Reconstitution of the Signal-Transduction Bicyclic Cascade Responsible for the Regulation of Ntr Gene Transcription in *Escherichia coli*†

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ABSTRACT: Nitrogen-regulation of gene transcription in *Escherichia coli* results from the regulation of the phosphorylation state of the enhancer-binding transcription factor NRI (NtrC). We examined the regulation of NRI phosphorylation in a reconstituted bicyclic cascade system containing four regulatory proteins: NRI, the signal-transducing uridylyltransferase/uridylyl-removing enzyme (UTase/UR), its substrate the signal transduction protein PII, and the kinase/phosphatase NRII (NtrB), which is a PII receptor that phosphorylates and dephosphorylates NRI. In this reconstituted system, the phosphorylation state of NRI was regulated reciprocally by the small molecule effectors glutamine, which prevented the accumulation of NRI−P, and 2-ketoglutarate, which caused accumulation of NRI−P. Regulation of the bicyclic system by glutamine was exclusively due to sensation and signal-transduction by the UTase/UR−PII monocycle, which was observed to function essentially as a glutamine-sensing apparatus. In contrast, regulation of NRI phosphorylation by 2-ketoglutarate, which binds to PII, was due to direct regulation of the NRII−PII interaction and the rate of NRI−P dephosphorylation. Thus, the PII protein transduces the glutamine signal to the NRII−NRI monocycle in the form of its uridylylation state and is also the receptor of the antagonistic 2-ketoglutarate signal, which blocks the activity of unmodified PII.

Escherichia coli, and many other bacteria, coordinates the assimilation of nitrogen with the assimilation of carbon so as to sustain balanced growth under a variety of environmental conditions (*1*). Previous work has suggested that glutamine serves as an intracellular signal molecule indicating nitrogen sufficiency (*1*, *2*) and 2-ketoglutarate serves as an intracellular signal molecule indicating carbon sufficiency (*1*). These antagonistic signals regulate the phosphorylation state of the enhancer-binding transcription factor NRI,[1] which in its phosphorylated form is the transcripitional activator of the Ntr (nitrogen-regulated) regulon (*3*). The Ntr regulon includes the *glnA* gene encoding glutamine synthetase (GS), the most important enzyme for the assimilation of ammonia, and genes encoding products that enable the cell to generate ammonia from organic nitrogen sources (reviewed in ref *4*). The PII signal transduction protein regulates the phosphorylation state of NRI and the transcription of Ntr genes indirectly, by stimulating the dephosphorylation of NRI−P by the kinase/phosphatase NRII [Figure 1 (*3*, *5*, *18*, *19*)]. Various lines of evidence suggest that NRII contains the NRI−P phosphatase activity and PII is an activator of that activity (*5*). The interaction of PII with NRII is prevented by the uridylylation of PII, catalyzed by uridylyltransferase/uridylyl-removing enzyme (UTase/UR, Figure 1). The modified form of PII, PII−UMP, is unable to bind to NRII

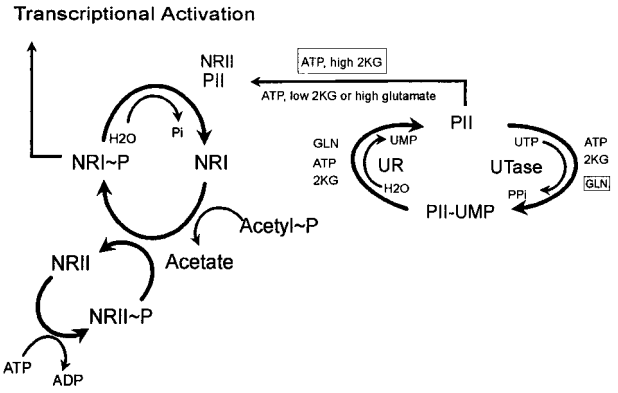

FIGURE 1: Signal transduction system responsible for the regulation of Ntr gene transcription. Effectors that act as activators are shown unboxed, while effectors that act as inhibitors are shown boxed. NRI and NRI−P, unphosphorylated and phosphorylated forms of the enhancer binding transcription factor; NRII, the kinase/phosphatase regulating NRI phosphorylation state; PII, PII−UMP, unmodified and uridylylated forms of the signal transduction protein controlling NRII phosphatase activity; UTase/UR, uridylyltransferase/uridylyl-removing enzyme controlling PII uridylylation state. GLN, glutamine; 2KG, 2-ketoglutarate.

(*6*) or activate the dephosphorylation of NRI−P (*7*). Thus, the UTase/UR regulates the activity of PII, which in turn regulates the phosphatase activity of NRII, forming the bicyclic cascade system shown in Figure 1. In previous models of nitrogen regulation, the antagonistic signals glutamine and 2-ketoglutarate were thought to act by regulating the PII uridylylation state (ref *8* and reviewed in refs *9* and *10*).

NRI and NRII are members of the response regulator and histidine kinase/phosphatase families of signal transduction proteins that form the two-component regulatory systems

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[1] Abbreviations: NRI, nitrogen regulator I, *glnG* (*ntrC*) product; NRI−P, phosphorylated NRI; PII, protein II (*glnB* product); NRII, nitrogen regulator II, *glnL* (*ntrB*) product; UTase/UR, uridylyltransferase/uridylyl-removing enzyme (*glnD* product); ATase, adenylyltransferase (*glnE* product); GS, glutamine synthetase (*glnA* product).

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(reviewed in refs *11* and *12*). These signal transduction systems use the phosphorylation of a conserved response regulator domain to control the activity of other protein domains, or use this phosphorylation as a step in a signal-transducing phosphorelay system (*13*). The histidine kinase/phosphatase NRII binds ATP and catalyzes its own phosphorylation on a histidine residue, and these phosphoryl groups are transferred to an aspartate residue on the response regulator NRI (*14*−*17*).

In addition to its phosphorylation by phosphotransfer from NRII−P, NRI may become phosphorylated directly by acetyl−phosphate [Figure 1 (*20*)], which is formed from acetyl−CoA and Pi by the enzyme phosphotransacetylase (*pta* product) and from acetate and ATP by the enzyme acetate kinase (*ackA* product; reviewed in ref *21*). A number of other response regulator proteins are also directly phosphorylated by acetyl−phosphate (*22*), and it has been suggested that this common use of acetyl−phosphate provides a means for communication between distinct two-component systems in the cell (*11*). NRII plays a major role in regulating the intracellular acetyl−phosphate concentration (*23*), probably by acting as a sink for phosphoryl groups under nitrogen-rich conditions by dephosphorylating NRI−P (in concert with PII). While both mechanisms for the phosphorylation of NRI must be present to achieve the high levels of NRI−P necessary for transcription of some Ntr promoters, either mechanism in isolation is able to provide sufficient NRI−P for the full activation of the nitrogen-regulated *glnA* promoter (*20*). This promoter, *glnAp2*, is endowed with a highly efficient enhancer capable of capturing NRI−P at low concentration (*24*, *25*). Genetic and physiological studies indicate that the tight negative regulation of *glnA* transcription by ammonia or glutamine requires NRII, as well as the UTase/UR and PII [Figure 1 (*26*)].

In cells deleted for *pta* and *ackA*, and therefore unable to synthesize acetyl−phosphate, the transcription of *glnA* is apparently regulated by the ratio of carbon to nitrogen (C. Chen, and A.J.N., unpublished data), as in wild-type cells (ref *1*; C. Chen, and A.J.N., unpublished data). The reconstituted enzymatic systems described in this paper were used to examine the mechanism of this antagonistic regulation by carbon and nitrogen signals occurring in the absence of acetyl−phosphate.

The uridylylation and deuridylylation of the PII protein by the UTase/UR have recently been studied in detail (*19*, *27*). Both uridylylation and deuridylylation reactions are activated by very low concentrations of 2-ketoglutarate and ATP, which bind to the PII protein (*27*). In addition, glutamine, which binds the enzyme, is a potent inhibitor of the uridylylation of PII and an activator of the uridylyl-removing reaction (*27*, *28*). Since the concentrations of 2-ketoglutarate required for the activation of the UTase and UR activities (*27*) are well below the reported intracellular concentrations of this compound [0.1−0.9 mM (*1*)], it was proposed that the UTase/UR−PII monocycle is likely to serve mainly as a glutamine-sensing apparatus in intact cells (*27*). Concentrations of 2-ketoglutarate within the physiological range did not inhibit the uridylylation and deuridylylation reactions (*27*).

The ability of PII to activate the phosphatase activity of NRII is also directly controlled by 2-ketoglutarate (*6*, *29*). Very low concentrations of 2-ketoglutarate (∼5 μM) greatly stimulated the PII-mediated activation of NRI−P dephosphorylation (*6*, *29*). However, higher concentrations of 2-ketoglutarate, such as the reported in vivo concentration of 0.1−0.9 mM, weakly stimulated the dephosphorylation of NRI−P (*6*, *29*).

Direct measurements of the binding of 2-ketoglutarate to the Ntr regulatory components and kinetic analysis of the UTase and UR reactions indicated that the PII protein is the sole receptor for 2-ketoglutarate (*6*, *27*). The binding of 2-ketoglutarate and a second effector, ATP, is synergistic, with each effector stimulating the binding of the other.

In this study, we first examined the regulation of the steady-state level of PII modification by 2-ketoglutarate and glutamine in a reconstituted PII−UTase/UR monocycle. Results of those studies indicated that the steady-state extent of PII modification was regulated mainly by the glutamine concentration. We then examined the role of 2-ketoglutarate and glutamine in regulating the extent of phosphorylation of NRI in a reconstituted bicyclic system containing the UTase/UR, PII, NRII, and NRI. Our results indicate that these effectors antagonistically regulate the accumulation of NRI−P and act by distinct mechanisms. The 2-ketoglutarate effects were mainly due to direct regulation of the interaction of unmodified PII with NRII, while glutamine effects were completely due to the effects of this compound on controlling the UTase/UR activities and the uridylylation state of PII.

MATERIALS AND METHODS

Purified Proteins. NRI was the generous gift of E. S. Kamberov and was purified as described previously (*19*). UTase/UR and PII were the generous gifts of M. R. Atkinson and were purified as described previously (*19*). NRII was the generous gift of P. Zucker and was purified as described previously (*19*). Each of these proteins is greater than 90% pure, as judged by their appearance on SDS−polyacrylamide gels (not shown), and is free of the other activities. Protein concentrations were determined by the Lowry method (*30*), with BSA as the standard. Previous work demonstrated that the Lowry assay provides an accurate estimation of protein concentration for these proteins (unpublished data). Concentrations of the PII protein were determined independently for each experiment by completely uridylylating an aliquot of PII and determining the moles of labeled UMP incorporated. This concentration value, which agreed closely with the nominal input based on the Lowry, was used to standardize the PII protein concentration for each experiment.

Reconstitution of the UTase/UR−PII Monocycle. The reaction mixtures contained 100 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 0.3 mg/mL BSA, 2-ketoglutarate as indicated, glutamine as indicated, 0.5 mM ATP, 0.5 mM UTP (α-^{32}P), 3 μM PII (trimer), and 0.3−0.6 μM UTase/UR (monomer) as indicated. Reactions were incubated at 30 °C, and aliquots were removed at the indicated times. These samples were spotted onto nitrocellulose filters, which were immersed immediately in 5% (w/v) TCA and washed extensively with this solvent, dried, and subjected to liquid scintillation counting in a Beckman LS3801 instrument. Previous studies have shown that under these conditions the acid-insoluble radioactivity is due to the uridylylation of PII by labeled [α-^{32}P]UTP (*19*, *31*). The uridylylation state of PII [expressed as the average number of uridylyl groups per PII trimer, n (n = 0−3)] was

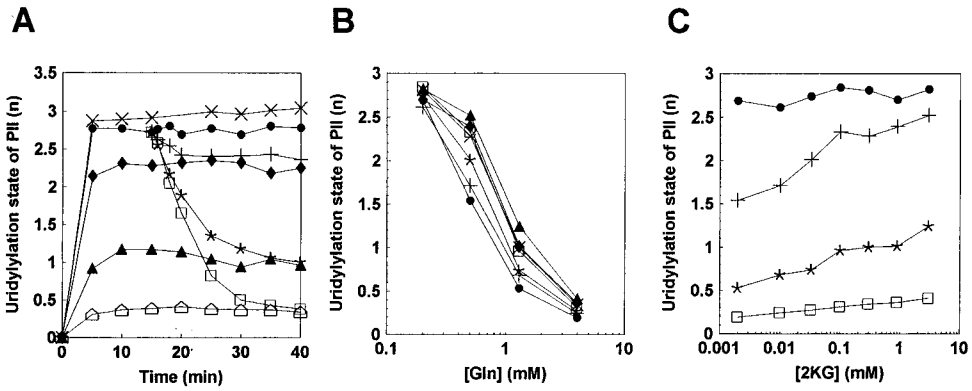

FIGURE 2: Reconstitution of the UTase/UR−PII monocycle. The reaction conditions were as described in the Materials and Methods. (Panel A) Regulation of PII uridylylation state by glutamine. The glutamine concentration was varied at the fixed 2-ketoglutarate concentration of 0.3 mM. Glutamine concentrations were (×) zero; (dot) 0.2 mM; (diamond) 0.5 mM; (triangle) 1.3 mM; (house) 4 mM. The curves indicated (+, asterisk, and square) show the results of addition of glutamine to final concentrations of 0.5, 1.3, and 4 mM, respectively, to reaction mixtures that initially contained 0.2 mM glutamine. (Panels B and C) Summary of the effect of glutamine and 2-ketoglutarate on the state of PII uridylylation. In panel B, glutamine was varied and 2-ketoglutarate was fixed at (dot) 2 uM; (+) 10 μM; (asterisk) 33 μM; (square) 0.1 mM; (×) 0.3 mM; (diamond) 0.9 mM; and (triangle) 3 mM. In panel C, 2-ketoglutarate was varied and glutamine was fixed at (dot) 0.2 mM; (+) 0.5 mM; (asterisk) 1.3 mM; and (square) 4 mM. The average number of covalently attached uridylyl groups per PII trimer (*n*) is shown on the *y*-axis; for panels B and C, the steady-state *n* value is shown.

determined by dividing the moles of UMP incorporated by the moles of PII protein. Under the assay conditions used here, the steady-state levels of PII uridylylation were achieved in a few minutes. Variation in the ATP concentration from 0.1 to 3 mM did not have a significant effect on the steady-state extent of PII uridylylation (data not shown). To investigate whether UTase activity decreased during the incubation period, 0.01 μM UTase/UR was preincubated in similar reaction mixtures lacking ATP and UTP at 30 °C for up to 40 min, after which the nucleotides were added and the initial velocity of PII uridylylation was measured. In these experiments, no loss of UTase activity was observed with preincubations of up to 40 min (data not shown). We also examined whether variations in the UTase/UR and PII concentrations would affect the steady-state level of PII uridylylation. Variation of the PII concentration from 0.3 to 3.0 μM and variation of the UTase/UR concentration from 0.06 to 0.3 μM did not significantly alter the steady-state extent of PII modification (data not shown).

Reconstitution of the UTase/UR−PII−NRII−NRI Bicycle. Conditions were similar to those described above, except that NRI and NRII were present. Concentrations were 15 μM NRI (dimers), 0.3 μM NRII (dimers), 0.5 μM PII, 0.3 μM UTase/UR, 1−2 mM ATP, and 1 mM UTP. Incubation was at 25 °C. [γ-^{32}P]ATP or [α-^{32}P]UTP was present, depending on whether the extent of NRI or PII covalent modification was measured. For reconstitution of the NRII−NRI monocycle, UTase/UR and UTP were omitted, and PII was added as indicated. In the bicycle, the effect of inclusion of NRI and NRII on the uridylylation state of PII was estimated (data not shown). The combination of 15 μM NRI and 0.3 μM NRII did not alter the steady-state extent of PII uridylylation significantly, although at these concentrations, NRI alone increased and NRII alone decreased the extent of PII modification ~10% when glutamine was 0.2 mM and 2-ketoglutarate was 0.3 mM.

RESULTS

To learn the mechanisms by which 2-ketoglutarate and glutamine regulate the phosphorylation state of NRI, we first reconstituted the UTase/UR−PII monocycle and examined the roles of these effectors in controlling the PII uridylylation state. We then reconstituted the UTase/UR−PII−NRII−NRI bicyclic system and again examined the roles of these effectors. Next, we reexamined the role of 2-ketoglutarate in regulating the dephosphorylation of NRI−P in a monocyclic system containing NRII, NRI, and PII. Finally, the role of the glutamine/2-ketoglutarate ratio in regulating the bicyclic system was examined.

The UTase/UR−PII Monocycle is a Glutamine-Sensing Apparatus. In a reconstituted UTase/UR−PII monocycle, the extent of PII uridylylation quickly reached a steady-state level that was dependent on the glutamine concentration and was essentially independent of the 2-ketoglutarate concentration at physiological concentrations of these effectors (Figure 2). An example of a typical experiment, in which the effect of glutamine was examined at the fixed 2-ketoglutarate concentration of 300 μM, is shown in Figure 2A. The steady state level of PII uridylylation, *n*, was characteristic of the glutamine concentration (PII is a trimer, so *n* may vary between 0 and 3). Once a steady state of uridylylation had been obtained, subsequent addition of glutamine quickly resulted in adjustment in the extent of PII uridylylation to the level characteristic of the new glutamine concentration (Figure 2A).

Prior studies indicated that 2-ketoglutarate and ATP are essential activators of both the UTase and UR activities and that these effectors bind to PII and PII−UMP but not to the UTase/UR (*27*, *31*, *32*). Those studies also indicated that, in the presence of the in vivo concentration of ATP, very low concentrations of 2-ketoglutarate were required for activation of both the uridylyltransferase and uridylyl-removing activities. Furthermore, higher concentrations of 2-ketoglutarate, similar to the concentrations reported in vivo, did not significantly inhibit either the uridylylation or the deuridylylation of PII. Those studies suggested that 2-ketoglutarate was unlikely to play a regulatory role on the UTase/UR monocycle (*27*). This hypothesis was tested in a series of experiments where the steady-state extent of PII uridylylation was measured in the presence of various concentrations of glutamine and 2-ketoglutarate (summarized

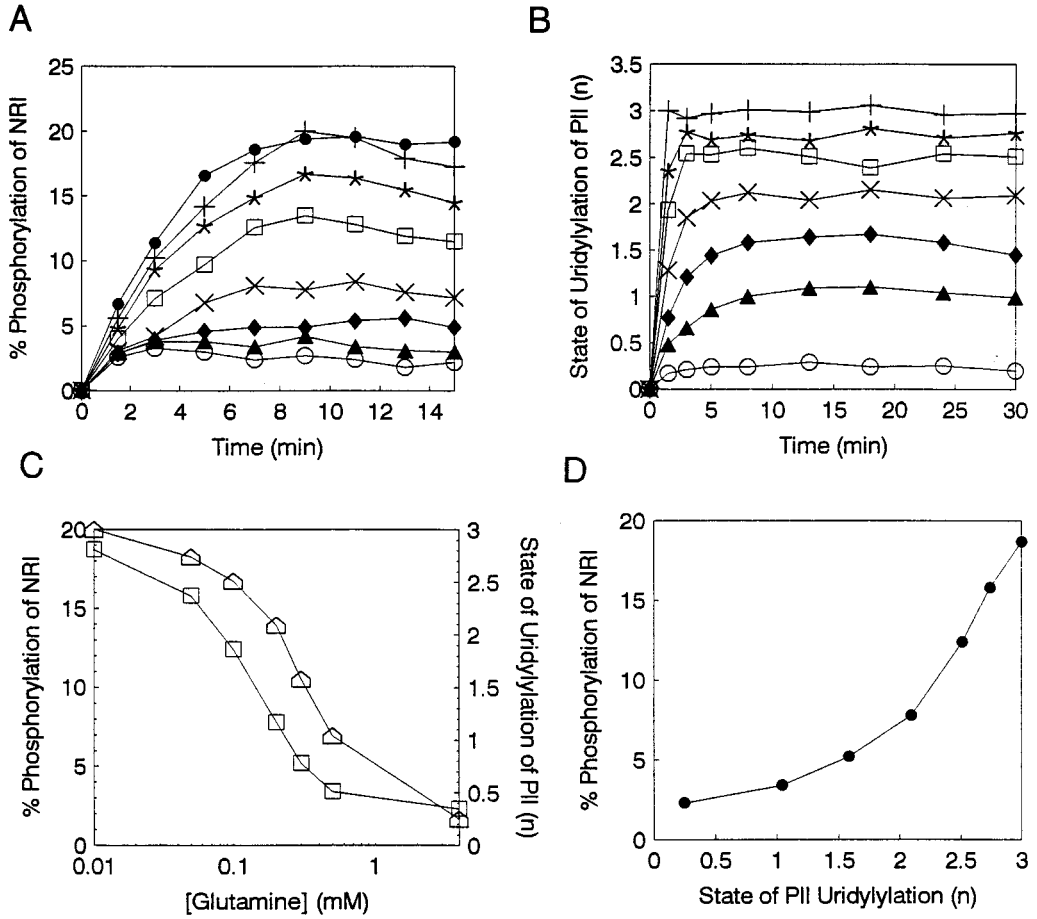

FIGURE 3: Reconstitution of the UTase/UR−PII−NRII−NRI bicycle. (A) Phosphorylation of NRI at various glutamine concentrations. The concentration of 2-ketoglutarate was 0.3 mM. (B) Uridylylation of PII at various glutamine concentrations. The experiments shown in panels A and B were performed side-by-side with identical conditions. For panels A and B the glutamine concentrations were (dot) no PII and 4 mM glutamine; (+) -glutamine; (*) 0.05 mM; (square) 0.1 mM; (×) 0.2 mM; (diamond) 0.3 mM; (triangle) 0.5 mM; (○) 4 mM. (Panel C) Effect of glutamine on the steady-state levels of NRI phosphorylation and PII uridylylation. Symbols are (square) % phosphorylation of NRI; (house) uridylylation state of PII (*n*). (Panel D) Relationship between uridylylation state of PII and phosphorylation state of NRI in the coupled system.

in Figure 2, panels B and C). Glutamine was a potent regulator of the extent of PII uridylylation at every 2-ketoglutarate concentration examined. In contrast, 2-ketoglutarate effects were mainly limited to concentrations of this effector well below the reported in vivo range (0.1−0.9 mM; Figure 2, panels B and C). These experiments show that when 2-ketoglutarate was within the physiological range of 0.1−0.9 mM (*1*), the UTase/UR−PII monocycle was a glutamine-sensing apparatus.

Regulation of the Phosphorylation State of NRI by Glutamine in a Reconstituted UTase/UR−PII−NRII−NRI Bicycle. Previous studies have demonstrated that when NRII and NRI are combined in the presence of ATP, NRI quickly becomes phosphorylated. The steady-state extent of this phosphorylation depends on the relative concentrations of NRII and NRI as well as on the ATP concentration (data not shown). In the experiments shown here, NRI and ATP were present in large excess primarily because, under these conditions, the extent of NRI phosphorylation was easily and accurately measured. Prior studies have also demonstrated that in the absence of the UTase/UR, the extent of NRI phosphorylation was insensitive to the presence of glutamine and/or 2-ketoglutarate, but was sensitive to the presence of PII, which caused dephosphorylation of NRI−P (*3*). This activity of PII was greatly stimulated by 2-ketoglutarate at low concentrations, but at higher 2-ketoglutarate concentrations, the steady-state extent of NRI phosphorylation was increased due to inhibition of NRI−P dephosphorylation (*6*, *29*).

The significance of the observations noted above became apparent when we examined the regulation of NRI phosphorylation in a reconstituted bicyclic system containing the UTase/UR, PII, NRII, and NRI. To monitor both the uridylylation of PII and the phosphorylation of NRI, duplicate reaction mixtures were assembled, which were identical in all respects except for the position of the radioactive label. In the bicyclic system, glutamine regulated both the extent of NRI phosphorylation (Figure 3A) and the extent of PII uridylylation (Figure 3B). The relationship between these covalent modifications was not proportional. At the conditions used, significant accumulation of NRI−P did not occur until PII had been largely converted to PII−UMP (Figure 3, panels C and D). This phenomenon is consistent with recent data showing that a single functional unmodified subunit in a PII trimer is sufficient for productive interaction of PII with its receptors (*32*).

In a series of additional experiments, we examined the extent of NRI phosphorylation in reaction mixtures containing unmodified PII at various concentrations but no UTase/UR (i.e., in the NRII−NRI monocyclic system) and in the

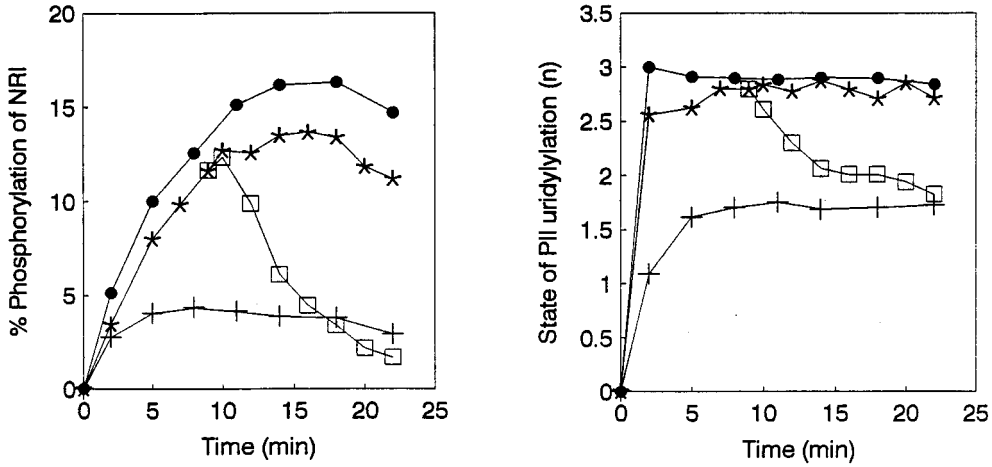

FIGURE 4: Response of the UTase/UR−PII−NRII−NRI bicyclic system to glutamine addition. The left panel shows the effect on NRI phosphorylation, and the right panel shows the effect on PII uridylylation. The experiments were performed side-by-side under identical conditions and differed only in which nucleotide was labeled. The concentration of 2-ketoglutarate was 0.3 mM. Initial glutamine concentrations were (dot) zero; (asterisk) 0.05 mM; (+) 0.3 mM. After 9 min, glutamine was added to a portion of the reaction mixtures containing 0.05 mM glutamine to bring the final concentration to 0.3 mM. These curves are indicated with (squares).

complete bicyclic system with various glutamine concentrations. Since the concentration of unmodified PII in the coupled system can be directly observed at the different glutamine concentrations (Figures 2 and 3), this permitted the comparison of the effectiveness of various concentrations of fully unmodified PII in the NRII−NRI monocycle with the effectiveness of PII in the coupled system, where unmodified and modified subunits are distributed randomly in trimers (*7*). These experiments indicated that the concentration of unmodified PII subunits regulated the extent of NRI phosphorylation (data not shown). That is, we could imitate the effects of providing a given glutamine concentration and the UTase/UR by providing instead the concentration of unmodified PII subunits found at that glutamine concentration (data not shown). This argues that unmodified PII subunits were equally effective in bringing about the dephosphorylation of NRI−P when distributed randomly in trimers with modified subunits or when present in fully unmodified trimers. It also suggests that the sole effect of glutamine in the coupled system is to bring about the deuridylylation of PII via its effects on the UTase and UR activities.

Further addition of glutamine to the bicyclic system at equilibrium resulted in the rapid reestablishment of new steady-state levels of NRI phosphorylation and PII uridylylation characteristic of the final glutamine concentration (Figure 4).

Regulation of the Phosphorylation State of NRI by 2-Ketoglutarate in a Reconstituted UTase/UR−PII−NRII−NRI Bicycle. Although variation of 2-ketoglutarate within the range 0.1−0.9 mM had little effect on the extent of PII uridylylation, such variation had a pronounced effect on the extent of NRI phosphorlyation in the coupled system (Figure 5). As shown, 2-ketoglutarate was antagonistic to glutamine and stimulated the accumulation of NRI−P (Figure 5A). While glutamine was the more potent regulator, 2-ketoglutarate clearly caused a 2−4-fold change in the extent of NRI phosphorylation (Figure 5A). In contrast, within this range of 2-ketoglutarate concentrations, variation of 2-ketoglutarate had no effect on the uridylylation state of PII (Figure 5B). Thus, in the bicyclic system, 2-ketoglutarate altered the extent

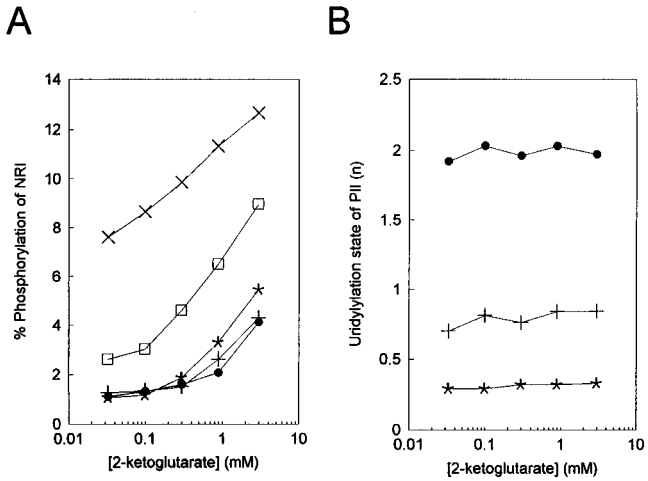

FIGURE 5: Regulation of NRI phosphorylation by glutamine and 2-ketoglutarate in a coupled UTase/UR−PII−NRII−NRI bicyclic system. (Panel A) Phosphorylation state of NRI was plotted as a function of the 2-ketoglutarate concentration. The different glutamine concentrations were (×) 0.08 mM; (square) 0.2 mM; (asterisk) 0.5 mM; (+) 1.3 mM; and (dot) 4 mM. (Panel B) Examination of the uridylylation state of PII in the coupled system at identical conditions. PII uridylylation state (*n*) was plotted as a function of the 2-ketoglutarate concentration; the three glutamine concentrations examined were (dot) 0.2 mM; (+) 0.5 mM; and (asterisk) 1.3 mM.

of NRI phosphorylation without altering the extent of PII uridylylation.

We examined whether the accumulation of NRI−P in response to 2-ketoglutarate was due to the effect of 2-ketoglutarate on the rate of NRI−P dephosphorylation (*6*). In the absence of the UTase/UR (i.e., in the NRII−NRI monocyclic system), 2-ketoglutarate resulted in a considerable decrease in the ability of PII to elicit the dephosphorylation of NRI−P (Figure 6), as observed previously (*6*). For comparison, the effect of various 2-ketoglutarate concentrations on the extent of NRI phosphorylation in the bicyclic system is shown at 0.2 mM glutamine (Figure 6). These reciprocal effects of 2-ketoglutarate occurred at the same concentration (Figure 6).

Regulation of the Phosphorylation State of NRI by the Ratio of Glutamine and 2-Ketoglutarate in a Reconstituted

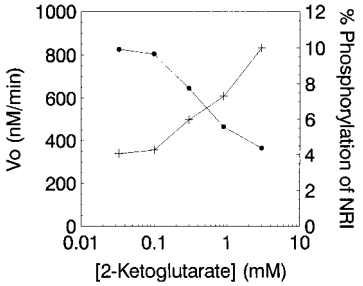

FIGURE 6: Comparison of the phosphorylation state of NRI in the coupled system and initial velocity of NRI−P dephosphorylation in the monocyclic system as a function of the 2-ketoglutarate concentration. Experiments were performed as described in the Materials and Methods. The % NRI phosphorylation data (+) is from the coupled system, and represents the mean value of the last three time points, when the system had achieved the steady state. For the initial velocity data (dot), from the NRI−NRII−PII monocyclic system, the initial rate of NRI−P dephosphorylation was corrected by subtraction of the rate obtained when PII was added in the absence of 2-ketoglutarate.

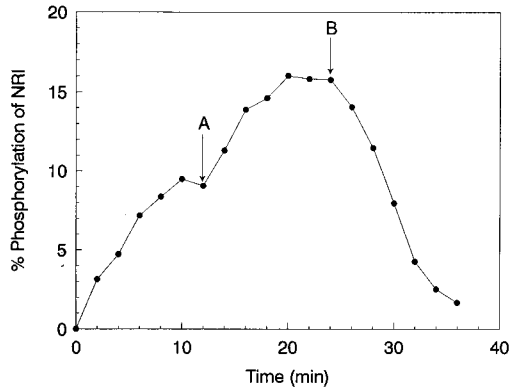

FIGURE 7: Response of the UTase/UR−PII−NRII−NRI bicycle to sequential addition of 2-ketoglutarate and glutamine. Conditions were as described in the Materials and Methods. At 0 time, 0.08 mM glutamine and 0.033 mM 2-ketoglutarate were present. After 12 min, additional 2-ketoglutarate was added to bring the final concentration to 3 mM (A). After an additional 12 min (i.e., at the 24 min point), the glutamine concentration was increased to 4 mM (B).

UTase/UR−PII−NRII−NRI Bicycle. In cells, the expression of *glnA* and the modification of GS are correlated with the ratio of the internal glutamine and 2-ketoglutarate concentrations (*1*). We further examined the antagonistic effect of 2-ketoglutarate and glutamine in the coupled system by observing the effect of sequential addition of these effectors (Figure 7). As shown, addition of a high concentration of 2-ketoglutarate to the system at steady-state resulted in the rapid further accumulation of NRI−P to a new higher steady-state level. Subsequent addition of a high glutamine concentration then resulted in a decrease in the extent of NRI phosphorylation (Figure 7).

DISCUSSION

Reconstitution of the UTase/UR Monocycle. In this paper, we extended our study of the UTase/UR−PII monocycle to include a characterization of the steady-state extent of PII modification in response to various concentrations of glutamine and 2-ketoglutarate. Our results indicated that PII uridylylation state was strongly regulated by glutamine at every concentration of 2-ketoglutarate examined. At physiological concentrations, 2-ketoglutarate was not an effective regulator of the PII uridylylation state. This arrangement was expected, based on kinetic analysis of the UTase and UR reactions (*27*). In our reconstituted monocycle, the transferase and uridylyl-removing activities were suitably balanced in the presence of physiological 2-ketoglutarate and glutamine concentrations, such that the monocycle served as a glutamine-sensing apparatus (Figure 2A). This result is not consistent with the widely held view that the extent of PII modification is regulated by the ratio of the concentrations of glutamine and 2-ketoglutarate (*9*). While 2-ketoglutarate had important regulatory effects on NRI phosphorylation state (this paper) and GS adenylylation state (*37*) in our reconstituted systems, these effects were not due to regulation of the PII uridylylation state.

Reconstitution of the UTase/UR−PII−NRII−NRI Bicyclic System. In our bicyclic system, the extent of NRI phosphorylation was regulated reciprocally by glutamine and 2-ketoglutarate. Regulation by glutamine was completely due to the regulation of PII uridylylation state by this effector. We could obtain the same level of NRI phosphorylation either by adding the UTase/UR and a given glutamine concentration to reaction mixtures or by providing instead the concentration of unmodified PII subunits characteristically found at that glutamine concentration in the presence of the UTase/UR. This suggests that the sole role of glutamine in the reconstituted bicycle is to regulate the concentration of unmodified PII subunits by its control of the UTase and UR reactions. In contrast, 2-ketoglutarate regulated the extent of NRI phosphorylation both in the presence and in the absence of the UTase/UR. This was due to regulation of the rate of NRI−P dephosphorylation by 2-ketoglutarate (*6*, *29*).

PII Is Subjected to Antagonistic Covalent Modification and Allosteric Regulation. The results discussed above indicate that PII transmits the antagonistic glutamine and 2-ketoglutarate signals by distinct mechanisms. We hypothesize that 2-ketoglutarate exerts its effect by direct allosteric modification of PII activity. Previous studies indicated that, among these proteins, only PII binds to 2-ketoglutate (*6*, *27*). The binding data and kinetics presented in the previous article in this issue suggest that the binding of a molecule of 2-ketoglutarate exerts strong negative cooperativity on the binding of additional molecules of this effector to PII (*27*). We hypothesize that, at elevated 2-ketoglutarate concentrations, this negative cooperativity is overcome and the fully liganded PII assumes a distinct conformation that does not interact efficiently with NRII (this paper) or ATase (*37*) but which interacts well with the UTase/UR (*27*). Previous studies have indicated that the interactions of PII with the UTase/UR, ATase, and NRII are subtly different and genetically separable (*31*). Comparisons of the results from this work and the next paper in this issue (*37*) suggest that the form of PII present at high 2-ketoglutarate concentration interacts somewhat better with NRII than it does with ATase.

Additional evidence for the allosteric regulation of PII activity by 2-ketoglutarate comes from the study of the uridylylation of PII in the presence of Mn^{2+} (*27*). In the presence of Mn^{2+}, the binding of PII to the enzyme requires ATP, but does not require 2-ketoglutarate. A significant rate of PII uridylylation is observed in the absence of 2-ketoglutarate, and this activity is strongly inhibited by 2-ketoglutarate at low concentration (2−10 μM). Above 33 μM, the inhibition by 2-ketoglutarate is reversed by increasing

its concentration, and is no longer significant when 2-ketoglutarate is ~1 mM (*27*). Therefore, this result also suggested that PII may exist in multiple forms, corresponding to occupancy by one or more than one molecule of 2-ketoglutarate, and that the dissociation constant for the second molecule of this ligand is considerably higher than that of the first to bind.

Interaction of Different Ligands with PII Permits Complex Combinatorial Effects on Signal Transduction. Although our hypothesis serves to explain the role of 2-ketoglutarate in our reconstituted systems, it may be a vast simplification (at best) of the situation in vivo. Previous binding studies have indicated that glutamate, 3-ketoglutarate, oxaloacetate, and other related molecules could compete with 2-ketoglutarate for binding to PII, albeit at considerably higher concentrations (*6*, *29*). Furthermore, while glutamate could stimulate the interaction of PII with NRII, it could not stimulate the uridylylation of PII by the UTase/UR (*6*). This suggests that these effectors bring about different PII conformations. In cells, many possible PII binding effectors may have cumulative and synergistic or antagonistic effects that are unanticipated at this time. Furthermore, signals of carbon status are apparently transmitted by redundant and overlapping mechanisms, including regulation by acetyl−phosphate (*20*). Many bacteria contain multiple PII proteins (*34*−*36*), and these may lead to a further refinement in regulation since different paralogues may be synthesized only under certain conditions, could respond differently to small molecule effectors, or may interact differently with the various PII receptors.

Evolution of the PII System. Since the interaction of PII with a receptor may be directly regulated by the binding of small molecule effectors to PII, it seems reasonable that this process evolved prior to the regulation of PII activity by covalent modification. Subsequently, the covalent modification of PII may have subjected the targeted receptors to additional beneficial regulation, such as regulation by glutamine in the *E. coli* Ntr system. PII proteins are found in a wide array of bacteria, and in some cases, a cognate UTase/UR homologue appears to be absent. Indeed, in some bacteria, PII activity is controlled by reversible phosphorylation instead of uridylylation (*33*). Since our results indicate that PII plays a key sensory role in Ntr signal transduction, we predict that other bacterial PII will also be shown to act as direct sensors of stimulating ligands, which allosterically regulate the PII activity.

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