Effect of Mutations in Escherichia coli glnL (ntrB), Encoding Nitrogen Regulator II (NRII or NtrB), on the Phosphatase Activity Involved in Bacterial Nitrogen Regulation* 

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We examined the effects of mutations in glnL, encoding the signal-transducing kinase/phosphatase nitrogen regulator II (NRII), on the regulated phosphatase activity of NRII. Three different glnL mutations result in altered NRII proteins that had phosphatase activity in the absence of PII. The most active of these contained an alteration of the site of NRII autophosphorylation, histidine 139, to asparagine (H139N). The phosphatase activity of the NRII-H139N protein was further stimulated by the PII protein and by ATP. This suggests that the PII protein is not directly involved in a catalytic step of the regulated phosphatase activity but rather plays a regulatory role. We also measured the effect on the regulated phosphatase activity of alterations at conserved residues in the kinase/phosphatase domain of NRII and the effect of deleting the non-conserved N-terminal domain of NRII. For this we used fusion proteins containing the Escherichia coli maltose-binding protein (MBP) linked to the protein of interest. A protein consisting of MBP linked to wild-type NRII was a less active kinase than was wild-type NRII but in the presence of PII had wild-type phosphatase activity. A protein consisting of MBP linked to just the C-terminal domain of wild-type NRII had kinase activity but lacked phosphatase activity. Alterations at the highly conserved residues Asp-287, Gly-289, and Gly-291 in NRII affected both activities. A fusion of MBP to the NRII-H139N protein lacked kinase activity but had phosphatase activity in the absence of PII. Thus, while the kinase and phosphatase activities of NRII could be genetically separated, some of the highly conserved residues in the C-terminal domain of NRII (Asp-287, Gly-289, Gly-291) are apparently important for both activities.

Nitrogen regulation of transcription in Escherichia coli and related bacteria results from the control of the intracellular concentration of the phosphorylated form of the transcription factor NRII (NtrC) (reviewed in Refs. 1–3). The unphosphorylated form of NRII is a dimeric DNA-binding protein, which is not able to activate transcription (4, 5). Upon phosphorylation, NRII is thought to form a tetramer or higher oligomer (6–8), binds in a highly cooperative fashion to sites located far from the observed promoter (−10), has an ATPase activity (6, 8, 11), and activates transcription by RNA polymerase containing glnA (4, 12). Among the target promoters is the glnAp2 promoter, which permits the glnA gene, encoding glutamine synthetase, to be expressed under conditions of nitrogen limitation (13).

In intact cells, NRII becomes phosphorylated upon interaction with either the phosphorylated form of the glnL (ntrB) product, NRII, or the phosphorylated metabolic intermediate acetyl phosphate (6, 14). NRII is autophosphorylated on residue histidine 139 in an ATP-dependent reaction, and these phosphoryl groups are transferred to NRII (14, 15, 24, 29). Phosphorylation of NRII occurs on an aspartyl residue (Asp-54) within its N-terminal domain (14–16), which apparently controls the activity of the C-terminal and central domains. The central domain contains the inherent ATPase and transcriptional activation activities (8), and the C-terminal domain is responsible for the interaction with DNA.

The acyl phosphate moiety in the phosphorylated form of NRII has a characteristic instability (t½) for hydrolysis at pH 7.0 of about 4–8 min (14, 15) that is considerably greater than that observed when the protein is denatured (t½ for hydrolysis of >3 h). This “self-catalyzed” dephosphorylation reaction has been called the autophosphatase activity (15). In cells grown with nitrogen excess, the dephosphorylation of NRII-P is regulated by a second activity referred to as the regulated phosphatase activity (4, 15, 17). The regulated phosphatase activity is observed when NRII interacts with NRII and the glnB product, PII, and results in the rapid dephosphorylation of NRII-P in vitro (4, 15, 18). This activity requires both NRII and PII, as mutations eliminating NRII abolish the negative regulation of glnA and mutations eliminating PII result in a severe defect in the negative regulation of glnA under nitrogen excess conditions (17, 19–22). In addition to the loss of function mutations noted above, point mutations in glnL, encoding NRII, have been identified that render NRII defective in negative regulation but do not affect the ability of NRII to positively regulate glnA expression (17, 19–23). Conversely, mutations in glnL have been identified that result in negative regulation of glnA even under conditions of nitrogen limitation or in the absence of PII (20, 22). One such mutation occurs as a result of the conversion of the site of NRII autophosphorylation, histidine 139 (μZE product); MBP-cNRII, fusion of the maltose-binding protein to the C-terminal domain of NRII; MBP-NRII, fusion of the maltose-binding protein to intact (full-length) NRII; NRII-H139N, NRII-G313A, NRII-G289A, and NRII-D287N, intact NRII except containing the indicated alteration (standard single-letter amino acid symbols).
to the non-phosphorylatable residue asparagine (H139N) (20, 24). These results support a model in which NRII is able to adopt two alternative conformations, one of which stimulates the dephosphorylation of NRI–P (17). The role of the PII protein according to this model is to regulate the equilibrium between the two different conformations of NRII. It should be noted, however, that PII has no apparent effect on the rate of NRII autophosphorylation in the absence of NRI (18). This may signify that PII does not interact with NRII in the absence of NRI or that the interaction of PII with NRII does not affect the autophosphorylation of NRII. In either case, there is no direct kinetic evidence for two alternative conformations of NRII (18).

Nitrogen regulation of the system is apparently due to the regulation of the activity of PII. Under conditions of nitrogen limitation, PII is converted to a uridylylated form by the product of glmD, a uridylyltransferase/uridylyl-removing enzyme (reviewed in Refs. 25 and 26). PII-UMP is unable to productively interact with NRII to elicit the regulated phosphatase activity (17, 33). Under nitrogen excess conditions, the glmD product removes the uridylyl groups from PII, which permits the interaction of PII with NRII. This results in the appearance of the regulated phosphatase activity, the dephosphorylation of NRI–P, and the cessation of transcriptional activation from nitrogen-regulated promoters such as glnAp2.

NRI and NRII are closely related to many other regulatory proteins, in both bacteria and eucaryotic cells, that utilize a common phosphorylation and dephosphorylation mechanism to regulate various cellular functions (for reviews see Refs. 3 and 27). In the aggregate, these systems have been called the two-component systems. In each case, one component (response regulator) contains a domain homologous to the N-terminal regulatory domain of NRI, which becomes phosphorylated on an aspartate residue. The other component (histidine kinase) contains a domain homologous to the C-terminal domain of NRII, which contains the site of NRII autophosphorylation and residues involved in the binding of ATP. This conserved domain is associated with additional unconserved domains that are thought to mediate the sensation of distinct stimuli that control the activities of each protein.

In this report, we characterize the regulated phosphatase activity in several mutant forms of NRII, using purified bacterial components. Our results suggest that PII had no catalytic role in this reaction but was strictly regulatory. Furthermore, the highly conserved histidine that is the site of NRII auto-phosphorylation (H139) was not required for the regulated phosphatase activity, indicating that this dephosphorylation reaction is not the reversal of the reaction in which NRI becomes phosphorylated. Finally, while the kinase and phosphatase activities of NRII could be genetically separated, some of the highly conserved residues in the C-terminal domain of NRII were apparently important for both activities.

**EXPERIMENTAL PROCEDURES**

**Purified Proteins**—NRI, PII, NRII, NRII-A129T (NRII2302), NRII-H138N, MBP-NRII (fusion of the maltose-binding protein to the N terminus of full-length, wild-type NRII), NRII-G131A, and NRII-ter291 (a truncated NRII resulting from a termination codon at codon 291) were purified as described previously (5, 12, 18, 28, 29). Each protein was greater than 90% pure, as judged by its appearance on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels, except for the NRII-G131A and NRII-ter291 mutant proteins, which were about 80-90% pure, as described previously (29). To construct a fusion protein consisting of the maltose-binding protein fused to the C-terminal domain of NRII (MBP-NRII), we placed an EcoRI site immediately upstream of the dephosphorylation of NRI-P (17). The role of the PI1 pro-tein according to this model is to regulate the equilibrium between the two different conformations of NRII. It should be noted, however, that PI1 has no apparent effect on the rate of NRII autophosphorylation in the absence of NRI (18). This may signify that PI1 does not interact with NRII in the absence of NRI or that the interaction of PI1 with NRII does not affect the autophosphorylation of NRII. In either case, there is no direct kinetic evidence for two alternative conformations of NRII (18).

**RESULTS**

**Dephosphorylation of NRI–P by the Combination of NRII and PII**—Using purified NRI, PII, and various mutant forms of NRII, we examined the PII-dependent dephosphorylation of NRI–P, referred to as the regulated phosphatase activity. It has been shown that the regulated phosphatase activity was observed only in the absence of PII when wild-type NRI was used (4, 18) and that the regulated phosphatase activity was not detectable when the NRII-A129T (NRII2302) mutant form of NRII was used in place of NRII (4, 18). Furthermore, PII did not affect the rate or extent of autophosphorylation of NRI nor did PII seem to affect the rate of transfer of phosphoryl groups.
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**Fig. 1. Effect of wild-type and mutant forms of NRII on the extent of NRI phosphorylation in the presence and absence of PII.** NRI (20 μM) was phosphorylated by the MBP-cNRII protein (250 nM) in the presence of 0.5 mM [γ-32P]ATP. After reaction for 25 min, the mixtures were split, and the aliquots received either buffer (○) or the indicated protein (final concentrations: 300 nM MBP-NRII; ▲, wild-type NRII; ●, MBP-NRII; ●, NRII-G313A). Panel A, effect of addition of NRII and various NRII mutant proteins. □, NRII-A129T (NRII28296); *, wild-type NRII; ▲, MBP-NRII; ●, NRII-G313A. Panel B, effect of simultaneous addition of PII (4 μM) and NRII or mutant NRII proteins. The symbols for the different NRII proteins are the same as in panel A; △ indicates a reaction mixture receiving only PII but no additional NRII. Panel C, effect of the NRII-ter291 protein on the phosphorylation of NRI. Symbols are as in panels A and B; ▼, NRII-ter291 protein in the absence of PII; △, NRII-ter291 protein in the presence of PII (4 μM). Different preparations of labeled ATP were used in the three experiments.

from NRII–P to NRI (18). Further background information on the assay can be obtained from Ref. 18.

We observed early in our work that the MBP-cNRII construct, in which the N-terminal domain of NRII (codons 1-110) was deleted and replaced by the maltose-binding protein, was able to become autophosphorylated and transfer phosphoryl groups to NRI but did not stimulate the dephosphorylation of NRI–P in the presence of PII. That is, MBP-cNRII specifically lacked the regulated phosphatase activity. The ability of MBP-cNRII to phosphorylate NRI and the inability of MBP-cNRII to dephosphorylate NRI–P in the presence of PII are shown in Fig. 1, A and B, respectively. Since the MBP-cNRII protein lacks the regulated phosphatase activity, we used this protein to phosphorylate NRI and then assessed whether other NRI proteins had the regulated phosphatase activity by adding them to reaction mixtures either alone or in combination with the PII protein (Fig. 1 and 2). As shown, the addition of NRII-A129T, NRII, or MBP-NRII to the reaction mixture in the absence of PII resulted in the further phosphorylation of NRI (Fig. 1A). Of these proteins, the MBP-NRII construct resulted in the smallest increase in the phosphorylation of NRI. In additional experiments we have observed that MBP-NRII is somewhat defective as a kinase. While the MBP-NRII protein readily autophosphorylates (29), the rate of transfer of the phosphoryl groups to NRI is slower than with wild-type NRII (data not shown). The addition of the NRII-G313A protein to an identical reaction mixture (another aliquot of the phosphorylation reaction mixture) resulted in the slow dephosphorylation of NRI–P. Thus, while the MBP-NRII protein is somewhat defective as a kinase, it was essentially identical to wild-type NRII with regard to the regulated phosphatase activity. The NRII-G313A protein was an even more potent phosphatase than NRII in the presence of PII. As observed previously, the NRII-A129T protein fails to interact productively with PII (Fig. 1B); thus, it lacks the regulated phosphatase activity. These results agree with the expectations based on previous data for NRII and NRII-A129T (4, 18) and indicate that the assay is suitable for characterization of the phosphatase activity of mutant forms of NRII.

Interestingly, three mutant forms of NRII (NRII-H139N, NRII-G313A, and NRII-ter291) brought about the dephosphorylation of NRI–P even in the absence of PII (Figs. 1 and 2). PII resulted in a further stimulation of the already rapid rate of dephosphorylation of NRI–P catalyzed by the NRII-H139N protein (Fig. 2). Only when the NRII-H139N protein was present at the very low concentration of 10 nM (30-fold lower than the concentration of mutant NRII proteins used in the experiment shown in Fig. 1) was the dephosphorylation of NRI–P strongly dependent on the presence of PII (Fig. 2). In contrast, the NRII-ter291 protein at the high concentration of 320 nM was a weak phosphatase in the absence of PII, and this activity was not significantly stimulated by the presence of PII (Fig. 1C).

The Phosphatase Activity of the NRII-H139N and NRII-G313A Proteins Is Stimulated by ATP—Previous experiments had indicated that the regulated dephosphorylation of purified NRI–P was stimulated by ATP (15). Dephosphorylation of the related OmpR response regulator protein by its cognate histidine kinase EnvZ was also shown to require ATP (32). We purified NRI–P as described before (14, 15); this involved producing NRI–P by incubation of NRI with NRII and ATP and separation of NRI–P from the other reaction components by chromatography on heparin-Sepharose in the presence of EDTA. As shown in Fig. 3, NRI–P prepared in this manner was relatively stable in the presence of EDTA but was rapidly dephosphorylated upon the addition of MgCl₂ due to the auto-phosphatase activity of NRI–P. The τₜ for spontaneous hydrolysis in the presence of MgCl₂ was longer in our experiments (~10 min) than observed previously (~4–8 min (14, 15)), since our experiments were performed at 27 °C and the earlier experiments had been performed at 37 °C. The dephosphorylation of purified NRI–P was stimulated by the NRII-H139N and NRII-G313A proteins, and this dephosphorylation was fur-
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A

B

FIG. 2. Phosphatase activity of the H139N protein in the presence and absence of PII. The experiment is similar to that shown in Fig. 1, except that much lower concentrations of the NRII-H139N protein were used. NRI was present at 20 μM, MBP-cNRII was at 250 μM, and ATP was at 0.5 mM. Panel A, effect of PII (4 μM) on the phosphatase activity of NRII-H139N protein when NRII-H139N was at 35 μM. ●, MBP-cNRII alone; ○, PII added in the absence of additional NRII; ×, NRII-H139N protein in the absence of PII; ⋆, NRII-139N + PII. Panel B, effect of PII (1.33 μM) on the phosphatase activity of NRII-H139N when NRII-H139N was at 10 μM. Symbols are as in panel A. Different preparations of labeled ATP were used in the two experiments.

Fig. 3. Dephosphorylation of purified NRI−P by the H139N and G313A proteins. Purified NRI−P was incubated at 27 °C in the presence of 1 mM EDTA, 10 mM MgCl₂, or the combination of MgCl₂ and indicated proteins (final concentration, 150 μM) + ATP (final concentration, 1 μM) as indicated. At the indicated times, samples were removed, and trichloroacetic acid-insoluble radioactivity was measured. Two different preparations of NRI−P were used for the experiments shown in the two panels.

Effect of Point Mutations at the Highly Conserved NRII-D287, NRII-G289, and NRII-G291 Residues on the Regulated Phosphatase Activity—In order to readily check the properties of additional NRII proteins altered at particular codons as a result of point mutations, we constructed fusions in which full-length altered NRII proteins were fused to the maltose-binding protein. Four different point alterations of NRII were checked as MBP fusions: G289A, G291A, D287N, and H139N. The first three of these are altered in highly conserved residues shared by the kinases of the two-component systems, while the H139N alteration changes the site of NRII autophosphorylation to a non-phosphorylatable amino acid. This last protein was constructed to serve as a control for the effects of fusing NRII to MBP, since we have characterized the phenotype of the unfused, native H139N protein (Figs. 2 and 3). The results of SDS-polyacrylamide gel electrophoresis of the purified fusion proteins used in these experiments are shown in Fig. 4A.

The various single amino acid alterations had a clearly discernible effect on the phosphorylation of NRII by the MBP-NRII fusion proteins (Fig. 4B). As expected, the H139N alteration resulted in the absence of detectable phosphorylation activity (Fig. 4B). The D287N alteration also essentially eliminated the ability to phosphorylate NRI (Fig. 4B). The G289A and G291A alterations adversely affected the phosphorylation activity but did not eliminate it (Fig. 4B).

The regulated phosphatase activity of these MBP-NRII fusion proteins in the presence of PII was examined. The MBP-NRII-H139N construct had phosphatase activity in excess of the wild type, while the other mutant forms of MBP-NRII were clearly defective in this activity (Fig. 4C). MBP-NRII-H139N had considerable phosphatase activity in the absence of PII, and PI1 stimulated this activity (Fig. 4D). Thus, both native H139N protein and the MBP-NRII-H139N fusion share the property of possessing phosphatase activity in the absence of PII, which is stimulated by PII.

ther stimulated by ATP (Fig. 3). This result suggests that the phosphatase activity seen with these two proteins in the absence of PII is the same activity observed with wild-type NRII in the presence of PII. Unlike previous results with EnvZ and OmpR, ADP could not substitute for ATP in this reaction (data not shown).
DISCUSSION

The mechanisms by which NRI becomes phosphorylated and dephosphorylated indirectly control the activation of transcription from nitrogen-regulated promoters. Previous work has indicated that NRI–P has an inherent instability (autophosphorylation) and can be dephosphorylated at a still faster rate by the concerted action of NRII and PI1 (regulated phosphatase activity). We examined the effects of mutations in NRII on the regulated phosphatase activity. While this regulated phosphatase activity required the simultaneous presence of PI1 when wild-type NRII was present, three different mutant forms of NRII (NRII-H139N, NRII-G313A, and NRII-ter291) stimulated the dephosphorylation of NRI–P in the absence of PI1. In the case of the NRII-G313A and NRII-ter291 proteins, which were only about 80–90% pure as judged by their appearance on stained gels, the possibility cannot be excluded that the observed phosphatase activity is due to a contaminating phosphatase. However, no such activity was observed with the other purified proteins, which were overproduced in and purified from the same genetic background. It is very unlikely that the phosphatase activity of the NRII-H139N construct was due to a contaminating activity, since both the native NRII-H139N protein and the MBP-NRII-H139N fusion protein had this activity. Furthermore, for the native NRIII-H139N and NRII-G313A proteins, this activity was stimulated by ATP, as had been previously shown for wild-type NRII (15). Thus, it seems that PI1 is not directly involved in the catalysis of the dephosphorylation of NRI–P but rather plays a regulatory role. Also, the conserved site of autophosphorylation, histidine 139, is not required for the regulated phosphatase activity, indicating that this phosphatase activity is not the reversal of the phosphotransfer reaction.

As noted above, the regulated phosphatase activity is stimulated by ATP (15). Our results with the NRII-H139N protein serve to clarify this finding somewhat, since we have previously shown that this protein does not become autophosphorylated in the presence of ATP (29). Thus, the ATP effect noted here, presumably the same phenomenon observed previously by Keener and Kustu (15), cannot be due to the autophosphorylation of NRII. We also examined the regulated phosphatase...
activity of NRII-G313A, previously considered to be defective in ATP binding based on its inability to become cross-linked to ATP under conditions where wild-type NRII and NRII-H139N were readily cross-linked to ATP (29). The rate of autophosphorylation of NRII-G313A is about 20-fold slower than NRII (29). In intact cells, the NRII-G313A protein was a very weak positive regulator of glnA in vivo but seemed to be unaffected in the PII-dependent negative regulation of glnA expression (20). We observed here that this protein is a better phosphatase than NRII in the presence of PII, and that it had considerable phosphatase activity in the presence of PII. This latter point was not obvious from the results using intact cells (20). The ATP dependence of this phosphatase activity suggests that this is the same activity observed with wild-type NRII + PII. Since the ATP concentration used here is 2 orders of magnitude higher than that used in the previous cross-linking studies (29), it is possible that the NRII-G313A protein is binding ATP in our experiments. The stimulation by ATP was not as great for the NRII-G313A protein as for the NRII-H139N protein (Fig. 3). However, there are many other alternative explanations for the ATP effect that remain unexcluded. For example, NRII may contain a second, low affinity, ATP-binding site that was not detected in our cross-linking experiments (29) yet is required for the regulated phosphatase activity. Alternatively, the ATP requirement for the regulated phosphatase activity may reflect the necessity of binding ATP to another component of the system, such as NRI–P. This is not implausible, since NRI–P is an ATPase and therefore must bind ATP (6, 8, 11).

Our previous work with intact cells indicated that NRII-H139N is a transdominant negative regulator of glnA in intact cells and a negative regulator even in the absence of PII (20). The results obtained here with the purified NRII-H139N protein are in excellent agreement with those results. In contrast, the results obtained here with the MBP fusion proteins containing the D287N, G289A, and G291A alterations are not consistent and C-terminal domains of NRII that result in the loss of the capacity to productively interact with PII (19, 20). Conceivably, the mutations in the conserved C-terminal domain of NRII having this property may affect the catalysis step.

One hypothesis to explain the mechanism of action of PII is that NRII exists in two alternative conformations, one of which is responsible for the regulated phosphatase activity (17). Apparently, NRII-H139N, NRII-G313A, and NRII-ter291 proteins can adopt the "phosphatase" conformation even in the absence of PII, and PII further shifts the equilibrium toward the "phosphatase" activity. Possibly, the same phenomenon occurs with wild-type NRII but is masked by the kinase activity of NRII. Unfortunately, there is still no direct biochemical evidence for these two alternative conformations. Another question regarding the regulated phosphatase activity is whether this activity represents an acceleration of the autophosphatase activity or is a separate activity. Future efforts will be directed toward resolving these issues.

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