Escherichia coli Glutamine Synthetase Adenylyltransferase (ATase, EC 2.7.7.49): Kinetic Characterization of Regulation by PII, PII-UMP, Glutamine, and α-Ketoglutarate†

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ABSTRACT: Glutamine synthetase adenylyltranferase (ATase, EC 2.7.7.49) catalyzes the adenylylation and deadenylylation of glutamine synthetase (GS), regulating GS activity. The adenylyltransferase (AT) reaction is activated by glutamine and by the unmodified form of the PII signal transduction protein and is inhibited by the uridylylated form of PII, PII-UMP. Conversely, the adenylyl-removing (AR) reaction is activated by PII-UMP and is inhibited by glutamine and by PII. Both AT and AR reactions are regulated by α-ketoglutarate, which binds to PII and PII-UMP. Here, we present a kinetic analysis of the AT and AR activities and their regulation. Both AT and AR reactions used a sequential mechanism of rapid equilibrium random binding of substrates and products. Activators and inhibitors had little effect on the binding of substrates, instead exerting their effects on catalysis. Our results were consistent with PII, PII-UMP, and glutamine shifting the enzyme among at least six different enzyme forms, two of which were inactive, one of which exhibited AR activity, and three of which exhibited AT activity. In addition to a site for glutamine, the enzyme appeared to contain two distinct sites for PII and PII-UMP. The PII, PII-UMP, and glutamine sites were in communication so that the apparent activation and inhibition constants for regulators depended upon each other. The binding of PII was favored by glutamine and its level reduced by PII-UMP, whereas glutamine and PII-UMP competed for the enzyme. α-Ketoglutarate, which acts exclusively through its binding to PII and PII-UMP, did not alter the binding of PII or PII-UMP to the enzyme. Rather, α-ketoglutarate dramatically affected the extent of activation or inhibition of the enzyme by PII or PII-UMP. A working hypothesis for the regulation of the AT and AR activities, consistent with all data, is presented.

ATase regulates the activity of glutamine synthetase (GS)[1] in response to intracellular signals of nitrogen status by catalyzing the reversible adenylylation of GS on tyrosine 397 (reviewed in ref *1*). The adenylylation of GS results in a reduction in GS activity, while deadenylylation of GS∼AMP restores full activity. These reactions play a role in both short-term and long-term adaptation to changes in nitrogen availability (*2*, *3*). Adenylylation and deadenylylation are distinct reactions; adenylylation utilizes ATP and produces GS∼AMP and PP_i, while deadenylylation is a phosphorolysis in which P_i and GS∼AMP are converted to GS and ADP. Since GS is a dodecamer of identical subunits, there are 13 possible states of adenylylation of the enzyme corresponding to 0−12 adenylyl groups per dodecamer. It is thought that the effects of adenylylation of subunits are independent and additive (*1*). Furthermore, it is thought that the rate of adenylylation and deadenylylation of subunits in the GS dodecamer is independent of the status of the other subunits. That is, adenylylation did not exhibit apparent processivity (*4*). The ATase provided the first example of signal transduction by reversible adenylylation (*5*, *6*) and provided an important example of signal transduction by a bicyclic cascade mechanism (*7*, *8*).

The 946-amino acid ATase, the product of *glnE*, contains two copies of a highly conserved motif shared by other nucleotidyltransferase enzymes, designated the NT domain (*9*), as well as a central region of ∼200 amino acids. The C-terminal NT domain contains the AT active site, and the N-terminal NT domain contains the AR active site (see ref *10*). Structure−function studies of the enzyme suggested that the enzyme contained two sites for PII/PII-UMP as well as a site for glutamine and that communication between the PII site and the glutamine site required interactions involving both NT domains (*10*). A hypothesis for the organization and regulation of the enzyme was presented (*10*). In this report, we use kinetic approaches to further investigate the regulation of the enzyme, with a specific emphasis on testing the roles of activators and inhibitors and their interactions and the number of PII/PII-UMP sites.

Earlier studies suggested that the ATase can adopt multiple enzyme conformations, one of which is inactive, one of

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[1] Abbreviations: ATase, glutamine synthetase adenylyltransferase, product of *glnE*; AT, adenylyltransferase activity of ATase; AR, adenylyl-removing (deadenylylation) activity of ATase; PII, signal transduction protein, product of *glnB*; PII-UMP, uridylylated form of PII; GS, glutamine synthetase, product of *glnA*; GS∼AMP, adenylylated form of GS; NT, nucleotidyltransferase domain; UTase/UR, uridylyltransferase/uridylyl-removing enzyme, product of *glnD*.

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which exhibits AR activity, and three of which exhibit AT activity (*8*, *11*). The AT reaction is activated synergistically by (unmodified) PII and by glutamine, and each of these activators reduces the apparent activation constant (K_{act}) for the other (*8*). This suggested that PII and glutamine stabilize each other's binding to the enzyme and promote the conversion of the enzyme to the form with high AT activity (*8*). In the presence of just one of these activators (PII or glutamine), the enzyme exhibits less potent AT activity, perhaps signifying that in the presence of just one of these activators, the enzyme is less stably converted to the conformation with AT activity or that it is converted to a form with reduced AT activity. Similarly, the AR activity requires PII-UMP as an essential activator (*8*), suggesting that PII-UMP might be required to convert the enzyme to the conformation with AR activity. Since the unactivated enzyme exhibits neither AT nor AR activity, it must be able to adopt an "inactive" conformation. An alternative explanation for the effects of activators and inhibitors is that these could more intimately affect the catalytic activities, for instance, by affecting the binding of substrates.

PII proteins are among the most widely distributed signal transduction proteins in nature; these small homotrimeric proteins bind to α-ketoglutarate and to ATP and regulate receptors that are signal transduction enzymes, such as ATase, or key metabolic enzymes (*12*). The activity of *Escherichia coli* PII for activation of ATase is regulated by the binding of α-ketoglutarate and ATP to PII (*13*). When the level of ATP is saturating, the binding of α-ketoglutarate displays negative cooperativity; the binding of the first effector molecule to the trimer inhibits further effector binding. At physiological concentrations of α-ketoglutarate, this anticooperativity is overcome, and the PII trimer is saturated with α-ketoglutarate. The ability of PII to activate the AT activity was best at concentrations of α-ketoglutarate corresponding to unsaturated PII trimers (*8*). Conversely, the ability of PII-UMP to activate the AR activity was best at concentrations of α-ketoglutarate that are expected to saturate the three α-ketoglutarate sites of the PII-UMP trimer (*8*, *13*). These observations suggested that α-ketoglutarate regulated the binding of PII and PII-UMP to ATase and, by analogy, to the other PII receptors (*8*). We also investigated this issue.

EXPERIMENTAL PROCEDURES

Purified Proteins. ATase and PII were purified as described previously (*8*), and preparations of UTase/UR described previously were used (*15*). GS was purified as described previously (*8*), using an overexpression system that was kindly provided by M. Atkinson in which the *E. coli glnA* structural gene was cloned into the pJLA503 expression plasmid (*16*). GS∼AMP was prepared as described in ref *10*, and the preparations of the ATMΔ and ATC1 mutant forms of ATase described previously (*10*) were used.

PII-UMP. The conditions included 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.3 mM α-ketoglutarate, 0.5 mM ATP, 2 mM UTP, 10 mM DTT, 10 μM PII, and 0.1 μM UTase/UR, and reaction mixtures were incubated at 30 °C for 40 min and then heated at 60 °C for 15 min to inactivate the UTase/UR. The samples were then purified via chromatography on small G-25 columns to exchange the buffer with 50 mM Tris-HCl (pH 7.5), 100 mM KCl, and 10% (w/v) glycerol. Aliquots of PII-UMP were examined by nondenaturing gel electrophoresis (*17*), where the extent of uridylylation was always greater than 98% as judged by visual inspection of Coomassie-stained gels.

Measurement of AT and AR Activities. These assays were essentially as described previously (*8*, *10*). For the AT assay, general conditions included 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 0.5 mM [α-^{32}P]ATP or as indicated, GS as indicated (concentration stated in dodecamers), 0.05 mM α-ketoglutarate or as indicated, and ATase as indicated. Other additions, such as regulators, products, or substrate analogues, were as indicated. Incubation was carried out at 30 °C, and aliquots at different times were spotted onto nitrocellulose filters and washed immediately in 5% TCA as described previously (*8*). Radioactivity on the filters was quantified by liquid scintillation counting, and initial velocities were determined by linear regression using KaleidaGraph. For the AR assay, the general conditions included 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, [^{32}P]GS∼AMP as indicated (stated as a monomer concentration), KP_i as indicated, 1 mM ATP or as indicated, 1 mM α-ketoglutarate or as indicated, ATase as indicated, 0.4 μM PII-UMP or as indicated, and other regulators as indicated. (GS∼AMP concentrations are stated as the monomer concentration because these concentrations were directly determined by measurement of the amount of ^{32}P incorporated into GS∼AMP; in all experiments, the GS∼AMP preparations were modified on >95% of the GS subunits.) Incubation was carried out at 30 °C, and at various times, samples were spotted into nitrocellulose filters and treated as described above, with initial velocities again determined using linear regression in KaleidaGraph.

Modeling. Modeling used the Mathematica program and the model described in the Supporting Information.

RESULTS

Both AT and AR Activities Utilized a Sequential Reaction Mechanism. The AT reaction catalyzes the conversion of GS and ATP to PP_i and GS∼AMP. Our assay directly measures the level of incorporation of labeled AMP onto GS and has fairly low noise (ranging from ∼0.1 to 1% of the activity observed when the enzyme is maximally activated). In earlier work, we failed to detect a covalent enzyme−AMP intermediate using [α-^{32}P]ATP as a substrate (in the presence and absence of GS), consistent with a sequential mechanism. To directly examine the mechanism, we measured the initial rate of GS adenylylation as one substrate was varied in the presence of different fixed concentrations of the second substrate [S1 vs S2 experiment, Figure 1 (*18*)]. For this, the AT activity was activated by PII in the absence of glutamine, with α-ketoglutarate at a concentration that favors the AT activity. ATP is one of the substrates, but it should be noted that it is also an activator in these reactions, as binding of ATP to PII appears to be required for all activities of PII (*13*). However, at the α-ketoglutarate concentration that was used, ATP binds much better to PII than to the substrate site of ATase, allowing us to observe its effects as a substrate. A pattern of clearly intersecting lines whose intersection point was to the left of the $1/v$ axis and very near the $1/S$ axis was obtained, indicating a sequential mechanism and suggesting

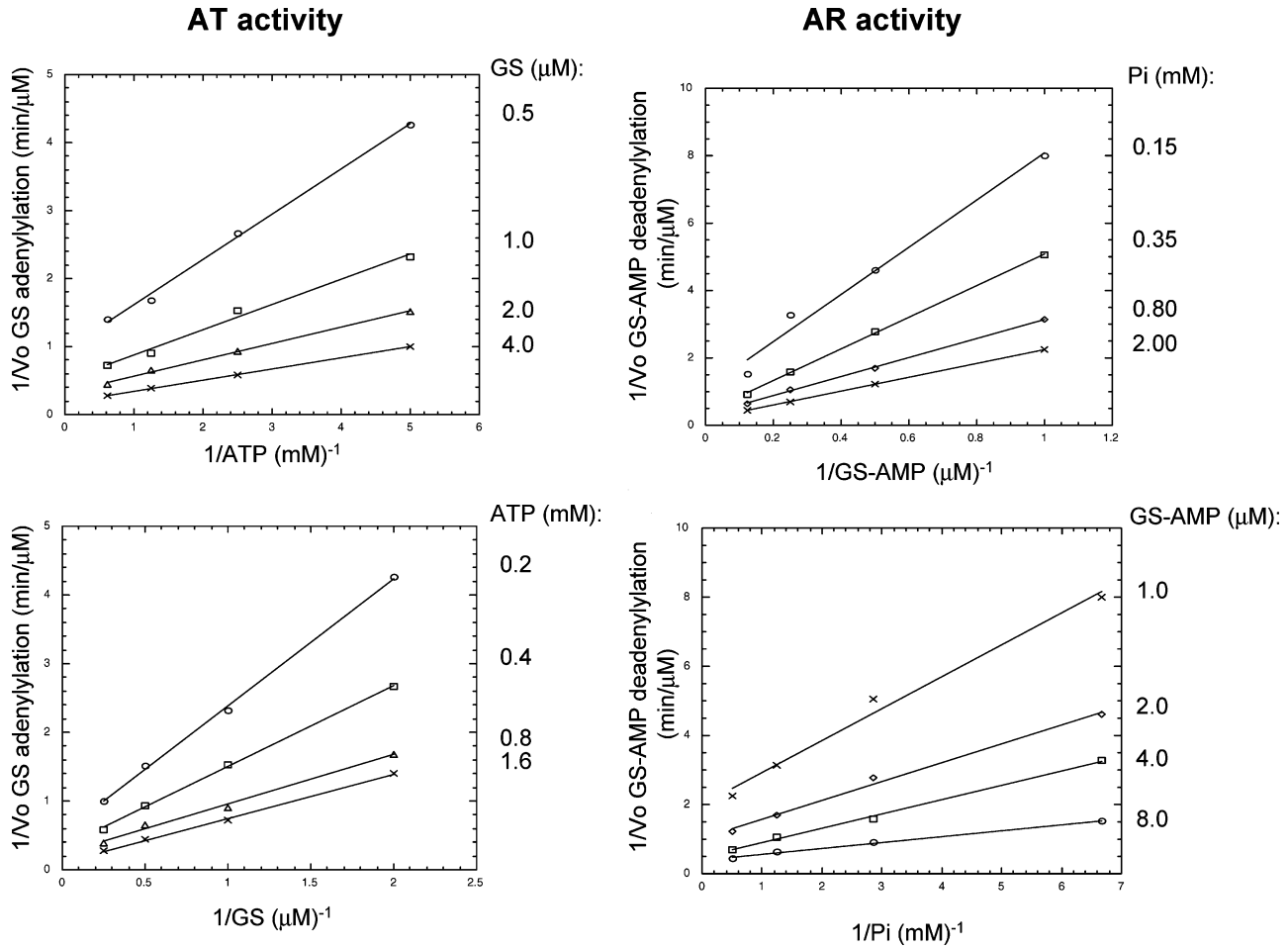

FIGURE 1: S1 vs S2 experiments indicated a sequential mechanism for the AT and AR activities. For the AT activity (left), the ATase concentration was 0.05 μM, the α-ketoglutarate concentration 0.05 mM, and the PII concentration 1 μM. GS concentrations were 0.5, 1, 2, and 4 μM (dodecamers), and ATP concentrations were 0.2, 0.4, 0.8, and 1.6 mM. For the AR activity (right), the ATase concentration was 0.03 μM, the PII-UMP concentration 0.3 μM, the ATP concentration 1 mM, and the α-ketoglutarate concentration 1 mM. KP_i concentrations were 0.15, 0.35, 0.8, and 2 mM, and GS∼AMP concentrations were 1, 2, 4, and 8 μM (subunit concentration).

that the substrates have a modest effect on each other's binding (*18*). The K_m for the substrates was directly measured under a few conditions, as summarized in Table 1. As shown, the substrates seemed to have a slightly antagonistic interaction (Table 1).

The AR activity catalyzes the conversion of GS∼AMP and P_i to GS and ADP. Our assay measures the rate of cleavage of labeled AMP from GS∼AMP and has relatively low noise (∼5% of the maximally activated rate). Prior studies failed to identify a covalent enzyme−AMP intermediate, and here we directly examined the AR mechanism by conducting the S1 versus S2 experiment in the presence of PII-UMP and the concentrations of α-ketoglutarate and ATP (1 mM each) that favor AR activity (Figure 1). Again, a pattern of intersecting lines was obtained, whose point of intersection is to the left of the $1/v$ axis and near the $1/S$ axis. This is consistent with a sequential mechanism and very modest substrate effects on each other (Figure 1). Direct K_m measurements for the AR reaction are presented in Table 1.

Product Inhibition Studies Suggested that both the AT and AR Reactions Utilized Rapid Equilibrium Random Binding of Substrates and Products. To help determine the order of substrate binding and product release, we examined the inhibition patterns of each substrate versus each product for both the AT and AR activities. In each case, we used the conditions described for the S1 versus S2 experiments, favoring the appropriate activity. In the case of the AT activity, we saw an apparent competitive pattern of inhibition for each product versus each substrate (Figure 2). The distinction between competitive and noncompetitive inhibition patterns is frequently difficult. Additional evidence of a random rapid equilibrium mechanism came from the observation that a high concentration of either substrate (GS or ATP) eliminated inhibition by either product (Figure S1 of the Supporting Information). For the AR activity, we again saw an apparent competitive inhibition pattern for each product versus each substrate (Figure 3), and we observed that high concentrations of P_i could block inhibition by either product (Figure S2 of the Supporting Information). For technical reasons, it was not practical to try to use very high GS∼AMP concentrations to block product inhibition. Thus, for both AT and AR activities, the data were consistent with a random rapid equilibrium mechanism. All inhibition patterns seemed to be linear, and there was no apparent evidence for dead-end complexes or substrate inhibition. Additional studies to confirm the mechanism would typically utilize analogues for each substrate. Unfortunately, we were unable to identify a suitable analogue for GS and GS∼AMP, but we did observe that GTP could inhibit the AT activity. This inhibition appeared to be competitive with ATP and

Table 1: Kinetic Parameters

AT Activity

experiment	[ATP] (mM)	[α-ketoglutarate] (mM)	[PII] (μM)	[ATase] (μM)	K_m (GS) (μM)[a]
052504	0.5	0.05	0.5	0.08	2.9
100505	0.5	0.05	0.5	0.1	3.5
100505	0.5	0.05	5.0	0.1	3.4
092805	3.5	0.05	5.0	0.05	6.0

AT Activity

experiment	[GS] (μM)[a]	[α-ketoglutarate] (mM)	[PII] (μM)	[ATase] (μM)	K_m (ATP) (mM)
092605	15	0.05	5.0	0.1	1.42
092605	1.5	0.05	5.0	0.1	0.75

$k_{cat} \sim 500\ min^{-1}$

AR Activity

experiment	[ATase] (μM)	[PII-UMP] (μM)	[α-ketoglutarate] (mM)	[ATP] (mM)	[GS∼AMP] (μM)[b]	K_m (KP_i) (mM)
013004	0.05	0.5	1	1	3.4	0.33

AR Activity

experiment	[ATase] (μM)	[PII-UMP] (μM)	[α-ketoglutarate] (mM)	[ATP] (mM)	[KP_i] (mM)	K_m (GS∼AMP) (μM)[b]
042205	0.02	0.4	1	1	5	9.0

$k_{cat} \sim 230\ min^{-1}$

[a] Dodecamer concentration. [b] Monomer (subunit) concentration.

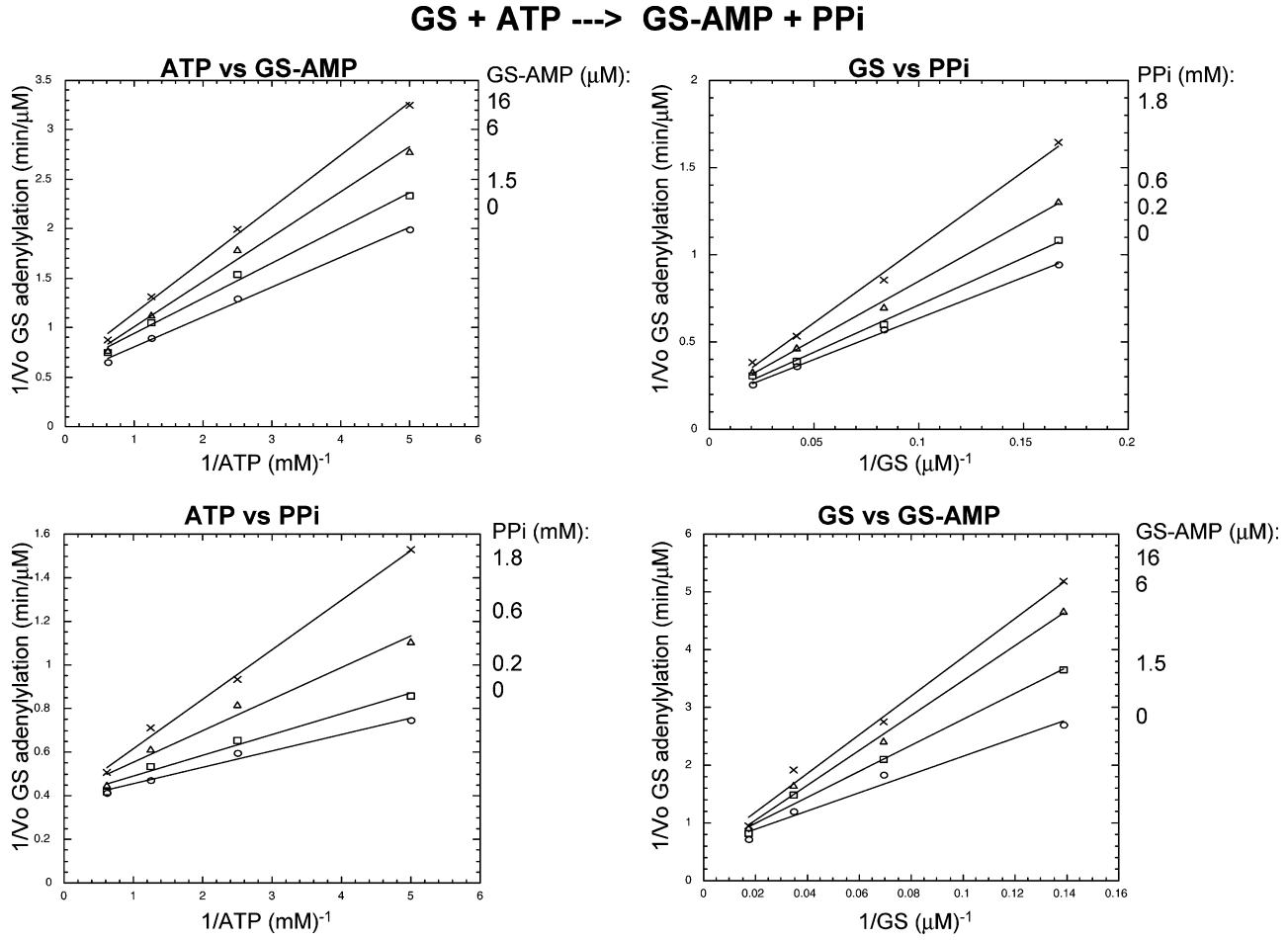

FIGURE 2: For the AT activity, each product was competitive with each substrate. Conditions were as follows. ATP vs GS∼AMP: 2 μM GS, 0.1 μM ATase, 0.05 mM α-ketoglutarate, and 0.5 μM PII. ATP vs PP_i: 1 μM GS (dodecamer concentration), 0.05 μM ATase, 0.05 mM α-ketoglutarate, and 0.5 μM PII. GS vs PP_i: 0.5 mM ATP, 0.04 μM ATase, 0.05 mM α-ketoglutarate, and 0.4 μM PII. GS vs GS∼AMP: 0.5 mM ATP, 0.12 μM ATase, 0.05 mM α-ketoglutarate, and 0.5 μM PII. For this experiment, the GS∼AMP concentration is stated as the dodecamer concentration.

noncompetitive with GS, consistent with the proposed random rapid equilibrium mechanism (not shown).

What Do the Activators and Inhibitors Do? Activators and inhibitors might regulate the binding of substrates or

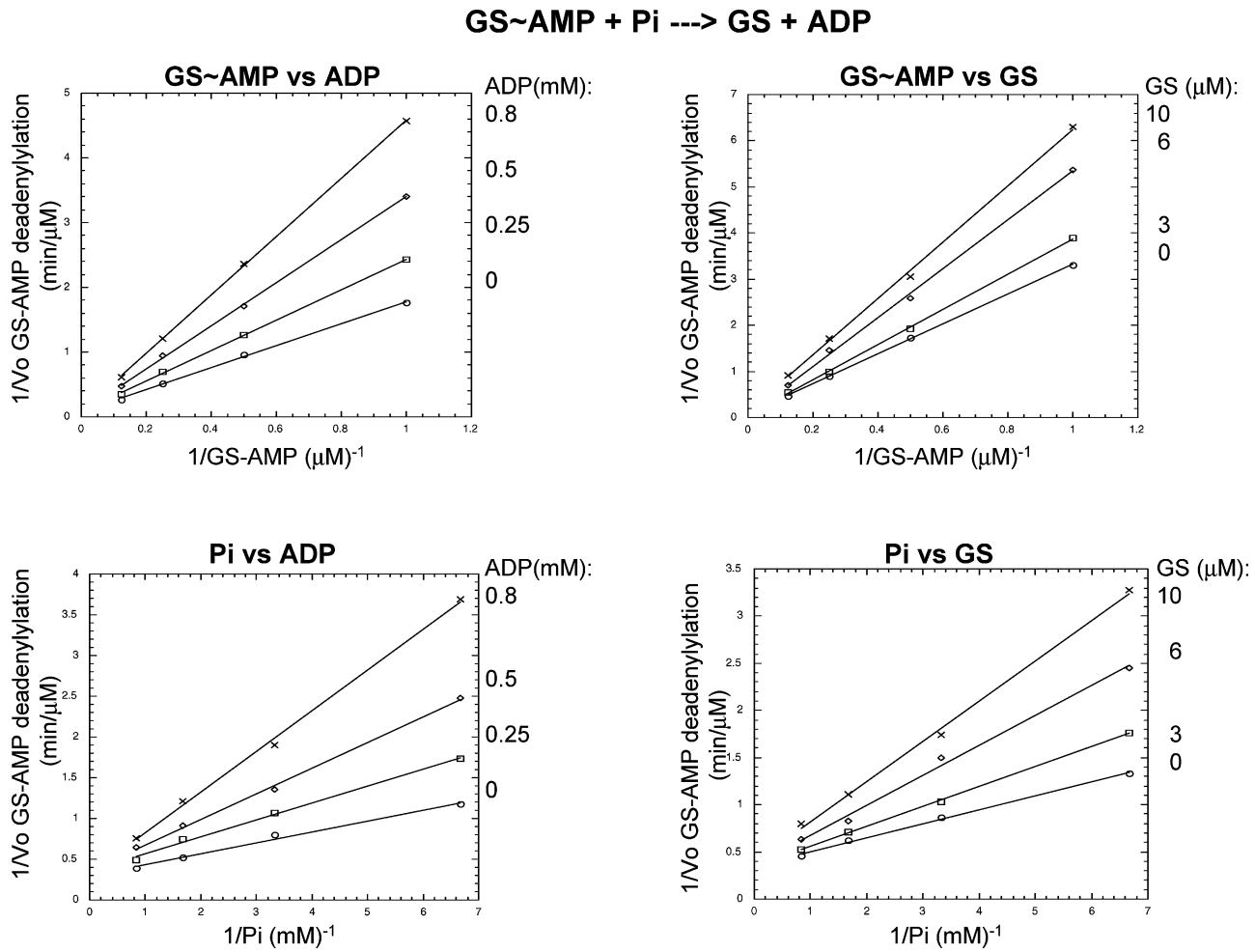

FIGURE 3: For the AR activity, each product was competitive with each substrate. For all experiments, the α-ketoglutarate concentration was 1 mM, the ATP concentration 1 mM, and the PII-UMP concentration 0.5 μM. Additional conditions were as follows. GS∼AMP vs ADP: 0.06 μM ATase and 0.3 mM KP_i. P_i vs ADP: 0.04 μM ATase and 3.2 μM GS∼AMP (monomer concentration). GS∼AMP vs GS: 0.05 μM ATase and 0.3 mM KP_i. P_i vs GS: 0.5 μM ATase and 3.2 μM GS∼AMP (monomer concentration).

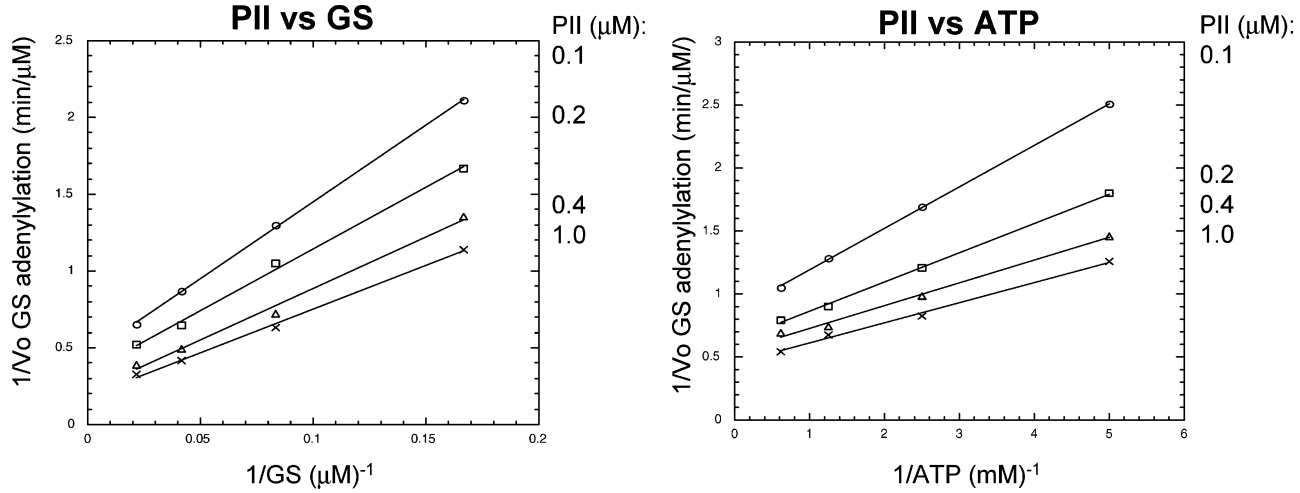

FIGURE 4: PII had little effect on the binding of the AT substrates. PII vs GS (left panel) conditions: 0.04 μM ATase, 0.5 mM ATP, and 0.05 mM α-ketoglutarate. PII concentrations were 0.1, 0.2, 0.4, and 1 μM. The 1/[GS] axis intercepts were −0.0445, −0.0418, −0.0319, and −0.0324, respectively. PII vs ATP (right panel) conditions: 0.04 μM ATase, 1 μM GS, and 0.05 mM α-ketoglutarate. PII concentrations were 0.1, 0.2, 0.4, and 1 μM. The 1/[ATP] axis intercepts were −2.604, −2.713, −2.993, and −2.819, respectively.

products, affect the rate of catalysis, or influence reaction rates in multiple ways. To investigate the role of activators and inhibitors in our initial rate studies, we examined the patterns of inhibition when each substrate was varied in the presence of different fixed concentrations of inhibitor(s) or activator(s), for both the AT and AR reactions (Figure 4 and Figures S3−S5 of the Supporting Information). For the AT activity, studies were conducted with either glutamine or PII as the sole activator. In all cases, both slope and $1/v_0$ intercept effects were observed, and the inhibitor or activator had little

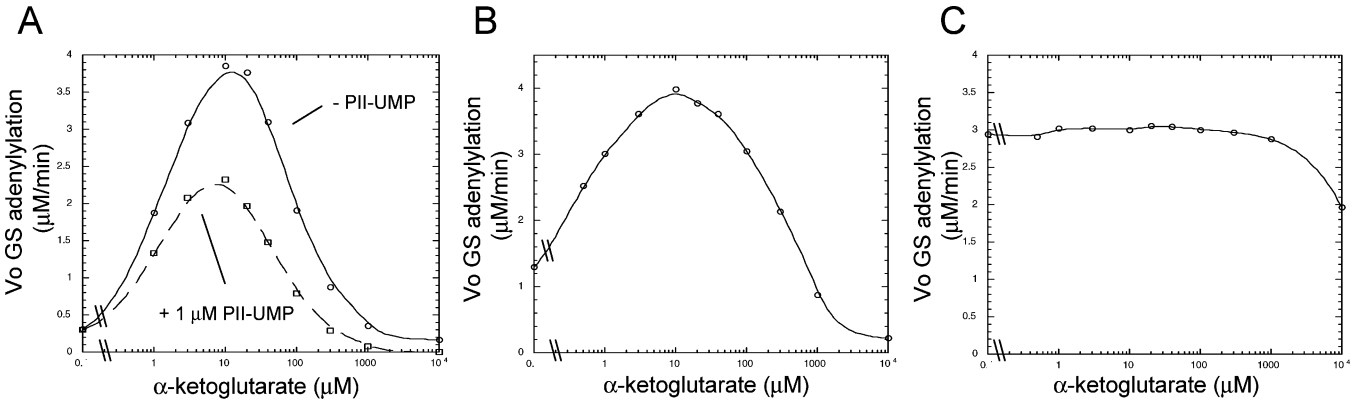

FIGURE 5: α-Ketoglutarate effects depend upon PII, PII-UMP, and glutamine. (A) Effect of α-ketoglutarate on the PII-activated AT activity. Conditions included 0.1 μM ATase, 3 μM GS, 0.5 mM ATP, and 0.5 μM PII: (top curve) without PII-UMP and (bottom curve) with 1 μM PII-UMP. (B) Effect of α-ketoglutarate on the PII-activated AT activity in the presence of a subsaturating concentration of glutamine. Conditions included 0.005 μM ATase, 3 μM GS, 0.5 mM ATP, 0.5 μM PII, and 2 mM glutamine. (C) α-Ketoglutarate had little effect on the PII activation of AT activity in the presence of a saturating glutamine concentration. Conditions included 0.005 μM ATase, 3 μM GS, 0.5 mM ATP, 0.5 μM PII, and 50 mM glutamine. In all three panels, the points plottted on the *Y*-axis were in the absence of α-ketoglutarate.

effect on the 1/*S* intercept of the Lineweaver−Burke plots. Intuitively, such results suggest that the activators and inhibitors cannot have significant effects on substrate binding, since the 1/*S* intercept is $-1/K_s$ as modified by the effects of activators and inhibitors (*18*). In the Supporting Information, we use the approach described by Segel (pp 274−291 of ref *18*) to examine the significance of the 1/*S* intercept for three different types of systems with activators and inhibitors; as expected, in all three cases, 1/*S* intercept effects were predicted when activators and inhibitors affected substrate binding.

Regulation by α*-Ketoglutarate.* Both AT and AR activities require α-ketoglutarate when they are activated by PII and PII-UMP, respectively, with low α-ketoglutarate concentrations favoring activation of the AT by PII and high α-ketoglutarate concentrations favoring activation of the AR by PII-UMP (*8*). When the initial rate of the AT reaction was measured with PII as the activator (in the absence of glutamine) and at various α-ketoglutarate concentrations, a biphasic response to α-ketoglutarate was observed (Figure 5A). Adenylylation was favored by a very low concentration of α-ketoglutarate (with an optimum effector concentration of ~10 μM), and activation by PII was nearly eliminated at 1 mM α-ketoglutarate. When PII-UMP was present, the AT activity was significantly inhibited (Figure 5A), with the inhibition most severe at high concentrations of α-ketoglutarate. This effect shifted the optimal α-ketoglutarate concentration to a lower value when PII-UMP was present (Figure 5A). The biphasic saturation profile for PII is consistent with its activation and inactivation by α-ketoglutarate.

When both glutamine and PII were present, the AT activity was strongly activated. When the concentration of glutamine was below its apparent K_{act} (in the absence of PII), the response to α-ketoglutarate was still biphasic, as seen previously (*8*), but the rate and saturation profile were broader because the ascending arm starts from a higher position because of activation by glutamine and PII in the absence of α-ketoglutarate (Figure 5B). However, when a saturating concentration of glutamine was present, the enzyme was fully activated in the absence of α-ketoglutarate, and α-ketoglutarate at very high concentrations provided only weak inhibition of the AT activity (Figure 5C). Thus, the interaction between PII and glutamine could compensate for inhibitory effects of α-ketoglutarate at high concentrations and eliminate the need for the activating effect of α-ketoglutarate at low concentrations.

The results described in the previous paragraph led us to examine directly whether PII and glutamine displayed an interaction in the absence of α-ketoglutarate and in the presence of very high α-ketoglutarate concentrations. In the absence of either glutamine or PII, the basal rate of the AT activity is insignificant. When PII and glutamine were present at the optimal α-ketoglutarate concentration, the AT rate was considerably greater than that obtained with either activator alone (Figure 6). In the absence of α-ketoglutarate and at very high α-ketoglutarate concentrations, the combination of PII and glutamine again resulted in rates higher than that explained by additive effects (Figure 5). Indeed, in these two cases, PII alone barely activated the ATase yet functioned in concert with glutamine. Furthermore, even when the ATase was saturated with glutamine (50 mM), PII was able to further activate the AT reaction (Figure 6).

The apparent K_{act} for PII activation of the AT reaction depended on the glutamine concentration (Table 2), as noted previously (*8*). In the presence of saturating glutamine concentrations, the apparent K_{act} for PII was 50−100-fold lower than that observed in the absence of glutamine (Table 2). By comparison, the apparent K_{act} for glutamine was reduced ~14-fold in the presence of a high concentration of PII (Table 2). Both of these K_{act} values should be modified by the same α factor (see Figure 12 below); that is, the synergy should be symmetrical, and thus, the discrepancy probably reflects the fact that it is easier to provide a saturating concentration of glutamine than of PII. Remarkably, although α-ketoglutarate dramatically regulated PII activation of the AT activity (e.g., Figure 5A), it did not have a significant effect on the PII apparent K_{act} (Table 2) when PII activation was examined in the absence of PII-UMP and glutamine. This result is not consistent with a hypothesis that α-ketoglutarate regulates the binding of PII to ATase, for in that case a dramatic effect on K_{act} would be expected.

The AR activity is activated by PII-UMP, and this activation requires α-ketoglutarate and is favored by a high concentration of α-ketoglutarate (*8*). We examined the

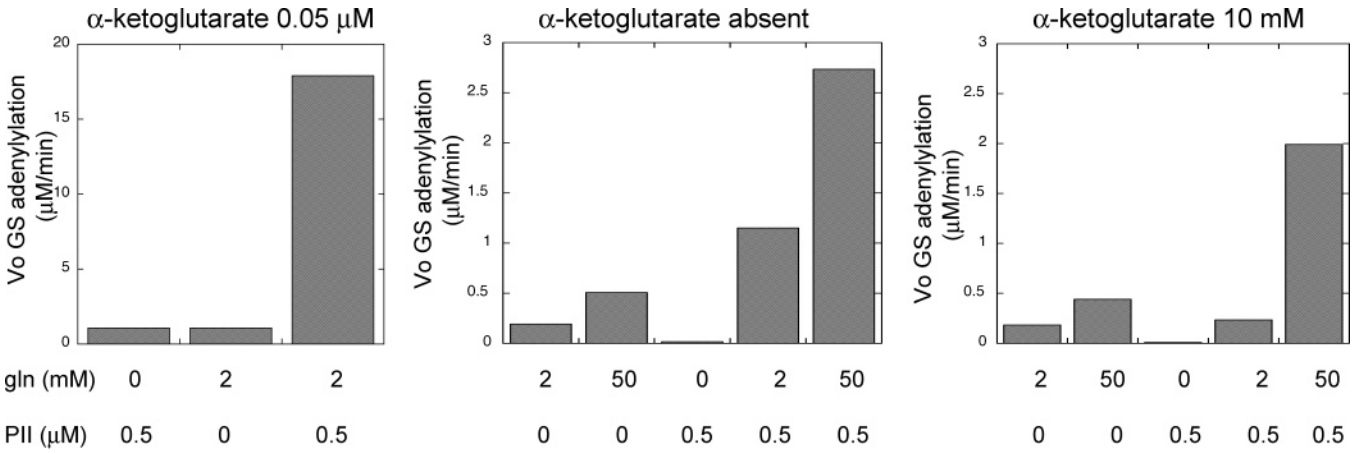

FIGURE 6: Interaction between PII and glutamine did not require α-ketoglutarate. For the experiment with α-ketoglutarate at 0.05 mM (left panel), the ATase concentration was 0.04 μM, the GS concentration 2.5 μM, and the ATP concentration 0.5 mM and the PII and glutamine concentrations were as indicated. For the experiments with α-ketoglutarate absent or at 10 mM (center and right panels), the ATase concentration was 0.005 μM, the GS concentration 3 μM, and the ATP concentration 0.5 mM, with PII and glutamine as indicated.

Table 2: Apparent K_{act} Values for PII and Glutamine Activation of the AT Activity under Various Conditions

PII Apparent K_{act} Values

experiment	[α-KG] (mM)	[glutamine] (mM)	[ATase] (μM)	[PII-UMP] (μM)	apparent K_{act} (μM)
010605	0.05		0.10		1.00
010605	1.00		0.40		2.00
010705	0.05		0.1		1.90
010705	1.00		1.20		2.50
011005	0.05	50	0.01		0.015
011005	1.00	50	0.01		0.024
022805	0.05	50	0.02	10.0	0.065
022205	1.0	7	0.02	3.0	2.00
030705	0.05		0.20	10	~6.00
030705	1.00		2.00	10	>40.0

Glutamine Apparent K_{act} Values

experiment	[α-KG] (mM)	[PII] (μM)	[PII-UMP] (μM)	[ATase] (μM)	apparent K_{act} (mM)
011405	0.05			0.06	7.00
011405	0.05	15		0.01	0.50
031505	0.05		10	0.10	>20
031505	1.00		10	0.10	>30
122304	1.00	5	10	0.06	9.00
022806	0.00	15		0.01	1.10
022806	0.05	15		0.01	0.45
022806	1.00	15		0.01	2.6

Table 3: PII-UMP Apparent K_{act} Values for Activation of the AR Activity

experiment	[α-KG] (mM)	[PII] (μM)	[glutamine] (mM)	[ATase] (μM)	K_{act} (μM)
022305	1.00			0.02	0.28
022305	0.02			0.02	0.35
030105	1.00	2.00		0.02	0.40
030105	1.00		10	0.02	0.50
041105	1.00	10		0.03	0.80
041105	1.00	10	10	0.04	>2.00
042105	1.00		50	0.03	0.85

apparent K_{act} for PII-UMP in the presence of high and low concentrations of α-ketoglutarate and found that α-ketoglutarate had a very minor effect on the PII-UMP apparent K_{act} (Table 3). Again, this observation is inconsistent with α-ketoglutarate regulating the binding of the activator (PII-UMP) to the enzyme.

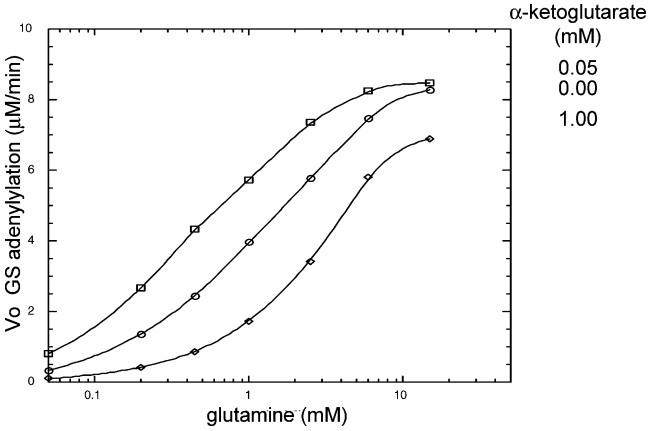

FIGURE 7: Glutamine apparent K_{act} values for activation of the AT activity depended on the α-ketoglutarate concentration when PII was present. Conditions included 0.01 μM ATase, 3 μM GS, 0.5 mM ATP, and 15 μM PII. α-Ketoglutarate concentrations were 0.05 (□), 0 (○), and 1 mM (◇), and the apparent glutamine K_{act} values were 0.45, 1.1, and 2.6 mM, respectively.

Despite the fact that α-ketoglutarate seemed to have little effect on the apparent K_{act} of PII and PII-UMP for the AT and AR reactions, this effector did affect the apparent K_{act} of glutamine for activation of the AT activity in the presence of PII (Figure 7). The effect of α-ketoglutarate was again biphasic, with the optimal effector concentration for enzyme activation corresponding to the lowest apparent K_{act} for glutamine. This is consistent with the hypothesis that PII facilitates the binding of glutamine to the enzyme, and α-ketoglutarate influences the interaction of PII with the enzyme. At the optimal α-ketoglutarate concentration, PII is most effective in converting the enzyme to the form that binds glutamine more avidly.

Although α-ketoglutarate did not have a dramatic effect on the PII and PII-UMP K_{act} when these activators were present separately, it clearly affected the interaction between PII and PII-UMP when both were present. At high α-ketoglutarate concentrations, PII-UMP was a much more effective inhibitor of the PII-activated AT reaction than it was at low α-ketoglutarate concentrations (Table 2), and correspondingly, α-ketoglutarate controlled the PII K_{act} in the presence of PII-UMP. A possible explanation for these data is that

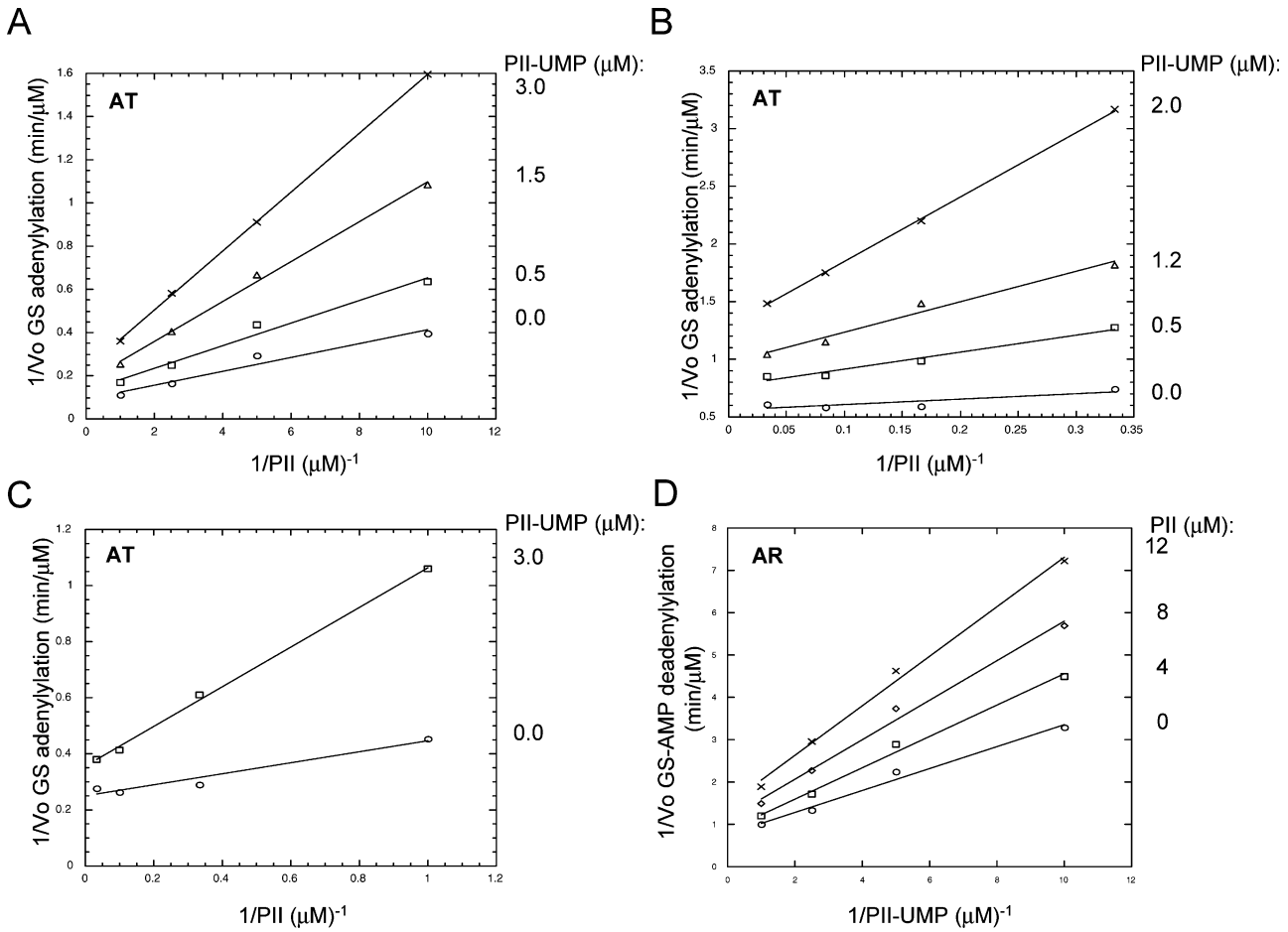

FIGURE 8: PII-UMP vs PII. (A) Inhibition of the PII-activated AT activity by PII-UMP at low α-ketoglutarate concentrations. Conditions included 0.02 μM ATase, 3 μM GS, 3 mM ATP, and 0.05 mM α-ketoglutarate. PII-UMP concentrations were 0, 0.5, 1.5, and 3 μM. (B) Inhibition of the PII-activated AT activity by PII-UMP at high α-ketoglutarate concentrations. Conditions included 0.3 μM ATase, 3 μM GS, 3 mM ATP, and 1 mM α-ketoglutarate. PII-UMP concentrations were 0, 0.5, 1.2, and 2 μM. (C) Inhibition of the PII-activated AT activity by PII-UMP at fixed free α-ketoglutarate concentrations. Conditions included 0.025 μM ATase, 3 μM GS, and 3 mM ATP, and the α-ketoglutarate concentration was equal to the PII and PII-UMP concentration + 20 μM. The bottom line is for data in the absence of PII-UMP, and the top line is for data with 3 μM PII-UMP. (D) Inhibition of the PII-UMP-activated AR activity by PII. Conditions included 0.06 μM ATase, 3 μM GS∼AMP, and 1 mM ATP. The KP_i concentration was 0.4 mM and the α-ketoglutarate concentration 1 mM. PII concentrations were 0, 4, 8, and 12 μM.

α-ketoglutarate, while not directly affecting the binding of either PII or PII-UMP to the enzyme, regulates the ability of PII and PII-UMP to convert the enzyme to the form that binds the other species less avidly. That is, the α factor describing the interaction of PII and PII-UMP (α_2 in Figure 12, below) must be sensitive to α-ketoglutarate.

Interactions of PII, PII-UMP, and Glutamine. To examine the interactions of PII and PII-UMP, we examined the effect of varying activator concentrations at different fixed inhibitor concentrations (A vs I experiment) for both the AT and AR reactions (Figure 8). In both cases, we saw a clearly noncompetitve inhibition pattern. In the case of the AT reaction, it could be considered that in experiments conducted at low α-ketoglutarate concentrations, the activator (PII) and inhibitor (PII-UMP) sequester the effector and thus alter the concentration of the free effector (Figure 8A). Of course, this cannot explain the noncompetitive inhibition patterns at high α-ketoglutarate concentrations (Figure 8B). To examine the interaction of PII and PII-UMP at low α-ketoglutarate concentrations, another experiment was performed in which the concentration of free α-ketoglutarate was kept constant throughout the experiment. Again, a noncompetitive inhibition pattern was obtained (Figure 8C). These results are not consistent with a hypothesis whereby PII and PII-UMP act from a single common site or where each converts the enzyme to a form that does not bind the other, for in that case we would expect a competitive inhibition pattern. For the AR experiment, α-ketoglutarate was present at a high concentration so that the noncompetitive inhibition by PII cannot be explained by titration of the effector (Figure 8D).

As noted above when focusing upon α-ketoglutarate effects, we also examined the interaction of PII and PII-UMP by determining the K_{act} for the activating species in the presence of the inhibiting species (Tables 2 and 3). For the AT activity, the influence of PII-UMP on the PII apparent K_{act} depended on the α-ketoglutarate concentration: at low α-ketoglutarate concentrations, an ∼6-fold increase in the apparent PII K_{act} was observed in the presence of 10 μM PII-UMP, and at high α-ketoglutarate concentrations, an ∼20-fold increase in the PII apparent K_{act} was observed when the PII-UMP concentration was 10 μM (Table 2). Considerably more modest effects were observed when we examined the effect of PII on the activation of the AR by PII-UMP at high α-ketoglutarate concentrations. In that case, PII increased the apparent PII-UMP K_{act} ∼2-fold (Table 3). We found it impractical to measure the effect of PII on the PII-

Table 4: AR Activity Inhibition Constants

PII Apparent K_i Values

experiment	[α-KG] (mM)	[PII-UMP] (μM)	[glutamine] (mM)	[ATase] (μM)	K_i (μM)
030205	1.00	0.40		0.04	8.00
030205	1.00	10		0.02	>80
012904	1.00	0.50		0.05	4.00
020204	1.00	0.50	50	0.05	0.80
041205	1.00	0.40	50	0.15	1.20
041205	1.00	0.10		0.10	10.0

Glutamine Apparent K_i Values

experiment	[α-KG] (mM)	[PII-UMP] (μM)	[PII] (μM)	[ATase] (μM)	K_i (mM)
013004	1.00	0.50	1.00	0.05	9.00
041905	1.00	0.40		0.05	23.00
041905	1.00	0.40	10.0	0.20	5.00
042105	1.00	10		0.01	~90.0

UMP K_{act} at low α-ketoglutarate concentrations, as it was difficult to assess the inhibition of the low AR activity under these conditions, and the experiment again requires careful control of the free α-ketoglutarate concentration to prevent the titration of the effector.

Additional information about the interaction of PII and PII-UMP comes from the examination of the inhibition of the AR by PII under different conditions (Table 4). The apparent inhibition constant for PII was increased ~10-fold when PII-UMP was present at a high concentration, consistent with the hypothesis that PII and PII-UMP act to force the enzyme into conformations that bind the other species less avidly.

In contrast to the noncompetitive inhibition patterns obtained in PII versus PII-UMP experiments, competitive patterns of inhibition were observed when glutamine and PII-UMP were in opposition (Figure 9). This was observed both for the inhibition of the glutamine-activated ATase activity by PII-UMP (Figure 9A) and for the inhibition of the PII-UMP-activated AR activity by glutamine (Figure 9B). While we think it is unlikely that the binding sites for PII-UMP and glutamine are identical, these results are consistent with a hypothesis that the enzyme can bind only one of these regulators and that the form bound by PII-UMP lacks the AT activity while the form bound by glutamine lacks the AR activity.

Further support for the idea that PII-UMP and glutamine binding are antagonistic comes from the examination of the regulation of ATC1, a truncated form of ATase that lacks the N-terminal AR domain (*10*). This truncated form of the ATase is activated by glutamine, but it is not activated by PII. Rather, PII and PII-UMP both inhibit the AT activity of ATC1, presumably by binding to a site corresponding to the natural PII-UMP site (*10*). We examined the inhibition of the ATC1 AT activity as the glutamine concentration was varied at several different fixed inhibitor (PII or PII-UMP) concentrations (A vs I experiments); for both PII and PII-UMP, the inhibition appeared to be competitive with glutamine (Figure 10A,B). Furthermore, the inhibition by PII and PII-UMP appeared to be additive, as if they were acting from a common site, as indicated by a series of parallel lines in the Dixon plot as one inhibitor was varied in the presence of different fixed concentrations of the other (Figure 10C). These results are consistent with glutamine (activator) and PII-UMP/PII (inhibitors) competing to convert the enzyme to forms that bind only either an activator or an inhibitor (Supporting Information).

In addition to the data presented above, direct measurement of the glutamine apparent K_{act} for activation of the AT activity in the presence and absence of PII-UMP indicated that PII-UMP increases the glutamine K_{act} ~2-fold when the α-ketoglutarate concentration was low and up to 4-fold when the α-ketoglutarate concentration was high (Table 2). For the PII-UMP-activated AR activity, a saturating glutamine concentration increased the apparent K_{act} for PII-UMP ~3-fold (Table 3). These results are consistent with the hypothesis of distinct enzyme forms that bind glutamine and PII-UMP (at the AR-activating site), but we were somewhat surprised by the relatively low magnitude of the effects on the apparent K_{act}.

Additional evidence of the competition between glutamine and PII-UMP can be discerned from the effect of the PII-UMP concentration on the apparent glutamine K_{inhib} for inhibition of the AR activity. When the AR activity was activated by fairly low concentrations of PII-UMP (0.4 or 0.5 μM), the glutamine apparent K_{inhib} was ~23 mM; this value was increased to ~90 mM when the PII-UMP concentration was increased to 10 μM (Table 4).

To begin to understand the interplay among PII-UMP, PII, and glutamine when all three of these regulators were present, we studied the activation and inhibition of the AT and AR activities. For the AT activity, the A versus I plots in the presence of a fixed level of the second A were nonlinear, reflecting the synergistic activation of the AT by PII and glutamine (Figure S6 of the Supporting Information). Although the inhibition of the AR by the combination of PII and glutamine was synergistic, it appeared to be decidedly less so than that was observed for activation of the AT activity (Figure S7 of the Supporting Information). Nevertheless, the synergy between glutamine and PII had an obvious effect on the apparent K_{act} and K_{inhib} for the AT and AR activities. For example, when the glutamine concentration was saturating and the α-ketoglutarate concentration was low, PII-UMP had no significant effect on the PII apparent K_{act} (Table 2). Furthermore, at less than saturating concentrations, glutamine could offset the effect of PII-UMP on the PII apparent K_{act} (Table 2). For the AR activity, the combination of PII and glutamine increased the apparent PII-UMP K_{act} ~6-fold, whereas either inhibitor alone had a fairly modest effect (Table 3). Also, PII and glutamine significantly reduced the apparent K_{inhib} for each other for inhibition of the AR activity; PII reduced the apparent K_{inhib} of glutamine ~4-fold, and glutamine decreased the apparent K_{inhib} of PII ~5-fold (Table 4). Taken together, these data are consistent with a hypothesis that PII and glutamine stabilize each other's binding and that glutamine and PII-UMP antagonize each other's binding, leading to partitioning of the enzyme into AT and AR active conformations. With regard to physiological significance, we note that the glutamine K_{act} that we observe in the absence of PII (Table 2) is significantly higher than the reported concentrations of glutamine in nitrogen-rich cells (*19*, *20*), suggesting that synergism with PII plays an important role in regulation of the enzyme by glutamine in vivo.

Additional Evidence of Independent PII and PII-UMP Sites from the ATMΔ Mutant Version of ATase. In ref *10*, we

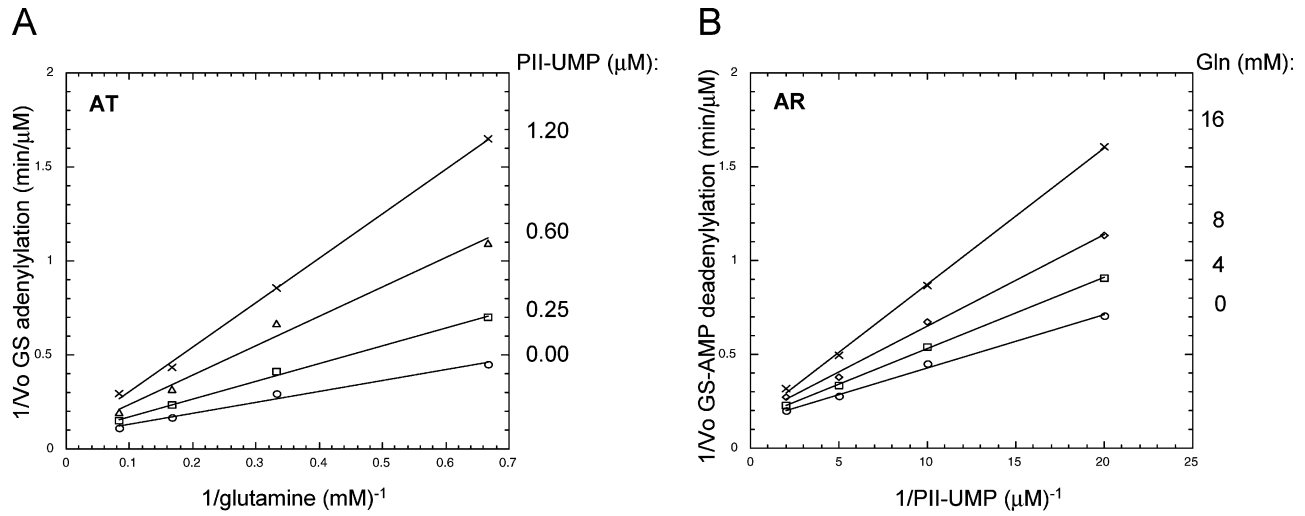

FIGURE 9: PII-UMP vs glutamine. (A) Inhibition of the glutamine-activated AT activity by PII-UMP. Conditions included 0.15 μM ATase, 2.5 μM GS, 0.5 mM ATP, and 1 mM α-ketoglutarate. PII-UMP concentrations were 0, 0.25, 0.6, and 1.2 μM. (B) Inhibition of the PII-UMP-activated AR activity by glutamine. Conditions included 0.04 μM ATase, 4 μM GS∼AMP, 1 mM ATP, 5 mM KP_i, and 1 mM α-ketoglutarate. The glutamine concentrations were 0, 4, 8, and 16 mM.

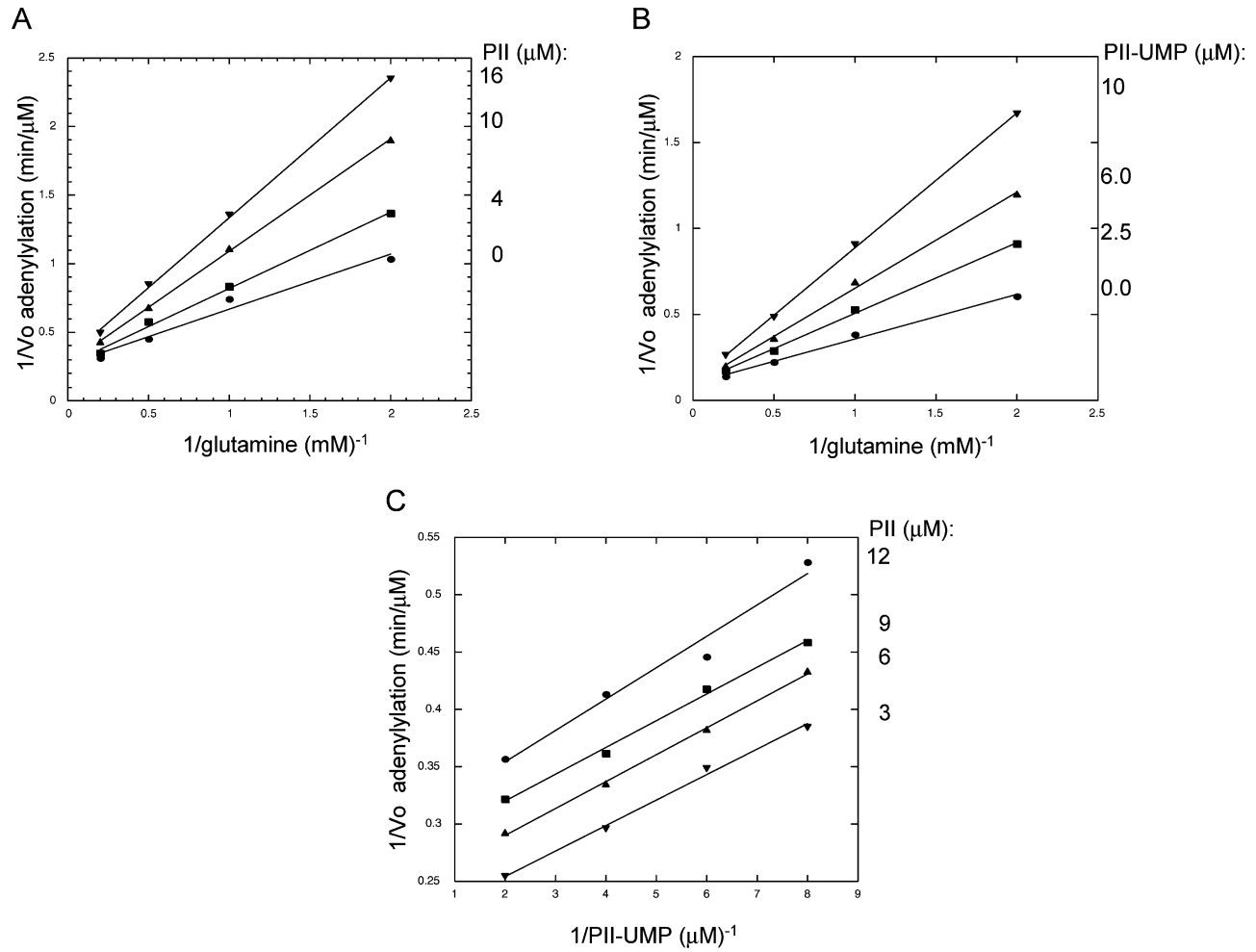

FIGURE 10: Inhibition of the glutamine-activated AT activity of the ATC1 enzyme by PII and PII-UMP. (A) Glutamine vs PII. Conditions included 0.1 μM ATC1, 2.5 μM GS, 1 mM ATP, and 1 mM α-ketoglutarate. PII concentrations were 0, 4, 10, and 16 μM. (B) Glutamine vs PII-UMP. Conditions included 0.1 μM ATC1, 2.5 μM GS, 1 mM ATP, and 1 mM α-ketoglutarate. PII-UMP concnetrations were 0, 2.5, 6, and 10 μM. (C) Dixon plot of inhibition of the glutamine-activated AT activity of the ATC1 enzyme by PII and PII-UMP. Conditions included 0.4 μM ATC1, 1 mM glutamine, 2.5 μM GS, 1 mM ATP, and 1 mM α-ketoglutarate. PII concentrations were 3, 6, 9, and 12 μM.

describe the ATMΔ enzyme, which is missing 122 amino acids from the central region linking the AR and AT domains (*10*). This enzyme lacked AR activity but had a significant AT basal activity, which was activated by glutamine and inhibited by PII and by PII-UMP. Interestingly, in the presence of glutamine, the level of inhibition by PII-UMP was greatly reduced, but inhibition by PII was not (*10*). Since the ATMΔ enzyme has significant basal AT activity, this

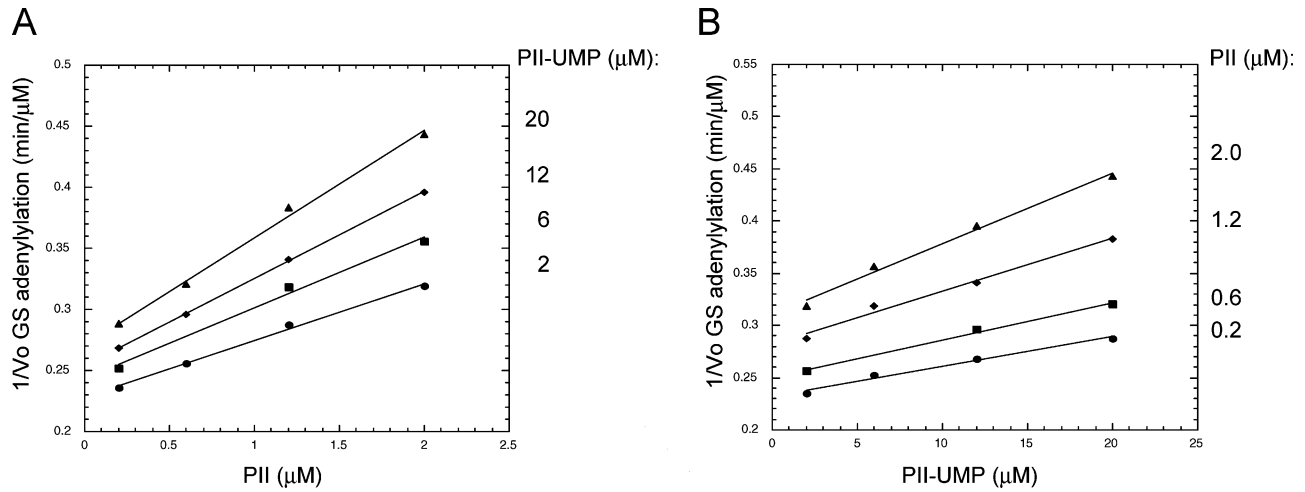

FIGURE 11: Inhibition of the basal AT activity of the ATMΔ enzyme by PII and PII-UMP. (A) Dixon plot for the inhibition by PII at different fixed concentrations of PII-UMP. Conditions included 0.16 μM ATMΔ, 3 μM GS, 1 mM ATP, and 1 mM α-ketoglutarate. The PII-UMP concentrations were 2, 6, 12, and 20 μM. (B) Dixon plot of the inhibition by PII-UMP at different fixed concentrations of PII. Conditions were as described for panel A, and the PII concentrations were 0.2, 0.6, 1.2, and 2.0 μM.

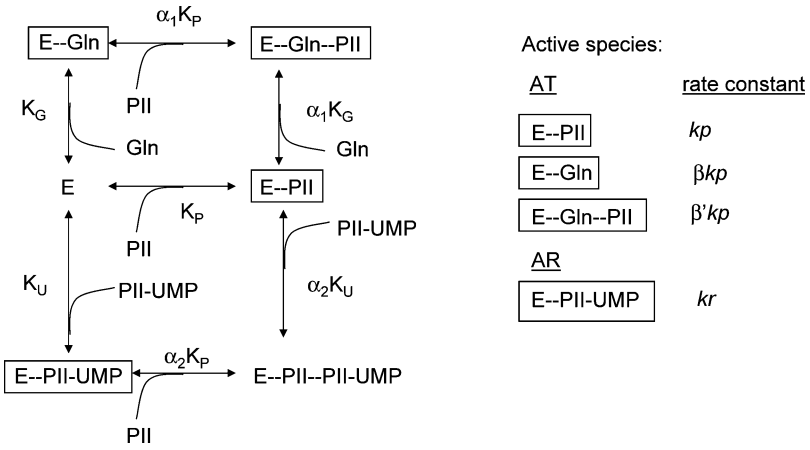

FIGURE 12: Model for the regulation of the AT and AR activities by PII, PII-UMP, and glutamine. The enzyme is denoted by E. Complexes with either AT or AR activity are boxed. α_1 describes the interaction of glutamine and PII, and α_2 describes the interaction of PII and PII-UMP. K_P, K_G, and K_U denote the dissociation constants for PII, glutamine, and PII-UMP, respectively. k_p and k_r denote the rate constants for the PII-activated AT activity and the PII-UMP-activated AR activity, respectively.

allowed the interaction between PII and PII-UMP to be studied in a simpler system. For example, if PII and PII-UMP bind to a common site or if their binding to the enzyme is strictly competitive such that only one could be bound at any given time, then the Dixon plot where one inhibitor is varied in the presence of different fixed concentrations of the other must be linear and give a series of parallel lines (Supporting Information). Conversely, a linear and noncompetitive pattern is expected in the Dixon plot when there are distinct sites for the two inhibitors, and they can both be simultaneously bound to the enzyme (Supporting Information). Indeed, more complex arrangements can be imagined, where each inhibitor binds, with different affinities, to two sites, that is, where the site specificity is relaxed. These also give rise to noncompetitive Dixon plots, but the lines can have various curvature depending upon the degree to which site specificity has been relaxed (Supporting Information). We therefore conducted the I_1 versus I_2 experiment for inhibition of the basal AT activity of the ATMΔ enzyme; the Dixon plots appeared to be linear, and there was a small but significant slope effect, that is, a noncompetitive pattern (Figure 11). This is consistent with PII and PII-UMP binding to independent sites with reasonable specificity in the absence of glutamine and suggests the existence of the ternary complex with both inhibitors bound to the enzyme.

We also examined the inhibition of the ATMΔ AT activity by PII and by PII-UMP in the presence of glutamine. Interestingly, PII provided approximately noncompetitive inhibition, as expected, except that there was a slight curvature to the lines (Figure S8A). On the basis of the $1/v_0$ intercepts and assuming that the parameters from the tables (obtained with the wild-type enzyme) apply, a value of α_1, describing the interaction of PII and glutamine, of ~0.05 can be extracted from the kinetic plot (see Figure 12 and Supporting Information). Thus, the ATMΔ mutant enzyme is rather curious in that the binding of the activator (glutamine) appears to make one of the inhibitors (PII) bind better. Inhibition by PII-UMP was nonlinear (Figure S8B). When the PII-UMP concentration was 5 μM, glutamine could almost completely overcome inhibition by PII-UMP. However, when the PII-UMP concentration was 15 μM, the inhibition was reminiscent of that seen with PII and perhaps was due to binding of PII-UMP to the natural PII site.

A Minimal Model for the Regulation of the AT and AR Activities. Since the activators and inhibitors did not appear to affect the binding of substrates yet had dramatic effects

in our initial rate studies where no product is present, their roles appeared to be to partition the enzyme among different conformations with different activities, and to influence each other's binding (Figure 12). Although the regulation of the AT and AR activities is complex, comfort is obtained from the constraint that the identical enzyme catalyzes both activities, and thus, the number of enzyme forms must be the same for both activities, leading to a minimum model with six enzyme species. The rate equations for the AT and AR activities derived from this model are presented in the Supporting Information. It should be noted that the effects of α-ketoglutarate are not included in the model; this effector probably affects α_1, α_2, k_p, k_r, and β' (Figure 12), making it difficult to globally fit the data from different experiments performed under different conditions. Nevertheless, in some cases, we could compare parameters extracted from kinetic plots to those determined directly. For example, data from Figure 9A suggest a K_G (K_{act}) for glutamine activation of the AT of 7.8 mM and a K_U (K_{inhib}) for PII-UMP inhibition of this activity of 0.35−0.5 μM, similar to the values from direct measurements (Tables 2 and 3). The data from Figure 9B suggest a K_U of ∼0.2 μM and a K_G of 11−15 mM, each differing from the direct measurements by ∼2-fold. Similarly, K_P and K_U extracted from panels B and D of Figure 8, 0.9 and 0.33 μM, respectively, were similar to the values obtained by direct measurement (Table 3).

Less appealingly, attempts to extract α_2 from kinetic plots, describing the interaction of PII and PII-UMP (Figure 12), have provided inconsistent results. For example, the data in panels A and D of Figure 8 provide fairly consistent estimates of α_2 between 1.2 and 1.9, but the data in Figure 8B provide a significantly higher estimate of this parameter of 6.8−12.9. To obtain another estimate of α_2, we measured the initial rate of the AT and AR reactions when the PII and PII-UMP concentrations were varied at a constant ratio and with a constant free α-ketoglutarate concentration. For both activities, a biphasic response to an increasing concentration of PII and PII-UMP was obtained (Figure S9 of the Supporting Information). Appealingly, simulation of the models using parameters from the tables also resulted in a biphasic response, but high α_2 values (∼10−20) were required for a good fit to the data (Figure S9C). Thus, the minimal model (Figure 12) does not fully capture the complexity of the interaction between PII and PII-UMP. We might expect that PII and PII-UMP can each bring about a variety of enzyme forms, depending on the α-ketoglutarate concentration as well as whether the other is bound and whether glutamine is bound.

Fitting Data to the Model and Estimation of Parameters. To probe α_1 describing the interaction of PII and glutamine and estimate the catalytic activity of the enzyme bound to PII, glutamine, or both, we examined the AT rate as the PII concentration was varied at different glutamine concentrations (A1 vs A2 experiment, Figure S10 of the Supporting Information). These data were globally fit reasonably well to the model as described in the Supporting Information (rms error = 0.227), providing the parameters listed in the figure legend of Figure S10. The K_G so determined (15.6 mM) is ∼2-fold higher than from direct measurement, and the K_P so determined, 0.18 μM, is ∼6-fold lower than that from direct measurement. The results predict that the synergy between glutamine and PII is due both to their effects on each other's binding ($\alpha_1 = 0.17$) and to the very high activity of the enzyme form bound by both effectors.

For further analysis of the model, we chose six experimental data sets, employing a variety of conditions, and globally fit the data to the model as described in the Supporting Information. Our goal was to see whether reasonably good fits could be obtained, to see whether parameters were in agreement with data from direct measurement, and, in particular, to obtain estimates of α_1 and α_2, describing the interactions of PII with glutamine and with PII-UMP, respectively.

To study the interaction of PII and PII-UMP, the experiments shown in panels A, B, and D of Figure 8 were globally fit (Figure S11 of the Supporting Information). In all cases, reasonably good fits were obtained, and given the different conditions that were used, the parameters extracted from the fit were reasonable when compared to those obtained from direct measurement. The value of α_2 from these fits ranged from 2.17 to 8.85, suggesting that PII and PII-UMP have significant effects on each other's binding.

To study the interaction of PII-UMP and glutamine, the data from panels A and B of Figure 9 were globally fit to the model. This is a simpler situation, since there is no interaction between PII and PII-UMP and the antagonism between glutamine and PII-UMP is competitive. These data were fit well by the model, and the extracted parameters were in good agreement with the values obtained by direct measurement (Figure S12 of the Supporting Information).

Thus, while the model described in Figure 12 is obviously a simplification and does not adequately describe the interactions of PII and PII-UMP with complex effects of α-ketoglutarate, other aspects of the model fit the experimental data reasonably well.

DISCUSSION

The ATase is a remarkably complex enzyme, with two distinct active sites and four regulatory species (PII, PII-UMP, glutamine, and α-ketoglutarate). One unexpected finding of our studies was that α-ketoglutarate, which controls PII activation of the AT activity and inhibition of the AR activity and also controls PII-UMP activation of the AR activity and inhibition of the AT activity, did not appear to regulate the binding of PII and PII-UMP to the enzyme. This was revealed as the absence of an effect of α-ketoglutarate on the K_{act} of PII and PII-UMP (Tables 2 and 3). Apparently, α-ketoglutarate regulated the ability of PII and PII-UMP to exert their influence at a postbinding step; that is, PII and PII-UMP functioned as dissociable regulatory subunits that mediated regulation by α-ketoglutarate. Since all PII proteins characterized so far bind α-ketoglutarate (*12*), this may be a regulatory property shared by other PII proteins and their receptors.

Another unexpected finding was that PII and PII-UMP did not appear to compete for a single site but, rather, appeared to act from distinct sites. This was revealed as noncompetitve inhibition patterns when PII and PII-UMP were in opposition, for both the AT and AR activities. One of these sites was apparently located on the fraction of the protein remaining in the ATC1 truncation, which removes the N-terminal NT (AR) domain, since binding of PII or PII-UMP to this species was able to inhibit the activation of its

AT activity by glutamine. Apparently, this is the site from which PII-UMP normally activates the AR activity and inhibits the AT activity, with the specificity of the site for PII-UMP relaxed due to the truncation of the rest of the ATase (*10*). For the wild-type enzyme, the linear noncompetitive patterns obtained in the A versus I experiments where PII and PII-UMP were in antagonism suggest that the two sites in the wild-type enzyme are fairly specific for PII and PII-UMP; otherwise, nonlinear patterns would be expected.

The ATMΔ mutant enzyme provided additional evidence for distinct PII and PII-UMP sites. Since this mutant enzyme is the result of an internal deletion, the mutation is unlikely to have created a site for PII or PII-UMP. The significant basal AT activity of the ATMΔ enzyme in the absence of glutamine allowed us to examine the interaction of PII and PII-UMP in a system with no activators, where the presence of at least two distinct sites and the presence of the ternary complex containing enzyme and both inhibitors could be inferred.

Although PII and PII-UMP did not compete for a single site, they did affect each other's binding to the enzyme. This was evident in the influence PII and PII-UMP had on the inhibition constant for the other. Increasing the concentration of the activator of either the AT or AR activity resulted in an increase in the apparent K_{inhib} of the inhibitor. Further evidence of an α factor greater than unity (α_2 in Figure 12) comes from the analysis of the kinetic plots and from global fitting of the data from activator versus inhibitor experiments where PII and PII-UMP were in opposition (Figure 9) and from experiments where PII and PII-UMP were held at a fixed ratio and varied simultaneously (Figure S9).

In contrast to the situation involving PII and PII-UMP, we were able to observe clear competition between PII-UMP and glutamine for the enzyme, for both AT and AR activities, suggesting that the enzyme exclusively binds either PII-UMP or glutamine. Most likely, PII-UMP and glutamine bind distinct sites and cause the enzyme to adopt alternative conformations. Interestingly, when the ATC1-truncated form of ATase was examined, either PII or PII-UMP competed with glutamine for the enzyme, and inhibition of the ATC1 ATase activity seemed to be due to additive effects of PII and PII-UMP binding. This is consistent with the hypothesis that ATC1 contains only the natural PII-UMP site, with reduced specificity. When the AT activity of the ATMΔ mutant enzyme was studied, glutamine appeared to stimulate the binding of PII to the enzyme. Thus, it appears that PII inhibits the ATMΔ mutant enzyme by binding to the natural PII site.

The ATase appears to be a highly coordinated enzyme in which all regulatory sites communicate. This is illustrated by the influence of α-ketoglutarate on the apparent K_{act} of glutamine for activation of the AT activity and on the effects of α-ketoglutarate and glutamine in experiments where both PII and PII-UMP were allowed to influence the AT and AR activities (Tables 2 and 3). We imagine that a given enzyme molecule is able to display AT or AR activity at any given moment, but never both (Figure 12). The coordination of enzyme activities was further studied in ref *10*, where we hypothesized that the C-terminal AT domain of the enzyme is involved in the activation of the AR activity of the N-terminal domain (*10*). This is consistent with the hypothesis that the PII-UMP site involved in activation of the AR activity of the N-terminal domain is located on or near the C-terminal AT domain.

The simplest scheme to account for the data available so far includes six enzyme forms (Figure 12). One of these forms, in which the enzyme is bound to neither PII, PII-UMP, nor glutamine, is inactive for both AT and AR activities. The three enzyme species bound by glutamine, by PII, and by both PII and glutamine exhibit AT activity, while the enzyme form bound by PII-UMP exhibits AR activity. As a high concentration of PII and PII-UMP could effectively inhibit the AR and AT activities, respectively, when these were activated by PII-UMP or PII, it seems that the enzyme form bound by both PII and PII-UMP exhibits neither activity. At this time, we do not know whether the enzyme is ever bound by PII or by PII-UMP at both of the PII/PII-UMP sites. However, in unpublished experiments, we did not observe significant inhibition of either the AT or AR activitiy at very high concentrations of the PII or PII-UMP activator, as might be expected if both sites could be bound by a single species, as long as effector molecules (ATP and α-ketoglutarate) were not subject to titration.

The scheme presented in Figure 12 provides possible explanations of why PII and glutamine were highly synergistic in activation of the AT activity but exhibited less synergy in the inhibition of the PII-UMP-activated AR activity. First, a high value for β' (Figure 12 and Figure S10) would amplify the effects of synergistic binding of PII and glutamine in activation of the AT activity. Second, when the AT activity is activated in the absence of PII-UMP, there is nothing to impede the fomation of the enzyme complex containing both PII and glutamine, but in the presence of PII-UMP, formation of this complex is impeded both by the competition between glutamine and PII-UMP for the enzyme and by PII-UMP converting the enzyme to a form that binds PII less avidly. The prediction is that the synergy between PII and glutamine for activation of the AT activity would be greatly reduced in the presence of PII-UMP, and although we did not systematically study this matter, data presented in Tables 2−4 and Figure S6 suggest this is the case.

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SUPPORTING INFORMATION AVAILABLE

Supporting Information for Kinetic Characterization of ATase. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Rhee, S. G., Bang, W. G., Koo, J. H., Min, K. H., and Park, S. C. (1988) Regulation of glutamine synthetase activity and its biosynthesis in *Escherichia coli*: Mediation by three cycles of covalent modification, in *Enzyme dynamics and regulation* (Chock, P. B., Huang, C. Y., Tsou, C. L., and Wang, J. H., Eds.) pp 136−145, Springer-Verlag, Berlin.
2. Kustu, S., Hirschman, J., Burton, D., Jelsko, J., and Meeks, J. C. (1984) Covalent modification of bacterial glutamine synthetase: Physiological significance, *Mol. Gen. Genet. 197*, 309−317.
3. Rhee, S. G., Chock, P. B., and Stadtman, E. R. (1989) Regulation of *Escherichia coli* glutamine synthetase, in *Advances in Enzymol-*

ogy (Meister, A., Ed.) Vol. 62, pp 37−92, John Wiley and Sons, New York.
4. Stadtman, E. R., Hohman, R. J., Davis, J. N., Wittenberger, M., Chock, P. B., and Rhee, S. G. (1980) Subunit interaction of adenylylated glutamine synthetase, *Mol. Biol. Biochem. Biophys. 32*, 144−156.
5. Kingdon, H. S., Shapiro, B. M., and Stadtman, E. R. (1967) Regulation of glutamine synthetase. VIII. ATP:glutamine synthetase adenylyltransferase, an enzyme that catalyzes alterations in the regulatory properties of glutamine synthetase, *Proc. Natl. Acad. Sci. U.S.A. 58*, 1703−1710.
6. Wulff, I., Mecke, D., and Holzer, H. (1967) Mechanism of the enzymatic inactivation of glutamine synthetase from *E. coli*, *Biochem. Biophys. Res. Commun. 28*, 740−745.
7. Adler, S. P., Purich, D., and Stadtman, E. R. (1975) Cascade control of *Escherichia coli* glutamine synthetase, *J. Biol. Chem. 250*, 6264−6272.
8. Jiang, P., Peliska, J. A., and Ninfa, A. J. (1998) The regulation of glutamine synthetase covalent modification revisited: Role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state, *Biochemistry 37*, 12802−12810.
9. Holm, L., and Sander, C. (1995) DNA polymerase β belongs to an ancient nucleotidyltransferase superfamily, *Trends Biochem. Sci. 20*, 345−347.
10. Jiang, P., and Ninfa, A. J. (2007) Structure−function analysis of glutamine synthetase adenylyltransferase (ATase, EC 2.7.7.49) of *Escherichia coli*, *Biochemistry 46*, 4117−4132.
11. Rhee, S. G., Park, R., Chock, P. B., and Stadtman, E. R. (1978) Allosteric regulation of monocyclic interconvertable enzyme cascade systems: Use of *Escherichia coli* glutamine synthetase as an experimental model, *Proc. Natl. Acad. Sci. U.S.A. 75*, 3138−3142.
12. Ninfa, A. J., and Jiang, P. (2005) PII signal transduction proteins: Sensors of α-ketoglutarate that regulate nitrogen metabolism, *Curr. Opin. Microbiol. 8*, 168−173.
13. Kamberov, E. S., Atkinson, M. R., and Ninfa, A. J. (1995) The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP, *J. Biol. Chem. 270*, 17797−17807.
14. Jaggi, R., van Heeswijk, W. C., Westerhoff, H. V., Ollis, D., and Vasudevan, S. G. (1997) The two opposing activities of adenylyltransferase reside in distinct homologous domains, with intramolecular signal transduction, *EMBO J. 16*, 5562−5571.
15. Jiang, P., Peliska, J. A., and Ninfa, A. J. (1998) Enzymological characterization of the signal-transducing uridylyltransferase/uiridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein, *Biochemistry 37*, 12782−12794.
16. Schauder, B., Blocker, H., Frank, R., and McCarthy, J. (1987) Inducible expression vectors incorporating the *Escherichia coli atpE* translational initiation region, *Gene 52*, 279−283.
17. Atkinson, M. R., Kamberov, E. S., Weiss, R. L., and Ninfa, A. J. (1994) Reversible uridylylation of the *Escherichia coli* PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor Nitrogen Regulator I (NRI or NtrC), *J. Biol. Chem. 269*, 28288−28293.
18. Segel, I. (1975) *Enzyme Kinetics*, John Wiley and Sons, New York.
19. Senior, P. J. (1975) Regulation of nitrogen metabolism in *Escherichia coli* and *Klebsiella aerogenes*: Studies with the continuous culture technique, *J. Bacteriol. 123*, 407−418.
20. Ikeda, T. P., Shauger, A. E., and Kustu, S. (1996) *Salmonella thyphimurium* apparently perceives external nitrogen starvation as internal glutamine limitation, *J. Mol. Biol. 259*, 589−607.

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