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Phosphorylation and Dephosphorylation of the NarQ, NarX, and NarL Proteins of the Nitrate-Dependent Two-Component Regulatory System of *Escherichia coli*

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The NarX, NarQ, and NarL proteins make up a nitrate-responsive regulatory system responsible for control of the anaerobic respiratory pathway genes in *Escherichia coli*, including nitrate reductase (*narGHJI*), dimethyl sulfoxide/trimethylamine-*N*-oxide reductase (*dmsABC*), and fumarate reductase (*frdABCD*) operons among others. The two membrane-bound proteins NarX and NarQ can independently sense the presence of nitrate and transfer this signal to the DNA-binding regulatory protein NarL, which controls gene expression by transcriptional activation or repression. To establish the role of protein phosphorylation in this process and to determine whether the NarX and NarQ proteins differ in their interaction with NarL, the cytoplasmic domains of NarX and NarQ were overproduced and purified. Both proteins were autophosphorylated in the presence of [γ-^{32}P]ATP and $MgCl_2$ but not with [α-^{32}P]ATP. Whereas these autophosphorylation reactions were unaffected by the presence of nitrate, molybdate, GTP, or AMP, ADP was an inhibitor. The phosphorylated forms of 'NarX and 'NarQ were stable for hours at room temperature. Each protein transferred its phosphoryl group to purified NarL protein, although 'NarQ-phosphate catalyzed the transfer reaction at an apparently much faster rate than did 'NarX-phosphate. In addition, NarL was autophosphorylated with acetyl phosphate but not with ATP as a substrate. NarL-phosphate remained phosphorylated for at least 3 h. However, addition of 'NarX resulted in rapid dephosphorylation of NarL-phosphate. In contrast, 'NarQ exhibited a much slower phosphatase activity with NarL-phosphate. These studies establish that the cytoplasmic domains of the two nitrate sensors 'NarX and 'NarQ differ in their ability to interact with NarL.

The expression of the anaerobic respiratory pathway genes for nitrate reductase (*narGHJI*), dimethyl sulfoxide reductase (*dmsABC*), and fumarate reductase (*frdABCD*) in *Escherichia coli* is regulated in response to nitrate availability and anaerobiosis (4, 17, 35). During anaerobic cell growth, this control ensures that *narGHJI* expression is optimal when nitrate is present and that the level of expression of the *dmsABC* and *frdABCD* operons remains low until nitrate is either depleted from the medium or is absent (see reference 6 for a review). The transcription of these respiratory genes is mediated by the anaerobic activator protein Fnr (18, 22, 35); gene expression is stimulated by 12- to 70-fold when oxygen is depleted from the cell's environment (6). Nitrate control of these respiratory operons is mediated by the products of three genes, *narX*, *narQ*, and *narL* (3, 7, 19, 20, 30, 32, 35, 36). On the basis of comparison of the amino acid sequences of these gene products with those of other prokaryotic regulatory proteins, NarX and NarQ were predicted to be sensor transmitter proteins that detect nitrate in the environment and interact with the response regulator protein NarL to activate or repress transcription of the anaerobic respiratory pathway genes.

The NarX and NarQ proteins have been shown to sense nitrate availability independently of each other (3, 32). However, it is not yet clear why the cell employs two nitrate-sensing proteins for achieving nitrate regulation. NarX and NarQ are similar in size and amino acid sequence and contain a conserved histidine residue common to other sensor transmitter proteins (2, 3, 8, 28, 32). Both sensors also contain two regions of hydrophobic amino acids in their N-terminal portion which are responsible for targeting the proteins to the cytoplasmic membrane (3, 32). The NarX protein was recently partially purified from the membrane fraction of *E. coli* cells and was shown to covalently attach phosphate which could be subsequently transferred to the NarL protein (37). Similar phosphotransfer activities have been previously demonstrated for other bacterial two-component regulatory systems (1, 5, 8–10, 15, 21, 23, 27, 29, 33, 39). The properties of the NarQ protein in this process have not yet been examined.

To establish whether the NarQ protein of *E. coli* can also function biochemically like NarX in protein phosphorylation and to define whether these two nitrate sensors act equivalently in their ability to autophosphorylate and to phosphorylate NarL, we purified the cytoplasmic domains of the NarX and NarQ proteins to homogeneity. Each sensor was shown to have autokinase activity, and each would transfer its phosphoryl group to purified NarL protein. However, NarL was phosphorylated at different apparent rates. The NarX and NarQ proteins were also shown to dephosphorylate NarL-phosphate; NarX had the faster phosphatase activity. Thus, the NarX and NarQ nitrate sensors differ in their ability to interact with NarL, which suggests a rationale for why the cell contains two seemingly redundant proteins.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. All strains used were derivatives of *E. coli* K-12. Strains CJ236 and MV1190 (Bio-Rad) were employed for site-directed mutagenesis experiments. M13KO7 (Bio-Rad) was used as a helper

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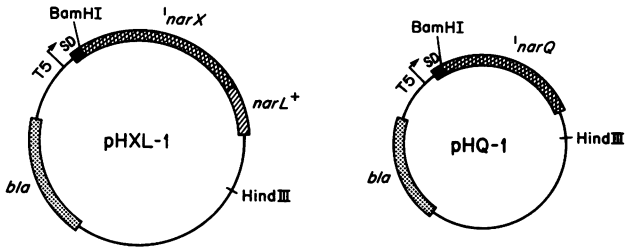

FIG. 1. Diagram of the *'narXL* and *'narQ* overexpression plasmids used for NarX, NarL, and NarQ protein production. The solid line represents the plasmid vector, the cross-hatched boxes indicate the *'narX*, and *'narQ* genes, and the hatched box indicates the *narL* gene, while the solid boxes indicate the codons for the N-terminal Met-$(His)_6$ residues. SD indicates the location of the Shine-Dalgarno sequence, while T5 and the bent arrow represent the T5 promoter location. The *bla* gene (dotted boxes) encodes β-lactamase.

phage in the site-directed mutagenesis experiments. Strain JM101 (Stratagene) was used for all *narX*, *narQ*, and *narL* expression experiments. To overexpress *narX*, a *Bam*HI restriction enzyme site was introduced into the *narX* gene at nucleotide position 566 with respect to position +1 of the start of *narX* translation by using the Bio-Rad site-directed mutagenesis kit. Phagemid pIS19, which contains the *narXL* genes inserted on a 5.0-kb *Hin*dIII fragment in pTZ19U (Bio-Rad), was used as the template for mutagenesis. The introduction of the *Bam*HI site changed the serine codon at amino acid position 190 to an aspartate codon and the alanine codon at position 191 to a proline codon. This *narX* mutant plasmid was designated pIS27. A 3.1-kb *Bam*HI-*Hin*dIII fragment containing the C-terminal region of *narX* and the entire *narL* gene was cloned into plasmid pQE10 (Qiagen) to create an in-frame fusion between the new N-terminal methionine and six histidine codons provided by pQE10 and aspartate codon 190 of *narX*. The resulting plasmid was designated pHXL1 (Fig. 1). The expression of the *'narX* and *narL*$^+$ genes on pHXL1 was controlled from an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible T5 promoter under control of the LacI repressor. Induction of cells containing plasmid pHXL1 with IPTG resulted in production of a Met-$(His)_6$-NarX hybrid protein containing the C-terminal 408 amino acids from position 190 to position 598 of the NarX protein (Fig. 2).

To create a *narQ* expression vector, a *Bam*HI restriction enzyme site was introduced by site-directed mutagenesis at nucleotide position 512 with respect to position +1 of the start

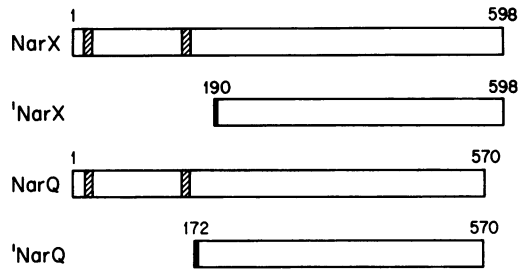

FIG. 2. Schematic diagram of the full-length and cytoplasmic domains of the NarX and NarQ proteins. The boxed areas denote the protein regions, the hatched regions indicate the predicted transmembrane domains, and the solid regions symbolize the Met-$(His)_6$ termini. The numbers above the boxes indicate positions of the N- and C-terminal amino acid residues.

of *narQ* translation; phagemid pRQ1000R (3) was used as a template for mutagenesis. Introduction of the *Bam*HI site into this plasmid, designated pRCQ44, did not result in any amino acid changes in the NarQ protein. A 1.4-kb *Bam*HI-*Hin*dIII fragment containing the C-terminal region of *narQ* was cloned into pQE11 (Qiagen) to create pHQ1 (Fig. 1). Like plasmid pHXL1, pHQ1 contains an IPTG-inducible T5 promoter and a ribosome-binding site for translation initiation and expression of the desired recombinant gene. The recombinant *'narQ* gene encodes an N-terminal Met-$(His)_6$ fused to Ile codon 172 (Fig. 2).

Cell growth. For plasmid, phage, and strain manipulations, cells were grown aerobically in Luria broth or on solid medium. When required, ampicillin and chloramphenicol were added to the medium at concentrations of 100 and 30 mg/liter, respectively. For production of the 'NarX, 'NarQ, and NarL proteins, 100- or 400-ml cultures containing strain JM101 with pHXL1 or pHQ1 were grown to late log phase (optical density at 600 nm, 0.7 to 0.9) at 37°C. IPTG was added to the culture medium at a final concentration of 2 mM, and cells were grown for an additional 3 h at 37°C. Bacteria were harvested by centrifugation at 4°C and stored at −20°C.

Purification of the 'NarX and 'NarQ proteins. Cells from a 100-ml induced culture (ca. 0.8 g [wet weight]) of JM101/pHXL1 or JM101/pHQ1 were resuspended in 3 ml of 50 mM Tris-HCl (pH 7.6) and sonicated for 2 min at 30-s intervals on ice (Ultrasonics Corp.). The membrane fraction was isolated by centrifugation at 100,000 × *g* for 30 min at 4°C and resuspended in 2 ml of Tris-urea buffer (50 mM Tris-HCl [pH 7.6], 8 M urea, 5 mM β-mercaptoethanol). The mixture was stirred slowly for 1 h at room temperature and then centrifuged at 100,000 × *g* for 30 min at 20°C to remove the lipid fraction. A Ni-nitrilotriacetic acid resin Qiagen column (3 ml) equilibrated with Tris-urea buffer was added to the urea-solubilized membrane proteins, and the suspension was gently mixed for 1 h at room temperature. The slurry was poured into a 10-ml column and washed with 10 ml of Tris-urea buffer. The 'NarX and 'NarQ proteins were eluted with 3 ml of Tris-urea buffer containing 0.5 M imidazole. The following protein refolding procedure was performed at 4°C. The eluted proteins were diluted in a stepwise fashion in Tris-Triton buffer (30 mM Tris-HCl [pH 7.6], 200 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100) until a protein concentration of about 0.2 mg/ml was reached. After each dilution, the protein solution was incubated for at least 6 h. The protein solution was then dialyzed without stirring against 2 volumes of Tris-Triton buffer. The dialysis buffer was changed until the urea concentration was below 0.25 M. The protein solution was then dialyzed once for 10 h with stirring against 100 volumes of Tris-Triton buffer. The purified 'NarX and 'NarQ proteins were stored at −70°C.

Purification of NarL. Cells of a 400-ml induced JM101/pHXL1 culture (about 3.7 g [wet weight]) were resuspended in 10 ml of 50 mM Tris-HCl (pH 7.6)–50 mM KCl and passed through the French press cell twice at a cell pressure of 537 lb/in^2. To shear chromosomal DNA, the cell lysate was sonicated for 30 s on ice and then centrifuged at 100,000 × *g* for 30 min to remove membrane material. The soluble fraction (98 mg of protein) was gently mixed with Tris-KCl buffer-equilibrated DE 52 (Whatman) anion-exchange resin for 1 h on ice. The protein-DE 52 slurry was then poured into a column (50-ml bed volume) and washed with 50 ml of Tris-KCl buffer. The DE 52 column wash was concentrated 40-fold with a Centriprep-10 filter (Amicon) and loaded in 0.2-ml portions onto a Superose 12 gel filtration column (Pharmacia) equilibrated with Tris-KCl buffer. All purification steps with the

exception of the gel filtration were performed at 4°C. Glycerol was added to the purified NarL protein to give a final concentration of 10% (vol/vol), and the purified NarL protein was stored at −70°C. Protein concentration was determined by the Coomassie brilliant blue G-250 assay (Bio-Rad) with bovine serum albumin as a standard.

Phosphorylation assays for 'NarX, 'NarQ, and NarL. Protein phosphorylation assays were carried out at room temperature in a 10-μl volume containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES [pH 8.0]), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM dithiothreitol. The reaction was initiated by addition of 25 μM [γ-^{32}P] ATP (3,000 Ci/mmol [NEN]), incubated for 15 min, and then stopped by the addition of 4 μl of sodium dodecyl sulfate (SDS) loading buffer (10 mM Tris-HCl [pH 8.0], 10% SDS, 20% β-mercaptoethanol, 0.02% bromphenol blue). The samples were immediately heated for 5 min at 85°C.

Phosphorylation time course experiments were performed as described above but with a 50-μl reaction volume. At the indicated time points, 5-μl samples were removed, transferred to 2 μl of SDS loading buffer, heated, and kept at room temperature until all samples were taken. Each sample (1 μl) was run on a 20% polyacrylamide–SDS gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). Autoradiography was performed to visualize the radioactive proteins. For quantitation of the radioactivity, the nitrocellulose membranes were stained with 0.1% Pelikan royal blue 4001 (Pelikan AG) in phosphate-buffered saline (15 mM Na_2HPO_4, 2 mM NaH_2PO_4 [pH 7.2], 125 mM NaCl)–1% acetic acid–0.3% Tween 20. Nitrocellulose slices containing protein bands were cut from the nitrocellulose membrane, and the radioactivity in each slice was determined with a Beckman liquid scintillation counter.

Synthesis of radioactive acetyl phosphate. Radioactive acetyl phosphate was prepared as described by E. R. Stadtman (34) with [*ortho*-^{32}P]phosphate (10 mCi/ml [NEN]). According to the described procedure, the purity of the synthesized [^{32}P]acetyl phosphate was approximately 60%.

NarL phosphorylation by acetyl phosphate. NarL phosphorylation by [^{32}P]acetyl phosphate was carried out in a 30-μl volume at 37°C. Purified NarL protein was incubated in 50 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$ at 37°C for 1 min, and the phosphorylation reaction was initiated by the addition of about 10 mM [^{32}P]acetyl phosphate. At indicated time points, 3-μl samples were removed and transferred to 1 μl of SDS loading buffer, heated, and kept at room temperature until all samples were taken. Each sample (1 μl) was run on a 20% polyacrylamide–SDS gel and transferred to a nitrocellulose membrane. Autoradiography was performed to visualize the radioactive proteins. The amount of [^{32}P]phosphate associated with the NarL protein was quantitated with the Molecular Dynamics Phosphorimager system.

NarL-phosphate dephosphorylation assay. The NarL-phosphate dephosphorylation assays were performed in a 20-μl reaction volume at 37°C. NarL protein was phosphorylated with [^{32}P]acetyl phosphate for 30 min as described above. To initiate the dephosphorylation of [^{32}P]NarL-phosphate, either 'NarX or 'NarQ protein was added and 3-μl samples were taken at the indicated time points. The samples were treated as described above, run on 20% polyacrylamide–SDS gels, and blotted to nitrocellulose, and autoradiography was performed to visualize the radioactive proteins.

Gel electrophoresis and Western blotting (immunoblotting). Protein samples were prepared in 2.5% SDS–5% β-mercaptoethanol–0.005% bromphenol blue (final concentration [glycerol was omitted]). After heating for 5 min at 85 to 90°C, 1 μl of the sample was run on a 20% polyacrylamide–SDS gel (PhastSystem; Pharmacia). Precast polyacrylamide gels and SDS buffer strips were purchased from Pharmacia. The proteins were either stained with Coomassie blue or transferred to nitrocellulose membranes (Schleicher and Schuell) with the PhastSystem blot transfer system (Pharmacia).

RESULTS

Overproduction of the 'NarX, 'NarQ, and NarL proteins. NarX and NarQ are cytoplasmic membrane proteins that each contain two hydrophobic regions of approximately 20 amino acids near their N termini (Fig. 2) (3). Their C-terminal domains are predicted to be exposed to the cell cytoplasm (3). Since the purification of intact membrane proteins to homogeneity is often difficult, we overproduced and purified the cytoplasmic domains of NarX and NarQ. The truncated 'NarX protein had the N-terminal 189 amino acids deleted, while 'NarQ lacked the N-terminal 171 amino acid residues (Fig. 2). On the basis of the sequence similarity of NarX and NarQ to other bacterial regulatory proteins, the truncated C-terminal 'NarX and 'NarQ proteins were each expected to possess kinase activity. The overproduction of the truncated 'NarX and 'NarQ proteins was achieved by constructing the plasmids pHXL1 and pHQ1, which contained the C-terminal regions of the *narX* and *narQ* genes, respectively, fused in frame to codons for one methionine and six histidine residues (Fig. 1). The expression plasmids contain the T5 phage promoter fused to two *lac* operator sequences in tandem in addition to a ribosome-binding site and the initiation codon for expression of the *'narX* and *'narQ* genes.

Purification of 'NarX and 'NarQ. To purify the 'NarX and 'NarQ proteins, *E. coli* JM101 carrying pHXL1 and pHQ1, respectively, was grown in the presence of IPTG to induce gene expression and cell growth was continued for an additional 3 h. Cell lysates were prepared, and the protein composition was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Upon IPTG induction of cells containing pHXL1, two new major protein bands with M_rs of 46,000 and 24,000 appeared. The larger protein band corresponded to the predicted M_r of the truncated 'NarX protein, and the smaller band corresponded to the predicted M_r of NarL (Fig. 3A, lanes 2 and 3). IPTG addition to cells containing pHQ1 resulted in the overexpression of a 'NarQ protein with a predicted M_r of 45,000 (Fig. 3C, lanes 2 and 3). After centrifugation of the cell lysates, the 'NarX and 'NarQ proteins were each detected in the particulate fraction rather than the soluble fraction (Fig. 3A, lanes 4 and 5, and 3C, lanes 4 and 5). Similar findings have been reported for other truncated sensor proteins (i.e., ArcB and VirA) (15, 16), and this phenomenon is likely associated with the formation of inclusion bodies. Since the 'NarX and 'NarQ proteins could not be extracted from the particulate fraction by using 1% Triton X-100, the proteins were solubilized in their denatured form with 8 M urea (Fig. 3A and C, lanes 6). With Ni-affinity chromatography, 'NarX was purified to near homogeneity (Fig. 3A, lane 7); 'NarQ was about 80% pure (Fig. 3C, lane 7). A protein with an apparent molecular weight of 29,000 copurified with 'NarQ during Ni-affinity chromatography. Binding to the Ni-affinity matrix requires several adjacent histidine residues, which are not frequently found in prokaryotic proteins. Since the production of the contaminating protein also appeared to be induced upon the addition of IPTG (compare lanes 2 and 3 in Fig. 3C), it is possible that this protein is a proteolytic cleavage product of 'NarQ. Because no autophosphorylation activity was associated with this protein, no further purification of 'NarQ was

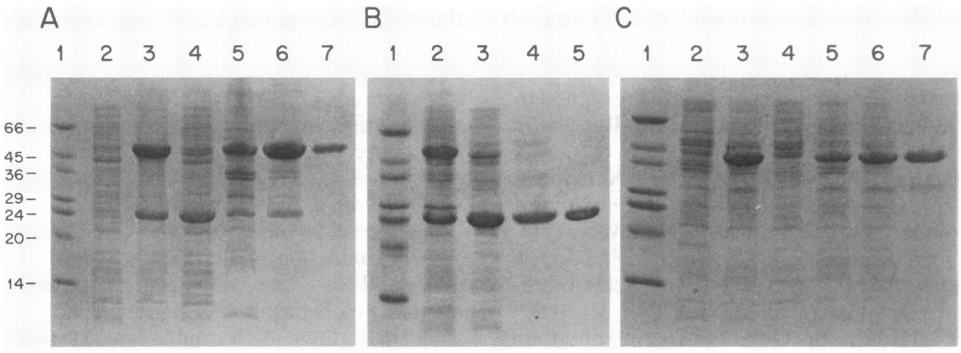

FIG. 3. Purification of the 'NarX, NarL, and 'NarQ proteins. Purification was performed as described in Materials and Methods. The protein composition at each purification step was analyzed on 20% polyacrylamide–SDS gels. (A) The samples and amounts of protein applied for 'NarX purification (by lane) are as follows: 1, molecular weight standard (1.5 μg); 2, cell lysate of JM101/pHXL1 grown in the absence of IPTG (2 μg); 3, cell lysate of JM101/pHXL1 grown in the presence of IPTG (2 μg); 4, soluble fraction (1.8 μg); 5, membrane fraction (1.5 μg); 6, solubilized membrane proteins in Tris-urea buffer after centrifugation (1 μg); and 7, eluate after Ni-affinity chromatography (0.2 μg). (B) The samples for NarL purification (by lane) are as follows: 1, molecular weight standard (1.5 μg); 2, cell lysate of JM101/pHXL1 grown in the presence of IPTG (2 μg); 3, soluble fraction (1.4 μg); 4, concentrated DE 52 column wash (1 μg); and 5, pooled fractions after Superose 12 gel filtration (0.3 μg). (C) The samples for 'NarQ purification (by lane) are as follows: 1, molecular weight standard (1.5 μg); 2, cell lysate of JM101/pHQ1 grown in the absence of IPTG (1.8 μg); 3, cell lysate of JM101/pHQ1 grown in the presence of IPTG (1.8 μg); 4, soluble fraction (1.5 μg); 5, membrane fraction (1.5 μg); 6, solubilized membrane proteins in Tris-urea buffer after centrifugation (1.2 μg); and 7, eluate after Ni-affinity chromatography (0.5 μg). The following molecular weight markers (given on the left in thousands) were used: α-lactalbumin (M_r, 14,400), trypsin inhibitor (M_r, 21,100), trypsinogen (M_r, 24,000), carbonic anhydrase (M_r, 29,000), glyceraldehyde-3-phosphate dehydrogenase (M_r, 36,000), egg albumin (M_r, 45,000), and bovine albumin (M_r, 66,000).

attempted. The 'NarX and 'NarQ proteins were refolded by slowly lowering the protein concentration to 0.2 mg/ml and then dialyzing in the presence of 5 mM dithiothreitol and 0.1% Triton X-100. The efficacy of the refolding procedure was monitored by observing the autophosphorylation activity of each protein; in their denatured form, the proteins do not exhibit autophosphorylation activity (data not shown). About 0.8 g (wet weight) of cells yielded about 8 mg of 'NarX and 5.6 mg of 'NarQ protein.

Purification of NarL. Upon IPTG induction of JM101 cells carrying pHXL1, the NarL protein was overexpressed in conjunction with 'NarX (Fig. 3B, lane 2). After cell breakage, about 70% of NarL fractionated to the soluble fraction (Fig. 3A, lanes 4 and 5, and 3B, lane 3). The NarL protein found in the particulate fraction was only loosely associated and could be washed off the membranes with 0.1% Triton X-100 (data not shown). The NarL protein in the soluble fraction was purified by DE 52 anion-exchange chromatography (Fig. 3B, lane 4). Under the conditions chosen, NarL did not bind to the DE 52 matrix and was about 90% pure after its elution. After Superose 12 gel filtration, no contaminating proteins could be detected in the NarL preparation by SDS-polyacrylamide gel electrophoresis (Fig. 3B, lane 5). When the NarL protein was reapplied to Superose 12, it eluted as a single peak with an M_r of 24,000 relative to the elution of reference proteins (data not shown). Thus the NarL protein was purified to homogeneity as a monomer. From 3.7 g (wet weight) of induced cells, about 5 mg of protein was obtained. The sequence of the N-terminal 25 amino acid residues of the purified NarL protein was analyzed and corresponded to amino acids 2 to 26 of the predicted NarL sequence (data not shown).

Autophosphorylation of 'NarX and 'NarQ. Purified 'NarX and 'NarQ proteins were incubated with 25 μM [γ-^{32}P]ATP and 5 mM $MgCl_2$, run on SDS-polyacrylamide gels, and blotted to nitrocellulose membranes. Autoradiography of the nitrocellulose membranes revealed that radioactivity was associated with each protein (Fig. 4, lanes 1). When [α-^{32}P]ATP was used, no labeling of the proteins occurred (Fig. 4, lanes 2), indicating that only the γ-phosphate group of ATP was being transferred to the 'NarX and 'NarQ proteins. The phosphorylation process was dependent on the presence of $MgCl_2$ in the reaction mixture (Fig. 4, lanes 3). The addition of nitrate or molybdate to the reaction mixture had no apparent effect on the phosphorylation of either the 'NarX or the 'NarQ proteins (Fig. 4, lanes 4 and 5). Addition of excess GTP and AMP did not affect the ATP-dependent autophosphorylation reaction (Fig. 4, lanes 6 and 8). However when 250 μM ADP was added to the reaction mixture, 'NarX autophosphorylation was inhibited completely and NarQ autophosphorylation was decreased by about 80% (Fig. 4, lanes 7). In the presence of [γ-^{32}P]ATP and $MgCl_2$, 'NarX and 'NarQ were autophosphorylated in a protein-dependent fashion (Fig. 5). About 0.2 mol of phosphate was incorporated per mol of 'NarX, while 'NarQ contained 0.06 mol of phosphate per mol of protein. The time course of 'NarX and 'NarQ phosphorylation was monitored,

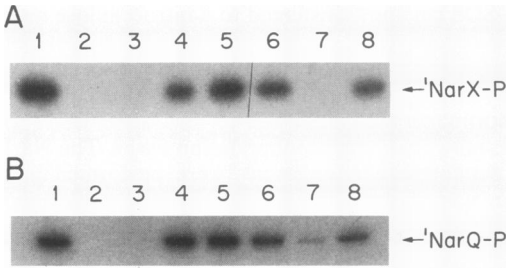

FIG. 4. Autophosphorylation of the 'NarX (A) and 'NarQ (B) proteins. P, phosphate. 'NarX (0.4 μM) and 'NarQ (2.2 μM) proteins were incubated at room temperature for 15 min as described in Materials and Methods. Reaction mixtures in lanes 1 and 3 to 8 contained 25 μM [γ-^{32}P]ATP, and those in lanes 1, 2, and 4 to 8 contained 5 mM $MgCl_2$. Lanes 2 contained 25 μM [α-^{32}P]ATP, and in lanes 3, $MgCl_2$ was omitted. Further additions (by lane) were as follows: 4, 5 mM $NaNO_3$; 5, 0.5 mM Na_2MoO_4; 6, 250 μM GTP; 7, 250 μM ADP; and 8, 250 μM AMP.

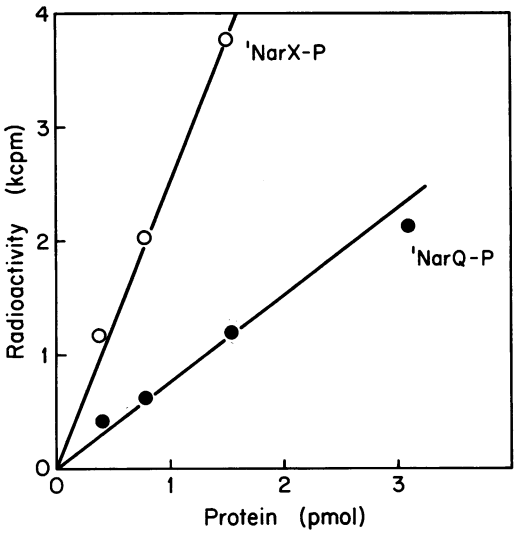

FIG. 5. Protein dependency of 'NarX and 'NarQ autophosphorylation. P, phosphate. Increasing amounts of purified 'NarX and 'NarQ were incubated with 25 μM [γ-^{32}P]ATP (12.5 kcpm/pmol) for 15 min. The proteins were run on 20% polyacrylamide–SDS gels and transferred to nitrocellulose membranes, and their radioactivity was determined with a Beckman liquid scintillation counter.

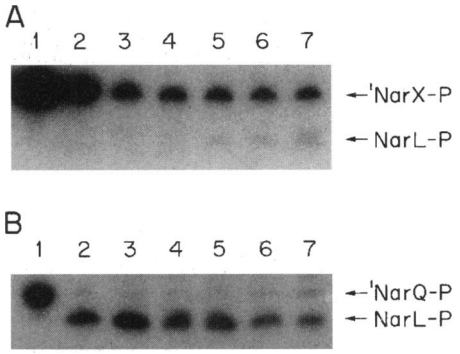

FIG. 7. Time course of NarL phosphorylation by 'NarX-phosphate and by 'NarQ-phosphate. P, phosphate. Purified 'NarX (0.7 μM) (A) and purified 'NarQ (0.7 μM) (B) were incubated with [γ-^{32}P]ATP for 15 min as described in Materials and Methods, and then NarL (20 μM) was added. At various time intervals (lanes: 2, 15 s; 3, 30 s; 4, 1 min; 5, 1.5 min; 6, 2 min; 7, 4 min), 5-μl samples were removed and added to 2 μl of SDS loading buffer (Materials and Methods). One microliter of each sample, containing 0.5 pmol of 'NarX or 'NarQ and 1.4 pmol of NarL (lanes 2 to 7), was run on SDS–20% polyacrylamide gels and transferred to nitrocellulose membranes, and radioactivity was visualized by autoradiography. Lanes 1 contained 'NarX (0.5 pmol) and 'NarQ (0.1 pmol) without NarL added.

and the results are shown in Fig. 6. The phosphorylations of 'NarX and 'NarQ were rapid and occurred at similar rates. After 2 min, about 70% of the maximal level of 'NarX phosphorylation had occurred and 'NarQ had been phosphorylated to about 50% of the final level. After 60 min, no significant increase in phosphorylation was detected (data not shown) relative to the level of phosphorylation seen by 15 min. Incubation of the proteins in the presence of 250 μM [γ-^{32}P]ATP did not increase the rate of autophosphorylation.

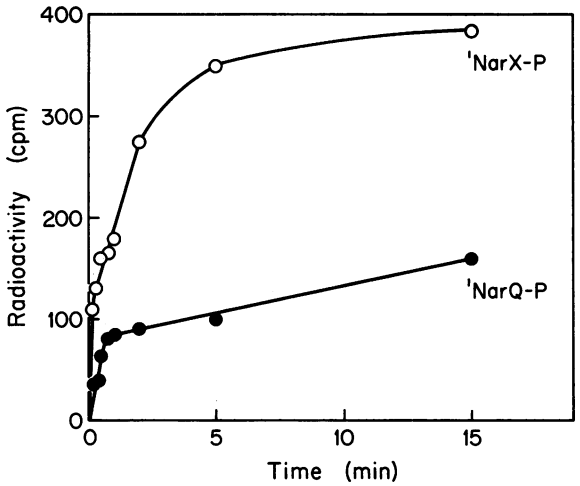

FIG. 6. Time course of 'NarX and 'NarQ autophosphorylation. P, phosphate. Purified 'NarX or 'NarQ protein (0.86 μM) was incubated in the presence of 25 μM [γ-^{32}P]ATP. At the indicated time, 5-μl samples were removed and added to 2 μl of SDS loading buffer (Materials and Methods). One microliter of the samples containing 0.6 pmol of each protein was run on SDS–20% polyacrylamide gels and transferred to nitrocellulose membranes, and their radioactivity was determined with a Beckman liquid scintillation counter.

The phosphorylated forms of both 'NarX and 'NarQ were stable for at least 5 h at room temperature, indicating that both proteins do not exhibit autophosphatase activity.

Phosphorylation of NarL by 'NarX-phosphate and 'NarQ-phosphate. The 'NarX and the 'NarQ proteins were individually incubated with [γ-^{32}P]ATP and 5 mM $MgCl_2$ for 15 min, and then NarL was added to the phosphorylation assay mixture. The addition of NarL to the phosphorylated 'NarX protein resulted in a distinctly different pattern of labeling relative to phosphate transfer from 'NarQ-phosphate to NarL. By 15 s after the addition of NarL to 'NarX-phosphate, 'NarX-phosphate underwent partial dephosphorylation with little detectable transfer of the phosphate group to NarL (Fig. 7A, lane 2). Only after 1.5 min did phosphorylated NarL protein become detectable in the autoradiogram (Fig. 7A, lane 5). Four minutes after the addition of NarL to NarX-phosphate, the amount of phosphorylated NarL did not further increase and the amount of 'NarX-phosphate decreased only slightly (Fig. 7A, lane 7). In contrast, phosphoryl transfer from NarQ-phosphate to NarL differed: by 15 s after NarL addition to 'NarQ-phosphate, 'NarQ-phosphate was almost completely dephosphorylated while phosphorylated NarL was detectable almost immediately (Fig. 7B, lane 2). The amount of NarL-phosphate remained constant after 4 min (Fig. 7B, lane 7). Thus, the phosphate transfer from 'NarQ-phosphate to NarL appears to occur at an much faster rate than that from 'NarX-phosphate to NarL.

Phosphorylation of NarL by acetyl phosphate. Several low-molecular-weight phosphorylated compounds were recently demonstrated to serve as substrates for phosphorylation of response regulatory proteins like NtrC and CheY (see reference 24 for a review). With the goal of establishing whether NarL could also be phosphorylated by a low-molecular-weight phosphate donor, purified NarL was incubated with 10 mM [^{32}P]acetyl phosphate. Phosphorylation of NarL was observed after 30 s and increased during a period of 60 min (Fig. 8). Phosphorylated NarL was stable at room temperature in the presence of [^{32}P]acetyl phosphate for at least 3 h, with only a

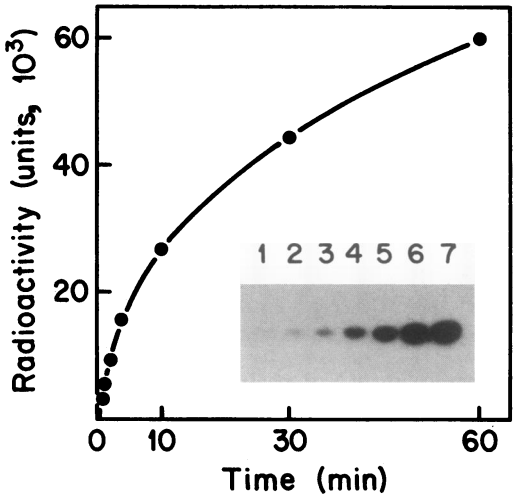

FIG. 8. Time course of NarL phosphorylation by [^{32}P]acetyl phosphate. NarL (80 μM) was incubated with about 10 mM [^{32}P]acetyl phosphate at 37°C in a volume of 30 μl. At various time points (lanes: 1, 30 s; 2, 1 min; 3, 2 min; 4, 4 min; 5, 10 min; 6, 30 min; 7, 60 min), 3-μl samples were removed and added to 1 μl of SDS loading buffer (Materials and Methods). One microliter of each sample was run on 20% polyacrylamide–SDS gel and transferred to nitrocellulose membranes. The amount of radioactivity associated with NarL-phosphate was determined with a Phosphorimager (graph), and the radioactivity was visualized by autoradiography (inset).

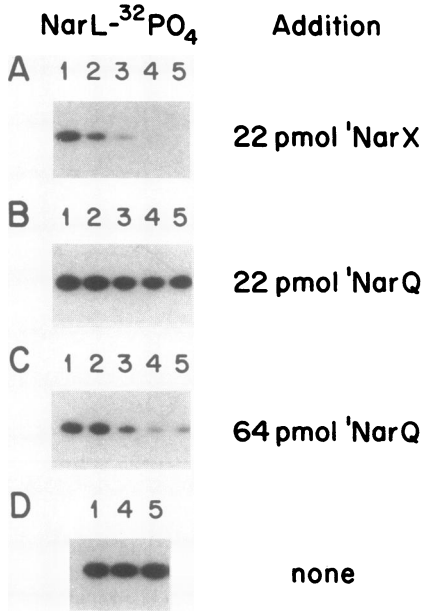

FIG. 9. Stability of NarL-phosphate and its dephosphorylation by 'NarX and by 'NarQ. NarL protein (805 pmol) which had been phosphorylated with [^{32}P]acetyl phosphate for 30 min was incubated with 22 pmol of 'NarX (A), 22 pmol of 'NarQ (B), 64 pmol of 'NarQ (C), and no addition (D) in a 20-μl reaction volume at 37°C. At various time intervals (lanes: 1, 15 s; 2, 1 min; 3, 5 min; 4, 10 min; 5, 30 min), 3-μl samples were removed and added to 1 μl of SDS loading buffer (Materials and Methods). Samples (1 μl) were run on 20% polyacrylamide–SDS gels and transferred to nitrocellulose membranes, and their radioactivity was visualized by autoradiography.

little increase in the amount of [^{32}P]phosphate associated with NarL (data not shown).

Dephosphorylation of NarL-phosphate by 'NarX and 'NarQ. To determine whether 'NarX and 'NarQ exhibit phosphatase activities which would regenerate NarL from its phosphorylated form, NarL protein which had been phosphorylated by [^{32}P]acetyl phosphate was incubated with either 'NarX or 'NarQ. Almost complete dephosphorylation of NarL-phosphate was observed after 5 min in the presence of 'NarX (Fig. 9A). In contrast, 'NarQ catalyzed the dephosphorylation of NarL-phosphate at a very slow rate; NarL-phosphate was decreased by about 10% after a 30-min incubation with 'NarQ (Fig. 9B). Dephosphorylation of NarL-phosphate by 'NarQ was more readily apparent when the 'NarQ protein concentration in the phosphatase assay was increased threefold (Fig. 9C). In a control experiment, NarL-phosphate in the absence of both 'NarX and 'NarQ was stable over a period of at least 30 min (Fig. 9D). Thus, 'NarX dephosphorylates NarL-phosphate at a significantly faster rate than does the 'NarQ protein.

DISCUSSION

The nitrate regulatory system in *E. coli* differs from those of many other bacterial two-component regulatory systems in that it has two sensor proteins, NarX and NarQ, that can each respond to nitrate independently of the other (3, 32). Strains with either *narX* or *narQ* deleted appear to be normal for nitrate regulation of *narG-lacZ*, *dmsA-lacZ*, and *frdA-lacZ* expression (3, 32). However, when both *narX* and *narQ* were deleted from the chromosome, nitrate induction of *narG-lacZ* expression and nitrate repression of both *dmsA-lacZ* expression and *frdA-lacZ* expression were abolished (3). To compare the biochemical properties of the two nitrate sensors, we overexpressed and purified the cytoplasmic regions of NarX and NarQ by use of recombinant DNA methods. These protein domains contain a conserved region and an associated histidine residue proposed to be involved in protein kinase activity (3). In this study, we demonstrate that the cytoplasmic domains of the NarX and the NarQ proteins are rapidly autophosphorylated and that each phosphoprotein can interact with the NarL protein to rapidly transfer its phosphoryl group to NarL. This experimental approach has been used to study kinase activities of other bacterial regulator proteins, including EnvZ (1, 12), VirA (11, 16), KdpD (26), and ArcB (15).

Both the 'NarX and 'NarQ proteins autophosphorylated at a similar initial rate, but more phosphate was found to be associated with 'NarX-phosphate (0.2 mol/mol) than with 'NarQ-phosphate (0.06 mol/mol). The autophosphorylation activities were not affected by the presence of nitrate, suggesting that the cytoplasmic domains are unable to sense this signal. GTP addition to the reaction mixture did not interfere with the phosphorylation reaction, indicating that GTP cannot serve as a phosphate donor for 'NarX and 'NarQ phosphorylation. When excess ADP was added to the autophosphorylation assay mixture, 'NarX phosphorylation was completely inhibited and 'NarQ phosphorylation was partly inhibited, which suggests that the phosphorylation reaction is reversible. Weiss and Magasanik showed the transfer of phosphate from NtrB-phosphate to ADP (39), which demonstrates the reversibility of the autophosphorylation reaction.

In the phosphotransfer assay, 'NarQ-phosphate exhibited a faster net transfer of phosphate to NarL (i.e., seconds) than 'NarX-phosphate (i.e., minutes). Thus, 'NarQ-phosphate appears to be the more efficient phosphate donor for NarL.

However, the difference in NarL phosphorylation by 'NarQ-phosphate versus 'NarX-phosphate is partly explained by the ability of 'NarX to dephosphorylate NarL-phosphate at a faster rate than does the 'NarQ protein (Fig. 9). During the phosphotransfer reaction from 'NarX-phosphate to NarL, the truncated 'NarX protein can immediately remove the phosphoryl group from NarL-phosphate whereas 'NarQ does not. Phosphatase activity has been demonstrated with several other sensor proteins, including EnvZ, PhoR, and DegS, as a means of controlling regeneration of the unphosphorylated response regulator (31). The autophosphorylation and phosphate transfer activities of the truncated NarX protein used in these studies are qualitatively similar to those exhibited by the full-length NarX protein (37), which had been partially purified from *E. coli* cytoplasmic membranes (37). Because the autophosphorylation activity of full-length NarX was also independent of the presence of nitrate, it was not possible to determine how the activity of NarX was modulated by nitrate.

The bacterial two-component regulatory systems exhibit the general theme of a single sensor protein that detects an environmental signal and then interacts with a response regulatory protein to affect gene regulation of motility (2, 31). The nitrate regulatory system is clearly different in employing two independent sensor proteins, NarX and NarQ, to modulate NarL-dependent control of gene expression. Several other two-component systems may also have similar complexity; a second sensor protein (PhoM) was described that could partially replace the major histidine kinase (PhoR) in the phosphate regulatory system (38). PhoR and PhoM were each shown to be able to phosphorylate the response regulator protein PhoB. Whereas PhoR responds to the phosphate concentration in the cell's environment, the signal that controls PhoM activity is unknown. It has been proposed that PhoM may be part of a different regulatory pathway than PhoR (38). Aerobic and anaerobic regulation in *E. coli* is mediated in part by the ArcA/ArcB two-component system (13). A second sensor protein, CpxA, can also interact with ArcA (13, 14) but in response to some signal other than that detected by ArcB. In a third example, the gene encoding a novel sensor protein, *barA*, was identified that shares a high degree of homology to both EnvZ and OmpR (25). When supplied on a high-copy-number plasmid, *barA* complemented an *envZ* deletion mutant. It is not clear whether BarA interacts with OmpR under physiological conditions or whether it may participate in cross-talk, either for controlling osmoregulation or some other cellular function.

The demonstration that NarL can be phosphorylated by acetyl phosphate (Fig. 8) raises several interesting considerations. First, NarL can be considered to be an autokinase that can use either the phosphorylated forms of the NarX and NarQ proteins or acetyl phosphate as a phosphate donor. Although the affinity of NarL for the different substrates has yet to be determined, NarX-phosphate and NarQ-phosphate appear to serve as better substrates for phosphorylation of NarL because a low concentration of either NarX-phosphate or NarQ-phosphate (0.5 μM) gave rapid labeling compared with when millimolar amounts of acetyl phosphate were used. The rate of NarL phosphorylation with either NarX- or NarQ-phosphate was considerably faster (seconds) than that with acetyl phosphate (minutes). Second, because acetyl phosphate is an important intermediate during fermentative metabolism in *E. coli*, it could conceivably function as a signal to control anaerobically expressed genes. The fact that NarL can be autophosphorylated with acetyl phosphate suggests that acetyl phosphate could also serve as a second signal in the nitrate regulatory circuit to integrate carbon flow with control of the electron transport pathway. Direct genetic evidence for this has yet to be established, although we have observed in vivo induction of *narG-lacZ* expression in cells lacking either NarX or NarQ if NarL levels are elevated (data not shown). This increased level of *narG-lacZ* expression was independent of whether or not nitrate was added to the culture medium. On the basis of the observation that acetyl phosphate served as a phosphate donor to CheY, NtrC, and PhoB in vitro, McCleary et al. recently proposed that acetyl phosphate is a global signal involved in both gene regulation and chemotaxis in *E. coli* (24).

Why does *E. coli* contain a two-component regulatory system that requires two sensor proteins for nitrate? From the in vitro studies described in this paper, the cytoplasmic domains of the NarX and NarQ proteins exhibit different NarL phosphotransferase and NarL-phosphate phosphatase activities. Different roles for NarX and NarQ may thus be proposed in nitrate control in which NarQ is the major phosphotransferase to generate active NarL (NarL-phosphate) when nitrate is present, whereas NarX serves as the major phosphatase of NarL-phosphate when nitrate is absent. However, it is not yet evident whether the kinase, phosphotransferase, and phosphatase activities of the intact membrane-bound forms of the NarX and NarQ proteins are like those exhibited by the cytoplasmic domains of NarX and NarQ (this study). It remains to be established if one or more of these biochemical activities are altered by the presence of their membrane domains and lipids and how they are modulated by nitrate. It is also conceivable that NarX and NarQ might sense different levels of nitrate in the cellular environment. By any of a number of plausible scenarios, NarX and NarQ appear to work in concert to ensure that activation and repression of NarL-dependent genes are coordinated for optimal modes of cellular respiration when nitrate is present or absent from the environment.

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