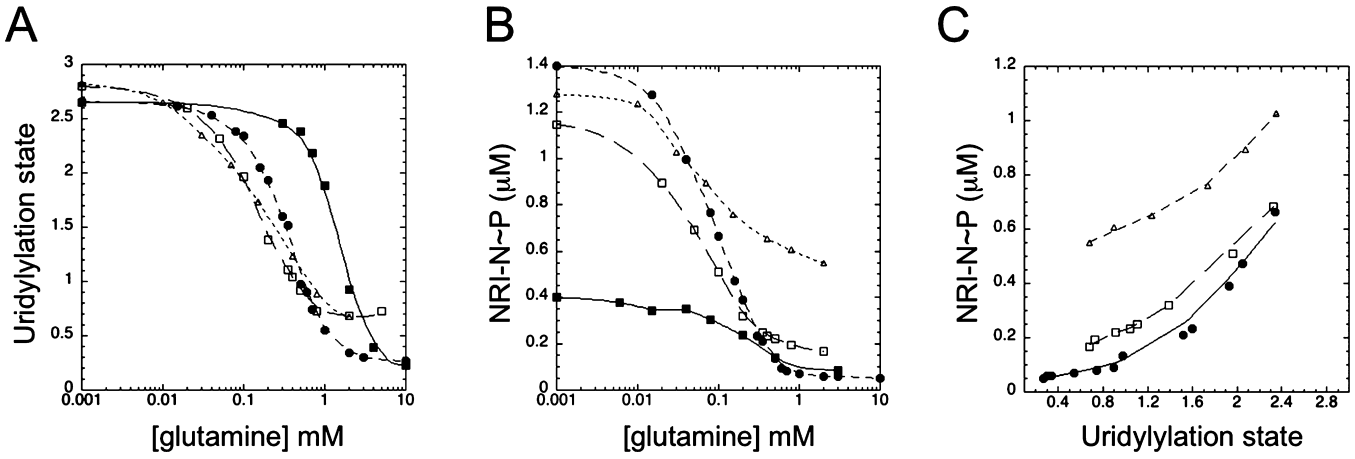

Figure 6. Comparison of bicyclic systems where the PII/NRII ratio was varied 25-fold. (A) Regulation of PII uridylylation state by glutamine. △, PII was 0.1 μM, NRII was 0.5 μM, and the sensitivity corresponded to $n_H \sim 1.11$; □, PII and NRII were both 0.1 μM, and the sensitivity corresponded to $n_H \sim 1.36$; ●, PII was 0.5 μM and NRII was 0.1 μM, and the sensitivity corresponded to $n_H \sim 1.55$; ■, reference sample where PII was 5 μM and NRII was 0.1 μM, and the sensitivity corresponded to $n_H \sim 2.13$. (B) Regulation of NRI phosphorylation state by glutamine. Symbols are as in panel A, sensitivities were as follows: △, 1.19; □, 1.26; ●, 1.34; ■, 1.06. (C) Relationship between the modification states of PII and NRI for the three systems where the NRII/PII ratio was varied 25-fold. For panels A and B, values plotted on the Y axis were obtained in the absence of glutamine.

measured the sensitivity of the response of NRI-N phosphorylation to PII in NRII-NRI monocycles where NRII was at 1 μM and NRI-N was at either 3 μM (nonsaturating) or 300 μM (saturating). As shown in Figure 4A, neither the sensitivity of the response nor the midpoint ($S_{0.5}$) were significantly influenced by the concentration of NRI-N; the estimated sensitivities corresponded to n_H of 1.3 and 1.46 at low and high NRI-N, respectively, and the $S_{0.5}$ was ~0.06 μM PII. Since the sensitivity was not altered as NRI-N was raised from a subsaturating level to a saturating level, both zero-order effects and substrate-inhibition effects were counter-indicated.

NRII is a bifunctional protein that mediates both the phosphorylation and dephosphorylation of NRI; since it is bifunctional, the relative levels of the NRII "kinase" and "phosphatase" activities are fixed. To examine the dependence of cycle amplitude, sensitivity, and $S_{0.5}$ on the kinetic parameters for the NRII activities, we examined the effects of adding the H139N mutant form of NRII to reconstituted monocycles that also contained wild-type NRII. NRII-H139N lacks the ability to become autophosphorylated due to conversion of the site of autophosphorylation, histidine 139, to the non-phosphorylatable asparagine. Yet, NRII-H139N retains the ability to bring about the dephosphorylation of

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NRI∼P, which is greatly stimulated by PII.[17] Thus, addition of this altered form of NRII to wild-type NRII in the reconstituted monocycle increases the rate of the NRI∼P dephosphorylation relative to the rate of NRI phosphorylation, while leaving regulation by PII intact. The addition of the NRII-H139N protein to NRII in the monocycle reduced the amplitude of the response to PII, and consequently the dynamic rage of the stimulus was also decreased, leading to a higher apparent sensitivity and a higher $S_{0.5}$ (Figure 4B). The effects on sensitivity and $S_{0.5}$ were mainly due to the decrease in the amplitude of the response brought about by an increase in the NRI∼P dephosphorylation activity (Figure 4B). Similarly, we could alter the relative levels of the activities of NRII by using a pair of altered NRII proteins, NRII-H139N and NRII-S227R, in place of wild-type NRII. The NRII-S227R altered protein is defective in binding to PII, such that it is autophosphorylation activity is essentially unregulated and its ability to bring about the dephosphorylation of NRI∼P is low.[59] When the NRII-H139N protein was combined with the NRII-S227R protein in a reconstituted monocycle, the amplitude of the response to PII was determined by the relative concentrations of the two monofunctional enzymes (Figure 4C,D). For example, when PII was absent, the level of NRI-N∼P was diminished as the level of the NRII-H139N enzyme was increased relative to the NRII-S227R enzyme (Figure 4C). This diminution in amplitude resulted in a increased sensitivity and in a decreased $S_{0.5}$ (Figure 4D); furthermore, sensitivity appears to have been increased in a relative sense as well, as discerned from a normalized plot of the responses (Figure 4E). When the ratio of altered NRII proteins was 0.2 H139N:1 S227R, the sensitivity of the system to PII corresponded to $n_H \sim 1.89$. This experiment was repeated on two occasions, and the PII sensitivities from those experiments corresponded to n_H of ∼1.74 and ∼1.91. These are higher sensitivities than we have obtained with wild-type NRII. Together, the results in Figure 4 suggest that bifunctionality of NRII fixes the relative levels of its two activities and by so doing helps determine the amplitude, sensitivity, and $S_{0.5}$ of the system. The wild-type system is apparently designed to produce a low sensitivity (graded) response to PII, since relatively minor alterations in catalytic rates produced more sensitive responses.

Reconstituted PII-UTase/UR-NRI-NRII Bicyclic System Produced a Graded Response to Glutamine and Was Not Robust to Changes in the Ratio of PII to NRII. The glutamine response profile for reconstituted PII-UTase/UR-NRI-NRII bicyclic systems varied greatly, depending on the concentration of NRII and PII (Figure 5A). Figure 5A summarizes the response profiles obtained under a number of conditions that will be discussed in this section; as evident from simple inspection of the figure, the amplitude of the responses (level of NRI-N∼P) as well as the level of NRI-N∼P at high glutamine levels was quite variable. Specifically, when PII was in excess relative to NRII, the amplitude of the response was low, while when NRII was in excess of PII, the level of NRI∼P at high glutamine was elevated. Yet, all of the response profiles in Figure 5A share the property of low sensitivity (sensitivities of the individual curves varied from $n_H \sim 1.05$ to $n_H \sim 1.25$ and are noted in the figure legend).

To examine whether the sensitivity of the glutamine response of the upstream PII-UTase/UR cycle contributed to the overall sensitivity of the glutamine response of the bicycle, we compared bicyclic systems in which PII was at 5 μM and NRII was at 0.1 μM with bicyclic systems where NRII was at 1 μM and PII was at 0.5 μM (Figure 5B). The inset to Figure 5B shows three repetitions of the latter experiment and two repetitions of the former experiment; in all cases good agreement was obtained for repeated experiments. As shown in Figure 5C, when PII was at 5 μM, the upstream PII-UTase/UR cycle produced an ultrasensitive response to glutamine ($n_H \sim 2.13$), whereas when PII was at 0.5 μM, the upstream PII-UTase/UR cycle produced a lower-sensitivity response to glutamine ($n_H \sim 1.5$). These results are consistent with the results for the isolated PII-UTase/UR monocycle as reported in Figure 2. Notably, in the bicyclic systems the sensitivity of the response of NRI phosphorylation to glutamine was nearly identical, regardless of whether the upstream cycle was operating at high or low sensitivity (Figure 5B). This can be easily discerned by comparing the normalized curves, which were nearly identical in shape (Figure 5B). However, despite the similar sensitivity of the responses, other features of the responses under the two conditions were distinct (Figure 5B, inset). Specifically, when PII was in large excess, the amplitude of NRI∼P was low, both in the absence of glutamine and throughout the range of glutamine concentrations examined. Conversely, when NRII was in excess over PII, the amplitude of NRI∼P was elevated in the absence of glutamine and throughout the range of glutamine concentrations examined, and a considerable basal level of NRI∼P was obtained even in the presence of high glutamine concentrations. Thus, the level of NRI∼P produced by the systems did not display robustness to changes in the concentration of PII and NRII.

To get a sense of the robustness of the system to relatively small changes in the concentration of PII and NRII, we compared three bicyclic systems where PII was 0.5 μM and NRII was 0.1 μM, where PII and NRII were both 0.1 μM, and where PII was 0.1 μM and NRII was 0.5 μM, such that the ratio of PII to NRII was varied 25-fold (Figure 6). For these three experiments, the uridylylation state of PII and the phosphorylation state of NRI were examined at the same glutamine concentrations, allowing direct comparison of coupling of the two cycles within the bicyclic system (Figure 6C). In all three of these conditions, the upstream PII-UTase/UR cycle operated with hyperbolic sensitivity (Figure 6A), and the overall responses of the bicyclic systems (change in NRI∼P in response to glutamine) also displayed hyperbolic sensitivity (Figure 6B). For comparison, we also show another experiment in Figure 6A,B, where PII was at 5 μM and NRII was at 0.1 μM. As already noted, under these conditions the upstream PII-UTase/UR cycle produced an ultrasensitive response to glutamine, while the response of the overall bicyclic system was hyperbolic. As shown in Figure 6A, the function of the upstream UTase/UR-PII cycle was not dramatically different when the PII/NRII ratio was varied 25-fold for the three conditions where this cycle was in its hyperbolic regime. By contrast, when the overall response of the bicyclic systems was examined (Figure 6B), the NRI∼P level was only similar when PII was equal to or in excess of NRII; when NRII was in excess over PII the system was characterized by elevated levels of NRI∼P, even at high glutamine concentration. The coupling between the two monocycles forming the bicyclic system was also different under the three conditions (Figure 6C). When PII was in excess, considerable modification of PII had to occur before NRI-P could rise, such that the replot of NRI-N∼P vs PII uridylylation state was nonlinear (Figure 6C). Conversely, when NRII was in excess of PII, the replot of NRI-N∼P vs PII uridylylation state was flatter, but did not extrapolate through

the origin (Figure 6C). Together, these data show that the bicyclic cascade is a fine-tuned system that is quite sensitive to even modest variations in the concentrations of NRII and PII.

DISCUSSION

Robustness of the Sensitivity of Responses by the UTase/UR-PII-NRII-NRI and UTase/UR-PII-ATase-GS Bicyclic Systems. The UTase/UR-PII monocycle is part of two bicyclic signal transduction systems. The UTase/UR-PII-ATase-GS bicyclic system controls the adenylylation state and catalytic activity of glutamine synthetase (GS), while the UTase/UR-PII-NRII-NRI bicyclic system, studied here, controls the phosphorylation state and activity of NRI. Prior work showed that the UTase/UR-PII-ATase-GS system produced a highly ultrasensitive response to glutamine, corresponding to $n_H > 5.0$, regardless of whether PII was present at high or low concentration.[24] In that system, a major source of ultrasensitivity was the mechanism coupling the upstream and downstream cycles: PII had to become completely deuridylylated upon glutamine stimulation before it could interact with and activate the AT domain of ATase. This coupling mechanism, together with multistep effects of glutamine regulating all four catalytic activities of the bicyclic system, resulted in a highly ultrasensitive response to glutamine by the bicycle, even when PII was at low concentration. In the work reported here, it was shown that the sensitivity of the response of the UTase/UR-PII monocycle to glutamine depended upon the PII concentration, and in particular there was a clear difference in sensitivity when PII was at 0.5 μM relative to when it was present at 3 μM. Since the prior work showed that at both these PII concentrations the UTase/UR-PII-ATase-GS bicycle was highly ultrasensitive,[24] we may conclude that the sensitivity of the response of the UTase/UR-PII monocycle only plays a minor role, if any, in the overall sensitivity of the bicycle controlling GS modification. In this paper, we examined the UTase/UR-PII-NRII-NRI bicyclic system and observed that the sensitivity of the upstream UTase/UR-PII monocycle had little, if any, effect on the sensitivity of the glutamine responses of the bicyclic system, which was always low. Our results show that an unregulated downstream cycle can reduce system sensitivity and that the overall system sensitivity is not simply the product of the sensitivities of the individual cycles. This is as predicted by Goldbeter and Koshland,[50] and by Chock and Stadtman.[60] In summary, for both branches of the linked bicyclic systems controlling GS and NRI activity, the sensitivity of the glutamine response appears to be fixed and relatively robust to changes in the PII concentration. For the GS branch of the system, the sensitivity of the glutamine response is fixed as highly ultrasensitive,[24] while for the NRI branch of the system the sensitivity of the glutamine response is fixed at (nearly) hyperbolic sensitivity.

Signal Amplification by the UTase/UR-PII-NRII-NRI Bicyclic System. The UTase/UR-PII-NRII-NRI bicyclic system provides a striking example of signal amplification provided by the downstream cycle of a bicyclic cascade, a possibility noted by Chock and Stadtman,[60] Goldbeter and Koshland,[35] and by Koshland et al.[51] When PII was at 0.5 μM, the glutamine $S_{0.5}$ of the UTase/UR-PII system was ~1.0 mM (Figure 2, Table 1). But, in the bicyclic UTase/UR-PII-NRII-NRI systems, the glutamine $S_{0.5}$ was ~0.06 mM (Figures 5 and 6). Thus, the downstream NRII-NRI monocycle lowered the $S_{0.5}$ ~14-fold, even though neither NRII nor NRI are directly regulated by glutamine. The signal amplification capability came from the bicyclic system design, as predicted by theory.[60] By contrast, previous studies of the UTase/UR-PII-ATase-GS bicyclic system, conducted under similar conditions as the studies reported here, did not observe large signal amplification: under conditions where the glutamine $S_{0.5}$ of the UTase/UR-PII system was ~1.0 mM, the glutamine $S_{0.5}$ of the UTase/UR-PII-ATase-GS bicycle was ~0.2−0.8 mM.[24] Thus, the NRI branch of the system displayed significantly greater signal amplification than did the GS branch of the system, suggesting that the GS branch of the system may be designed to act at higher glutamine concentrations than does the NRI branch of the system. We argue that the difference in signal amplification by the two linked bicyclic systems is a physiologically significant design feature of the system, consistent with the biological functions of the system. As the cellular glutamine concentration falls, the regulation of GS adenylylation state occurs almost immediately, at fairly high internal glutamine concentration. Only if this activation of GS fails to satisfy the cellular requirements for glutamine and the internal glutamine concentration falls significantly lower is NRI activated and the nitrogen scavenging program executed.

PII-Dependent Sensitivity of the UTase/UR Monocycle. The UTase/UR-PII cycle responded to glutamine with variable sensitivity, depending upon the PII concentration. Theory suggested that systems with bifunctional enzymes with tightly coupled reciprocal regulation should not be able to display zero-order ultrasensitivity. However, reciprocal regulation of the UT and UR activities of the bifunctional UTase/UR is not tightly coupled. Another mechanism that may increase the sensitivity of responses of a covalent modification cycle is substrate inhibition of a converter enzyme activity. We observed that the UT activity was subject to substrate inhibition by PII, providing a possible explanation for the dependence of sensitivity on PII concentration. Prior studies of the UT and UR activities showed that both activities are subject to product inhibition, and that both activities have distinct K_m and K_d for PII and PII-UMP.[28] Product inhibition is expected to reduce the sensitivity of responses and may be responsible for preventing bistability by this cycle. Differences in the K_m of the opposing activities as well as differences in the inhibition constants for substrates and products may contribute to shifts in the $S_{0.5}$ as PII concentration was varied.[50]

The UTase/UR-PII-NRII-NRI Bicyclic System Produced a Graded Response of NRI Phosphorylation State to Glutamine. By contrast to the UTase/UR-PII monocycle, where sensitivity depended on the PII substrate concentration, the PII response of the NRII-NRI monocycle was always of low sensitivity regardless of the NRI concentration. We are fairly certain that the ability of NRII to bring about the phosphorylation of NRI-N was saturated in our experiments when NRI-N was 300 μM and NRII was 1 μM, because when compared to results obtained with 3 μM NRI-N and 1 μM NRII, a 100-fold increase in the NRI-N concentration only resulted in a 5-fold increase in NRI-N~P levels (Figure 4A). However, we have no data on whether or not the phosphatase activities involved in dephosphorylation of NRI-N~P were saturated in our experiments, or even if these activities are able to be saturated. NRI~P catalyzes its own dephosphorylation, and it has been hypothesized that the role of the NRII-PII complex may be to stimulate this autophosphatase activity.[20] If NRI~P is both enzyme and substrate in the dephosphorylation reaction, traditional ideas about enzyme active site saturation may not be applicable; such concerns should also extend to

systems containing G proteins, which also catalyze their own inactivation. Gomez-Uribe and colleagues have conducted theoretical studies of covalent modification cycles where either, both, or neither of the two antagonistic converter-enzyme activities are able to become saturated.[61] Although further work will be necessary to confirm that that NRI~P dephosphorylation cannot be saturated, the NRII-NRI system appears to correspond to the "signal transduction regime" described by Gomez-Uribe et al., in which a linear response to a broad range of stimulatory effector concentrations is obtained due to saturation of the kinase activity and unsaturation of the phosphatase activity.[61]

Early modeling work on covalent modification cycles noted that the amplitude, sensitivity, and midpoint of responses were controlled in part by the rates of the antagonistic converter enzyme activities.[60] In the atypical NRII-NRI monocycle, NRII mediates both the phosphorylation and dephosphorylation of NRI and thus could be considered to be equivalent to a bifunctional converter enzyme. Because of this bifunctionality, the rates of the antagonistic converter enzyme activities are fixed relative to one another. We could alter this relationship simply by adding a monofunctional form of NRII to monocycles that also contained wild-type NRII, or by using a pair of monofunctional altered forms of NRII in place of the wild-type bifunctional protein. Using this method, we had no problem assembling systems that displayed higher sensitivity responses to PII than did the wild-type system; these systems also differed in amplitude and midpoint of responses. These results were consistent with the prior theory[62] and suggest that the wild-type system has been selected to produce a low-sensitivity response.

The UTase/UR-PII-NRII-NRI Bicyclic System Is a Fine-Tuned System. Our experimental results directly demonstrate that the UTase/UT-PII-NRII-NRI bicyclic system displayed limited robustness to variations in the PII/NRII ratio. This lack of robustness is consistent with the positive and negative feedback loops present in the genetic circuitry, which bring about changes in the levels of NRII and the PII-like protein GlnK as cells experience changes in their nitrogen status. When cells are growing in an ammonia-rich environment, they have elevated internal glutamine levels. A consequence of this is that PII is mainly unmodified, and the action of PII and NRII keeps the concentration of NRI~P low, which in turn results in a low concentration of NRII and NRI within the cell. If the cell should become limited for ammonia, the internal glutamine concentration will fall and PII will become modified by the UT activity of the UTase/UR. Under these conditions, the highly ultrasensitive UTase/UR-PII-ATase-GS system will provide an initial, dramatic response to restore the internal glutamine concentration. However, if sufficient ammonium is not present, this initial response will fail and the glutamine concentration will fall further, allowing the level of NRI~P to rise. One consequence of this rise in NRI~P is that the cellular concentrations of NRII and NRI increase, due to activation of the *glnALG* operon by NRI~P. As long as the level of NRII does not become greater than the concentration of PII, PII will be able to control the level of NRI~P. However, if nitrogen limitation is persistent, the level of NRII will eventually exceed PII, and the ability of the system to reduce NRI~P concentration in the presence of glutamine becomes compromised (consistent with the *in vitro* results of Figures 5 and 6). If there were no way to amplify PII activity, the system would become stuck in a state with high NRI~P and constitutive expression of nitrogen-regulated genes. This does not happen because the cell contains the PII-like GlnK, that becomes expressed from a nitrogen regulated promoter in nitrogen-limited cells.[48,49] Our results are consistent with the hypothesis that one role of GlnK is to control the concentration of NRI~P when the level of NRII has been amplified due to nitrogen limitation.[15,49]

E. coli has the remarkable ability to maintain homeostasis of the ratio of α-ketoglutarate to glutamine at a wide range of environmental conditions.[33,63] This robust metabolic homeostasis is an emergent property of the cells, that results from the combined actions of the UTase/UR-PII-NRII-NRI bicyclic system, the UTase/UR-PII-ATase-GS system, and likely other regulatory systems.[64] Although the cells display robust metabolic homeostasis, the individual systems that contribute to this homeostasis may individually display limited robustness, and indeed saturation of the outputs of the individual systems may serve as the trigger for activation of other systems. This seems to be the case for the two bicyclic systems controlling GS activity and Ntr gene transcription; the former system operates at higher glutamine concentrations then does the latter, allowing the systems to function at different physiological states to contribute to the robust metabolic homeostasis.

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Notes

The authors declare no competing financial interest.

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