Escherichia coli PII Signal Transduction Protein Controlling Nitrogen Assimilation Acts As a Sensor of Adenylate Energy Charge *in Vitro*†

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ABSTRACT: PII signal transduction proteins are among the most widely distributed signaling proteins in nature, controlling nitrogen assimilation in organisms ranging from bacteria to higher plants. PII proteins integrate signals of cellular metabolic status and interact with and regulate receptors that are signal transduction enzymes or key metabolic enzymes. Prior work with *Escherichia coli* PII showed that all signal transduction functions of PII required ATP binding to PII and that ATP binding was synergistic with the binding of α-ketoglutarate to PII. Furthermore, α-ketoglutarate, a cellular signal of nitrogen and carbon status, was observed to strongly regulate PII functions. Here, we show that in reconstituted signal transduction systems, ADP had a dramatic effect on PII regulation of two *E. coli* PII receptors, ATase, and NRII (NtrB), and on PII uridylylation by the signal transducing UTase/UR. ADP acted antagonistically to α-ketoglutarate, that is, low adenylylate energy charge acted to diminish signaling of nitrogen limitation. By individually studying the interactions that occur in the reconstituted signal transduction systems, we observed that essentially all PII and PII-UMP interactions were influenced by ADP. Our experiments also suggest that under certain conditions, the three nucleotide binding sites of the PII trimer may be occupied by combinations of ATP and ADP. In the aggregate, our results show that PII proteins, in addition to serving as sensors of α-ketoglutarate, have the capacity to serve as direct sensors of the adenylylate energy charge.

About 35 years ago, D. E. Atkinson and colleagues introduced the concept of adenylylate energy charge (EC[1]), as a measure of the energy available for metabolism (*1*). EC is defined as ([ATP] + 1/2 [ADP])/([ATP] + [ADP] + [AMP]); in bacteria, where the concentration of AMP is nearly constant and very low, the main component of EC is the ratio of ADP/ATP (D/T ratio). Retrospective calculation of EC from classical earlier studies (*2*) shows that at a variety of steady-state growth conditions resulting in different growth rates, the EC of *Escherichia coli* was relatively invariant. These results have been confirmed by more recent studies using a variety of cell types and tissues, suggesting that in general, cell growth occurs when the EC is about 0.8−0.95 (*3*−*6*). Upon starvation of bacteria for a carbon source, the EC falls, gradually at first and then more rapidly, and EC levels in the range of 0.1−0.6 have been reported in various studies of starved cells (e.g., refs *1* and *5*). Re-supply of starved cells with a carbon source results in the restoration of the EC co-incident with the resumption of growth (*1*). Similarly, during the diauxic growth of bacteria on a mixture of glucose and lactose, the EC falls upon exhaustion of the glucose as growth stops and rises again as lactose begins to be utilized and growth resumes (*3*). Nitrogen status also has a dramatic effect on EC in bacteria, with nitrogen limitation reducing the EC (*7*). Thus, in addition to being a principal regulator of cellular metabolism, the EC may serve as a cellular signal of environmental conditions.

PII proteins are among the most widely distributed signal transduction proteins in nature, with representatives in the Archaea, bacteria, eukaryotic algae, and higher plants (reviewed in refs *8*−*10*). These small proteins (subunits of 112 amino acids in eubacteria) are trimers that regulate signal transduction enzymes or key enzymes of nitrogen metabolism. In *E. coli*, PII is regulated by reversible uridylylation, catalyzed by the signal transduction enzyme UTase/UR (E.C.2.7.7.59), and interacts with and regulates at least three other receptors: ATase (E.C. 2.7.7.49), NRII (NtrB), and AmtB (reviewed in refs *11*, *12*). ATase (glutamine synthetase adenylyltransferase/adenylyl-removing enzyme) catalyzes the reversible adenylylation of glutamine synthetase (GS) on a conserved tyrosine residue, regulating GS. In *E. coli*, GS is the key enzyme for the assimilation of ammonia, the favored nitrogen source, and ATase plays an important role in allowing the cell to rapidly change GS activity in response to changes in the availability of ammonia (*13*). ATase also participates in the long-term adaptation to conditions (*14*).

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[1] Abbreviations: EC, energy charge; PII, signal transduction protein, product of *glnB*; PII-UMP, uridylylated form of the PII protein; NRI, transcriptional activator of Ntr genes, product of *glnG*; NRI-P, phosphorylated form of NRI; NRI-N, N-terminal domain of NRI that contains the site of phosphosphorylation; NRI-N-P, phosphorylated form of NRI-N; NRII, signal transduction protein encoded by glnL, regulates the phosphorylation and dephosphorylation of NRI; ATase, glutamine synthetase adenylyltransferase (E.C.2.7.7.49), product of *glnE*; UTase/UR, uridylyltransferase/uridylyl-removing enzyme (E.C.2.7.7.59), product of *glnD*; GS, glutamine synthetase, product of *glnA*; GS-AMP, adenylylated form of GS.

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The ATase enzyme and GS regulation by reversible covalent adenylylation are widely observed in bacteria, and for certain bacteria, ATase is essential for viability (*15*). NRII (NtrB) is the transmitter component of the NRI/NRII two-component signal transduction system controlling the transcription of nitrogen-regulated (Ntr) genes. NRII brings about the phosphorylation of the enhancer-binding transcription factor NRI (NtrC) and activation of Ntr gene transcription in the absence of PII and brings about the dephosphorylation of NRI∼P and inactivation of Ntr gene transcription when complexed to PII (reviewed in ref *11*).

Classical early studies of nitrogen regulation in *E. coli* showed that the level of α-ketoglutarate in *E. coli* and *Klebsiella aerogenes* depended on nitrogen status (*16*) and that α-ketoglutarate was a potent regulator of the glutamine synthetase (GS) modification cascade (*17*) of which PII forms a part. Later studies using PII from a variety of organisms showed that PII proteins bind to ATP and to α-ketoglutarate; in most cases, the binding of these effectors is highly synergistic (reviewed in ref *18*). The *E. coli* PII protein bound ATP very avidly in the presence of α-ketoglutarate, leading to the suggestion that ATP probably did not play a regulatory role *in vivo* (*19*, *20*). Conversely, the properties of α-ketoglutarate binding to PII clearly suggested physiological significance (*19*, *20*). When ATP was at physiological levels, the binding of the first molecule of α-ketoglutarate to the PII trimer was quite avid but inhibited the binding of additional molecules of α-ketoglutarate. This negative cooperativity was overcome at physiological concentrations of α-ketoglutarate such that at the high end of the physiological range, α-ketoglutarate was saturating, and three molecules of the effector were bound per PII trimer. The form of PII bound to a single molecule of α-ketoglutarate/trimer was very effective in activating ATase and NRII in reconstituted systems, whereas the form of PII bound with 2 or 3 molecules of α-ketoglutarate/trimer had a reduced or no ability to activate ATase and NRII (*19*, *21*−*23*). Since none of the other components of the reconstituted *E. coli* signal transduction systems was able to bind α-ketoglutarate at physiological concentrations, these studies established PII as the α-ketoglutarate sensor in the reconstituted systems and led to the suggestion that PII proteins serve as sensors of α-ketoglutarate *in vivo* (reviewed in ref *18*).

The *E. coli* PII protein is controlled, indirectly, by glutamine concentration. Uridylyltransferase/uridylyl-removing enzyme (UTase/UR) is a signal transduction enzyme that catalyzes the reversible uridylylation of PII (*17*, *20*, reviewed in ref *24*). The uridylyltransferase (UT) activity of the UTase/UR is strongly inhibited by glutamine, and the uridylyl-removing (UR) activity of the UTase/UR is activated by glutamine. In a reconstituted UTase/UR-PII system, the steady-state level of PII uridylylation reflected the glutamine concentration; at physiological concentrations of α-ketoglutarate, the steady-state levels of PII modification in the UTase/UR-PII reconstituted system were little affected by α-ketoglutarate (*20*). Thus, it was hypothesized that UTase/UR is responsible for transmitting information on the glutamine status to PII (*20*).

The regulation of GS adenylylation state and NRI phosphorylation state was studied in reconstituted bicyclic signal transduction systems (*21*, *22*). In a UTase/UR-PII-NRII-NRI system, the phosphorylation state of NRI was regulated antagonistically by α-ketoglutarate and by glutamine, with the former favoring phosphorylation by decreasing the ability of PII to stimulate the dephosphorylation of NRI (the nitrogen limitation response) and the latter favoring dephosphorylation by increasing the concentration of un-modified PII (the nitrogen excess response). In a UTase/UR-PII-ATase-GS system, the adenylylation state of GS was also regulated antagonistically by α-ketoglutarate and glutamine, with α-ketoglutarate favoring GS de-adenylylation (activation) and glutamine favoring GS adenylylation (inactivation). These studies showed that the reconstituted signaling systems had the capacity to process antagonistic signals (glutamine and α-ketoglutarate) by different mechanisms and to calculate an appropriate output signal (reviewed in ref *11*). The prior studies were conducted at fixed ATP concentration and in the absence of ADP (except as generated by the various system reactions) and thus did not address the issue of adenylate energy charge.

The current study was motivated in part by the work of G. P. Roberts and colleagues extending to more than a decade ago (*25*−*27*), where the regulation of PII-receptors in the photosynthetic bacteria *Rhodospirillum rubrum* was studied. Specifically, Roberts and colleagues presented evidence that PII proteins mediated signals of nitrogen and energy status and that these two signals appeared to be independent of one another on the basis of the kinetics of adaptations of intact cells (*25*, *27*). The conclusion from that work was that *Rhodospirillum rubrum* PII proteins were somehow transducing a signal of energy status, and it was hypothesized that darkness (energy deprivation) resulted in an ATP-deficient form of PII (*27*). Having the reconstituted *E. coli* signal transduction systems in hand, we investigated whether the *E. coli* PII protein transduced signals of energy status *in vitro* and whether the ability of PII to bind ATP reflected an ability to sense the EC or the D/T ratio. We observed that ADP was a powerful regulator of PII and PII-UMP. In the reconstituted signal transduction systems, low adenylylate energy charge acted to reduce signaling of nitrogen limitation (α-ketoglutarate). By individually studying the interactions of PII and PII-UMP that occur in the reconstituted systems, we observed that essentially all of these interactions were influenced by the presence of ADP. Some of our experiments suggested that under certain conditions, the three nucleotide-binding sites of the PII trimer may be bound by mixtures of ATP and ADP. Together, these results show that PII proteins in addition to serving as sensors of α-ketoglutarate also have the capacity to serve as direct sensors of the adenylylate energy charge or, more specifically, its chief component, the D/T ratio.

EXPERIMENTAL PROCEDURES

Purified Proteins. GS, ATase, UTase/UR, PII, NRII, and NRI-N preparations described previously were used (*20*, *28*−*30*). GS-AMP was prepared from purified GS, and PII-UMP was prepared from purified PII as described (*22*). PII proteins from *Arabidopsis thaliana* and *Synechococcus elongatus* were the kind gift of Stephen Atkins, and their preparation will be described in detail elsewhere.

Reconstituted PII-UTase/UR-ATase-GS Bicyclic System. The reactions were conducted essentially as described before (*22*). Briefly, the conditions were 50 mM Tris-Cl at pH 7.5,

100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 1 μM GS (dodecamer concentration) or as indicated, 0.5 μM PII (trimer concentration) or as indicated, 0.04 μM UTase/UR or as indicated, 1 mM KPi, 1 mM [α-^{32}P]-ATP or as indicated, 0.5 mM UTP, and ADP, α-ketoglutarate, and glutamine as indicated. Reactions were initiated by the addition of ATP and UTP, incubated at 30 °C, and aliquots were taken at time intervals and spotted onto nitrocellulose filters, which were soaked immediately in 5% (w/v) TCA and washed thoroughly in 5% TCA to remove unincorporated nucleotides. The radioactivity on the filters was quantified by liquid scintillation counting.

Reconstituted PII/PII-UMP-ATase-GS Monocyclic System. The conditions were similar to those used for the PII-UTase/UR-ATase-GS bicyclic system described above, except that UTase/UR and UTP were omitted, and PII-UMP was present at 0.5 μM or as indicated.

Reconstituted PII-UTase/UR Monocyclic System. Conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 1.0 μM PII or as indicated, UTase/UR as indicated, 0.5 mM ATP or as indicated, 0.5 mM [α-^{32}P] UTP, 0.3 mM α-ketoglutarate or as indicated, and glutamine as indicated. Reactions were initiated by the addition of UTP and ATP and incubated at 30 °C. Aliquots were removed at time intervals and spotted onto nitrocellulose filters, which were washed in 5% TCA and counted by liquid scintillation.

Reconstituted PII-UTase/UR-NRII-NRI Bicyclic System. Conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 30 μM NRI-N (monomer concentration), 0.3 μM NRII (dimer concentration), 0.1 μM UTase/UR or as indicated, 0.5 mM UTP, 0.5 mM [γ-^{32}P] ATP, and PII, α-ketoglutarate, glutamine, and ADP as indicated. Reactions were initiated by the addition of a mixture containingATP and UTP and incubated and analyzed as described above.

Reconstituted NRII-NRI Monocyclic System. Conditions were as in the PII-UTase/UR-NRII-NRI bicyclic system, except that UTase/UR and UTP were omitted.

AT Activity of ATase. Conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 3 μM GS (dodecamer), 0.5 μM PII or as indicated, ATase as indicated, 0.5 mM [α-^{32}P] ATP or as indicated, and α-ketoglutarate, glutamine, and ADP as indicated. Reactions were initiated by the addition of ATP and incubated and analyzed as described above. The initial velocity was determined by linear regression using KaleidaGraph.

AR Activity of ATase. Conditions were similar to those used to measure AT activity, except [^{32}P]-GS-AMP (concentration stated as monomer concentration of adenylylated subunits) was used in place of GS, unlabeled ATP was present at 1 mM, PII-UMP replaced PII as indicated, and KPi was at 5 mM or as indicated.

UT Activity of UTase/UR. Conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 0.5 mM [α-^{32}P] UTP, 0.04 μM UTase/UR or as indicated, 10 μM PII or as indicated, 0.5 mM ATP or as indicated, and α-ketoglutarate, glutamine, and ADP as indicated.

UR Activity of UTase/UR. Conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, [^{32}P] PII-UMP as indicated (monomeric concentration), 0.04 μM UTase/UR or as indicated, 0.5 mM ATP or as indicated, and α-ketoglutarate, glutamine, and ADP as indicated.

Dephosphorylation of NRI-N∼P. Conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, [^{32}P]-NRI-N∼P as indicated (monomeric concentration), 0.1 μM NRII or as indicated, 0.1 μM PII or as indicated, and α-ketoglutarate and ADP as indicated. Preparation of [^{32}P]-NRI-N∼P was briefly as follows: 60 μM NRI-N was mixed with 0.5 μM MBP-NRII111 (which consists of maltose-binding protein fused to amino acids 111−349 of NRII and lacks the phosphatase activities of NRII) and 0.5 mM ATP [γ-^{32}P] at 25 °C for 30 min, then purified on a small Sephadex G-25 column to remove unincorporated nucleotides and the other small molecules. Fractions containing NRI-N∼P were stored at −20 °C until use.

Estimation of Errors. For the estimation of reaction initial velocities (measurement of AT, AR, UT, UR, NRII autophosphorylation, NRI-N phosphorylation, and NRI-N∼P dephosphorylation), experiments typically included 4−5 time points in the period where the production of product was linear. From these data, the initial velocity was determined by linear regression using KaleidaGraph; errors were typically well below 10%, on the basis of the residuals from the linear regression. In our experiments, kinetic constants were estimated directly from secondary plots (rate vs concentration); there were typically at least 6 data points in the range providing the transition from minimimum to maximum rate. We estimate errors from the secondary plots to be on the order of 10% or less, on the basis of the residuals when a smooth curve is drawn through the data points. For three of the experiments in Table 1, we repeated the exact experiment on different days and obtained results for the estimated kinetic constant that differed by less than 15%. However, in the past we have seen slightly higher errors using the same methods, such that final kinetic constants from repeated experiments may vary by up to 50% (i.e., a *Kact* of 2 μM and a *Kact* of 1 μM were obtained when the same measurement was repeated on different days (*29*)).

For the experiments with reconstituted systems (monocycles and bicycles), final steady-state levels of protein modification (adenylylation of GS, uridylylation of PII, or phosphorylation of NRI-N) were observed to vary by less than 10% in repeated experiments on different days.

Binding of ATP, ADP, and α-Ketoglutarate to PII. A micro centrifugation/filtration method was used, as described previously (*19*, *20*). For ATP binding to PII, conditions were 50 mM Tris-Cl at pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, or as indicated, and PII, ATP [γ-^{32}P], ADP, and α-ketoglutarate as indicated. For ADP binding to PII, conditions were similar, except that the labeled species was ^{14}C-ADP. For α-ketoglutarate binding to PII, uniformly labeled ^{14}C-α-ketoglutarate was the labeled species. Incubation of reaction mixtures was at room temperature. The equation for modeling a single class of binding sites was as follows:

$$L_B = \frac{(L_T + R_T + K_D) - ((L_T + R_T + K_D)^2 - 4R_T L_T)^{1/2}}{2}$$

where L_B is bound ligand, L_T is total ligand, and R_T is the

Table 1: Parameters for Activation and Inhibition of the AT and AR Activities of ATase

Apparent *Kact* for PII Activation of the AT Activity

experiment	α-KG[a] (mM)	ADP (mM)	ATase (μM)	ATP (mM)	*Kact* (μM)[b]
041206	0	0	0.3	0.5	7.0
040706	0	0.5	0.05	0.5	1.5
121306	0	0	0.3	3.0	10.0[c]
041206	1	0.5	0.3	0.5	0.8
010605[d]	0.05	0	0.1	0.5	1.00

Apparent *Kact* for ADP Activation of the AT Activity

experiment	α-KG[a] (mM)	PII (μM)	ATase (μM)	ATP (mM)	Gln (mM)	*Kact* (mM)[b]
032106	0	0.5	0.05	0.5	0	0.23[e]
090806	0	0.5	0.02	0.5	0.5	0.10
041406	0	0.5	0.05	3.0	0	>1.2
040506	0.01	0.5	0.025	0.5	0	0.125
040506	1.0	0.5	0.05	0.5	0	1.10[f]

Apparent *Kact* for PII-UMP Activation of the AR Activity

experiment	α-KG[a] (mM)	ATase (μM)	ATP (mM)	ADP (mM)	*Kact* (μM)[b]
062806	1.0	0.03	1.0	1.0	0.85
022305[d]	1.0	0.02	0.5	0	0.28

Apparent *Kinhib* for ADP Inhibition of the AR Activity (Including Product Inhibition Effects)

experiment	α-KG[a] (mM)	PII-UMP (μM)	ATase (μM)	ATP (mM)	*Kinhib* (mM)[b]
060706	0.01	0.4	0.09	1.0	0.29
060706	1.0	0.4	0.09	1.0	0.86

[a] α-ketoglutarate. [b] For a discussion of errors of measurements, please see the Experimental Procedures section. [c] A repetition of this experiment on a different date resulted in a *Kact* value of 9 μM. [d] Data from ref 29 for comparison purposes. [e] A repetition of this experiment on a different date resulted in a *Kact* value of 0.20 mM. [f] A repetition of this experiment on a different date resulted in a *Kact* value of 1.0 mM.

concentration of sites (i.e., protein concentration X 3 for the trimeric PII). The equation for modeling three classes of sites was as follows:

$$L_B = \frac{P_T(K_{D2}K_{D3}L_F + 2K_{D3}L_F^2 + 3L_F^3)}{K_{D1}K_{D2}K_{D3} + K_{D2}K_{D3}L_F + K_{D3}L_F^2 + L_F^3}$$

where the symbols are as described above, and P_T is the total protein concentration, L_F is the concentration of free ligand, and K_{D1}, K_{D2}, and K_{D3} are the three dissociation constants for the three different classes of sites. The equation for modeling two classes of sites was identical, with K_{D2} and K_{D3} being equal. Variations of data in the binding assays were generally less than 15% from experiment to experiment, with higher levels of binding resulting in smaller relative error and lower levels of binding having higher relative error.

RESULTS

ADP Had a Dramatic Effect on the Adenylylation State of Glutamine Synthetase in a Reconstituted UTase/UR-PII-GS-ATase Bicyclic Signal Transduction System. The adenylylation state of glutamine synthetase (GS) is regulated by a linked-bicyclic signal transduction system, in which the UTase/UR controls the uridylylation state of PII in one cycle, and PII and PII-UMP influence the ATase that controls GS adenylylation state in the other (Figure 1A). The ATase enzyme contains two distinct active sites, one of which catalyzes the adenylyltransferase (AT) reaction [GS + ATP → GS-AMP + PPi] and the other of which catalyzes the adenylyl-removing (AR) reaction [GS-AMP + Pi → ADP + GS] (*24*, *28*, *29*, *31*). The regulation of the ATase is quite complex, as the AT activity is synergistically activated by PII and glutamine and is inhibited by PII-UMP, while the AR activity is activated by PII-UMP and is inhibited by PII and glutamine (Figure 1A). Additionally, α-ketoglutarate regulates the activity of PII and PII-UMP, with low concentrations of this effector favoring PII action and high concentrations of this effector favoring PII-UMP action (Figure 1A). Interestingly, recent kinetic and structure/function studies of the enzyme indicate that PII and PII-UMP act from independent sites and that α-ketoglutarate does not regulate the binding of the enzyme by PII or PII-UMP (*29*). Rather, PII and PII-UMP appear to act as dissociable regulatory subunits that convey information on the α-ketoglutarate level.

Prior studies with our reconstituted system containing highly purified UTase/UR, PII, ATase, and GS showed that the GS adenylylation state was rapidly altered in response to alterations in the concentrations of α-ketoglutarate and glutamine (*22*). Here, we examined the effect of ADP on the steady-state level of GS adenylylation in the reconstituted bicyclic system. In one series of experiments, a variety of initial α-ketoglutarate and glutamine conditions (leading to known adenylylation states) were used, and the effect of sequential additions of regulatory molecules were explored. An example of this type of experiment is shown in Figure 1B, where the effects of ADP and α-ketoglutarate were explored in a system that had a low concentration of glutamine (0.5 mM) present throughout the experiment and initially had a very low concentration of α-ketoglutarate present (0.05 mM). As shown, the GS adenylylation state was rapidly and reversibly altered by sequential additions of α-ketoglutarate and ADP, with the former causing a decrease and the latter an increase in the GS adenylylation state (Figure 1B). These results, qualitatively similar to those observed when α-ketoglutarate and glutamine were pitted against one another in the reconstituted system (*22*), suggested that ADP and α-ketoglutarate were antagonistic signals. In another series of experiments, we started with conditions favoring deadenylylation of GS and examined the effect of ADP on the steady-state level of GS adenylylation. ADP was observed to have a dramatic effect on adenylylation state and at millimolar concentration shifted GS from nearly completely unadenylylated to nearly completely adenylylated (Figure 1C).

In additional experiments with the reconstituted bicyclic system, we saw that glutamine signaling was intact in the presence of ADP and that glutamine acted synergistically with ADP. At very low concentrations of glutamine, such as 0.2 mM, the ability of ADP to antagonize α-ketoglutarate was reduced, relative to its ability when glutamine was at 0.5 mM, as in Figure 1B. Furthermore, the steady-state level of GS adenylylation also depended on the concentrations of glutamine and α-ketoglutarate present; as the glutamine concentration increased, lower concentrations of ADP were more potent in promoting GS adenylylation (Figure 1C). These results suggested that ADP was a signaling molecule

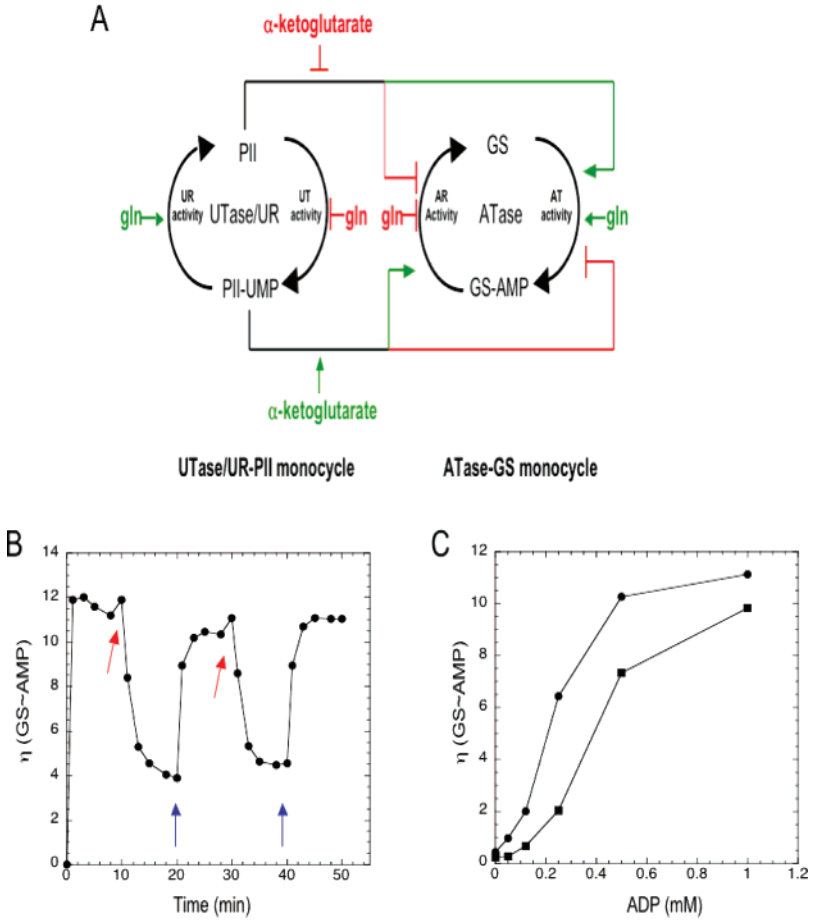

FIGURE 1: ADP acts as a signal molecule controlling GS adenylylation state in a reconstituted UTase/UR-PII-ATase-GS bicyclic signal transduction system. A. Model for the circuit topology based on prior data. Green lines with arrowheads signify activation of the targeted activity; red lines with flat heads signify inhibition of the targeted activity. B. Effect of sequential additions of ADP and α-ketoglutarate to the reconstituted bicyclic system. The initial conditions were 1 μM GS (dodecamer), 0.1 μM ATase, 0.5 μM PII, 0.04 μM UTase/UR, 1 mM [α-^{32}P] ATP, 0.5 mM UTP, 1 mM KPi, 0.05 mM α-ketoglutarate, and 0.5 mM glutamine, with other conditions as described in Experimental Proccedures. At 10 min, α-ketoglutarate was increased to 0.3 mM; at 20 min, ADP was added to 0.25 mM; at 30 min, α-ketoglutarate was further increased to 1.3 mM, and at 40 min, ADP was further increased to 1 mM. Red arrows indicate the times of α-ketoglutarate addition, and blue arrows indicate the times of ADP addition. η stands for the average number of subunits in the GS dodecamer that were adenylylated; since GS is a dodecamer, the maximum η value is 12, signifying complete adenylylation. C. Influence of ADP concentration on the steady-state level of GS adenylylation in the reconstituted bicyclic system. The conditions were 1 μM GS, 0.04 μM ATase, 1 μM PII, 0.1 μM UTase/UR, 1 mM KPi, 1 mM [α-^{32}P] ATP, 0.5 mM UTP, 0.3 mM α-ketoglutarate, and glutamine and ADP as indicated. Symbols: ●, 0.5 mM glutamine; ■, 0.3 mM glutamine.

that worked synergistically with glutamine and antagonistically with α-ketoglutarate and that the signaling system was producing an appropriate output based on all three signaling molecules (ADP, α-ketoglutarate, and glutamine). To explore further how ADP might influence the sensation of α-ketoglutarate and glutamine, we studied the regulation of the uncoupled monocycles that form the bicyclic system.

ADP Had a Dramatic Effect on the GS Adenylylation State in a Reconstituted PII/PII-UMP-ATase-GS Monocyclic System. To study the effect of signaling molecules on the ATase-GS monocycle (Figure 1A, right), we provided PII and PII-UMP at fixed concentrations and left out the UTase/UR. As for the coupled bicyclic system, the adenylylation and de-adenylylation of GS were initiated by the addition of ATP, and the systems came to a steady-state level of GS adenylylation, reflecting the regulatory species present. Under conditions strongly favoring GS de-adenylylation, and thus a low steady-state level of GS adenylylation, ADP was observed to have a dramatic effect on GS adenylylation state, increasing adenylylation about 10-fold (Figure 2A). Furthermore, the stimulation by ADP was antagonized by α-ketoglutarate (Figure 2A) and was synergistic with glutamine (Figure 2B). On the basis of a recent study of the ATase and its control by PII and PII-UMP (*29*), these observations suggested that ADP somehow exerted its effect via PII or PII-UMP, or both. In an additional experiment, we examined the effect of ADP under conditions that were less biased toward de-adenylylation, where glutamine was also present at 1 mM. Glutamine acts synergistically with PII to activate GS adenylylation and to inhibit the activation of de-adenylylation by PII-UMP (*29*; Figure 1A). Under these conditions, ADP again had a dramatic effect on GS adenylylation state, with possibly a biphasic response revealed by a diminished adenylylation state at the highest ADP concentration tested (Figure 2A). The synergy between ADP (at 0.5 mM) and glutamine was further explored; when ADP was present, the reconstituted system displayed a sharper response to glutamine, which occurred at lower concentrations of glutamine (Figure 2C). In the aggregate, these results suggest that ADP either activated the AT activity of ATase, inhibited the AR activity of ATase, or did both. Furthermore, the effects of ADP clearly depended on the concentrations

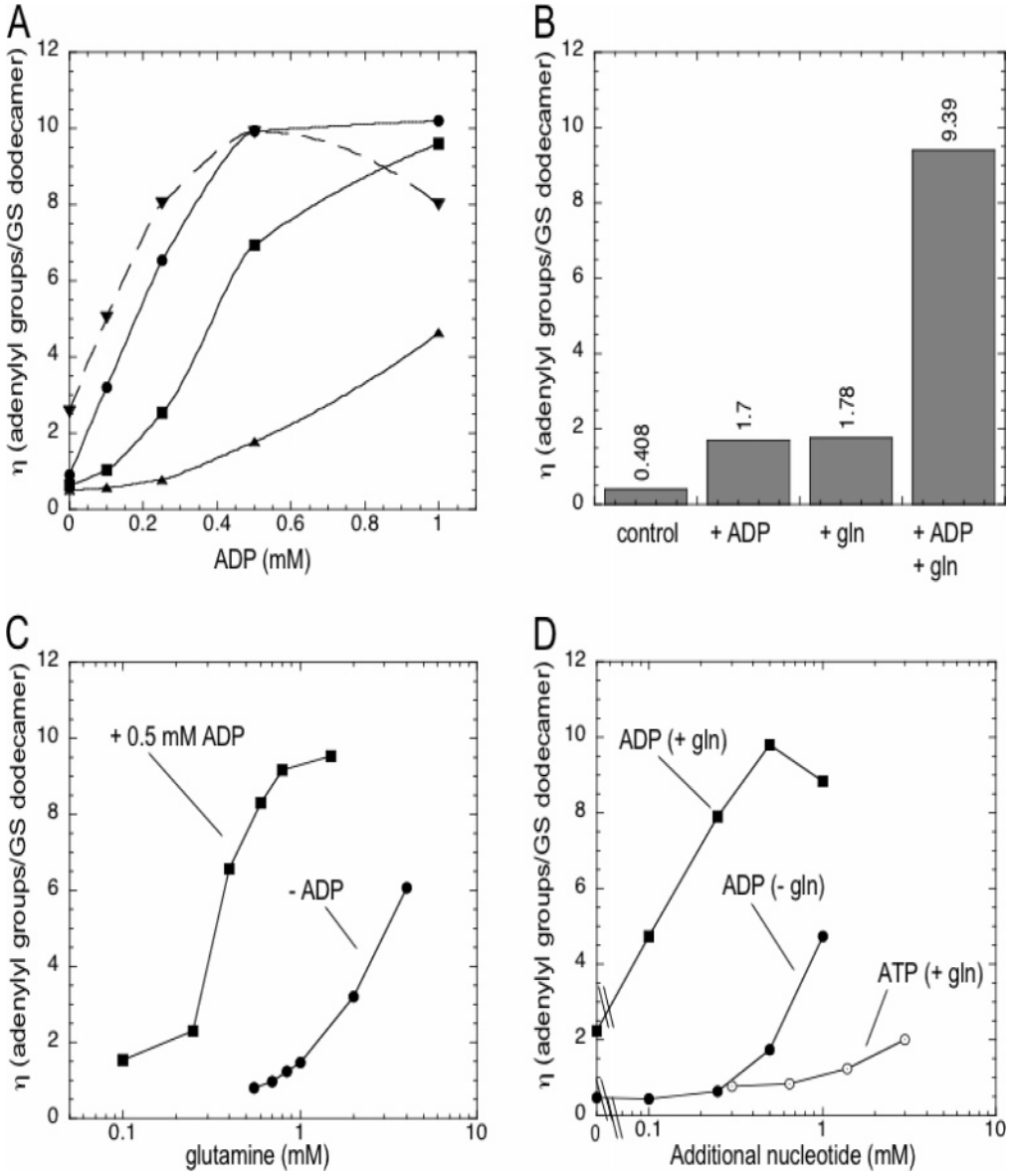

FIGURE 2: Effect of ADP on the adenylylation state of glutamine synthetase in a reconstituted PII/PII-UMP-ATase-GS monocyclic system. A. Steady-state levels of GS adenylylation were affected by ADP. The conditions were 1 μM GS, 0.04 μM ATase, 2 mM [α-^{32}P] ATP, 1 mM KPi, 0.5 μM PII, 0.5 μM PII-UMP, α-ketoglutarate, and glutamine as indicated, and other conditions were as described in Experimental Procedures. Symbols: ●, 0.1 mM α-ketoglutarate; ■, 0.3 mM α-ketoglutarate; ▲, 1 mM α-ketoglutarate; ▼, 1 mM α-ketoglutarate + 1 mM glutamine. B. Synergy between ADP and glutamine for stimulating GS adenylylation. The conditions were 1 μM GS, 0.04 μM ATase, 2 mM [α-^{32}P] ATP, 1 mM KPi, 0.5 μM PII, 0.5 μM PII-UMP, 1 mM α-ketoglutarate, 0.5 mM ADP where indicated, and 1 mM glutamine where indicated. C. ADP alters glutamine sensation in the reconstituted monocyclic system. The conditions were as in panel B, except that glutamine was varied as indicated. ●, control (− ADP); ■, 0.5 mM ADP. D. Comparison between the effects of adding ADP and increasing the concentration of ATP in the reconstituted monocyclic system. The conditions were 1 μM GS, 0.04 μM ATase, 1 mM KPi, 0.5 μM PII, 0.5 μM PII-UMP, 1 mM α-ketoglutarate, and [α-^{32}P] ATP and glutamine as indicated. Symbols: ●, varied ADP with ATP present at the fixed concentration of 2 mM; ■, varied ADP with ATP present at the fixed concentration of 2 mM and with glutamine at 1 mM; ○, varied ATP with 1 mM glutamine present and no ADP present.

of α-ketoglutarate and glutamine present. Given the dramatic effects of ADP in the ATase-GS monocycle, it is likely that most of the effects of ADP observed in the bicyclic system were due to effects on the ATase-GS monocycle.

Since there was 2 mM ATP and up to 1 mM ADP present in the experiments shown in Figure 2A−C, we performed a series of control experiments with the ATase-GS monocyclic system to determine whether simply increasing the ATP concentration gave a similar increase in GS adenylylation state. It did not (Figure 2D); under conditions where GS adenylylation state was low (1 mM α-ketoglutarate and 1 mM glutamine), increasing the ATP concentration up to 3 mM resulted in only a slight increase in GS adenylylation state. Under the same conditions, ADP significantly increased the GS adenylylation state even in the absence of glutamine, and when combined with glutamine, the synergistic action of both effectors resulted in a dramatic increase in GS adenylylation state. Thus, the effect seemed to require ADP.

ADP Acted through PII to Activate the Adenylylation Activity of ATase. To further study the mechanism of signaling by ADP in the ATase-GS monocycle, we examined the individual AT and AR reactions that form the monocycle. The AT activity of ATase can be activated by PII or by glutamine and is synergistically activated by their combina-

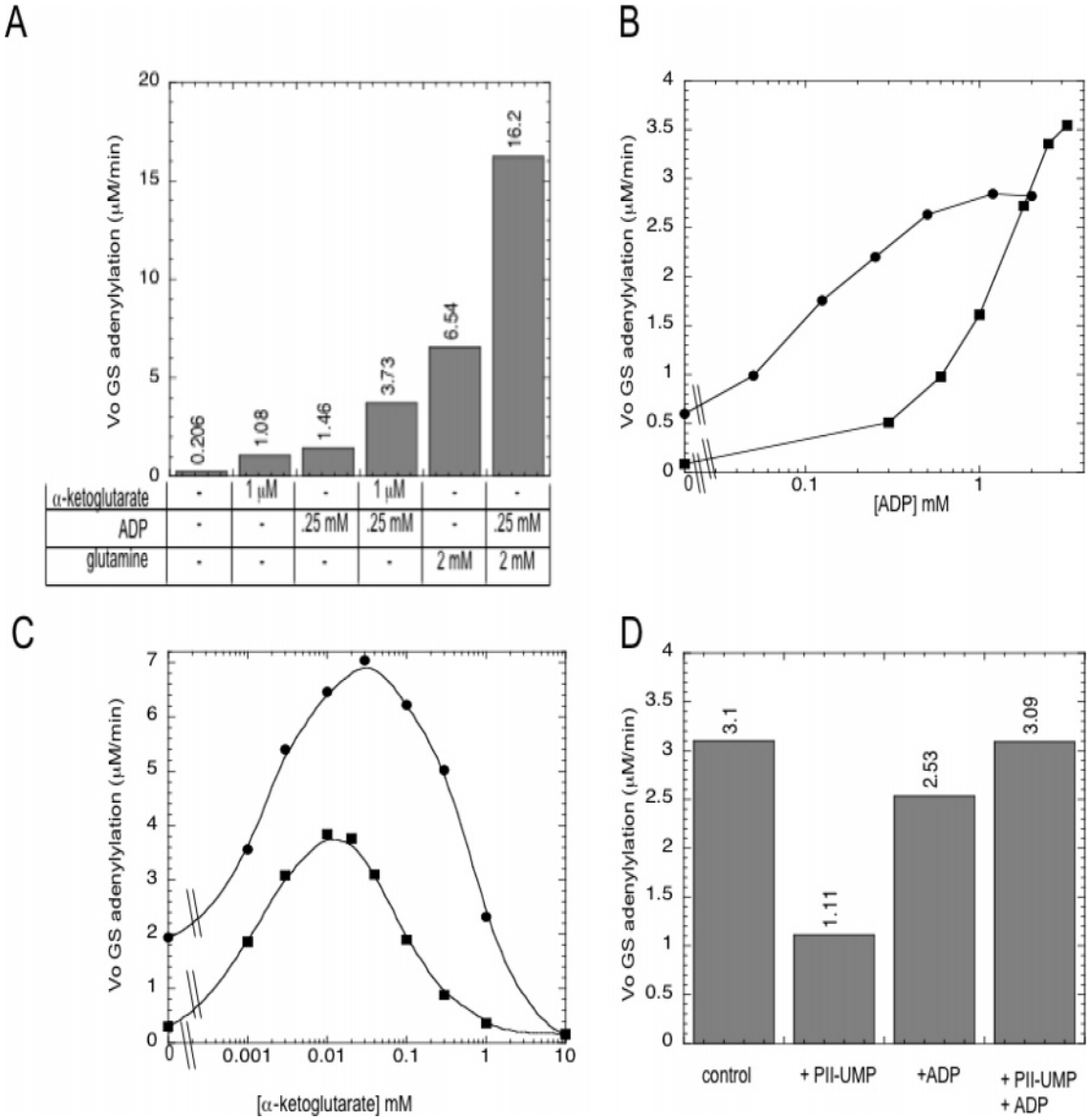

FIGURE 3: ADP acts through PII to activate the AT activity of ATase. A. The AT activity was activated by ADP. Conditions were 3 μM GS, 0.05 μM ATase, 0.5 mM [α-^{32}P] ATP, 0.5 μM PII, 1 μM α-ketoglutarate as indicated, 2 mM glutamine as indicated, and 0.25 mM ADP as indicated. B. α-Ketoglutarate affected the ADP apparent *Kact*. Conditions were 3 μM GS, 0.5 mM [α-^{32}P] ATP, and 0.5 μM PII. Symbols: ●, 0.025 μM ATase and 0.01 mM α-ketoglutarate; ■, 0.05 μM ATase and 1 mM α-ketoglutarate. C. ADP alters PII-mediated regulation of the AT activity by α-ketoglutarate. Conditions were 3 μM GS, 0.5 mM [α-^{32}P] ATP, and 0.5 μM PII. Symbols: ●, 0.05 μM ATase and 0.5 mM ADP; ■, 0.1 μM ATase and no ADP. D. ADP-abolished PII-UMP inhibition of the glutamine-activated AT activity. Conditions were 3 μM GS, 0.05 μM ATase, 0.5 mM [α-^{32}P] ATP, 1 mM α-ketoglutarate, 7 mM glutamine, 1 mM ADP as indicated, and 1 μM PII-UMP as indicated.

tion (*22*, *29*; Figure 1A). We observed that ADP was an activator of the AT activity, but only when PII was present. When the AT activity was activated by glutamine alone, ADP was a weak inhibitor of the AT activity. This is reasonable, as ADP may act as a poor analogue for the substrate, ATP. In one experiment, 10% inhibition of the AT rate was provided by 0.5 mM ADP when ATP was also 0.5 mM (not shown). In another experiment, ADP at 1 mM provided 19% inhibition of the AT rate when ATP was 0.5 mM (see below; Figure 3D). Thus, in the absence of PII, ADP was not an activator of the AT activity but was instead a weak inhibitor.

Remarkably, in the presence of PII, ADP was a potent activator of the AT activity of ATase (Figure 3A). This activation was observed even in the absence of α-ketoglutarate and was synergistic with glutamine and with α-ketoglutarate at very low concentrations (1 μM) (Figure 3A). Also, α-ketoglutarate had a dramatic effect on the apparent *Kact* of ADP (Figure 3B and Table 1). On the basis of our current understanding of ATase regulation (*29*), these results strongly implicated PII as a target of ADP action. The apparent *Kact* of ADP for activation of the AT activity was increased dramatically when ATP was present at high concentrations (Table 1). Together, these observations suggested that ADP acted by binding to PII and that ATP and ADP may compete for binding to PII.

Prior studies showed that α-ketoglutarate provided biphasic regulation of the AT activity when PII was present along with ATP as the sole nucleotide (*22*, *29*; Figure 3C); the optimal concentration of α-ketoglutarate presumably corresponds to that at which the PII trimers were bound by a single molecule of α-ketoglutarate. Here, we observed that a similar biphasic response to α-ketoglutarate was obtained when ATP

and ADP were present at equal concentration, except that the optimal α-ketoglutarate concentration was shifted from 10 μM to 30 μM under the conditions used (Figure 3C). Also, the response curve was asymmetric because of activation by ADP in the absence of α-ketoglutarate, and the overall rates were higher due to activation by ADP (Figure 3C; note that different enzyme concentrations were used to obtain comparable rates). This shows that in the presence of ADP, PII was still a potent sensor of α-ketoglutarate but that its sensory properties and signaling were altered.

Prior studies showed that PII, in the presence of ATP and α-ketoglutarate, activated the AT activity with an apparent *Kact* of ~1−2 μM (*29*). Also, when ATP was the sole nucleotide, the PII apparent *Kact* was not affected by α-ketoglutarate within the physiological range, although the extent of AT activation decidedly was, suggesting that α-ketoglutarate (in this range) did not alter the binding of ATase by PII (*29*). Here, we examined PII activation of AT activity in the presence of ADP, ATP, and α-ketoglutarate and observed a PII *Kact* of ~0.8 μM (Table 1), which is not significantly different from the prior results when just ATP and α-ketoglutarate were present. This suggests that the major effects of ADP on PII activation of the AT activity were probably not due to greatly improved binding of PII to ATase.

As already noted, the AT activity of ATase is activated by glutamine, by PII, and is synergistically activated by the combination of glutamine and PII. Prior work demonstrated that the AT activity of ATase was undetectable in the absence of both ATase activators, PII and glutamine (*22*). That is, there was no detectable basal AT activity in the absence of an activator. During the course of this work, we observed the activation of AT activity by PII when ATP was 0.5 mM or 3 mM in the absence of α-ketoglutarate or ADP (Table 1 and Figure 3A). When ATP was at 0.5 mM, a PII *Kact* of ~7 μM was obtained (Table 1), and *kcat*′ was ~5/min. When ATP was 3.0 mM, a PII *Kact* of ~10 μM was observed (Table 1), and *kcat*′ was ~15.2/min. By comparison, the *kcat*′ for similar conditions, except with α-ketoglutarate present and at optimal concentration, was ~300−400/min. Thus, PII in the presence of ATP and the absence of α-ketoglutarate was a poor activator of the AT activity of ATase. In contrast, α-ketoglutarate was not required for PII to serve as activator of the AT activity of ATase when ADP was present (Figure 3A); the PII apparent activation constant under these conditions was ~1.5 μM (Table 1), about 5-fold lower than that observed with ATP alone, implying that ADP might improve the PII-ATase interaction.

ADP also influenced the inhibition of the glutamine-activated AT activity by PII-UMP (Figure 3D). Under conditions where PII-UMP provided ~64% inhibition of the AT rate, the addition of ADP completely eliminated inhibition by PII-UMP. Part of this apparent loss of inhibition may be due to ADP activation of a trace amount of un-modified PII contaminating the PII-UMP preparation. However, since the PII-UMP preparation was assessed as being ~98% modified (not shown) and given the low concentration of PII-UMP used in the experiment, it seems that most of the observed effect was due to ADP antagonizing the inhibitory activity of PII-UMP. The level of unmodified PII in the experiment, ~20 nM, is far below its *Kact* in the presence of ADP (~1.5 μM, Table 1). Thus, in addition to acting through PII to activate the AT activity, ADP also eliminated the inhibition of the AT activity by PII-UMP.

Table 2: Parameters for the Activation of the UT and UR Activities of UTase/UR

Apparent *Kact* for ADP Activation of the UT Activity

experiment	α-KG[a] (mM)	PII (μM)	UTase/UR (μM)	UTP (mM)	ATP (mM)	*Kact* (μM)[b]
072806	0.03	10	0.04	0.5	0	45
072806	3.00	10	0.04	0.5	0	40

Apparent *Kact* for Activation of the UT Activity by α-Ketoglutarate

experiment	UTP (mM)	PII (μM)	UTase/UR (μM)	ADP (mM)	ATP (mM)	*Kact* (μM)[a]
072706	0.5	5.0	0.04	0.5	0.0	130.0
080807	0.5	5.0	0.02	0.0	0.5	3.5
[a]	0.04	4.0	0.01	0.0	0.5	5.0

Apparent *Kact* for Activation of the UR Activity by ADP

experiment	α-KG[a] (mM)	PII−UMP (μM)	UTase/UR (μM)	ATP (mM)	*Kact* (μM)[b]
051506	0.03	6.49	2.0	0	70
051506	3.00	6.49	2.0	0	70

[a] Data from ref 20, for comparison. [b] For a discussion of errors of measurements, please see the Experimental Procedures section.

ADP Inhibits the AR Activity of ATase. ADP is a product of AR activity, and prior work showed that it is an inhibitor that acts competitively with either substrate (GS-AMP and Pi) (*29*). Here, we observed that the ADP apparent K_{inhib} was dependent on α-ketoglutarate concentration, which implies a role for PII-UMP (Table 1). Furthermore, ADP appeared to increase the PII-UMP *Kact*, that is, diminish the binding of PII-UMP to the enzyme (Table 1). Thus, both PII-UMP-dependent and -independent mechanisms of inhibition were observed, with the PII-UMP-dependent mechanism(s) likely due to ADP binding to PII-UMP.

ADP Reduced the Extent of PII Uridylylation in a Reconstituted PII-UTase/UR Monocyclic System and Rendered the PII Uridylylation State Sensitive to the Concentration of α-Ketoglutarate. Prior studies showed that uridylylation and de-uridylylation of PII by UTase/UR requires α-ketoglutarate and a PII-binding activating adenosine nucleotide, such as ATP, ADP, or AMP-PNP (*20*), and we confirmed this (not shown). The highest rates of both the uridylylation (UT) reaction and the uridylyl-removing (UR) reaction were obtained when ATP was the activating nucleotide (not shown). For both uridylylation and de-uridylylation reactions, α-ketoglutarate had little effect on the ADP apparent *Kact* values, which were well within the physiological range of ADP concentrations (Table 2). Notably, when ADP was the sole activating nucleotide for the UT reaction, the apparent *Kact* of α-ketoglutarate was significantly raised relative to the *Kact* when ATP was the activating nucleotide (Table 2). (This is reasonable since ATP promotes the binding of α-ketoglutarate to PII, while, as we show below, ADP does not.) High concentrations of α-ketoglutarate greatly increased the ability of ADP to activate the UR reaction. These observations are again consistent with α-ketoglutarate and ADP exerting their effects through PII and PII-UMP.

To investigate which of these effects of ADP were most important, we examined the steady-state level of PII modi-

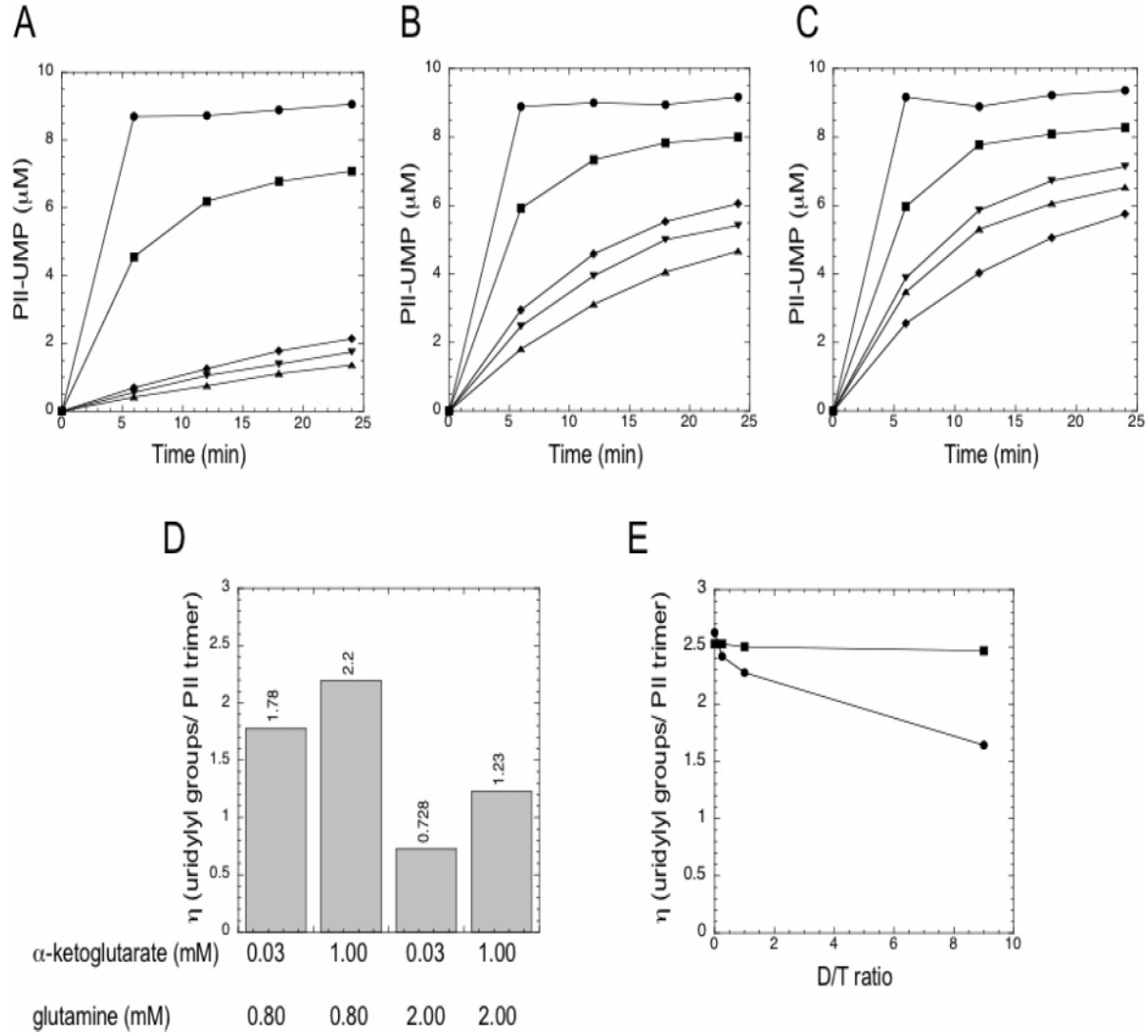

FIGURE 4: Effect of ADP on the PII-UTase/UR monocycle. A−C. ADP renders PII uridylylation state sensitive to the concentration of α-ketoglutarate. Conditions were 3 μM PII, 0.5 mM [α-^{32}P] UTP, 0.3 μM UTase/UR, 0.8 mM glutamine, α-ketoglutarate at 0.03 mM (panel A), 0.3 mM (panel B), or 3 mM (panel C), and ATP or ADP as indicated. Symbols: ●, control (− glutamine and + 0.5 mM ATP); ■, + 0.5 mM ATP; ◆, + 0.5 mM ADP; ▼, + 0.15 mM ADP; ▲, + 0.05 mM ADP. D. Both α-ketoglutarate and glutamine regulated PII uridylylation state when ADP was the activating adenylylate nucleotide. Conditions were 1 μM PII, 0.3 μM UTase/UR, 0.5 mM ADP, 0.5 mM [α-^{32}P] UTP, and α-ketoglutarate and glutamine as indicated. E. Ratio of ADP to ATP (D/T ratio) affects PII uridylylation state at low concentration of α-ketoglutarate. Conditions were 3 μM PII, 0.4 μM UTase/UR, 0.5 mM [α-^{32}P] UTP, 0.8 mM glutamine, α-ketoglutarate as indicated, and ATP + ADP equal to 1 mM. Symbols: ●, 0.03 mM α-ketoglutarate; ■, 0.3 mM α-ketoglutarate.

fication in a reconstituted UTase/UR-PII monocycle system. In this system, both uridylylation of PII and deuridylylation of PII-UMP occur, and the steady-state uridylylation state reflects the regulation of these activities by the regulatory molecules present. For the current experiments, an intermediate level of glutamine was present so that it would be possible to observe either increases or decreases in the PII uridylylation state. Under the conditions chosen, when ATP was 0.5 mM and glutamine was absent, the steady-state level of PII uridylylation corresponded to complete uridylylation, and a somewhat lower steady-state level of PII uridylylation was obtained in the presence of glutamine, as intended (Figure 4A−C). As before (*22*), the level of PII uridylylation under these conditions was little altered as α-ketoglutarate was varied 100-fold between 0.03 mM and 3 mM (Figure 4A−C).

Remarkably, exactly the opposite result was obtained when ADP was used as the activating nucleotide in place of ATP. Under these conditions, α-ketoglutarate was a potent regulator of PII uridylylation state, favoring PII uridylylation (Figure 4A−C). At low α-ketoglutarate, ADP as the activating nucleotide resulted in a dramatically lower PII uridylylation state than did ATP (Figure 4A), whereas at high α-ketoglutarate, ADP as the activating nucleotide resulted in a level of PII uridylylation only slightly lower than that obtained when ATP was the activating nucleotide (Figure 4B and C). The net effect is thus that the uridylylation state was strongly regulated by α-ketoglutarate when ADP was the activating nucleotide. In another experiment where ADP was the sole adenylylate nucleotide, clear effects of α-ketoglutarate were observed at both low and high glutamine concentrations (Figure 4D). Thus, ADP can alter the interactions of PII with UTase/UR and α-ketoglutarate. However, we observed in several additional experiments that when both ATP and ADP were present, the effects of ADP on PII uridylylation state and its control by α-ketoglutarate were

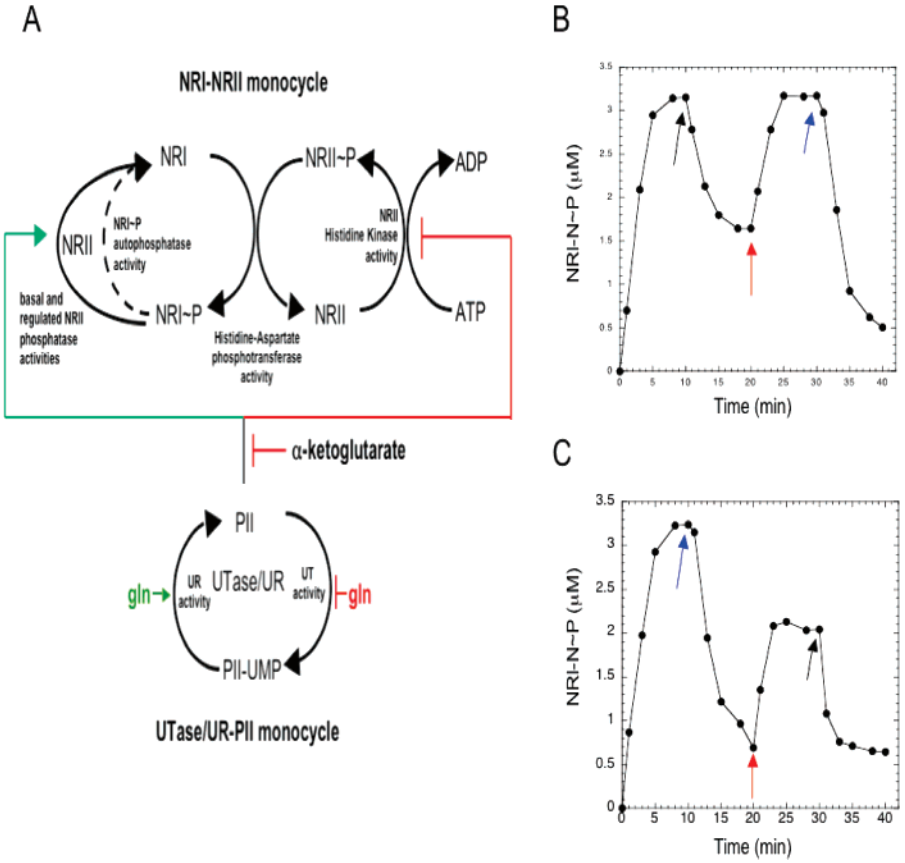

FIGURE 5: ADP acted as a signaling molecule in a reconstituted PII-UTase/UR-NRI-NRII bicyclic signal transduction system. A. Model for the circuit topology on the basis of prior data. Green lines with arrowheads signify activation of the targeted activity; red lines with flat heads signify inhibition of the targeted activity. B and C. Sequential additions of α-ketoglutarate, glutamine, and ADP change the NRI phosphorylation state in the reconstituted bicyclic system. The initial conditions were 30 μM NRI-N (monomer), 0.3 μM NRII, 0.2 μM PII, 0.1 μM UTase/UR, 0.5 mM [γ-^{32}P] ATP, 0.5 mM UTP, 0.1 mM α-ketoglutarate, and 0.1 mM glutamine. In panel B at 10 min, ADP was added to 0.5 mM; at 20 min, α-ketoglutarate was increased to 10 mM; and at 30 min, glutamine was increased to 2 mM. In panel C at 10 min, glutamine was raised to 1 mM; at 20 min, α-ketoglutarate was raised to 10 mM; and at 30 min, ADP was added to 0.5 mM.

muted. As an example, in one experiment where adenylylate nucleotide (ATP + ADP) concentration was held constant and the ratio of ADP/ATP was varied from 0 to 9, the D/T ratio was only regulatory when α-ketoglutarate was low, and even then, significant effects were only observed when the D/T ratio was 1.0 or greater (Figure 4E). Thus, the dramatic effects of ADP in the bicyclic PII-UTase/UR-ATase-GS system were observed under conditions where significant changes in PII uridylylation states was not observed in the UTase/UR-PII monocycle. Therefore, in the bicycle experiments, ADP seemed to be exerting its effects mainly on the ATase-GS monocycle.

ADP Affects The Phosphorylation State of NRI in a Reconstituted NRI-NRII-PII-UTase/UR Bicyclic Signal Transduction System. To determine the generality of ADP effects on PII, we examined another PII/PII-UMP-dependent regulatory circuit. The phosphorylation state of the enhancer-binding transcription factor NRI is regulated by a linked bicyclic signal transduction system, in which UTase/UR controls the uridylylation state of PII in one cycle, and PII regulates the autophosphorylation of NRII that leads to NRI phosphorylation and regulates the dephosphorylation of NRI~P by NRII in the other cycle (Figure 5A) [reviewed in ref *11*]. NRII mediates both the phosphorylation and dephosphorylation of NRI, but both reactions are catalyzed by NRI itself (*30*). For the phosphorylation reaction, NRII phosphorylates itself on a histidine residue using ATP and producing ADP, and NRI catalyzes its own phosphorylation using the NRII phospho-histidine as its substrate. NRI-P slowly catalyzes its own dephosphorylation, an activity referred to as the autophosphatase activity. NRII, in the absence of PII, is a weak activator of the NRI-P autophosphatase activity (*32*), we will refer to this activity of NRII as its basal phosphatase activity (Figure 5A). When NRII is complexed to PII, it is a very potent activator of the autophosphatase activity of NRI-P, an activity that has been referred to as the regulated phosphatase activity of NRII (*30, 33, 34*).

Prior studies with our reconstituted system containing highly purified UTase/UR, PII, NRII, and NRI showed that the NRI phosphorylation state was rapidly altered in response to alterations in the concentrations of α-ketoglutarate and glutamine (*21*). Here, we examined the effect of ADP on the steady-state level of NRI phosphorylation in the reconstituted bicyclic system. For convenience, we used the N-terminal domain of NRI, which is phosphorylated and dephosphorylated normally by PII and NRII (*23*, *30*), and adjusted the concentrations of PII and NRII in the reconstituted system such that intermediate levels of NRI-N phosphorylation were obtained (typically ~15% phosphorylation of the NRI-N as a maximum level). In one series of experiments, a variety of initial α-ketoglutarate and glutamine conditions were used, and the effect of sequential additions of regulatory molecules were explored. An example of this

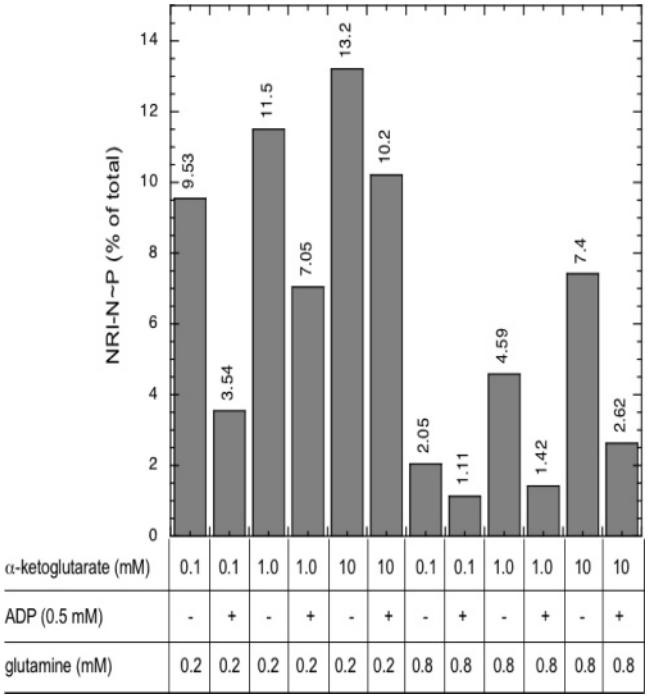

FIGURE 6: ADP affected NRI phosphorylation state in a reconstituted PII-UTase/UR-NRI-NRII bicyclic signal transduction system. The conditions were 30 μM NRI-N, 0.3 μM NRII, 0.2 μM PII, 0.1 μM UTase/UR, 0.5 mM [γ-^{32}P] ATP, 0.5 mM UTP, and ADP, glutamine, and α-ketoglutarate as indicated. The level of NRI phosphorylation is shown as the percentage of the total NRI-N concentration.

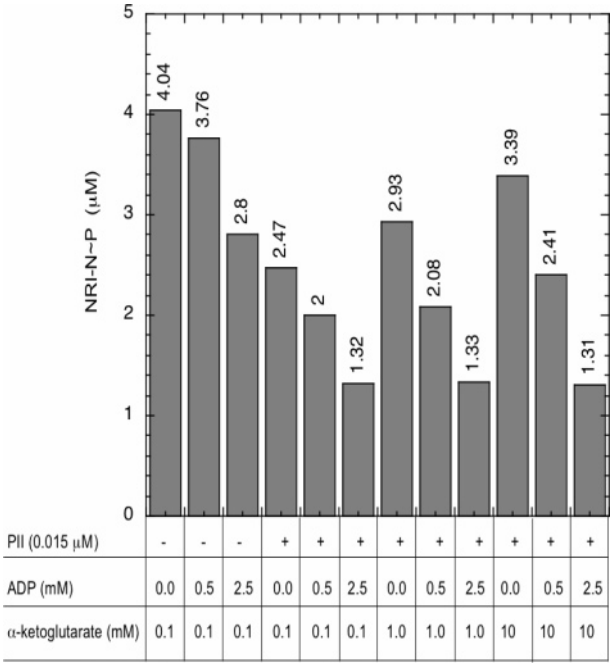

FIGURE 7: ADP affects NRI phosphorylation state in a reconstituted NRII-NRI monocyclic system. Conditions were 30 μM NRI-N (monomer), 0.3 μM NRII, 0.5 mM [γ-^{32}P] ATP, and PII, ADP, and α-ketoglutarate as indicated. The control for absence of PII was performed at 0.1 mM α-ketoglutarate only, as additional experiments (not shown) indicated that α-ketoglutarate had no effect in the absence of PII.

type of experiment is shown in Figure 5B, where the effects of ADP and α-ketoglutarate were examined in a system that initially had a low concentration of glutamine (0.1 mM) and α-ketoglutarate (0.1 mM). As shown, NRI-N was rapidly phosphorylated under these conditions, reaching steady state in about 10 min. The NRI phosphorylation state was rapidly decreased upon addition of ADP to 0.5 mM, then rapidly increased upon increasing α-ketoglutarate to 10 mM, and then rapidly decreased by increasing glutamine to 2 mM (Figure 5B). These results, qualitatively similar to those observed when α-ketoglutarate and glutamine were pitted against one another in the reconstituted system (*21*), suggested that ADP and α-ketoglutarate were antagonistic signals. In additional experiments, we observed that the transitions between steady states were rapid regardless of the order of addition of the regulatory small molecules (ADP, α-ketoglutarate, and glutamine) (e.g., Figure 5C).

Using the bicyclic NRI-NRII-PII-UTase/UR system, we examined the steady-state level of NRI-N phosphorylation in the presence of various α-ketoglutarate, glutamine, and ADP concentrations. ADP had a significant effect on the extent of NRI phosphorylation in the reconstituted bicycle, acting to reduce NRI-N phosphorylation state at both high and low concentrations of α-ketoglutarate and glutamine (Figure 6). As before, α-ketoglutarate acted to increase the phosphorylation state of NRI-N in the reconstituted bicyclic system, and glutamine, working through UTase/UR to increase the concentration of unmodified PII, acted to decrease it (Figure 6). The effects of ADP were most dramatic when the concentration of α-ketoglutarate was low (Figure 6). These experiments show that the steady-state level of NRI-N phosphorylation in the reconstituted bicyclic system responded to all three regulatory molecules (α-ketoglutarate, glutamine, and ADP). In another experiment, we saw that the addition of ADP to 0.25 mM had no significant effect in the absence of PII, regardless of the α-ketoglutarate concentration (not shown). Also, α-ketoglutarate had no effect on NRI phosphorylation state in the absence of PII (not shown). Since α-ketoglutarate and ADP acted antagonistically in the bicyclic system, the results suggested the possibility that the binding of ADP to PII counteracted the effect of α-ketoglutarate at high concentration.

ADP Affects the Steady-State Level of NRI Phosphorylation in a Reconstituted NRI-NRII Monocycle. To study the effect of signaling molecules on the regulation of the NRI-NRII monocycle, we provided PII at fixed concentration and left out UTase/UR. In these reconstituted systems, the phosphorylation and de-phosphorylation of NRI were initiated by the addition of ATP, and the systems rapidly came to a steady-state level of NRI phosphorylation, reflecting the regulatory species present. Again, for convenience, we used the isolated N-terminal domain of NRI, present at large excess, and manipulated the concentrations of NRII and PII such that intermediate levels of phosphorylation were observed.

In the monocyclic system, ADP was observed to have a negligible effect in the absence of PII when present at 0.5 mM but had a significant effect in the absence of PII when present at 2.5 mM (Figure 7). When PII was present, ADP, even at 0.5 mM, significantly reduced the phosphorylation state of NRI-N (Figure 7). As before, α-ketoglutarate acted to raise the phosphorylation state of NRI-N in the monocyclic system when ADP was absent. ADP acted to block this effect of α-ketoglutarate in the monocyclic system (Figure 7). Thus,

these experiments with the monocyclic NRI-NRII system are consistent with the results with the larger bicyclic system and suggest that most of the ADP effects observed in the experiments with the bicyclic system were due to effects on the NRI-NRII monocycle.

ADP Was A Potent Activator of the Regulated Phosphatase Activity of NRII. As before, we further investigated the role of ADP by examining the individual reactions that constitute the monocyclic system. To directly focus on NRI-P dephosphorylation, we used a well-studied assay where the release of labeled phosphoryl groups from NRI-N-^{32}P was measured and avoided the presence of ATP to prevent the replacement of labeled phosphoryl groups with unlabeled ones. We observed/confirmed that the weak basal phosphatase activity of NRII did not require any nucleotides but was modestly stimulated by the ATP analogue AMP-PNP and by ADP, with ADP providing the greater stimulation (not shown). We also confirmed that the powerful regulated phosphatase activity required α-ketoglutarate in combination with an activating nucleotide such as ADP or AMP-PNP and that α-ketoglutarate, even at high concentrations, was unable to activate the regulated phosphatase activity in the absence of an activating nucleotide (not shown). We observed that at a low concentration of α-ketoglutarate (0.01 mM), AMP-PNP was a better activator than was ADP, while the reverse was seen at a high concentration of α-ketoglutarate (1 mM). This led us to study the issue in detail, where it was observed that ADP and AMP-PNP were activators with distinct α-ketoglutarate optima (Figure 8A). When both nucleotides were present, the maximal rate of the regulated phosphatase activity was obtained at an intermediate concentration of α-ketoglutarate (Figure 8A). Thus, nucleotides and α-ketoglutarate appeared to constitute interacting signals that controlled the regulated phosphatase activity. ADP had the effect of keeping the regulated phosphatase activity high (and thus the extent of NRI phosphorylation low) at elevated α-ketoglutarate. In additional experiments, we examined whether other nucleotide diphosphates or AMP could stimulate the regulated phosphatase activity and found that they could not (not shown). The effect was specific to ADP. Remarkably, at their optimal concentrations of α-ketoglutarate, the apparent *Kact* for ADP and AMP-PNP were similar: ~0.25 mM (Table 3). By providing various combinations of AMP-PNP and ADP at the fixed total nucleotide concentration of 3 mM, we observed that the rate of the regulated phosphatase activity was regulated by the adenylylate energy charge or D/T ratio *in vitro*, with the caveat that AMP-PMP is an ATP analogue that may have binding properties different from those of ATP (Figure 8B). Under the conditions used (1 mM α-ketoglutarate, as in nitrogen-starved cells), decreasing energy charge correlated with an increased rate of NRI-N-P dephosphorylation, as long as both nucleotides were present.

ADP Was also an Inhibitor of the NRII Autophosphorylation Activity. As expected, ADP, a product of NRII autophosphorylation, inhibits the rate of NRII autophosphorylation. The experiments with the monocyclic system, already described, indicated that in the absence of PII, ADP at 0.5 mM had a small effect on the NRI-N phosphorylation state (Figure 7), suggesting that significant inhibition of NRII autophosphorylation was not occurring under these conditions. This may be because in the reconstituted monocyclic and bicyclic systems, the vast excess of NRI-N pulls the equilibrium toward NRI-N phosphorylation. We directly studied NRII autophosphorylation by measuring the rate of incorporation of the label from ATP into NRII, as described previously (*32*). When ATP was 0.5 mM, ADP at 0.25 mM provided about 30% inhibition of the autophosphorylation rate, regardless of α-ketoglutarate concentration (not shown). Thus, the effects of ADP on the extent of NRI phosphor-

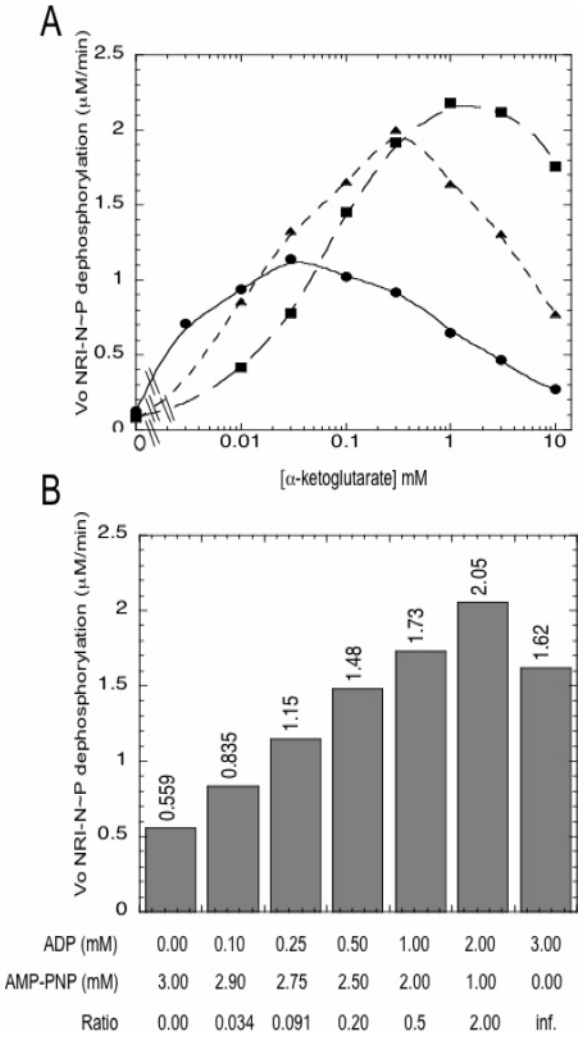

FIGURE 8: Rate of the regulated phosphatase activity is influenced by ADP. A. The regulated phosphatase activity was differentially regulated by α-ketoglutarate depending on whether ADP or AMP-PNP was the activating nucleotide. All experiments contained 0.1 μM NRII and 0.1 μM PII, and α-ketoglutarate was varied as indicated. Symbols: ●, 0.89 μM [^{32}P] NRI-N~P and 1 mM AMP-PNP; ■, 0.89 μM [^{32}P] NRI-N~P and 0.5 mM ADP; ▲, 0.73 μM [^{32}P] NRI-N~P and 1 mM AMP-PNP + 0.5 mM ADP. B. The regulated phosphatase activity is influenced by the ratio of ADP to AMP-PNP. Conditions were 0.73 μM [^{32}P] NRI-N~P, 0.1 μM NRII, 0.1 μM PII, 1 mM α-ketoglutarate, and the sum of ADP + AMP-PNP concentrations equal to 3 mM as indicated.

Table 3: Activation of the Dephopshorylation of NRI-N~P by ADP and AMP-PNP

experiment	α-KG (mM)	NRII (μM)	PII (μM)	NRI-N-^{32}P (μM)	activator	*Kact* (mM)[a]
042106	1.0	0.1	0.1	0.73	ADP	0.25
042406	0.1	0.1	0.1	0.73	AMP-PNP	0.21

[a] For a discussion of errors of measurement, please see the Experimental Procedures section.

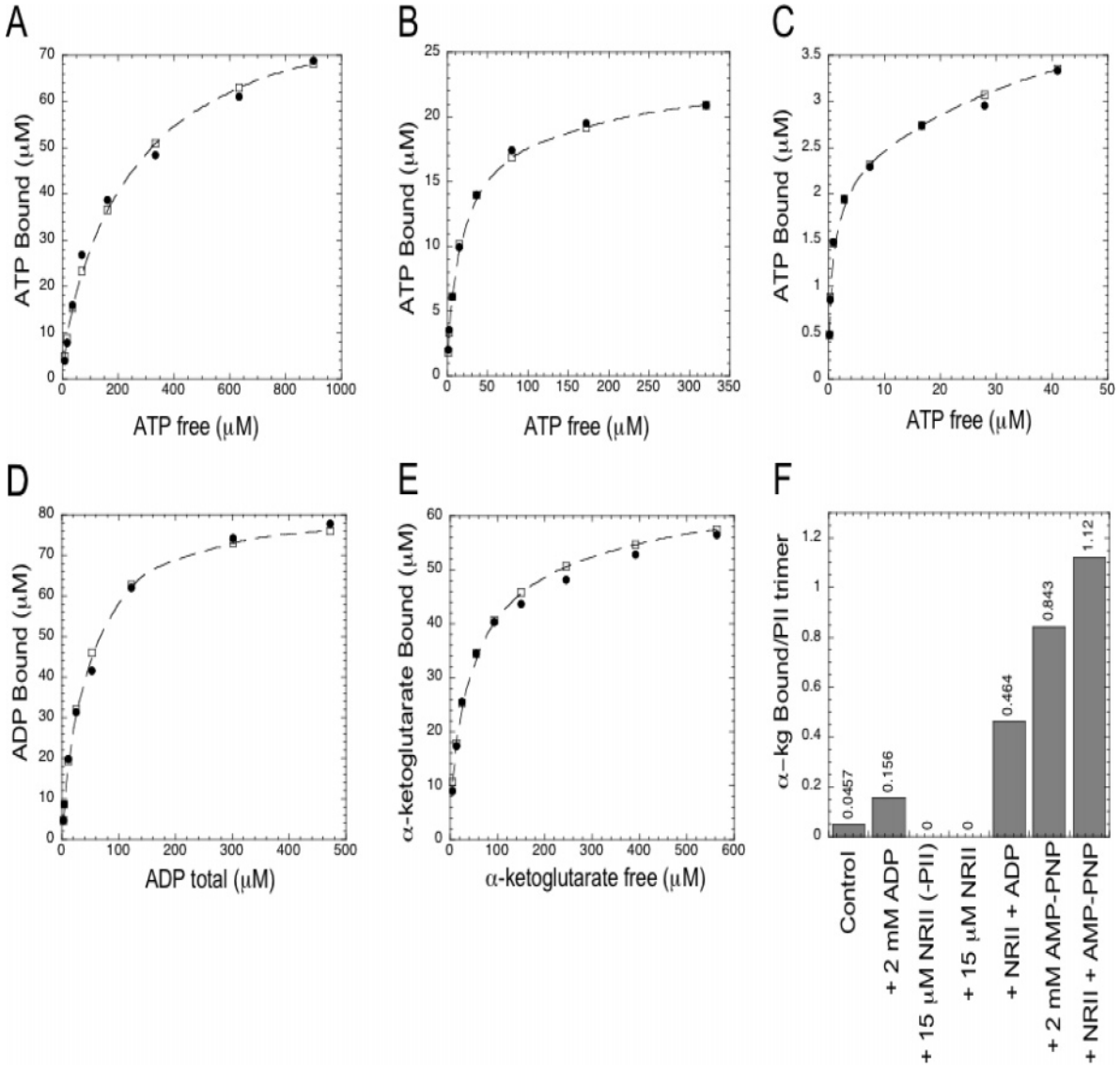

FIGURE 9: Binding of ATP, ADP, and α-ketoglutarate to PII. Symbols: experimental data, ●; calculated data from models, □ and dashed lines. A. Binding of ATP to PII in the absence of α-ketoglutarate. PII was 27.4 μM. B. Binding of ATP to PII in the presence of 0.05 mM α-ketoglutarate. PII was 9.75 μM. C. Binding of ATP to PII in the presence of 2 mM α-ketoglutarate. PII was 2.0 μM. D. Binding of ADP to PII in the absence of α-ketoglutarate. PII was 27.4 μM. E. Binding of α-ketoglutarate to PII in the presence of 4 mM ATP. PII was 30 μM. F. Effect of NRII on the binding of α-ketoglutarate to PII. PII was 30 μM, and α-ketoglutarate was 154.1 μM. Additions were as indicated.

ylation in the reconstituted monocyclic and bicyclic systems seemed to be due mainly to the increase in the rate of the regulated phosphatase activity, but when the autophosphorylation of NRII was studied in isolation, ADP had a significant inhibitory effect.

Binding of ATP, ADP, and α-Ketoglutarate to PII. Prior results indicated that the binding of ATP and α-ketoglutarate to PII was synergistic (*19*). Here, we studied the binding of ATP to PII in the absence of α-ketoglutarate, conditions under which the binding of ATP is greatly reduced, as well as in the presence of an intermediate concentration of α-ketoglutarate (50 μM) and a high concentration of α-ketoglutarate (2 mM). As before, we used a micro-centrifugation method that measures the depletion of unbound ^{32}P-ATP from the reaction mixtures (*19*).

In the absence of α-ketoglutarate, the binding of ATP to PII was suggestive of multiple classes of sites, with negative cooperativity (Hill coefficient $\eta_H = 0.82$; Figure 9A); these data were best fit by a model with three classes of sites, with *Kd* values of 50 μM, 250 μM, and 520 μM (Figure 9A). The data were also fit reasonably but less well by a model with two classes of sites, with *Kd* values of 42 μM and 420 μM (not shown), but could not be acceptably fit to a model with a single class of sites (not shown). In the presence of 50 μM α-ketoglutarate, the binding of ATP was again suggestive of multiple classses of sites with negative cooperativity ($\eta_H = 0.59$); in this case, the best fit of the data was to a model with three classes of sites with *Kd* values of 5 μM, 35 μM, and 1000 μM (Figure 9B). Significantly worse fits were obtained when we tried to fit these data to models with a single class of sites or two classes of sites (not shown). When α-ketoglutarate was present at 2 mM, the binding of ATP was again suggestive of multiple classses of sites; in this case, the best fit of the data was to a model with three classes of sites with *Kd* values of 0.36 μM, 30 μM, and 300 μM ($\eta_H = 0.37$; Figure 9C). An acceptable fit

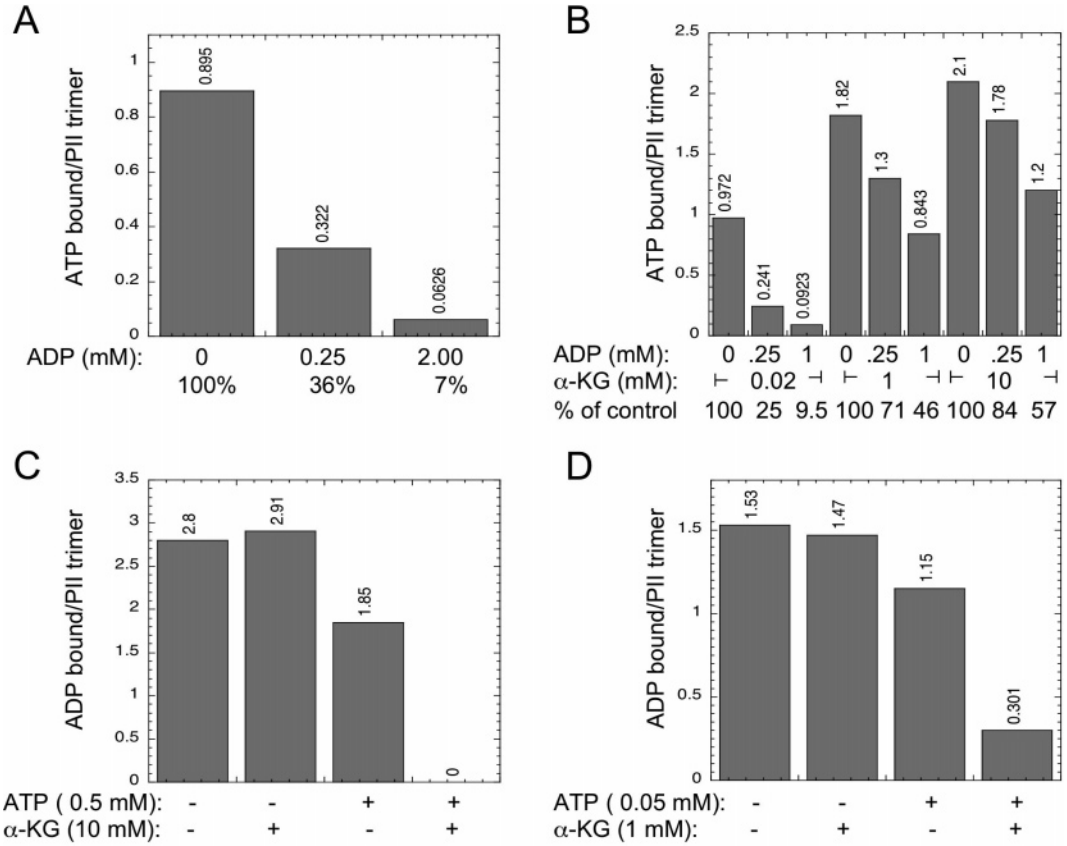

FIGURE 10: Competition betweeen ADP and ATP for binding to PII. ADP reduces the binding of ATP to PII. A. ADP blocks ATP binding to PII. PII was 38 μM and ATP was 150 μM, with ADP varied as indicated. α-ketoglutarate was not present. B. ADP influence on ATP binding to PII was affected by α-ketoglutarate. PII at 8.3 μM and 50 μM ATP were used, and ADP and α-ketoglutarate were varied as indicated. C. ATP blocks ADP binding to PII. PII was 27.4 μM, and ADP was 514 μM. D. ATP blocks ADP binding to PII. PII was 10 μM and ADP was 56 μM.

of these data was also obtained using a model with two classes of sites with *Kd* values of 0.36 μM and 60 μM (not shown). Thus, multiple classes of ATP sites were observed regardless of the α-ketoglutarate concentration, and synergism with α-ketoglutarate resulted in over a 100-fold difference in *Kd* for the binding of the first molecule of ATP. The negative cooperativity of ATP binding was also increased by α-ketoglutarate.

In contrast to the situation obtained with ATP, the binding of ADP to PII was not suggestive of multiple classes of sites (Figure 9D); in this case, the data were well fit by a model with a single class of sites and a *Kd* of 38 μM ($\eta_H = 1.01$). In another experiment, a similar result was obtained, and the data suggested a *Kd* of 32 μM ($\eta_H = 0.99$). We examined whether α-ketoglutarate had any influence on the binding of ADP and did not observe a significant effect. Thus, it seems that ADP binding is not synergistic with α-ketoglutarate binding, at least for *E. coli* PII (see below for information on PII proteins from other organisms).

We also examined the binding of α-ketoglutarate to PII in the presence of 4 mM ATP; as before, we used a microcentrifugation method that measures the depletion of unbound uniformly labeled ^{14}C-α-ketoglutarate from the reaction mixtures (*19*). In the presence of 4 mM ATP, the binding of α-ketoglutarate suggested multiple classes of sites with negative cooperativity ($\eta_H = 0.49$; Figure 9E). These data were fit best by a model with three classes of sites, with *Kd* values of 12 μM, 120 μM and 5000 μM (Figure 9E). These data could not be acceptably fit to models with a single class of sites or two classes of sites (not shown).

Curiously, the combination of ADP and α-ketoglutarate strongly activated the PII-dependent regulated phosphatase activity of NRII (Figure 8A), and this regulated phosphatase activity absolutely required α-ketoglutarate. Yet α-ketoglutarate did not show significant synergism with ADP in binding to PII. We therefore examined the effect of NRII on the binding of α-ketoglutarate to PII (Figure 9F). While neither ADP nor NRII, when alone, could significantly increase the binding of α-ketoglutarate by PII, the combination of ADP and NRII significantly increased the binding of α-ketoglutarate (Figure 9F). The increase in α-ketoglutarate binding provided by the combination of ADP and NRII was about half that provided by AMP-PNP (Figure 9F). This concerted action of ADP and the PII receptor NRII probably explains α-ketoglutarate-dependent PII effects in the presence of ADP as the sole activating nucleotide.

ADP Blocked ATP Binding to PII and Was Less Effective When α-Ketoglutarate Was High. We next examined the competition of ATP and ADP for the nucleotide-binding sites of PII in the presence of various concentrations of α-ketoglutarate. When we used a labeled ATP concentration corresponding approximately to the saturation of the first binding site of each trimer (150 μM) in the absence of α-ketoglutarate, ADP at 0.25 mM provided 64% reduction in the binding of ATP, while ADP at 2 mM resulted in 93% reduction in the binding of ATP (Figure 10A). Given the

small size of the PII protein and structural data for the *T. thermophilus* PII protein (*35*), ATP and ADP are likely competing for a common site in these experiments and/or reduce each other's binding from different subunits of the PII trimer. For experiments where α-ketoglutarate was present, PII was at 8.3 μM, and labeled ATP was at 50 μM, again corresponding to approximate saturation of the first binding site/PII trimer when α-ketoglutarate was at 0.02 mM (Figure 10B). Under these conditions, ADP was a potent competitor of ATP binding in the presence of a low concentration of α-ketoglutarate but was a relatively poor competitor of ATP binding in the presence of a high concentration of α-ketoglutarate (Figure 10B). These results are consistent with the idea that ADP and ATP bind to common sites on PII and that α-ketoglutarate increases the ability of ATP to bind to PII. In additional experiments, we attempted to determine the IC_{50} of ADP for ATP binding in the presence of various concentrations of α-ketoglutarate, using conditions (800 μM ATP) that resulted in near-saturation of PII with ATP. A difficulty of these experiments was that when α-ketoglutarate was 2 mM, it was not possible to add a sufficient concentration of ADP to eliminate ATP binding (not shown); rough estimations of IC_{50} from three such experiments ranged from 15 to 22 mM (not shown). In contrast, when α-ketoglutarate was 0.1 mM under the same conditions, the ADP IC_{50} was approximately 3.5 mM (not shown).

We also examined the ability of ATP to compete with labeled ADP for binding to PII. Under conditions where labeled ADP was nearly saturating (514 μM), α-ketoglutarate had no effect on ADP binding, and ATP at 0.5 mM was only able to displace approximately 1 of the three ADP bound to PII (Figure 10C). But when α-ketoglutarate was saturating and ATP was at 0.5 mM, ADP was completely displaced from PII (Figure 10C). Under conditions corresponding to the binding of 1.5 molecules of ADP per PII trimer (56 μM), ATP at 50 μM resulted in only a 25% reduction in ADP binding (Figure 10D). But when α-ketoglutarate was present, the same concentration of ATP provided an 80% reduction in the binding of ADP (Figure 10D). These data clearly show that ADP and α-ketoglutarate did not directly inhibit each other's binding to PII and that the apparent antagonism between them was due to the intermediation of ATP's binding to PII.

Nucleotide Binding Properties of Other PII Proteins. The results presented above led us to survey the binding of ATP and ADP to other proteins of the PII family. When the GlnK protein of *E. coli* was examined, ATP bound poorly in the absence of α-ketoglutarate, ATP binding was significantly improved in the presence of α-ketoglutarate, and the presence of α-ketoglutarate reduced the ability of ADP to displace bound ATP (Figure 11A). The first two of these properties had been demonstrated before (*36*). Also, ADP bound GlnK well in the absence of α-ketoglutarate and was not affected by α-ketoglutarate, and ATP was a poor competitor in the absence of α-ketoglutarate but was a better competitor in the presence of α-ketoglutarate (Figure 11B). These properties are similar to those of *E. coli* PII. A cyanobacterial PII protein (*Synechococcus*) and a plant PII protein (*Arabidopsis*) showed different properties. For both of these proteins, α-ketoglutarate was synergistic with both ATP and with ADP (Figure 11C−F). For the *Arabidopsis* PII protein, ATP and ADP also displayed synergy (Figure 11E and F). Thus, in the case of the *Arabidopsis* protein, it was directly evident that both ADP and ATP were simultaneously bound to the PII trimer.

DISCUSSION

Our experiments show that the remarkable PII signal transduction protein, which integrates antagonistic signals provided by α-ketoglutarate and glutamine in reconstituted bicyclic systems, also acted as an energy charge sensor in the reconstituted systems. In these systems, reduced energy charge (increased D/T ratio, brought about by the addition of ADP to systems that contained ATP) was synergistic with the signal of nitrogen sufficiency (glutamine) and antagonistic to the signal of nitrogen limitation (α-ketoglutarate). Thus, the adenylylation state of glutamine synthetase was increased upon addition of ADP to a reconstituted UTase/UR-PII-ATase-GS bicyclic system, and the phosphorylation state of NRI-N was decreased upon addition of ADP to a reconstituted UTase/UR-PII-NRII-NRI-N bicyclic system. Studies of the individual monocycles and reactions that comprise the bicyclic systems showed that the targets of ADP action were PII and PII-UMP and that essentially all interactions of PII with the three signal transduction enzymes in our systems (ATase, UTase/UR, and NRII) were altered by the presence of ADP, as were the sensation of α-ketoglutarate and glutamine. Thus, ADP is an important regulatory molecule that controls both the adenylylation state of GS and the phosphorylation state of NRI by controlling the interactions of PII with its receptors and the regulatory small molecules α-ketoglutarate and ATP.

Since ATP is a substrate for the GS adenylylation reaction and for the phosphorylation of NRI, many of the experiments here studying the effects of ADP were of necessity conducted in the presence of ATP. Certain data suggest that under some conditions, both ATP and ADP were bound to PII simultaneously. For example, our direct measurements of binding, where unlabeled ADP was present in vast excess over labeled ATP, showed that even when present at 0.25 mM, ADP was not able to block the binding of ATP by PII. Yet, when this same concentration of ADP was present in the reconstituted systems along with much higher concentrations of ATP, very significant effects were seen. The most likely explanation is that in the reconstituted systems, both nucleotides were simultaneously bound to PII trimers. Similarly, we observed that the maximal rate of the NRII regulated phosphatase activity occurred when PII had available a combination of ADP and the ATP analogue AMP-PNP, as opposed to having either nucleotide alone. For the case of the activation of the AT activity of ATase by PII, we observed that a low concentration of α-ketoglutarate was synergistic with ADP (Figure 3A); since α-ketoglutarate increases ATP binding to PII, this synergy likely reflects the simultaneous binding of ATP and ADP to the same PII molecule. Finally, ATP and ADP stimulated each other's binding to the *Arabidopsis* PII protein, directly showing that ATP and ADP can simultaneously occupy the same PII molecule. Thus, the signaling mechanism may prove to be quite complex. The binding of ATP and ADP by PII has been studied structurally, with the conclusion that the same nucleotide-binding pocket of the protein is responsible for binding both nucleotides (*35*, *37*, *38*). Binding of ATP and ADP to this

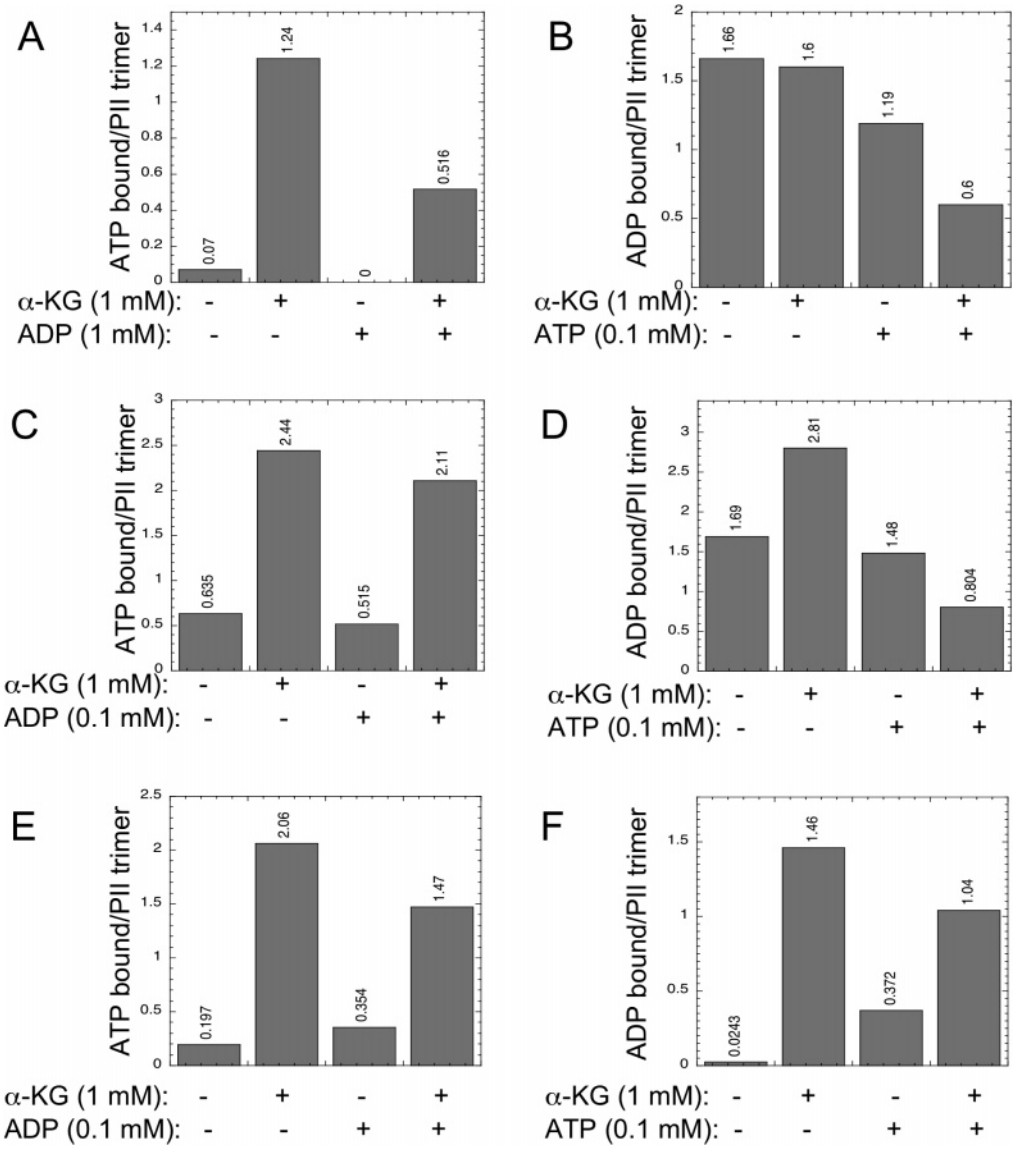

FIGURE 11: Binding of ATP and ADP by various PII-like proteins. A. Binding of ATP by *E. coli* GlnK. GlnK was 10 μM, and ATP was 50 μM. B. Binding of ADP by *E. coli* GlnK. GlnK was 10 μM, and ADP was 105.4 μM. C. Binding of ATP by *Synechococcus elongatus* PII. PII was 10 μM, and ATP was 100 μM. D. Binding of ADP by *Synechococcus elongatus* PII. PII was 10 μM, and ADP was 105.4 μM. E. Binding of ATP by *Arabidopsis thaliana* PII. PII was 10 μM, and ATP was 100 μM. F. Binding of ADP by *Arabidopsis thaliana* PII. PII was 10 μM, and ADP was 105.4 μM.

site apparently results in different PII conformations that influence the binding of α-ketoglutarate, the consequences of α-ketoglutarate binding, and interactions of PII with its receptors.

The current results and prior results show that in the reconstituted signal transduction systems, sensation by PII was remarkably complex, with the D/T ratio, α-ketoglutarate, and glutamine acting in interdependent ways to control the PII output signal. Our current model of the interactions of PII with its small molecule ligands includes the following features:

(a) Both ATP and α-ketoglutarate display negative cooperativity for binding to sites on the PII trimer, while ADP does not display negative cooperativity for binding to PII.

(b) ATP and ADP either compete for the same site or inhibit each other's binding from different sites on the PII trimer, or both.

(c) α-Ketoglutarate and ATP show synergy in binding to PII but only for binding to the first site on the trimer for each ligand. Once the first site for each ligand is occupied, the negative cooperativity for occupancy of the additional sites is increased, relative to that seen with either ligand in isolation.

(d) ADP and α-ketoglutarate do not directly interfere with each other's binding to PII; the observed antagonism between ADP and α-ketoglutarate is mediated by ATP binding to PII.

(e) In the absence of ATP, ADP may stimulate the binding of one molecule of α-ketoglutarate to the PII trimer, when NRII is present. That is, the combination of the PII receptor, NRII, and ADP approximates the action of ATP in stimulating the binding of the first molecule of α-ketoglutarate to the PII trimer.

Our results with reconstituted signal transduction systems show that PII has the capacity to serve as a sensor of cellular EC. Whether this capacity reflects a physiologically significant regulatory mechanism is unclear at this point. Since the binding of adenylylate nucleotide and α-ketoglutarate seem

to be common features of all PII proteins, studies with PII proteins from other sources may reveal additional biochemical examples of PII regulation by the D/T ratio and provide further support for the hypothesis. It has been observed that cyanobacterial PII binds its receptor N-acetyl glutamate kinase (NAGK) in the presence of ATP but not in the presence of ADP (*39*). The hypothesis that PII senses cellular EC is consistent with the physiological challenges facing nitrogen-starved cells. The GS reaction consumes one ATP per molecule of ammonia assimilated. The execution of the Ntr program of gene expression in response to nitrogen limitation is energetically expensive. Finally, the scavenging for nitrogen and assimilation of reduced nitrogen from poor nitrogen sources are also frequently energetically expensive processes. This is very dramatically so in those organisms that directly fix N_2 using dinitrogenase and dinitrogenase reductase, a capacity that *E. coli* lacks. Thus, a role of cellular EC in nitrogen assimilation control is not unreasonable.

The work of Roberts and colleagues focusing upon *Rhodospirrilum rubrum* clearly suggested that PII proteins transduced a signal of energy status (*25*−*27*). There is no equivalent strong evidence for a PII role in energy status sensation for the non-photosynthetic bacterium *E. coli*, mainly because the physiological issue has not been studied in detail, and the necessary experimental design is not nearly as elegant as with a photosynthetic organism, where one can simply shut off the light source while measuring receptor activities. It would be highly desirable to study the issue biochemically with a reconstituted *R. rubrum* system and physiologically in *E. coli*.

A number of studies based upon the extraction of nucleotides from cells have suggested that the ATP concentration is ~3 mM and that this level is maintained at a variety of growth rates. Since most enzymes and signal transduction proteins bind ATP with dissociation constants well below 3 mM, the likelihood of regulation by ATP or EC would seem remote. A recent re-evaluation of the cellular ATP level using intact cells confirmed that ATP is relatively stable during growth regardless of growth rate but suggested that the concentration of free (unbound) ATP in cells is significantly less than 1 mM (*40*). If this is true, the experiments reported here used a range of nucleotide concentrations that span the *in vivo* concentrations of ATP and ADP and thus are likely to correspond with physiologically significant processes.

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