Phosphorylation and Dephosphorylation Catalyzed in Vitro by Purified Components of the Nitrate Sensing System, NarX and NarL*

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The regulation of specific gene expression by nitrate in Escherichia coli is mediated by the NarX/NarQ-NarL system. Based on sequence homologies with a family of two-component regulatory systems in bacteria, NarL has been identified as a putative response regulator while NarX and NarQ were proposed to be alternative membrane-associated sensors that activate NarL in the presence of nitrate. To investigate the interaction of NarX and NarL in vitro, both proteins were purified from overproducing strains. Purified NarX was rapidly labeled when incubated with [γ-32P]ATP but not with [α-32P]ATP in a reaction that required Mg2+ but was unaffected by nitrate. Incubation of the labeled NarX with purified NarL resulted in the transient phosphorylation of NarL. Both the phosphorylation and dephosphorylation of NarL required Mg2+, and neither reaction was affected by the presence of nitrate. NarL-phosphate, stabilized by the addition of EDTA, ran as a monomer on gel filtration. Dephosphorylation of the isolated NarL-phosphate required the addition of both Mg2+ and the NarX protein. The relative stabilities of the phosphorylated forms of the two proteins at different pH values were consistent with the proposal that, in analogy to other related two-component regulatory systems, NarX and NarL were phosphorylated on specific histidine and aspartate residues, respectively.

Although specific cis-acting sequences have been identified in NarL-responsive promoters (9, 10), it has not been possible to demonstrate with crude extracts either the phosphorylation of NarL or the specific binding of NarL to these sequences. Therefore, to establish conditions for the activation of NarL in vitro we purified both NarX and NarL from overproducing strains and demonstrate here that phosphorylation and dephosphorylation of NarL are mediated by NarX.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The host strain for all plasmids was MV1190 (Alac-pro AB, zhi sup E, Δnar-loc A) 306/Tn10 (ref') (F': tra D36, pro AB, lac I* Δ2M15) obtained from Bio-Rad. Plasmids for overproducing NarX and NarL were constructed by inserting an EcoRI to BamHI fragment from the plasmid pDR540 (12) between the EcoRI and BamHI sites in pMW74 and inserting an XbaI linker with stop codons in each frame between the BglII sites in narL to terminate translation of the narL gene after the first 25 codons. To overproduce NarL, the StyI site in pMW74, 8 base pairs upstream of the narL start codon, was converted to a BamHI site, and the EcoRI to BamHI tac promoter fragment was inserted as above to give pMW745.

Purification of NarX and NarL—Overproduced NarX was purified from a 1-liter culture of MV1190(pMW747) grown aerobically to 100 Klett units in L-broth containing ampicillin (100 μg/ml). 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and after 4 h of further aerobic growth the cells were harvested, washed, and frozen. Membranes, prepared as described previously (11), were suspended in 7 ml of 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, and 2% Triton X-100. After 1 h on ice the suspension was centrifuged for 15 min at 17,000 × g. The supernatant was added to a 1.5 × 25-cm DEAE-Sephadex column equilibrated with 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 0.1% Triton X-100 and eluted with a 400-ml gradient from 0.1 to 0.4 M NaCl. In the same buffer, fractions containing NarX were identified by assaying for phosphorylation of the overproduced 87-kDa protein.

Overproduced NarL was purified from the cell pellet obtained from a 2-liter culture of MV1190(pMW745) grown and induced as above. The pellet was suspended and fractionated into membranes and a cytoplasmic fraction (11). The cytoplasmic fraction was added to a 2.5 × 15-cm DEAE-Sephalcel column equilibrated with 50 mM Tris-HCl, pH 8.0, and 1 mM DTT and was eluted with 0.1 M NaCl in the same buffer. Fractions containing NarL were identified by SDS-polyacrylamide gel electrophoresis, the peak tubes were pooled, and protein was precipitated by 60% ammonium sulfate. The supernatant was dissolved in 1 ml of 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 5% glycerol, and 0.4 ml was added to a 1 × 55-cm Sephadex G-75 column equilibrated and eluted with the same buffer. Fractions containing NarL were identified as above.

Dephosphorylation Catalyzed in Vitro by Purified Components of the Nitrate Sensing System, NarX and NarL

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1 The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol.

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RESULTS AND DISCUSSION

In preliminary experiments with a strain that overproduced both NarX and NarL, we were unable to detect the phosphorylation of either NarX or NarL with $\gamma^{32}$P]ATP in crude extracts. However, with strains that overproduced NarX alone, phosphorylation of a protein with the expected size of NarX was detected in membrane preparations, and this phosphorylation appeared to be suppressed by the addition of a soluble extract containing overproduced NarL.

To facilitate the purification of NarX and NarL away from possible interfering activities, plasmids were constructed with each of the components under the control of the tac promoter (12), so that each could be induced to high levels by the addition of IPTG to growing cells. NarX, solubilized from the membrane fraction of strain MV1190(pMW747), was purified on DEAE-Sephacel by following the autophosphorylation activity with $\gamma^{32}$P]ATP (Fig. 1, A and B). Purified NarX migrated to a position on SDS-polyacrylamide gel electrophoresis corresponding to 67 kDa, and N-terminal sequence analysis yielded a sequence through 10 residues that was consistent with the deduced NarX gene product (4). The purified preparation contained only faintly stained contaminating protein bands (Fig. 1A, lane 4), but two proteins appeared to become significantly phosphorylated when incubated with $\gamma^{32}$P]ATP (Fig. 1B). One corresponded to the overproduced 67-kDa NarX protein. The other was a 36-kDa protein that was mostly resolved from NarX during chromatography but still contaminated the most purified fractions.

**FIG. 1. Purification of NarX and NarL.** A, cultures of MV1190 and MV1190(pMW747) were grown to 106 Klett units, and then MV1190(pMW747) was divided into two cultures. IPTG was added to MV1190 and one of the MV1190(pMW747) cultures. After 1 h the three cultures were harvested, membranes were prepared, and aliquots were analyzed on a 15% SDS-polyacrylamide gel stained with Coomassie Blue. The samples and amount of protein added to each lane were: lane 1, membranes of MV1190 after induction with IPTG (32 pg); lane 2, membranes of MV1190(pMW747), uninduced (30 pg); lane 3, membranes of MV1190(pMW747) after induction with IPTG (30 pg); lane 4, purified NarX (see “Experimental Procedures”) (10 pg). B, autoradiogram of the same preparations in A after incubation with $\gamma^{32}$P]ATP for 15 min as described under “Experimental Procedures” and run on the same SDS-polyacrylamide gel. Lanes 1–4, incubations contained the same protein samples in the same amounts as panel A except that 0.3 mg of purified NarX was used for lane 4. C, purification of NarL was described under “Experimental Procedures.” Aliquots were analyzed on a 12.5% SDS-polyacrylamide gel stained with Coomassie Blue. The samples and amounts of protein applied to each lane were: lane 1, the cytoplasmic fraction (20 pg); lane 2, the pooled fractions from DEAE-Sephadex chromatography (9 pg); lane 3, sample after Sephadex G-75 chromatography (3 pg). Molecular weight markers for A, B, and C were prelabeled phosphorylase b (96,000), glibinate dehydrogenase (55,000), lactate dehydrogenase (36,000), carboxic anhydrase (29,000), $\beta$-lactoglobulin (18,400), and cytochrome c (12,400) from Diversified Biotech.

The phosphorylated form of purified NarX, produced by incubation with $\gamma^{32}$P]ATP and then separated from the ATP by gel filtration, was stable for over 60 min when incubated with Mg$^{2+}$ at 25 °C (Fig. 2, lanes 1–7). When purified NarL was added (lanes 8–14), the $^{32}$P rapidly disappeared from the NarX band accompanied by the phosphorylation and then rapid dephosphorylation of NarL. Significantly, the phosphorylated 36-kDa component that contaminated the NarX preparation was stable in both incubations, indicating that it did not contribute to either the phosphorylation or the dephosphorylation of NarX or NarL.

**Fig. 2. Phosphorylation of NarL by NarX-phosphate.** Radioactively labeled phosphorylated NarX was prepared by incubating purified NarX (1.2 $\mu$m) in a total volume of 300 $\mu$m of 105 al of TEGD containing 5 mM MgCl$_2$ and 28 nM $\gamma^{32}$P]ATP for 30 min at room temperature. The reaction mixture was chromatographed on a 0.7 x 7.5-cm Sephadex G-50 column equilibrated with 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 0.1% Triton X-100 and eluted with the same buffer. 77 $\mu$m of phosphorylated NarX, essentially free of $\gamma^{32}$P]ATP, was incubated in 105 $\mu$m of TEGD containing 5 mM MgCl$_2$, with or without NarL (2 $\mu$m), and 15 $\mu$m aliquots were withdrawn at the indicated times, added to 4 $\mu$m of sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis. Autoradiograms show the incubations without NarL (lanes 1–7) and with NarL (lanes 8–14).

**Fig. 3. pH stability of the phosphoryl groups of phosphorylated NarX and NarL.** Purified NarX (1.2 $\mu$m) was phosphorylated as described under “Experimental Procedures.” After 14 min of incubation NarL was added (2 $\mu$m) and the reaction stopped after 1 min with 0.1 volume of 10% SDS. The pH was adjusted to the indicated values according to Fors et al. (17) and incubated at 43 °C for 60 min. The samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.
no effect on the rates of the phosphorylation or dephosphorylation of NarL while EDTA (50 mM) inhibited both the transfer of phosphate from NarX to NarL and the subsequent dephosphorylation of NarL (data not shown).

To compare the relative properties of the phosphorylated forms of NarX and NarL, samples of an incubation mixture that contained the phosphorylated forms of both proteins were incubated at different pH values after the addition of 

The gel was dried and autoradiographed.

As predicted by analogy to other two-component regulatory systems studied in bacteria (3–5), these studies demonstrate that NarX acts as an autokinase that transfers the resulting phosphoryl group to NarL and, as found with several other systems, it also enhances the rate of dephosphorylation of NarL-phosphate. NarL appears to be monomeric in both the unphosphorylated and phosphorylated forms. The isolation of stable NarL-phosphate should facilitate DNA binding studies and the determination of the role of NarL in the regulation of specific gene transcription.

Although it is assumed that the activity of NarX in the whole cell is regulated in some way by nitrate, we were unable to detect any effect of nitrate on the activities of purified NarX. A full understanding of how nitrate regulates the phosphorylation will most likely require identification or reconstitution of a nitrate-dependent form of NarX in the cell membrane.

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