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A Source of Ultrasensitivity in the Glutamine Response of the Bicyclic Cascade System Controlling Glutamine Synthetase Adenylylation State and Activity in *Escherichia coli*

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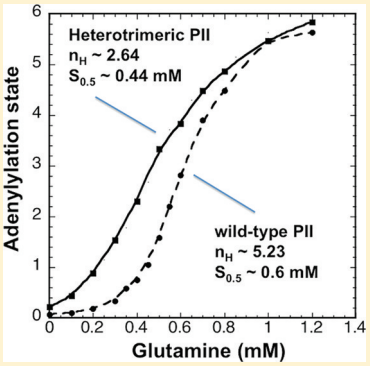
Supporting Information

ABSTRACT: Glutamine synthetase (GS) activity in *Escherichia coli* is regulated by reversible adenylylation, brought about by a bicyclic system comprised of uridylyltransferase/uridylyl-removing enzyme (UTase/UR), its substrate, PII, adenylyltransferase (ATase), and its substrate, GS. The modified and unmodified forms of PII produced by the upstream UTase/UR-PII cycle regulate the downstream ATase-GS cycle. A reconstituted UTase/UR-PII-ATase-GS bicyclic system has been shown to produce a highly ultrasensitive response of GS adenylylation state to the glutamine concentration, but its composite UTase/UR-PII and ATase-GS cycles displayed moderate glutamine sensitivities when examined separately. Glutamine sensitivity of the bicyclic system was significantly reduced when the trimeric PII protein was replaced by a heterotrimeric form of PII that was functionally monomeric, and coupling between the two cycles was different in systems containing wild-type or heterotrimeric PII. Thus, the trimeric nature of PII played a role in the glutamine response of the bicyclic system. We therefore examined regulation of the individual AT (adenylylation) and AR (deadenylylation) activities of ATase by PII preparations with various levels of uridylylation. AR activity was affected in a linear fashion by PII uridylylation, but partially modified wild-type PII activated the AT much less than expected based on the extent of PII modification. Partially modified wild-type PII also bound to ATase less than expected based upon the fraction of modified subunits. Our results suggest that the AT activity is only bound and activated by completely unmodified PII and that this design is largely responsible for ultrasensitivity of the bicyclic system.



One of the most common mechanisms of enzyme regulation in nature is by reversible covalent modification, and accordingly, considerable effort has been invested in learning the properties of covalent modification cycles and cascades comprised of linked covalent modification cycles. In many covalent modification cycles, stimulatory effectors control one or both of the antagonistic converter-enzymes of the cycle, such that the modification state of the cycle substrate protein reflects the level of stimulation. An important feature of these cycles is that the response to the stimulatory effector can occur over a very narrow range of stimulus concentration, corresponding to a Hill-type equation with a Hill coefficient $n_H > 1$, in which case we refer to the response as ultrasensitive, or the response can occur over a broader range of stimulus concentrations, in which case the response is characterized as hyperbolic ($n_H = 1$) or subsensitive ($n_H < 1$).[1] An ultrasensitive response is desirable in situations where switchlike behavior of the system is needed, while a subsensitive response is desireable in situations where the system is required to provide a graded response over a broad range of stimulation. Theoretical and experimental work has established some of the mechanisms that control the sensitivity of responses of covalent modification cycles. For example, ultrasensitive responses can occur when the substrate protein of the cycle is present in high enough concentration to saturate both of the converter-enzymes of the cycle (zero-order ultrasensitivity[2,3]) as long as the two converter-enzyme activities of the cycle are found on different proteins as opposed to on a bifunctional enzyme.[4,5] Ultrasensitive responses can also result when the stimulatory effector acts to coherently regulate both of the antagonistic converter-enzyme activities of a cycle or multiple steps in a cascade of linked cycles (multistep ultrasensitivity[6,7]). Substrate inhibition of one or both of the converter-enzymes of a covalent modification cycle can also result in ultrasensitive responses,[8] and a related phenomenon is ultrasensitvity arising from the requirement to covalently modify the substrate protein on multiple sites for it to be active.[9] Conversely, product inhibition of the converter-enzyme activities, very high *Km* for the converter-enzymes, sloppy control of the converter-enzyme activities by the stimulatory effector, and the presence of a bifunctional converter-enzyme all act to favor hyperbolic and subsensitive responses (e.g., refs 2, 4, 5, and 10). In this article, we demonstrate another mechanism that results in an ultrasensitive response in a cascade of covalent modification cycles.

The experimental system we study is a cascade of two linked covalent modification cycles responsible for the regulation of glutamine synthetase activity in *Escherichia coli* (ref 11, Figure

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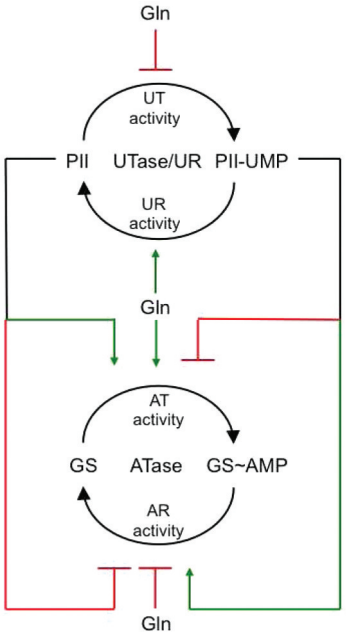

Figure 1. Signal transduction system controlling glutamine synthetase (GS) adenylylation state. In the upstream cycle, the UT activity of UTase/UR converts PII to PII-UMP, while the UR activity of UTase/UR converts PII-UMP to PII. In the downstream cycle, the AT activity of ATase converts GS to GS~AMP, while the AR activity of ATase converts GS~AMP to GS. Regulatory interactions are depicted wih green arrows for enzyme stimulation and red lines for enzyme inhibition. Glutamine (Gln) coherently regulates all four of the converter-enzyme activities, as shown. PII and PII-UMP act antagonistically to regulate the activities of ATase.

1). Glutamine synthetase (GS) is the most important enzyme for nitrogen assimilation in *E. coli* and sits at a key metabolic branch point; like many such enzymes in nature its activity is tightly controlled by both short-term and long-term control systems. The covalent modification system depicted in Figure 1 is responsible for the short-term control of GS activity, while another system provides long-term control of GS activity by controlling the expression level of the enzyme. GS is a dodecameric protein, and the activity of each subunit appears to be independently regulated by adenylylation, which inactivates the subunit.[12] The adenylylation and deadenylylation of GS are catalyzed by a bifunctional enzyme, ATase, that has separate AT and AR active sites located on separate domains.[13−15] The activities of ATase are regulated by glutamine and by the PII signal transduction protein in its unmodified and uridylylated forms (Figure 1). PII acts synergistically with glutamine to stimulate the AT activity of ATase, while PII-UMP activates the AR activity of ATase. Regulation of ATase involves shifting the enzyme between different forms, such that activation of either activity occurs concurrently with reduction in the antagonistic activity.[14,16] The reversible uridylylation of PII is catalyzed by another bifunctional enzyme, uridylyltransferase/uridylyl-removing enzyme (UTase/UR), that has separate UT and UR active sites located on separate domains.[17] The UT and UR activities of the UTase/UR are regulated coherently by glutamine,[18] which binds to a separate sensory domain (ref 17, Figure 1). The coupled UTase/UR-PII-ATase-GS bicyclic system is responsible for integrating (at least) three distinct stimuli to control GS activity.[11,19] As noted above, glutamine regulates each of the converter-enzyme activities. The system also senses the level of α-ketoglutarate,[20] an important signal of cellular metabolic status, and adenylylate energy charge,[19] an important signal of cellular energy status.[21] Sensation and integration of these stimuli are due to their binding to the PII protein and control of PII interactions with its various receptors.[19,22,23] In the studies reported here, we will study the system responses to glutamine under conditions where α-ketoglutarate and adenylylate energy charge are held constant, as we have done previously.[19,24]

The UTase/UR-PII-ATase-GS bicyclic system has been reconstituted from purified components,[24] and the enzymology of the individual reactions has been studied.[13−15,18] The bicyclic system provides an ultrasensitive response to glutamine when adenylylate energy charge and α-ketoglutarate are fixed.[19,24] The sources of this ultrasensitivity have not been explored experimentally, although theory argues that zero-order ultrasensitivity does not contribute, since both enzymes are bifunctional.[4,5] Glutamine acts to coherently regulate all four enzymatic steps in the cascade, although the regulation of the AR and UR activities by glutamine is quite weak, and thus multistep effects likely contribute to the ultrasensitivity of the system.

The UTase/UR-PII monocycle produces a change in PII uridylylation state in response to glutamine; this response is modestly ultrasensitive, cooresponding to a Hill coefficient of $n_H \sim 2.0$.[25] The trimeric PII protein can become modified once on each subunit, on residue tyrosine 51, and therefore the modification state of PII trimers may vary between zero and three. Two lines of evidence suggest that the trimeric nature of the PII protein is not related to the sensitivity of the glutamine response by the reconstituted UTase/UR-PII monocycle. First, the uridylylation and deuridylylation of PII subunits in the trimers are by completely distributive (nonprocessive) reactions; thus, partially modified PII trimers containing one or two uridylyl groups were readily evident during a time course of PII uridylylation.[26] Second, it is possible to form a "functionally monomeric" form of PII by creating heterotrimers in which two of the subunits are unable to interact with receptors (such as UTase/UR) while the third subunit is wild-type and has normal interactions. These heterotrimers were formed *in vitro* starting with wild-type and altered forms of PII by gentle denaturation and renaturation of trimers.[27] The mutant form of PII used for our studies interacts normally with the PII ligands (α-ketoglutarate and ATP/ADP) and displays normal intersubunit signaling within the PII trimer but is unable to contact receptors.[23,25,27] Use of heterotrimeric PII containing these subunits along with wild-type subunits allowed an approximation of a system in which PII was monomeric. When the UTase/UR-PII monocycle was reconstituted with heterotrimeric PII in place of wild-type PII, the sensitivity of the response to glutamine was not significantly different than when wild-type PII was used (ref 25, $n_H \sim 2.0$). The reasons for the ultrasensitivity of the UTase/UR-PII cycle have not been demonstrated experimentally. Coherent regulation of both activities by glutamine probably plays a role, although regulation of the UR activity by glutamine is very weak. We will show elsewhere that PII exerts strong substrate inhibition on the UT activity, which has the potential to contribute to the sensitivity of the response.[8] Regardless of the cause of the modest ultrasensitivity of the glutamine response of the UTase/UR-PII monocycle, we will show that the sensitivity of the response of the bicyclic system is much greater than the sensitivity of the UTase/UR-PII monocycle, and thus factors in

addition to the modest ultrasensitivity of the UTase/UR-PII monocycle contribute to the ultrasensitivity of the bicyclic system. Here, we identify one of the mechanisms responsible for the ultrasensitivity of the linked bicyclic system.

MATERIALS AND METHODS

Purified Proteins and General Assay and Analysis Methods. The preparations of PII, PII-Δ47-53, NRII, NRI-N-K104Q, UTase/UR, ATase, AT-NDN, AT-CDN, and GS were described previously.[13,14,18,22−24,26,34] Measurement of steady-state levels of protein uridylylation, adenylylation, and phosphorylation were as described[14,19,33] and employed the appropriate radiolabeled substrates (α-[^{32}P]-ATP, 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 nitrocellulose filters or Whatman p81 cellulose phosphate filters, which were washed extensively to remove unincorporated labeled and counted by liquid scintillation, as before.[14] In all cases, when steady state values are reported, these were determined by taking several time points and verifying that the steady state had been attained. 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 simply by calculating the ratio log 81/log(S_{10}/S_{90}), as described.[2,28] The sensitivities were independently estimated on two occasions with no significant variation.

Reconstituted Bicyclic Systems. Standard conditions were 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL bovine serum albumin (BSA), 0.5 μM GS, 0.1 μM ATase, PII as indicated (0.5, 5, or 36 μM for wild-type PII; 0.2 or 0.5 μM for heterotrimeric PII), UTase/UR (at 0.5 μM when PII was 5 μM, at 0.8 μM when PII was 36 μM, and at 0.05 μM when PII was at 0.5 μM or when heterotrimeric PII was used), 1.0 mM ATP [α-^{32}P in experiments where GS adenylylation was measured], 0.5 mM UTP [α-^{32}P in experiments where PII uridylylation was measured], 1 mM KP_i, and 0.1 mM α-ketoglutarate or as indicated, with incubation at 30 °C. As described previously,[19] reactions were initiated by addition of nucleotides and allowed to proceed until the level of protein labeling had attained the steady state. Incorporation of label into protein was measured by spotting reaction aliquots onto filters and washing away unbound label in a 5% TCA solution. Filters were then counted in a liquid scintillation counter; data were corrected for background which was always insignificant, values for several time points at the steady state were averaged, and converted to concentration values.

An important aspect of our studies is the use of heterotrimeric PII in reconstituted bicyclic systems and enzyme assays. The heterotrimeric PII preparation is stated in text as the concentration of PII trimers that would contribute the same number of wild-type PII subunits. Thus, if both are present at 0.5 μM, the identical number of wild-type subunits are present, but in the case of the heterotrimeric sample they are mixed with mutant subunits in heterotrimers. We used a ratio of 6 mutant to 1 wild-type to form heterotrimers, using a well-studied mutant (Δ47-53) that has a small deletion right at the point where PII contacts receptors (e.g., refs 23 and 25). We empirically estimate from experience that the Δ47-53 subunits and wild-type subunits exchange with little or no bias (e.g., ref 23), in which case our 6:1 ratio ensures that there will be a tiny amount of wild-type trimers remaining, as well as detectable levels of trimers with two wild-type subunits and one mutant subunit. Furthermore, in our mixture there will always be present the large concentration of PII-Δ47-53 trimers, since these re-form as the heterotrimers are being produced. We directly tested whether adding PII-Δ47-53 trimers affected the function of a bicyclic system that contained wild-type PII and found that it was innocuous (Figure S1). This is as expected based on past studies of the protein and because our reconstituted systems all contained 0.3 mg/mL BSA such that changes in protein concentration caused by adding more or less PII were relatively insignificant. We also compared the activation of ATase by wild-type PII and heterotrimeric PII and found that these have similar affinity for ATase, although the wild-type protein activated ATase to ∼2-fold higher levels of activity (Figure S2).

Reconstituted ATase-GS Monocycle. General conditions and procedures were as in the bicyclic systems, except that UTase was absent, and PII and PII-UMP were provided in fixed concentration. Our PII preparations are purified from cells lacking UTase/UR and appear to be completely unuridylylated. For production of PII-UMP, a time course of uridylylation was conducted as in Figure 4A, and upon complete uridylylation of PII the PII-UMP was recovered by brief heating to kill the UTase/UR followed by gel filtration into buffer, concentration, and quantitation.[14] Conditions for PII-UMP production were 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.2 mM α-ketoglutarate, 0.5 mM AMP-PNP [to ensure no modification of PII with adenylyl groups], 1 mM UTP, 30 μM PII, and 0.1 μM UTase/UR, with incubation at 30 °C. Conditions were the same for production of modified heterotrimeric PII, except that PII was 5 μM and the UTase/UR was 0.02 μM. To recover partially modified PII-UMP, samples were taken and processed as above at intermediate time points. To have preparations that are unlabeled, while still roughly knowing the extent of modification, side-by-side experiments were carefully performed, in which one tube contained labeled UTP and the other contained unlabeled UTP. As for the reconstituted bicycles, the ATase-GS monocyclic systems were preincubated in the absence of the nucleotides, and reactions were started by addition of the nucleotide mixture.

Measurement of Adenylylation and Deadenylylation Rates. Conditions for the AT assay were 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL bovine serum albumin (BSA), 0.01 mM α-ketoglutarate, 0.5 mM ATP, AT-NDN enzyme at either 0.5 or 1 μM nominal concentration which, depending on the enzyme batch, seemed to be equivalent, and either PII or partially modified PII at 0.5 μM, or heterotrimeric PII preparation at 0.2 μM or 0.4 μM. Importantly, although only one concentration of heterotrimeric PII is shown in the main text, a nearly linear response to PII modification was seen with heterotrimeric PII at both concentrations (Figure S3). For deadenylylation rate studies, GS-^{32}P-AMP was prepared as described[14] by adenylylation using ATase. Conditions for deadenylylation were as for adenylylation, but also 1 mM KP_i, and α-ketoglutarate was 1 mM, and instead of the AT-NDN enzyme reactions contained 0.1 or 0.25 μM AT-CDN enzyme, and instead of GS contained either 11.7 or 7.5 μM GS-AMP, and contained either 0.1 μM of partially modified wild-type PII trimers or 0.1 μM partially modified heterotrimeric PII. The experiments were performed in duplicate, and the error bars show standard deviations.

Nondenaturing Gel Electrophoresis Assay for Binding of PII to NRII and ATase. Experiments were as described[13] and used 14% polyacrylamide nondenaturing gels lacking SDS.

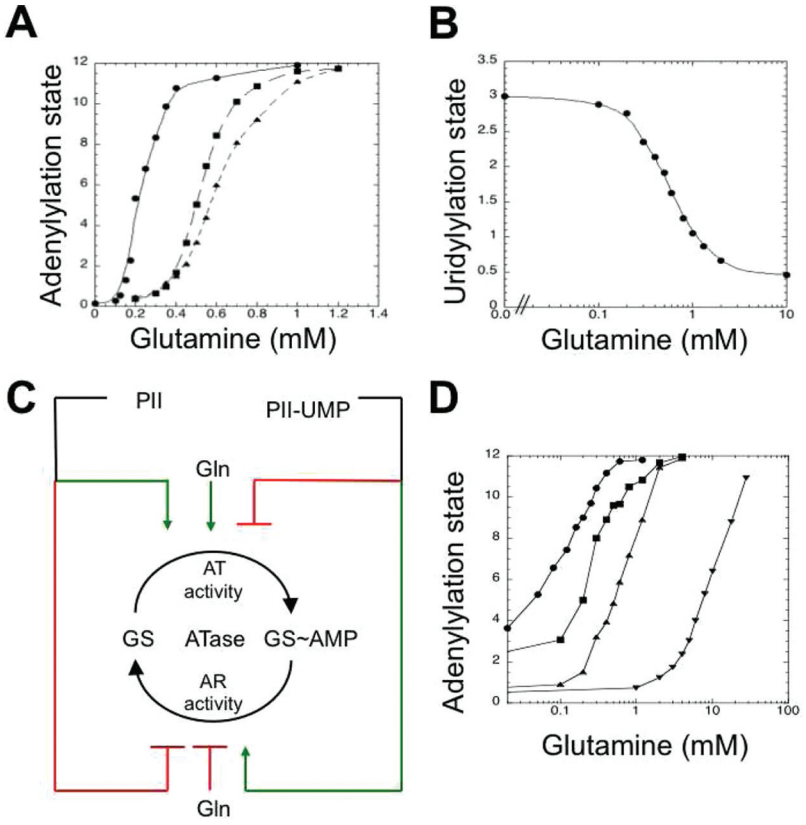

Figure 2. Reconstituted monocyclic and bicyclic signal transduction systems. (A) UTase/UR-PII-ATase-GS bicyclic systems respond to glutamine with high ultrasensitivity. The three systems depicted contained PII at 36 μM (●), 5 μM (■), and 0.5 μM (▲), and steady-state GS adenylylation state was measured at various glutamine concentrations. (B) The UTase/UR-PII cycle within the reconstituted UTase/UR-PII-ATase-GS bicyclic system did not display high ultrasensitivity. The depicted system contained 0.5 μM PII. (C) Reconstituted ATase-GS monocyclic system. In this system, UTase/UR is absent, and PII and PII-UMP were provided at fixed concentrations. Regulatory interactions are depicted wih green arrows for enzyme stimulation and red lines for enzyme inhibition. (D) Reconstituted ATase-GS monocyclic systems were not highly ultrasensitive at various ratios of PII and PII-UMP. (●) PII was 0.4 μM and PII-UMP was 0.1 μM. The sensitivity cooresponded to $n_H \sim 1.39$, and the $S_{0.5}$ was 0.14 mM glutamine. (■) PII was 0.2 μM and PII-UMP was 0.3 μM. The sensitivity cooresponded to $n_H \sim 1.58$, and the $S_{0.5}$ was 0.24 mM glutamine. (▲) PII was 0.08 μM and PII-UMP was 0.42 μM. The sensitivity cooresponded to $n_H \sim 2.11$, and the $S_{0.5}$ was 0.63 mM glutamine. (▼) PII was absent and PII-UMP was at 0.3 μM. The sensitivity cooresponded to $n_H \sim 1.83$, and the $S_{0.5}$ was 8.20 mM glutamine.

The separating gel conditions and running buffer were 25 mM Tris-Borate, pH 7.5, 0.5 mM ATP, 1 mM $MgCl_2$, 0.03 mM α-ketoglutarate, and 10 mM glutamine, at 4 °C (in a cold room). Gels were prerun at 100 V for 1 h prior to application of samples. Samples containing the indicated proteins (10 μL) were incubated at room temperature for 10 min, after which 2 μL of a 5X loading buffer was added, and they were loaded onto the gel. Gels were run at 150 V for 40 min, after which the voltage was increased to 230 V for 50 min. Gels were exposed to X-ray film overnight at −20 °C (without an intensifyng screen), then gently thawed, stained with Coomassie Brilliant Blue R250, and photographed.

RESULTS

Reconstituted UTase/UR-PII-GS-ATase Bicyclic System Displayed a Highly Ultrasensitive Response to Glutamine. Although it had been observed that the reconstituted UTase/UR-PII-ATase-GS bicyclic system brought about the interconversion of GS and GS~AMP over a narrow range of glutamine concentrations, the sensitivity of this system had not been quantified. We examined the glutamine response of reconstituted bicyclic systems that contained the PII protein at either 0.5, 5, or 36 μM (Figure 2A); this concentrations range corresponds to systems that are unsaturated to saturated for PII. In all three cases the bicyclic systems produced an ultrasensitive response (Figure 2A). To estimate sensitivities, we used a graphical method to determine the range of glutamine concentrations required to shift the system from 10% of the full response to 90% of the full response. This range can be used to estimate the Hill coefficient.[2,28] When PII was 36 μM, the sensitivity of the glutamine response cooresponded to $n_H \sim 4.48$, and the $S_{0.5}$ was 0.225 mM glutamine; when PII was 5 μM, the sensitivity cooresponded to $n_H \sim 6.46$ and the $S_{0.5}$ was 0.530 mM glutamine; when PII was 0.5 μM, the sensitivity cooresponded to $n_H \sim 5.23$ and the $S_{0.5}$ was 0.6 mM glutamine (Figure 2A). Using the same conditions and with PII at 0.5 μM, we also examined the change in PII uridylylation state in the UTase/UR-PII part of the bicyclic system as glutamine was varied; the sensitivity of the glutamine response cooresponded to $n_H \sim 1.95$, and the $S_{0.5}$ was 0.55 mM glutamine (Figure 2B). Thus, the sensitivity of the glutamine response of the UTase/UR-PII cycle within the context of the bicyclic system was quite similar to that obtained when it was examined in the absence of the downstream ATase-GS cycle.[25] This is not surprising, as we used conditions where the downstream cycle converter-enzyme, ATase, was present at low enough concentration so as to not sequester a significant fraction of the PII/PII-UMP and cause retroactive effects on the upstream cycle.[29]

A Reconstituted ATase-GS Monocyclic System Did Not Display Significant Ultrasensitivity in Its Glutamine Responses. Since the bicyclic system displayed a highly ultrasensitive response to glutamine ($n_H \sim 5$), while the upstream UTase/UR-PII cycle only displayed modest ultrasensitivity ($n_H \sim 2$), we expected the downstream ATase-GS monocycle to show an ultrasensitive response when it was examined in the absence of the UTase/UR-PII cycle. However, this did not prove to be the case. We examined ATase-GS monocyclic systems where PII and PII-UMP were provided at fixed concentrations (Figure 2C); we note that in these experiments the PII preparation was fully unmodified on all three subunits of the PII trimers, while the PII-UMP preparation consisted of trimers that, almost entirely, were modified on all three subunits of the trimers. That is, there were almost no partially modified PII trimers in the experiments. Under these conditions, strong responses to glutamine were obtained at a variety of different PII/PII-UMP ratios (Figure 2D). As expected in this system, when PII was high relative to PII-UMP, there was some adenylylation of GS even in the absence of glutamine since PII can directly active the AT activity (Figure 2D). Remarkably, at all ratios of PII/PII-UMP, the response of the monocyclic system to glutamine cooresponded to $n_H \sim 1.5$ (see the figure legend for the sensitivities of each curve). Note that, as expected from theory,[30] changing the ratio of PII and PII-UMP changes the catalytic rates of the antagonistic activities and by so doing shifted the $S_{0.5}$ of the system (Figure 2D). Together, the results in Figure 2 show that while the coupled bicyclic system displayed high ultrasensitivity, neither the UTase/UR-PII cycle nor the ATase-GS cycle, by itself, displayed high ultrasensitivity.

Trimeric Nature of PII Contributed to the Sensitivity of the UTase/UR-PII-ATase-GS Bicyclic System. Prior results had shown that the sensitivity of the glutamine response of the UTase/UR-PII monocycle was not significantly different when PII was replaced by heterotrimeric, functionally monomeric, PII trimers.[25] For comparisons of heterotrimeric PII to wild-type PII trimers, we state the concentration of the heterotrimeric PII preparation as the concentration of wild-type PII trimers that would contribute the same number of wild-type PII subunits. Thus, for comparisons where both wild-type PII trimers and the heterotrimer preparation were present at 0.5 μM, an identical number of wild-type subunits were present, but in the case of the heterotrimeric sample they were mixed with mutant subunits in heterotrimers, such that there are ~2.5-fold as many PII molecules that contain an active subunit (Materials and Methods). Prior mathematical modeling suggested that PII subunits were about 2.5-fold better able to activate the PII receptor NRII when in heterotrimers, as opposed to when they are in native trimers.[23,27] We have hypothesized that this effect reflects the fact that while in native trimers, only one of the three PII subunits can activate a receptor at any time, while upon distribution into heterotrimers, all of the wild-type subunits can act simultaneously.[27] The increase in the number of molecules containing an active subunit is because each heterotrimer molecule only contains ~1 wild-type subunit, while the wild-type trimers each contain three subunits. The value of ~2.5 (as opposed to 3) takes into account that not all PII subunits survive the formation of heterotrimers, and as noted, a small fraction of the trimers in the mixture contain more than a single wild-type subunit.[25,27] Here, we examined the effect of using heterotrimeric PII in place of wild-type PII in the bicyclic system. Under the conditions of our experiments, where a 6:1 ratio of mutant/wild-type subunits was used to form heterotrimers (Materials and Methods), we expect the distribution of wild-type subunits in the heterotrimer populations to be as follows: 85% of the wild-type subunits are expected to be found in trimers with 2 mutant subunits, 14% of the wild-type subunits are expected to be found in trimers with one other wild-type subunit and one mutant subunit, and 1% of the wild-type subunits are expected to be found in trimers with two other wild-type subunits.[25] For comparisons where wild-type PII was at 0.5 μM and the heterotrimer preparation was at 0.2 μM, aproximately the same number of PII molecules are present with at least 1 active subunit. We compared the glutamine responses of bicyclic systems that had wild-type PII at 0.5 μM and PII heterotrimers at either 0.5 or 0.2 μM (Figure 3). Remarkably, the sensitivity

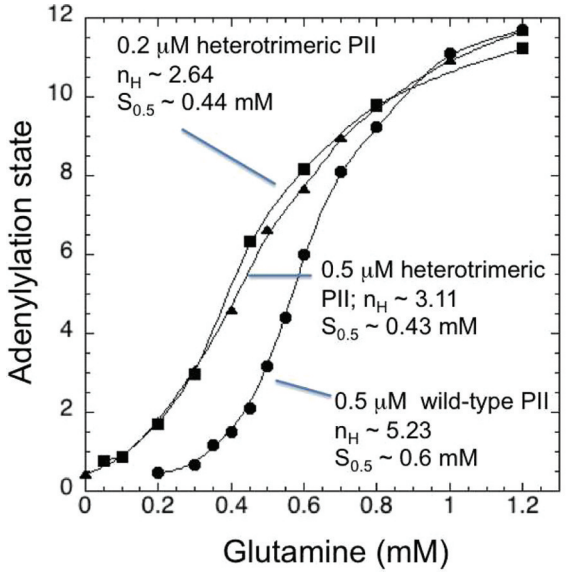

Figure 3. Trimeric nature of PII contributed to the sensitivity of the UTase/UR-PII-ATase-GS bicyclic system. The steady-state glutamine responses are shown for bicyclic systems containing wild-type PII at 0.5 μM (●), heterotrimeric PII at 0.2 μM (■), and heterotrimeric PII at 0.5 μM (▲).

and $S_{0.5}$ of the glutamine response of the bicyclic system was significantly reduced when heterotrimeric PII was used, relative to when wild-type PII was used, and the results were quite similar for both concentrations of heterotrimeric PII (Figure 3). When 0.5 μM wild-type PII was used, the sensitivity of the glutamine response corresponded to $n_H \sim 5.23$ and the $S_{0.5}$ was 0.6 mM glutamine (Figure 3). When heterotrimeric PII was used, sensitivities corresponded to $n_H \sim 2.64$ (for 0.2 μM heterotrimeric PII) and $n_H \sim 3.11$ (for 0.5 μM heterotrimeric PII), with S_{50} of ~0.43 in both cases (Figure 3). In another experiment, we observed a similar response of the system containing heterotrimeric PII at 0.5 μM. Thus, a considerable fraction of the sensitivity of the glutamine response, perhaps as much as half, was lost upon replacement of PII with heterotrimeric PII. These results showed that the trimeric nature of wild-type PII played a role in the ultrasensitivity of the bicyclic system.

Relationship between PII Modification and GS Modification in Reconstituted Bicyclic Systems Containing Wild-Type or Heterotrimeric PII. To examine both the modification of GS and modification of PII in bicyclic systems,

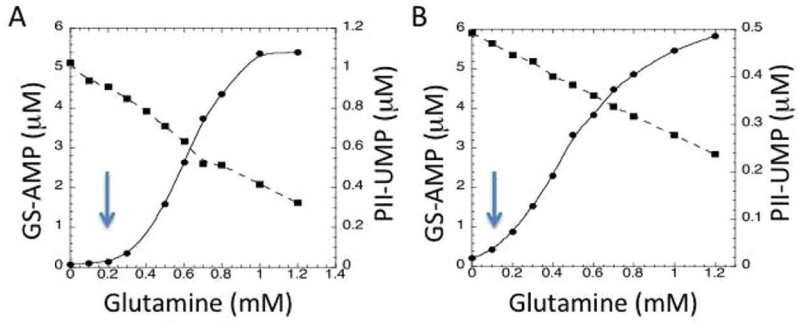

Figure 4. Coupling between the two monocycles of the bicyclic system was different in systems containing wild-type PII and heterotrimeric PII. The steady-state levels of GS adenylylation (solid line) and PII uridylylation (dashed line) were determined in duplicate reaction mixtures that contained either labeled ATP (to measure GS adenylylation) or UTP (to measure PII uridylylation). (A) Results with systems containing 0.5 μM wild-type PII. (B) Results with systems containing 0.2 μM heterotrimeric PII. The arrow points to a region of the GS adenylylation state curve that is discussed in the text.

we used duplicate reaction mixtures that were identical, except in the identity of the radioactive nucleotide. In one mixture the radioactive label was on the α-position of UTP, to measure PII uridylylation, while in the other mixture the radioactive label was on the α-position of ATP, to measure GS adenylylation. This procedure allowed us to monitor the coupling of the two cycles forming the bicyclic system, as before.[31] Here, we examined whether the coupling between the two cycles was altered when PII was replaced by heterotrimeric PII, and we observed that the coupling was significantly altered (Figure 4). When wild-type PII (0.5 μM) was used in the bicyclic system, GS adenylylation state was nearly zero in the absence of glutamine, and the PII uridylylation state corresponded to ~73% of the PII subunits. As glutamine was increased, steady-state GS adenylylation state was not increased until the steady-state level of PII uridylylation had fallen significantly (Figure 4A). This is highlighted with a blue arrow in Figure 4A; this nonlinearity of the adenylylation response to PII-UMP deuridylylation is responsible for the high ultrasensitivity of the glutamine response in this system. By contrast, when heterotrimeric PII (0.2 μM) was used in the bicyclic system, the steady-state adenylylation state of GS increased concurrently, in a nearly linear fashion, as the steady-state uridylylation level of PII decreased, in response to glutamine increase (Figure 4B). In these experiments utilizing heterotrimeric PII at 0.2 μM, GS adenylylation state was slightly above zero in the absence of glutamine, and the PII uridylylation state corresponded to ~83% of the wild-type subunits in the sample. Increasing glutamine resulted both in a decrease in PII uridylylation state and in a concurrent increase in GS adenylylation state (Figure 4B). These experiments therefore suggested that the difference in sensitivities of bicyclic systems containing PII and heterotrimeric PII was due to different coupling between the component monocycles in the two systems.

Regulation of the AR and AT Activities by Partially Modified Preparations of PII. The results presented so far suggested that partially modified PII trimers, containing both modified and unmodified subunits, might have properties that contribute to the sensitivity of the bicyclic system. We therefore produced populations of PII trimers with various extents of uridylylation, using both wild-type PII and heterotrimeric PII. Of course, the population of partially modified heterotrimeric PII is simply a mixture of trimers with one modification and trimers with zero modifications, as the heterotrimers can only be modified on their (approximately) one wild-type subunit. But, in the population of partially modified wild-type PII trimers, trimers with both modified and unmodified subunits will be present, and the average uridylylation per trimer will reflect the fraction of total subunits that are uridylylated.[26] To produce partially modified PII populations, we conducted a time-course experiment in parallel using two reaction mixtures, one of which contained α-^{32}P-UTP and the other of which contained unlabeled UTP. The radioactive mixture was used to monitor the extent of uridylylation (Figure 5A,B) and to produce material for experiments where labeled and partially modified PII was required. The nonradioactive samples were used for experiments in which nonradioactive and partially modified PII was required. To stop the uridylylation process at various time points, samples were briefly heated to 60 °C, which completely inactivated UTase/UR but did not discernibly affect PII or PII-UMP, as before.[14]

To measure the ability of partially modified PII preparations to activate the AR activity of ATase, an altered version of the ATase was used, which contained inactivating alterations in the AT active site.[13] This enzyme, AT-CDN, has been shown to have strong AR activity and tight regulation of the activity by PII-UMP, PII, and glutamine but to completely lack AT activity. We measured the initial rate of release of labeled adenylyl groups from GS-AMP, as previously, in reaction mixtures that contained partially modified (unlabeled) PII populations, where the PII was either wild-type (Figure 5C) or heterotrimeric (Figure 5D). In both cases, the initial rate of GS-AMP deadenylylation showed a linear relationship with the fractional modification of the PII population (Figure 5C,D). This suggests that for wild-type trimers PII-UMP subunits activated the AR activity of AT-CDN independently of the uridylylation status of the other subunits within the PII trimer.

To measure the ability of partially modified PII preparations to activate the AT activity of ATase, another altered version of ATase was used that contained inactivating alterations in the AR active site.[13] This enzyme, AT-NDN, has been shown to have strong AT activity and tight regulation of the AT activity by PII, PII-UMP, and glutamine but to completely lack AR activity. We measured the rate of incorporation of labeled adenylyl groups into GS, as previously, in reaction mixtures that contained partially modified (unlabeled) PII populations, where PII was either wild-type (Figure 5E) or heterotrimeric (Figure 5F, also Figure S3). When wild-type PII was used, a nonlinear response of AT activation to PII modification was observed (Figure 5E). That is, partially modified PII activated ATase

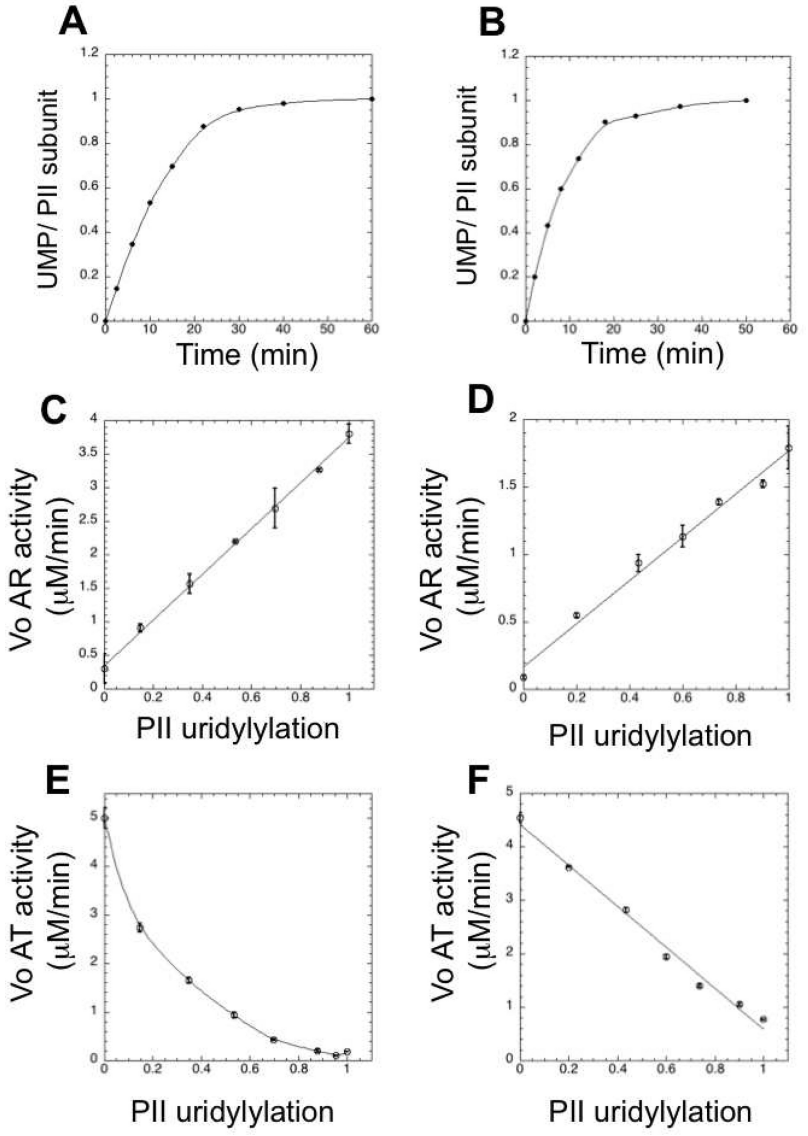

Figure 5. Activation of the AT and AR activities of ATase by partially modified populations of PII. (A) Time course of PII uridylylation for the experiment used to produce partially modified wild-type PII. (B) Time course of PII uridylylation for the experiment used to produce partially modified heterotrimeric PII. (C) Initial rate of the AR activity activated by partially modified populations of wild-type PII trimers. Measurements were conducted as described in Materials and Methods and contained 11.7 μM adenylylated GS subunits, 0.1 μM AT-CDN enzyme, and 0.1 μM of the partially modified PII preparations. PII populations were used with fraction modification of 0, 0.147, 0.348, 0.535, 0.697, 0.876, and 1.0. All reactions were conducted in duplicate, and error bars show the standard deviations (some errors were so small that their error bars are not readily evident). (D) Initial rate of the AR activity activated by partially modified populations of heterotrimeric PII trimers. Measurements were conducted as described in Materials and Methods and contained 7.5 μM adenylylated GS subunits, 0.25 μM AT-CDN enzyme, and 0.1 μM of the partially modified heterotrimeric PII preparations. PII populations were used with fraction modification of 0, 0.199, 0.433, 0.600, 0.697, 0.737, 0.902, and 1.0. All reactions were conducted in duplicate, and error bars show the standard deviations. (E) Initial rate of the AT reaction activated by partially modified wild-type PII populations. Reactions were conducted as in Materials and Methods and contained 2.5 μM GS dodecamers, 0.5 μM AT-NDN enzyme, and 0.5 μM of the partially modified PII. PII populations were used with fraction modification of 0, 0.147, 0.348, 0.535, 0.697, 0.876, 0.952, and 1.0. All reactions were conducted in duplicate, and error bars show the standard deviations (some errors were so small their error bars are not readily evident). (F) Initial rate of the AT reaction activated by partially modified heterotrimeric PII populations. Reactions were conducted as in Materials and Methods and contained 2.5 μM GS dodecamers, 1.0 μM AT-NDN enzyme, and 0.4 μM of the partially modified PII. PII populations were used with fraction modification of 0, 0.199, 0.433, 0.600, 0.737, 0.902, and 1.0. All reactions were conducted in duplicate, and error bars show the standard deviations.

discernibly less well than expected, based upon the fraction of the PII population that was uridylylated. This nonlinearity strongly suggests that unmodified PII subunits within trimers that contain modified subunits could not activate the AT as well as could unmodified subunits in completely unmodified PII trimers. By contrast to these results, when heterotrimeric PII was used, the ability of PII to activate the AT displayed a linear relationship with the fractional modification of the PII population (Figure 5F, Figure S3), as expected. Because of the high accuracy of the AT assay, the distinction between the shapes of the curves in Figure 5E,F is unambiguous. Therefore, we hypothesize that activation of AT required (or was vastly better with) fully unmodified PII trimers, while activation of AR

required PII-UMP subunits, but it did not matter whether the other subunits within the PII trimer were uridylylated or not.

Our hypothesis predicts that if the activation of the AT activity of ATase is examined, a mixture of completely unmodified PII trimers and completely modified PII-UMP trimers will behave differently than a population of PII trimers with the same average level of uridylylation, but where partially modified PII trimers are present. Specifically, our hypothesis predicts that partially modified PII trimers will be much less able to activate the AT than expected based upon the fractional modification of PII. We directly tested this prediction in the series of experiments shown in Figure 6. To study the activation

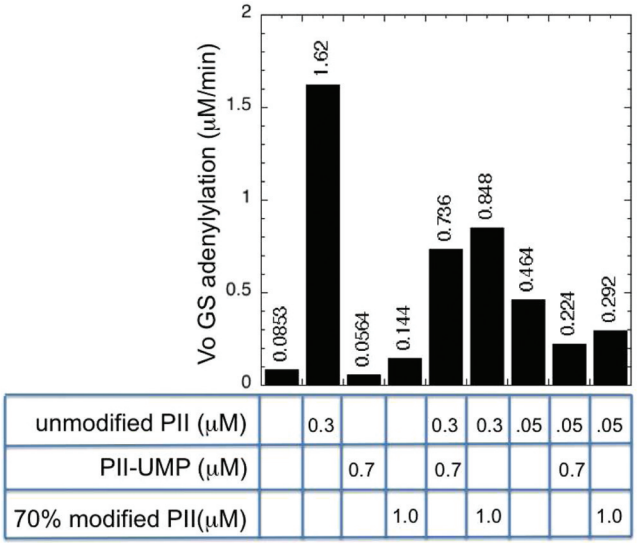

Figure 6. Comparison of AT activation by partially modified and unmodified PII trimers. Initial rates of GS adenylylation were determined as in Materials and Methods, in reactions containing 2.5 μM GS dodecamers, 0.3 μM AT-NDN enzyme, and the indicated concentrations of wild-type unmodified PII trimers, wild-type completely modified PII-UMP trimers, and a population of PII trimers with 70% modification.

of the AT activity, we again used the AT-NDN protein that has AT activity but lacks AR activity. We used a partially modified PII population where the extent of modification was ~65−70% and compared this to a mixture of completely modified and completely unmodified PII trimers. Fully unmodified PII trimers strongly activated the AT activity, while fully modified PII trimers failed to activate AT; notably, the mixture of 0.3 μM PII and 0.7 μM PII-UMP trimers provided strong activation of AT, although the presence of the PII-UMP was clearly inhibitory, as expected.[14] By comparison, the 70% modified PII population, when present at 1.0 μM, was barely able to active the AT activity and in fact inhibited activation by fully unmodified PII (Figure 6). To get a sense of the difference between the different PII preparations and the limits of our assay method, we also examined the ability of fully unmodified PII at the low concentration of 0.05 μM to activate the AT activity in the presence of 0.7 μM fully modified PII-UMP trimers (Figure 6). Fully unmodified PII at 0.05 μM activated AT discernibly better than did the 70% modified PII population at 1 μM, which had a 6-fold greater number of unmodified PII subunits (Figure 6). Thus, a significant difference in the ability of unmodified PII subunits to activate AT was observed, depending upon whether these subunits were in fully unmodified trimers or not.

In a separate experiment, we explored whether the ability of partially modified PII to activate the AT could be restored upon its deuridylylation. An aliquot of the ~70% partially modified PII sample was incubated in the presence of a mutant form of UTase/UR, containing the D107N alteration, that completely inactivates the UT activity but has only a minor effect on the UR activity. As a control, another aliquot of the partially modified PII sample was incubated in the absence of the D107N-UTase/UR enzyme. After suitable time to allow deuridylylation of PII-UMP in the sample containing enzyme, both samples were heated to 60 °C to inactivate the UTase/UR, and the ability of the PII to activate the AT activity of AT-NDN was examined (Figure 7). In the absence of PII, the initial

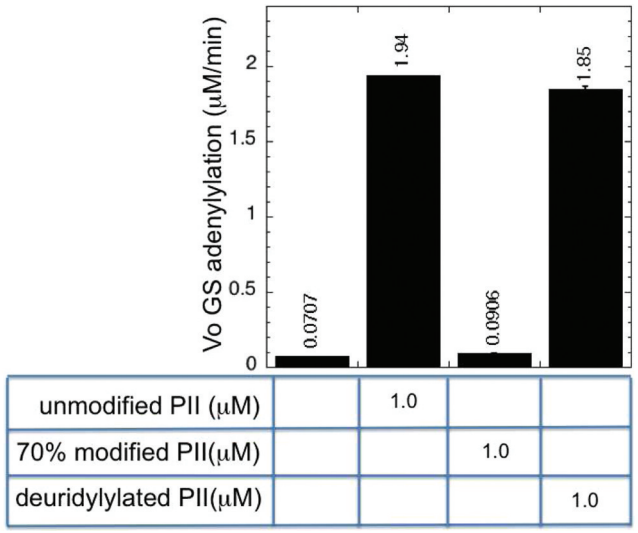

Figure 7. Deuridylylation of partially modified PII restored its ability to activate the AT activity of ATase. Initial rates of GS adenylylation were determined as in Materials and Methods, in reactions containing 2.5 μM GS dodecamers, 0.3 μM AT-NDN enzyme, and the indicated concentrations of wild-type unmodified PII trimers, a population of PII trimers with 70% modification, and a population of PII trimers that initially was 70% modified but had been treated with UTase/UR-D107N to deuridylylate the PII ("deuridylylated PII"). The experiments with 70% modified PII and with "deuridylylated PII" were performed in duplicate, and the barely visible error bars show the raw error in the duplicates.

rate of GS adenylylation by AT-NDN was insignificant, whereas strong activation was seen by 1 μM wild-type unmodified PII (Figure 7). As before, the partially modified PII preparation with ~70% modification displayed minimal ability to activate the AT activity of AT-NDN (Figure 7). But upon deuridylylation of the partially modified PII, its ability to activate the AT activity was restored (Figure 7). This experiment shows that the partially modified PII preparation was not artifactually ruined somehow during its preparation and was only unable to activate the AT activity of ATase because it was uridylylated.

We used the partially modified PII populations created in this work to also examine the effect of PII uridylylation on the regulation of the activities of another cellular PII receptor, NRII. Prior work showed that unmodified PII inhibited the autophosphorylation of NRII, and that the complex of PII and NRII brought about the very rapid dephosphorylation of the

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enhancer-binding transcription factor NRI~P.[32,33] Fully modified PII-UMP trimers do not bind to NRII.[26] We measured the initial rate of NRI~P dephosphorylation in reaction mixtures containing partially modified populations of wild-type and heterotrimeric PII (Figure S4A); both PII preparations behaved similarly and displayed a slightly nonlinear relationship between PII uridylylation and the ability to activate NRI~P dephosphorylation. The initial rate of NRII autophosphorylation was measured indirectly, by measuring the phosphorylation of a mutant form of the N-terminal domain of NRI that can rapidly take phosphoryl groups from NRII and is highly stable when phosphorylated.[34] When this mutant form of NRI was present at huge excess, the rate of its phosphorylation reflected the NRII autophosphorylation rate. We observed a linear relationship between the uridylylation of wild-type PII and the ability of the PII population to inhibit NRII autophosphorylation (Figure S4B). Together, these results suggest that, analogous to the relationship between PII-UMP and the AR activity, subunits of PII interacted with NRII (nearly) the same, regardless of the modification status of the other subunits in the trimer.

Binding of Partially Modified PII to ATase and NRII.

Prior studies showed that the interaction between ATase and PII and the interaction between NRII and PII could be easily detected using an assay based upon nondenaturing gel electrophoresis.[13] However, the same assay did not detect the interaction between PII-UMP and ATase, even though PII-UMP activates the AR activity of ATase.[13] Here, we used the radioactively labeled partially modified wild-type PII preparations to study the binding of ATase and NRII by partially modified PII trimers. We used a radioactively labeled sample in which about 60−70% of the PII subunits were modified. An appealing aspect of the approach is that the label is on the uridylyl groups; thus, if the label is found in a bound species, there is no ambiguity that the bound species has at least one uridylyl group; also, the bound species can be simply detected by staining, such that the level of radioactivity and staining can be compared. An example of typical results is shown in Figure 8. Unmodified PII trimers form a dense (unlabeled) band, while modification of PII resulted in its labeling and in its faster migration in the gel. It is possible to cleanly separate the different modified forms of PII trimers in similar gels,[26] but the gels in the current study were optimized for protein interactions between larger species and do not completely resolve the different modified forms of PII-UMP (Figure 8). Our ATase preparation forms a characteristic pair of closely spaced bands on nondenaturing gels.[13] ATase bound a discernible fraction of the PII in both the unmodified sample and the partially modified sample (Figure 8A), but in the latter case, very little of the label was found in the PII-ATase complex (compare parts A and B of Figure 8). In essence, ATase selectively bound the unmodified PII trimers from the partially modified population of trimers. By contrast, NRII bound to PII in both the unmodified and partially modified samples, and in the latter case a very significant amount of radioactivity was found in PII-NRII complexes. But when a fully modified PII-UMP population was examined, neither NRII nor ATase was bound to any significant extent (Figure 8A,B). These results show that NRII binds to unmodified and partially modified PII trimers, while ATase bound well to unmodified PII but hardly bound to partially modified PII trimers.

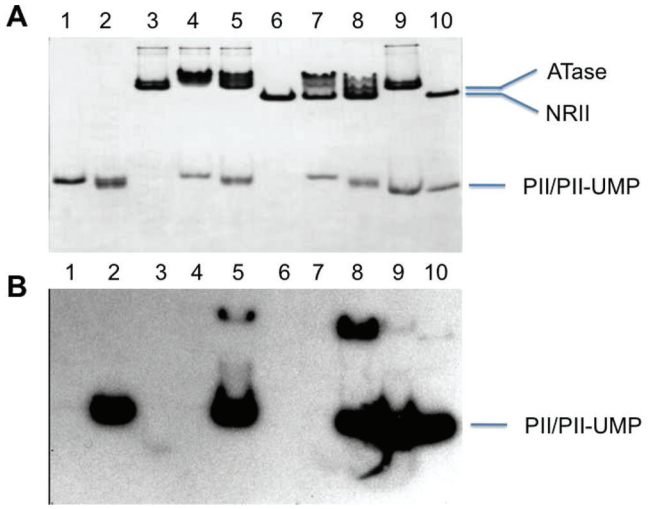

Figure 8. Nondenaturing gel electrophoresis assay for the binding of partially modified PII trimers to ATase and NRII. A 14% polyacrylamide gel lacking SDS was used, as in Materials and Methods. Panel A shows an image of the gel staining pattern using Coomassie Brilliant Blue R-250. Panel B shows an autoradiograph of the same gel. The lanes contained (from left): lane 1, 6 μM unmodified PII trimers; lane 2, 6 μM population of 70% modified PII trimers; lane 3, 6 μM ATase; lane 4, 6 μM ATase + 6 μM unmodified PII trimers; lane 5, 6 μM ATase + 6 μM partially modified PII trimers; lane 6, 6 μM NRII dimers, lane 7, 6 μM NRII + 6 μM unmodified PII; lane 8, 6 μM NRII + 6 μM partially modified PII trimers; lane 9, 6 μM ATase + 6 μM completely modified PII-UMP trimers; lane 10, 3 μM NRII and 3 μM fully modified PII-UMP trimers.

DISCUSSION

Our results demonstrate a general mechanism that can bring about ultrasensitive responses in a cascade of covalent modification cycles, namely, having a requirement for the complete modification or demodification of an oligomeric species for the activation of a downstream converter-enzyme activity. The mechanism in essence consists of imposing a high kinetic order interaction at one of the points of coupling between the two cycles in the cascade. In our case, the oligomeric protein involved, PII, was a trimer; even though there were only three subunits, the requirement for its complete deuridylylation for ATase activation brought about a very significant increase is system sensitivity, relative to the analogous system containing heterotrimeric (and functionally monomeric) PII. Conceivably, even stronger effects will be obtained in other natural systems where the oligomeric species is a tetramer or higher-order oligomer. In our system, the requirement for fully unmodified PII for the activation of ATase helped to produce a switchlike response to glutamine. This effect is likely exagerated by the great synergy of fully unmodified PII and glutamine in activating the AT activity.[14] Thus, as the steady-state level of fully unmodified PII trimers increases in the presence of glutamine, there is a dramatic increase in GS adenylylation state.

The mechanism providing ultrasensitivity based on the high kinetic order interaction at the coupling point of the two cycles is superficially reminiscent of how ultrasensitivity arises in systems where the substrate protein must be phosphorylated on two different sites to be active.[9] However, in the latter case, the increase in sensitivity is experienced within the cycle containing the multiply modified protein and is due to a form of substrate inhibition.[9] Here we describe a case where PII trimers were

multiply modified in one cycle, and due to the nature of coupling between cycles, this resulted in an increase in the sensitivity of response of the downstream cycle. Interestingly, in the system we studied there were two distinct coupling points between the two cycles, since both PII and PII-UMP products of the upstream cycle regulated activities of the downstream cycle. But only one of these interactions appeared to be of high kinetic order, namely the AT-PII interaction. By contrast, the AR-PII-UMP interaction did not appear to be of high kinetic order. Thus, a single high kinetic order interaction at a coupling point between cycles was enough to increase system sensitivity; we speculate that an even more dramatic effect might be obtained in circuits where both (or all) coupling points between cycles involve interactions with high kinetic order.

Our reconstituted bicyclic systems give insights into how bacterial cells regulate the activity of a key metabolic enzyme in response to metabolite levels. Although the system has been studied for a long time (e.g., ref 35), many features of the system still are not understood. For example, we observed here that the midpoint of the glutamine response ($S_{0.5}$) of the UTase/UR-PII-ATase-GS bicyclic system was shifted upon variation of the (total) PII concentration (Figure 2A); it also seemed to be the case that the bicyclic system displayed the highest ultrasensitivity at an intermediate concentration of (total) PII (Figure 2A). The reasons for these phenomena remain to be determined. The occurrence of such phenomena show a capacity for a memory or learning function by the system, if signaling results in the increase or decrease in the concentration of the component coupling cycles. Such a change in a critical component of the system would result in differences in the initial response and in subsequent responses to the identical stimulation. The GS regulatory system has just such a feature, since a protein closely related to PII is expressed upon stimulation of the system by falling glutamine levels.[36,37]

The nature of the binding interactions between PII and the site from which it activates the AT activity are of interest. One possibility is that modification of a PII subunit alters the conformation of other subunits in the trimer, spoiling their ability to interact with ATase. In that case, it would be of interest to know which residues of PII are involved in communicating this intersubunit signaling to ATase and whether we can measure other differences in intersubunit signaling within PII trimers[23] that result from uridylylation of one PII subunit. It does not seem likely that ATase interacts with all three subunits of PII simultaneously to detect their uridylylation status because heterotrimeric PII containing only a single PII T-loop capable of interacting with ATase was able to activate the AT activity. But the heterotrimeric PII was not as strong an activator of the AT activity as was wild-type PII (Figure S2), suggesting that the T-loops of the PII subunits that do not contact AT participate in facilitating AT activiation, perhaps by helping the productive PII subunit maintain the active conformation. Other PII receptors do interact with all three PII subunits simultaneously;[38] thus, PII engages in mechanistically distinct interactions. We previously conducted cross-linking studies to identify the general region on the ATase protein to which PII bound.[13] Fortunately, our uniquely positioned cross-linker in those studies had a rather long spacer arm and was positioned some distance away from the site of PII uridylylation. Apparently, the cross-linker in those studies did not mimic the effects of uridylylation in any way, or we might not have been able to detect the cross-linking. We also obtained cross-linking of highly uridylylated PII-UMP to ATase in prior studies.[13] In retrospect, that cross-linking was likely to reflect binding to the PII-UMP site of ATase, since addition of even one uridylyl group to PII appears to spoil its ability to interact with the PII site.

It is not clear why *E. coli* should require a very highly ultrasensitive response of GS adenylylation state to changes in glutamine concentration or even whether the response is truly ultrasensitive *in vivo*. Of course, studies with reconstituted systems by definition cannot explain the actions of unknown additional components that may exist and contribute to the *in vivo* behavior of the system. If the bicyclic system is highly ultrasensitive *in vivo*, perhaps this provides important benefits to the cell when signaling dynamics are considered. In our study, we measured steady-state responses of the reconstituted systems to different constant glutamine levels. By contrast, *in vivo* the short-term control system must rapidly adjust GS activity level in response to sudden environmental changes and must be able to accommodate a wide variation in GS protein levels.[39] The requirement to accurately respond to a dynamically changing stimulatory effector and to be robust to changes in the level of GS may have contributed to the evolution of a highly ultrasensitive signaling system.

The distinction between regulation of NRII, which is regulated by partially modified trimers as well as completely unmodified trimers, and regulation of the AT activity of ATase, which is only activated by completely unmodified PII trimers, likely has biological significance. This difference may ensure that as the cellular glutamine concentration falls and PII becomes partially modified, the inactivation of GS by adenylylation stops abruptly, while, by contrast, the ability of PII to block Ntr gene transcription is only modestly affected. This, in turn, may ensure that the short-term regulatory system functions to control GS activity before the long-term system begins to adjusts the cellular level of the GS protein.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GS, glutamine synthetase (product of the *gln A* gene); ATase, glutamine synthetase adenylyltransferase (product of the *gln E* gene); AT activity, adenylyltransefrase activity of ATase; AR activity, adenylyl-removal activity of ATase; UTase/UR, uridylyltransferase/uridylyl-removing enzyme (product of the *gln D* gene); UT activity, uridylyltransferase activity of UTase/UR; UR activity, uridylyl-removing activity of UTase/UR; PII, signal transduction protein (product of the *gln B* gene); PII-UMP, uridylylated form of the PII protein; NRII, nitrogen regulator II, also known as NtrB (product of the *gln L* (*ntrB*)

gene); NRI, nitrogen regulator I, also known as NtrC (product of the *gln G* (*ntrC*) gene).

REFERENCES

(1) Ferrell, J. E. Jr. (1996) Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem. Sci. 21*, 460−466.

(2) Goldbeter, A., and Koshland, D. E. (1981) An amplified sensitivity arising from covalent modification in biological systems. *Proc. Natl. Acad. Sci. U. S. A. 78*, 6840−6844.

(3) Meinke, M. H., Bishop, J. S., and Edstrom, R. D. (1986) Zero-order ultrasensitivity in the regulation of glycogen phosphorylase. *Proc. Natl. Acad. Sci. U. S. A. 83*, 2865−2868.

(4) Ortega, F., Acerenza, L., Westerhoff, H. V., Mas, F., and Cascante, M. (2002) Product dependence and bifunctionality compromise the ultrasensitivity of signal transduction cascades. *Proc. Natl. Acad. Sci. U. S. A. 99*, 1170−1175.

(5) Mutalik, V. K., Shah, P., and Vankatesh, K. V. (2003) Allosteric interactions and bifunctionality make the response of glutamine synthetase cascade system of *Escherichia coli* robust and ultrasensitive. *J. Biol. Chem. 278*, 26327−26332.

(6) Koshland, D. E. Jr., Goldbeter, A., and Stock, J. B. (1982) Amplification and adaptation in regulatory and sensory systems. *Science 217*, 220−225.

(7) Chock, P. B., and Stadtman, E. R. (1977) Superiority of interconvertable enzyme cascades in metabolic regulation: analysis of multicyclic systems. *Proc. Natl. Acad. Sci. U. S. A. 74*, 2766−2770.

(8) Guidi, G. M., Carlier, M.-F., and Goldbeter, A. (1998) Bistability in the isocitrate dehydrogenase reaction: an experimentally based theoretical study. *Biophys. J. 74*, 1229−1240.

(9) Markevich, N. I., Hoek, J. B., and Kholodenko, B. N. (2004) Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. *J. Cell Biol. 164*, 353−359.

(10) Cardenas, M. L., and Cornish-Bowden, A. (1989) Characterisrtics necessary for an interconvertible enzyme cascade to generate a highly sensitive response to an effector. *Biochem. J. 257*, 339−345.

(11) Ninfa, A. J., Jiang, P., Atkinson, M. R., and Peliska, J. A. (2000) Integration of antagonistic signals in the regulation of nitrogen assimilation in *Escherichia coli*. *Curr. Top. Cell. Regul. 36*, 31−75.

(12) Kingdon, H. S., and Stadtman, E. R. (1967) Two *E. coli* glutamine synthetases with different sensitivities to feedback effectors. *Biochem. Biophys. Res. Commun. 27*, 470−473.

(13) Jiang, P., Pioszak, A. A., and Ninfa, A. J. (2007) Structure/function analysis of glutamine synthetase adenylyltransferase (ATase, E.C. 2.7.7.49) of *Escherichia coli*. *Biochemistry 46*, 4117−4132.

(14) Jiang, P., Mayo, A. E., and Ninfa, A. J. (2007) *Escherichia coli* glutamine synthetase adenylyltransferase (ATase, E.C. 2.7.7.49): Kinetic characterization of regulation by PII, PII-UMP, glutamine, and α-ketoglutarate. *Biochemistry 46*, 4133−4146.

(15) Jiang, P., and Ninfa, A. J. (2009) Reconstitution of *Escherichia coli* glutamine synthetase adenylyltransferase from N-terminal and C-terminal fragments of the enzyme. *Biochemistry 48*, 415−423.

(16) Rhee, S.-G., Park, R., Chock, P. B., and Stadtman, E. R. (1978) Allosteric regulation of monocyclic interconvertable enzyme cascade systems: use of *Escherichia coli* glutamine synthetase as an experimental model. *Proc. Natl. Acad. Sci. U. S. A. 75*, 3138−3142.

(17) Zhang, Y., Pohlmann, E. L., Serate, J., Conrad, M. C., and Roberts, G. P. (2010) Mutagenesis and functional characterization of the four domains of GlnD, a bifunctional nitrogen sensor protein. *J. Bacteriol. 192*, 2711−2721.

(18) Jiang, P., Peliska, J. A., and Ninfa, A. J. (1998) Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. *Biochemistry 37*, 12782−12794.

(19) Jiang, P., and Ninfa, A. J. (2007) Escherichia coli PII signal transduction protein controling nitrogen assimilation acts as a sensor of adenylate energy charge *in vitro*. *Biochemistry 46*, 12979−12996.

(20) Kamberov, E. S., Atkinson, M. R., and Ninfa, A. J. (1995) The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP. *J. Biol. Chem. 270*, 17797−17807.

(21) Chapman, A. G., Fall, L., and Atkinson, D. E. (1971) Adenylate energy charge in *Escherichia coli* during growth and starvation. *J. Bacteriol. 108*, 1072−1086.

(22) Jiang, P., and Ninfa, A. J. (2009) Alpha-ketoglutarate controls the ability of the *Escherichia coli* PII signal transduction protein to regulate the activities of NRII (NtrB) but does not control the binding of PII to NRII. *Biochemistry 48*, 11514−11521.

(23) Jiang, P., and Ninfa, A. J. (2009) Sensation and signaling of alpha-ketoglutarate and adenylate energy charge by the *Escherichia coli* PII signal transduction protein require cooperation of the three ligand-binding sites within the PII trimer. *Biochemistry 48*, 11522−11531.

(24) Jiang, P., Peliska, J. A., and Ninfa, A. J. (1998) The regulation of *Escherichia coli* glutamine synthetase revisited: role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. *Biochemistry 37*, 12802−12810.

(25) Ventura, A. C., Jiang, P., Van Wassenhove, L., Del Vecchio, D., Merajver, S. D., and Ninfa, A. J. (2010) Signaling properties of a covalent modification cycle are altered by a downstream target. *Proc. Natl. Acad. Sci. U. S. A. 107*, 10032−10037.

(26) Atkinson, M. R., Kamberov, E. S., Weiss, R. L., and Ninfa, A. J. (1994) Reversible uridylylation of the Escherichia coli PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC). *J. Biol. Chem. 269*, 28288−28293.

(27) Jiang, P., Zucker, P., and Ninfa, A. J. (1997) Probing interactions of the homotrimeric PII signal transduction protein with its receptors by use of PII heterotrimers formed *in vitro* from wild-type and mutant subunits. *J. Bacteriol. 179*, 4354−4360.

(28) Taketa, K., and Pogell, B. M. (1965) Allosteric inhibition of rat liver fructose 1, 6-diphosphatase by Adenosine 5′-Monophosphate. *J. Biol. Chem. 240*, 651−662.

(29) Ventura, A. C., Sepulchre, J. A., and Merajver, S. D. (2008) A hidden feedback in signalling cascades is revealed. *PLoS Comput. Biol. 4*, e1000041.

(30) Stadtman, E. R., and Chock, P. B. (1977) Superiority of interconvertable enzyme cascades in metabolite regulation: analysis of monocyclic systems. *Proc. Natl. Acad. Sci. U. S. A. 74*, 2761−2765.

(31) Jiang, P., Peliska, J. A., and Ninfa, A. J. (1998) Reconstitution of the signal-transduction bicyclic cascade responsible for the regulation of Ntr gene transcription in *Escherichia coli*. *Biochemistry 37*, 12795−12801.

(32) Jiang, P., and Ninfa, A. J. (1999) Regulation of autophosphorylation of *Escherichia coli* nitrogen regulator II by the PII signal transduction protein. *J. Bacteriol. 181*, 1906−1911.

(33) Jiang, P., Atkinson, M. R., Srisawat, C., Sun, Q., and Ninfa, A. J. (2000) Functiuonal dissection of the dimerization and enzymatic activities of *Escherichia coli* nitrogen regulator II and their regulation by the PII protein. *Biochemistry 39*, 13433−13449.

(34) Pioszak, A. A., and Ninfa, A. J. (2004) Mutations altering the N-terminal receiver domain of NRI (NtrC) that prevent dephosphorylation by the NRII-PII complex in *Escherichia coli*. *J. Bacteriol. 186*, 5730−5740.

(35) Mecke, D., Wulff, K., and Holzer, H. (1966) Characterization of a glutamine synthetase inactivating enzyme from *Escherichia coli*. *Biochem. Biophys. Res. Commun. 24*, 452−458.

(36) vanHeeswijk, W. C., Stegeman, B., Hoving, S., Molenaar, D., Kahn, D., and Westerhoff, H. V. (1995) An additional PII in *Escherichia coli*: a new regulatory protien in the glutamine synthetase cascade. *FEMS Microbiol. Lett. 132*, 153−157.

(37) Atkinson, M. R., and Ninfa, A. J. (1998) Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. *Mol. Microbiol. 29*, 431−447.

(38) Llacer, J. L., Contreras, A., Forchhammer, K., Marco-Marin, C., Gil-Ortiz, F., Maldonado, R., Fita, I., and Rubio, V. (2007) The crystal structure of the complex of PII and acetylglutamate kinase reveals how

PII controls the storage of nitrogen as arginine. *Proc. Natl. Acad. Sci. U. S. A. 104*, 17644−17649.
(39) Hart, Y., Madar, D., Yuan, J., Bren, A., Mayo, A. E., Rabinowitz, J. D., and Alon, U. (2011) Robust control of nitrogen assimilation by a bifunctional enzyme in *E. coli*. *Mol. Cell 41*, 117−127.