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# α-Ketoglutarate Controls the Ability of the *Escherichia coli* PII Signal Transduction Protein To Regulate the Activities of NRII (NtrB) but Does Not Control the Binding of PII to NRII[†]

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ABSTRACT: PII signal transduction proteins are among the most widely distributed signaling proteins in nature; these proteins are direct sensors of α-ketoglutarate and adenylylate energy charge and control receptors that are signal transduction proteins, metabolic enzymes, or permeases involved in nitrogen metabolism. Prior studies showed that α-ketoglutarate regulated the ability of PII to control the activities of glutamine synthetase adenylyltransferase (ATase) but did not affect the ability of PII to bind to ATase. Here, we show that a similar pattern of α-ketoglutarate regulation was obtained with another PII receptor, the two-component system transmitter protein NRII (NtrB). Although α-ketoglutarate was required for the binding of PII to NRII, PII bound to NRII equally well as the concentration of α-ketoglutarate was varied through its physiological range. Variation of the concentration of α-ketoglutarate through its physiological range provided dramatic regulation of the ability of PII to activate the phosphatase activity of NRII and controlled the ability of PII to inhibit the autophosphorylation of NRII. Thus, PII control of NRII activities could be dissected into distinct binding and regulation steps, and when present in its physiological concentration range, α-ketoglutarate apparently played a role in only the latter step.

PII signal transduction proteins are among the most widely distributed signal transduction proteins in nature, being found in bacteria, archaea, and plants, where they regulate metabolic enzymes, permeases, and signal transduction proteins involved in various aspects of nitrogen assimilation control (*1*, *2*). In *Escherichia coli*, PII regulates two signal transduction proteins, glutamine synthetase adenylyltransferase (ATase)[1] and NRII (NtrB), and serves as the platform for the integration of at least three distinct signals that are used for the assessment of cellular nitrogen status, namely, α-ketoglutarate, the ratio of ATP to ADP (a measure of the adenylylate energy charge), and glutamine (*3*, *4*). Of these stimuli, α-ketoglutarate and the ratio of ATP to ADP are directly sensed by PII, while information about the cellular glutamine level is conveyed by PII in the form of its reversible uridylylation, catalyzed by the glutamine-controlled uridylyltransferase/uridylyl-removing enzyme (UTase/UR) (*3*).

In *E. coli*, PII acts through NRII to regulate the transcription of nitrogen-regulated genes, by controlling the phosphorylation state of the enhancer-binding transcription factor NRI, also known as NtrC (*5*) (Figure 1). NRI and NRII are members of a large family of related signaling systems known as the two-component signal transduction systems that share a common signaling mechanism based on reversible protein phosphorylation (*6*). Phosphorylation of the N-terminal receiver domain of NRI results in the oligomerization of the dimeric protein into hexamers (or heptamers); this form of the protein binds tightly to enhancer sequences, has potent ATPase activity, and activates transcription by $\sigma^{54}$-RNA polymerase, which it contacts by a DNA looping mechanism (*7*−*9*). The dephosphorylation of NRI-P results in its deoligomerization and loss of its ability to activate transcription. For the experiments in this work, the isolated N-terminal "receiver" domain of NRI was used (NRI-N). Prior studies showed that the N-terminal domain of NRI was phosphorylated and dephosphorylated normally but does not oligomerize and lacks the ATPase activity (*10*, *11*). Furthermore, this small domain of NRI is highly soluble and can be added to reaction mixtures at very high concentrations.

Both the phosphorylation and dephosphorylation of NRI (and NRI-N) are brought about by NRII, but the activities are not typical kinase and phosphatase activities (Figure 1). NRII (also known as NtrB) binds to ATP and phosphorylates itself on a highly conserved histidine residue (*12*, *13*). This is termed the autophosphorylation reaction. These phosphoryl groups are then transferred to a conserved aspartate residue in NRI in a reaction catalyzed by NRI (*14*). NRI may be considered a phosphoprotein phosphatase whose transient covalent intermediate activates transcription (*14*). NRI-P has a significant rate of spontaneous dephosphorylation, which is lost upon denaturation of the protein; this activity is termed the autophosphatase activity (*11*, *12*) (Figure 1). The rate of NRI-P dephosphorylation

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[1]Abbreviations: PII, signal transduction protein encoded by *glnB*; NRII, signal transduction protein encoded by *glnL* (*ntrB*) and the transmitter protein of the NRII−NRI two-component signal transduction system; NRI, signal transduction protein encoded by *glnG* (*ntrC*) and the receiver protein of the NRII−NRI two-component signal transduction system; NRI-N, N-terminal domain of NRI, containing the site of its reversible phosphorylation; 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.

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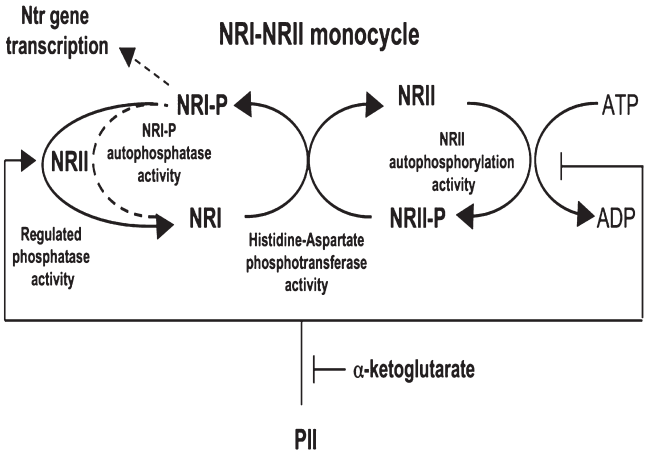


FIGURE 1: Enzymatic activities catalyzing the phosphorylation and dephopshorylation of NRI and NRII, and their regulation by PII and α-ketoglutarate. NRII binds ATP and phosphorylates itself in a reaction producing ADP (NRII autophosphorylation activity). These phosphoryl groups are transferred to NRI by the histidine-aspartate phospho-transfer activity, which is believed to reside in NRI. NRI-P is slowly dephopshorylated by the NRI-P autophosphatase activity (dotted line). In the absence of PII, NRII weakly stimulates the autophosphatase activity of NRI-P. The complex of PII and NRII powerfully activates the autophosphatase activity of NRI-P, bringing about the rapid dephosphorylation of NRI-P. This is termed the regulated phosphatase activity. As depicted, PII is an activator of the regulated phosphatase activity and an inhibitor of NRII autophosphorylation. At physiological concentrations, an increasing α-ketoglutarate concentration weakens the ability of PII to activate the regulated phosphatase activity and inhibit the autophosphorylation activity.

is vastly increased by the binding of the complex of PII and NRII to NRI-P (*15*). This activity is termed the regulated phosphatase activity. Studies of mutant forms of NRI suggest that the regulated phosphatase activity represents the activation of the slow autophosphatase activity by the NRII−PII complex (*16*). Thus, the relationship may be somewhat analogous to that of the GAP proteins that interact with G-proteins to increase the rate of GTP hydrolysis. In summary, NRII brings about the activation (phosphorylation) of NRI when it is not in a complex with PII and brings about the rapid inactivation (dephosphorylation) of NRI-P when it is in a complex with PII. Finally, the binding of PII to NRII also inhibits the autophosphorylation of NRII (Figure 1) (*17*).

As already noted, the PII protein is responsible for the sensation of α-ketoglutarate and adenylylate energy charge (*3*, *4*). The homotrimeric PII protein has three binding sites for α-ketoglutarate and three sites that bind ATP or ADP. In this study, we focus our attention on the mechanism by which α-ketoglutarate controls the ability of PII to regulate NRII activities, and we therefore excluded signaling of energy charge by using saturating concentrations of ATP or its analogue, AMP-PNP, in our experiments. We excluded glutamine signaling by using completely unmodified PII in our experiments. Prior work showed that under these conditions, the ability of PII to control ATase and NRII activities is regulated by α-ketoglutarate, which binds to PII, and that the binding of α-ketoglutarate to PII demonstrates strong anticooperativity. The high-affinity (first) site of the trimer displays a dissociation constant of ~0.01 mM; for sites 2 and 3, one can only estimate binding affinity, which must be at least 1 order of magnitude higher, due to the sensitivity of the binding assay (*18*, *19*). The form of PII that lacks bound α-ketoglutarate is a very poor activator of ATase and NRII (in the absence of ADP and adenylylate energy charge signaling). The form of PII that contains a single molecule of α-ketoglutarate per trimer is a very effective regulator of ATase and NRII activities, while the form of PII saturated with three molecules of α-ketoglutarate per trimer is significantly less potent as a regulator of ATase and NRII. Consequently, rate−saturation plots of rates of ATase or NRII activities versus α-ketoglutarate concentration are biphasic for reactions controlled by PII, with the PII effect (activation or inhibition) being most dramatic at low concentrations of α-ketoglutarate and reduced at higher concentrations of α-ketoglutarate (*4*, *5*, *17*, *19*). Several lines of investigation suggested that the reduction in receptor activities at elevated α-ketoglutarate concentrations was not due to nonspecific effects; for example, similar molecules were without significant effects in the system, and the biphasic response to α-ketoglutarate was shifted to higher concentrations when a mutant PII with a reduced level of α-ketoglutarate binding was used (*17*). Thus, it appears that a significant role of α-ketoglutarate (when ATP was saturating) was to weaken the activation or inhibition of enzyme activities by PII. Since the α-ketoglutarate concentration in intact cells [0.1−0.9 mM (*20*)] varies over the range of concentrations at which α-ketoglutarate overcomes the anticooperativity of binding to PII and PII becomes saturated, it seems that under many conditions in cells, PII would be bound to at least one molecule of α-ketoglutarate, and the cellular function of PII is to signal the α-ketoglutarate concentration by shifting between its singly liganded and fully liganded forms. This role of α-ketoglutarate is depicted in Figure 1, where α-ketoglutarate is shown as an inhibitor of PII functions.

We note that the experimental system discussed above and used previously, where ATP was saturating, is focused only on α-ketoglutarate signaling through PII. In cells, PII is also a sensor of adenylylate energy charge, through its binding of both ADP and ATP at its nucleotide binding sites (*4*). ATP and α-ketoglutarate exert strong synergy on each other's binding, while the binding of ADP and α-ketoglutarate is independent. ADP binds to PII much more avidly than does ATP. A functional consequence of this arrangement is that ADP antagonizes the effects of α-ketoglutarate, while ATP and α-ketoglutarate act in concert. Furthermore, the ability of PII to regulate its receptors is directly influenced by the nature of the nucleotides bound as well as by α-ketoglutarate. For example, at high α-ketoglutarate concentrations, maximal activation of the NRII-regulated phosphatase activity occurred when PII was liganded by a mixture of AMP-PNP and ADP (*4*). Those observations suggested that PII may adopt numerous conformations as it integrates infomation about its ligands, and to avoid this complexity, we conducted studies here and earlier with saturating ATP or its analogue, AMP-PNP. Thus, the signaling by α-ketoglutarate studied here reflects only a portion of the physiological signaling process. Specifically, the signaling by α-ketoglutarate studied here corresponds to that occurring in energy-replete cells (such as cells growing on glucose) containing the *glnD* mutation, as the concentration of the preferred nitrogen source, ammonia, is varied. Physiological measurements have shown that considerable regulation of *glnA* transcription occurs in *glnD* mutant cells under those conditions (*21*); this regulation required PII and NRI phosphorylation and is apparently due to α-ketoglutarate signaling through PII to control the phosphorylation state of NRI.

Interestingly, studies with purified components showed that α-ketoglutarate did not appear to regulate the binding of PII to ATase but instead seemed to control the ability of PII to control

ATase after it had bound. ATase is a bifunctional enzyme consisting of two antagonistic enzymes tethered together and coordinately regulated to minimize futile cycling (*22*). The adenylyl transferase (AT) activity of ATase catalyzes the adenylylation of glutamine synthetase (GS), while the adenylyl-removing (AR) activity of the ATase catalyzes the deadenylylation of GS-AMP. PII activates the AT activity of ATase and inhibits the AR activity of ATase, and in each case, α-ketoglutarate controls the extent of PII effects. The activation constant ($K_{act}$) for PII activation of the AT activity or inhibition of the AR activity was essentially the same, regardless of the α-ketoglutarate concentration (*23*). That is, α-ketoglutarate controlled only the extent of the PII effect, not the PII concentration at which regulation occurred (*23*). These results were consistent with direct studies of the binding of PII and ATase (*22*). The complex of PII and ATase could be visualized on nondenaturing gels, where binding required small molecule ligands be present in the gel and running buffer. In gel electrophoresis experiments, binding of PII to ATase required α-ketoglutarate when ATP was the only nucleotide present, but binding was not significantly different at high and low concentrations of α-ketoglutarate. The results of additional binding assays for the interaction of ATase and PII were consistent with the results obtained by nondenaturing gel electrophoresis; the binding of PII and ATase in gel filtration columns and cross-linking between PII and ATase occurred equally well at high and low concentrations of α-ketoglutarate (*22*). Those results were interpreted as indicating that PII bound to ATase more or less equally well regardless of the α-ketoglutarate concentration but acted powerfully only to regulate ATase activities when it contained a single liganded α-ketoglutarate per trimer (*23*).

Here, we examine the mechanism by which PII mediates α-ketoglutarate regulation of NRII, with the goal of discerning whether α-ketoglutarate affects the binding of PII to NRII or, as with ATase, acts at a step after the initial binding of PII to NRII. Prior efforts to specifically cross-link PII to NRII showed that cross-linking required α-ketoglutarate and that similar levels of cross-linking were obtained as the concentration of α-ketoglutarate was varied through the range at which dramatic regulation of NRII activity occurred (*24*). Those results are consistent with α-ketoglutarate being required for binding of PII to NRII, but not playing a role in controlling the extent of binding once above the concentration that enables binding. However, the irreversibility and the fairly slow kinetics of the cross-linking reaction raise the issue of whether cross-linking is the appropriate assay to use to study the α-ketoglutarate dependence of binding of PII to NRII. In this report, we examine the binding of PII to NRII directly, using nondenaturing gel electrophoresis, and characterize the activation and inhibition constants for PII regulation of NRII activities at different concentrations of α-ketoglutarate. We find that under conditions where α-ketoglutarate provided dramatic PII-mediated control of NRII activities, it had little effect on the binding of PII to NRII. Thus, as with PII control of ATase, α-ketoglutarate seemed to act at a step after the initial binding of PII to NRII.

## MATERIALS AND METHODS

*Purified Proteins*. The preparations of NRII, NRI-N, NRI-N(K104Q), and PII used here were described previously (*10*, *24*).

*Nondenaturing gel electrophoresis* was conducted as described previously (*22*). 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 (0.5 mM ATP, 1 mM $MgCl_2$, and either 0.03 or 5.0 mM α-ketoglutarate, 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 other components 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.

*Regulated Phosphatase Activity Assay*. NRI-N-$^{32}$P was prepared as described previously (*25*). The initial rate of NRI-N-P dephosphorylation was measured at 25 °C in reaction mixtures that contained NRI-N-$^{32}$P at 2.09 μM or as indicated, 0.025 μM NRII, 1 mM AMP-PNP, and either 0.03 or 10 mM α-ketoglutarate, as indicated. Briefly, dephosphorylation was initiated by addition of NRII, and samples were removed at various times and spotted onto nitrocellulose filters, which were washed extensively in TCA to remove unincorporated phosphoryl groups. Filters were then counted by liquid scintillation counting. To determine the PII activation constant, the rate of NRI-N-P dephosphorylation was determined at different concentrations of PII, as indicated. For each rate determination, a time course was run and linear regression was used to estimate the initial rate from early samples of the time courses, during which the rate visually appeared to be linear. Day-to-day variations using these methods may range from 10 to 15% (*4*, *5*, *10*, *25*), although in many cases smaller variations were obtained.

*Phosphorylation of NRI-N(K104Q)*. For Figure 3A, the initial rate of phosphorylation was determined at 25 °C, with 4 μM NRII, 0.5 mM [γ-$^{32}$P]ATP, 40 μM PII, 240 μM NRI-N(K104Q), 0.3 mg/mL bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM KCl, with the α-ketoglutarate concentration varied as indicated. The phosphorylation of NRI-N(K104Q) was initiated by addition of the labeled ATP, and at various times, samples were spotted onto nitrocellulose filters, washed, and counted as described above. In Figure 3B, three time courses of NRI-N(K104Q) phosphorylation are shown. For these experiments, the reactions were conducted at 25 °C and were as in panel A except that the NRI-N(K104Q) concentration was 100 μM and the NRII concentration was 2, 4, or 8 μM, as indicated. For the experiments shown in Figure 3B, all components except NRI-N(K104Q) were preincubated for 4 min at 25 °C (to allow autophosphorylation of NRII), after which the reactions were started by the addition of NRI-N(K104Q). The time courses shown in Figure 3C are similar to those in Figure 3B, except without the preincubation phase. Conditions were like those described for panels A and B, except the NRI-N(K104Q) concentration was 120 μM and the reaction mixtures contained either 4 μM NRII and 0.04 mM α-ketoglutarate or 2 μM NRII and 0.03 mM α-ketoglutarate, as indicated. In Figure 3D, the time course of NRI-N(K104Q) phosphorylation was measured under different preincubation and incubation conditions, to determine the effect of order of addition of components. For these experiments, both the preincubation and incubation were conducted at 25 °C, and the final conditions were 100 μM NRI-N(K104Q), 8 μM NRII, 30 μM PII, 0.5 mM [γ-$^{32}$P]ATP,

0.3 mg/mL bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM KCl, with the α-ketoglutarate concentration being 0.03 or 10 mM, as indicated. Preincubation mixtures contained buffer and salts, NRII, PII, and ATP, with or without α-ketoglutarate, as indicated, and reactions were initiated by addition of NRI-N(K104Q) with or without α-ketoglutarate, as indicated. For the determination of the rate of NRI-N(K104Q) phosphorylation as a function of its concentration in Figure 3E, reactions were conducted at 25 °C and mixtures contained buffer and salts, with 2 $\mu$M NRII, 0.5 mM [$\gamma$-$^{32}$P]ATP, and 0.3 mg/mL bovine serum albumin, with NRI-N(K104Q) at the indicated concentrations. To equalize the glycerol concentration of all reaction mixtures, the storage buffer for NRI-N(K104Q) was used to balance the additions such that all samples had an identical contribution of the NRI-N storage buffer. This storage buffer consisted of 50 mM Tris-HCl (pH 7.5), 200 mM KCl, and 50% (v/v) glycerol. Reactions were initiated by addition of labeled ATP, and initial rates were determined from the early portions of time courses, where the reactions were visually linear, as described above. For the experiment shown in Figure 3F, initial rates of NRI-N(K104Q) phosphorylation were determined with 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 60 $\mu$M NRI-N(K104Q), 1 $\mu$M NRII, 0.5 mM [$\gamma$-$^{32}$P]ATP, 0.3 mg/mL bovine serum albumin, and 0.03 or 10 mM α-ketoglutarate, as indicated, and PII as indicated.

*NRII Autophosphorylation Assay*. NRII autophosphorylation was assessed as described previously (*26*). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 4 $\mu$M NRII, 0.5 mM [$\gamma$-$^{32}$P]ATP, and 0.3 mg/mL bovine serum albumin. For the experiment shown in Figure 4A, in which the dependence on α-ketoglutarate concentration was examined, reaction mixtures contained 40 $\mu$M PII and the α-ketoglutarate concentration was varied as indicated. For the experiments shown in Figure 4B, the α-ketoglutarate concentration was either 0.05 or 10 mM, as indicated, and the PII concentration was varied as indicated. Reactions were conducted at 25 °C and initiated by addition of the labeled ATP; at various times, samples were removed and spotted onto nitrocellulose filters, which were washed in 0.1 M $Na_2CO_3$ to remove unincorporated label and counted by liquid scintillation counting. As described previously, initial rates were obtained by linear regression of the data for early samples of the time courses, where the reactions visually appeared to be linear.

## RESULTS

*α-Ketoglutarate Controlled the Ability of PII To Activate the Regulated Phosphatase Activity of NRII but Did Not Alter the PII Activation Constant ($K_{act}$) for Activation of NRII by PII*. The PII-activated regulated phosphatase activity of NRII can be easily measured by following the rate of dephosphorylation of NRI-N-$^{32}$P in the absence of ATP and presence of a saturating amount of AMP-PNP, where no phosphorylation occurs. It is possible to provide NRI-N-$^{32}$P at a high concentration relative to that of NRII, such that the rate of release of label from NRI-N-$^{32}$P is linear for sufficient time to allow accurate measurement of initial rates (*25*). A replot of the initial rate of NRI-N-$^{32}$P dephosphorylation as a function of PII concentration (that is, a determination of the PII $K_{act}$), at low and high concentrations of α-ketoglutarate, is shown in Figure 2. Under the conditions used in these experiments, the basal phosphatase activity of NRII alone, which occurs in the absence of PII, accounts for a few percent of the observed rates, and in the absence of α-ketoglutarate, PII had no discernible effect (not shown). When the α-ketoglutarate concentration was 0.03 mM, PII was a 2.5-fold better activator than when the α-ketoglutarate concentration was 10 mM, but the concentration of PII required to activate NRII was essentially unchanged [apparent $K_{act}$ values of ~0.1 and ~0.15 $\mu$M, respectively (Figure 2)]. The estimated $K_d$ for PII binding to NRII from the experiments at 0.03 mM α-ketoglutarate is ~0.09 $\mu$M, reasonably close to estimates of 0.05 $\mu$M from previous studies (*25*).

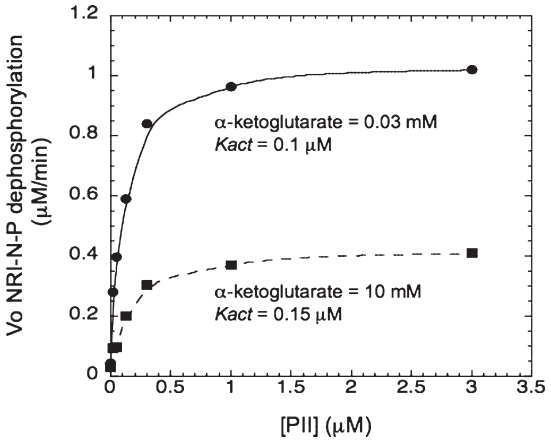


FIGURE 2: α-Ketoglutarate controlled the $k_{cat}$ of the PII-activated regulated phosphatase activity but did not significantly alter the PII activation constant ($K_{act}$). The initial rate of NRI-P dephosphorylation was measured at a fixed NRII concentration and various PII concentrations, as described in Materials and Methods. As shown, the ability of PII to activate was saturable at both high and low concentrations of α-ketoglutarate. Under the conditions shown, the $k_{cat}$ of the regulated phosphatase activity was regulated ~2.5-fold by α-ketoglutarate, while the activation constant for PII ($K_{act}$) was similar at high and low concentrations of α-ketoglutarate.

*α-Ketoglutarate Regulation of PII Inhibition of NRII Autophosphorylation*. The direct study of autophosphorylation rates, even for relatively slow reactions such as the NRII autophosphorylation, is intrinsically difficult because of the terminal nature of the reaction. It is possible to study the autophosphorylation reaction and PII regulation by using reaction conditions under which NRI-N dephosphorylates NRII as soon as it becomes autophosphorylated, and the rate of NRI-N phosphorylation is limited only by the rate of NRII autophosphorylation. This can be achieved, for example, by having NRI-N in large excess over NRII under conditions where the phospho-transfer reaction from autophosphorylated NRII to NRI-N is much faster than the rate of NRII autophosphorylation. To further clarify our studies, we used a mutant form of NRI-N containing the K104Q alteration that lacks both the autophosphatase activity and the ability to be dephosphorylated by the complex of NRII and PII [i.e., when phosphorylated, it is not a substrate for the regulated phosphatase activity (*16*)]. Thus, PII activity in this experimental system was limited to inhibition of the NRII autophosphorylation reaction. When the rate of NRI-N(K104Q) phosphorylation was measured in the presence of PII and NRII as a function of α-ketoglutarate concentration, biphasic regulation by α-ketoglutarate was obtained, as expected (Figure 3A). In the absence of α-ketoglutarate, PII had little effect on NRII, and the maximal rate of NRI-N(K104Q) phosphorylation was obtained (dotted line, Figure 3A). At 0.03 mM α-ketoglutarate, ~90% inhibition of phosphorylation was obtained, and this level of inhibition was gradually reduced

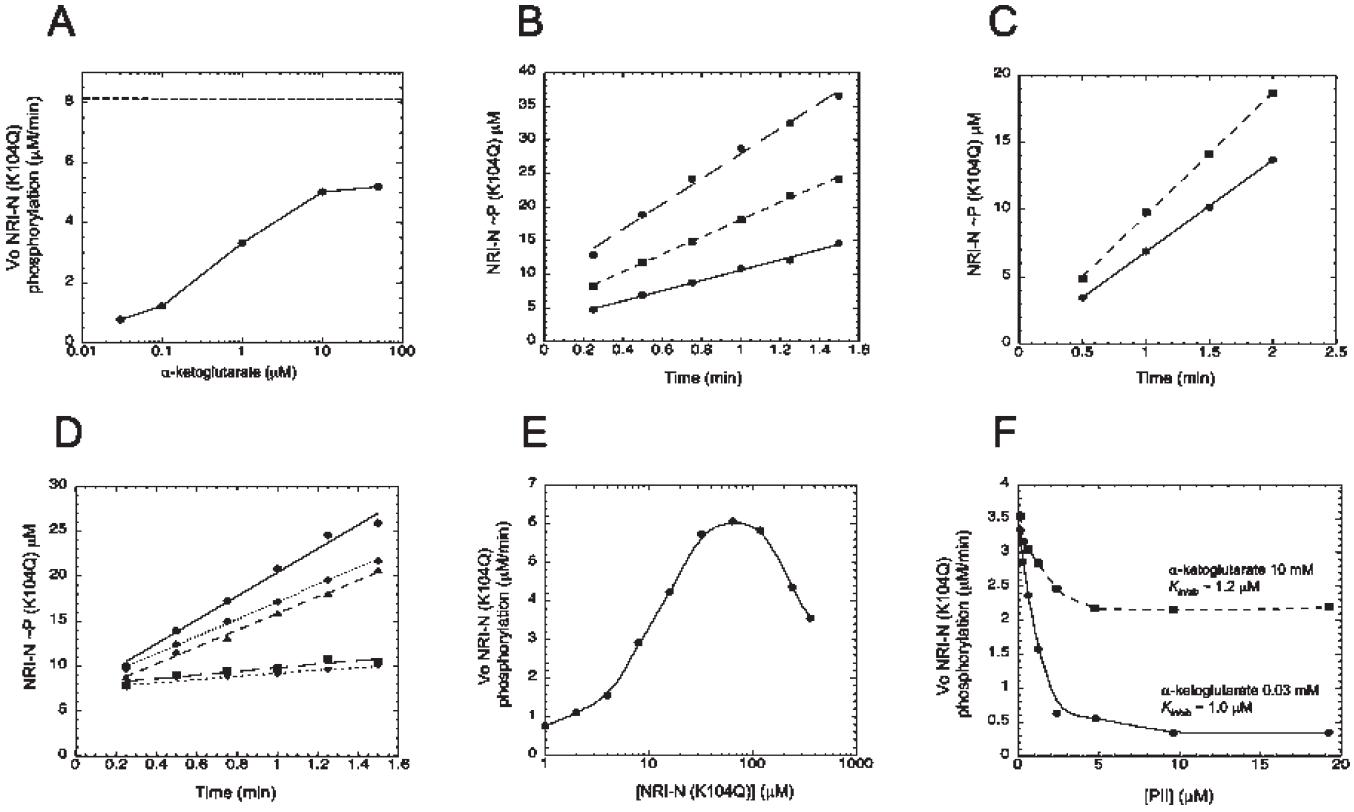


FIGURE 3: Indirect measurement of NRII autophosphorylation, and its regulation by PII and α-ketoglutarate, indicated that α-ketoglutarate controlled the ability of PII to inhibit NRII autophosphorylation but did not affect the PII inhibition constant ($K_{inhib}$). (A) α-Ketoglutarate provides biphasic regulation of the phosphorylation of NRI-N(K104Q) in reaction mixtures with fixed PII and NRII concentrations. Reaction conditions were as described in Materials and Methods and were such that the rate of NRII autophosphorylation limited the rate of NRI-N(K104Q) phosphorylation. α-Ketoglutarate regulated the ability of PII to inhibit NRII autophosphorylation; in the absence of α-ketoglutarate, NRII autophosphorylation was not inhibited (···). As shown, at low concentrations of α-ketoglutarate, severe inhibition was obtained, and this inhibition was weakened as the α-ketoglutarate concentration was increased. (B) When NRII autophosphorylation occurred in a preincubation step and NRI-N(K104Q) was subsequently added, the progress curves for NRI-N(K104Q) phosphorylation did not extrapolate back through the origin. Reactions were conducted as described in Materials and Methods: (—) 2 $\mu$M NRII, (---) 4 $\mu$M NRII, and (– – –) 8 $\mu$M NRII. (C) When there is no preincubation step, the progress curves for NRI-N(K104Q) phosphorylation extrapolated through the origin. Reactions were conducted as described in Materials and Methods: (---) 4 $\mu$M NRII and 0.04 mM α-ketoglutarate and (—) 2 $\mu$M NRII and 0.03 mM α-ketoglutarate. (D) α-Ketoglutarate control of NRI-N(K104Q) phosphorylation required conditions limited by the NRII autophosphorylation rate. The progress of NRI-N(K104Q) phosphorylation was followed in reaction mixtures containing fixed PII and NRII, as described in Materials and Methods. In all cases, NRII, PII, and ATP were preincubated for 10 min, which was sufficient time to allow for NRII autophosphorylation, even under conditions where PII would be able to inhibit the rate of autophosphorylation. Reactions were then started by addition of excess NRI-N(K104Q), and the progress of NRI-N phosphorylation was measured under these conditions, where NRII autophosphorylation was limiting: (—) α-ketoglutarate absent both during preincubation and after addition of NRI-N (no inhibtion of NRII autophosphorylation by PII); (···) preincubation in the absence of α-ketoglutarate and α-ketoglutarate added to 10 mM along with addition of NRI-N; (---) preincubation with 10 mM α-ketoglutarate, followed by addition of NRI-N; (– – –) preincubation with 0.03 mM α-ketoglutarate, followed by addition of NRI-N; and (...) preincubation in the absence of α-ketoglutarate, followed by α-ketoglutarate being added to a final concentration of 0.03 mM along with NRI-N. (E) Dependence of the rate of NRI-N(K104Q) phosphorylation on its concentration in experiments with fixed NRII. Reactions were as described in Materials and Methods; $V_{max}$ under the conditions used was ~6.3 $\mu$M/min, and "$K_m$" for NRI-N(K104Q) was ~10.7 $\mu$M. Note that rates were slower in this experiment than in others due to an increased glycerol concentration in this experiment (Materials and Methods), and significant substrate inhibition was found at high concentrations of NRI-N(K104Q). (F) α-Ketoglutarate regulated the extent of NRI-N(K104Q) phosphorylation in reaction mixtures containing NRII and PII but did not influence the PII inhibition constant ($K_{inhib}$). Reactions were conducted as described in Materials and Methods: (---) 10 mM α-ketoglutarate and (—) 0.03 mM α-ketoglutarate.

to ~65% inhibition as the α-ketoglutarate concentration was increased to 50 mM (Figure 3A). Since the NRI-N(K104Q) protein had not been extensively studied, control experiments were performed to verify that the relationship between autophosphorylation and phospho-transfer rates was as expected. To examine whether the autophosphorylation of NRII was limiting under the conditions used, the initial rate of NRI-N(K104Q) phosphorylation was examined as a function of NRII concentration, in experiments where NRII was preincubated to allow it to become autophosphorylated before addition of NRI-N(K104Q) (Figure 3B). The levels of NRI-N(K104Q) phosphorylation in this experiment did not extrapolate to the origin, and increasing levels of NRII resulted in higher rates of NRI-N(K104Q) phosphorylation. Under similar conditions when NRI-N(K104Q) was present at the outset such that NRII did not have an opportunity to accumulate in an autophosphorylated form, the levels of NRI-N(K104Q) did extrapolate to the origin (Figure 3C). The simplest explanation for these results is that the rate of NRII autophosphorylation was limiting, as expected (*16*). In a related set of experiments, we also examined the effect of order of addition of α-ketoglutarate to reaction mixtures. In Figure 3D, we preincubated NRII and PII with ATP and then added NRI-N(K104Q) in excess. When α-ketoglutarate was absent, addition of NRI-N(K104Q) led to its rapid phosphorylation, as the rate of autophosphorylation of NRII was not inhibited by PII. However, when α-ketoglutarate was present at a

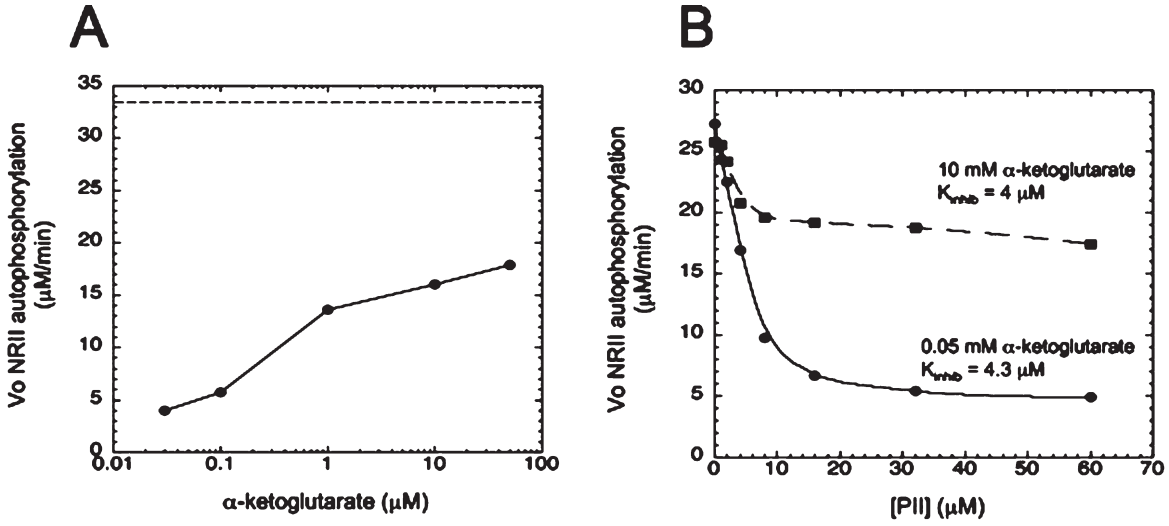


FIGURE 4: Direct measurement of NRII autophosphorylation, and its regulation by PII and α-ketoglutarate, indicated that α-ketoglutarate controlled the ability of PII to inhibit NRII autophosphorylation but did not affect the PII inhibition constant ($K_{inhib}$). (A) α-Ketoglutarate provided biphasic regulation of NRII autophosphorylation in reaction mixtures containing a fixed PII concentration. Reactions were conducted as described in Materials and Methods. In the absence of α-ketoglutarate, PII failed to inhibit NRII autophosphorylation (···). At low concentrations of α-ketoglutarate, PII provided effective inhibition of NRII autophosphorylation, and this inhibition was weakened as the concentration of α-ketoglutarate was increased. (B) α-Ketoglutarate regulated the rate of NRII autophosphorylation in reaction mixtures containing NRII and PII but did not influence the PII inhibition constant ($K_{inhib}$): (---) 10 mM α-ketoglutarate and (—) 0.05 mM α-ketoglutarate.

concentration of 0.03 mM, addition of NRI-N(K104Q) did not result in its phosphorylation, as NRII autophosphorylation was effectively inhibited by PII (Figure 3D). When α-ketoglutarate was present at a concentration of 10 mM, the rate of NRI-N(K104Q) phosphorylation was hardly inhibited. Thus, biphasic regulation by α-ketoglutarate was again obtained. The effects of α-ketoglutarate were the same in the experiment, regardless of whether it was present from the outset or added along with NRI-N(K104Q) (Figure 3D). This is as expected if the rate of small molecule binding and unbinding is much more rapid than the protein interactions or catalytic steps, and if α-ketoglutarate effects were only important under conditions where NRII autophosphorylation were limiting (such as after the addition of NRI-N(K104Q). For our experiments, we wanted NRI-N to be in excess, such that reactions were limited only by NRII autophosphorylation. We observed that the rate of NRI-N(K104Q) phosphorylation was increased as its concentration was increased to ~100 μM, after which there was significant inhibition of phosphorylation (Figure 3E). This seemed to be genuine substrate inhibition; perhaps unphosphorylated NRI-N(K104Q) bound to unphosphorylated NRII and inhibited its autophosphorylation. The substrate constant $K_M$ seemed to be ~11 μM (deduced from the ascending part of the curve in Figure 3E). To avoid substrate inhibition, we used NRI-N(K104Q) at 60 μM to determine the PII $K_{inhib}$ at different α-ketoglutarate concentrations, in experiments where the NRII concentration was 1 μM. Under these conditions, α-ketoglutarate had a dramatic effect on the ability of PII to inhibit NRI-N(K104Q) phosphorylation but had no significant effect on $K_{inhib}$ [~1 and ~1.2 μM at 0.03 mM α-ketoglutarate and 10 mM α-ketoglutarate, respectively (Figure 3F)]. Thus, the binding of PII to NRII was not significantly affected by α-ketoglutarate under conditions where the NRII autophosphorylation reaction was limiting, the phospho-transfer reaction was faster than the autophosphorylation reaction, significant substrate inhibition by NRI-N was not a factor, and PII inhibition of NRII autophosphorylation activity was controlled by α-ketoglutarate.

*Direct Examination of the NRII Autophosphorylation Rate*. Although, as already mentioned, studies of terminal activities, such as autophosphorylation, are intrinsically difficult and consume a considerable amount of enzyme, we also directly measured PII inhibition of autophosphorylation and α-ketoglutarate's role in this process as a check on the methods and results of the preceding section. When PII was present in excess (40 μM) over NRII (4 μM), α-ketoglutarate was a potent regulator of the rate of NRII autophosphorylation (Figure 4A). In the absence of α-ketoglutarate, PII did not inhibit NRII autophosphorylation and the maximal rate was attained [Figure 4A (···)]; a similar result was obtained when PII was omitted (not shown). At low concentrations of α-ketoglutarate, PII was a potent inhibitor of autophosphorylation, but at high concentrations of α-ketoglutarate, PII was a less effective inhibitor of autophosphorylation (Figure 4A). These results were as expected on the basis of prior studies. Even though α-ketoglutarate provided strong control of PII inhibition of NRII autophosphorylation, it had no significant effect on the PII inhibition constant ($K_{inhib}$). In the experiment shown in Figure 4B, the NRII concentration was 4 μM, autophosphorylation reactions were conducted at 25 °C, and the PII $K_{inhib}$ was ~4 μM at 10 mM α-ketoglutarate and ~4.3 μM at 0.05 mM α-ketoglutarate. In another experiment conducted with 2 μM NRII at 0 °C, where the extent of inhibition was reduced relative to that at 25 °C, PII displayed a $K_{inhib}$ of 1.2 μM at 0.03 mM α-ketoglutarate and a $K_{inhib}$ of 1.0 μM at 10 mM α-ketoglutarate (not shown). In yet another experiment conducted with 5 μM NRII at 0 °C, PII displayed a $K_{inhib}$ of 4 μM at 0.03 mM α-ketoglutarate and a $K_{inhib}$ of 5.5 μM at 10 mM α-ketoglutarate. Thus, we did not discern a significant difference in the PII $K_{inhib}$ values at different α-ketoglutarate concentrations in experiments where α-ketoglutarate had a very significant effect on the extent of inhibition by PII.

*PII Bound NRII Equally Well at High and Low α-Ketoglutarate Concentrations*. We directly examined the binding of PII to NRII using nondenaturing gel electrophoresis. For these experiments, binding required the presence

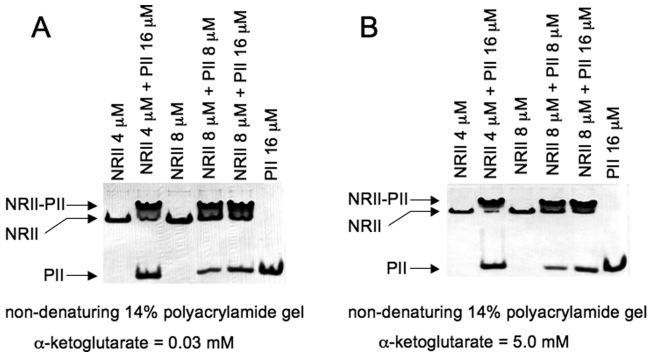


FIGURE 5: Nondenaturing gel electrophoresis binding assay for the interaction of PII and NRII. Procedures are described in Materials and Methods; the concentrations of proteins in the initial incubation mixtures are shown above each gel lane. A constant volume of each sample was loaded, such that the proportions of proteins in the gel parallel their initial concentrations. (A) Reaction mixtures, gel, and running buffer contained 0.03 mM α-ketoglutarate. (B) Reaction mixtures, gel, and running buffer contained 5.0 mM α-ketoglutarate. The bands corresponding to PII, NRII, and the complex of PII and NRII are indicated.

of α-ketoglutarate in the initial incubation mixtures, as well as in the gels and the electrophoresis buffer (not shown). However, the binding of PII to NRII seemed to occur approximately equally when the α-ketoglutarate concentration was 0.03 or 5 mM (Figure 5). Indeed, binding of PII to NRII appeared to be slightly improved at 5 mM α-ketoglutarate, relative to 0.03 mM α-ketoglutarate, but the band consisting of the complex seemed to have a slightly reduced relative mobility (Figure 5). We will show elsewhere that under the conditions used here, the band corresponding to the PII−NRII complex, when excised from the gel, contained both NRII and PII and that in these experiments, each NRII dimer bound to two PII trimers.

## DISCUSSION

The simplest explanation for the data shown here is that α-ketoglutarate did not affect the binding of PII to NRII but controlled the ability of PII to activate the NRII-regulated phosphatase activity or inhibit NRII autophosphorylation once PII was bound to NRII. We measured the $K_{act}$ and $K_{inhib}$ of PII for the activation of the NRII-regulated phosphatase activity and the autophosphorylation activity, respectively. While these $K_{act}$ and $K_{inhib}$ are not binding constants ($K_d$), in the absence of highly unusual behavior such as conformational memory or sticky behavior of binding partners, they should be related to $K_d$ in some simple way. Whatever their relationship to $K_d$, $K_{act}$ and $K_{inhib}$ did not appreciably change under conditions where α-ketoglutarate had dramatic effects on NRII activity. Thus, either α-ketoglutarate did not affect the binding of PII to NRII, or this effector changed the activation and inhibition mechanism such that identical $K_{inhib}$ and $K_{act}$ were obtained by coincidence. Yet, even this unlikely explanation would fail to explain the results of direct visualization of the binding of PII to NRII using nondenaturing gel electrophoresis, where it was observed that α-ketoglutarate was required for binding of PII to NRII, and that similar complex formation was obtained at high and low concentrations of α-ketoglutarate. Thus, we must reject the hypothesis that variation of the α-ketoglutarate concentration through its physiological range controls the binding of PII to NRII. Instead, α-ketoglutarate apparently controls the ability of PII to regulate NRII activities at a step that occurs after the binding of PII to NRII. Since a similar pattern of regulation by α-ketoglutarate was observed when the PII-mediated activation of the adenylyltransefrase activity of ATase was examined (*23*), it is possible that this describes a general mechanism for the transduction of α-ketoglutarate signals by PII proteins, at least when PII is saturated with ATP as it was in our experiments.

If it is true that α-ketoglutarate controls the ability of PII to activate or inhibit receptor activities in a postbinding step, then PII must contain distinct structural elements involved in initial receptor binding and in control of receptor activities. These structural elements need not be distant from one another on the PII surface or stable in the absence of the binding partner, and indeed, α-ketoglutarate may exert its effects by controlling the flexibility or other dynamical aspects of PII conformational transitions. We earlier showed that conservative amino acid substitutions in the large T-loop of PII could alter the relative ability of PII to control ATase and NRII (*27*). For example, the E50Q substitution within the T-loop improved the ability of PII to activate NRII, relative to its ability to activate ATase, while the Y51F substitution improved the ability of PII to activate ATase relative to its ability to activate NRII. We will show elsewhere that these mutations in the T-loop appear to affect the ability of the altered PII proteins to bind to NRII and ATase; specifically, they significantly altered the $K_{act}$ for PII activation of ATase and NRII activities. [Thus, we could detect variations in the PII $K_{act}$ for activation of receptors, using assays similar to those used here (ref *27* and unpublished data).] Furthermore, a small internal deletion of seven amino acids from the apex of the T-loop resulted in an altered form of PII that failed to bind to either NRII or ATase (*27*). From these results, we concluded that some portions of the T-loop of PII appear to be directly involved in the binding of PII to NRII and ATase (*27*). This conclusion was strongly supported by cross-linking studies, where certain positions within the T-loop were found to become cross-linked to NRII and ATase (*22*, *24*). A working hypothesis is that α-ketoglutarate controls the conformation of the T-loop such that, after binding to the receptor, PII is either able or unable to make contacts necessary for the regulation of receptor activities. Since the regulatory effects of α-ketoglutarate are obtained when PII trimers make the transition between singly liganded and saturated states, it seems that intramolecular signaling between the subunits of the PII trimer controls the conformation of the T-loops and, consequently, the ability to regulate receptor activities. The observed anticooperativity of α-ketoglutarate binding to the three sites of the PII trimer is consistent with a strong conformational coupling of the subunits within the PII trimer.

A paradigm for the activation mechanism discussed above is provided by the control of transcription initiation by RNA polymerase at certain gene promoters in bacteria. The process of transcription initiation involves a complex sequence of conformational transitions by both the multisubunit RNA polymerase and the DNA template, leading to the formation of an active "open" transcription complex in which the two strands of the DNA have been melted and are bound within two separate channels on the enzyme surface. Deficiencies at a variety of points along the way may result in weak promoters that require activator proteins to enable transcription; some promoters fail to successfully bind to (recruit) RNA polymerase, while other promoters bind RNA polymerase quite avidly but are defective in one or more of the isomerization steps leading to the open transcription complex (*28*). For the latter class of promoters,

activator proteins may act to stimulate the rate of transcription by increasing the rate of the limiting isomerization step (*7*−*9*, *28*). In some cases, these activators do not influence the initial binding of RNA polymerase to the promoter DNA sequence but act solely to accelerate the limiting isomerization step (*7*−*9*, *28*). In the case at hand, PII bound to its receptor NRII equally well at both high and low concentrations of α-ketoglutarate, but its ability to regulate receptor activities at high concentrations of α-ketoglutarate was weakened. Apparently, after binding to NRII, PII activates the regulated phosphatase activity and inhibits the autophosphorylation activity by bringing about NRII conformational transitions that otherwise would be very infrequent. Its ability to bring about those conformational transitions is in turn regulated by α-ketoglutarate.

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