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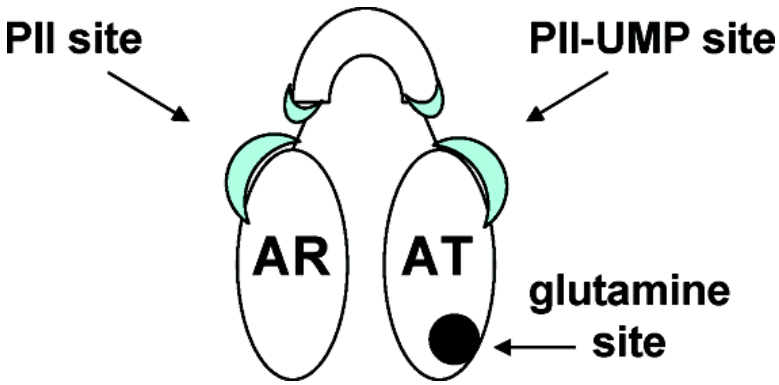

Structure−Function Analysis of Glutamine Synthetase Adenylyltransferase (ATase, EC 2.7.7.49) of *Escherichia coli*†

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ABSTRACT: Glutamine synthetase adenylyltransferase (ATase) regulates the activity of glutamine synthetase by adenylylation and deadenylylation in response to signals of nitrogen and carbon status: glutamine, α-ketoglutarate, and the uridylylated and unmodified forms of the PII signal transduction protein. ATase consists of two conserved nucleotidyltransferase (NT) domains linked by a central region of ∼200 amino acids. Here, we study the activities and regulation of mutated and truncated forms of ATase. Our results indicate the following. (i) The N-terminal NT domain contained the adenylyl-removing (AR) active site, and the C-terminal NT domain contained the adenylyltransferase (AT) active site. (ii) The enzyme contained a glutamine binding site, and glutamine increased the affinity for PII. (iii) The enzyme appeared to contain multiple sites for the binding of PII and PII-UMP. (iv) Truncated versions of ATase missing the C-terminal (NT) domain lacked both AT and AR activity, suggesting a role for the C-terminal NT domain in both activities. (v) The purified C-terminal NT domain and larger polypeptides containing this domain had significant basal AT activity, which was stimulated by glutamine. These polypeptides were indifferent to PII and PII-UMP, or their ATase activity was inhibited by either PII or PII-UMP. (vi) Certain point mutations in the central region or an internal deletion removing most of this part of the protein eliminated the AR activity and eliminated activation of the AT activity by PII, while not eliminating the binding of PII or PII-UMP. That is, these mutations in the central region appeared to destroy the communication between the PII and PII-UMP binding sites and the AT and AR active sites. (vii) Certain mutations in the central region of ATase appeared to dramatically improve the binding of glutamine to the enzyme. (viii) While the isolated AT and AR domains of ATase bound poorly to PII and PII-UMP, these domains bound PII and PII-UMP significantly better when linked to the central region of ATase. Together, our results indicate a highly coordinated enzyme, in which the AT and AR domains participate in each other's regulation and distant regulatory sites are in communication with each other. A model for the regulation of ATase by glutamine, PII, and PII-UMP consistent with all data is presented.

ATase,[1] product of *glnE*, regulates the activity of glutamine synthetase (GS) in response to intracellular signals of nitrogen status, by catalyzing the adenylylation and deadenylylation of GS (*1*, *2*). The enzyme participates in both short-term and long-term adaptation to nitrogen status (*3*, *4*). Under nitrogen-excess growth conditions, GS is adenylylated by ATase, resulting in low GS activity. Conversely, under nitrogen-limiting conditions, GS∼AMP is deadenylylated by ATase, restoring GS activity. ATase allows rapid alteration of GS activity in response to dramatically changing environments (*4*). The enzyme is widely distributed in bacteria, as is regulation of glutamine synthetase by reversible covalent adenylylation, and in some bacteria, the enzyme is essential for cellular viability (*5*).

Historically, ATase was one of the first signal transduction enzymes identified (*1*, *2*, *6*), and it provided the first example of reversible nucleotidylylation as a signal transduction mechanism (*7*). The study of ATase regulation in *Escherichia coli* also provided one of the first examples of a bicyclic signal transduction system (*8*−*10*; Figure 1A). One cycle of this bicyclic system consists of the uridylyltransferase/uridylyl-removing enzyme (UTase/UR, product of *glnD*) and the PII signal transduction protein. Under physiological conditions, UTase/UR controls the PII uridylylation state in response to the glutamine concentration (*11*). PII and PII-UMP regulate the antagonistic adenylylation (AT) and adenylyl-removing (AR) activities of ATase (*12* and references cited therein). Specifically, PII activates the AT activity and PII-UMP the AR activity, and each also inhibits the antagonistic activity (Figure 1A). In addition, glutamine directly activates the AT activity and inhibits the AR activity (Figure 1A). A signal antagonistic to glutamine is provided by α-ketoglutarate, which acts through PII and PII-UMP (*13*). A low level of α-ketoglutarate favors the action of PII, and a high level of α-ketoglutarate (Figure 1A) favors the action

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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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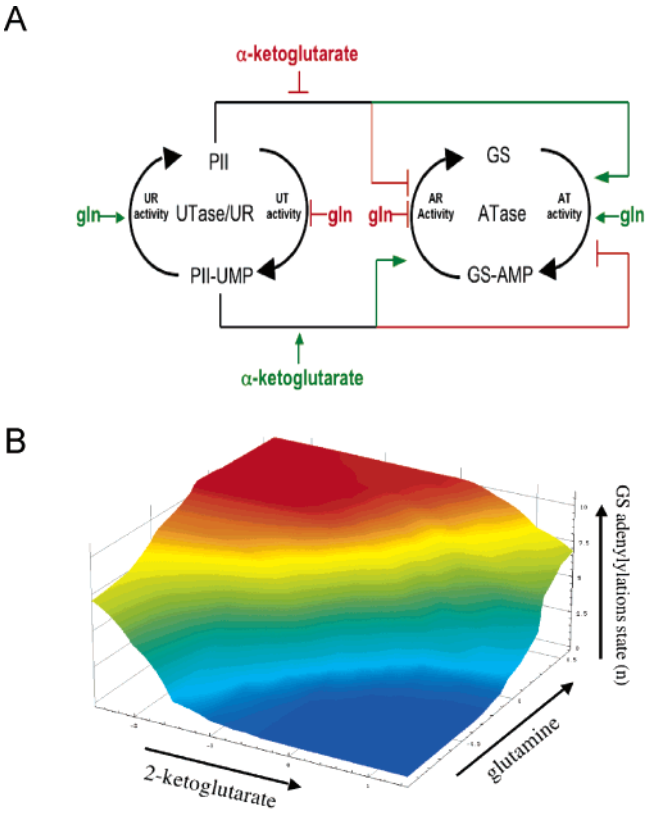

FIGURE 1: Bicyclic signal transduction system regulating GS activity. (A) Topology of the network. The bicycle is comprised of two linked monocycles. In the UTase/UR−PII monocycle, the PII protein is converted between unmodified and uridylylated forms by the UT and UR activities of the UTase/UR protein. Glutamine (gln) activates the UR activity and inhibits the UT activity. In the ATase−GS monocycle, the GS enzyme is converted between unmodified and adenylylated forms by the AT and AR activities of the ATase protein. Glutamine (gln) activates the AT activity and inhibits the AR activity. PII also activates the AT activity and inhibits the AR activity. PII-UMP activates the AR activity and inhibits the AT activity. α-Ketoglutarate acts upon PII and PII-UMP; a low concentration of this effector favors the action of PII and inhibits the action of PII-UMP, while a high concentration of this effector inhibits the action of PII and favors the action of PII-UMP. (B) Integration of antagonistic α-ketoglutarate and glutamine signals by the reconstituted bicyclic system. Data are from ref *12*.

of PII-UMP. The bicyclic system has been reconstituted using purified components, and the reconstituted system provided rapid alteration of the GS adenylylation state in response to alterations in the concentrations of glutamine and α-ketoglutarate and reproducibly "calculated" an appropriate GS adenylylation state in response to effectors [e.g., Figure 1B (*12*)]. The question of how exactly PII, PII-UMP, α-ketoglutarate, and glutamine bring about the regulation of the AT and AR activities of ATase remains.

The adenylylation (AT) activity of ATase catalyzes the addition of AMP to tyrosine 397 of GS, with the release of pyrophosphate (*7*). This reaction is activated synergistically by (unmodified) PII and by glutamine; either effector alone can partially activate the enzyme, and each effector reduces the apparent K_{act} of the other effector (*12*). The ability of PII to activate ATase is regulated by the synergistic binding of α-ketoglutarate and ATP to PII. PII activity is highest when the trimeric protein is bound by three molecules of ATP and a single molecule of α-ketoglutarate (*12*, *13*). At physiological ATP concentrations, the binding of α-ketoglutarate to PII displays negative cooperativity such that the binding of the first molecule of α-ketoglutarate inhibits further effector binding. This negative cooperativity is overcome as the level of α-ketoglutarate is increased through its physiological range such that the PII trimers become saturated with three effector molecules (*12*). The form of PII saturated with three molecules of α-ketoglutarate per trimer is unable to activate the AT activity, and thus, as the concentration of α-ketoglutarate is increased through its physiological concentration range, it blocks the activation of the AT activity by PII (*12*). The AT activity is inhibited by PII-UMP in the presence of PII and glutamine, and a high level of α-ketoglutarate improves the ability of PII-UMP to inhibit the AT activity (*12* and references cited therein).

The AR activity catalyzes the phosphorolysis of GS∼AMP to produce GS and ADP (*14*). In vitro, the activity requires PII-UMP as an essential activator and is weakly inhibited by PII and by glutamine (*12*). The activation of the AR activity by PII-UMP requires α-ketoglutarate and is favored by high concentrations of α-ketoglutarate. Thus, as the concentration of α-ketoglutarate is increased through its physiological range, it both inhibits the ability of PII to activate the AT activity and improves the ability of PII-UMP to activate the AR activity (Figure 1A).

Bioinformatic analysis indicated that the 946-amino acid ATase contains two copies of a highly conserved motif shared by other nucleotidyltransferase enzymes, designated the NT domain (*15*, *16*; Figure 2). The NT domains utilize a two-metal mechanism (usually Mg^{2+} ions) and contain a glycine-rich loop followed by a pair of acidic amino acids, usually aspartates, which participate in chelation of the Mg^{2+} ions. The two Mg^{2+} ions serve to position the substrate and stabilize the pentavalent phosphate transition state, respectively (*17*, *18*). The two NT domains in ATase are related to each other, as well as to the single NT domain found within the UTase/UR (*15*, *16*).

Vasudevan and colleagues have examined the effect of producing two "halves" of the ATase as discrete polypeptides (*15*). The C-terminal polypeptide, consisting of residues 425−946 of ATase, displayed AT activity that was regulated by glutamine but was reported to be unaffected by PII (*15*). An N-terminal polypeptide, consisting of residues 1−423 of ATase, was expressed and studied in crude fractions (0−50% ammonium sulfate cut from a crude extract). It was reported that this polypeptide had AR activity that was activated by PII-UMP and inhibited by PII and unaffected by glutamine (*15*). On the basis of these observations, it was concluded that the two opposing activities of ATase reside in the distinct NT domains, with the N-terminal domain catalyzing the AR activity and the C-terminal NT domain catalyzing the AT activity (*15*). Furthermore, it was concluded that a single PII/PII-UMP site maps within the polypeptide of residues 1−423, while a glutamine binding site maps in the polypeptide of residues 425−946 (*15*).

Here, we further examine the structure−function relationships of the ATase and its mechanism of control. Some of our results are consistent with the results of Vasudevan and colleagues. Specifically, we show by site-specific mutagenesis of the putative active sites that the N-terminal domain of ATase contains the AR active site and the C-terminal domain of ATase contains the AT active site. We also

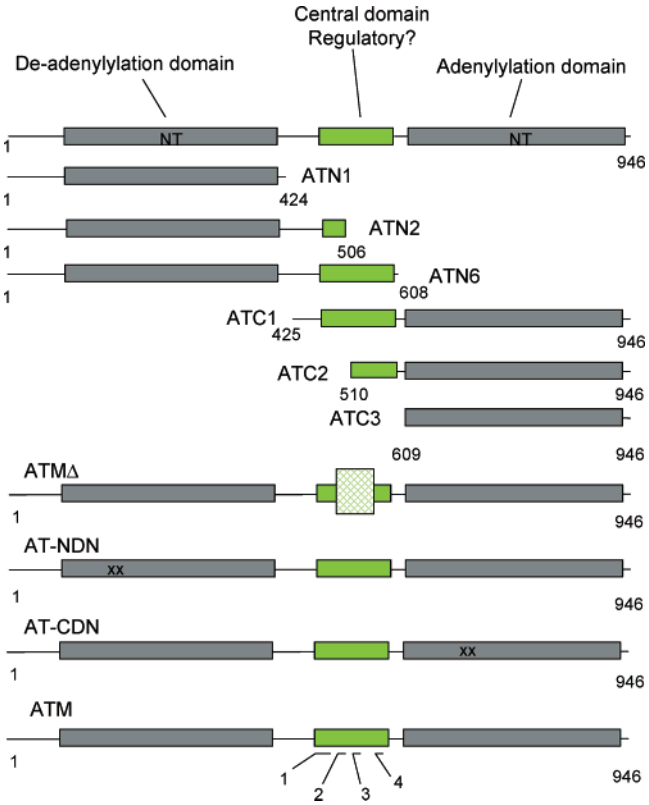

FIGURE 2: Schematic depiction of the ATase domain structure and mutant and truncated versions of ATase studied here. Numbers at the beginning and end of each species indicate the first and last amino acid present, respectively. The cross-hatched box depicts the deleted region in ATMΔ. The ATM series of enzymes contain clusters of point mutations in different parts of the central region, as described in the text. Specifically, ATM1 contains the D463N, P467A, and L469G mutations, ATM2 the R499A, R501A, D505N, P509A, and L511G mutations, ATM3 the L525G, R527A, and I528G mutations, and ATM4 the R571A, P573A, and L575G mutations.

observed that the enzyme contains a glutamine site that probably maps to the C-terminal AT domain. However, other results shown here are not in agreement with the conclusions of Vasudevan and colleagues (*15*). Our results suggest that the enzyme contains multiple PII/PII-UMP sites, and we demonstrate conclusively that the enzyme contains a PII/PII-UMP site on the C-terminal side of position 425. In the aggregate, our results show that the enzyme is highly coordinated, with the AT and AR domains participating in each other's regulation and communication among the PII, PII-UMP, and glutamine sites. A hypothesis for the organization and regulation of the enzyme is presented.

EXPERIMENTAL PROCEDURES

Recombinant DNA Techniques. The wild-type *glnE* gene encoding ATase was amplified from *E. coli* strain YMC10 by PCR, using the primers listed in Table 1, and cloned into pALTER-1 (Promega), and its sequence was verified by DNA sequencing. AT-NDN (D173N and D175N), AT-CDN (D701N and D703N), ATM1 (D463N, P467A, and L469G), ATM2 (R499A, R501A, D505N, P509A, and L511G), ATM3 (L525G, R527A, and I528G), and ATM4 (R571A, P573A, L575G) were constructed using the pALTER mutagenesis kit (Promega), following the instructions of the vendor and using the mutagenic oligonucleotides listed in Table S1 of the Supporting Information. The mutations in ATM1, ATM2, ATM3, and ATM4 were chosen to test the role of portions of the central region of ATase that bear some resemblance to the β-hairpin element of the C-terminal domain of NRII, implicated as possibly forming part of the PII-binding site of NRII (*19*). ATMΔ was constructed as follows. The pALTER mutagenesis kit was used to introduce two restriction sites for XhoI into the wild-type gene, using the primers listed in Table S1. The mutated gene was digested with XhoI and ligated with DNA ligase to form the desired internal deletion mutation. Truncated forms of the ATase were formed by PCR using the primers listed in Table S1. All mutated alleles and truncated alleles were verified by DNA sequencing. The wild-type allele, alleles containing point mutations, and truncated alleles were cloned into pJLA503 for hyperexpression of the protein products (*20*). Hyperexpression of proteins from the pJLA503-based plasmids was induced by a temperature shift to 43 °C and used strain Ec (*21*), which contains a null mutation in *glnE* and thus lacks endogenous ATase.

Purified Proteins. Preparations of GS, NRII, PII, and PII-(E44C/C73S) obtained previously were used (*12*, *22*, *23*). GS∼AMP was freshly prepared for each series of experiments as described previously (*12*). For purification of ATase and mutant forms of ATase, cells containing the hyperexpressed protein were harvested and stored as a frozen cell paste at −80 °C. In all cases, overexpression was sufficient to permit purification of the desired proteins to be monitored by SDS−polyacrylamide gel electrophoresis.

Purification of ATase, AT-CDN, and AT-NDN was conducted briefly as follows. Cells were resuspended in 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1 mM DTT, and 200 mM KCl (TGDK buffer), disrupted by sonication, and clarified by centrifugation. A 25−50% ammonium sulfate cut was made, and the precipitate was collected by centrifugation, resuspended in TGD buffer (TGDK lacking KCl), diluted with TGD, and applied to a DE-52 column (Whatman) equilibrated with TGD buffer. Proteins were eluted with a 0 to 0.6 M KCl gradient in TGD buffer; ATase and the mutated forms of ATase were eluted at ∼310−350 mM KCl. Peak fractions were then pooled and fractionated on a Bio-Gel A0.5M gel filtration column equilibrated in TGDK buffer. Peak fractions from the gel filtration column were pooled and fractionated on Phenyl Sepharose CL-4B (Sigma) as follows. The column was equilibrated in 40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 4 mM 2-mercaptoethanol, and 1 M $(NH_4)_2SO_4$ (TEMN buffer). The pooled sample from gel filtration was brought to 25% saturated $(NH_4)_2SO_4$ and applied to the column. The column was then washed with a gradient of TEMN buffer to TEM buffer [as TEMN but lacking $(NH_4)_2SO_4$]. ATase and mutant forms of ATase remained bound to the column during this elution. ATase and mutant forms of ATase were then eluted with a gradient from 0 to 50% ethylene glycol in TEM buffer. Peak fractions were pooled and dialyzed against storage buffer [TGDK buffer, except with 50% (v/v) glycerol]. Purification of ATMΔ, ATM1, ATM2, ATM3, and ATM4 was similar, except that a 25−60% saturation ammonium sulfate cut was used after sonication, the phenyl-Sepharose chromatography step was omitted, and the pooled peak from the gel filtration step was dialyzed against storage buffer. Purification of truncated proteins ATC1, ATN1, ATN2, and ATN6 was as

described above, using a 25−50% ammonium sulfate cut as the first step and omitting the phenyl-Sepharose step. Purification of ATC2 was conducted as described for the other truncated proteins, except that the protein did not stick to DE-52, and was recovered from the flow-through fraction. Purification of ATC3 was briefly as follows. Cells were disrupted by sonication, and the polypeptide was fractionated with the pellet. The pellet was resuspended in 6 M urea and slowly stirred at 4 °C overnight. Insoluble material was removed by centrifugation, and the supernatant was dialyzed against TGD buffer. The dialysate was clarified by centrifugation; a 0−50% $(NH_4)_2SO_4$ cut was made, and the resuspended pellet was subjected to gel filtration on Sephadex G-75. ATC3 fractionated into two peaks, corresponding to the excluded fraction and presumably the monomeric polypeptide. The latter was highly purified and dialyzed directly into storage buffer.

Preparation of PII-UMP and PII (E44C/C73S)-UMP was as follows. A 10 mL reaction mixture contained 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 25 mM $MgCl_2$, 2 mM DTT, 300 μM α-ketoglutarate, 500 μM ATP, 2 mM UTP, 10 μM trimer PII (E44C/C73S), and 0.1 μM monomer UTase/UR. The reaction was started by adding UTP and the mixture incubated at 37 °C for 25 min. The reaction was stopped by adding EDTA to a final concentration 25 mM. The UTase/UR and PII-UMP were separated on a MonoQ column (Pharmacia FPLC) run at room temperature using a gradient from 0 to 1 M KCl in TGD buffer. PII-UMP was not separated from PII during this procedure but was well separated from UTase/UR; PII-UMP eluted later than UTase/UR at ∼300 mM KCl. The peak PII-UMP fractions were pooled and heated to 60 °C for 5 min to kill any remaining UTase/UR activities, then concentrated using a 10 kDa molecular mass cutoff Ultrafree-15 centrifugal filter device (Millipore), and stored at −20 °C.

Labeling of PII (E44C/C73S) and PII (E44C/C73S)-UMP with a cross-linker was exactly as described previously (*22*) except that only the noncleavable TFPAM-3 cross-linker was used. TFPAM-3 was purchased from Molecular Probes. In some cases, 10% (v/v) glycerol was added to the final PII-TF3 preparation, and it was stored at −20 °C for later use. This did not appear to result in any diminished cross-linking ability.

Cross-Linking Reactions. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 500 μM ATP as indicated, 20 μM, 50 μM, or 10 mM α-ketoglutarate as indicated, 5 μM dimer NRII, 2 μM wild-type ATase, 5 μM monomer ATase truncations, and 10 μM trimer PII or PII-UMP as indicated. As indicated, reaction mixtures also contained 200 μg/mL bovine serum albumin (Sigma). Reaction volumes were typically 20−30 μL, and reactions were carried out in 96-well microtiter plates on ice. UV light was from a hand-held source placed directly on the 96-well plate on ice for exposure for 20 min. Reactions were stopped by addition of SDS loading buffer. Aliquots were run on 13.5% SDS−polyacrylamide gels and the protein bands visualized by Coomassie Brilliant Blue R250 staining. In some cases, aliquots were run on SDS−polyacrylamide gels and transferred to nitrocellulose for immunoblotting, as indicated. Note that phenylazide cross-linkers such as TFPAM-3 must be used in the presence of an excess of nucleophilic buffer, such as Tris, to prevent nonspecific cross-linking, and we observed that 50 mM Tris-HCl, as used here, appeared to be a suitable buffer concentration.

Immunoblotting. Blots were probed with anti-PII that was generously provided by W. van Heeswick (*24*). The crude antibody was further purified as follows. A 250 mL Luria Broth/chloramphenicol culture of strain BKc, lacking both PII and GlnK, was grown overnight at 37 °C. Cells were harvested by centrifugation and resuspended in 50 mL of Tris-buffered saline (TBS) [20 mM Tris-HCl (pH 7.5) and 137 mM NaCl]. The suspension was sonicated on ice to break open the cells. Polyclonal anti-PII serum was added to the lysate (1:5000 dilution), and the mixture was stirred for 1 h at room temperature to allow contaminating antibodies to adsorb to the cellular protein. The mixture was cleared by centrifugation, and the supernatant was saved. Tween 20 was added to a final concentration of 0.1% (v/v), and the extract-treated antibody was stored at 4 °C. Blots were visualized by enhanced chemiluminescence (ECL) using the Amersham ECL kit according to the manufacturer's directions.

Nondenaturing Gel Electrophoresis Assay for PII−ATase Binding. The incubation mix contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 50 μM α-ketoglutarate or as indicated, 10 mM glutamine or as indicated, and proteins as indicated. After incubation at room temperature for 10 min, the samples were mixed with gel loading buffer [50 mM Tris-HCl (pH 7.5) and 10% (v/v) glycerol] and subjected to electrophoresis on 14% nondenaturing polyacrylamide gels. The stacking gel contained 62.5 mM Tris-HCl (pH 7.6), and the resolving gel contained 187.5 mM Tris-HCl (pH 7.5). The running buffer consisted of 25 mM Tris-borate (pH 7.5), 1 mM $MgCl_2$, 0.5 mM ATP, 30 μM α-ketoglutarate (or as indicated), and 10 mM glutamine (or as indicated). The gels were prerun at 100 V for 50−75 min to balance the small molecule components of the gels and buffer. After the electrophoretic run, gels were stained with Coomassie Brilliant Blue R-250 to visualize the protein bands.

To determine if the presumed complex bands on the nondenaturing gels contained both ATase and PII, the bands were extracted and analyzed via SDS−PAGE as follows. The native gels were stained briefly with Coomassie Brilliant Blue R-250 in 10% (v/v) acetic acid and then briefly destained with 50% (v/v) methanol, using several changes of the destaining solution. The visualized bands were excised and soaked in 0.2× SDS loading buffer and neutralized with 1 M Tris-HCl (pH 9.0). (A minimal amount of the Tris-HCl was added, until the bromophenol blue pH indicator in the SDS loading buffer turned blue.) The gel slices were shaken at 37 °C for 16 h to extract the proteins, and the gel extracts were analyzed on 14% polyacrylamide−SDS gels. After the samples had been stained with Coomassie Blue, the bands were quantified by densitometry using Image Quant software (Molecular Dynamics).

Gel Filtration Chromatography Assay for PII−ATase Binding. The incubation mixture contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 72 μM α-ketoglutarate, 42 μM PII, 6 μM ATase, and glutamine as indicated. After incubation at room temperature for 15 min, the sample was loaded onto a Sephadex G-100 column (∼120 mL bed volume) equilibrated with 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM ATP, and

30 μM α-ketoglutarate and eluted with the same buffer at 4 °C. Fractions containing proteins were analyzed by SDS−PAGE with Coomassie Blue staining.

Adenylyltransferase (AT) Assays. The assay included 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 25 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin, 0.5 mM [α-^{32}P]ATP, and the indicated concentrations of glutamine, α-ketoglutarate, PII (expressed as the trimer concentration), glutamine synthetase (GS, expressed as the dodecamer concentration), and ATase or a mutant form of ATase. Incubation was carried out at 30 °C for the indicated times, and samples were analyzed by either the nitrocellulose filter assay as described previously (*12*) or SDS−polyacrylamide gel electrophoresis followed by autoradiography.

Adenylyl Removing (AR) Assays. GS was adenylylated as described above, with GS at 2 μM, enzyme (ATase or ATC1 at 2 μM or ATC3 at 1 μM), and glutamine (10 mM when ATase was used, 1 mM when ATC1 was used, and no glutamine when ATC3 was used). No PII was present during the adenylylation reactions used to prepare GS∼AMP for use as a substrate in the AR assays. After a 20 min incubation at 30 °C, the reaction mixture was incubated at 60 °C for 10 min. This treatment irreversibly inactivates the ATase without affecting the ability of GS∼AMP to serve as a substrate in the AR reaction. The reaction mixtures were then passed over Sephadex G-25 minicolumns to separate GS∼AMP (and the inactivated ATase) from the small molecules of the adenylylation reaction mixture. The extent of GS modification was typically greater than 95%, as estimated by comparing the amount of ^{32}P incorporated into GS∼AMP with the quantity of GS used in the adenylylation reaction. GS∼AMP concentrations are stated as the monomer concentration, reflecting the concentration of the incorporated α-phosphoryl group from ATP.

Reaction conditions for the AR assay were like those for the ATase reaction, except that GS∼AMP was used in place of GS, PII and glutamine were absent except where their inhibition was studied, ATP was unlabeled, the α-ketoglutarate concentration was 1 mM, the KP_i concentration was 5 mM, and PII-UMP was present as indicated. Reactions were analyzed by the nitrocellulose filter method (*12*).

Glutamine Synthase Assay. Conditions were as described previously (*15*), using GS∼AMP at 0.152 μM (dodecamer), PII∼UMP at 2 μM, and either ATase at 0.001 μM or AT N-terminal fragments at 5 μM.

RESULTS

Binding of PII to ATase Was Stimulated by Glutamine. Prior results indicated that the two activators of the AT activity, PII and glutamine, acted synergistically; the apparent K_{act} for either activator was reduced in the presence of the other (*12*). However, neither this observation nor the simple fact that glutamine activates the AT activity and inhibits the AR activity of ATase indicates that ATase itself contains a regulatory site for glutamine. This is because the substrates for adenylylation and deadenylylation (GS and GS∼AMP) contain a binding site for glutamine (the GS product site) such that all glutamine effects on ATase activities could be due to binding of glutamine to GS and GS∼AMP. The K_{act} for glutamine for the AT activity is sufficiently high (∼7 mM for the wild-type enzyme) that it was not practical to directly assess the binding of glutamine to either GS or ATase. Kinetic methods can be used to distinguish whether an activator binds to the enzyme or to the substrate (*25*), but in the case of the ATase reaction, these kinetic methods are impractical. The substrate for adenylylation, GS, is a dodecamer of ∼600 kDa and difficult to deliver in huge excess. More importantly, at very low substrate (GS) levels, the measurement of the GS∼AMP product becomes unreliable. We therefore examined whether ATase contained a glutamine regulatory site indirectly, by examining whether glutamine affected the binding of PII to ATase in the absence of GS.

Three different binding assays were used to study the interaction of PII and ATase, based upon gel filtration chromatography, nondenaturing gel electrophoresis, and protein cross-linking. Since these assays have a different physical basis, it is expected that different results should be obtained, depending on the properties of the protein−protein interaction. In the gel filtration assay, as soon as a complex becomes dissociated, the components begin to separate from one another such that rebinding is limited. Also, in the gel filtration assay, the concentration of complexes and the dissociated proteins is slowly reduced by diffusion. In the nondenaturing gel electrophoretic mobility shift assay, the complex moves through the gel, which consists of small "chambers" where rebinding of dissociated components may occur. Such rebinding should be favored when the two individual components move through the gel at a similar rate and its level reduced when the mobility of the separate components is vastly different. Finally, the cross-linking assay, which has been described previously for the interaction of PII and NRII (*22*), results in a covalent linkage between ATase and PII or PII-UMP. There is no dissociation of complexes in this assay, and cross-linked complexes accumulate over time. We found that glutamine was required to observe the binding of PII and ATase in a gel filtration assay (Figure 3) and that glutamine was not required but greatly enhanced the binding of PII to ATase in a nondenaturing gel electrophoresis assay (Figure 4). Glutamine was not required for the cross-linking of PII to ATase (see below), and thus, the cross-linking assay appears to be the most sensitive of the three assays. Nevertheless, prior control experiments for the cross-linking procedure (*22*) and experiments to be described later suggest that even this assay depends on the presence of a bona fide PII/PII-UMP binding site.

In the gel filtration assay (Figure 3), mixtures of PII and ATase were fractionated on Sephadex G-100, which completely separated the two proteins when glutamine was not present in the incubation mixture and throughout the column. Binding of PII to ATase was only observed when glutamine was in the incubation mixture as well as equilibrated in the column. This binding can be deduced in two ways; the ATase protein eluted in an earlier fraction, and some PII was clearly observable coeluting with ATase and shortly behind the ATase peak.

The nondenaturing gel electrophoresis assay (protein electrophoretic mobility shift assay) readily detected the complex of PII and ATase (Figure 4). Under the conditions that were used, the trimeric PII migrated as a well-defined band, while the monomeric ATase migrated as a closely spaced doublet band. The complex of the two proteins

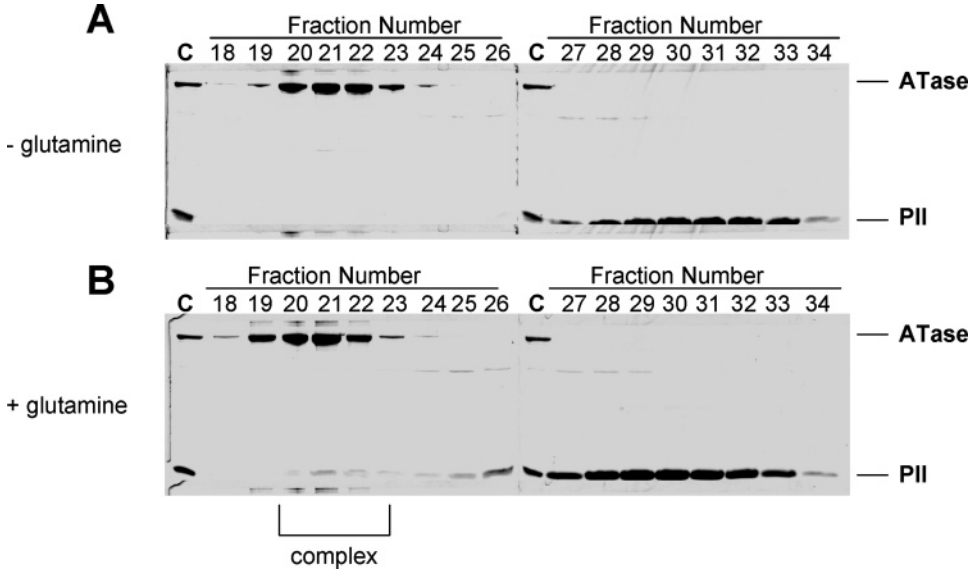

FIGURE 3: Gel filtration chromatography assay for the interaction of PII and ATase. PII (42 μM) and ATase (6 μM) were incubated in the presence or absence of glutamine and subjected to fractionation on a Sephadex G-100 column, as described in Experimental Procedures. (A) Elution profile in the absence of glutamine. (B) Elution profile in the presence of glutamine. The gel lanes marked C are control lanes containing PII and ATase.

migrated with significantly reduced mobility. This complex formed a very sharp and darkly stained band when the incubation mixture and the gel and electrophoresis buffer contained glutamine (10 mM, Figure 4A). In the absence of glutamine, binding of PII to ATase could be detected, but the complex did not form a sharp band (Figure 4B). Instead, a smeared and somewhat diffuse band with a ragged top edge was reproducibly obtained. Thus, glutamine seemed to be required for formation of a stable complex that could migrate as a discrete band.

To demonstrate that the complex observed in the electrophoretic mobility shift assay indeed consisted of PII and ATase, we excised this complex from the nondenaturing gel, extracted the proteins from the gel slice, and subjected the mixture to separation by SDS−PAGE. As expected, the complex consisted of PII and ATase (Figure 4C,D). To estimate the stoichiometry of the complex, we examined the distribution of PII and ATase in complexed and uncomplexed form when present at various concentrations (Figure 4C and data not shown). When present at a 1:1 ratio, most of the ATase was found in the complex, and the mobility of the complex was not altered when PII was in significant excess (Figure 4C). This suggests that the complex resulted from a 1:1 interaction. Furthermore, the amount of PII that was present in the complex could be estimated from the amount of PII extracted from the gel slice containing the complex (Figure 4D), which also suggested that the stoichiometry was 1:1 under the conditions that were used.

Since glutamine was required for detection of the interaction of PII and ATase by the gel filtration chromatography assay and clearly influenced the binding of PII to ATase in the electrophoretic mobility shift assay, both of which were conducted in the absence of GS, we conclude that ATase does indeed contain a glutamine site. In additional experiments to be published elsewhere, we observed that the nondenaturing gel electrophoresis assay for protein interactions described here was also useful for detecting other protein interactions. For example, we have used the assay to study the interaction of *E. coli* PII and GlnK with NRII (NtrB) and the interaction of *Arabidopsis thaliana* PII with NAGK (manuscripts in preparation). Later in this report, we will show that the assay can be used to detect interactions between different truncated versions of ATase. For each interaction, optimization of conditions is required, yet neither the gel filtration method nor the gel electrophoresis method captures all PII interactions; we were not able to detect the interaction of PII-UMP with ATase by either method.

Cross-Linking of PII and PII-UMP to ATase. For these studies, a mutant form of PII with a unique cysteine at position 44 of the T-loop (C73S/E44C) was used (*22*). A heterobifunctional cross-linker with a UV-activatable phenyl azide at one end and a maleamide at the other was attached to this cysteine, as described previously (*22*), and the ability of PII and PII-UMP so labeled to become cross-linked to ATase upon UV exposure was investigated. UV-dependent cross-linking of PII and PII-UMP to ATase was clearly observed, even in the presence of excess BSA (Figure 5). The cross-linking of PII to ATase did not require glutamine, α-ketoglutarate, or ATP, was stimulated by ATP and α-ketoglutarate at 20 μM, and was not strongly inhibited by α-ketoglutarate at 10 mM (Figure 5A). Similarly, cross-linking of PII-UMP to ATase required UV exposure and could be observed in the presence of excess BSA but was only modestly stimulated by ATP and α-ketoglutarate and was unaffected by glutamine (Figure 5B). Thus, in both cases, the cross-linking seemed to be specific but only weakly regulated by the small molecule effectors. To be certain that our PII-UMP preparation was not contaminated with a significant fraction of unmodified PII, we both examined the preparation by nondenaturing gel electrophoresis (where it appeared to be completely modified; not shown) and tested the ability of the material to become cross-linked to NRII, which is readily cross-linked to unmodified PII (*22*). Via this functional assay, the PII-UMP preparation seemed to be fully modified, as no cross-linking to NRII was detected (Figure 5B).

Construction and Purification of Altered Forms of ATase. The ATase consists of two conserved NT domains separated

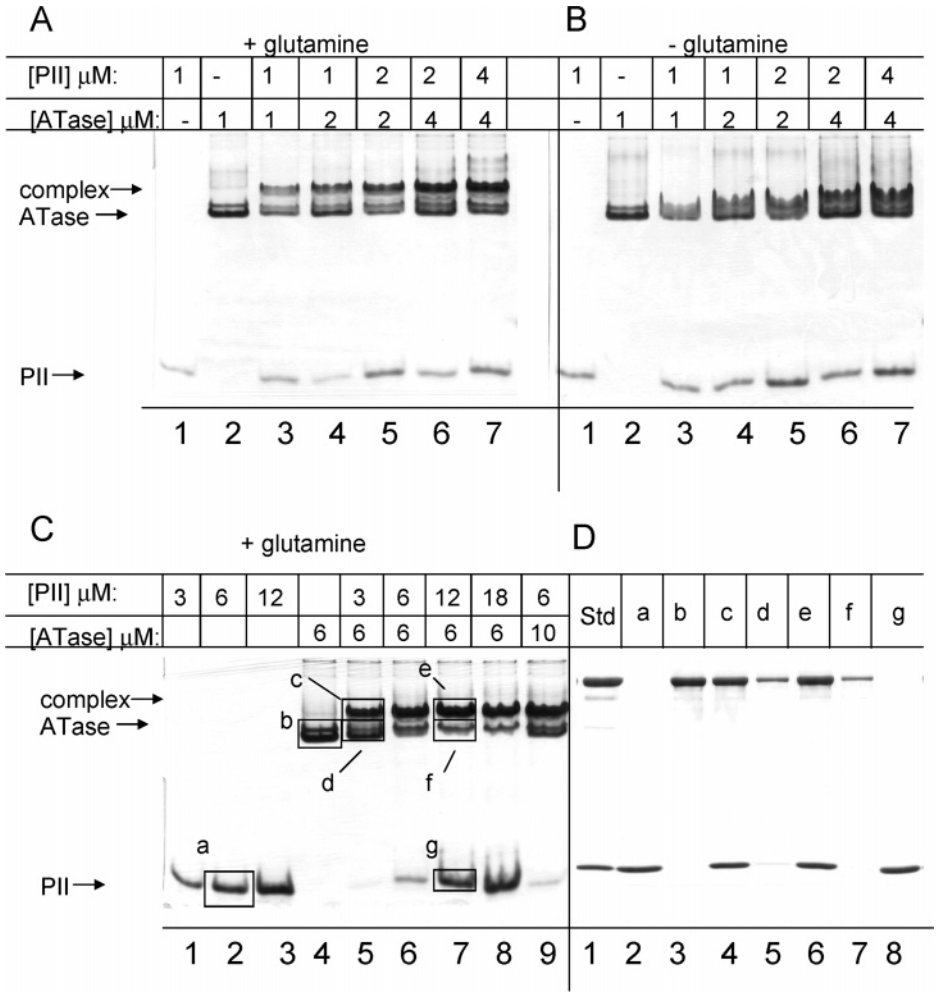

FIGURE 4: Nondenaturing gel electrophoresis assay for the interaction of PII and ATase. Gels were prepared and protein mixtures fractionated as described in Experimental Procedures; the gels used here contained 30 μM α-ketoglutarate. (A) Complexes were formed and fractionated in the presence of 10 mM glutamine. (B) Complexes were formed and fractionated in the absence of glutamine. The samples for panels A and B were as follows: (1) PII (1 μM), (2) ATase (2 μM), (3) ATase and PII (both at 1 μM), (4) ATase and PII (2 and 1 μM, respectively), (5) ATase and PII (both at 2 μM), (6) ATase and PII (4 and 2 μM, respectively), and (7) ATase and PII (both at 4 μM). (C) Purification of the ATase−PII complex from a nondenaturing gel. The gel was prepared, and samples were prepared and fractionated as described in Experimental Procedures; the gel contained 10 mM glutamine and 50 μM α-ketoglutarate: (1) PII (3 μM), (2) PII (6 μM), (3) PII (12 μM), (4) ATase (6 μM), (5) ATase and PII (6 and 3 μM, respectively), (6) ATase and PII (both at 6 μM), (7) ATase and PII (6 and 12 μM, respectively), (8) ATase and PII (6 and 18 μM, respectively), and (9) ATase and PII (10 and 6 μM, respectively). Bands cooresponding to PII, ATase, or the complex were excised from the gel as indicated by small boxes and extracted from the gel slices as described in Experimental Procedures. (D) SDS−14% polyacrylamide gel analysis of extracted proteins: (1) control sample with ATase and PII and (a−g) extracts from the excised bands indicated in panel C.

by a central region of ~200 amino acids (Figure 2). We hyperexpressed and purified altered forms of ATase with either truncations, an internal deletion, or clusters of point mutations, as depicted in Figure 2. The three N-terminal and C-terminal truncations were designed to include various portions of the central region of ATase, and the four clusters of point mutations were focused on different portions of the central region. The internal deletion mutation removes 122 amino acids from the middle of the ~200-amino acid central region. Finally, each of the putative NT active sites was separately inactivated by mutation of both of the highly conserved aspartates involved in the two-metal mechanism to asparagine. The purified proteins that were obtained are shown in Figure S1 of the Supporting Information. The truncated forms of ATase ranged from ~80−90% pure (ATN1 and ATC1) to ~40% pure (ATC3). The mutations altering the active sites (CDN and NDN) did not dramatically affect purification, but the other mutations altering the central region of ATase affected the expression and/or purification of the these proteins; final preparations of these proteins were less pure than the wild-type protein (Figure S1).

Effect of Mutations at the Active Sites. The active sites for the highly conserved NT domains can be readily recognized from the primary sequence; we engineered the replacement of the two conserved aspartate resides with asparagine within each NT domain. For the N-terminal NT domain, aspartates 173 and 175 were converted, while for the C-terminal NT domain, aspartates 701 and 703 were converted, yielding the enzymes AT-NDN and AT-CDN, respectively (Figure 2). The purified AT-CDN enzyme lacked any discernible AT activity (data not shown) but had AR activity that was only slightly lower than that of the wild type in the presence of PII-UMP (Figure 6A). Like the wild-type enzyme, the AT-CDN AR activity was inhibited by PII and glutamine, and their concerted effect did not display obvious synergy (Figure 6A). In additional experiments, we

Table 1: Kinetic Parameters of Mutant Enzymes Defective in AT or AR Active Sites

AT Activity of ATNDN and the Wild Type

experiment	[α-KG] (mM)	PII	Gln	enzyme	parameter
101205	0.05	+		0.2 μM ATNDN	PII K_{act} = 1.2 μM
101205	0.05		+	0.12 μM ATNDN	Gln K_{act} = 7.5 mM
011405	0.05		+	0.06 μM wild type	Gln K_{act} = 7.0 mM[a]
010605	0.05	+		0.1 μM wild type	PII K_{act} = 1.0 μM[a]
010705	0.05	+		0.1 μM wild type	PII K_{act} = 1.9 μM[a]

AR Activity of ATCDN and the Wild Type

experiment	[α-KG] (mM)	PII-UMP	Gln	enzyme	parameter
101705	1	+		0.02 μM ATCDN	PII-UMP K_{act} = 0.1 μM
101705	1	+		0.02 μM wild type	PII-UMP K_{act} = 0.3 μM
111005	1	0.4 μM	+	0.04 μM ATCDN	Gln K_{inhib} = 52 mM
041905	1	0.4 μM	+	0.05 μM wild type	Gln K_{inhib} = 23 mM

[a] Data from ref *26*.

observed that the AR activity of AT-CDN required PII-UMP, as did the wild-type enzyme (not shown), and that the apparent K_{act} of PII-UMP for activation of the AR activity was reduced for the AT-CDN enzyme relative to the wild type (Table 1). Also, the apparent K_{inhib} for glutamine inhibition of the AR activity was modestly increased relative to that of the wild type (Table 1). Thus, alteration of the C-terminal domain active site seemed to make PII-UMP bind better and glutamine bind worse.

The AT-NDN enzyme had no discernible AR activity (not shown) but retained AT activity that was synergistically activated by PII and glutamine (Figure 6B) and weakly inhibited by PII-UMP (data not shown), like the wild-type enzyme. In additional experiments, we saw that the apparent K_{act} values for glutamine (7.5 mM) and PII (1.2 μM) were similar to that of the wild-type enzyme (Table 1). Thus, the AT activity of the AT-NDN enzyme appeared to be regulated normally.

Both AT-NDN and AT-CDN appeared to bind PII like wild-type ATase in the nondenaturing gel electrophoresis assay (Figure S2 of the Supporting Information).

Effect of a 122-Amino Acid Deletion in the Central Region of ATase. The ATMΔ enzyme is missing amino acids 456−577 from the central region of ATase (Figure 2). This enzyme completely lacked AR activity (not shown) but retained AT activity (Figure 7). The ATMΔ enzyme exhibited high basal AT activity in the absence of any activators, and this basal activity was activated ∼5-fold by glutamine at 10 mM (Figure 7). Remarkably, the AT activity of the ATMΔ enzyme was inhibited by PII as well as by PII-UMP, regardless of whether α-ketoglutarate was at a low or high concentration (Figure 7A). That is, the mutation altering ATase converted PII from an activator to an inhibitor. Inhibition of the AT activity of the ATMΔ enzyme by PII was unaltered when glutamine was present at 10 mM, but inhibition by PII-UMP was greatly weakened in the presence of glutamine (Figure 7A). In another experiment, we observed that the inhibition by the combination of PII and PII-UMP was less than additive with reference to the inhibition by either PII or PII-UMP alone (Figure 7B). These observations suggested that PII and PII-UMP might exert their inhibitory effects from independent sites. In ref *26*, kinetic analysis of the inhibition of the ATMΔ enzyme by PII and PII-UMP is presented, which is consistent with this conclusion (*26*). The ATMΔ enzyme appeared to bind PII normally in the gel electrophoresis assay (Figure S2) and was cross-linked to PII and to PII-UMP like the wild-type enzyme in the cross-linking assay (Figure S3 of the Supporting Information). Thus, the central region of ATase was not essential for PII/PII-UMP binding, but its deletion appeared to disprupt the communication between the PII/PII-UMP site(s) and the active sites, as well as the communication between the glutamine and PII sites. In another experiment, we observed that the apparent glutamine K_{act} for activation of the AT activity of ATMΔ was ∼0.3 mM, a remarkable 23-fold below that of the wild-type enzyme (Table 2). Thus, the mutation also appeared to have a significant effect on binding of glutamine to the enzyme.

Clustered Point Mutations Affecting the Central Region of the ATase. Four different mutated enzymes containing mutations in the central region were examined; each of these contains several point mutants clustered into a small portion of the central region (Figure 2). Our original purpose in forming these mutations was to test portions of the central region of ATase that bear some resemblance to the β-hairpin element of the C-terminal domain of NRII, implicated as possibly forming part of the PII-binding site of NRII (*19*). The mutant ATM1 contains the D463N, P467A, and L469G mutations. This enzyme had approximately one-half to one-third of the AR activity observed with the wild-type enzyme, and this AR activity was inhibited by PII and by glutamine like the wild-type enzyme (Figure 8 and Table 2). The ATM1 enzyme also had approximately one-half to one-third of the AT activity when compared to the wild-type enzyme, and this activity was regulated normally by glutamine, PII, and PII-UMP (Figure 9 and Table 2). The enzyme appeared to bind PII normally in the gel electrophoresis assay (Figure S2) and was cross-linked to PII and PII-UMP in the cross-linking assay (Figure S3). Thus, alteration of residues 463, 467, and 469 did not result in dramatic alterations of the ATase activities or their regulation.

The ATM2 enzyme contains the alterations R499A, R501A, D505N, P509A, and L511G. This enzyme completely lacked AR activity (not shown) but retained AT activity (Figure 9). Like the ATMΔ enzyme, ATM2 exhibited significant basal AT activity in the absence of any activators, approximately equal to the activity obtained with the wild-type enzyme in the presence of 1 mM glutamine (not shown). This AT activity was strongly activated by glutamine, and reminiscent of the situation with the ATMΔ

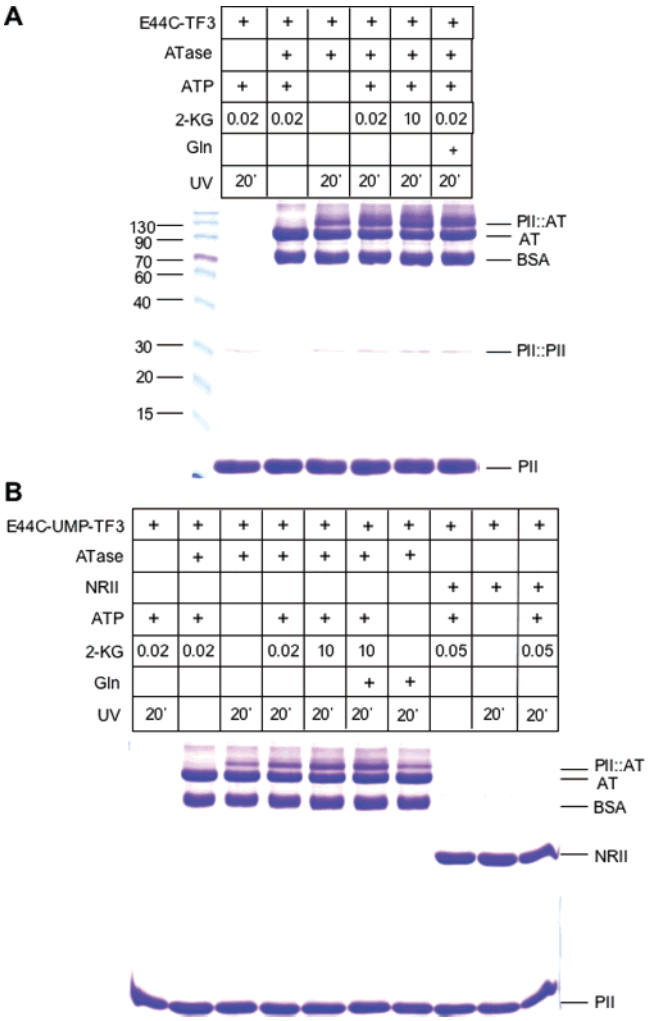

FIGURE 5: Cross-linking of ATase and PII or PII-UMP, using PII and PII-UMP containing a heterobifunctional cross-linker uniquely positioned at residue 44. Experiments were conducted as described in Experimental Procedures. The 2-KG (α-ketoglutarate) concentration is expressed in millimolar, and Gln (glutamine), where present, was at a concentration of 10 mM. PII:AT designates the complex formed between PII (A) or PII-UMP (B) and ATase. AT designates un-cross-linked ATase. BSA designates bovine serium albumin. PII designates PII (A) or PII-UMP (B). PII:PII designates PII cross-linked to PII. (A) Cross-linking of PII (E44C-TF3) to ATase. (B) Cross-linking of PII-UMP (E44C-TF3) to ATase. NRII was used as a control since it cross-links readily to PII and does not bind to PII-UMP; the results with NRII thus show that the PII-UMP preparation did not contain a significant concentration of uridylylated PII.

protein, either PII or PII-UMP inhibited the enzyme (Figure 9). The ATM2 enzyme retained some ability to bind PII as indicated by the gel electrophoresis assay (Figure S2) and the cross-linking assay (Figure S3). However, it should be noted that in the gel electrophoresis assay, the appearance of the ATase−PII complex band resembled that typically seen in the absence of glutamine (Figure S2), yet the glutamine apparent K_{act} for the AT activity of ATM2 was significantly lower than that observed with the wild-type enzyme (Table 2). The mutations in ATM2 apparently block communication between the glutamine and PII sites. The ATM2 protein was also slightly defective in cross-linking to PII-UMP (Figure S3).

The ATM3 enzyme contains the L525G, R527A, and I528G mutations. This enzyme proved quite difficult to purify due to a low expression level, and the preparation we used was only ~40% pure (Figure S1). The enzyme seemed to completely lack AR activity but retained AT activity (Figure 9). Basal AT activity in the absence of any activators was discernible; this AT activity was strongly activated by glutamine and weakly activated by PII, and the degree of synergy between the two activators was lower than that observed for the wild-type protein (Figure 9). The AT activity of the ATM3 enzyme was poorly inhibited by PII-UMP (Figure 9). The enzyme seemed to bind PII normally in the gel electrophoresis assay, but because of the poor purity of the enzyme, this conclusion is tentative (Figure S2). Additional studies indicated that the apparent K_{act} for glutamine was significantly lower than that observed with the wild-type enzyme, and furthermore, the glutamine K_{act} was not decreased in the presence of PII (Table 2), unlike the situation obtained with the wild-type enzyme (*12*). Thus, the mutations in ATM3 appear to strengthen the binding of glutamine, weaken the binding of PII-UMP, and alter the communication between the PII site and the AT active site, as well as eliminating the AR activity.

The ATM4 enzyme contains the alterations R571A, P573A, and L575G. This enzyme exhibited approximately one-eighth of the AR activity seen with the wild type; its AR activity was inhibited by glutamine and PII (Figure 9). Inhibition of the AR activity by glutamine seemed to be more effective than that observed with the wild-type enzyme, and the apparent K_{inhib} for glutamine was a remarkable 0.4 mM, or ~50-fold below that observed with the wild-type enzyme (Table 2). The apparent PII-UMP K_{act} for activation of the AR activity was increased relative to that of the wild-type enzyme (Table 2). The enzyme displayed discernible basal AT activity in the absence of any activators, and the AT activity was strongly activated by glutamine (Figure 9). The activation of the enzyme by glutamine seemed to saturate at 1 mM glutamine (not shown), and the apparent K_{act} for glutamine was a remarkable 0.4 mM, or ~20-fold lower than that obtained with the wild-type enzyme (Table 2). The enzyme bound normally to PII in the gel electrophoresis assay (Figure S2) and was cross-linked to PII normally in the cross-linking assay (Figure S3), but cross-linking to PII-UMP may be modestly defective (Figure S3). Thus, the main effect of the mutations in ATM4 was to dramatically strengthen the binding of glutamine to the enzyme, as well as increasing the basal and glutamine-activated AT activity.

Activities of Truncated Forms of ATase. The three C-terminal fragments of ATase that we purified, ATC1, ATC2, and ATC3, contained various portions of the central region of ATase and the C-terminal NT domain (Figure 2 and Figure S1). All three of these polypeptides lacked AR activity (not shown) and had AT activity that was activated by glutamine (shown for ATC1 in Figure 10 and Table 2). For polypeptide ATC1, consisting of residues 425−946, these results were expected, as it should be similar to a polypeptide produced previously (*15*). The ATC1 and ATC3 polypeptides had discernible basal AT activity in the absence of any activators (not shown), and these enzymes were strongly activated by glutamine. ATC2 was activated by glutamine in a manner similar to that of the wild-type enzyme (not shown).

Interestingly, the glutamine-stimulated AT activity of ATC1 was weakly inhibited by PII and by PII-UMP, and concerted inhibition by PII and PII-UMP seemed to be less

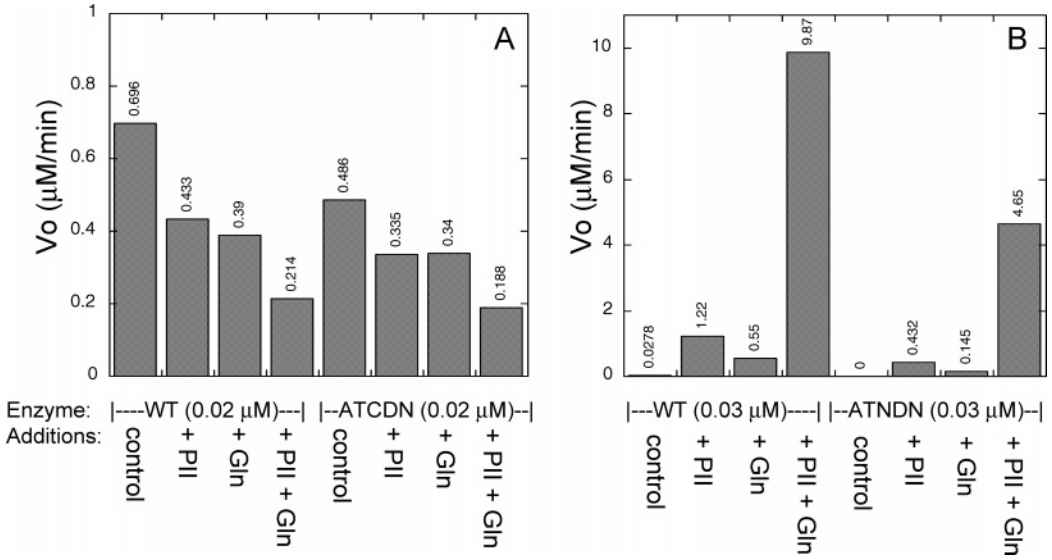

FIGURE 6: Comparison of wild-type and mutant enzymes altered in one of the putative active sites. (A) Comparison of the AR activity of ATase and ATCDN. Reactions were conducted as described in Experimental Procedures with the following concentrations: 1.36 μM GS∼AMP, 0.5 mM ATP, 5 mM KP_i, 1 mM α-ketoglutarate, and 0.5 μM PII-UMP. PII, where indicated, was at a concentration of 2 μM, and glutamine, where indicated, was at a concentration of 10 mM. (B) Comparison of the AT activity of ATase and ATNDN. Reactions were conducted as described in Experimental Procedures with the following concentrations: 3 μM GS, 0.5 mM ATP, and 0.05 mM α-ketoglutarate. PII, where indicated, was at a concentration of 1 μM, and glutamine, where indicated, was at a concentration of 1 mM.

Table 2: Kinetic Parameters of Mutant and Truncated Enzymes

	AT activity		AR activity	
enzyme	Gln K_{act} (mM)	PII K_{act} (μM)	Gln K_{inhib} (mM)	PII-UMP K_{act} (μM)
wild type	7.0[a]	1.0	23[a]	0.3
ATC1	5.5	—[b]	—[c]	—[c]
ATC2	1.4	—[b]	—[c]	—[c]
ATC3	1.5	—[b]	—[c]	—[c]
ATM1	9.0		∼23	0.27
ATM2	2.7	—[b]	—[c]	—[c]
ATM3	1.5	1.3	—[c]	—[c]
ATM3 with PII	1.4		—[c]	—[c]
ATM4	0.4		0.4	1.0
ATMΔ	0.3	—[b]	—[c]	—[c]

[a] Data from ref *26*. [b] The enzyme is not activated by PII. [c] The enzyme lacks AR activity.

than additive (Figure 10). In additional experiments, the apparent K_{inhib} values for PII-UMP and PII were 8.5 and 13 μM, respectively, indicating poor binding of the enzyme by PII and PII-UMP. We observed that inhibition by PII did not require α-ketoglutarate and was relatively insensitive to the α-ketoglutarate concentration, being slightly increased at 1 mM α-ketoglutarate (not shown). At a high glutamine concentration (50 mM), inhibition by PII and by PII-UMP was significantly weakened, again, regardless of the α-ketoglutarate concentration (Figure 10). These observations are explored further in ref *26*, where kinetic analysis of the ATC1 AT activity suggested that PII and PII-UMP inhibit competitively with glutamine and that both PII and PII-UMP act from a common site (*26*). Thus, ATC1 seemed to interact with PII and PII-UMP, indicating the presence of a PII/PII-UMP site that is between the C-terminus and position 425. However, we could not detect the interaction using the gel electrophoresis assay (not shown). Binding of ATC1 to PII and PII-UMP was readily observed in the cross-linking assay (Figures S4 and S5 of the Supporting Information).

The ATC2 polypeptide contains a portion of the central region of ATase and the C-terminal AT domain. This polypeptide had low basal AT activity, which was activated by glutamine (not shown). The enzyme was completely unaffected by PII and PII-UMP (not shown) and displayed a glutamine apparent K_{act} that was significantly lower than that displayed by wild-type ATase (Table 2). We could not detect binding to PII or PII-UMP by a gel electrophoresis assay (not shown), or by the cross-linking assay when Coomassie Blue-stained gels were used to monitor cross-linking (Figures S4 and S5).

The ATC3 polypeptide consists of essentially just the AT domain. The enzyme lacked AR activity and displayed a discernible basal AT activity, which was further activated by glutamine (not shown). Again, the apparent K_{act} for glutamine was lower than the expected value (Table 2). The enzyme seemed to be completely indifferent to the presence of PII and PII-UMP (not shown). Binding of PII or PII-UMP was not detected by the gel electrophoresis assay or by the cross-linking asssay when Coomasie Blue-stained gels were used to monitor cross-linking (not shown).

The N-terminal polypeptides, ATN1, ATN2, and ATN6, were also purified. Contrary to the expectations based on a previous report (*15*), we could not measure AR activity with any of our truncated versions, nor could we detect AR activity with another truncated version (1−423) that was identical to that reported previously (not shown). Because of this, we attempted to measure AR activity in crude extracts, both by examining the regulation of GS activity, as reported previously (*15*), and by directly examining the deadenylylation of labeled GS∼AMP. We were unable to detect AR activity by either assay for any of the ATN constructs, including the construct identical to that reported previously (*15*). As expected, none of the N-terminal polypeptides displayed AT activity (not shown). The largest of the N-terminal polypeptides, ATN6 (residues 1−608),

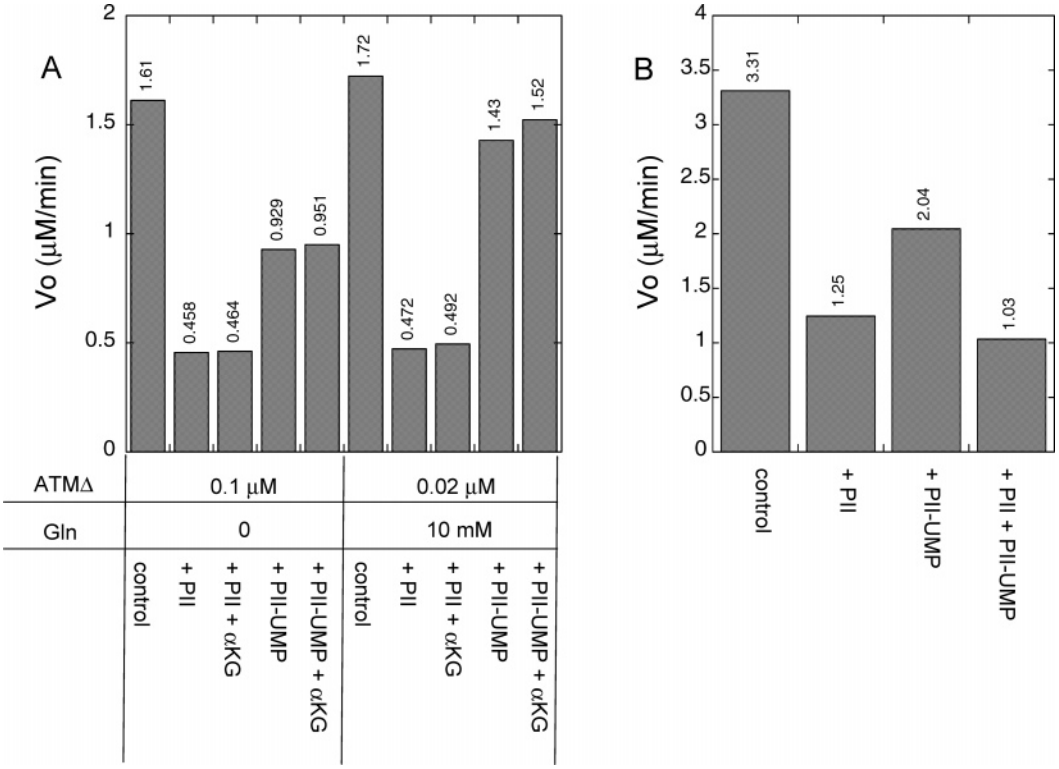

FIGURE 7: AT activity of ATMΔ. (A) Basal and glutamine-activated AT activity of ATMΔ, and inhibition by PII and PII-UMP. Reactions were conducted as described in Experimental Procedures with the following concentrations: 0.1 or 0.02 μM ATMΔ, as indicated, 2.5 μM GS , 0.5 mM ATP, and 0.05 mM α-ketoglutarate, except where indicated, in which case it was 1 mM. PII, when present, was at a concentration of 10 μM, and PII-UMP, when present, was at a concentration of 10 μM. (B) Inhibition by PII and PII-UMP is less than additive. Reactions were conducted as described in Experimental Procedures with the following concentrations: 2.5 μM GS, 0.5 mM ATP, 0.2 μM ATMΔ, and 0.05 mM α-ketoglutarate. PII, when present, was at a concentration of 5 μM, and PII-UMP, when present, was at a concentration of 10 μM. The fraction of activity remaining was as follows: control (100%), +PII (37.6%), +PII-UMP (61.5%), and +PII and PII-UMP (31.0%).

clearly bound PII in the gel electrophoresis assay, while ATN1 and ATN2 failed to bind PII in this assay (not shown). The ATN6 and ATN2 polypeptides were readily cross-linked to PII in the cross-linking assay; ATN6 was also cross-linked to PII-UMP, while ATN2 was defective in cross-linking to PII-UMP (Figures S4 and S5).

Cross-Linking of PII and PII-UMP to Truncated Forms of ATase. The cross-linking experiments discussed so far employed Coomassie Blue-stained gels for monitoring cross-linking. To have a more sensitive assessment of cross-linking of PII and PII-UMP to ATase and the various ATase truncations, and to confirm the identity of the bands seen on Coomassie-stained gels, we used an immunoblotting method to detect the formation of the cross-linked complexes (Figure 11). Using this method, a very dense band corresponding to the cross-linked complex was obtained when wild-type ATase was used (Figure 11), and as expected on the basis of the results with Coomassie-stained gels (Figures S4 and S5), the ATN6 polypeptide was well cross-linked to both PII and PII-UMP. By comparison, all of the truncation polypeptides with the exception of ATN6 exhibited weak but clearly discernible cross-linking to PII (Figure 11). The ATC1, ATN1, and ATN2 polypeptides also exhibited clear cross-linking to PII-UMP, while the ATC2 and ATC3 polypeptides exhibited very weak cross-linking to PII-UMP (Figure 11). Since the ATN1 and ATC3 polypeptides consist mainly of the two conserved NT domains, these results suggest that PII and PII-UMP weakly interacted directly with the NT domains.

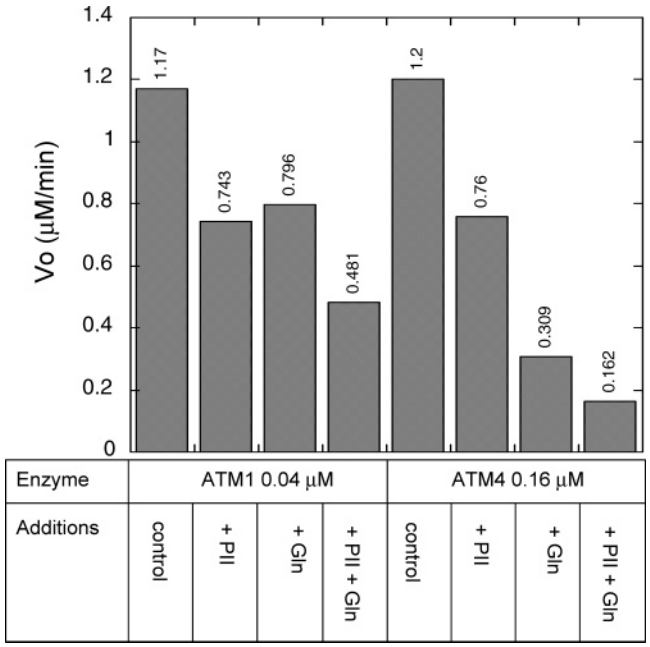

FIGURE 8: AR activity of the ATM1 and ATM4 enzymes. Reactions were conducted as described in Experimental Procedures with the following concentrations: 1.36 μM GS∼AMP, 0.5 mM ATP, 5 mM KP_i, 1 mM α-ketoglutarate, and 0.5 μM PII-UMP. For the ATM1 experiment, PII, when present, was at a concentration of 2 μM, and glutamine, when present, was at a concentration of 10 mM. For the ATM4 experiment, PII, when present, was at a concentration of 0.5 μM and glutamine, when present, was at a concentration of 1 mM.

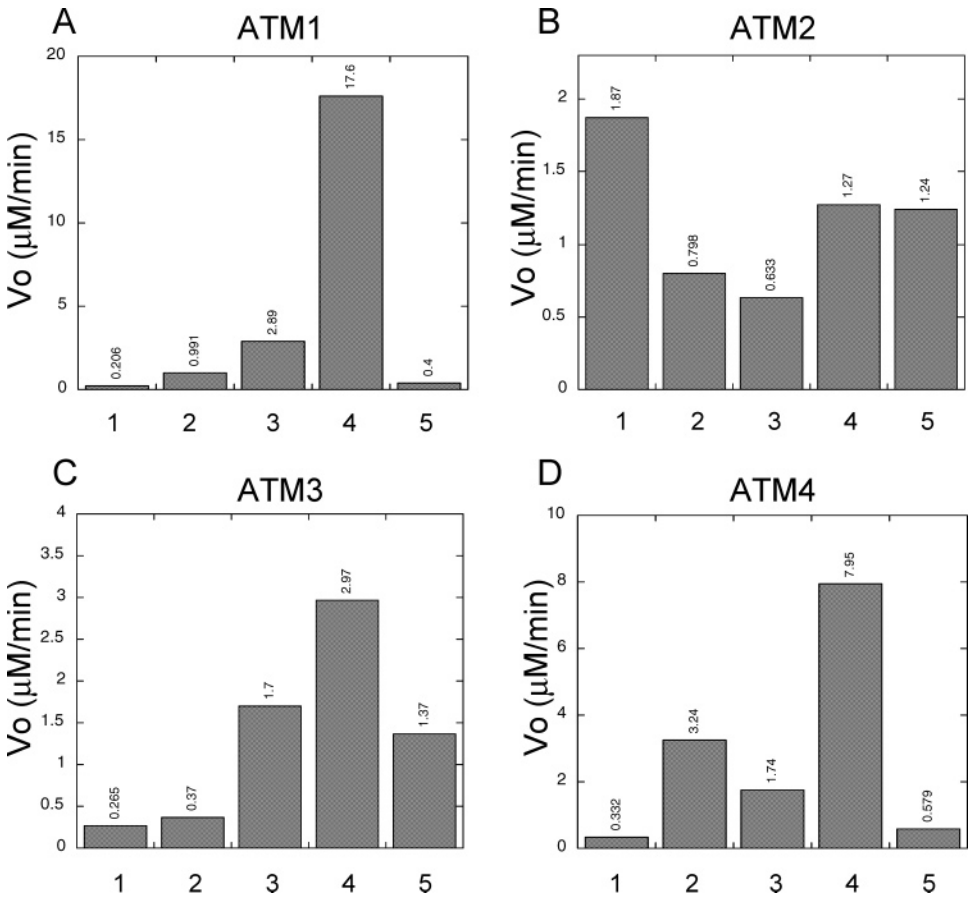

FIGURE 9: AT activity of the ATM1, ATM2, ATM3, and ATM4 enzymes. Reactions were conducted as described in Experimental Procedures with the following concentrations: 2.5 μM GS, 0.5 mM ATP, and 0.05 mM α-ketoglutarate or as indicated. (A) AT activity of ATM1. The enzyme was at a concentration of 0.06 μM. The samples were as follows: (1) control, (2) with PII (5 μM), (3) with glutamine (9 mM), (4) with 5 μM PII and 9 mM glutamine, (5) with 9 mM glutamine, 10 μM PII-UMP, and the α-ketoglutarate concentration increased to 1 mM. (B) AT activity of ATM2. The enzyme was at a concentration of 0.02 μM, and all reaction mixtures contained 3 mM glutamine. The samples were as follows: (1) control, (2) with 10 μM PII, (3) with 10 μM PII and the α-ketoglutarate concentration increased to 1 mM, (4) with 10 μM PII-UMP, and (5) with 10 μM PII-UMP and the α-ketoglutarate concentration increased to 1 mM. (C) AT activity of ATM3. The enzyme was present at a concentration of 0.03 μM. The samples were as follows: (1) control, (2) with PII (5 μM), (3) with glutamine (1.5 mM), (4) with 5 μM PII and 1.5 mM glutamine, and (5) with 1.5 mM glutamine, 10 μM PII-UMP, and the α-ketoglutarate concentration increased to 1 mM. (D) AT activity of ATM4. The enzyme was present at a concentration of 0.04 μM. The samples were as follows: (1) control, (2) with PII (5 μM), (3) with glutamine (0.4 mM), (4) with 5 μM PII and 0.4 mM glutamine, and (5) with 0.4 mM glutamine, 10 μM PII-UMP, and the α-ketoglutarate concentration increased to 1 mM.

Interactions of Truncated Forms of ATase. Because all of the N-terminal truncations examined here lacked AR activity, the possibility that these polypeptides were denatured or improperly folded existed. To explore this possibility, we examined the interactions of the truncated polypeptides with each other, using the gel electrophoresis mobility shift assay. No combination of polypeptides that contained overlapping segments was found to interact, nor was an interaction discernible between ATN1 and ATC1 or between ATN2 and ATC2 (not shown). However, in the presence of glutamine, a strong interaction was detected between ATN6 (the longest N-terminal truncation) and ATC3 (the shortest C-terminal truncation), which together comprise the ATase (Figure S6 of the Supporting Information). This interaction did not appear to prevent interaction of ATN6 with PII, and indeed, a complex could be excised from the mobility shift gels that contained ATN6, ATC3, and PII (Figure S6). Thus, ATC3 and ATN6 display specific interaction with each other, implying that they are both at least partially native. However, as with ATN6, the complex of ATC3 and ATN6 did not display AR activity (not shown).

DISCUSSION

The ATase provides an example of a bifunctional enzyme with antagonistic activities, each of which is regulated by inhibitors and activators (*27*). Here, we focus on the regulation of the activities by PII, PII-UMP, and glutamine and on locating the sites from which these effectors work. Our results suggest that the enzyme is even more complex than previously envisioned (*15*, *28*). A hypothesis consistent with the available data is presented in Figure 12. We hypothesize that the enzyme contains two sites for the binding of PII and PII-UMP, one associated with each of the NT domains. The central region of ATase either forms a portion of these sites or regulates the binding of PII and PII-UMP to these sites. We hypothesize that the binding of PII is favored by the N-terminal PII/PII-UMP site and that the binding of PII-UMP is favored by the C-terminal PII-UMP/PII site. Glutamine binds to a site that is probably located on the C-terminal NT domain. All of the sites communicate with each other; in the case of the glutamine and PII sites, this communication requires proper interaction of the AT and AR domains of ATase. We hypothesize that

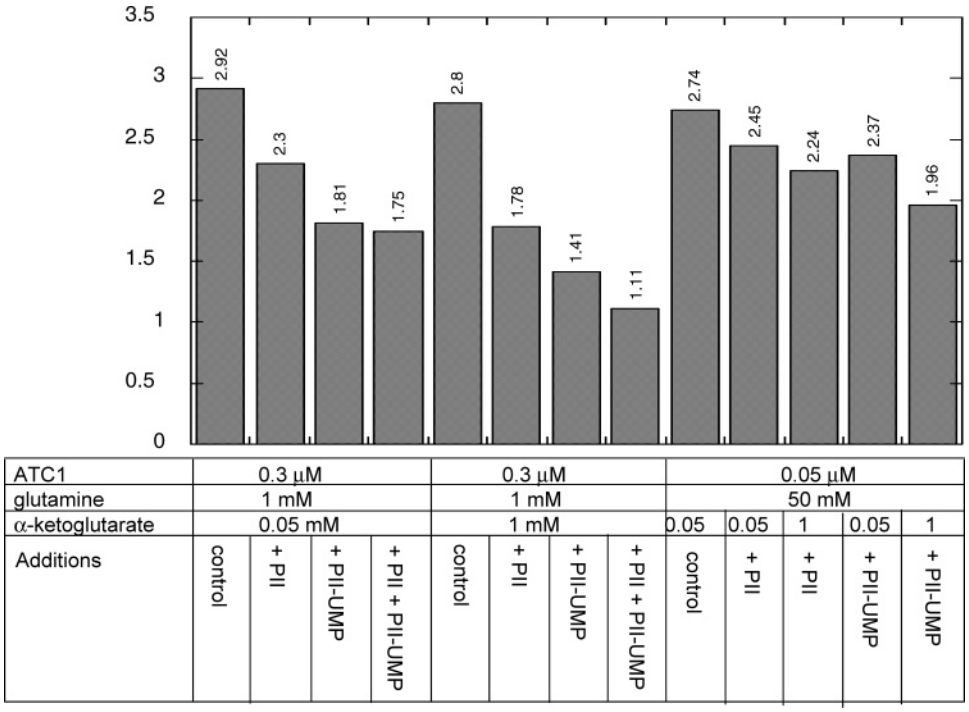

FIGURE 10: Inhibition of ATC1 AT activity by PII and PII-UMP. Reactions were conducted as described in Experimental Procedures with the following concentrations: 2.5 μM GS and 0.5 mM ATP. PII, when present, was at a concentration of 10 μM, and PII-UMP, when present, was at a concentration of 10 μM.

the AT and AR activities are mutually antagonistic, that the C-terminal AT domain must contribute to or activate the AR activity of the N-terminal NT domain, and that this interaction can be disrupted by mutations in the central region of ATase.

While none of the points discussed above have been proven, our data were consistent with this hypothesis and inconsistent with the previous hypothesis (*15*). A suggestion for multiple PII/PII-UMP sites comes from the ATMΔ enzyme, where both PII and PII-UMP inhibited the AT activity in a less than additive fashion, with only inhibition by PII-UMP eliminated by glutamine. Furthermore, the ATM2 and ATM3 mutant enzymes were clearly defective in communication between the PII site and the ATase active site (and glutamine site). Since these enzymes have mutations in the central regions of the ATase, this suggests that the PII site and AT active site may not reside on the same domain. In the cross-linking assay, binding of PII to polypeptides ATC1 and ATN6 could be clearly observed on Coomassie-stained gels. Since these two peptides contain the central region of ATase, it would seem from these data that PII and PII-UMP bind to the central region. However, deletion of most of the central region in the ATMΔ enzyme did not eliminate the binding of PII and PII-UMP. Furthermore, when immunoblotting was used to examine the products of cross-linking, we observed weak cross-linking of PII and PII-UMP to the isolated NT domains. Therefore, it seems likely that the binding sites are located on the NT domains themselves and that the central region either forms a part of the sites or regulates the binding of PII/PII-UMP to the sites. Since the ATMΔ protein seems to have two sites for PII/PII-UMP, the 122 deleted central amino acids of the central region of ATase probably do not contain either site. Thus, we hypothesize that the wild-type enzyme has two PII/PII-UMP sites, as depicted in Figure 12. In ref *26*, these observations were followed up with a kinetic analysis of the ATase that also suggested the presence of multiple PII/PII-UMP sites (*26*).

Since the ATase substrate, GS, has a binding site for glutamine, we studied the effect of glutamine on binding of PII to the ATase to examine whether ATase also contained a site for glutamine. Glutamine affected the binding of ATase to PII in the absence of GS, making it likely that the ATase does have a glutamine site. The synergistic activation of the AT activity by glutamine and PII (*12* and references cited therein) seems to be due, at least in part, to the effect of glutamine on PII binding. The glutamine site on ATase that facilitates PII binding may or may not be the same site from which glutamine activates the AT activity. If it is the same site, it is likely that this site is on the C-terminal NT domain, as polypeptide ATC3 exhibited AT activity that was strongly activated by glutamine. This conclusion is consistent with previous structure−function studies of the ATase (*15*).

Mutations in the central region could eliminate the AR activity and result in elevated basal AT activity in the absence of any activators or block the activation of the AT by PII. Thus, the central region played an important role in communication between the domains. Since the ATC series of truncated enzymes exhibited AT activity while none of the N-terminal polypeptides exhibited AR activity, it seems that the C-terminal AT domain is sufficient for the AT activity and also plays a role in the activation of the AR activity. PII-UMP is an essential activator of the AR activity. We hypothesize that PII-UMP activates the AR activity by binding to a PII-UMP/PII site on the C-terminal domain and that this domain must then interact with the N-terminal domain for the AR activity to occur. Since mutations in the central region can block communication between the PII and glutamine sites, we hypothesize that a PII/PII-UMP site is located on the N-terminal AR domain (Figure 12).

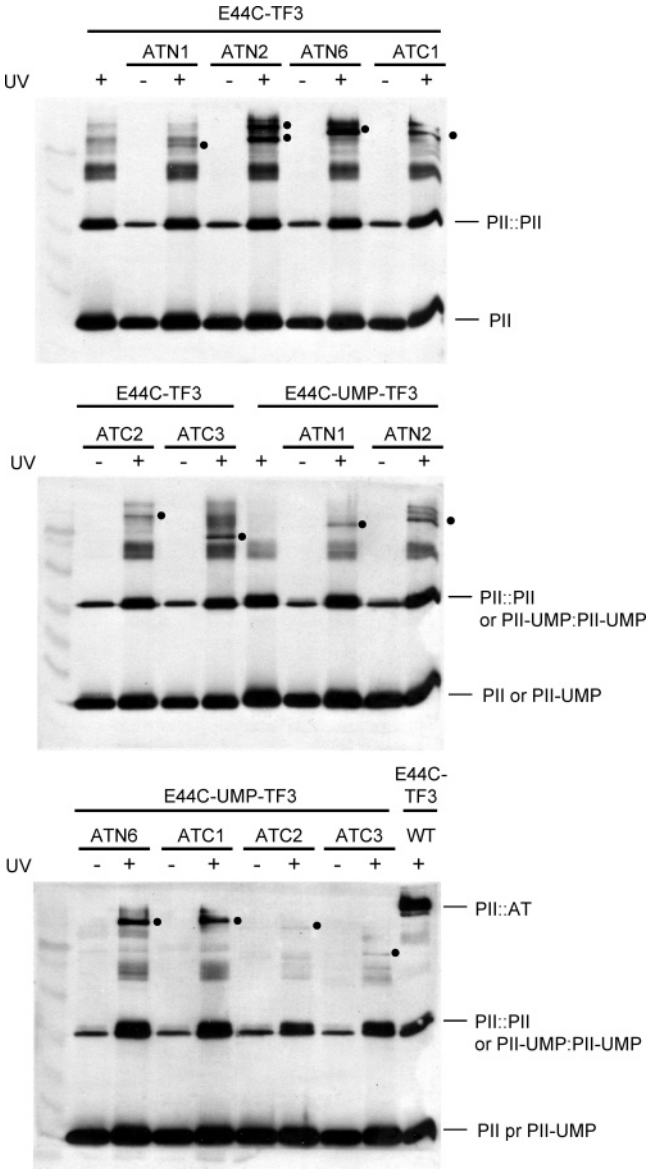

FIGURE 11: Cross-linking of PII and PII-UMP to truncated forms of ATase, as detected by immunoblotting with anti-PII. The reactions and immunoblotting were performed as described in Experimental Procedures. Cross-linked species are marked with black dots.

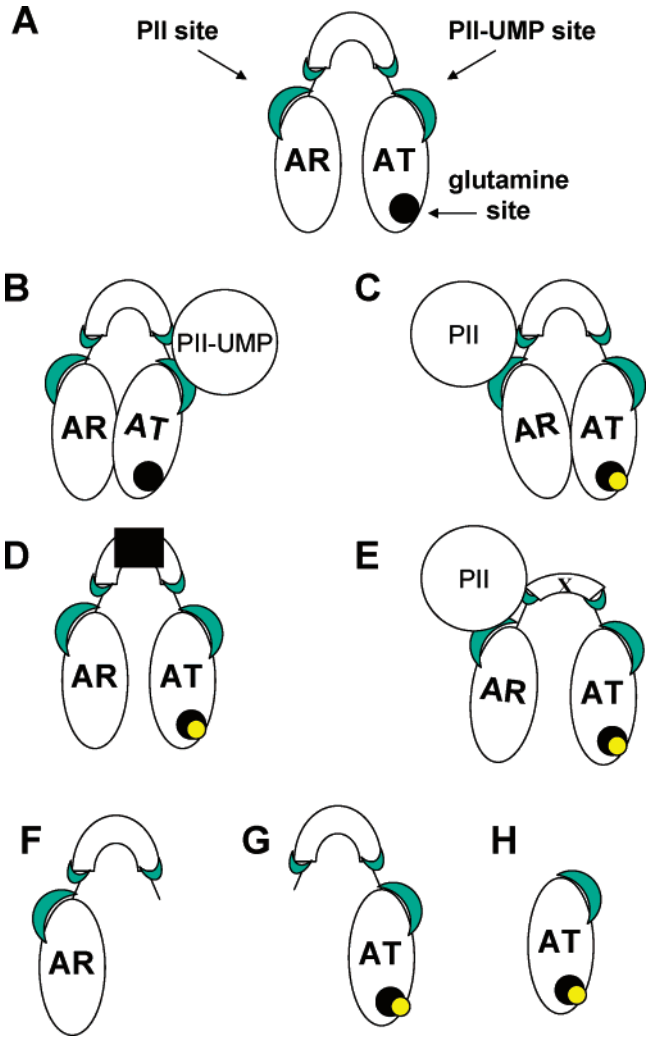

FIGURE 12: Hypothesis for the organization of the catalytic and regulatory sites in ATase. (A) Inactive form of the enzyme in the absence of regulators. The proposed PII and PII-UMP sites are shaded, and the proposed glutamine site is a black circle. (B) PII-UMP activation of the AR activity. (C) PII and glutamine activation of the AT activity. Glutamine is depicted as a lightly shaded circle within the glutamine site. (D) Deletion of 122 amino acids from the middle of the central region destroys the AR activity and alters the interaction of the AT and AR domains. (E) Mutations in the central region may destroy communication between the PII and glutamine sites. (F) Deletion of the C-terminal AT domain also eliminates AR activity. (G) Deletion of the N-terminal AR domain does not eliminate the AT activity, but it can no longer be activated by PII. (H) The isolated AT domain has AT activity that is regulated by only glutamine.

Interestingly, PII-UMP and glutamine appeared to be directly antagonistic, as if they competed for the enzyme. For example, a high concentration of glutamine blocked inhibition of the ATC1 AT activity by PII-UMP or PII, and a high concentration of glutamine blocked inhibition of the ATMΔ AT activity by PII-UMP (but not by PII). These observations are described further in the following paper, where kinetic analysis indicated that glutamine and PII-UMP compete for binding the enzyme (*26*). Furthermore, we show in that work that PII and PII-UMP are likely to act from a common site to inhibit the AT activity of ATC1 and that glutamine acts competitively with both PII and PII-UMP binding to this site (*26*). Thus, ATC1 seems to contain the PII-UMP site, whose specificity for PII-UMP was reduced by truncation of the rest of the protein. Interestingly, the mutations in the ATM4 enzyme both elevated the PII-UMP apparent K_{act} for the AR activity and reduced the glutamine apparent K_{act} for the AT activity. It seems that in the ATM4 enzyme, the C-terminal AT domain conformation is altered in a way that affects both PII-UMP and glutamine binding. Conversely, but less dramatically, alteration of the AT active site within the C-terminal AT domain decreased the PII-UMP K_{act} for the AR activity and increased the glutamine K_{inhib} for the AR activity. These observations suggested that both the PII-UMP site and glutamine site may map to the C-terminal AT domain.

As already noted, the glutamine activation of the AT activity of the ATC3 polypeptide suggests that the glutamine site maps to the C-terminal AT domain of ATase. It is remarkable then that all of the C-terminal truncations and three of the four enzymes altered in the central region of ATase exhibited reduced apparent K_{act} values for glutamine

activation of the AT activity; in some cases, these apparent K_{act} values were quite dramatically reduced. This observation suggests that the glutamine site in the native enzyme is in communication with the other domains of the protein; presumably, the mutations in the central part of the ATase alter the glutamine site indirectly by altering the interactions of the N-terminal and C-terminal domains.

According to our hypothesis, PII and PII-UMP act as activators by binding to the domain with the antagonistic activity; that is, PII activates the AT by binding to the AR domain, and PII-UMP activates the AR by binding to the AT domain (Figure 12). We imagine that this binding alters the conformation of the bound domain, and this is communicated to the other domain by direct interaction of the NT domains. Direct interaction between the ATase domains is consistent with our observation of a strong and specific interaction between the ATN6 and ATC3 polypeptides. Presumably, PII and PII-UMP binding also inhibits the activity of the domain to which it is bound. This is consistent with the observation that PII and PII-UMP inhibited the AT activity of the ATC1 polypeptide.

Prior studies have established that α-ketoglutarate binds to PII and to PII-UMP and regulates the ability of PII and PII-UMP to activate the AT and AR reactions (*12*, *13*). The simplest explanation for those results is that α-ketoglutarate regulates the ability of PII and PII-UMP to bind to ATase (*29*). Although we did not focus on the role of α-ketoglutarate in this work, we did observe that this effector had little effect on cross-linking of PII and PII-UMP to ATase. This observation is described further in the following paper, where kinetic analysis suggested that the binding of PII and PII-UMP to ATase was not altered by α-ketoglutarate. Rather, this effector seemed to act at a postbinding step to regulate the activity of PII and PII-UMP (*26*).

The ATase is a complex enzyme that has resisted definition for more than 30 years. Our hypothesis makes several predictions that may be directly tested in future experiments. For example, mapping of the contacts of PII and PII-UMP with the enzyme, for instance, by using "zero-spacer" cross-linking and classical peptide mapping, should reveal two different PII/PII-UMP binding sites, and furthermore, when the intact enzyme is used, the N-terminal site should have a greater affinity for PII while the C-terminal site should have a greater affinity for PII-UMP. Our hypothesis also predicts that the synergy between glutamine and PII results from the ability of either effector to propagate a conformational change that alters the site on the opposite domain. Also, the contacts between the AT and AR domains with each other or with the central region should be different in the absence and presence of PII-UMP, as we hypothesize that in the later situation the AT domain plays a role in activating the AR domain.

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SUPPORTING INFORMATION AVAILABLE

Oligodeoxyribonucleotide primers used in this study (Table S1), SDS−PAGE analysis of purified proteins (Figure S1), nondenaturing gel electrophoresis assay for the interaction of PII with mutated forms of ATase (Figure S2), cross-linking of ATase and mutated forms of ATase to PII and PII-UMP (Figure S3), cross-linking of PII to ATase and truncated forms of ATase (Figure S4), cross-linking of PII-UMP to ATase and truncated forms of ATase (Figure S5), and interaction between ATN6 and ATC3 as detected by nondenaturing gel electrophoresis (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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