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

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ABSTRACT: The PII signal transduction protein regulates the transcription of nitrogen-regulated genes by controlling the kinase and phosphatase activities of NRII. We used a cross-linking approach to study the interaction of the T-loop of the PII protein with NRII. Cross-linking of PII to NRII required ATP and 2-ketoglutarate, allosteric effectors known to control PII activity, and was not affected by the presence of excess nonspecific proteins such as bovine serum albumin. The purified cross-linked species appeared to consist mainly of PII trimers in which one of the three subunits was cross-linked to a single subunit of the NRII dimer; this complex had the phosphatase activity characteristic of the un-cross-linked PII−NRII complex, and had significant phosphatase activity in the absence of 2-ketoglutarate, suggesting that once PII was tethered to NRII the active conformation was stabilized. Studies with truncated forms of NRII indicated that the purified N-terminal "sensory" domain of NRII was not cross-linked to PII, nor was a polypeptide consisting of NRII residues 1−189. In contrast, polypeptides containing the kinase domain of the transmitter module of NRII (residues 190−349) were cross-linked to PII in an ATP- and 2-ketoglutarate-dependent reaction. These results indicate that PII controls NRII by interaction with the conserved kinase domain of the transmitter module.

The two-component regulatory systems comprise the largest family of related signal transduction systems in nature, with representatives in bacteria, archaea, lower eukaryotes, and plants (for reviews, see refs *1* and *2*). These signal transduction systems regulate target activities by a conserved phospho-transfer mechanism involving the transfer of phosphoryl groups from ATP to conserved histidine residues on "transmitter" proteins to conserved aspartate residues on "receiver" proteins. In *Escherichia coli*, the cellular responses to nitrogen starvation, osmotic stress, and other physiological processes are regulated by such binary systems. In other cases, phosphoryl groups are passed from transmitter to receiver, then to another histidine residue on yet another type of conserved protein domain, and from there to the aspartate on yet another receiver protein, forming a phospho relay. For both variations of the basic theme, the phosphorylation state of the ultimate receiver controls the activity of the regulatory target, which is usually RNA polymerase at specific gene promoters. Stimuli may act by increasing or decreasing the rate of flow of phosphoryl groups to the ultimate receiver, or by stimulating the dephosphorylation of the ultimate receiver.

The dimeric transmitter proteins bind ATP and phosphorylate themselves on a conserved histidine motif by a trans-intramolecular mechanism in which one subunit phosphorylates the opposing subunit in the dimer (*3*−*6*). From this site, phosphoryl groups are transferred to the receiver in a reaction that is probably catalyzed by the receiver (*7*, *8*). Many receiver proteins can also be phosphorylated directly by metabolic intermediates such as acetyl phosphate (*9*, *10*). In addition to their kinase activity, many transmitter proteins display a phosphatase activity that brings about the rapid dephosphorylation of the phosphorylated receiver (*11*). Thus, the transmitter protein may be both a positive and negative regulator of the phosphorylation state of the receiver in intact cells or reconstituted systems (*12*, *13*). When the counterpoised kinase and phosphatase activities of the transmitter are balanced, rheostat-like control of the phosphorylation state of the receiver may be obtained (*13*, *14*).

The transmitter proteins consist of the conserved transmitter module typically flanked by one or more nonconserved domains. Most frequently, an unconserved N-terminal membrane-spanning domain is found, fused to the transmitter module. In some cases, such as the two Nar transmitter proteins (NarX and NarQ), there is a considerable periplasmic domain involved in the detection of the extracellular signals (*15*). In other cases, such as FixL, the membrane-spanning part serves only to anchor the protein to the membrane, and a cytoplasmic N-terminal domain is involved in sensation (*16*). These N-terminal domains may regulate the balance of the kinase and phosphatase activities of the associated transmitter module (*14*, *17*). In the case of the enteric bacteria chemotaxis system, the CheA transmitter lacks phosphatase activity, and stimuli control the rate of CheA autophosphorylation by acting through transmembrane receptors and an adaptor protein. The receptors and adaptor interact with a

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segment of CheA at the C-terminal side of the transmitter module (*18*). Finally, it has recently been observed that factors may interact directly with the transmitter module itself to regulate its activities (*19*). Thus, signals may impinge upon the transmitter module in a variety of ways. Interestingly, there are examples in nature of proteins that consist entirely of the conserved transmitter module, and in these cases, regulation must be either strictly at the level of synthesis or due to factors that interact with the transmitter module itself.

The transmitter module consists of two domains separated by a short linker. The N-terminus of these is a dimerization/phospho-transfer domain bearing the site of transmitter autophosphorylation (*20*−*22*); the C-terminal is a kinase domain with an ATP site related to Hsp90 proteins (*23*, *24*). The dimerization/phospho-transfer domain may also be responsible for the phosphatase activity of the transmitter module (*22*, *25*).

The NRII/NRI[1] (NtrB/NtrC) two-component system plays a key role in the regulation of nitrogen assimilation in enteric bacteria (for a review, see ref *26*). The response to nitrogen starvation includes the activation of transcription initiation of several genes, including the structural gene for glutamine synthetase, *glnA*. The transcriptional activator, NRI∼P, consists of three domains. The N-terminal domain is the conserved receiver domain; the central domain is involved in transcriptional activation, and the C-terminal domain is responsible for binding the enhancer elements upstream from nitrogen-regulated promoters. The phosphorylation and dephosphorylation of the receiver domain of NRI are catalyzed by the NRII transmitter protein, which thus indirectly controls the rate of transcription initiation by controlling the concentration of NRI∼P (*11*). The kinase and phosphatase activities of NRII are reciprocally regulated by the binding of the PII signal transduction protein to NRII. The binding of PII to NRII activates the phosphatase activity of NRII and decreases the rate of NRII autophosphorylation (*27*). The interaction of PII with NRII is regulated by signals of intracellular nitrogen and carbon status. Nitrogen status controls the uridylylation state of PII due to reciprocal regulation of the activities of the bifunctional uridylyltransferase/uridylyl-removing enzyme (product of *glnD*) by glutamine; the uridylylation of PII when the level of glutamine is low prevents the interaction of PII with NRII (*28*, *29*). Carbon status regulates the interaction of unmodified PII with NRII by affecting the availability of 2-ketoglutarate, a key allosteric effector controlling the binding of PII to NRII and its other receptors (*28*, *30*, *31*). A low concentration of 2-ketoglutarate, resulting in binding of a single molecule of 2-ketoglutarate to the trimeric PII, favors the interaction of PII with NRII. A high concentration of 2-ketoglutarate, resulting in the binding of three molecules of this effector to the trimeric PII, reduces the level of interaction of PII with NRII (*28*, *30*, *32*). This allosteric regulation of PII by 2-ketoglutarate may represent a mechanism for the regulation of NRII activities by carbon status (for a review, see ref *26*). Thus, antagonistic signals of carbon and nitrogen status are integrated by PII and communicated to NRII, controlling the phosphorylation state of NRI and thus the expression of nitrogen-regulated genes.

NRII consists of the transmitter module linked to a small N-terminal domain bearing a PAS motif. PAS motifs are sometimes involved in the binding of sensory ligands such as heme (*16*) and in other cases are involved in the interactions between domains (*33*). In this report, we describe studies of the interaction of PII and NRII using a protein cross-linking approach. Our results indicated that PII regulated the kinase and phosphatase activities of NRII by binding to the kinase domain of the transmitter module of NRII. In the accompanying paper (*22*), functional dissection of the activities of NRII and their regulation by PII also suggested that PII interacted with the conserved transmitter module of NRII.

MATERIALS AND METHODS

Construction of Site-Specific Mutants of glnB, Encoding Altered PII Proteins. Mutations were introduced into *glnB* by oligonucleotide-based mutagenesis with the Altered Sites II *in vitro* mutagenesis kit (Promega), used according to the manufacturer's directions. The native cysteine 73 of PII was mutated to serine, and subsequent cysteine mutants were introduced at positions 44 and 50 in the C73S background. All constructs were sequenced over the entire coding region with a Sequenase kit (Amersham-USB) to ensure that only the desired mutations were introduced. The mutagenic primers were 5′-GTCGATACCTCTGTCGATACC-3′ (C73S), 5′-GGCCATACCTGTCTGTACCGCGGC-3′ (E44C), and 5′-GTACCGCGGCGCGTGTTATATGGTGG-3′ (E50C). Overexpression plasmids were constructed for the *glnB* alleles as described previously (*35*). This involved subcloning a *glnB Nde*I−*Bam*HI fragment into similarly digested pJLA503. These plasmids were transformed into strain RB9060 (Δ*glnB*) (*12*), with selection for ampicillin resistance. Growth of cultures for protein purification was carried out in LB medium at 30 °C, and induction was carried out by raising the temperature to 44 °C as described previously (*35*).

Purified Proteins. Purification of wild-type PII was carried out as described previously (*36*) except that the hydroxylapatite step was replaced with a phenyl-Sepharose step. The purification involved ammonium sulfate fractionation and chromatography on DE52 (Whatman), Sephadex G-75 (Pharmacia), and phenyl-Sepharose (Pharmacia). The progress of purification was followed by examining aliquots of fractions by SDS−PAGE. Briefly, conditions for phenyl-Sepharose were as follows. The pooled Sephadex G-75 peak fractions were precipitated with ammonium sulfate at 60% saturation, resuspended in a minimal volume of buffer lacking ammonium sulfate, loaded directly onto a phenyl-Sepharose column equilibrated in buffer containing 0.8 M ammonium sulfate, and eluted with a decreasing gradient of ammonium sulfate. PII eluted from the column in a broad peak centered at approximately 0.5 M ammonium sulfate. Purification of altered PII proteins was the same except all buffers contained 1 mM DTT. Typical yields from 4 L

[1] Abbreviations: PII, signal transduction protein encoded by *glnB*; NRII and NtrB, signal transduction protein encoded by *glnL* (*ntrB*); NRI and NtrC, signal transduction protein encoded by *glnG* (*ntrC*); NRI∼P, phosphorylated form of NRI; 2-KG, 2-ketoglutarate; DTT, dithiothreitol (Cleland's reagent); BSA, bovine serum albumin; TFPAM-SS1, *N*-[2-[[2-[[(4-azido-2,3,5,6-tetrafluoro)benzoyl]amino]ethyl]-dithio]ethyl]maleimide; TFPAM-3, *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide; PEAS, *N*-[(2-pyridyldithio)ethyl]-4-azidosalicylamide.

cultures, with pooling of only highly enriched fractions at each step, were ~100 mg of PII protein. The proteins were judged to be about 90% pure by SDS−PAGE analysis with Coomassie brilliant blue staining. NRII was purified as described previously (*35*), except that Sephadex G-100 (Pharmacia) was used for the gel filtration step and the storage buffer for NRII contained no DTT. Our NRII preparation is about 90% pure, and contains a minor contaminant of ~88 kDa. Preparations of polypeptides derived from NRII described in the accompanying paper were used (*22*). Protein concentrations were determined by the methods of Lowry (*37*) or Bradford (*38*) with BSA as the standard. Hemiphosphorylated NRII~^{32}P was prepared by autophosphorylation of NRII as described previously (*34*), followed by chromatography on a PD10 Sephadex G-25 column (Pharmacia) to remove nucleotides.

Labeling of Altered PII Proteins with Cross-Linkers. The cross-linking reagents *N*-[2-[[2-[[(4-azido-2,3,5,6-tetrafluoro)benzoyl]amino]ethyl]dithio]ethyl]maleimide (TFPAM-SS1), *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM-3), and *N*-[(2-pyridyldithio)ethyl]-4-azidosalicylamide (PEAS) are described elsewhere (*39*−*41*) and were purchased from Molecular Probes. Cross-linkers were resuspended into pure DMSO and the stock solutions stored at −20 °C in the dark. For TFPAM-SS1 and TFPAM-3, labeling conditions included 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10% (v/v) DMSO, 200 μM monomer PII, and 4 mM cross-linker. For PEAS, labeling conditions were the same except the pH was 8.4. All labeling reactions were carried out in the dark at room temperature overnight. Cross-linker-labeled proteins were separated from the free cross-linker using a PD10 Sephadex G-25 column (Pharmacia) run in the dark in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, and 1 mM EDTA. Labeled proteins were stored protected from light at 4 °C and used within a few days. Protein concentrations were determined by the method of Bradford (*38*).

Cross-Linking Reactions. For PII−TFPAM-SS1 and PII−PEAS, conditions included 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP as indicated, 50 μM 2-ketoglutarate as indicated, 5 μM NRII dimer or NRII truncations, and 10 μM cross-linker-labeled PII trimer. For PII−TFPAM-3, conditions were the same except the reaction mixtures contained 2 mM DTT, and the nonhydrolyzable ATP analogue AMP-PNP was used in place of ATP where indicated. Total reaction volumes were typically 20−50 μL. All components except labeled PII were mixed in a 96-well plate and incubated at room temperature for 10 min. Cross-linker-labeled PII was added, and a further 10 min incubation on ice was carried out in the dark. Cross-linking was initiated by exposure to long-wave UV light from a hand-held UV source (model UVGL-25 Mineralight Lamp, UV at 254 and 366 nm, 115 V, 60 Hz, 0.16 A, UVP Inc., San Gabriel, CA) placed directly onto the 96-well plate on ice. UV exposure times were as indicated. Reactions were stopped by addition of SDS gel loading buffer with or without reducing agent, as indicated, and analyzed by 15% SDS−polyacrylamide gels, or stopped with 120 mM glycine (pH 9.0) and examined on 10% nondenaturing polyacrylamide gels.

Polyacrylamide Gel Electrophoresis. Nondenaturing gel electrophoresis was as described previously (*22*, *29*). These gels were prerun at 100 V and 4 °C for 15 min before loading. Samples were loaded in 10% glycerol and 0.04% bromophenol blue, and the gels were run at 100 V and 4 °C for 2.5 h. Nondenaturing gels were stained with Coomassie brilliant blue R250. SDS−polyacrylamide gel electrophoresis was as described previously (*43*). The 5-fold concentrated SDS loading buffer was 250 mM Tris-HCl (pH 6.8), 50% glycerol (v/v), 10% SDS (w/v), and 0.1% bromophenol blue. DTT or β-mercaptoethanol was added to samples as indicated. Urea−polyacrylamide gels were as described previously (*34*).

Nondenaturing Gel Electrophoresis Purification of the PII−NRII Cross-Linked Complex. Cross-linking reactions were carried out as described above, using PII(C73S/E44C)−TFPAM-3 and ATP or AMP-PNP as indicated. The reactions were stopped by addition of 120 mM glycine (pH 9.0), and aliquots of the reaction mixtures were run on a nondenaturing 10% polyacrylamide gel at 4 °C. The gel was lightly stained with Coomassie brilliant blue R250 and briefly destained with a 50% methanol/10% acetic acid solution. The gel was soaked in water briefly to remove the acetic acid. The protein bands representing the cross-linked complexes formed in the presence of ATP and AMP-PNP were excised from the gel with a razor blade, and the gel slices were placed in the wells of a 15% SDS−polyacrylamide gel. The gel slices were overlaid with 5× SDS loading buffer and allowed to incubate for ~15 min before running the gel. The gel was run at ~50 V through the stacking gel and then at 150 V through the resolving gel. Samples from the cross-linking reactions and control reactions were run on the same SDS−polyacrylamide gel for comparison.

Gel Filtration Chromatography Purification of the PII−NRII Cross-Linked Complex. A 2.1 mL cross-linking reaction was carried out using PII(C73S/E44C)−TFPAM-3, AMP-PNP, and NRII under the conditions described above. This was done using 14 wells of the 96-well plate, each containing 150 μL of the reaction mixture. After UV exposure for 20 min, the aliquots were combined and DTT was added to a final concentration of 10 mM. The bulk of the reaction mixture was loaded onto a 150 mL Sephadex G-100 column equilibrated in 50 mM Tris-HCl (pH 7.5), 200 mM KCl, and 1 mM DTT and eluted at room temperature using the same buffer. Fractions were collected and placed on ice soon after elution from the column. Fractions were analyzed by SDS and nondenaturing polyacrylamide gel electrophoresis. Peak fractions containing the complex were combined and concentrated using a 10 kDa molecular mass cutoff Ultrafree-15 centrifugal filter device (Millipore). The protein concentration of the complex was determined by the method of Bradford (*38*), and the molarity was estimated using a molecular mass corresponding to one PII trimer and one NRII dimer (~114 kDa). The purified complex was stored on ice at 4 °C and did not appear to lose activity over a period of 1 week.

Activity of Mutant PII Proteins. The kinase and phosphatase activity of NRII was measured as described previously (*11*, *35*). Briefly, NRI (15 μM) was incubated with NRII (0.3 μM) at 25 °C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μM 2-ketoglutarate, and 0.5 mM [γ-^{32}P]ATP. After 20 min, wild-type or mutant PII proteins were added to a final concentration of 0.3 μM. Aliquots were spotted on nitrocellulose filters at the indicated times and washed in 5% TCA. Errors using this assay method were generally

≤10%. [^{32}P]NRI was assessed by liquid scintillation counting of washed filters.

Phosphatase Activity of the PII(C73S/E44C)−TFPAM-3−NRII Cross-Linked Complex. Phosphatase activity of the purified cross-linked complex was assayed as described above, with the following modifications. MBP−CT111 was used in place of NRII to phosphorylate NRI. Previous studies showed that MBP−CT111, consisting of maltose-binding protein fused to residues 111−349 of NRII, lacks phosphatase activity (*42*). Also, 2-ketoglutarate was omitted from the initial reaction mixture and added as indicated with PII and NRII or the cross-linked complex. Protein concentrations were 8.5 μM NRI, 0.17 μM MBP−CT111, and PII, NRII, and cross-linked PII::NRII complex as indicated. The cross-linked complex was also assayed for phosphatase activity using [^{32}P]NRI as the substrate, as described (*22*, *42*). [^{32}P]-NRI was prepared as described (*22*) except the storage buffer contained 1 mM DTT. Briefly, reaction mixtures containing 12 μM NRI, 0.3 μM MBP−CT111, 0.1 mM [γ-^{32}P]ATP, and 10 mM $MgCl_2$ were incubated at 25 °C for 30 min to allow phosphorylation of NRI. [^{32}P]NRI was separated from small molecule reaction components by gel filtration on a PD10 Sephadex G-25 column. The phosphatase assay was carried out as in the accompanying paper (*22*). Briefly, the reaction mixtures contained 0.18 μM monomer [^{32}P]NRI, 1 mM AMP-PNP, 10 mM $MgCl_2$, 0.3 mg/mL BSA, 50 μM 2-ketoglutarate where indicated, and PII, NRII, and the cross-linked PII−NRII complex as indicated. Reaction mixtures were incubated at 25 °C; at the indicated times, aliquots were spotted on nitrocellulose filters, and TCA-precipitable radioactivity was measured by liquid scintillation counting. Errors using this assay method were generally ≤15−20%.

Autophosphorylation of the PII−NRII Cross-Linked Complex. Reactions were performed at 25 °C, and the mixtures contained the PII(C73S/E44C)−TFPAM-3−NRII complex (1.33 μM) or NRII (1.33 μM) in 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 μM 2-ketoglutarate, and 0.5 mM [γ-^{32}P]ATP. Reactions were started by addition of [γ-^{32}P]ATP and stopped at indicated times by addition of SDS gel loading buffer. Samples were run on a 15% SDS−polyacrylamide gel and the labeled protein bands visualized by autoradiography.

RESULTS

To study the interaction between PII and NRII, we used a chemical cross-linking approach that has previously been used to examine the interactions of the subunits of the F1 ATPase of *E. coli* (*39*, *40*) and the interaction of the complex of cAMP and CAP (CRP) with RNA polymerase of *E. coli* (*41*). An overview of the approach is shown in Figure 1. Previous genetic and biochemical studies have demonstrated that an exposed loop of PII known as the T-loop interacts with NRII and the other known PII receptors of *E. coli* (*36*). We engineered unique cysteine residues at specific positions in the T-loop to allow the labeling of PII with heterobifunctional cross-linking reagents. One end of the cross-linker is thiol reactive and reacts specifically with the unique cysteine engineered onto the T-loop of PII. The other end of the cross-linker is activated upon UV exposure and reacts nonspecifically. Thus, regardless of where PII touches NRII, the cross-linker can form a covalent bond linking PII to NRII.

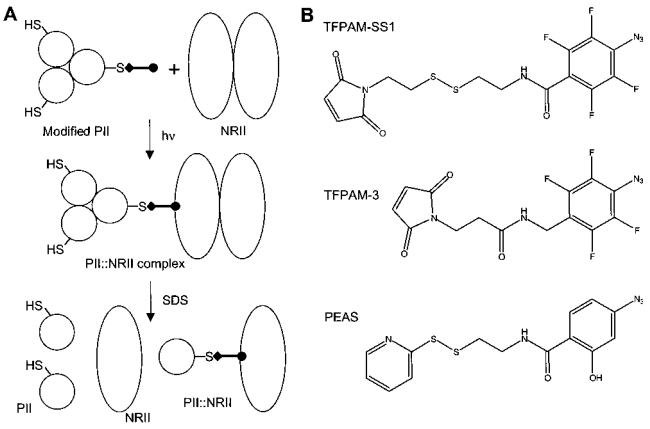

FIGURE 1: Overview of the cross-linking approach. (A) Cross-linking of PII to NRII. A PII trimer is shown, which has been modified to contain a single cysteine residue on the T-loop of each subunit. The cysteine residue has been modified with a heterobifunctional cross-linking reagent such as TFPAM-3. The diamond end represents a maleimide that reacts specifically with thiols. The circle represents an aryl azide, which is photoactivatable and reacts nonspecifically. The modified PII trimer is incubated with dimeric NRII to which it binds. UV exposure activates the aryl azide group, and a covalent bond is formed tethering PII to NRII. Under denaturing conditions, such as in SDS−polyacrylamide gel electrophoresis, the tethered complex gives rise to the three species that are shown. For simplicity, we have shown a single PII subunit labeled with a cross-linker. It is likely that other subunits within the trimer are labeled as well, although our labeling efficiency was not 100%. We only depict the major cross-linked species; PII trimers linked to more than one NRII dimer or NRII dimers linked to more than one PII trimer may also be formed. (B) Structure of cross-linkers used in this study. The cross-linkers are heterobifunctional and photoactivatable. The maleimide group of TFPAM-SS1 and TFPAM-3 and the pyridyl disulfide group of PEAS react specifically with thiols. The aryl azide moieties of TFPAM-SS1, TFPAM-3, and PEAS are activated upon exposure to UV light and react nonspecifically. TFPAM-SS1 and PEAS also contain an internal disulfide bond that can be cleaved with reducing agents.

Wild-type PII contains a single cysteine at position 73. This was altered to serine by site-specific mutagenesis, and subsequent mutations were constructed in the C73S background. Glutamate 44 and glutamate 50 were chosen for mutation to cysteine on the basis of their positions in the crystal structure of PII (*44*) and on the basis of available genetic and biochemical data. The side chains of both glutamates are solvent-exposed, projecting from the T-loop of PII. Previous studies showed that mutation of glutamate 50 to glutamine had no effect on the interaction of PII with NRII (*36*). Glutamate 44 was chosen for study because it lies on the surface of PII, and because studies from our laboratory on the related GlnK protein suggest that this part of GlnK is involved in the interaction of GlnK with NRII (*45*, *46*; Q. Sun, A. Tseng, M. R. Atkinson, and A. J. Ninfa, unpublished data).

The cross-linking reagents that were used are shown in Figure 1B. TFPAM-SS1 and TFPAM-3 feature a maleimide group that reacts specifically with thiols by addition of the thiol across the double bond of the maleimide, forming a thioether. PEAS features a pyridyl disulfide group that undergoes disulfide exchange with thiols. All three cross-linkers feature an aryl azide that becomes activated upon exposure to UV light and reacts nonspecifically. The fluorinated aryl azides of TFPAM-SS1 and TFPAM-3 form aryl nitrenes upon photolysis, which form C−H bond

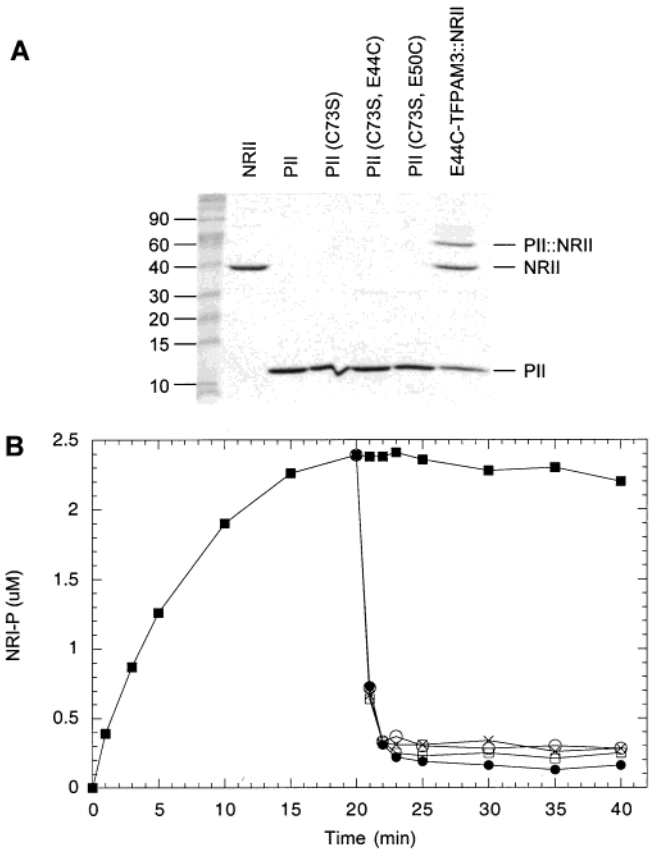

FIGURE 2: Activity of altered PII proteins. (A) SDS gel of purified proteins used in this study. Two micrograms of purified proteins was mixed with SDS loading buffer containing 2 mM β-mercaptoethanol and run on a 15% gel at 200 V for 1 h. The gel was stained with Coomassie brilliant blue R250. Shown are NRII, PII, altered PII proteins, and the PII−NRII cross-linked complex that was purified in this study. The molecular masses of the markers are shown in kilodaltons. (B) Activity of wild-type and mutant PII proteins. Phosphorylation reaction mixtures containing 15 μM NRI, 0.3 μM NRII, 0.5 mM [γ-^{32}P]ATP, and 50 μM 2-ketoglutarate were incubated at 25 °C for 20 min. At 20 min, wild-type or mutant PII proteins were added to final concentrations of 0.3 μM to analyze their effect on the phosphorylation state of NRI. At the indicated times, samples were removed and spotted onto nitrocellulose filters and analyzed as described in Materials and Methods: (■) buffer control, (●) wild-type PII, (□) PII(C73S), (×) PII(C73S/E44C), and (○) PII(C73S/E50C).

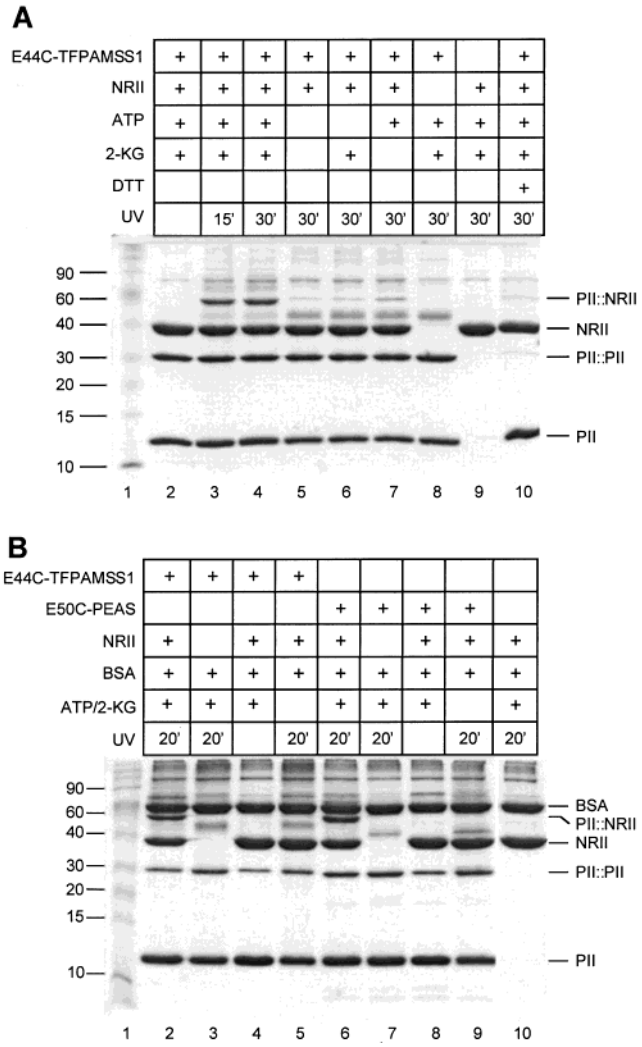

FIGURE 3: Cross-linking of PII to NRII requires ATP and 2-ketoglutarate and is not affected by BSA. (A) PII(C73S/E44C)−TFPAM-SS1 cross-linking to NRII. Cross-linking reactions were as described in Materials and Methods, and the mixtures contained 10 μM trimer PII(C73S/E44C)−TFPAM-SS1, 5 μM dimer NRII, 0.5 mM ATP, and 50 μM 2-ketoglutarate as indicated. Reaction mixtures were exposed to long-wave UV light for the indicated times (minutes) and reactions stopped by addition of SDS loading buffer that lacked reducing agent. Samples were run on a 15% SDS−polyacrylamide gel. The gel was stained with Coomassie brilliant blue R250. For the sample in lane 10, DTT was added to a final concentration of 100 mM before loading. Lane 1 shows markers with molecular masses in kilodaltons. (B) PII(C73S/E44C)−TFPAM-SS1 and PII(C73S/E50C)−PEAS cross-linking to NRII in the presence of BSA. Conditions were as described for panel A, except the reaction mixtures also contained 0.3 mg/mL BSA. Reaction mixtures were exposed to long-wave UV light for 20 min where indicated and reactions stopped by addition of SDS gel loading buffer that did not contain reducing agent. Samples were analyzed by 15% SDS−polyacrylamide gel electrophoresis, and the gel was stained with Coomassie brilliant blue R250.

insertion products (*47*; for reviews, see refs *48* and *49*). The simple aryl azide of PEAS forms a nitrene upon photolysis, which undergoes ring expansion and reacts with nucleophiles (*48*). In addition to these features, TFPAM-SS1 and PEAS contain an internal disulfide that can be cleaved with reducing agents (Figure 1B).

Activity of Mutant PII Proteins. The mutant PII proteins described above were overexpressed in *E. coli* and purified (Figure 2A). The mutant proteins behaved essentially the same as wild-type PII during purification. DTT was used in all buffers to prevent aggregation of the proteins. All mutant proteins were able to activate the phosphatase activity of NRII nearly as well as wild-type PII (Figure 2B).

Cross-Linking of PII to NRII. PII(C73S/E44C) and PII(C73S/E50C) were labeled with the cross-linkers TFPAM-SS1 and PEAS overnight at room temperature in the dark. The labeled proteins were separated from the free cross-linker by gel filtration. PII(C73S) did not become labeled, confirming that the cross-linkers do not react nonspecifically with the protein (data not shown). Figure 3A shows typical results of cross-linking reactions of PII(C73S/E44C)−TFPAM-SS1 with NRII. Proteins were mixed together in the presence or absence of the small molecule effectors ATP and 2-ketoglutarate and exposed to long-wave UV light for various times. Reactions were stopped by addition of SDS gel loading buffer and analyzed on a nonreducing 15% SDS−polyacrylamide gel.

Lane 2 of Figure 3A shows the result of incubating PII(C73S/E44C)−TFPAM-SS1 and NRII in the absence of UV exposure. PII(C73S/E44C)−TFPAM-SS1 migrated as two species corresponding to a monomer and a dimer. This is due to the fact that our labeling was not complete, and the free cysteines formed disulfide bonds. The band just below 90 kDa is a contaminant in the NRII preparation. Lanes 3 and 4 show the reaction products after UV exposure for 15 and 30 min, respectively. An additional prominent band was apparent that corresponds to the size of a PII subunit linked to a NRII subunit. Additional experiments showed that UV exposure beyond 30 min did not result in an increased yield of the cross-linked species (data not shown). Lane 5 shows that the level of PII−NRII cross-linked species was greatly reduced when reactions were conducted in the absence of the PII allosteric effectors ATP and 2-ketoglutarate. Lanes 6 and 7 show the results of single omissions of ATP and 2-ketoglutarate, respectively. Removal of either effector greatly reduced the level of cross-linking of PII to NRII, suggesting that the cross-linking is specific to the PII−NRII interaction and is not adventitious. Lane 8 shows the result when PII(C73S/E44C)−TFPAM-SS1 was exposed to UV light in the absence of NRII. Note that high-molecular mass species were obtained; these were less evident when NRII was present (compare lanes 8 and 4), but were still evident when the small molecules were omitted (compare lanes 8 and 5). Thus, the binding of NRII to PII suppressed PII−PII cross-linking. It was not surprising that the trimeric PII could cross-link to itself as the cross-linkers were on the highly flexible T-loop of PII which projects above the surface of the PII trimer (*44*), and since some PII trimers may have been linked to other PII trimers through disulfide bonds of unlabeled cysteines. Lane 9 shows that NRII in isolation did not exhibit any UV-induced cross-linking. Lane 10 shows that the PII−NRII cross-linked species disappeared upon addition of 100 mM DTT immediately prior to gel loading, as we would expect from the cleavable TFPAM-SS1 cross-linker. The magnitude of the band corresponding to PII−PII dimers was also greatly reduced upon addition of DTT; this result taken together with the fact that the PII−PII dimer was present in the absence of UV exposure indicates that this species was a dimer formed by a disulfide bond between unlabeled cysteines of PII subunits.

Similar results were obtained with PII(C73S/E50C)−PEAS cross-linking to NRII (data not shown). Remarkably consistent results were obtained in numerous experiments using PII(C73S/E44C)−TFPAM-SS1 and PII(C73S/E50C)−PEAS, where ∼5−10% of the NRII subunits became cross-linked to PII as estimated from the intensity of bands on gels. Cross-linking of PII(C73S/E50C)−TFPAM-SS1 and PII(C73S/E44C)−PEAS to NRII was also examined (data not shown). In the former case, diminished cross-linking was seen in most experiments, but in one experiment, cross-linking similar to that shown in Figure 3A was obtained. This suggests that the variability in results with this PII cross-linker pair may be in part due to inconsistencies in the labeling of PII(C73S/E50C) with TFPAM-SS1. Cross-linking activity of PII(C73S/E44C) that had been labeled with PEAS was poor in multiple experiments. This may also reflect the poor labeling efficiency of PII(C73S/E44C) with PEAS.

As a further test of the specificity of the cross-linking reactions, we assessed the effect of the presence of BSA at a high concentration (0.3 mg/mL, Figure 3B). Results for both PII(C73S/E44C)−TFPAM-SS1 and PII(C73S/E50C)−PEAS are shown. Lanes 2 and 6 show that in the presence of BSA both labeled PII proteins were cross-linked to NRII; lanes 5 and 9 show that this cross-linking was effector-dependent. The labeled PII protein also did not appear to cross-link to BSA. Lane 10 shows the result of incubating BSA and NRII in the presence of UV light. The high-molecular mass bands are present in the BSA preparation; their appearance did not require UV exposure (lanes 4 and 8).

Gel Purification of the Cross-Linked Complex. Since our PII proteins containing unique T-loop cysteine residues form high-molecular mass multimers in the absence of DTT and the cleavable cross-linkers TFPAM-SS1 and PEAS are cleaved by DTT, we used the noncleavable cross-linker TFPAM-3 and conducted the cross-linking reactions in the presence of 2 mM DTT. This was necessary, as studies of the phosphatase activity of the cross-linked complex required the complex uncontaminated by un-cross-linked PII. Studies of the autophosphorylation activity of the cross-linked species required that the NRII not be phosphorylated, necessitating purification of the complex from reaction mixtures that lacked ATP. Previous studies have shown that ATP is an allosteric effector of PII that binds synergistically with 2-ketoglutarate (*28*, *35*) and that the nonhydrolyzable analogue AMP-PNP could substitute for ATP in activating the interaction of PII with NRII (*22*). Thus, we examined the cross-linking of PII(C73S/E44C)−TFPAM-3 to NRII in the presence of 2 mM DTT and AMP-PNP (Figure 4). Denaturing and nondenaturing gel electrophoresis of cross-linking reaction products indicated that AMP-PNP could substitute for ATP in activating cross-linking, and that in reaction mixtures containing DTT the cross-linked species was the major species with a molecular mass greater than that of NRII (Figure 4). For PII(C73S/E44C)−TFPAM-3 cross-linking, we observed a small amount of UV light-induced PII−PII dimers. PII(C73S/E50C)−TFPAM-3 was also cross-linked to NRII in the presence of AMP-PNP and DTT, but not as efficiently as was PII(C73S/E44C)−TFPAM-3 (data not shown).

To characterize the components of the cross-linked PII(C73S/E44C)−TFPAM-3−NRII complex, cross-linking reactions were conducted in the presence of 2 mM DTT, as in Figure 4, and aliquots of the reactions were subjected to nondenaturing gel electrophoresis, as in Figure 4B. After very brief staining of the gel to permit visualization of the bands, the bands corresponding to the cross-linked complex were excised from the gel with a razor blade, and the central portion of this band was subjected to SDS−PAGE (Figure 5). For comparison, aliquots of the cross-linking reaction mixtures and control reaction mixtures were run on the same gel. As shown, the purified complex formed in the presence of ATP or AMP-PNP appeared to consist of PII trimers linked to NRII dimers via a single covalent attachment, as free NRII and PII subunits were present along with the cross-linked PII−NRII subunits (Figure 5).

Since gel purification of the cross-linked complex resulted in the recovery of both the branched species and unaltered NRII subunits, we used this method to assess whether there was a bias in the cross-linking of subunits when hemiphosphorylated NRII was used in place of unphosphorylated

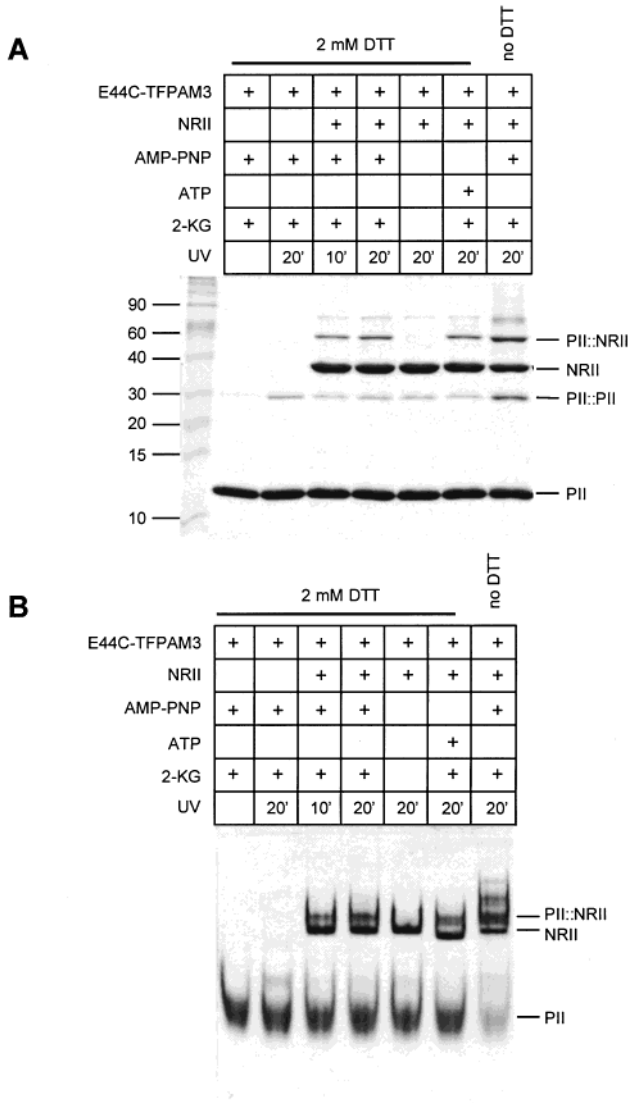

FIGURE 4: AMP-PNP can substitute for ATP in activating cross-linking, and DTT prevents the formation of high-molecular mass PII multimers. Cross-linking reactions were as described in Materials and Methods, and the mixtures contained 10 μM trimer PII(C73S/E44C)−TFPAM-3, 5 μM dimer NRII, 0.5 mM AMP-PNP or ATP, 50 μM 2-ketoglutarate, and 2 mM DTT as indicated. Reaction mixtures were exposed to long-wave UV light for the indicated times (minutes). (A) SDS−PAGE analysis of PII(C73S/E44C)−TFPAM-3 cross-linking to NRII in the presence of AMP-PNP and DTT. Aliquots of cross-linking reaction mixtures were stopped by addition of SDS loading buffer and analyzed on a 15% SDS−polyacrylamide gel, which was stained with Coomassie brilliant blue R250. Lane 1 shows markers with molecular masses in kilodaltons. (B) Nondenaturing polyacrylamide gel analysis of PII−NRII cross-linking in the presence of AMP-PNP and DTT. Portions of the cross-linking reactions were stopped by addition of 120 mM glycine (pH 9.0). Samples were made 10% glycerol and 0.04% bromophenol blue to facilitate loading and were analyzed on a 10% nondenaturing polyacrylamide gel run at 4 °C. The gel was stained with Coomassie brilliant blue R250.

NRII. Previous studies showed that autophosphorylation of NRII results in an asymmetrical form of the enzyme, where the phosphorylation of the second subunit proceeds with a 70-fold diminished equilibrium constant (*34*). Hemiphosphorylated NRII∼^{32}P was formed, purified, and characterized as previously described (*34*). Urea gel analysis showed that the NRII∼^{32}P preparation was almost exclusively hemiphosphorylated (data not shown). Cross-linking reactions were

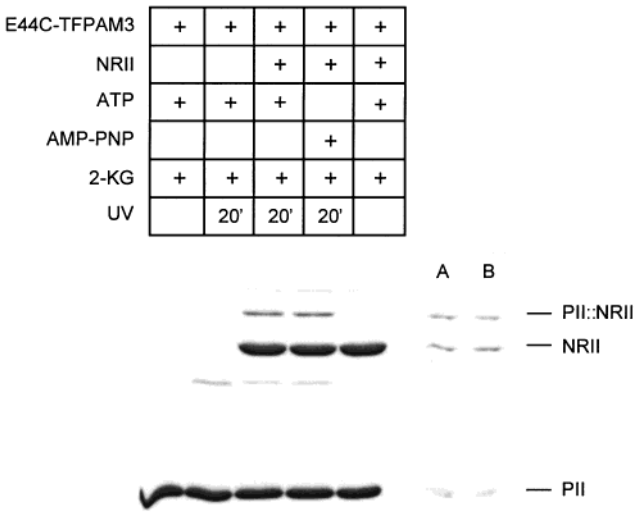

FIGURE 5: Purification of the cross-linked complex by nondenaturing polyacrylamide gel electrophoresis. Cross-linking reactions were as in Materials and Methods, and mixtures contained 10 μM trimer PII(C73S/E44C)−TFPAM-3, 5 μM dimer NRII, 2 mM DTT, 0.5 mM ATP or AMP-PNP as indicated, and 50 μM 2-ketoglutarate. Reaction mixtures were exposed to UV light for 20 min; reactions were stopped by addition of 120 mM glycine (pH 9.0), and mixtures were analyzed on a 10% nondenaturing polyacrylamide gel run at 4 °C. The gel was stained with Coomassie brilliant blue R250. The bands on the nondenaturing gel corresponding to the cross-linked complexes were excised from the gel, trimmed to include only the central part of the bands, and placed in the wells of a 15% SDS−polyacrylamide gel. Lanes labeled A and B show the cross-linked species formed in the presence of ATP and AMP-PNP, respectively. The gel slices were overlaid with 5× SDS loading buffer and allowed to incubate for ∼15 min before running the gel. The first five lanes show aliquots of the cross-linking reactions for comparison. The gel was stained with Coomassie brilliant blue R250. The image shows different portions from the same SDS−polyacrylamide gel.

conducted using hemiphosphorylated NRII∼^{32}P and PII(C73S/E44C)−TFPAM-3 as described above; the cross-linked complex was purified from a nondenaturing gel, and the complex components were characterized by denaturing SDS−PAGE and autoradiography. Both the unaltered NRII band and the band corresponding to the branched species had similar radioactivities (data not shown), indicating no discernible bias in the cross-linking of the unphosphorylated and phosphorylated subunits of hemiphosphorylated NRII.

Purification of the Cross-Linked PII−NRII Complex. To study the activities of the cross-linked complex, we purified this complex from a large cross-linking reaction mixture by gel filtration on Sephadex G-100 (Materials and Methods). We performed a large-scale cross-linking reaction with PII(C73S/E44C)−TFPAM-3 and NRII, with results similar to those shown in Figure 4. Approximately 800 μg of NRII was used in a 2.1 mL reaction mixture containing 0.5 mM AMP-PNP, 50 μM 2-ketoglutarate, and 2 mM DTT. After UV exposure for 20 min, DTT was added to a final concentration of 10 mM, and the bulk of the reaction mixture was fractionated on a 150 mL Sephadex G-100 column in the presence of 1 mM DTT. The cross-linked complex eluted just ahead of dimeric NRII, with the two peaks slightly overlapping, and well ahead of un-cross-linked PII trimers (Figure 6). This again suggests that the cross-linked species probably consists of a single PII trimer linked to a single NRII dimer. Such a species would have a molecular mass

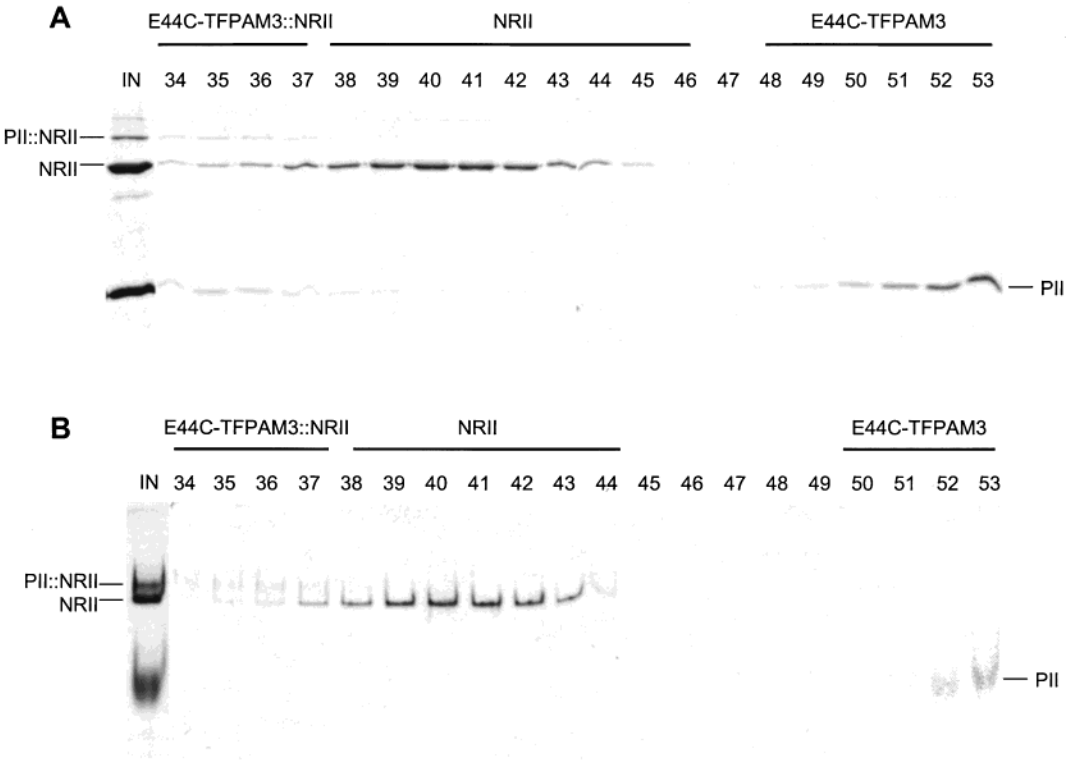

FIGURE 6: Gel filtration chromatography purification of the PII−NRII cross-linked complex. (A) SDS−PAGE analysis of Sephadex G-100 fractions. A 2.1 mL cross-linking reaction was carried out as in Materials and Methods. The reaction mixture contained 10 μM trimer PII(C73S/E44C)−TFPAM-3, 5 μM dimer NRII, 0.5 mM AMP-PNP, 50 μM 2-ketoglutarate, and 2 mM DTT. Long-wave UV exposure was for 20 min on ice at which point DTT was added to a final concentration of 10 mM. The sample was loaded onto a 150 mL Sephadex G-100 column run in 50 mM Tris-HCl (pH 7.5), 200 mM KCl, and 1 mM DTT at room temperature. Aliquots (16 μL) of fractions were analyzed on 15% SDS−polyacrylamide gels, which were stained with Coomassie brilliant blue R250. Fraction numbers are shown above the gels. Lane 1 shows input from a typical reaction. Fractions 31−35 were pooled and spin concentrated. Figure 2A shows the final concentrated preparation of the complex. (B) Nondenaturing polyacrylamide gel analysis of fractions. The same amounts of fractions as in panel A were run at 100 V for 2.5 h on 10% nondenaturing polyacrylamide gels at 4 °C. The gels were stained with Coomassie brilliant blue R250. Note that the native proteins do not stain as well as the SDS-denatured proteins. Positions of PII(C73S/E44C)−TFPAM-3, NRII, and the PII(C73S/E44C)−TFPAM-3−NRII cross-linked complex are shown.

of roughly 114 kDa. Dimeric NRII has a molecular mass of about 77 kDa. The cross-linked complex must form a relatively compact structure as it was not eluted in the void of the G-100 column and partially overlapped with the NRII peak. On SDS−polyacrylamide gels, the complex displayed three species as expected (Figure 2A), corresponding to PII and NRII subunits and the two types of subunits linked together.

Fractions 31−35 were pooled and spin concentrated. Fractions 31−33 are not shown in Figure 6 as the image reproduced poorly due to the small amount of protein in these fractions. The final yield of complex was roughly 50 μg. Nondenaturing gel analysis of the concentrated complex revealed that the preparation was slightly contaminated with free NRII (data not shown), but this does not affect the assays for phosphatase activity. Importantly, free PII was not evident in the preparation (data not shown).

Activities of the Cross-Linked PII−NRII Complex. Autophosphorylation of the PII−NRII Cross-Linked Complex. The ability of the cross-linked complex to become autophosphorylated was examined. An autophosphorylation reaction was carried out using [γ-^{32}P]ATP, and the reactions were analyzed by SDS−PAGE and autoradiography (Figure 7). Both species corresponding to the unlinked NRII subunit and the PII−NRII linked subunits became phosphorylated. This demonstrates that the complex was indeed active as a kinase

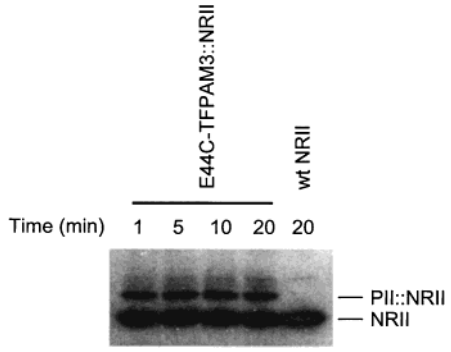

FIGURE 7: Autophosphorylation of the PII(C73S/E44C)−TFPAM3−NRII cross-linked complex. The purified complex was assayed for autophosphorylation activity as described in Materials and Methods. Reaction mixtures contained 0.5 mM [γ-^{32}P]ATP, 50 μM 2-ketoglutarate, and 1.33 μM PII(C73S/E44C)−TFPAM-3−NRII complex or NRII as indicated. Reaction mixtures were incubated at 25 °C for the indicated times and reactions stopped by addition of SDS gel loading buffer. Samples were run on a 15% SDS−polyacrylamide gel, and the radioactive protein bands were visualized by autoradiography.

and that the presence of PII did not prevent the phosphorylation of the cross-linked subunit of NRII. This experiment does not address the issue of whether there is a bias in the phosphorylation of the two subunits once PII is tethered to the enzyme. This is because the purified complex contained a small amount of free NRII, and additionally, the complex

appeared to undergo very slow subunit exchange with NRII dimers (data not shown). Subunit exchange could potentially give rise to NRII homodimers from the pure cross-linked PII−NRII complex. However, prior studies showed that PII is a stable trimer that is only dissociated by urea at high concentrations (*50*); thus, subunit exchange cannot result in the formation of un-cross-linked PII trimers from our purified cross-linked complex.

Phosphatase Activity of the PII−NRII Complex. The purified cross-linked complex was tested for the phosphatase activity characteristic of the un-cross-linked PII−NRII complex. The first assay method that was used was similar to that in Figure 2B. In this case, however, we used MBP−CT111 as the kinase instead of wild-type NRII. MBP−CT111 retains the ability to phosphorylate NRI while lacking the phosphatase activity. This allowed us to examine the phosphatase activity of the PII−NRII cross-linked complex in an assay measuring the balance between the kinase and phosphatase activities. Figure 8A shows the results of a typical assay. As expected, the addition of PII to the reaction mixture containing NRI and MBP−CT111 had no effect on the phosphorylation state of NRI (data not shown; *22*, *42*). The addition of PII and NRII (0.085 μM each) in the presence of 2-ketoglutarate resulted in the expected dephosphorylation of NRI∼P (Figure 8A). Addition of the PII−NRII cross-linked complex at twice the concentration (0.17 μM) in the presence of 2-ketoglutarate resulted in slightly more rapid dephosphorylation of NRI∼P than that realized with un-cross-linked PII and NRII. We know that the complex preparation is slightly contaminated with free NRII, however, and thus, the complex is probably more active than is evident in this experiment. The dephosphorylation of NRI∼P by the same amount of cross-linked complex in the absence of 2-ketoglutarate was examined; interestingly, the complex was still active, although to a lesser extent. This suggests that the presence of PII tethered to NRII is in part sufficient to induce the phosphatase conformation of NRII even in the absence of 2-ketoglutarate.

We next tested the phosphatase activity of the complex using NRI∼^{32}P as the substrate. This assay is different from the previous assay in that it directly measures the phosphatase activity in the absence of competing kinase activity. Reaction mixtures contained the nonhydrolyzable ATP analogue AMP-PNP to allow direct examination of the phosphatase activity. Figure 8B shows the results of a typical assay. NRI∼P had a slow rate of dephosphorylation in the absence of added proteins, and the addition of NRII (0.15 μM) alone had no effect on this rate. Addition of PII and NRII (0.15 μM each) in the presence of 2-ketoglutarate resulted in the dephosphorylation of NRI∼P. The PII−NRII cross-linked complex at twice the concentration (0.3 μM) in the presence of 2-ketoglutarate was about as active as PII and NRII in bringing about NRI∼P dephosphorylation. In the absence of 2-ketoglutarate, the cross-linked complex was still active, although to a lesser extent.

The results presented so far suggest that the PII(C73S/E44C)−TFPAM-3−NRII complex represents the physiologically relevant form of PII bound to NRII. Efficient formation of this complex required 2-ketoglutarate and ATP (or AMP-PNP), and the complex had the ability to bring about the dephosphorylation of NRI∼P.

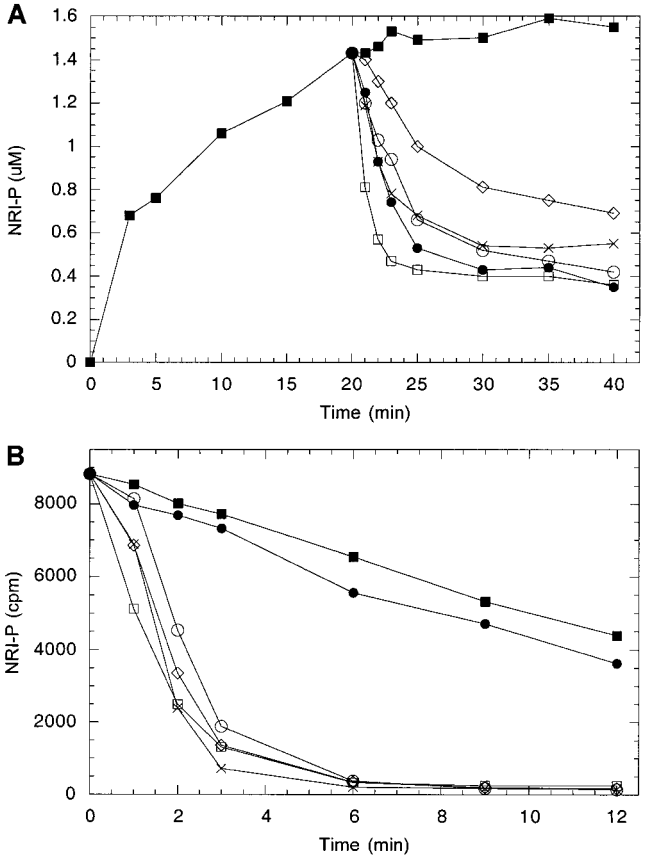

FIGURE 8: Phosphatase activity of the PII−NRII cross-linked complex. (A) Phosphorylation assay of NRI with MBP−CT111 and the cross-linked complex. Reaction mixtures initially contained 8.5 μM NRI, 0.17 μM MBP−CT111, 10 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP, 1 mM DTT, and 50 μM 2-ketoglutarate except where indicated. After 20 min, PII, NRII, and the cross-linked complex were added as indicated. Samples were analyzed as in Materials and Methods: (■) buffer control, (●) PII and NRII at 0.085 μM each, (□) 0.17 μM PII(C73S/E44C)−TFPAM-3−NRII complex, (×) 0.17 μM PII(C73S/E44C)−TFPAM-3−NRII complex without 2-ketoglutarate, (○) 0.057 μM PII(C73S/E44C)−TFPAM-3−NRII complex, and (◇) 0.057 μM PII(C73S/E44C)−TFPAM-3−NRII complex without 2-ketoglutarate. (B) Phosphatase activity of PII−NRII cross-linked complex using NRI∼^{32}P as the substrate. Reactions were as in Materials and Methods, and mixtures contained 0.18 μM NRI∼^{32}P monomer, 1 mM AMP-PNP, 10 mM $MgCl_2$, 50 μM 2-ketoglutarate except where indicated, and PII, NRII, and PII(C73S/E44C)−TFPAM-3−NRII as indicated. Reaction mixtures were incubated at 25 °C, and at the indicated times, aliquots were spotted on nitrocellulose filters, which were washed in 5% TCA and counted by liquid scintillation counting: (■) buffer control, (●) 0.15 μM NRII, (□) NRII and PII at 0.15 μM each, (×) 0.3 μM PII(C73S/E44C)−TFPAM-3−NRII complex, (○) 0.3 μM PII-(C73S/E44C)−TFPAM-3−NRII complex with no 2-ketoglutarate, and (◇) 0.1 μM PII(C73S/E44C)−TFPAM-3−NRII complex.

Mapping the Site of Interaction between PII and NRII. We mapped the site of interaction between PII and NRII at the domain level using polypeptides derived from NRII. These polypeptides are described in the accompanying paper (*22*). NT110 consists of the N-terminal domain of NRII, and NT189 consists of the N-terminal domain and four-helix bundle (central domain) of NRII. CT111 and CT126 are slightly different versions of the NRII transmitter module, containing the four-helix bundle (central domain) and C-terminal kinase domain of NRII. CT190 consists of the C-terminal kinase domain and the linker connecting this

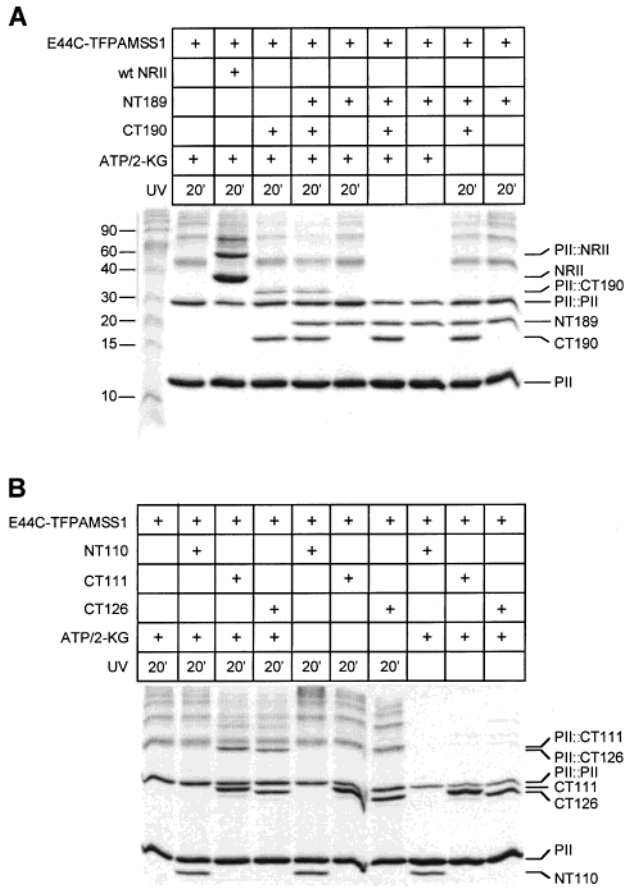

FIGURE 9: PII(C73S/E44C)−TFPAM-SS1 cross-linking to domains of NRII. (A) PII(C73S/E44C)−TFPAM-SS1 cross-links to the C-terminal domain of NRII and not to the N-terminal domain. Conditions were as in Materials and Methods. Reaction mixtures contained 10 μM trimer PII(C73S/E44C)−TFPAM-SS1, 5 μM dimer NRII or NRII truncations, 0.5 mM ATP, and 50 μM 2-ketoglutarate as indicated. NRII truncations were treated as dimers for purposes of concentration. Long-wave UV exposure was for 20 min where indicated, and reactions were stopped by addition of SDS gel loading buffer that did not contain any reducing agent. Samples were run on a 15% SDS−polyacrylamide gel. The gel was stained with Coomassie brilliant blue R250. (B) PII(C73S/E44C)−TFPAM-SS1 cross-linking to various domains of NRII. Conditions were the same as for panel A except different NRII truncations were analyzed. The samples were analyzed on a 15% SDS−polyacrylamide gel, and the gel was stained with Coomassie brilliant blue R250.

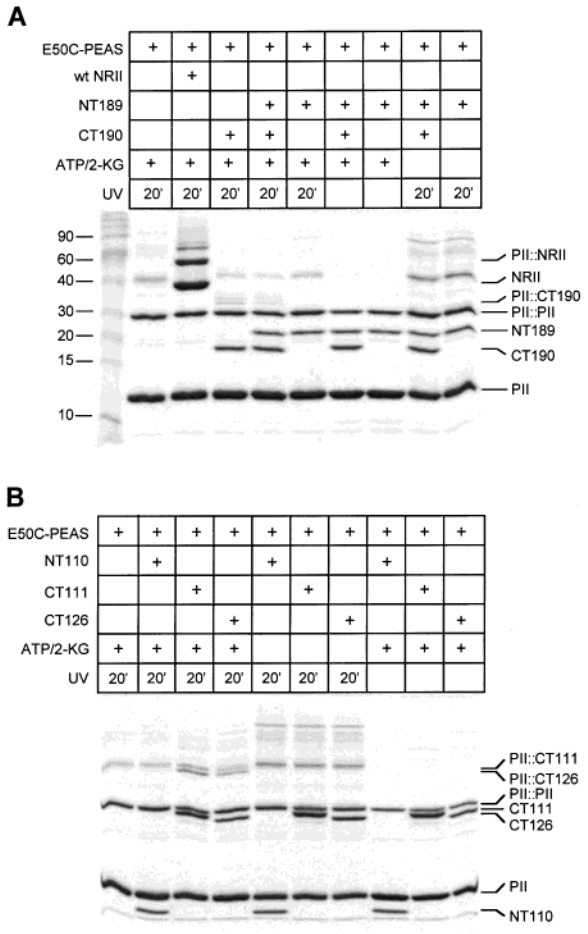

FIGURE 10: PII(C73S/E50C)−PEAS cross-linking to domains of NRII. (A) PII(C73S/E50C)−PEAS cross-links to the C-terminal domain of NRII and not to the N-terminal domain. Conditions were as in Materials and Methods. Reaction mixtures contained 10 μM trimer PII(C73S/E50C)−PEAS, 5 μM dimer NRII or NRII truncations, 0.5 mM ATP, and 50 μM 2-ketoglutarate as indicated. NRII truncations were treated as dimers for purposes of concentration. Long-wave UV exposure was for 20 min where indicated, and reactions were stopped by addition of SDS gel loading buffer that did not contain any reducing agent. Samples were run on a 15% SDS−polyacrylamide gel. The gel was stained with Coomassie brilliant blue R250. (B) PII(C73S/E50C)−PEAS cross-linking to additional domains of NRII. Conditions were the same as for panel A except different NRII truncations were analyzed. The samples were analyzed on a 15% SDS−polyacrylamide gel, and the gel was stained with Coomassie brilliant blue R250.

domain to the central domain. The cross-linking of these polypeptides to PII(C73S/E44C)−TFPAM-SS1 (Figure 9) and PII(C73S/E50C)−PEAS (Figure 10) was examined. The polypeptides that lacked the C-terminal kinase domain (NT110 and NT189) were not cross-linked to PII. However, each of the polypeptides that contained the C-terminal kinase domain (CT111, CT126, and CT190) was UV-cross-linked to PII in an ATP- and 2-ketoglutarate-dependent reaction. Since CT190 consists of just the kinase domain and the linker connecting this domain to the central domain of NRII, these results indicated that PII interacted with the C-terminal kinase domain of the NRII transmitter module. Interestingly, two cross-linking products with slightly different electrophoretic mobilities were obtained when PII(C73S/E50C)−PEAS was cross-linked to CT190, suggesting that two different branched species were formed by cross-linking (Figure 10A).

DISCUSSION

PII is a key regulator of nitrogen assimilation in *E. coli*, which acts in part by regulating the kinase and phosphatase activities of NRII. Previous studies indicated that the binding of PII to NRII is regulated by ATP and 2-ketoglutarate, small molecule effectors of PII, and that the binding of PII to NRII results in the activation of the phosphatase activity of NRII. Also, previous results have established that the T-loop of PII is involved in the interaction of PII with NRII and the other PII receptors. Here, we showed that cross-linking of the T-loop of PII to NRII required ATP (or the nonhydrolyzable analogue AMP-PNP) and 2-ketoglutarate, and that the level of this cross-linking was not diminished by the presence of BSA. Also, cross-linking of PII to BSA was not observed under similar conditions. These results suggested that the cross-linking of PII to NRII in our experiments was

due to the physiologically important interaction of PII and NRII. In further support of this conclusion, we observed that the purified cross-linked complex had NRI∼P phosphatase activity, whereas neither the PII nor the NRII used to form this cross-linked complex had such activity in isolation. Finally, the cross-linked complex had significant 2-ketoglutarate-independent phosphatase activity, which is a novel activity not seen with the starting materials or in numerous experiments with purified PII and NRII at the concentrations used in our experiments. Thus, this novel activity was likely due to the cross-linked complex. In prior work, 2-ketoglutarate-independent PII activity was observed using very high concentrations of PII (*11*, *30*). We interpret this as indicating that in the absence of 2-ketoglutarate, PII may infrequently obtain the conformation required for interaction with NRII. One explanation for the 2-ketoglutarate-independent phosphatase activity of our cross-linked complex is that when tethered to NRII, the effective concentration of PII is very high, greatly increasing the probability of the necessary interaction. Also, in the cross-linked complex, the active conformation of the PII T-loop may be stabilized by interaction with NRII. Alternatively, the cross-linked complex may bind 2-ketoglutarate so tightly that the effector was retained during gel filtration and spin concentration. If so, then this is a novel property of the cross-linked complex that is not observed with PII (*28*).

To examine the site in NRII contacted by the PII T-loop, we examined the cross-linking of PII to purified domains of NRII. The studies in the accompanying paper (*22*) showed that these domains of NRII were stable during hyperexpression and purification. For NT189, CT111, CT126, and CT190, the polypeptides displayed activities further indicating they were properly folded. For example, NT189, which contains the N-terminal and central domains of NRII, could form heterodimers with wild-type NRII and could be phosphorylated by NRII, CT111, CT126, and CT190. The CT111 and CT126 polypeptides, which essentially consist of just the transmitter module of NRII, were dimeric and able to become autophosphorylated, and were able to phosphorylate NRI as well as NT189 and the isolated central domain of NRII (*22*). CT190, corresponding to essentially just the kinase domain of the transmitter module, was able to phosphorylate the isolated central domain of NRII. Thus, these polypeptides appear to be valid tools for studying the interaction of PII with NRII. The results in the accompanying paper showed that PII regulated the autophosphorylation of CT111 and CT126, and regulated the transphosphorylation activity of CT190 (*22*). PII also regulated the weak phosphatase activity of CT111 and CT126 (*22*). Thus, activity assays indicated that PII interacts with CT190. Our cross-linking results are completely consistent with the activity assays, and thus, the two studies support each other. Each of the polypeptides that contained the kinase domain of NRII was UV-cross-linked to the T-loop of PII in an effector-dependent reaction; NT110, NT189, and BSA, lacking the kinase domain of NRII, were not UV-cross-linked to PII. While our results therefore show that the T-loop of PII interacts with the kinase domain of NRII, they do not exclude the possibility that other portions of PII interact with other portions of NRII. However, the level of cross-linking of PII to CT190, relative to that seen with NRII, suggests that CT190 binds PII similarly to intact NRII, which would imply that most or all of the determinants for PII binding lie within the CT190 polypeptide.

Our demonstration that PII interacts with the kinase domain of NRII raises the issue of how PII can distinguish between the 32 related kinase domains present in the cytoplasm of *E. coli* (*1*) and raises the possibility that PII may interact with some of the other related kinase domains present in the cell. Such regulation could contribute to the coordination of carbon and nitrogen metabolism with other physiological processes.

PII proteins are the most widely distributed of all signal transduction proteins, being found in the archaea, bacteria, and plants (for a review, see ref *51*). In many cases, organisms contain PII proteins but lack a transmitter protein specifically resembling NRII. Our results suggest that another transmitter protein kinase domain, or a related ATP-binding domain, may be the target of PII action in such cases. Our results, along with the recent results with the unrelated FixT protein (*19*), show that two-component systems may be regulated by factors that target the transmitter kinase domain. Additional factors may also exist, which regulate the activity of the kinase domains and coordinate their activities.

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