The homotrimeric PII signal transduction protein of *Escherichia coli* interacts with two small-molecule effectors, 2-ketoglutarate and ATP, regulates two protein receptors, the kinase/phosphatase nitrogen regulator II (NRII) and the glutamine synthetase (GS) adenylyltransferase (ATase), and is subject to reversible uridylylation, catalyzed by the uridylyltransferase/uridylyl-removing enzyme (UTase/UR). The site of PII uridylylation, Y51, is located at the apex of the solvent-exposed T-loop (E. Cheah, P. D. Carr, P. M. Sufolk, S. G. Vasudevan, N. E. Dixon, and D. L. Ollis, Structure 2:981–990, 1994), and an internally truncated PII lacking residues 47 to 53 formed trimers that bound the small-molecule effectors but were unable to be uridylylated or activate NRII and ATase (P. Jiang, P. Zucker, M. R. Atkinson, E. S. Kamberov, W. Tirasophon, P. Chandran, B. R. Schefke, and A. J. Ninfa, J. Bacteriol. 179:4342–4353, 1997). We investigated the ability of heterotrimers containing Δ47-53 and wild-type subunits to become uridylylated and activate NRII and ATase. Heterotrimers were formed by denaturation and renaturation of protein mixtures; when such mixtures contained a fivefold excess of Δ47-53 subunits, the wild-type subunits were mostly redistributed into trimers containing one wild-type subunit and two mutant subunits. The resulting population of trimers was uridylylated and deuridylylated by UTase/UR, stimulated the phosphatase activity of NRII, and stimulated adenylylation of GS by ATase. In all except the ATase interaction, the activity of the hybrid trimers was greater than expected based on the number of wild-type subunits present. These results indicate that a single T-loop region within a trimer is sufficient for the productive interaction of PII with its protein receptors. We also formed heterotrimers containing wild-type subunits and subunits containing the G89A alteration (P. Jiang, P. Zucker, M. R. Atkinson, E. S. Kamberov, W. Tirasophon, P. Chandran, B. R. Schefke, and A. J. Ninfa, J. Bacteriol. 179:4342–4353, 1997). The G89A mutant form of PII does not bind the small-molecule effectors, does not interact with UTase or with NRII, and interacts poorly with ATase. Heterotrimers formed with a 10/1 starting ratio of G89A to wild-type subunits interacted with UTase/UR and ATase to a lesser extent than expected based on the number of wild-type subunits present but activated NRII slightly better than expected based on the number of wild-type subunits present. Thus, intersubunit interactions within the PII trimer can adversely affect the activity of wild-type subunits and may affect the interactions with the different receptors in a variable way. Finally, we formed heterotrimers containing Δ47-53 and G89A mutant subunits. These heterotrimers were not uridylylated, did not interact with NRII, and interacted with the ATase to the extent expected based on the number of G89A subunits present. Thus, the G89A subunits, which contain an intact T-loop region, were not “repaired” by inclusion in heterotrimers along with Δ47-53 subunits.

The homotrimeric PII signal transduction protein plays a key role in the regulation of glutamine synthetase (GS) expression and activity and in the regulation of transcription of the Ntr regulon, by virtue of its interactions with the kinase/phosphatase nitrogen regulator II (NRII or NtrB), GS adenylyltransferase (ATase), and the signal-transducing uridylyltransferase/uridylyl-removing enzyme UTase/UR (reviewed in references 7, 8, and 10). PII is regulated by reversible uridylylation on Tyr 51, catalyzed by the UTase/UR. The uridylylation and deuridylylation reactions are regulated by small-molecule effectors that bind either to UTase/UR (glutamine) or to PII (ATP or 2-ketoglutarate) (4, 5). Both uridylylated and deuridylylated forms of PII interact with the ATase, the former stimulating the deadenylylation activity and the latter stimulating the adenylylation activity of ATase. The unuridylylated form of PII stimulates the phosphatase activity of NRII.

The crystal structure of PII has been solved (2), revealing a prominent solvent-exposed loop, the T-loop, containing at its apex the site of PII uridylylation. This loop and the clefts flanking it have been suggested as the site of interaction with the PII receptors (2), and mutational studies have supported this hypothesis (3). In an accompanying study, we constructed and characterized a mutant form of PII in which the apex of the T-loop was removed by genetic deletion of amino acids 47 to 53 of PII. This mutant form of PII formed trimers and bound the small-molecule effectors ATP and 2-ketoglutarate but could not be uridylylated or activate NRII or ATase (3). We also constructed another mutant form of the PII protein in which glycine at position 89 was converted to alanine (G89A). This alteration is distant from the apex of the T-loop, mapping within the B-loop of the molecule (2). Even though the G89A protein contains an intact T-loop region and forms trimers, it neither binds the small-molecule effectors nor interacts with the deadenylylation activity of ATase. The uridylylated form of PII stimulates the phosphatase activity of NRII.
Within a trimer by different subunits. Previous results have indicated that the uridylylation of wild-type PII was noncooperative, that is, that the probability of a subunit becoming uridylylated was not greatly influenced by the uridylylation state of the other two subunits within the trimer. This conclusion was based on the observation that PII trimers containing a single or two uridylylated groups were readily apparent and accumulated early in time course studies of PII uridylylation (1). Furthermore, it was observed that partially uridylylated PII trimers were able to activate NRII (1). Thus, uridylylation of a PII subunit does not result in a conformational change that alters the probability of unuridylylated subunits within the trimer becoming uridylylated. Also, uridylylation of subunits within the trimer does not result in a conformational change that prevents unuridylylated subunits within the same trimer from interacting with NRII. Finally, all subunits of a PII trimer need not simultaneously interact with NRII for NRII activation. The uridylylation-and-deuridylylation cycle of PII and its effect on the interaction of PII with NRII offer no simple explanation as to why the highly evolved PII protein is trimeric.

In the present study, we examined the minimal requirements for the interaction of PII with its protein receptors by forming mixed trimers containing one wild-type subunit and two mutant subunits. Previous work suggested that the T-loop was a critical component for the interaction of PII with its protein receptors, but the small-molecule effectors for deuridylylation of PII, such as ATP, this would indicate that docking required only a single intact T-loop within the trimer, implicating the functional unit of PII to be the individual subunit. Similarly, we examined the feasibility of this approach for the study of intersubunit interactions within the PII trimer. If intersubunit interactions within the trimer play little role in regulating the activity of PII subunits, then inclusion of wild-type subunits into heterotrimers along with G89A subunits should have little influence on the activity of the wild-type subunits. Conversely, if such heterotrimers are able to be uridylylated and activate NRII and ATase, this would indicate that docking required only a single intact T-loop within the trimer, implicating the functional unit of PII to be the individual subunit.

Also, we were interested in whether the small-molecule effectors must be bound by the same subunit that contains the uridylylation activity of PII trimers within the trimer by different subunits.

**MATERIALS AND METHODS**

**Purified proteins.** The preparations of PII, PII-G89A, PII-D47-53, NRII, NRI, UTase/UR, ATase, and GS used are described in the accompanying work (3). **Denaturation and renaturation of PII.** The method of denaturation and renaturation of PII and its development were similar to those described previously for NRII (9). The denaturing conditions were 100 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, 100 mM KCl, 1 mM glutathione, 0.5 mM 2-ketoglutarate, bovine serum albumin (0.5 mg/ml), 40 mM [γ-³²P]ATP, 4.5 mM wild-type PII subunits (monomer concentration) as indicated, 22.5 or 45.0 µM mutant PII subunits as indicated, and 0.01 µM UTase/UR. The mixtures were incubated at 30°C, and reactions were initiated by the addition of UTP. Aliquots were removed at various times and spotted onto nitrocellulose filters, which were washed extensively in 5% trichloroacetic acid. The filters were dried, and trichloroacetic acid-precipitable counts were determined by liquid scintillation counting. For conditions where the concentration of UTase/UR was 0.04 µM and 1.0 mM MnCl₂ and 6 mM glutamic acid were added after an initial uridylylation phase, as described previously (1).

**NRI dephosphorylation assay.** The assay was performed essentially as described previously (3). The reaction mixtures contained 50 mM Tris-Cl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 0.5 mM [γ-³²P]ATP, 50 mM 2-ketoglutarate, 18 µM NRI, and 0.3 µM NRII. Reactions were initiated by the addition of ATP and incubated at 25°C. After 20 min of incubation, PII was added to 0.1 µM (trimer concentration), as indicated in the figures, and mutant PII subunits were added (trimer concentration) as indicated in the figures. Sampling and counting were performed as in the uridylylation assay.

**Adenylylation assay.** The adenylylation assay was performed as described previously (3). The reaction mixtures contained 50 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, 5 mM glutamine, 1 mM GS dodecamer, 5 mM ATase, 0.6 µM PII (monomer concentration) as indicated in the figures, mutant PII (monomer concentration) as indicated in the figures, and 0.5 mM [α-³²P]ATP. Reaction mixtures were incubated at 30°C and initiated by the addition of ATP. Sampling and counting were performed as in the UTase assay.

**RESULTS**

Formation of hybrid trimers containing wild-type and D47-53 mutant subunits. Non-denaturing gel electrophoresis easily resolves wild-type PII and D47-53 mutant homotrimers (3) (Fig. 1A, lanes 1 and 2). When each type of trimer was subjected to the urea denaturation-renaturation protocol described in Materials and Methods, the appropriate homotrimeric species were observed (Fig. 1A, lanes 3 and 4), indicating that the denaturation-renaturation protocol does not irreversibly destroy the trimer. As expected, when wild-type and D47-53 trimeric species were observed (Fig. 1A, lanes 6 to 8). When the proportion of wild-type to mutant trimers in the starting mixtures was 1:1, the two novel species were observed to be approximately equal in quantity (Fig. 1A, lane 6). When the proportion of wild-type to mutant subunits in the starting mixture was decreased, the band corresponding to the wild-type homotrimer was progressively diminished, and the proportion of the faster-migrating of the two novel species (novel species B [Fig. 1A]) was increased. When the starting ratio of wild-type to mutant trimers was 1:5, the band corresponding to the wild-type homotrimer was not evident (Fig. 1A, lane 8).

Denaturing SDS-PAGE, on the other hand, resolves wild-type and D47-53 mutant subunits, albeit less well than non-denaturing gel electrophoresis resolves the homotrimers (Fig. 1B, lane 2). The bands corresponding to the renatured homotrimers and the two novel species formed with a 1:1 starting ratio were excised from a non-denaturing gel and subjected to denaturing SDS-PAGE (Fig. 1B). As shown, only either wild-type or mutant subunits were obtained from their respective homotrimers, but the novel species contained both types of subunits (Fig. 1B, lanes 5 and 6), in the expected disproportional amounts. To confirm the expected disproportionality of the subunits recovered from the novel species, the experiment was repeated.
untreated proteins, wild-type and mutant homotrimers that had been put through the denaturation-renaturation protocol in isolation, and a 1:5 mixture of the proteins that had been treated separately and then combined (mixture). Previous results indicated that the Δ47-53 mutant protein used here, as untreated homotrimer, was entirely unable to be uridylylated by UTase/UR or to activate NR II or ATase (3).

**Uridylylation of wild type:Δ47-53 hybrid trimers.** As shown in Fig. 2A, the uridylylation of the untreated wild-type protein was slightly inhibited by the presence of a five-times-higher concentration of the untreated mutant trimer. When the wild-type protein was subjected to the denaturation-renaturation protocol in isolation, its uridylylation was decreased by ~60%, and a similar decrease was observed with the mixture of wild-type and mutant proteins that had been treated separately. The Δ47-53 mutant protein, when subjected to the protocol in isolation, was entirely without activity, indicating that the denaturation-renaturation protocol itself does not activate the mutant. Surprisingly, the heterotrimers were uridylylated considerably better than the treated wild-type protein and nearly as well as the untreated wild-type protein (Fig. 2A). In additional experiments, we observed that the uridylylated heterotrimers were rapidly deuridylylated by the Mn2+-dependent, glutamine-stimulated UR activity of UTase/UR (data not shown).

**Activation of the phosphatase activity of NR II by wild type:Δ47-53 hybrid trimers.** As shown in Fig. 2B, addition of wild-type PII to reaction mixtures containing NR II and phosphorylated NR II (NR II ∼P) resulted in the rapid dephosphorylation of NR II ∼P, as expected. This PII activity was diminished when the wild-type protein was put through the denaturation-renaturation protocol in isolation, and similar activity was obtained from the mixture of wild-type and mutant proteins that had been put through the protocol separately and then combined. As in the UTase assay, the mutant protein, when subjected to the protocol in isolation, was entirely without activity, indicating that the denaturation-renaturation protocol itself does not activate the mutant. As shown, the heterotrimers were able to activate the NR II phosphatase activity nearly as well as the sample containing untreated wild-type trimers, and considerably better than the wild-type protein that had been treated in isolation.

**Activation of the adenylylation activity of ATase by wild type:Δ47-53 hybrid trimers.** The ATase enzyme had a basal capacity to adenylylate GS in the absence of PII (Fig. 2C), as expected, and the presence of wild-type untreated PII homotrimers greatly stimulated this activity (Fig. 2C), also as expected. Treatment of the wild-type protein in isolation resulted in a reduction of this stimulatory capacity, and comparable stimulatory capacity was obtained when the wild-type and mutant proteins were treated separately and then mixed (Fig. 2C). As in the UTase and phosphatase assays presented above, treatment of the mutant protein in isolation did not alter the inactivity of this protein to activate the ATase (Fig. 2C). The sample containing heterotrimers was able to activate the ATase about as well as the wild-type protein that had been treated in isolation.

**Formation of hybrid trimers containing G89A subunits and either wild-type or Δ47-53 subunits.** Unlike the Δ47-53 protein, which was easily distinguished from the wild-type by nondenaturing gel electrophoresis (Fig. 1), the G89A homotrimer has a mobility on nondenaturing gels that is only slightly less than that of the wild-type (Fig. 3, lanes 1 and 3). When the G89A protein was put through the denaturation-renaturation protocol in isolation, trimers with mobility identical to that of the starting material were obtained (Fig. 3, lane 2). When the G89A protein was put through the denaturation-renaturation protocol in isolation, trimers with mobility identical to that of the starting material were obtained (Fig. 3, lane 2). When the G89A protein was put through the denaturation-renaturation protocol in isolation, trimers with mobility identical to that of the starting material were obtained (Fig. 3, lane 2).
FIG. 2. Characterization of the activity of wild type:Δ47-53 heterotrimers and protein mixtures. In all experiments, the treatment protocol, when indicated, was with 6 M urea. (A) Uridylation by UTase/UR. Where indicated, wild-type PII subunits (4.5 μM) and Δ47-53 subunits (22.5 μM) were present. In all cases, 4-μl aliquots were examined; thus, a total of 18 pmol of wild-type PII subunits was present in each sample, where indicated. Thus, the maximum extent of uridylation obtained was about 60% of the available sites. In all experiments, the concentration of UTP was 40 μM and that of UTase-UR was 0.01 μM. Symbols are as follows (ratios of wild-type to Δ47-53 subunits are given in parentheses): dot, untreated wild type; asterisk, treated wild type; square, heterotrimers formed (1:5); multiplier, mixture treated separately (1:5); and diamond, Δ47-53 treated in isolation. (B) Activation of the NRII-regulated phosphatase. Protein samples were the same as in panel A. Where indicated, wild-type subunits were present at a concentration of 1.5 μM. In all experiments, NRII (0.3 μM dimer), NRI (18 μM dimer), ATP (0.5 mM), and 2-ketoglutarate (50 μM) were used, and 4-μl aliquots were examined. Thus, each sample contained a total of 72 pmol of NRI dimers, and the maximum extent of NRII phosphorylation in the experiment was ~17% of the dimers, or 8.5% of the total sites. Symbols are as follows (ratios of wild-type to Δ47-53 subunits are given in parentheses): dot, control reaction containing no PII; plus sign, untreated wild type; asterisk, treated wild type; square, heterotrimers formed (1:5); multiplier, mixture treated separately (1:5); and diamond, Δ47-53 treated in isolation. (C) Activation of the adenylylation activity of ATase. Protein samples were the same as in panels A and B. Where indicated, the concentration of wild-type subunits was 0.6 μM and that of Δ47-53 subunits was 3.0 μM. In all experiments, ATase (5 nM monomer) and GS (1 μM dodecamer) were used. In all cases, 4-μl aliquots were examined; thus, each sample contained 4 pmol of GS dodecamer, or 48 pmol of GS subunits, and the maximum extent of modification observed was ~35% of the available sites. Symbols are as follows: dot, no PII added; plus sign, untreated wild-type PII; asterisk, treated wild type; square, heterotrimers; multiplier mixture of proteins treated separately; diamond, Δ47-53 treated in isolation.
tion of trimers had an even greater ability to activate the NRII phosphatase activity (Fig. 4B).

**Activation of the adenylylation activity of ATase by wild type:G89A hybrid trimers.** As shown previously, the G89A protein has a slight capacity to activate the ATase, corresponding to about 5% of the wild-type activation capacity (3). When a 10-fold excess of G89A protein is then compared to the wild type, we would expect the 10-fold greater amount of the G89A protein to have about half the activation capacity of the wild-type. As shown in Fig. 4C, when the wild-type and the G89A protein were put through the denaturation-renaturation protocol in isolation and the wild-type and a 10-fold greater amount of the G89A protein were compared for the ability to activate the ATase, the activity of the G89A protein was slightly lower than expected. That is, the treatment seemed to decrease the activity of the G89A subunits to a greater extent than it did the wild-type subunits. Also, when the samples that had been treated in isolation were then combined in a wild-type-to-G89A-subunit ratio of 1:10, the activity of the mixture was only a little greater than the activity of the wild-type-treated sample alone (Fig. 4C). The population of trimers resulting from treatment of the wild-type and G89A proteins together at a ratio of 1:10 (heterotrimers) had slightly less activity than expected, based on the activity of the equivalent mixture of proteins treated in isolation and then combined. That is, treatment of these proteins together resulted in a modest loss of ATase-activating activity.

**Activity of G89A:Δ47-53 hybrid trimers.** We also examined the activity of heterotrimers formed between the G89A and Δ47-53 proteins when these were present at a starting ratio of 1:5. These heterotrimers were not uridylylated (data not shown). Furthermore, these hybrid trimers lacked the ability to stimulate NRII phosphatase (Fig. 4B) or ATase (Fig. 4C).

**DISCUSSION**

Little is known concerning the interaction of PII with its protein receptors and the mechanisms by which PII activates NRII and ATase. In this study, we addressed the fundamental issue of the minimal requirements for the docking of PII to its receptors and the activation of these receptors, by examining the activity of heterotrimers, formed in vitro, containing various combinations of wild-type and mutant PII subunits. We observed that when the denaturation-renaturation protocol described in Materials and Methods was used with wild-type and Δ47-53 subunits or with G89A and Δ47-53 subunits, heterotrimers containing a distribution of subunits were formed as expected based on the starting ratios of the different subunits. That is, the different PII subunits we used seemed to be redistributed into trimers randomly. We also put G89A and wild-type subunits through the denaturation-renaturation protocol together so as to form heterotrimers, but in this case it was not possible to document the formation of hybrids, since the wild-type and G89A subunits are of similar size and charge. Nevertheless, the data in Fig. 4A suggest that in this case also, heterotrimers were formed. Thus, our experiments indicate that PII trimers can be disassociated and reformed by treatment with urea and dialysis. Such treatment was not, however, without some loss of activity (Fig. 2), and thus future studies should address the issue of optimizing the recovery of activity, for example by using different denaturants or otherwise tweaking the protocol.

Previous structural and genetic studies have implicated the large solvent-exposed T-loop of PII as playing a key role in the interaction with all three receptors (2, 3). Mutations altering the specificity of PII for the protein receptors have been iden-
tified within this part of PII, and the Δ47-53 form of PII, which lacks the apex of the loop and thus lacks the site of uridylylation, was shown to completely lack the ability to activate NRII and ATase (3). Since PII is trimeric, the docking of PII with its receptors and the activation of the dimeric NRII or ATase (quaternary structure unknown) could have required or have been stimulated by the simultaneous contact of more than one T-loop region with the receptors. Our experiments eliminated this possibility, since we observed that PII heterotrimers containing, on average, one wild-type subunit and two Δ47-53 mutant subunits could be uridylylated even better than the equivalent wild-type sample or protein mixture, could activate NRII even better than the control samples, and could activate the ATase about as well as the control samples. Thus, a single T-loop region within a PII trimer is sufficient for the productive interaction of PII with its protein receptors.

We do not currently know whether one PII T-loop region interacts with one or both subunits of NRII to activate NRII, or, in the former case, whether both subunits of the NRII dimer must bind a PII T-loop for NRII to be activated. However, the possibility that the interaction of more than one T-loop from a single PII trimer with the NRII dimer contributes significantly to the activation of NRII seems to be excluded by the results of Fig. 2B. If both subunits of NRII had to be contacted for activation, and a single PII trimer could provide the contacts, then the redistribution of wild-type subunits into trimers containing two Δ47-53 subunits should have resulted in decreased NRII activation, for in that case two different PII trimers, as opposed to a single PII trimer, would have to contact NRII. But such redistribution did not decrease the activation of NRII; in fact, it improved it (Fig. 2B). This suggests that the activation of NRII occurs by the same kinetic mechanism for both the treated wild-type sample (and the treated mixture) and the heterotrimer sample. We hypothesize that under the conditions of our assays, the concentration of NRII is limiting such that virtually no PII trimers simultaneously contact more than one NRII dimer. Then, redistribution of wild-type subunits into heterotrimers with two Δ47-53 subunits should yield (at most) a threefold increase in PII activity.

Another possibility is that the stimulatory effect of Δ47-53 subunits in the heterotrimers discussed above is due to an altered conformation promoted by the Δ47-53 subunits. Determination of the structure of the Δ47-53 homotrimer may yield some clues in this regard, as perhaps will the analysis of slightly different truncations within the loop region. Furthermore, since many PII mutations are available, including the G89A mutation (3), use of subunits bearing the Δ47-53 truncation and additional mutations elsewhere in PII may yield insights in future experiments.

Note that the experiments presented here do not address the issue of whether portions of PII other than the loop also form specific contacts with the protein receptors, or the number of subunits that form such contacts if they exist. In the accompanying study (3), we identified an alteration far from the T-loop region (T83N, within the B-loop) that diminished the interaction of PII with ATP and with all three receptors, but most seriously affected the interaction of PII with ATase. Whether this alteration in receptor specificity is due entirely to conformational and allosteric effects of the T83N alteration on the structure of the T-loop or whether the ATase requires a specific contact with the B-loop of PII that is destroyed by the T83N alteration is unknown. In additional experiments to determine whether the T-loop region of PII was all that was necessary for uridylylation and the activation of ATase or NRII, we chemically synthesized several different peptides representing various versions of the PII loop region, including circular peptides approximating the size and sequence of the loop region (unpublished studies). None of those peptides demonstrated any activity or the capacity to inhibit PII activity in any assay we have performed (unpublished data), and thus we do not include those data in this work. Anyone interested in the identities of those peptides should contact us.

Heterotrimers formed such that they contained two G89A subunits and one wild-type subunit were also examined. The G89A mutation alters the B-loop of PII. In this case, uridylylation seemed to be diminished, relative to the equivalent controls, the ability to activate NRII seemed to be stimulated, relative to the controls, and the activation of ATase seemed to be slightly diminished, relative to the equivalent controls. These results indicate that adverse subunit interactions within the wild-type;G89A heterotrimers in some cases decrease the activity of the wild-type subunits, particularly in the uridylylation assay. Since the G89A subunits have an intact T-loop region, we cannot discern which fraction, if any, of the activity of wild type:G89A heterotrimers is due to G89A subunits that have been activated by inclusion in heterotrimers with the wild-type subunit.

It is curious that the mixture of G89A and wild-type homotrimers that was treated in isolation and then combined had a greater ability to activate NRII than did the treated wild-type protein (Fig. 4B). That is, the presence of a 10-fold excess of treated G89A homotrimers, which were themselves inactive (Fig. 4B), increased the activity of the treated wild-type protein. Note that this phenomenon was not observed in the experiment of Fig. 2B, when a fivefold excess of treated Δ47-53 subunits was present. It seems likely that this stimulatory effect of treated G89A homotrimers was due to something unrelated to the signal-transduction function of PII; for example, some fraction of the treated proteins may adhere to the walls of the reaction tubes and be lost, and the very large excess of the inactive G89A protein (but not the slightly smaller excess of the treated Δ47-53 protein) may provide protection from this inactivation. These observations suggest that the renatured samples resulting from our protocol may be conformationally different from the untreated homotrimers and highlight the necessity of comparing the activities of the samples containing heterotrimers to that of the analogous control mixture containing the identical concentrations of proteins that had been treated separately and then combined. In the experiment shown in Fig. 4B, it is evident that the G89A:wild type heterotrimer sample had only slightly more activity than the corresponding protein mixture.

Binding of the small-molecule effectors ATP and 2-ketoglutarate greatly stimulates the ability of PII to become uridylylated and activate NRII (4, 5). The stoichiometry of small-molecule effector binding, the location of the effector-binding sites, and the effects of fractional occupancy of sites within the PII trimer are unknown or uncertain (4, 5). Particularly curiously, a low concentration of 2-ketoglutarate has been noted to be more effective than a higher concentration of 2-ketoglutarate in promoting the productive interaction of PII with NRII (4, 5), but these high and low concentrations of 2-ketoglutarate were equally effective in stimulating the productive interaction of PII with UTase (4). Since our denaturation-renaturation protocol results in active PII, this technique may prove useful in addressing some of these issues in future studies. For example, the G89A alteration results in the complete inability to bind the small-molecule effectors (3), and this may in fact explain the defect of this protein in interactions with all three receptors (3). It would be of interest to know whether the binding of small-molecule effectors by Δ47-53 or wild-type

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subunits is diminished upon distribution into heterotrimers containing two G89A subunits and whether the productive interaction of such wild type:G89A heterotrimers with NRII is inhibited by 2-ketoglutarate at high concentrations. Alternatively, the G89A protein may be unable to undergo a conformational change necessary for interaction with the receptors that results from effector binding, and its inclusion within hybrid trimers may not affect the binding of small-molecule effectors by the wild-type subunits but may limit the ability of these to undergo the necessary conformational change. Future experiments in which the G89A alteration and other alterations in this part of PII known to affect the binding of effectors (3) are combined in the same subunit with the Δ47-53 truncation may yield further insights, as noted earlier.

Also, future studies should establish whether the binding of small-molecule effectors is affected by the presence of urea at concentrations that promote reassortment of subunits. Such studies may reveal that the monomeric form of the protein retains the ability to bind the small-molecule effectors, and thus that the effector binding sites are not composed of residues at the subunit interfaces, as was shown earlier for NRII (9).

We also examined the effect of including G89A and Δ47-53 subunits in heterotrimers. Neither of these, as homotrimers, are uridylylated or activate the NRII or ATase to a significant extent (3). The G89A protein contains a normal loop region, while the D47-53 protein binds the small-molecule effectors normally. However, these functions could not complement in trans within the heterotrimer, since heterotrimers formed from these subunits were completely inactive.

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