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†

Peng Jiang‡ and Alexander J. Ninfa*

Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

Received July 8, 2009; Revised Manuscript Received October 29, 2009

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)3 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 ς52-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

3Abbreviations: 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 2.7.7.49) and product of the glnE gene; GS, glutamine synthetase and product of the glnA gene.

†To whom correspondence should be addressed. A.J.N.: e-mail, aninfa@umich.edu; phone, (734) 763-8065; fax, (734) 764-3509. P.J.: e-mail, pejiang@umich.edu; phone, (734) 763-8064; fax, (734) 764-3509.

‡Supported by Grant GM59637 from the National Institute of General Medical Sciences to A.J.N.
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 information 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 NRII 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 adenyl transferase (AT) activity of ATase catalyzes the adenyllylation of glutamine synthetase (GS), while the adenyl-removing (AR) activity of 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 MgCl₂, 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 MgCl₂, 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 NRII-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 NRII-N(K104Q), 0.3 mg/mL bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 100 mM KCl, with the α-ketoglutarate concentration varied as indicated. The phosphorylation of NRII-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 NRII-N(K104Q) phosphorylation are shown. For these experiments, the reactions were conducted at 25 °C and were as in panel A except that the NRII-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 NRII-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 NRII-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 NRII-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 NRII-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 NRII-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 MgCl₂, 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 μM NRII, 0.5 mM [γ-32P]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 MgCl₂, 100 mM KCl, 60 μM NRI-N(K104Q), 1 μM NRII, 0.5 mM [γ-32P]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 MgCl₂, 100 mM KCl, 4 μM NRII, 0.5 mM [γ-32P]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 μM 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 Na₂CO₃ 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 (Kact) 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 NRII-N-32P in the absence of ATP and presence of a saturating amount of AMP-PNP, where no phosphorylation occurs. It is possible to provide NRII-N-32P at a high concentration relative to that of NRII, such that the rate of release of label from NRII-N-32P is linear for sufficient time to allow accurate measurement of initial rates (25). A plot of the initial rate of NRII-N-32P dephosphorylation as a function of PII concentration (that is, a determination of the PII Kact), 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 Kact, values of ∼0.1 and ∼0.15 μM, respectively (Figure 2)). The estimated Kact for PII binding to NRII from the experiments at 0.03 mM α-ketoglutarate is ∼0.09 μM, reasonably close to estimates of 0.05 μM from previous studies (25).

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 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.
levels of NRII resulted in higher rates of NRI-N(K104Q) phosphorylation in this experiment did not extrapolate back through the origin. Reactions were conducted as described in Materials and Methods: (---) 2 μM NRII, (---) 4 μM NRII, and (---) 8 μM 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 μM NRII and 0.04 mM α-ketoglutarate and (---) 2 μM NRII and 0.03 mM α-ketoglutarate. (D) α-Ketoglutarate regulated the extent 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 inhibition of NRII autophosphorylation by PII); (---) α-ketoglutarate in the absence of α-ketoglutarate and α-ketoglutarate added to 10 μM along with addition of NRI-N; (---) 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; \( F_{\text{max}} \) under the conditions used was \( \sim 6.3 \mu M/min \), and \( K_m \) for NRI-N(K104Q) was \( \sim 10.7 \mu M \). 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 \( (K_{\text{inhib}}) \). Reactions were conducted as described in Materials and Methods: (---) 10 mM α-ketoglutarate and (---) 0.03 mM α-ketoglutarate.

The levels of NRI-N(K104Q) phosphorylation in this experiment did not extrapolate back through the origin. Reactions were conducted as described in Materials and Methods: (---) 2 μM NRII, (---) 4 μM NRII, and (---) 8 μM 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 μM NRII and 0.04 mM α-ketoglutarate and (---) 2 μM NRII and 0.03 mM α-ketoglutarate. (D) α-Ketoglutarate regulated the extent 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 inhibition of NRII autophosphorylation by PII); (---) α-ketoglutarate in the absence of α-ketoglutarate and α-ketoglutarate added to 10 μM along with addition of NRI-N; (---) 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; \( F_{\text{max}} \) under the conditions used was \( \sim 6.3 \mu M/min \), and \( K_m \) for NRI-N(K104Q) was \( \sim 10.7 \mu M \). 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 \( (K_{\text{inhib}}) \). Reactions were conducted as described in Materials and Methods: (---) 10 mM α-ketoglutarate and (---) 0.03 mM α-ketoglutarate.

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 (6). 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
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 NRII-N(K104Q) phosphorylation was hardly inhibited. Thus, biphasic regulation by α-ketoglutarate was again obtained. The effect 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 NRII-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 NRII-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 NRII-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 $K_{inhb}$ 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 NRII-N(K104Q) phosphorylation but had no significant effect on $K_{inhb}$ of ∼1 and ∼12 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 NRII-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 ($K_{inhb}$). In the experiment shown in Figure 4B, the NRII concentration was 4 μM, autophosphorylation reactions were conducted at 25 °C, and the PII $K_{inhb}$ 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 $K_{inhb}$ of 1.2 μM at 0.03 mM α-ketoglutarate and a $K_{inhb}$ of 10 μM at 10 mM α-ketoglutarate (not shown). In yet another experiment conducted with 5 μM NRII at 0 °C, PII displayed a $K_{inhb}$ of 4 μM at 0.03 mM α-ketoglutarate and a $K_{inhb}$ of 5.5 μM at 10 mM α-ketoglutarate. Thus, we did not discern a significant difference in the PII $K_{inhb}$ 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
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 $K_{\text{act}}$ and $K_{\text{inhib}}$ of PII for the activation of the NRII-regulated phosphatase activity and the autophosphorylation activity, respectively. While these $K_{\text{act}}$ and $K_{\text{inhib}}$ 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 $K_d$, $K_{\text{act}}$ and $K_{\text{inhib}}$ 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 $K_{\text{inhib}}$ and $K_{\text{act}}$ 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 adenylyltransferase 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 $K_{\text{act}}$ for PII activation of ATase and NRII activities. [Thus, we could detect variations in the PII $K_{\text{act}}$ 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.

ACKNOWLEDGMENT

We thank Patrick O’Brien and David Ballou for helpful suggestions.

REFERENCES

1. Ninfa, A. J., and Atkinson, M. R. (2000) PII signal transduction proteins. Trends Microbiol. 8, 172–179.
2. Ninfa, A. J., and Jiang, P. (2005) PII signal transduction proteins: Sensors of α-ketoglutarate that regulate nitrogen metabolism. Curr. Opin. Microbiol. 8, 168–173.
3. Ninfa, A. J., Jiang, P., Atkinson, M. R., and Peliska, J. A. (2000) Integration of antagonistic signals in the regulation of bacterial nitrogen assimilation. Curr. Top. Cell. Regul. 36, 32–76.
4. Jiang, P., and Ninfa, A. J. (2007) The Escherichia coli PII signal transduction protein controlling nitrogen assimilation acts as a sensor of adenylate energy charge in vitro. Biochemistry 46, 12976–12986.
5. Jiang, P., Peliska, J., and Ninfa, A. J. (1998) Reconstitution of the signal transduction bicyclic cascade responsible for the regulation of Ntr gene transcription in Escherichia coli. Biochemistry 37, 12795–12801.
6. Stock, J. B., Ninfa, A. J., and Stock, A. M. (1989) Protein phosphorylation and the regulation of adaptive responses in bacteria. Microbiol. Rev. 53, 450–490.
7. Ninfa, A. J., Reitzer, L. J., and Magasanik, B. (1987) Initiation of transcription at the bacterial glnAp2 promoter by purified Escherichia coli components is facilitated by enhancers. Cell 50, 1039–1046.
8. Popham, D. L., Szeto, D., Keener, J., and Kustu, S. (1989) Function of a bacterial activator protein that binds to transcriptional enhancers. Science 243, 629–635.
9. Su, W., Porter, S., Kustu, S., and Echols, H. (1990) DNA-looping and enhancer activity: Association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter. Proc. Natl. Acad. Sci. U.S.A. 87, 5504–5508.
10. Pioszak, A. A., and Ninfa, A. J. (2003) How do the domains of NRII collaborate in the PII-activated phosphatase activity of Escherichia coli NRII (NtrB)? Biochemistry 42, 8885–8899.
11. Keener, J., and Kustu, S. (1988) Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: Roles of the conserved amino-terminal domain of NTRC. Proc. Natl. Acad. Sci. U.S.A. 85, 4976–4980.
12. Weiss, V., and Magasanik, B. (1988) Phosphorylation of Nitrogen Regulator I of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 85, 8919–8923.
13. Ninfa, A. J., and Bennett, R. L. (1991) Identification of the Site of Autophosphorylation of the Bacterial Protein Kinase:Phosphatase NRII. J. Biol. Chem. 266, 6888–6893.
14. Sanders, D. A., Gillee-Castro, B. L., Burlingame, A. L., and Koshland, D. E., Jr. (1992) Phosphorylation site of NtrC, a protein phosphatase whose covalent intermediate activates transcription. J. Bacteriol. 174, 5117–5122.
15. Ninfa, A. J., and Magasanik, B. (1986) Covalent modification of the glnA product, NR1A, by the phosphatase of the PII signal transduction system of the glnALG operon in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 83, 5909–5913.
16. Pioszak, A. A., and Ninfa, A. J. (2003) Genetic and biochemical analysis of the PII-dependent phosphatase activity of Escherichia coli NRII. J. Bacteriol. 185, 1299–1315.
17. Jiang, P., and Ninfa, A. J. (1999) Regulation of the autophosphorylation of Escherichia coli NRII by the PII signal transduction protein. J. Bacteriol. 181, 1906–1911.
18. Kamberov, E. S., Atkinson, M. R., and Ninfa, A. J. (1995) The Escherichia coli PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP. J. Biol. Chem. 270, 17797–17807.
19. Jiang, P., Peliska, J. A., and Ninfa, A. J. (1998) The regulation of glutamine synthetase covalent modification revisited: Role of 2-ketoglutarate in the regulation of glutamine synthetase adenyllylation state. Biochemistry 37, 12802–12810.
20. Senior, P. J. (1975) Regulation of nitrogen metabolism in Escherichia coli and Klebsiella aerogenes: Studies with the continuous-culture technique. J. Bacteriol. 137, 407–418.
21. Atkinson, M. R., and Ninfa, A. J. (1998) Role of the glnK gene in nitrogen-regulation of gene transcription in Escherichia coli. Mol. Microbiol. 29, 431–447.
22. Jiang, P., Pioszak, A. A., and Ninfa, A. J. (2007) Structure/function analysis of glutamine synthetase adenylyltransferase (ATase, EC 2.7.7.49) of Escherichia coli. Biochemistry 46, 4117–4132.
23. Jiang, P., Mayo, A. E., and Ninfa, A. J. (2007) Escherichia coli glutamine synthetase adenylyltransferase (ATase, EC 2.7.7.49): Kinetic characterization of regulation by PII, PII-UMP, glutamine, and α-ketoglutarate. Biochemistry 46, 4133–4146.
24. Pioszak, A. A., Jiang, P., and Ninfa, A. J. (2000) The Escherichia coli PII signal transduction protein regulates the activities of the two-component system transmitter protein NRII by direct interaction with the kinase domain of the transmitter module. Biochemistry 39, 13450–13461.
25. Jiang, P., Atkinson, M. R., Srisawat, C., Sun, Q., and Ninfa, A. J. (2000) Functional dissection of the dimerization and enzymatic activities of Escherichia coli nitrogen regulator II and their regulation by the PII protein. Biochemistry 39, 13433–13449.
26. Jiang, P., and Ninfa, A. J. (2000) Asymmetry in the autophosphorylation of the two-component system transmitter protein NRII (NtrB) of Escherichia coli. Biochemistry 39, 5058–5065.
27. Jiang, P., Zucker, P., Atkinson, M. R., Kamberov, E. S., Tirasophon, W., Chandran, P., Schefke, B. R., and Ninfa, A. J. (1997) Structure/function analysis of the PII signal transduction protein of Escherichia coli: Genetic separation of interactions with protein receptors. J. Bacteriol. 179, 4342–4353.
28. McClure, W. R. (1985) Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. 54, 171–204.