The Regulation of *Escherichia coli* Glutamine Synthetase Revisited: Role of 2-Ketoglutarate in the Regulation of Glutamine Synthetase Adenylylation State†

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ABSTRACT: The regulation of *Escherichia coli* glutamine synthetase (GS) by reversible adenylylation has provided one of the classical paradigms for signal transduction by cyclic cascades. Yet, many mechanistic features of this regulation remain to be elucidated. We examined the regulation of GS adenylylation state in a reconstituted system containing GS, adenylyltransferase (ATase), the PII signal transduction protein that controls ATase, and the uridylyltransferase/uridylyl-removing enzyme (UTase/UR), which has a role in regulating PII. In this reconstituted bicyclic cascade system, the adenylylation state of GS was regulated reciprocally by the small molecule effectors 2-ketoglutarate and glutamine at physiological effector concentrations. By examination of the individual regulatory monocycles and comparison to the bicyclic system and existing data, we could deduce that the only sensors of 2-ketoglutarate were PII and PII−UMP. At physiological conditions, we observed that the main role of 2-ketoglutarate in bringing about the deadenylylation of GS was to inhibit GS adenylylation, and this was due to the allosteric regulation of PII activity. Glutamine acted as an allosteric regulator of both ATase and UTase/UR. We also compared the regulation of GS adenylylation state to the regulation of phosphorylation state of the transcription factor NRI (NtrC) in a reconstituted bicyclic system containing NRI, the bifunctional kinase/phosphatase NRII (NtrB), PII, and the UTase/UR. This comparison indicated that, at a fixed 2-ketoglutarate concentration, the regulation of GS adenylylation state by glutamine was sharper and occurred at a higher concentration than did the regulation of NRI phosphorylation. The possible biological implications of this regulatory arrangement are discussed.

Glutamine synthetase (GS)[1] plays a key role in nitrogen assimilation in *Escherichia coli*, and its activity is controlled in at least three distinct ways. The enzyme is regulated by feedback inhibition by a number of nitrogenous metabolites (*1*, *2*). In addition, the enzyme is regulated by reversible covalent adenylylation (refs *3* and *4*; reviewed in refs *5* and *6*). The adenylylated form of the enzyme is inactive, but may be readily activated by deadenylylation. Finally, the structural gene for GS, *glnA*, is subjected to transcriptional regulation (reviewed in refs *7* and *8*). In the aggregate, these regulatory mechanisms ensure that GS activity is precisely controlled and can be rapidly adjusted in response to environmental stimuli.

The stimuli controlling GS activity include carbon and nitrogen signals, which are antagonistic. In the presence of a good carbon source, nitrogen starvation results in a high intracellular concentration of deadenylylated GS, while nitrogen excess results in a low intracellular concentration of GS, which is highly adenylylated (reviewed in ref *8*). However, when growth is limited by the carbon source, the activation of GS does not occur until starvation for nitrogen is so severe as to become growth limiting. Also, GS becomes activated without alteration of nitrogen availability when carbon-limited cells are transferred to conditions of carbon excess (M. R. Atkinson, C. Chen, and A.J.N., unpublished data). Simple experiments such as these indicate that cells balance the assimilation of carbon and nitrogen.

Experiments using the continuous culture technique have suggested that the key regulatory stimuli are the intracellular concentrations of 2-ketoglutarate and glutamine, since these were best correlated with the regulation of GS adenylylation state (*9*). Also, previous work with the purified signal-transduction components responsible for nitrogen regulation suggested that these effectors played a key role in controlling both GS adenylylation state (*10*, *11*) and the transcription of nitrogen-regulated genes (*12*−*14*). Measurements with intact cells indicated that 2-ketoglutarate levels ranged 0.1−0.9 mM (*9*) and that glutamine ranged from less than 0.3 mM to several millimolar (*15*) in vivo at the frequently used growth conditions. Thus, in the study reported here using purified components, we focused on elucidating the mechanisms of regulation by these effectors at these concentrations.

The signal transduction system responsible for regulation of GS adenylylation state and the transcription of nitrogen-regulated genes is depicted in Figure 1. Transcription of nitrogen-regulated genes is controlled by a bicyclic cascade consisting of four proteins: the enhancer-binding transcrip-

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[1] Abbreviations: ATase, adenylyltransferase, product of *glnE*; UTase/UR, uridylyltransferase/uridylyl-removing enzyme, product of *glnD*; PII, PII signal transduction protein, product of *glnB*; NRII, nitrogen regulator II or NtrB, product of *glnL* (*ntrB*); NRI, nitrogen regulator I or NtrC, product of *glnG* (*ntrC*); GS, glutamine synthetase, product of *glnA*; BSA, bovine serum albumin.

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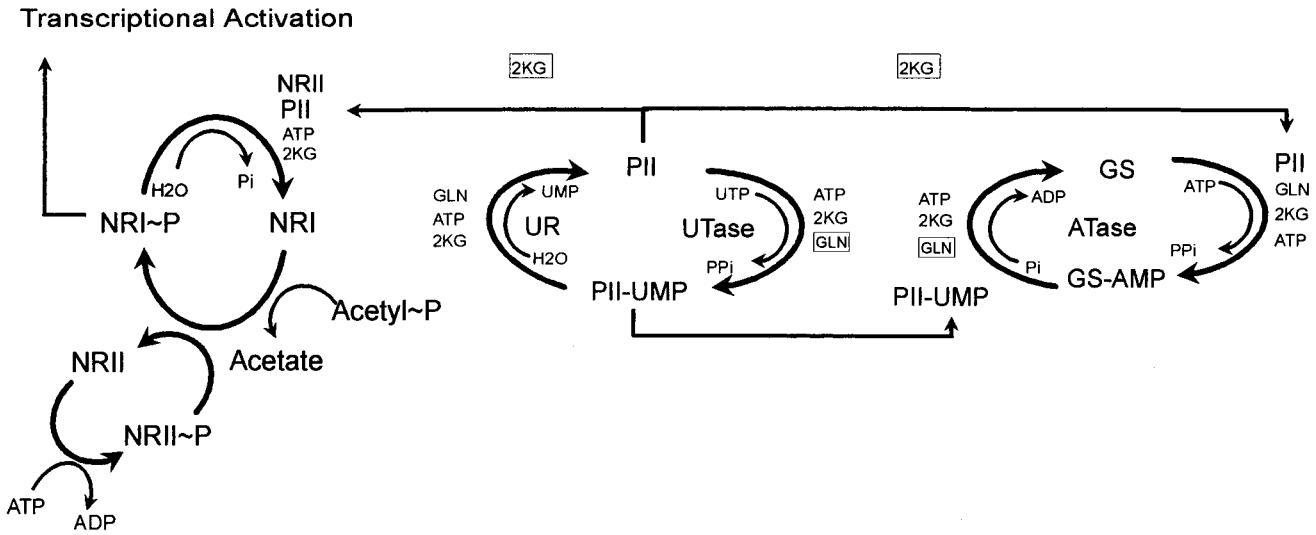

FIGURE 1: Signal transduction system controlling glutamine synthetase adenylylation state and the transcription of the Ntr regulon. NRI (NtrC) is the product of *glnG* (*ntrC*). This enhancer-binding transcription factor activates transcription only when it is phosphorylated. NRII (NtrB) is the product of *glnL* (*ntrB*). It is a kinase that phosphorylates itself and transfers phosphoryl groups to NRI. NRI is also phosphorylated by transfer of phosphoryl groups from acetyl phosphate. NRI−P is dephosphorylated in a reaction that requires NRII and PII and is activated by ATP and a low concentration of 2-ketoglutarate. A high concentration of 2-ketoglutarate (shown boxed) inhibits this reaction. PII is the product of *glnB*. It is reversibly uridylylated by the bifunctional uridylyltransferase/uridylyl-removing enzyme (UTase/UR) encoded by *glnD*. ATP and 2-ketoglutarate activate both UTase and UR activities; glutamine inhibits the UTase (shown boxed) and activates the UR. The bifunctional adenylyltransferase (ATase, product of *glnE*) catalyzes the adenylylation of glutamine synthetase (GS, product of *glnA*) and the deadenylylation of GS−AMP. The adenylylation reaction is activated by PII, while the deadenylylation reaction requires PII−UMP. Both of these reactions are stimulated by 2-ketoglutarate and ATP, and we show here that at high concentrations 2-ketoglutarate inhibited the adenylylation reaction (shown boxed).

tion factor NRI (NtrC), the kinase/phosphatase NRII (NtrB), the PII signal transduction protein, which regulates NRII, and the uridylyltransferase/uridylyl-removing enzyme (UTase/UR), which plays a role in regulating PII activity (reviewed in refs *7* and *8*). The activity of NRI is controlled by reversible phosphorylation; the activator of nitrogen-regulated gene transcription is the phosphorylated form of this protein (*16*). NRI is phosphorylated by NRII, and by direct transfer of phosphoryl groups from acetyl phosphate (refs *17* and *18*; Figure 1, left). The concentration of NRI−P is controlled by regulation of its dephosphorylation, catalyzed by the complex of PII and NRII (*16*). Various lines of evidence suggest that the role of PII in this process is strictly regulatory (*19*). The interaction of PII with NRII is greatly favored by the small molecule effectors 2-ketoglutarate and ATP, which bind to PII (*12*). However, at concentrations within the physiological range, 2-ketoglutarate is an inhibitor of NRI−P dephosphorylation (*12*−*14*). This is due to allosteric regulation of PII by 2-ketoglutarate. At low concentrations of this effector, a single molecule is bound by the PII trimer (*12*, *20*), activating its interaction with NRII. However, higher concentrations of this effector apparently result in the binding of additional molecules of effector to PII, altering its conformation and reducing its ability to bind NRII (ref *12*; E. S. Kamberov and A.J.N., unpublished data).

The activity of PII is also regulated by reversible uridylylation, catalyzed by the UTase/UR (refs *10*, *11*, and *21*−*23*; Figure 1, center). The uridylylated form of PII cannot activate the dephosphorylation of NRI−P (*23*), because it cannot bind to NRII (ref *12*; E. S. Kamberov and A.J.N., unpublished data). Both uridylylation and deuridylylation of PII require ATP and 2-ketoglutarate (*10*, *11*, *20*); however, these processes are not adversely affected by 2-ketoglutarate at high concentration (*12*, *20*). Glutamine inhibits the uridylylation of PII and stimulates the deuridylylation of PII−UMP (*10*−*12*, *20*, *24*). Thus, under conditions of nitrogen starvation, the intracellular concentration of glutamine will be low and PII will be mostly converted to PII−UMP, permitting the accumulation of NRI−P. If carbon is plentiful, the action of any PII molecules escaping uridylylation will be inhibited by the binding of multiple molecules of 2-ketoglutarate to PII. Conversely, cells grown with nitrogen excess will have a high intracellular concentration of glutamine, and PII will be maintained in its unmodified form. This PII will activate the dephosphorylation of NRI−P by NRII, and by so doing prevent the transcription of nitrogen-regulated genes. If the carbon source is poor, the resulting low intracellular concentration of 2-ketoglutarate will result in highly active PII bound by only a single molecule of 2-ketoglutarate, further reducing the level of NRI−P. The model presented above is supported by the results of experiments in which the bicyclic cascade was reconstituted from purified components (*14*).

GS adenylylation state is regulated by a bicyclic cascade consisting of the UTase/UR, PII, adenylyltransferase (ATase), and GS. The ATase catalyzes the adenylylation of GS and deadenylylation (phosphorolysis) of GS−AMP (refs *3*, *4*, and *25*; Figure 1). The adenylylation reaction is stimulated by PII and by glutamine, while the deadenylylation reaction requires 2-ketoglutarate, ATP, and PII−UMP (reviewed in refs *5* and *6*). We will show here that the adenylylation reaction was also stimulated by 2-ketoglutarate when this effector was present at low concentration in the presence of PII. In previous models for the regulation of GS adenylylation state, the main role of 2-ketoglutarate was thought to be as both an activator of the deadenylylation reaction and an inhibitor of the adenylylation reaction (reviewed in refs *5*−*7*). Since PII acts as the sensor of 2-ketoglutarate in

the NRI−NRII−UTase/UR−PII bicycle, we reexamined whether PII and PI−UMP also mediated the regulation of GS adenylylation state by 2-ketoglutarate and, if so, how this regulation was obtained. The results of our study suggest that PII and PII−UMP were the sole receptors for 2-ketoglutarate in the regulation of GS adenylylation state, and that, in contrast to the widely accepted earlier conclusion, the main role of 2-ketoglutarate at physiological conditions was to inhibit the adenylylation of GS.

MATERIALS AND METHODS

Purified Proteins. NRI was purified as described previously (*24*). PII and the UTase/UR were purified as described (*24*) and were the generous gift of M. R. Atkinson. NRII was purified as described (*24*) and was the generous gift of P. Zucker. ATase and GS were purified as described previously (*25*, *26*) and were the generous gift of P. Chandran. Each of these proteins is at least 90% pure and free from the other activities.

Measurement of Adenylylated GS and Uridylylated PII. The extent of protein modification was monitored by measurement of the extent of incorporation of radioactive nucleoside monophosphate into TCA-insoluable material, as described previously (*14*, *20*, *24*). Initial rates were estimated by computer fitting of progress curves using the ENZFIT program (*20*), and by direct estimation from progress curves, using a ruler. Steady-state levels of GS and PII modification were determined by averaging the values for the last several points of progress curves, where visual inspection indicated that a steady-state level of modification had been obtained. The levels of protein modification are designated n, which stands for the average number of modified subunits/molecule. Since GS is a dodecamer, *n* may vary from 0 to 12; PII is a trimer, and thus in this case, *n* may vary from 0 to 3.

Conditions for the Coupled GS-ATase-PII−UTase/UR Bicycle. 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, 10 mM KPi, 1.33 μM GS (dodecamers), 0.1 μM ATase (or as indicated, nominal monomer concentration), 0.5 μM PII (or as indicated, trimers), and 0.04 μM UTase/UR (or as indicated, nominal monomer concentration). ATP and UTP were 1 and 0.5 mM, respectively. Either α-[^{32}P]ATP or α-[^{32}P]UTP was included, depending on whether GS adenylylation or PII uridylylation was measured. 2-Ketoglutarate and glutamine were as indicated. Incubation was at 30 °C. Control experiments indicated that the presence of GS at 1.33 μM and ATase at 0.1 μM did not significantly alter the steady-state levels of PII uridylylation at the conditions used in our experiments (data not shown).

Conditions for the Adenylylation Reaction. Reactions were as above except that KPi, UTP, and the UTase/UR were omitted, and the labeled nucleotide was always ATP (0.5 mM). GS was 1 μM (dodecamers), and ATase concentration was varied as indicated to ensure that the initial rates were sufficiently slow to permit their accurate measurement.

Preparation of [^{32}P]GS−AMP. Reaction mixtures contained 100 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, 100 mM KCl, 10 mM glutamine, 1 mM α-[^{32}P]ATP, 10 μM GS dodecamers, and 2 μM ATase. The reaction mixture was incubated at 30 °C for 20 min and then at 60 °C for 10 min to inactivate the ATase. The mixture was then subjected to gel filtration chromatography on Sephadex G-25 to remove the unincorporated ATP and other small molecules. Under these reaction conditions, GS adenylylation was essentially complete, and the heating at 60 °C for 10 min completely inactivated the ATase while not altering the activity of GS or GS−AMP [as determined by the γ-glutamyl transferase assay (*22*)] or the adenylylation/deadenylylation properties (data not shown).

Conditions for the Deadenylyation Reaction. 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, 10 mM KPi, 0.5 mM ATP (or as indicated), 0.5 μM PII−UMP (or as indicated), 2-ketoglutarate as indicated, 2.7−8 μM [^{32}P]GS−AMP (nominal monomer concentration judged from the specific activity of the label), and ATase as indicated. Incubation was at 30 °C. The ratios of substrate to enzyme in these experiments were chosen to permit accurate determination of initial reaction velocities.

Conditions for the NRI−NRII−PII−UTase/UR Bicycle. 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 mM γ-[^{32}P]ATP, 15 μM NRI, 0.3 μM NRII, 0.5 μM PII, 0.3 μM UTase/UR, and various concentrations of glutamine and 2-ketoglutarate, as indicated. Incubation was at 25 °C, and the steady-state levels of NRI−P were determined as described (*14*) by measurement of TCA-insoluble radioactivity.

RESULTS

To learn the mechanisms by which 2-ketoglutarate and glutamine regulate the adenylylation state and activity of GS, we first examined the role of these effectors in controlling the steady-state level of GS adenylylation in a reconstituted UTase/UR−PII-ATase-GS bicyclic cascade system. Next, we examined the role of these effectors in regulating the initial rates of GS adenylylation and deadenylylation by ATase. Finally, the regulation by gluamine of the two bicyclic cascades regulating the transcription of nitrgen regulated genes (*14*), and the adenylylation state of GS was compared.

Regulation of GS Adenylylation State in a Reconstituted Bicyclic System. The effect of the small molecule effectors 2-ketoglutarate and glutamine on the adenylylation state of GS and the uridylylation state of PII were examined in a bicyclic system containing GS, ATase, PII, and UTase/UR. For these studies, the protein components were present at their likely in vivo concentrations corresponding to cells grown under nitrogen-limiting conditions (Materials and Methods). Reaction mixtures initially contained unadenylylated GS and unmodified PII, but quickly reached a steady state in which the extent of GS adenylylation depended on the 2-ketoglutarate and glutamine concentrations and the extent of PII modification depended on the glutamine concentration. A typical set of progress curves in which the extent of GS modification was measured at fixed glutamine and various 2-ketoglutarate concentrations is presented in Figure 2A. The reciprocal regulation of GS adenylylation state by 2-ketoglutarate and glutamine permitted sequential deadenylylation and adenylylation of GS upon sequential addition of these effectors (Figure 2B). The strong antagonistic effect of these effectors was evident from the series

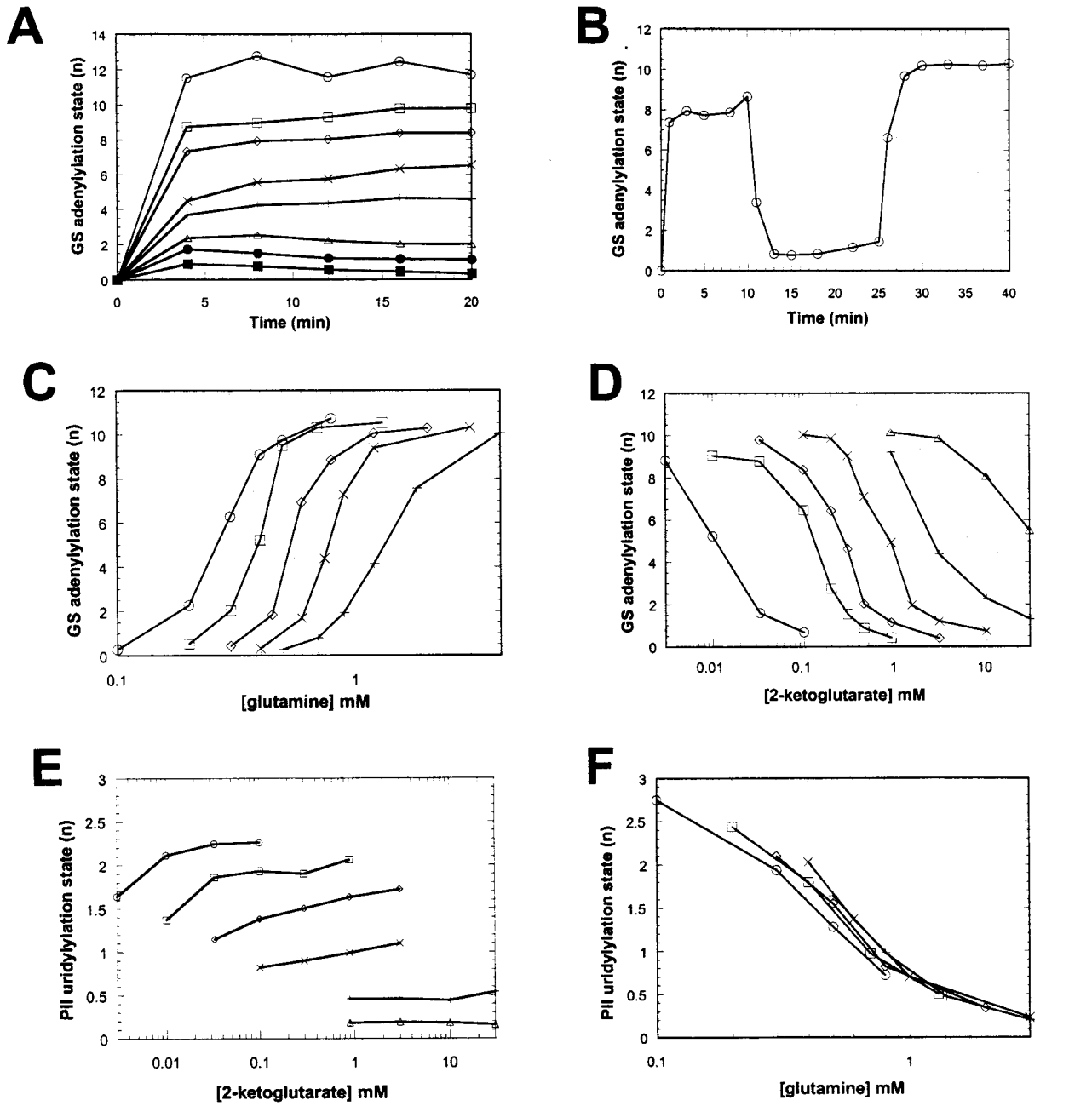

FIGURE 2: Regulation of GS adenylylation state and PII uridylylation state by 2-ketoglutarate and glutamine in the bicyclic ATase-GS−UTase/UR−PII system. (A) Adenylylation of GS at 0.5 mM glutamine and various concentrations of 2-ketoglutarate as follows: (unfilled circle) control experiment with 4 mM glutamine with no 2-ketoglutarate and no UTase/UR; (unfilled square) 0.033 mM 2-ketoglutarate; (unfilled diamond) 0.1 mM 2-ketoglutarate; (×) 0.2 mM 2-ketoglutarate; (+) 0.3 mM 2-ketoglutarate; (unfiled triangle) 0.45 mM 2-ketoglutarate; (filled circle) 0.9 mM 2-ketoglutarate; (filled square) 3 mM 2-ketoglutarate. (B) Timecourse of GS adenylylation. The initial conditions were 0.5 mM glutamine and 0.033 mM 2-ketoglutarate. After 10 min, 2-ketoglutarate was increased to 0.9 mM. After 25 min, glutamine was increased to 3 mM. As shown, GS adenylylation state could be reversibly altered by these sequential additions. (C) Effect of glutamine on GS adentylylation at various 2-ketoglutarate concentrations, as follows: (unfilled circles) 0.033 mM; (unfilled square) 0.1 mM; (unfilled diamonds) 0.3 mM; (×) 0.9 mM; (+) 3.0 mM. (D) Effect of 2-ketoglutarate on GS adenylylation state at various glutamine concentrations, as follows: (unfilled circle) 0.2 mM; (unfilled square) 0.35 mM; (unfilled diamonds) 0.5 mM; (×) 0.75 mM; (+) 1.3 mM; (unfilled triangle) 4.0 mM. (E) Effect of 2-ketoglutarate on PII unridylylation state at various glutamine concentrations, as follows: (unfilled circle) 0.2 mM; (unfilled square) 0.35 mM; (unfilled diamonds) 0.5 mM; (×) 0.75 mM; (+) 1.3 mM; (unfilled triangles) 4 mM. (F) Effect of glutamine on PII uridylylation state at various 2-ketoglutarate concentrations, as follows: (unfilled circle) 0.033 mM; (unfilled square) 0.1 mM; (unfilled diamond) 0.3 mM; (×) 0.9 mM; (+) 3 mM.

of experiments summarized in Figure 2, panels C and D. It was evident that GS adenylylation state was more sensitive to the glutamine concentration than to the 2-ketoglutarate concentration, as noted before (*5*), but that 2-ketoglutarate also played an important role (Figure 2D). These results are consistent with the regulation of GS adenylylation state by glutamine and 2-ketoglutarate observed in intact cells (*1*).

Unlike the modification of GS, the uridylylation of PII was not greatly affected by the concentration of 2-ketoglutarate in the bicyclic system under these conditions (Figure 2E). However, the extent of PII uridylylation was regulated by the glutamine concentration (Figure 2F). Thus, in our

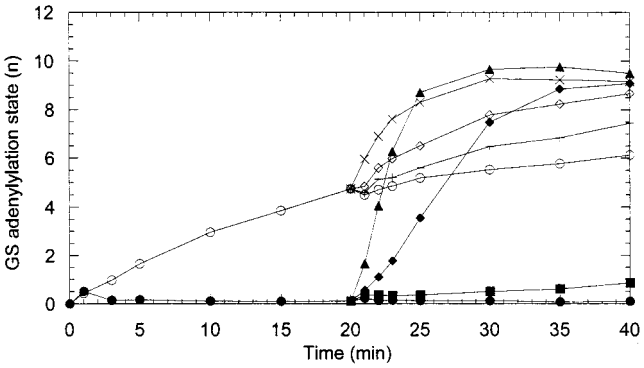

FIGURE 3: Comparison of the bicyclic GS-ATase-PII−UTase/UR system with the uncoupled system lacking PII and PII−UMP. All experiments were performed with 2-ketoglutarate fixed at 0.3 mM. Reaction conditions were as in the Materials and Methods, except that the ATase was 0.4 μM, PII was either 2 μM or absent, as indicated, and the UTase/UR was 0.4 μM. Elevated concentrations of PII and converter enzymes were used in this experiment to permit the steady state to be obtained within the time-course of the experiment. The initial reaction mixtures contained either 0.2 mM glutamine (unfilled circles) or 0.2 mM glutamine and PII (filled circles). After 20 min incubation, glutamine was increased to the following final concentrations: (filled square, +) 0.5 mM; (filled diamond, unfilled diamond) 1.3 mM; (filled triangle, X) 4 mM.

reconstituted system, the UTase/UR monocycle was mainly acting as a glutamine-sensing apparatus, while the dramatic effects of 2-ketoglutarate on GS adenylylation state were mainly due to effects on the adenylylation/deadenylylation monocycle. This result, also noted in the previous article in this issue (*14*), is not consistent with the widely accepted hypothesis that 2-ketoglutarate is a regulator of the uridylylation state of PII (*7*).

To investigate whether the UTase/UR and ATase monocycles were indeed coupled in our reaction mixtures, we examined the corresponding covalent modification states in individual monocycles as well as the effect of omitting the PII signal transduction protein, which couples the cycles in the bicyclic system. Previous results have indicated that in the absence of PII, glutamine stimulates the adenylylation of GS (*5*, *6*). Also, in the absence of PII−UMP the deadenylylation of GS does not occur (reviewed in refs *5* and *6*), and GS should eventually become highly adenylylated regardless of the concentrations of 2-ketoglutarate and glutamine. A typical experiment to test the dependence of our reconstituted bicycle on PII, which couples the cycles, is presented in Figure 3. In the coupled system containing PII, GS adenylylation state was low when the glutamine concentration was low, since PII−UMP was available to activate the deadenylylation reaction, and increased rapidly upon subsequent addition of a higher concentration of glutamine (Figure 3). In the uncoupled system lacking PII, glutamine stimulated the adenylylation of GS, but even at low glutamine concentration, GS slowly became adenylylated. Thus, in our complete system, PII was required to prevent the adenylylation of GS when the concentration of glutamine was low.

Regulation of the ATase/GS Monocycle. We reexamined the regulation of the adenylylation and deadenylylation of GS in monocyclic ATase-GS systems lacking the UTase/UR and containing either PII or PII−UMP. To study the adenylylation reaction, reaction mixtures initially contained unmodified GS, while GS−AMP was used to study the deadenylylation reaction. Initial rates of adenylylation or deadenylylation were measured under various conditions. For these experiments, protein concentrations and ratios were used that permitted accurate measurement of initial reaction rates.

Table 1: Factors Affecting the Adenylyation of GS[a]

line	conditions	V_o (μM/min)
	Experiment 1[b]	
1.	control	0.57
2.	16 μM 2KG	2.18
3.	10 mM 2KG	0.18
4.	20 mM glutamate	0.99
	Experiment 2[c]	
5.	control	0.16
6.	1 mM GLN	0.59
7.	1 mM GLN/20 μM 2KG	0.59
8.	1 mM GLN/10 mM 2KG	0.52
9.	0.5 μM PII	0.18
10.	0.5 μM PII/20 μM 2KG	0.75
11.	0.5 μM PII/10 mM 2KG	0.14
12.	0.5 μM PII/1 mM GLN	1.65
13.	0.5 μM PII/1 mM GLN/20 μM 2KG	8.61
14.	0.5 μM PII/1 mM GLN/10 mM 2KG	0.59
15.	0.5 μM PII/1 mM GLN/20 μM 2KG/ 0.5 μM PII−UMP	6.38
16.	0.5 μM PII/1 mM GLN/20 μM 2KG/ 5 μM PII−UMP	4.57
17.	0.5 μM PII-Q39E[d]	0.15
18.	0.5 μM PII-Q39E/1 mM GLN	1.66
19.	0.5 μM PII-Q39E/1 mM GLN/20 μM 2KG	2.02
20.	0.5 μM PII-Q39E/1 mM GLN/10 mM 2KG	2.17

[a] Experiments were performed as in Materials and Methods, except as noted for the individual experiments. GLN signifies glutamine and 2KG signifies 2-ketoglutarate. [b] Conditions were 1 μM GS (dodecamers), 0.04 μM ATase, 0.5 mM ATP, 0.5 μM PII (trimers), 1 mM GLN, 25 mM $MgCl_2$, with additions as indicated. [c] Conditions were 1 μM GS (dodecamers), 0.12 μM ATase, 0.5 mM ATP, 25 mM $MgCl_2$, with additions as indicated. [d] PII-Q39E is an altered form of the PII protein that contains glutamine at position 39 instead of the typical glutamate.

Regulation of GS Adenylylation. The effect of various components on the initial rate of GS adenylylation was reexamined (Table 1). Since ATP is a substrate in this reaction, all experiments were performed with saturating ATP. Neither 2-ketoglutarate nor glutamate were stimulatory individually (data not shown). PII at 0.5 μM was not stimulatory by itself (Table 1, line 9), but a 10-fold higher concentration afforded slight stimulation (data not shown). Similarly, the combination of PII with either 20 μM 2-ketoglutarate (Table 1, line 10) or 20 mM glutamate (data not shown) provided little stimulation of GS adenylylation. The combination of PII with 10 mM 2-ketoglutarate was slightly inhibitory (Table 1, line 11). Glutamine at 1 mM (in the middle of the physiological range) provided weak stimulation when added alone, and this activation was not significantly diminished by further addition of 2-ketoglutarate (Table 1, lines 6−8) or glutamate (not shown). These experiments indicated that neither 2-ketoglutarate nor PII had an appreciable effect on GS adenylylation in the absence of glutamine.

Dramatic activation of GS adenylylation was observed with the combination of glutamine, PII, and a low concentration (20 μM) of 2-ketoglutarate (Table 1, line 13). This activation was far greater than the additive effects of these components added individually. Glutamate at very high

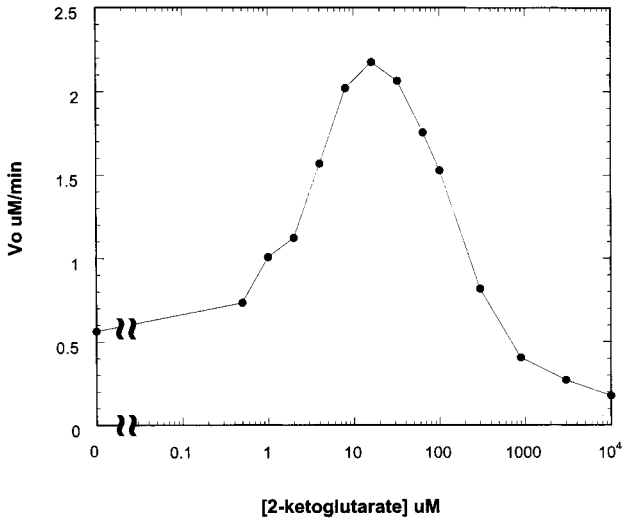

FIGURE 4: Effect of 2-ketoglutarate on the initial rate of GS adenylylation in a GS-ATase-PII system. Reaction conditions were as in the Materials and Methods, with ATase at 0.04 μM, PII at 0.5 μM, and glutamine at 1 mM. The peak initial velocity occurred at 16 μM 2-ketoglutarate.

concentration (20 mM) also provided significant stimulation when present in combination with glutamine and PII (Table 1, line 4). However, significant activation of GS adenylylation was not observed when a high concentration of 2-ketoglutarate (10 mM) was combined with glutamine and PII; instead a slight inhibition was observed (Table 1, line 14). That is, GS adenylylation was much more rapid in the presence of a low concentration of 2-ketoglutarate than in the presence of a high concentration of this effector. PII−UMP could inhibit the activation of GS adenylylation by the combination of PII, 2-ketoglutarate, and glutamine (Table 1, compare lines 15 and 16 with line 13).

To investigate whether activation of GS adenylylation by 2-ketoglutarate was due to sensation by PII, we took advantage of a mutant form of PII defective in binding 2-ketoglutarate. We previously characterized a mutant PII, containing the alteration Q39E, that was defective in binding of 2-ketoglutarate (*26*). In the absence of 2-ketoglutarate, this protein activated GS adenylylation as well as did wild-type PII, when glutamine was present (Table 1, line 18). However, unlike wild-type PII, when 20 μM 2-ketoglutarate was present, the Q39E protein did not dramatically activate adenylylation (Table 1, line 19). When 2-ketoglutarate was 10 mM, wild-type PII did not activate GS adenylylation, but the Q39E mutant form of PII activated as well as it did at 20 μM 2-ketoglutarate (Table 1, line 20). These data with the PII-Q39E protein suggest that both the activation of GS adenylylation at 20 μM 2-ketoglutarate and the inhibition of this reaction when 2-ketoglutarate was 10 mM were mediated by PII, and not due to direct interaction of 2-ketoglutarate with the ATase.

The activation and inhibition of the initial rate of GS adenylylation was further examined in a series of reaction mixtures containing fixed PII and glutamine and various concentrations of 2-ketoglutarate spanning the physiological range of this effector (Figure 4). The effect of 2-ketoglutarate was clearly biphasic, with an optimal 2-ketoglutarate concentration of ~16 μM. Above this concentration, an increase in the 2-ketoglutarate concentration strongly inhibited the initial rate of GS adenylylation. Thus, although 2-ketoglutarate at low concentration can stimulate GS adenylylation, in the physiological range 2-ketoglutarate is a potent inhibitor of GS adenylylation.

Table 2: Factors Affecting the Deadenylylation of GS−AMP[a]

line	conditions	V_o (nM/min)
	Experiment 1[b]	
1.	control	0.0
2.	0.5 μM PII−UMP/0.5 mM ATP	54.1
3.	0.5 μM PII−UMP/0.3 mM 2KG	77.8
4.	0.5 μM PII−UMP/0.5 mM ATP/0.3 mM 2KG	1741.1
	Experiment 2[c]	
5.	0.5 mM ATP/1 mM 2KG	0.0
6.	0.5 μM PII−UMP	34.1
7.	0.5 μM PII−UMP/0.5 mM ATP/1 mM 2KG	2063.2
8.	0.5 μM PII−UMP/0.5 mM ATP/20 mM glutamate	156.0
9.	5 μM PII/0.5 mM ATP/1 mM 2KG	118.6
10.	0.5 μM PII−UMP/0.5 mM ATP/1 mM 2KG/ 1 mM GLN	1867.2
11.	0.5 μM PII−UMP/0.5 mM ATP/1 mM 2KG/ 0.5 μM PII	1674.7
12.	0.5 μM PII−UMP/0.5 mM ATP/1 mM 2KG/ 5 μM PII	677.0
	Experiment 3[d]	
13.	control	1476.1
14.	0.25 mM GLN	1523.4
15.	1 mM GLN	1375.2
16.	4 mM GLN	1229.9
17.	15 mM GLN	908.5
18.	0.5 μM PII	1412.1
19.	0.5 μM PII/0.25 mM GLN	1415.5
20.	0.5 μM PII/1 mM GLN	1280.3
21.	0.5 μM PII/4 mM GLN	1146.6
22.	0.5 μM PII/15 mM GLN	699.8

[a] Experiments were performed as in the Materials and Methods, except as noted for the individual experiments. GLN signifies glutamine and 2KG signifies 2-ketoglutarate. GS−AMP is stated as the monomer concentration. [b] Conditions were 2.7 μM GS−AMP, 0.1 μM ATase, 0.5 μM PII−UMP, 10 mM KPi, 25 mM $MgCl_2$, and additives as indicated. [c] Conditions were 6 μM GS−AMP, 0.035 μM ATase, 10 mM KPi, 25 mM gCl_2, and additives as indicated. [d] Conditions were 6 μM GS−AMP, 0.03 μM ATase, 0.5 μM PII−UMP, 0.5 mM ATP, 1 mM 2KG, 10 mM KPi, 25 mM $MgCl_2$, and additives as indicated.

Regulation of GS Deadenylylation. To understand the contribution of the deadenylylation reaction to the regulation of GS adenylylation state, we reexamined the roles of effectors in regulating this activity. The deadenylylation of GS was dramatically activated by the combination of PII−UMP, ATP, and 2-ketoglutarate (Table 2, lines 4 and 7). Omission of either 2-ketoglutarate or ATP reduced the rate of GS−AMP deadenylylation greater than 20-fold (Table 2, lines 2 and 3). Glutamate, at 20 mM, provided slight activation when in place of 2-ketoglutarate (Table 2, line 8). PII could inhibit the activation of GS−AMP deadenylylation by the combination of PII−UMP, 2-ketoglutarate, and ATP (Table 2, lines 11 and 12). When a very high concentration of PII was added to reaction mixtures in the absence of PII−UMP, slight activation could be observed when ATP and 2-ketoglutarate were present (Table 2, line 9). Glutamine was a weak inhibitor of the deadenylylation reaction in the physiological range (0.25−4 mM), whether PII was present or not (Table 2, lines 10 and 13−22).

Since the binding of 2-ketoglutarate and ATP to PII−UMP is synergistic (*20*), we examined whether these effectors synergistically activated GS−AMP deadenylylation. In the

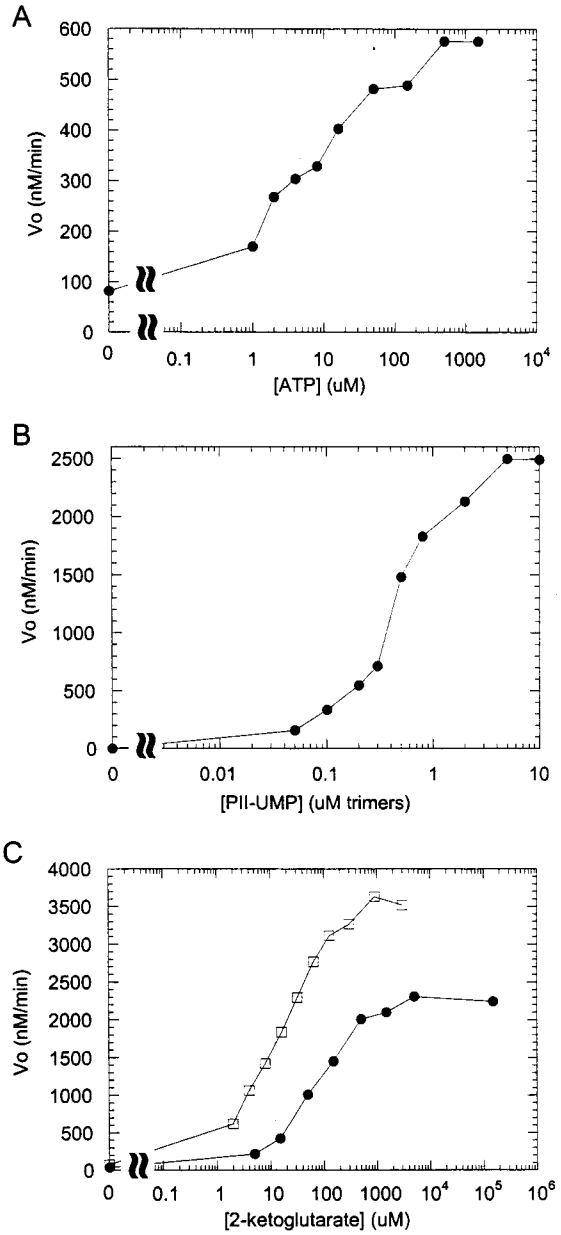

FIGURE 5: Deadenylylation of GS−AMP by ATase is activated by ATP, PII−UMP, and 2-ketoglutarate. The conditions were as in the Materials and Methods, except as noted. (A) Activation of GS−AMP deadenylylation by ATP. GS−AMP was 5.7 μM (monomers), ATase was 0.015 μM, PII was 0.5 μM, and 2-ketoglutarate was 2 mM. K_{act} for ATP under these conditions was estimated at 6 μM. (B) Activation by PII−UMP. GS−AMP was 8 μM (monomers), ATase was 0.025 μM, and 2-ketoglutarate was 1 mM. The K_{act} for PII−UMP under these conditions was ~0.4 μM. (C) Activation by 2-ketoglutarate. GS−AMP was 7 μM (monomers), ATase was 0.05 μM, PII−UMP was 0.5 μM, and ATP was either 30 μM (filled circles) or 0.5 mM (unfilled squares). The K_{act} was 16 μM when ATP was 0.5 mM and ~100 μM when ATP was 30 μM.

deadenylylation reaction, the observed K_{act} for ATP was ~6 μM when 2-ketoglutarate was saturating (Figure 5A). This value corresponds well to the previously measured dissociation of ATP from PII−UMP under these conditions [$K_d = 4$ μM (*20*)] and to the K_{act} observed for activation of the UR activity of the UTase/UR [$K_{act} = 5-6$ μM with 2-ketoglutarate at 2 mM (*20*)]. When ATP and 2-ketoglutarate were saturating, the K_{act} for PII−UMP was 0.4 μM (Figure 5B). The K_{act} for 2-ketoglutarate was dependent on the ATP concentration; at the saturating concentration of 0.5 mM ATP, the K_{act} was ~16 μM, whereas a K_{act} of ~100 μM was obtained when ATP was 30 μM (Figure 5C). These K_{act} values, and the synergy between ATP and 2-ketoglutarate, correspond well with the K_{act} values obtained for activation of the UR activity of the UTase/UR (*20*) as well as K_D values determined in direct binding studies at comparable conditions (*20*). The regulation of the deadenylylation reaction by 2-ketoglutarate was not noticiably biphasic and (at saturating ATP) was nearly saturated in the range 0.1−0.9 mM 2-ketoglutarate (Figure 5C). Yet, in the coupled system at high glutamine concentration, excellent regulation was observed by 2-ketoglutarate at considerably higher concentration (Figure 2D), and these effects could not be attributed to effects on the UTase/UR−PII monocycle (Figure 1E).

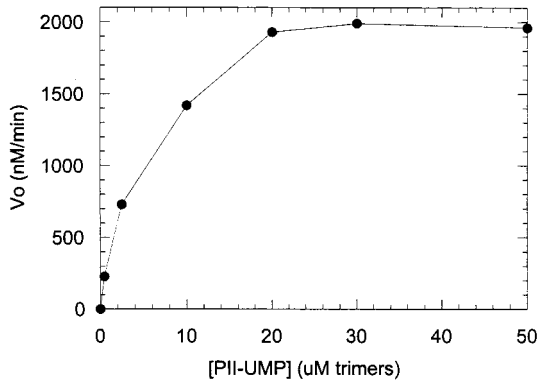

FIGURE 6: Activation of the deadenylylation reaction by 2-ketoglutarate is due to a single site for this effector. The condtitions were as described in the Materials and Methods, with GS−AMP at 6 μM (monomers), ATase at 0.08 μM, and 2-ketoglutarate at 1 μM. PII−UMP was varied as indicated, and the concentrations of KCl (154 mM) and glycerol (5%) was identical in all reaction mixtures.

To examine whether two distinct and essential 2-ketoglutarate sites were involved in the activation of the deadenylylation reaction, such as a site on PII and a site on the enzyme, we examined the initial rate of deadenylylation in reaction mixtures containing a very low concentration of 2-ketoglutarate (1 μM) and various concentrations of PII−UMP. If distinct essential 2-ketoglutarate sites were involved, then PII−UMP at high concentration should inhibit the rate of deadenylylation by sequestering the activator. However, PII−UMP, even in great excess, failed to inhibit the initial rate of GS−AMP deadenylylation (Figure 6). This result suggests that PII−UMP was the sole site of 2-ketoglutarate binding, and that the unliganded form of PII−UMP did not compete with the complex of PII−UMP and 2-ketoglutarate for binding the enzyme. Alternatively, 2-ketoglutarate may bind very tightly to ATase, such that it cannot be competed off with excess PII−UMP. However, we were unable to detect the binding of 2-ketoglutarate to the ATase, whereas its binding to PII−UMP was readily demonstrated (*20*). Thus, the data are consistent with the hypothesis that 2-ketoglutarate binds only to PII−UMP in the activation of GS−AMP deadenylylation.

Comparison of the Regulation of GS Adenylylation State by Glutamine to the Regulation of NRI Phosphorylation State by Glutamine in Reconstituted Systems. In the previous article in this issue, we described a reconstituted system in

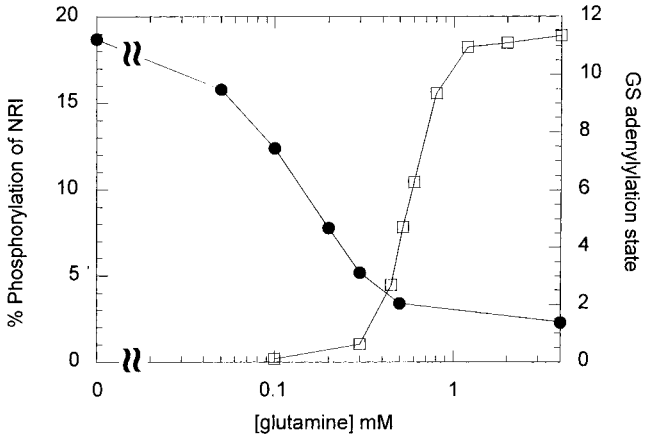

FIGURE 7: Comparison of the regulation of GS adenylylation state by glutamine in the GS-ATase-UTase/UR−PII bicyclic system to the regulation of NRI phosphorylation state by glutamine in the NRI−NRII−UTase/UR−PII bicyclic system. Conditions were as described in the Materials and Methods, with 2-ketoglutarate fixed at 0.3 mM. Symbols are (filled circle) % phosphorylation of NRI; (unfilled squares) GS adenylylation state.

which the phosphorylation state of the transcription factor NRI was regulated by glutamine and 2-ketoglutarate (*14*). This reconstituted system contained the UTase/UR, PII, the kinase/phosphatase NRII, and NRI. In this system, glutamine regulated NRI phosphorylation indirectly, by regulating the extent of PII uridylylation, while 2-ketoglutarate regulated the rate of NRI−P dephosphorylation by controlling the ability of PII to interact with NRII.

The effect of glutamine on the reconstituted NRI−NRII−UTase/UR−PII bicyclic system was compared to its effect on the GS-ATase-UTase/UR−PII bicyclic system. As shown in Figure 7 at a fixed concentration of 2-ketoglutarate (0.3 mM), regulation by glutamine was sharper and occurred at higher glutamine concentrations in the GS-ATase-UTase/UR−PII bicycle.

DISCUSSION

Hypothesis for the Role of 2-Ketoglutarate in the Regulation of GS Adenylylation State. The regulation of GS adenylylation state by 2-ketoglutarate and glutamine in our reconstituted bicyclic system mirrors the observations of Senior (*1*) in his study using intact cells. Therefore, our experimental system is useful for elucidating the mechanisms by which these effectors act. Various experimental data suggest that PII and PII−UMP were the only sensors of 2-ketoglutarate in our reconstituted GS adenylylation/deadenylylation system. The activation of GS adenylylation by PII, ATP, and 2-ketoglutarate was strongly reminiscent of the activation of NRI−P dephosphorylation by these activators (*12*−*14*), including the biphasic effect of 2-ketoglutarate variation (Figure 4), and the observation that glutamate at high concentration mimicked the effects of 2-ketoglutarate at low concentration (*12*−*14*). The activation of GS−AMP deadenylylation by PII−UMP, ATP, and 2-ketoglutarate, including the synergy between ATP and 2-ketoglutarate, were similar to the activation of PII−UMP deuridylylation by ATP and 2-ketoglutarate (ref *20*; Figure 5). The apparent K_{act} values observed here for ATP and 2-ketoglutarate also corresponded well to the K_d values for binding of of 2-ketoglutarate and ATP by PII and PII−UMP under comparable conditions (*20*). Finally, a mutant PII protein defective in the binding of 2-ketoglutarate eliminated the regulation of GS adenylylation by this effector (Table 1, lines 17−20). Therefore, we must conclude that the 2-ketoglutarate effects observed in our reconstituted ATase-GS−PII−UTase/UR bicyclic system were due to the binding of this effector by PII and PII−UMP. This conclusion is strengthened by our observation that 2-ketoglutarate acts at a single site to activate the GS−AMP deadenylylation reaction (Figure 6).

The results of our studies, when compared to previous data, suggest that, at physiological concentrations, 2-ketoglutarate regulates GS adenylylation state solely by allosteric regulation of the activity of PII. This effector has been reported to vary from 0.1 to 0.9 mM in cells (*9*). At these concentrations, the main role of 2-ketoglutarate in the regulation of GS adenylylation state was to inhibit the adenylylation reaction (Figures 4 and 5C). Indeed, variation of 2-ketoglutarate within this range had little effect on the rate of GS−AMP deadenylylation in the presence of ATP and PII−UMP; that process was essentially saturated at these effector concentrations (Figure 5). Thus, in intact cells, the PII/PII−UMP ratio is likely to be the most important regulator of the GS−AMP deadenylylation reaction, and the main role of 2-ketoglutarate is likely to be regulation of the adenylylation reaction. A different conclusion was obtained from many previous studies of GS adenylylation regulation, namely, 2-ketoglutarate was thought to play an important role in the regulation of GS−AMP deadenylylation (reviewed in refs *5* and *6*).

The current study and previous studies (*14*, *20*) suggest that at physiological conditions the UTase/UR is regulated mainly by the glutamine concentration and is insensitive to the 2-ketoglutarate concentration. However, the productive interaction of PII with the ATase and NRII receptors was clearly regulated by 2-ketoglutarate at physiological concentrations (*12*−*14*; this study). Optimal interaction with these receptors occurred when the 2-ketoglutarate concentration corresponded to that resulting in the binding of a single molecule of effector to the PII trimer (*20*). [PII displays a strong negative cooperativity in the binding of 2-ketoglutarate, which is relieved upon uridylylation (*20*).] At physiological concentrations of 2-ketoglutarate, which are much higher, the primary role of this effector was to prevent the productive interaction of PII with the ATase and NRII receptors, apparently by overcoming the negative cooperativity and filling two low-affinity sites in the PII trimer, altering the conformation of PII. In the case of NRII, the binding of NRII to PII is diminished at high 2-ketoglutarate concentration (ref *12*; E. S. Kamberov and A.J.N., unpublished data), and this could explain the control of the ATase-PII interaction as well. Glutamate, which can bind to the 2-ketoglutarate site of PII (*12*), can activate the productive interaction of PII with NRII (*12*, *13*) and ATase (this study), but not with the UTase/UR (*12*). Since other related small molecule effectors can also bind the 2-ketoglutarate site of PII (*12*), the regulation of PII activity in cells may be quite complex.

In summary, the antagonism between 2-ketoglutarate and glutamine in the regulation of GS adenylylation state and transcriptional regulation of the Ntr regulon is due to the effects of glutamine on the UTase/UR and ATase enzymes,

and the effect of 2-ketoglutarate, and possibly other small molecule effectors such as glutamate, on the conformation and activity of PII.

Relationship between the Regulation of GS Adenylylation State and the Regulation of Transcription of the Ntr Regulon. The UTase/UR−PII monocycle plays a key role in the regulation of NRII and ATase, and thus regulates both GS adenylylation state and the transcription of the Ntr regulon. However, there are important differences in the regulatory circuitry for these two systems. In the GS-ATase-PII−UTase/UR bicycle (Figure 1, right), PII and PII−UMP play antagonistic roles by activating the adenylylation and deadenylylation activities of ATase, respectively. Also, glutamine acts at two points in the GS-ATase-PII−UTase/UR bicycle, by regulating both ATase and UTase/UR. In contrast, in the NRI−NRII−UTase/UR−PII bicycle (Figure 1, left), PII−UMP has no direct role and glutamine acts at a single point, by regulating the UTase/UR. Furthermore, prior work has indicated that the interaction of PII with the ATase and NRII is different. For example, mutant forms of PII have been isolated that are specifically defective in the interaction with each of these receptors (*26*). Thus, it may be anticipated that the regulation of GS adenylylation state and the regulation of NRI phosphorylation state may respond differently to small molecule effectors, despite the use of the common sensory components PII and UTase/UR (Figure 1).

We observed that at a fixed concentration of 2-ketoglutarate (near the center of the physiological range), the regulation of GS adenylylation state was sharper and occurred at a higher concentration of glutamine than did the regulation of NRI phosphorylation state (Figure 7). Further work with the reconstituted bicyclic and monocyclic systems will be required to understand the mechanisms responsible for the different sensitivity to glutamine. The complexity of the ATase enzyme, which contains distinct transferase and adenylyl-removal domains (*25*), is only beginning to emerge. Nevertheless, our work provides the experimental system by which the issue of the sensitivity to glutamine may be addressed.

It is not known whether the difference in the sensitivity of GS adenylylation state and NRI phosphorylation state to glutamine observed in these reconstituted system also occurs in cells. A shallow response of NRI phosphorylation state to glutamine concentration may be required for appropriate control of the Ntr regulon. Expression of different Ntr genes is triggered by different NRI−P concentrations, depending on the promoter structure (reviewed in ref *8*). These differences in promoter sensitivity to NRI−P are due to differences in the quality and position of the enhancer and silencer sequences, differences in the occupancy of the promoters by σ^{54}-RNA polymerase, and other factors such as topography of the DNA. Among the promoters activated most efficiently by NRI−P is the *glnAp2* promoter, and activation of this promoter results in elevation of GS, NRII, and NRI, although not in equal proportions. A smooth and gradual regulation of NRI phosphorylation state by glutamine may be required to permit the partial activation of the Ntr regulon in response to intermediate conditions of nitrogen limitation. In contrast, sharp regulation of GS adenylylation state may be important for preventing the unnecessary activation of Ntr gene transcription under conditions where the activation of GS could alleviate the cellular glutamine deficiency.

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