11522 *Biochemistry* **2009,** *48,* 11522–11531
DOI: 10.1021/bi9011594

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Sensation and Signaling of α-Ketoglutarate and Adenylylate Energy Charge by the *Escherichia coli* PII Signal Transduction Protein Require Cooperation of the Three Ligand-Binding Sites within the PII Trimer[†]

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Received July 8, 2009; Revised Manuscript Received October 22, 2009

ABSTRACT: PII proteins are sensors of α-ketoglutarate and adenylylate energy charge that regulate signal transduction proteins, metabolic enzymes, and permeases involved in nitrogen assimilation. Here, purified *Escherichia coli* PII and two of its receptors, ATase and NRII, were used to study the mechanisms of sensation by PII. We assembled heterotrimeric forms of PII from wild-type and mutant subunits, which allowed us to assess the role of the three binding sites for α-ketoglutarate and adenylylate nucleotide in the PII trimer. Signaling of α-ketoglutarate and adenylylate energy charge by these heterotrimeric PII proteins required multiple binding sites for these effectors, and the ligand-binding sites on different subunits could influence the function of a single subunit interacting with a receptor, implying communication between PII subunits. Wild-type and heterotrimeric forms of PII were also used to examine the effects of α-ketoglutarate and ADP on PII activation of the adenylyltransferase (AT) activity of ATase. Previous work showed that when ATP was the sole adenylylate nucleotide, α-ketoglutarate controlled the extent of PII activation but did not alter the PII activation constant (K_{act}). We show that ADP affected both the PII K_{act} and the extent of activation by PII. When ATP was present, ADP dramatically reduced the K_{act} for wild-type PII, and this effect was antagonized by α-ketoglutarate. Consequently, when ATP was present, the antagonism between ADP and α-ketoglutarate allowed each of these effectors to influence the PII K_{act} for activation of ATase. A study of heterotrimeric forms of PII suggested that the major part of the ability of ADP to improve the binding of PII to ATase required multiple nucleotide binding sites and intersubunit communication. We also used nondenaturing gel electrophoresis to investigate the effect of ADP and α-ketoglutarate on the binding of PII to ATase and NRII. These studies showed that ATase and NRII differ in their requirements for interaction with PII, and that under the appropriate conditions, the antagonism between α-ketoglutarate and ADP allowed each of these effectors to influence the binding of PII to receptors.

The remarkable PII[1] signal transduction proteins are homotrimers of 112 amino acid subunits that function to integrate diverse signals and produce an output signal that controls cellular physiology (*1*). These small and highly conserved proteins are among the most widely distributed signaling proteins in nature and control metabolic enzymes, permeases, and signal transduction proteins involved in various aspects of nitrogen regulation (*2*−*4*). Studies of the *Escherichia coli* PII protein showed that it is the direct sensor of adenylylate energy charge and of α-ketoglutarate, a signal of carbon and nitrogen status (*5*−*10*). PII senses α-ketoglutarate by possessing a highly specific binding site on each subunit of the homotrimer, and it senses adenylylate energy charge by possessing a binding site on each subunit that can be occupied by either ADP or ATP. In *E. coli*, PII also transduces a signal of intracellular glutamine levels, by means of its reversible uridylylation and deuridylylation catalyzed by the glutamine-controlled uridylyltransferase/uridylyl-removing enzyme (UTase/UR) (*6*, *11*, *12*). Thus, in *E. coli*, PII serves as the platform for the integration of three signals: α-ketoglutarate, adenylylate energy charge, and glutamine (*10*, *13*). The UTase/UR enzyme is limited to certain bacteria, and certain other bacteria possess different enzyme systems for the reversible phosphorylation or adenylylation of PII (discussed in ref *2*). It has been hypothesized that sensation of α-ketoglutarate and adenylylate energy charge represent ancient and highly conserved functions of PII, while transmission of a glutamine signal or other signals by reversible covalent modifications of PII represents a more recent modification to the core regulatory system (*3*).

Structural studies of PII and extensive analysis of the properties of mutant forms of PII have provided some insight into the mechanisms of its function. Crystal structures have shown that PII trimers form a squat barrel, with three prominent loops termed the T-loop, B-loop, and C-loop (e.g., refs *14* and *15*). Projecting from one end of the barrel are the three T-loops, the largest structural features of the PII trimer, responsible for interactions with receptors (*16*). Near the apex of the T-loop is the site at which PII becomes reversibly uridylylated in *E. coli*,

[†]Supported by Grant GM59637 from the National Institute of General Medical Sciences to A.J.N.

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[1]Abbreviations: PII, signal transduction protein encoded by *glnB*; NRII, signal transduction protein encoded by *glnL* (*ntrB*) and transmitter protein of the NRII−NRI two-component signal transduction system; NRI, signal transduction protein encoded by *glnG* (*ntrC*) and receiver protein of the NRII−NRI two-component signal transduction system; UTase/UR, signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.49) and product of the *glnD* gene; ATase, signal-transducing glutamine synthetase adenylyltransferase (EC 2.7.7.39) and product of the *glnE* gene; GS, glutamine synthetase and product of the *glnA* gene.

 Published on Web 10/30/2009

residue Y51 (*17*). The T-loop seems to be highly flexible and has been crystallized in numerous conformations, many of which are apparently due to nonphysiological interactions within the crystals (e.g., ref *15*). Possibly, this loop forms a stable structure only when in complex with its receptors (*15*). Mutagenesis studies have shown that alterations of this loop can alter the relative affinity of PII for its receptors, and that a small deletion of the apex of the T-loop, removing residues 47−53 (Δ47−53), eliminated the binding of PII to its receptors (*16*), yet this deletion did not alter the binding of ATP or α-ketoglutarate by PII (*16*).

A second structural feature of PII, called the B-loop, is on the outer surface of the barrel and is involved in binding of ATP and ADP (*10*, *18*, *19*). Unlike the flexible T-loop, the B-loop is a stable structural element that has been studied in the presence and absence of nucleotides (*14*, *18*, *19*). Mutations in this part of PII have the ability to affect all functions of PII; in this study, we use the G89A substitution, which is particularly severe and eliminates both nucleotide binding and the binding of α-ketoglutarate (*16*). It has been suggested that the direct effect of the mutation was on nucleotide binding and that the loss of α-ketoglutarate binding reflected an indirect effect of the mutation (*16*). The G89A alteration in *E. coli* PII also eliminated all regulatory functions of PII (as would be expected if α-ketoglutarate and an adeylylate nucleotide were not bound), and the mutant protein appeared to be completely unable to bind to the PII receptors, as judged by the inability of the mutant PII to compete with wild-type PII for the receptors (*16*, *20*).

Direct measurement of the binding of ^{32}P- or ^{14}C-labeled ATP, ADP, and α-ketoglutarate to *E. coli* PII has revealed some features of effector binding (*5*, *6*, *10*). The binding of ATP and ADP saturates at three molecules per trimer, with the two adenylylate nucleotides competing for a common site (*10*). ADP binds to PII significantly better than ATP does in the absence of α-ketoglutarate (*10*). α-Ketoglutarate binds poorly to PII in the absence of adenylylate nucleotide and also binds poorly to PII in the presence of ADP as the sole adenylylate nucleotide; however, α-ketoglutarate binds very avidly to PII in the presence of ATP, and α-ketoglutarate and ATP each provide strong synergy for the binding of the other (*5*, *6*). When the ATP concentration is saturating, the binding of α-ketoglutarate displays strong anticooperativity, such that the first molecule binds with a K_d in the low micromolar range and the binding of the second and third molecules occurs with a K_d ~1−2 orders of magnitude higher (*5*, *6*). (Measurement of high dissociation constants is difficult when one is measuring the depletion of the labeled species from solution, as was done in the cited studies, because of the practical limitations in the concentrations of PII that can be used in the experiments.) By comparison to cellular levels of α-ketoglutarate and adenylylate nucleotides (*21*, *22*), it seems that in vivo, PII trimers will always be saturated by nucleotides and will be bound by at least one molecule of α-ketoglutarate under all physiological conditions, with α-ketoglutarate overcoming the anticooperativity and saturating the PII trimers as it reaches the high end of its physiological concentration range. That is, the anticooperativity of α-ketoglutarate binding provides a shallow response to physiological concentrations of α-ketoglutarate, a desirable feature for an accurate biological sensor.

The binding properties of α-ketoglutarate are reflected in the signaling of this effector by PII. Under conditions where PII is saturated with ATP and bound by a single molecule of α-ketoglutarate, such as when this effector is at the low end of its physiological range, PII is a potent activator of the adenylyltransferase (AT) activity of ATase and of the regulated phosphatase activity of NRII (NtrB), and it is a potent inhibitor of the adenylyl-removing (AR) activity of ATase and of the autophosphorylation activity of NRII (*7*−*10*, *23*). However, under conditions where α-ketoglutarate is at the high end of its physiological range and PII is saturated with three molecules of this effector, PII is a very weak regulator of these receptor activities (*7*−*10*, *23*). A striking manifestation of this regulation by α-ketoglutarate is obtained when receptor activities are measured in vitro and the α-ketoglutarate concentration is varied over a broad range extending from zero to beyond that needed to saturate PII with effector. In such experiments, receptor activity displays a biphasic response to α-ketoglutarate, first increasing and then decreasing as the α-ketoglutarate concentration is increased. The decrease in receptor activity at high α-ketoglutarate concentrations seems to suggest that as PII undergoes the transition from being bound by a single molecule of α-ketoglutarate to being bound by three molecules of α-ketoglutarate, its signaling activity becomes reduced. This relationship between the binding properties of α-ketoglutarate and its regulatory properties will be further studied in this work.

Several results have suggested that a single PII T-loop must contact the receptors for PII to regulate receptor activities. Cross-linking studies in which PII bearing heterobifunctional cross-linking agents at specific residues on its T-loops was cross-linked to receptors suggested that a single T-loop of PII interacted with the receptor (*24*, *25*). More powerful proof of this point comes from the study of PII heterotrimers. It is possible to dissociate the subunits of the PII trimer with urea and to re-form trimers by dialysis of the urea (*20*); this allows the formation of heterotrimers containing different subunits in various proportions. When wild-type subunits were distributed into trimers containing two mutant Δ47−53 subunits, the resulting heterotrimers were able to regulate ATase and NRII, even though they contained only a single functional T-loop on their single wild-type subunit (*20*). Since PII touches its receptors with a single T-loop, the signaling by α-ketoglutarate discussed in the previous paragraph implies that intersubunit signaling within the PII trimer is used to control the T-loop of the PII trimer that contacts the receptor. This hypothesized role of intersubunit signaling will also be tested here.

Additional experiments with heterotrimers explored the properties of the G89A subunits. When wild-type PII subunits were distributed into heterotrimers containing two copies of the G89A subunits, the resulting heterotrimers were able to regulate PII receptors (*20*). This revealed that the G89A subunits were not intrinsically inhibitory, but heterotrimers containing mixtures of G89A and Δ47−53 subunits were unable to activate PII receptors (*20*). Those results suggested that the nonmutated T-loop of the G89A subunits could not be activated "in trans" within the trimer by the α-ketoglutarate- and ATP-bound Δ47−53 subunits; that is, the small molecule effectors must be bound to the subunit that touches the receptor. When this finding is combined with the results already discussed, the picture that emerges is that within the PII trimer, the subunit binding the receptor must be bound by the small molecule effectors, and the other subunits of the trimer can influence its activity by intersubunit signaling when they bind to small molecule effectors.

The mechanism by which α-ketoglutarate regulates the ability of PII to control its receptors was examined by studying the binding of PII to its receptors and by measuring the effect of

α-ketoglutarate on the extent of receptor activation and the concentration of PII required for activation (PII K_{act}). Curiously, α-ketoglutarate (in its physiological range) did not affect the ability of PII to bind to NRII or ATase, nor did this effector alter the K_{act} of PII for activation of the AT activity of ATase or the regulated phosphatase activity of NRII (*23*, *25*, *26*). However, under the same conditions, dramatic regulation of these receptor activities was observed. Thus, it was suggested that α-ketoglutarate affects the ability of PII to activate the receptors at a step after the initial binding of PII to the receptor (*23*, *26*).

The sensation and signaling of adenylylate energy charge by PII have been studied less completely. It has long been known that adneylylate energy charge is a regulatory signal in essentially all cell types (*27*, *28*); in *E. coli* and other bacteria where the AMP concentration is very low and constant, the adenylate energy charge is dominated by the ratio of ADP to ATP. ADP has a dramatic effect on the regulatory properties of PII (*10*); for example, in the presence of ATP, the addition of ADP greatly enhanced the ability of PII to activate the AT activity of ATase and the regulated phosphatase activity of NRII (*10*). Indeed, the best activation of these receptor activities by PII occurred when a mixture of ADP and ATP (or the ATP analogue AMP-PNP) was present. This result suggested that sensation and signaling of adenylylate energy charge by PII require the coordinated activity of all three nucleotide binding sites of the PII trimer (*10*). Since ATP and ADP compete for common sites, ATP and α-ketoglutarate bind to PII synergistically, and ADP and α-ketoglutarate bind to PII without synergy, ADP and α-ketoglutarate acted antagonistically with respect to one another in the regulation of receptor activities in the presence of ATP (*10*). In the final section of this work, we study the mechanism by which ADP influences the activities of PII in the presence of α-ketoglutarate and ATP. We observed that ADP influenced both the binding of PII to its receptors and the extent of activation of the receptors by PII; that is, ADP decreased the PII K_{act} and increased the receptor k_{cat}. Furthermore, a direct assay for binding showed that ADP affected the binding of PII to its receptors, and that in the presence of ADP, α-ketoglutarate acted to control the binding of PII to ATase and NRII.

MATERIALS AND METHODS

Purified Proteins. The preparations of the purified proteins used here have been described previously (*20*, *23*, *26*). PII heterotrimers were prepared as described previously (*20*). Briefly, PII proteins were combined in the indicated ratios, and freshly prepared urea was added to a final concentration of 6 M; the reaction mixtures were incubated at 37 °C for 1 h, after which the urea was dialyzed out at 4 °C overnight, using a vast volume excess of dialysate. Since the wild-type and Δ47−53 subunits cause their respective trimers to migrate in gels with discernible differences, heterotrimer preparations with wild type and Δ47−53 subunits were examined by nondenaturing gel electrophoresis to confirm the subunit composition of the major species in the protein mixtures. Heterotrimers containing mainly one wild-type subunit and two copies of the Δ47−53 subunits were formed via incubation of the proteins at a 1:6 ratio. In this heterotrimer preparation, the vast majority of the protein was the completely mutant trimers, as expected, with ~90% of the wild-type subunits being incorporated into trimers with two mutant subunits, ~10% of the wild-type subunits being incorporated into trimers with one mutant subunit, and no detectable presence of wild-type homotrimers (which, nevertheless, must be present at a very low concentration). This mixture of species was used in the experiments as the heterotrimer sample. Heterotrimers containing mainly one wild-type subunit and two copies of the G89A subunits were formed by the same procedure, with the proteins at a 1:10 wild type:G89A ratio. Again, the mixture of species was used in the experiments as the heterotrimer sample. Prior results showed that nondenaturing gel electrophoresis cannot separate the different trimeric species containing these types of subunits (*20*), so these heterotrimers were not examined by gel electrophoresis; however, prior results showed that the G89A subunits readily formed heterotrimers with Δ47−53 subunits (even though the trimers had no activity), suggesting no inability to form heterotrimers.

AT Assay. The initial rate of GS adenylylation by ATase was measured as described previously (*23*). Briefly, purified unadenylylated GS and ATase were incubated under the indicated conditions, and the adenylylation of GS was initiated by addition of [α-^{32}P]ATP. At various times, samples were removed and spotted onto nitrocellulose filters, which were washed in TCA to remove unincorporated counts, dried, and counted by liquid scintillation. Initial rates were obtained from linear regression of the data from early parts of the time courses, where the reactions visually appear to be linear. Because multiple data points were used to estimate initial rates and the assays were conducted with labeled nucleotide mixtures with high specific activity, the reproducibility of the assay is excellent (*23*). Variations between experiments on a given day typically average only a few percent, and variations between experiments conducted on separate days may be 10−15%, although on many occasions smaller variations were obtained. The specific conditions for each experiment are provided in the figure legends.

Nondenaturing Gel Electrophoresis Was Conducted As Described Previously (25). Briefly, the resolving gels were 14% polyacrylamide (29:1 acrylamide:bisacrylamide ratio) and contained 187.5 mM Tris-HCl buffer (pH 7.5). Stacking gels contained 62.5 mM Tris-HCl (pH 6.8), and the acrylamide content was 5%. To equilibrate small molecule effectors in the gel (1 mM $MgCl_2$, and α-ketoglutarate, ATP, and ADP as indicated), gels were prerun in running buffer [25 mM Tris-borate (pH 7.5)] that contained the small molecule effectors for 45 min to 1 h prior to loading. Reaction mixtures containing the various proteins at their indicated concentrations and ATP, ADP, and α-ketoglutarate as indicated were preincubated at room temperature for 10 min in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM KCl. Electrophoresis was conducted at 4 °C and 200 V in a mini-gel format for ~2 h, after which gels were stained with Coomassie Brilliant Blue R-250 and photographed.

RESULTS

Signaling of Physiological Concentrations of α-Ketoglutarate Involved Multiple Effector Binding Sites and Intersubunit Signaling within the PII Trimer. Prior results showed that when ATP is the sole adenylylate nucleotide and at a saturating concentration, binding of α-ketoglutarate to PII provided biphasic control of the AT activity of ATase (*5*, *8*, *10*, *23*). At low concentrations of α-ketoglutarate, PII was a potent activator of the AT activity of ATase, but as the concentration of α-ketoglutarate was increased through its physiological range, the ability of PII to activate the AT activity was weakened (*5*, *8*,

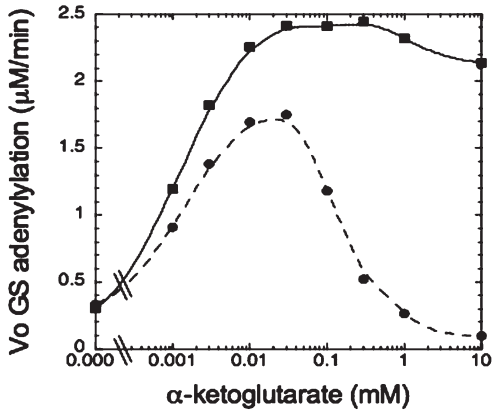

FIGURE 1: Sensation and signaling of α-ketoglutarate by heterotrimeric forms of PII. Initial rates of GS adenylylation by ATase (AT activity) were measured as described in Materials and Methods. Conditions were 2.5 μM GS, 0.1 μM ATase, 0.5 mM [α-^{32}P]ATP, PII heterotrimers with the concentration of wild-type subunits at 3 μM, and α-ketoglutarate as indicated: (●) heterotrimers containing one wild-type subunit and two Δ47−53 subunits and (■) heterotrimers containing one wild-type subunit and two G89A subunits.

10, *23*). We tested the hypothesis that the asymmetric form of PII bound to a single effector molecule is a potent activator of the AT activity, while the symmetrically bound, saturated, form of the PII trimer is a poor activator of the AT activity. Specifically, we examined whether the presence of multiple α-ketoglutarate binding sites and intersubunit signaling were required for physiological concentrations of α-ketoglutarate to weaken the ability of PII to activate the AT activity. Heterotrimeric PII containing one wild-type subunit and two copies of the Δ47−43 subunit contain only a single functional T-loop able to interact with receptors (on the wild-type subunit) but display normal binding of α-ketoglutarate and ATP and have three binding sites for each effector (*16*, *20*). Conversely, heterotrimeric PII containing one wild-type subunit and two copies of the G89A subunit have three unaltered T-loops but are unable to bind α-ketoglutarate and ATP on the mutant subunits. Consequently, these heterotrimers possess only a single effector binding site for each of ATP and α-ketoglutarate, on their wild-type subunit. The hypothesis predicts that the heterotrimers containing the Δ47−53 subunits should properly signal physiological concentrations of α-ketoglutarate, since their binding of ATP and α-ketoglutarate is normal. Furthermore, since these heterotrimers have only a single T-loop able to interact with the ATase, proper signaling of α-ketoglutarate by these heterotrimers would imply intersubunit signaling that communicates the status of the three effector binding sites to the single functional T-loop. Conversely, the heterotrimers containing two of the G89A subunits are predicted to exhibit a monophasic response, where low concentrations of α-ketoglutarate improve the ability of PII to activate the AT activity but high concentrations of α-ketoglutarate fail to weaken the ability of PII to activate the AT activity. This is because the heterotrimers containing two G89A subunits, having only a single site for binding nucleotide and a single α-ketoglutarate site on their one wild-type subunit, become trapped in the active, asymmetric, form even at physiological α-ketoglutarate concentrations.

The predictions of the hypothesis noted above were experimentally tested, and each of the predictions was met (Figure 1). Heterotrimers containing a wild-type subunit and two copies of the Δ47−53 subunit displayed a normal, biphasic response to α-ketoglutarate, quite similar to prior results with wild-type PII trimers (*8*, *23*). The descending phase of this biphasic regulation corresponds to the physiological response to an increasing α-ketoglutarate concentration. In contrast, heterotrimers containing a wild-type subunit and two copies of the G89A subunit displayed a monophasic response to α-ketoglutarate; physiological concentrations of α-ketoglutarate were unable to weaken the ability of PII to activate the AT activity (Figure 1). These results therefore provide strong support for the hypothesis that intersubunit signaling is involved in the signaling of high concentrations of α-ketoglutarate.

Signaling of Decreasing Adenylylate Energy Charge Required Multiple Adenylylate Nucleotide Binding Sites within the PII Trimer and Intersubunit Signaling. Prior results showed that either ADP or ATP could bind to the nucleotide binding site of PII and that wild-type heterotrimers could sense the ratio of ATP to ADP, a reflection of the adenylate energy charge (*10*). Decreasing adenylylate energy charge, caused by addition of ADP to reaction mixtures that contain a fixed concentration of ATP, resulted in an increased level of activation of the AT activity of ATase by PII (*10*). This is an appealing experimental system because in the absence of PII, ADP was a weak inhibitor of GS adenylylation by ATase (*23*). Thus, increases in the AT activity upon addition of ADP were due to increased levels of activation of ATase by PII, acting against the intrinsic inhibitory effect of ADP on ATase (*10*). Prior studies also showed that ADP and physiological concentrations of α-ketoglutarate acted antagonistically in the PII-mediated regulation of the AT activity. That is, PII served as the platform for the integration of these antagonistic signals (*10*). We used wild-type PII and the two heterotrimeric forms of PII described above to examine the role of multiple adenylylate nucleotide binding sites in response to decreasing adenylylate energy charge and to examine the antagonism between α-ketoglutarate and ADP in more detail.

The responses of the wild-type and heterotrimeric forms of PII to decreasing adenylylate energy charge are shown in Figure 2, where the initial rate of GS adenylylation by ATase (AT activity) was measured at fixed PII, ATase, α-ketoglutarate, and ATP concentrations, with the ADP concentration varied. When the α-ketoglutarate concentration was low (0.03 mM), wild-type PII and both of the heterotrimeric forms of PII were potent activators of the AT activity of ATase, consistent with prior results and the results shown in Figure 1 (Figure 2A). Addition of ADP under these conditions resulted in a dramatic increase in the level of activation of the AT activity by wild-type PII, with 0.1 mM ADP providing a 3-fold increase in AT activity and 1.0 mM ADP providing a 5-fold increase in AT activity (Figure 2A). The effects of ADP were less dramatic, but still significant, when heterotrimers containing one wild-type subunit and two copies of the Δ47−53 subunit were examined. In that case, addition of ADP to a concentration of 0.1 mM resulted in a 2-fold increase in the level of AT activation, and an increase in the ADP concentration to 1.0 mM resulted in a 4-fold increase in the level of AT activation. By contrast, the corresponding experiments using the heterotrimers with one wild-type subunit and two copies of the G89A subunit resulted in only 1.2- and 1.4-fold increases in the level of AT activation, respectively. Thus, a significant part of the response to decreasing adenylylate energy charge at a low fixed α-ketoglutarate concentration required the presence of multiple adenylylate nucleotide binding sites on the PII trimer. The 4-fold response displayed by the heterotrimers containing two of the Δ47−53 subunits suggests that multiple nucleotide

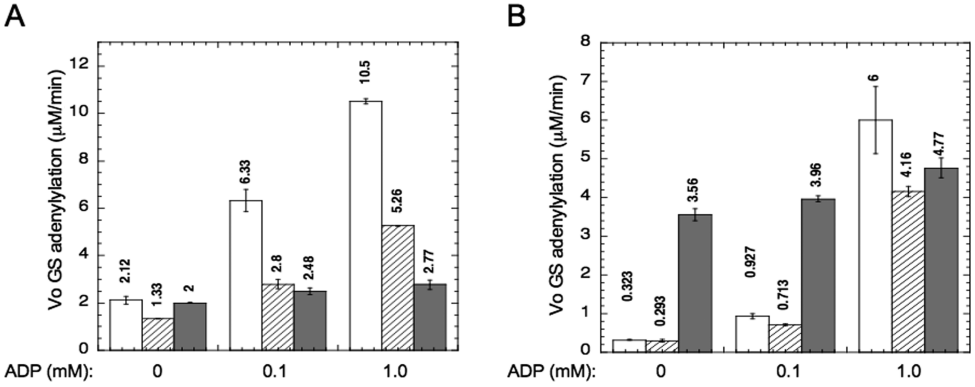

FIGURE 2: Sensation and signaling of adenylylate energy charge and α-ketoglutarate by wild-type and heterotrimeric forms of PII. The initial rate of GS adenylylation by ATase (AT activity) was measured as described in Materials and Methods. (A) Signaling of decreasing adenylylate energy charge at a low (0.03 mM) α-ketoglutarate concentration. Decreasing adenylylate energy charge was caused by addition of ADP to reaction mixtures with a fixed concentration of ATP. Conditions were 2.5 μM GS, 0.5 mM [α-^{32}P]ATP, 0.1 μM ATase, 1.5 μM wild-type PII subunits, 0.03 mM α-ketoglutarate, and ADP as indicated: (white bars) wild-type PII, (hatched bars) heterotrimers containing one wild-type subunit and two Δ47−53 subunits, and (gray bars) heterotrimers containing one wild-type subunit and two G89A subunits. (B) Signaling of decreasing adenylylate energy charge at a high (1 mM) α-ketoglutarate concentration. Conditions and symbols are as for panel A, except that the concentration of ATase was doubled (to 0.2 μM) to partially compensate for the low AT activity under these conditions.

binding sites collaborated to control the activity of the single functional T-loop, implying intersubunit communication of the status of the nucleotide sites.

At a high fixed concentration of α-ketoglutarate (1 mM), responses to an increasing ADP concentration were shifted to higher ADP concentrations, as expected from prior work (*10*). In the absence of ADP, PII and the heterotrimers containing Δ47−53 subunits were poor activators of the AT activity when the concentration of α-ketoglutarate was high, consistent with prior work and the results depicted in Figure 1, whereas the heterotrimers containing the G89A subunits were potent activators of ATase, since their ability to activate the AT activity could not be inhibited by high α-ketoglutarate concentrations, as shown in Figure 1. For the wild-type PII homotrimers, addition of ADP to a final concentration of 0.1 mM resulted in a 2.9-fold increase in the level of AT activation, while addition of ADP to a final concentration of 1 mM resulted in a 18.6-fold increase in the level of AT activation (Figure 2B). This large increase in the level of AT activation cooresponds to nearly complete antagonism of the inhibition of AT activation provided by high α-ketoglutarate concentrations. The heterotrimers containing Δ47−53 subunits displayed a similar pattern of regulation; addition of ADP to a final concentration of 0.1 mM resulted in a 2.4-fold increase in the level of AT activation, while an increase in the concentration of ADP to 1 mM resulted in a 14.2-fold increase in the level of AT activation (Figure 2B). By contrast, when the corresponding experiments were conducted using heterotrimers with the G89A subunits, increases of 1.1- and 1.3-fold were obtained (Figure 2B). Thus, again, a major part of signaling of decreasing adenylylate energy charge required multiple nucleotide binding sites on the PII trimer.

Lack of an Effect of α*-Ketoglutarate on the PII Activation Constant* (K_{act}) *for Activation of the AT Activity of ATase*. Prior results showed that although α-ketoglutarate strongly affected the ability of PII to activate the AT activity of ATase, it did not significantly alter the activation constant (K_{act}) for activation by PII. These results have been interpreted as indicating that α-ketoglutarate, within its physiological range, does not affect the binding of PII to ATase but regulates the ability of PII to activate the AT activity at a postbinding step (*23*). Here, we examined the activation constants for activation of the AT activity by the heterotrimeric forms of PII containing G89A or Δ47−53 subunits, at high (1 mM) and low (0.05 mM) concentrations of α-ketoglutarate (Figure 3). For both heterotrimeric forms of PII, α-ketoglutarate did not significantly alter the PII K_{act} (Figure 3). Consistent with the results shown in Figure 1, α-ketoglutarate had very significant effects on the level of activation by heterotrimers containing the Δ47−53 subunits; this may be discerned by considering the different scales used for the *Y* axis for the different panels in Figure 3.

ADP Increased both the Level of Binding of PII to ATase and the Extent of Activation of the AT Activity. Unlike α-ketoglutarate, which affected the extent of activation of the AT activity by PII without significantly altering the PII activation constant, ADP acted to both reduce the PII K_{act} and increase the extent of activation by PII (Figure 4). Prior work with wild-type PII homotrimers showed that in the presence of ATP and absence of α-ketoglutarate, a K_{act} of 7−10 μM was obtained and the extent of activation was minimal (*10*). When ADP was present in the absence of α-ketoglutarate, a PII K_{act} of 1.5 μM was observed and PII was a strong activator (*10*). Similarly, when ATP was present along with α-ketoglutarate, an activation constant of 1.5 μM was observed, whether the α-ketoglutarate concentration was high or low (*23*). To examine more completely the effects of ADP on the binding of PII to ATase, we studied the effect of α-ketoglutarate and ADP on the PII activation constant, in experiments where ATP was present at the fixed concentration of 0.5 mM. Remarkably, the K_{act} for wild-type PII homotrimers was 0.06 μM, when ADP was present at a concentration of 1 mM and α-ketoglutarate was present at a concentration of 0.05 mM (Figure 4A); this concentration is approximately 25-fold lower than the corresponding K_{act} in the absence of ADP (*23*). In the presence of 1 mM ADP and 1 mM α-ketoglutarate, the K_{act} for wild-type PII homotrimers was ~0.2 μM (Figure 4B). Thus, α-ketoglutarate antagonized the effect of ADP on the PII K_{act}. Because of this antagonism, under the conditions used, both α-ketoglutarate and ADP influenced the PII K_{act}.

The effect of ADP on the K_{act} for wild-type PII could be due to ADP binding to the subunit of PII that touches ATase, ADP binding to the other subunits of the PII trimer and signaling this binding through intersubunit interactions, or a combination of these mechanisms. To examine this issue more completely, the

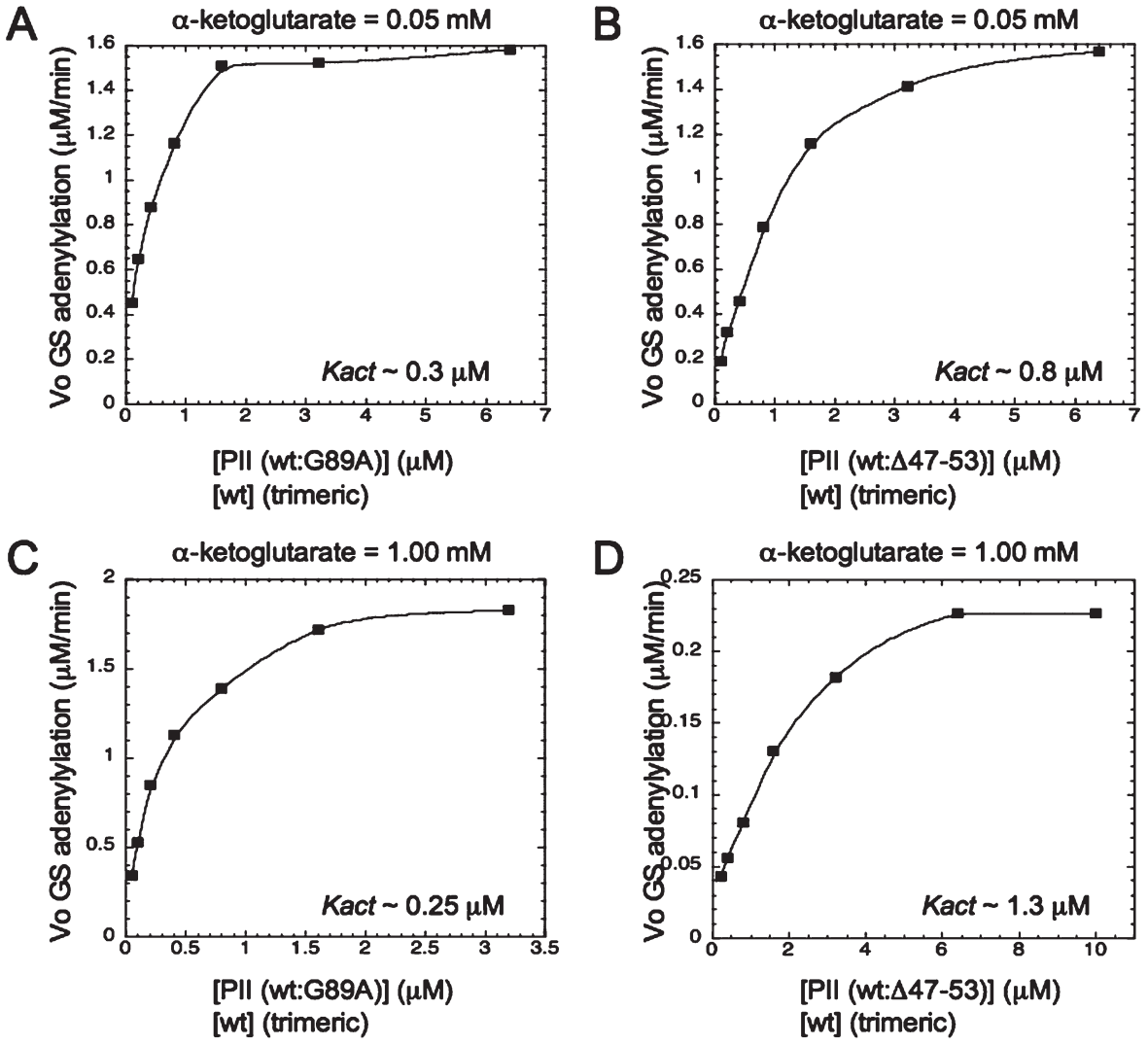

FIGURE 3: PII activation constants (K_{act}) for activation of the AT activity of ATase. The initial rate of GS adenylylation by ATase (AT activity) was measured as described in Materials and Methods, with 2.5 μM GS, 0.1 μM ATase, 0.5 mM [α-^{32}P]ATP, α-ketoglutarate at 0.05 or 1.0 mM, as indicated, and PII as indicated. As noted in Materials and Methods, the PII heterotrimer samples used were a mixture of species, consisting mainly of the innocuous mutant homotrimers along with the heterotrimers. The concentration of PII heterotrimers (*X*-axis) is expressed as the concentration of wild-type PII trimers used to assemble the quantity of heterotrimers (i.e., one-third the concentration of the wild-type subunits). This allows a comparison of the activity and activation constant of the heterotrimer samples with the published activity and activation constant of wild-type PII homotrimers. (A) Activation by heterotrimers containing G89A subunits, at a low α-ketoglutarate concentration. (B) Activation by heterotrimers containing Δ47−53 subunits, at a low α-ketoglutarate concentration. (C) Activation by heterotrimers containing G89A subunits, at a high α-ketoglutarate concentration. (D) Activation by heterotrimers containing Δ47−53 subunits, at a high α-ketoglutarate concentration.

heterotrimeric forms of PII were examined. The heterotrimeric form containing one wild-type subunit and two G89A subunits, and thus with a single nucleotide binding site per trimer, displayed a K_{act} of 0.3 μM in the absence of ADP (Figure 3) and a K_{act} of 0.15 μM in the presence of ADP (Figure 4). Variations of this magnitude are above the noise of the experimental method, but their interpretation is difficult. Perhaps the single wild-type subunit of these heterotrimers is a slightly better activator of the AT activity when bound to ADP as opposed to when it is bound by ATP and α-ketoglutarate. In contrast, when the heterotrimers containing Δ47−53 subunits were examined, a K_{act} of 0.8 μM in the absence of ADP (Figure 3) was reduced to 0.18 μM in the presence of ADP (Figure 4). This 4.4-fold difference suggests that multiple nucleotide binding sites and intersubunit signaling were required for ADP to have large effects on the PII K_{act}. We note that in the absence of ADP, the heterotrimers containing G89A subunits had a significantly lower K_{act} for activation of ATase than did the heterotrimers containing Δ47−53 subunits, but when ADP was present, both types of heterotrimers had the same K_{act} (ca. Figures 3 and 4).

To examine whether ADP affected the extent of PII activation of the AT activity of ATase, we measured the initial rate of GS adenylylation under conditions similar to those used in the preceding experiments (2.5 μM GS, 0.05 μM ATase, 0.5 mM [α-^{32}P]ATP, and 0.05 mM α-ketoglutarate, with or without 1 mM ADP) but with PII present at a saturating concentration of 20 μM. Under these conditions, the initial rate of the AT activity was 1.38 μM/min, corresponding to a k_{cat} of ~28 min^{-1}. In the cooresponding experiment in which ADP was present at a concentration of 1 mM, the initial rate of the AT activity was 4.61 μM/min (k_{cat} ~ 92 min^{-1}), or ~3.35-fold elevated relative to that in the experiment without ADP. The initial rate of GS adenylylation was essentially the same (4.78 μM/min) in a control experiment where the PII concentration was reduced to 1.5 mM, proving that the PII was indeed saturating by more than 1 order

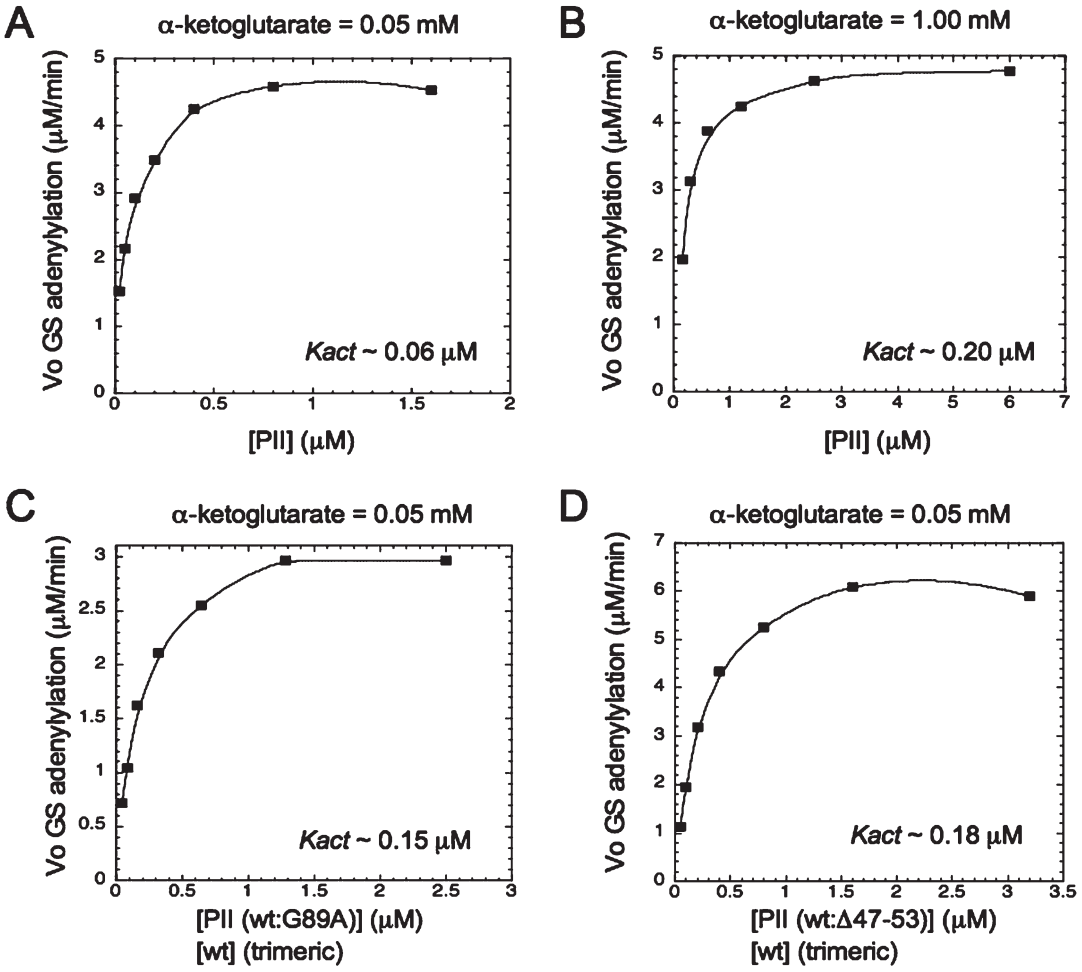

FIGURE 4: PII activation constants (K_{act}) for activation of the AT activity of ATase in the presence of ADP. The initial rate of GS adenylylation by ATase (AT activity) was measured as described in Materials and Methods, with 2.5 μM GS, 0.5 mM [α-^{32}P]ATP, and the indicated concentrations of ATase, α-ketoglutarate, ADP, and PII. The concentration of PII is expressed as the homotrimer concentration, while the concentration of the heterotrimeric species is expressed as the concentration of wild-type PII trimers used to assemble the quantity of heterotrimers (i.e., one-third the concentration of the wild-type subunits). (A) Activation by wild-type PII homotrimers. Conditions were 0.025 μM ATase, 0.05 mM α-ketoglutarate, and 1 mM ADP. (B) Activation by wild-type PII homotrimers. Conditions were 0.08 μM ATase, 1 mM ADP, and 1 mM α-ketoglutarate. (C) Activation by heterotrimers containing G89A subunits. Conditions were 0.1 μM ATase, 0.05 mM α-ketoglutarate, and 1 mM ADP. (D) Activation by PII heterotrimers containing Δ47−53 subunits. Conditions were 0.1 μM ATase, 0.05 mM α-ketoglutarate, and 1 mM ADP.

of magnitude. Thus, ADP increased the extent of activation by PII even when PII was present at a saturating concentration.

The Antagonism between α-Ketoglutarate and ADP Allowed Each of These Effectors To Influence the Binding of PII to ATase and NRII. We used nondenaturing gel electrophoresis to study the binding of ATase and NRII to PII, as described previously (*25*, *26*). Prior studies with this binding assay indicated that small molecule regulators of binding needed to be present while proteins were incubated to form complexes, as well as in the nondenaturing gels and electrophoresis buffer used to resolve the complexes. Thus, separate gels must be used for each binding condition to be studied. We earlier used these methods to show that ATase and NRII bound to PII in the presence of ATP and α-ketoglutarate, and that binding was equivalent at high and low concentrations of α-ketoglutarate (*25*, *26*). Here, we extend our focus to consider the effects of ADP on the binding of PII to ATase and NRII. In a control experiment, we observed that in the absence of α-ketoglutarate and ADP, but in the presence of ATP, PII failed to discernably bind to either ATase or NRII (Figure 5A). However, in the absence of ATP and α-ketoglutarate, and in the presence of ADP, PII bound to ATase, although binding to NRII was not detectable (Figure 5B). This result shows that ATase and NRII differ in their abilities to bind the differently liganded forms of PII. When ADP was present as the sole adenylylate nucleotide along with a low α-ketoglutarate concentration (0.03 mM), ATase bound well to PII, but the binding of NRII to PII was barely discernible (Figure 5C). Elsewhere, it was shown that with ATP and this concentration of α-ketoglutarate, PII readily bound to NRII (*26*). When ADP was present as the sole nucleotide along with a high concentration of α-ketoglutarate (1 mM), PII bound avidly to both ATase and NRII (Figure 5D). Thus, the current data, in combination with prior data, show that under the appropriate conditions both ADP and α-ketoglutarate influenced the binding of PII to ATase and NRII.

DISCUSSION

The reversible uridylylation and deuridylylation of PII by the UTase/UR is known to proceed in a distributive (nonprocessive) fashion, and furthermore, only one functional T-loop of the PII trimer must contact the PII receptors to provide receptor regulation (*16*, *29*). These observation raise the question of why

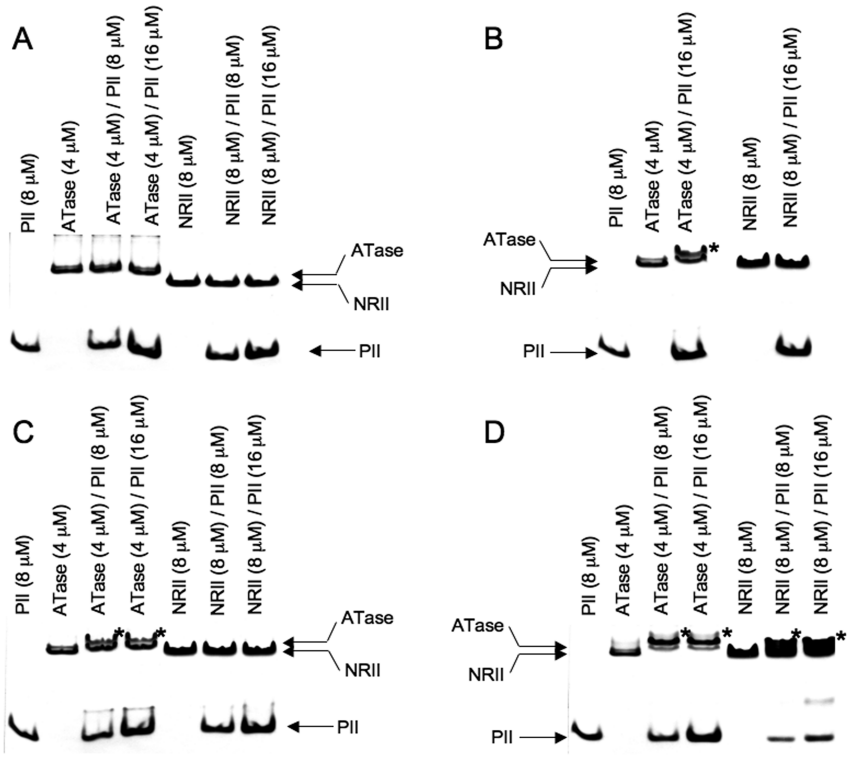

FIGURE 5: Nondenaturing gel electrophoresis analysis of the binding of PII to ATase and NRII. Procedures were as in Materials and Methods; the concentrations of proteins in the initial incubation mixtures are shown above each gel lane. A constant volume of each sample was loaded, such that the proportions of proteins in the gel parallel their initial concentrations. (A) Reaction mixtures, gel, and running buffer contained 0.5 mM ATP and lacked α-ketoglutarate and ADP. (B) Reaction mixtures, gel, and running buffer contained 0.5 mM ADP and lacked ATP and α-ketoglutarate. (C) Reaction mixtures, gel, and running buffer contained 0.5 mM ADP and 0.03 mM α-ketoglutarate and lacked ATP. (D) Reaction mixtures, gel, and running buffer contained 0.5 mM ADP and 1 mM α-ketoglutarate and lacked ATP. The bands corresponding to PII, NRII, and ATase are indicated, and complexes are marked with an asterisk.

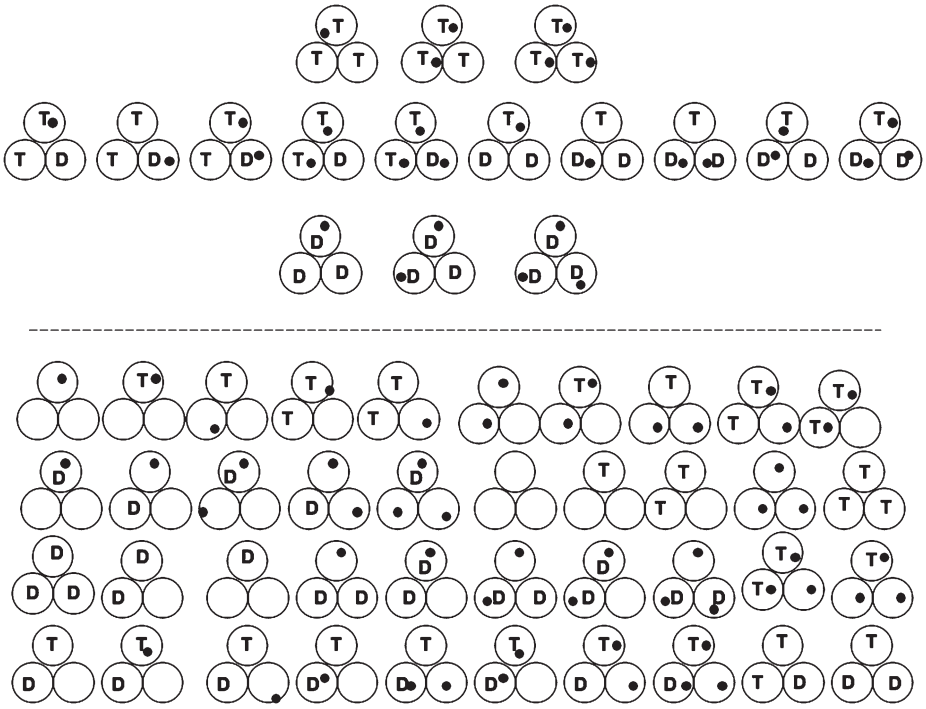

FIGURE 6: Hypothetical signaling states of PII. The PII trimer is schematically depicted as three linked circles. Black dots correspond to bound α-ketoglutarate molecules. T and D denote bound ATP and ADP, respectively. As shown above the dotted line, there are 16 different forms of PII in which all three nucleotide binding sites of the trimer are occupied and at least one molecule of α-ketoglutarate is bound. In all, there are 56 possible liganded states of the PII protein (species above and below the dotted line).

the very widely distributed PII protein maintained a trimeric structure through evolution. Here, we show that the trimeric structure of PII and the interactions between PII subunits played an essential role in the key functions of PII, namely, the sensation

and signaling of α-ketoglutarate levels and the ADP:ATP ratio. Our results were consistent with multiple effector binding sites within the PII trimer cooperating to control the output signal of a single T-loop in the PII trimer. Furthermore, in combination with prior results, our results suggest that the anticooperativity of α-ketoglutarate binding to PII (*5*) is an essential feature of the PII sensation mechanism. Presumably, the K_d values for the three α-ketoglutarate sites in the PII trimer have been optimized to provide the appropriate level of anticooperativity; if so, the PII proteins' binding properties likely reveal the physiological range of free α-ketoglutarate concentrations under the conditions where PII functions. With regard to adenylylate energy charge sensation, in combination with prior work, our results show that the main part of the sensory and signaling capacity required cooperation of the multiple effector binding sites of the PII trimer. Recently, it was shown that a fusion protein consisting of a PII protein (GlnK) from *Methanococcus jannaschii*, with an insertion of a form of GFP into its T-loop, acted as a sensor of adenylylate energy charge in vivo and in vitro (*30*). Our results predict that if these PII subunits were used to form a heterotrimer with subunits similar to the *E. coli* Δ47−53 subunits used here, the sensation of adenylylate energy charge would still occur. We expect that the relative K_d for ADP and ATP for PII proteins from different organisms will reflect the signaling requirements of the host organism and ecological niche.

Since there are a limited number of effector sites in the PII trimer, there are a limited number of regulatory states of the molecule. If it is true that there is a single site on each subunit for ATP or ADP and a single site for α-ketoglutarate, then there are a total of 56 possible forms of the PII trimer differing in their liganded status (Figure 6). If we hypothesize that under physiological conditions all of the nucleotide binding sites will have either ATP or ADP bound, and that each PII trimer will be bound by at least one molecule of α-ketoglutarate, then the maximum number of different signaling forms of PII would be reduced to 16 (Figure 6, top). This is still a large number of signaling states considering the size of the PII protein; it is certainly greater than the number provided by other systems (*31*). Of course, some of the 16 hypothetical different forms of PII shown at the top of Figure 6 may be similar in activity, such that the actual number of distinct signaling states is lower. However, as all of the signaling states are directly related to one another by stepwise changes in the state of effector binding to PII, under any cellular condition a variety of the different PII forms will be found at appreciable concentrations to provide a blended response. This blended response is made possible by the regulation of numerous molecules of the receptors by the pool of PII trimers and by the antagonistic activities of these bifunctional receptors that allow fine control of the steady state signaling output. Given the wide distribution of PII proteins in nature, it seems that a switch with a small number of signaling states, such as 16 states, is suitable for the integration of α-ketoglutarate and adenylylate energy charge signals.

ACKNOWLEDGMENT

We thank Patrick O'Brien for reading an early version of the manuscript.

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