Nitrates and Nitrites Control of Respiratory Nitrate Reduction in
Denitrifying Pseudomonas stutzeri by a Two-Component
Regulatory System Homologous to NarXL of Escherichia coli

ELISABETH HÄRTIG, ULRIKE SCHIEK, KAI-UWE VOLLACK, AND WALTER G. ZUMFT*

Lehrstuhl für Mikrobiologie der Universität zu Karlsruhe, Karlsruhe, Germany.

Received 10 February 1999/Accepted 6 April 1999

Bacterial denitrification is expressed in response to the concurrent exogenous signals of low-oxygen tension and nitrate or one of its reduction products. The mechanism by which nitrate-dependent gene activation is effected was investigated in the denitrifying bacterium Pseudomonas stutzeri ATCC 14405. We have identified and isolated from this organism the chromosomal region encoding the two-component sensor-regulator pair NarXL and found that it is linked with the narG operon for respiratory nitrate reductase. The same region encodes two putative nitrate or nitrite translocases, NarK and NarC (the latter shows the highest similarity to yeast [Pichia] and plant [Nicotiana] nitrite transporters), and the nitrate-regulated transcription factor, DnrE, of the FNR family. The roles of NarX and NarL in nitrate respiration were studied with deletion mutants. NarL activated the transcription of narG, narK, and dnrE but did not affect the denitrification regulons for the respiratory substrates nitrite, nitric oxide, and nitrous oxide. The promoters of narG, narK, and dnrE carry sequence motifs, TACYYM, which correspond to the NarL recognition sequence established for Escherichia coli. The cellular response toward nitrate and nitrite was mediated by the sensor protein NarX, which discriminated weakly between these oxyanions. Our data show that the NarXL two-component regulatory system has been incorporated into the bacterial denitrification process of P. stutzeri for selective regulation of nitrate respiration.

Denitrification by prokaryotes is part of the global nitrogen cycle, where it is responsible for the balance of the nitrogen budget of the biosphere. In a pathway of four reaction steps, nitrate is successively reduced via nitrite, nitric oxide (NO), and nitrous oxide (N2O) to dinitrogen. Denitrification genes are usually expressed in response to nitrate or nitrite and a low oxygen level (although aerobic denitrification exists in specialized cases) for a review, see reference 45). This requires the activation of sensory devices and signal transduction pathways by these respiratory substrates or their reduction products. We were interested in the mechanisms by which a denitrifying bacterium that is deprived of oxygen and shifted to N2O utilization senses nitrate or nitrite and activates genes for anaerobic respiration.

In Escherichia coli the transcription factor NarL is an important regulator in cellular bioenergetics. NarL activates the operon for respiratory nitrate reductase, narGHJI, and other operons of ancillary systems required for nitrate respiration. At the same time, the factor acts as a repressor of operons for alternative modes of respiration. NarL is part of a two-component regulatory system, NarXL (reviewed in reference 13). The sensor-regulator pair is duplicated in NarQP, which exhibits a specificity toward target genes somewhat different from that of NarXL. Putative NarX and NarL homologs, requiring functional analysis, have surfaced as the result of projects to sequence the genomes of Haemophilus influenzae, Neisseria gonorrhoeae, Yersinia pestis, Bacillus subtilis, and Pseudomonas aeruginosa. Here we identify by a targeted approach the narXL genes of the denitrifying bacterium Pseudomonas stutzeri and study their phenotypic manifestations in deletion strains. The function of NarXL is to activate the operon encoding the initiator reaction for denitrification, i.e., respiratory nitrate reduction, but not to act as a global regulatory system for the overall denitrification process.

(Preliminary accounts of this work have been presented previously [21, 45].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type P. stutzeri (ATCC 14405), the mutant strain MK21, which is a spontaneously Smr mutant but otherwise represents wild-type traits, and the MK21 derivatives MRL118 (narG Δ narK Smr) and MRX119 (narG Δ narK Δ narC Smr) were cultivated at 30°C in synthetic medium with asparagine and citrate as major ingredients (10). The construction of the mutant strains MRL118 and MRX119 has been described previously (22). E. coli DH10B and XLI-Blue MR were grown at 37°C in Luria-Bertani medium. Where necessary, kanamycin, ampicillin, or streptomycin was added at a final concentration of 50, 100, or 200 μg/ml, respectively. Cultures from which total RNA was prepared were grown in a 1-liter flask equipped with baffles and filled with 500 ml of medium. The optical density at 660 nm upon inoculation was about 0.3. The shaker speed of the gyratory incubator used was set at 240 rpm. Initial air saturation was estimated to be about 95% with a Clark-type electrode. Samples of oxygen-respiring cells were drawn after 3 h. For a shift to denitrifying conditions, cells were induced by nitrate (1 g/liter) for 1 h under O2 limitation by decreasing the shaker speed to 120 rpm, which lowered air saturation to about 0.5%. Full anaerobiosis is not required for the expression of the denitrification system of P. stutzeri provided that nitrate or nitrite is present (26). Samples for RNA extraction were drawn from cell suspensions that had reached an optical density of about 0.6 at 660 nm.

Purification of nitrate reductase and immunoblotting. Nitrate reductase from P. stutzeri was solubilized by heat and purified as described previously (6). The subunits were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the large subunit, NarG, was blotted onto a polyvinyl difluoride membrane. An N-terminal sequence, NRKQGEFADGHGETR, was provided that nitrate or nitrite is present (26). Samples for RNA extraction were drawn from cell suspensions that had reached an optical density of about 0.6 at 660 nm.

For immunoblot analysis of enzymes, aerobically grown cells were transferred to fresh medium and induced for 8 h under O2 limitation with sodium nitrate (1 g/liter) or sodium nitrite (0.5 g/liter). Cells were harvested by centrifugation and washed twice with 25 mM Tris-HCl (pH 7.5)–10 mM MgCl2. They were suspended in the same buffer without MgCl2 and broken in the cold by sonication (Branson). The supernatant from centrifugation for 20 min at 39,000 × g was used as a cell extract for SDS-PAGE (27). Proteins were transferred to a nitrocellulose membrane by semidry electrophoresis. For Western blotting (38), poly-
clonal antisera were raised against the purified oxidoreductases. Quantitation was done by scanning laser densitometry with an ImageMaster scanner and software (Amersham Pharmacia Biotech). Protein concentration was determined by the Lowry procedure with bovine serum albumin as the standard.

**Cloning of the narXL region.** A narL fragment was amplified from genomic DNA of strain MK21 with the primer pair 5'-AAAAAGCTTGAGGACCAACC SM-3' and 5'-TCGAGATCTARGTARCCTGCG-3', designed from conserved NarL and NarP sequences and observing the codon preference of *P. stutzeri* genes. The restriction sites HindIII and EcoRI (bolificated nucleotides in primer sequences) were added to allow the subsequent cloning of the PCR product. The PCR was carried out at an annealing temperature of 55°C. Amplification products were separated by electrophoresis, blotted onto a nylon membrane, and hybridized (16) with the narP probe of *E. coli*. A 285-bp fragment was isolated, cloned into pBluescript II SK (+) to give plasmid pBSnarL, and verified by sequencing as being homologous to narL from *E. coli*.

A genomic cosmid library of wild-type *P. stutzeri* was constructed with the SuperCos1 vector and *E. coli* XLI-Blue MR as the host (Stratagene). DNA was purified as a CSCI gradient and partially digested with SmaI under conditions that yielded fragments of 30 to 50 kb. These fragments were cloned into the BamHI site of the vector by following the protocol of the supplier. Packaging was performed by using the GigapackIII XL packaging extract (Stratagene). For screening of the library by colony hybridization, we used an internal narL probe of 219 nucleotides, which was amplified from plasmid pBSnarL with the primers 5'-GGCTGACGCTGCTGATCTG-3' and 5'-GCACATGGCTGCTGCG-3'. For digoxigenin (DIG) labeling of probes, the PCR mixture was made up to contain 7 μM DIG-11-dUTP.

**Cloning of a narL fragment, gene probes, and nucleic acid manipulations.** We translated the amino acid sequence GEFADGH, obtained from N-terminal phorilation, NarL and NarP proteins have the conserved sequence GFSPIPAM, encoded at the 5' ends of the narG genes. Reverse transcription was initiated from the primer 5'-AATCCAG AGGTGCGATGGCACC-3' complementary to the 5' end of the narG coding region. The sequencing reaction was performed with the same primer. The primer extension products and the sequencing reactions were analyzed on a 6% denaturing polyacrylamide gel. For mapping the narL transcript initiation sites, we used the primer 5'-AAATCCAG AGGTGCGATGGCACC-3' and the same conditions as for narG.

**RESULTS**

**Isolation of the narXL region and linkage with the narG operon.** We based our strategy for the isolation of narXL from *P. stutzeri* on designing primers on the basis of a comparison of the amino acid sequences of NarL and NarP from *E. coli* (20, 31, 34) with those of the hypothetical NarP protein from *H. influenzae*, encoded by the open reading frame (ORF) HI10726 (19). In addition to the domains for DNA binding and phosphorylation, NarL and NarP proteins have the conserved sequences I(V)DDHPL(M) and GADGYL. These regions were translated into a degenerate primer pair to be used to amplify a narL fragment from genomic DNA of *P. stutzeri* (see Materials and Methods). A 285-bp fragment was detected with the narP probe of *E. coli* among the amplification products. This PCR fragment was isolated and served as a template for the preparation of a genuine narL probe of 219 nucleotides. The probe was used to locate the narL gene in a genomic library on cosmids g279. Genes encoding NarXL of *E. coli* are clustered with the *narG* operon and narK, encoding a putative nitrite transporter. To explore a possible linkage of narXL with narG in *P. stutzeri*, we hybridized cosmids g279 with the narG probe; narG was found on this cosmids also.

Having established a linkage of the two *nar* functions, we determined a double-stranded sequence of about 8 kb by using sequence-derived primers. The physical map of the *narXL* region is shown in Fig. 1. The *narL*-encoding ORF spans 657 bp. It is followed by ORF235, which has no noteworthy similarity with current sequence entries in data banks. The next ORF encodes the transcription factor DnrE, which belongs to the Dnr subfamily of the AraC family. It is followed by ORF31, which has no noteworthy similarity with those of the hypothetical NarP protein from *P. stutzeri* and *P. stutzeri* genes. The restriction sites

![Diagram](https://example.com/diagram.png)
product of two narK genes of *P. aeruginosa*, NarK<sub>a</sub> and NarK<sub>b</sub> (accession no. Y15252), shows that NarK<sub>a</sub> is homologous to NarK of *P. stutzeri*, whereas NarK<sub>b</sub> shows more similarity to the deduced nitrate or nitrite transporters NarK<sub>p</sub>, NasA<sub>p</sub>, and NarT of the gram-positive bacteria *B. subtilis* (11, 29) and *Staphylococcus carnosus* (17). In principle, the presence of two transporters would satisfy the requirement of movement of nitrate from the periplasm to the cytoplasm and the opposite translocation of nitrite. This makes the existence of NarC and NarK and their roles in denitrification and perhaps also nitrate assimilation an intriguing prospect. The third product of the *narK* region, encoded by ORF134, is weakly similar to the so-called conserved protein MTH153 of *Methanobacterium thermoautotrophicum* (accession no. AE000803) and the hypothetical protein ORF138 of *Wolinella succinogenes* (accession no. AJ000662), both of unknown function.

**Properties of the derived NarL and NarX proteins.** NarL of *P. stutzeri* (NarLP<sub>p</sub>) consists of 218 amino acids, Mₘ 24,378; the protein has 51 and 47% positional identity with the *E. coli* proteins NarL and NarP, respectively. Because of the slightly higher amino acid identity with NarL of *E. coli* (NarLEc) and its function as regulator of the *narG* operon, we termed the newly isolated *P. stutzeri* gene narL. Figure 2 shows an alignment of NarLP<sub>p</sub> with homologous proteins. The crystal structure of NarLEc became known recently (3, 4). The high similarity of NarLP<sub>p</sub> with the *E. coli* protein allows predictions of secondary structure as shown in Fig. 2.

The NarLP<sub>p</sub> residues Asp13, Asp14, Asp59, and Lys109 correspond to a set of conserved amino acids found in response regulator proteins. The aspartic acid residues form an acidic pocket which is part of the phosphoryl acceptor chemistry (30), whereas the C region is a common feature of sensor proteins NarL and NarP, respectively. Because of the slightly higher amino acid identity with NarL of *E. coli* (NarLEc) and its function as regulator of the *narG* operon, we termed the newly isolated *P. stutzeri* gene narL. Figure 2 shows an alignment of NarLP<sub>p</sub> with homologous proteins. The crystal structure of NarLEc became known recently (3, 4). The high similarity of NarLP<sub>p</sub> with the *E. coli* protein allows predictions of secondary structure as shown in Fig. 2.

The derived NarX polypeptide of *P. stutzeri* (NarXP<sub>p</sub>) consists of 648 amino acids, Mₘ 71,791. NarXP<sub>p</sub> has 31% positional identity each with NarX and NarQ of *E. coli*. Hydrophathy analysis and transmembrane prediction suggest two membrane-spanning regions (TM1 and TM2 [Fig. 2]) that delimit an internal periplasmic domain and a cytosolic-terminal cytoplasmic domain. The latter exhibits the conserved regions, termed H, N, and D from the presence of key amino acids in these regions, which are characteristic for the histidine protein kinase family (36). The asparagine and histidine residues, which were identified by site-directed mutagenesis to be essential for kinase activity of NarXEc (8), are present in NarXP<sub>p</sub>. The conserved histidyl residue, which is subject to autophosphorylation, resides within the H region. In addition to the common characteristics of the members of the kinase family, the periplasmic P region, also known as the P-box, and the cytoplasmic C region, a stretch of conserved residues intercalated between the H and N regions, are conserved in nitrate- and nitrite-responsive sensory kinases. The P region is involved in binding of and distinguishing between nitrate and nitrite (7, 43), whereas the C region is a common feature of sensor proteins and is thought to be important in conferring specificity on sensor-response regulator interaction (30). NarXP<sub>p</sub> is C-terminally extended vis-à-vis NarX and NarQ from other sources. Certain sensor proteins with similarity to NarXP<sub>p</sub>, such as FixL, PhoR, EnvZ, and CpxA, also have extended C termini that show no sequence conservation.

**NarX senses nitrate and nitrite, with some preference for nitrate.** Nitrate and nitrite are the principal substrates for denitrification, and at a low oxygen tension, both induce the complete pathway of four consecutive enzymatic reactions. We were interested, therefore, in the specificity of NarXP<sub>p</sub> toward both substrates. We used the previously constructed narX deletion mutant MRX119, which has a 520-bp internal *Eco*47III-HindIII fragment replaced by a kanamycin resistance cassette (Fig. 1), to study by immunochromical means the expression of the structural genes for nitrate reductase, cytochrome *cd*, nitrite reductase, NO reductase, and *N₂O* reductase. MRX119 and the control strain MK21 were induced to denitrify nitrate or nitrite as described in Materials and Methods. The protein pattern of cell extracts was analyzed by SDS-PAGE, and the four denitrifying reductases were detected with polyclonal antisera (Fig. 3). Nitrate and nitrite both induced the synthesis of nitrate reductase in the wild type. Nitrite was about half as active an inducer as nitrate, as judged from the amount of NarG detected by Western blot analysis. The level of nitrate reductase was strongly reduced in MRX119 irrespective of which growth-supporting N oxide was present, with NarH falling below the detection limit. Lack of expression of nitrate reductase at wild-type levels in the narX mutant with nitrate as a substrate indicated that the nitrite signal is also processed by NarXP<sub>p</sub>. Induction of the other three denitrification enzymes elicited by either nitrate or nitrite was not affected by the disruption of narX; and they were present at wild-type levels (Fig. 3). The weak expression of nitrate reductase in MRX119 may depend on an alternative N oxide-responsive regulatory system, for which indirect evidence exists (22).

**NarL acts selectively in denitrification as a transcriptional activator of the narG operon.** To study the role of NarL, we used the mutant MRL118 and monitored the expression of the four structural reductase genes for denitrification at the mRNA level. narL of MRL118 lacks a 358-bp *Hin*II-*Avi*I fragment and carries a Km<sup>+</sup> marker instead (Fig. 1). For comparative studies of gene expression, mutant MRX119 was included. If narL is organized as an operon and NarL cannot be replaced by a homologous component, the phenotypes of narL and narX mutants should be indistinguishable.

We used an internal fragment from narG as a probe to detect transcription from the narG operon of *P. stutzeri* in Northern blot analysis. The operon from this bacterium has not yet been sequenced. However, nar sequences from *P. aeruginosa* (accession no. Y15252), *Paracoccus denitrificans* GB17 (subjective synonym, *Thiosphaera pantotropha*) (Q56356), *Mycobacterium tuberculosis* (O66559), *Thermus thermophilus* (Y10124), *B. subtilis* (X91819), *S. carnosus* (AF029225), and *Streptomyces coelicolor* (AL031515) show that without exception, nitrate-respiring and denitrifying bacteria, both gram negative and gram positive, have the nitrate reductase structural genes *narG*, *narH*, and *narI*, as well as a chaperone-like protein, encoded by...
narG, in an invariant narGHJI gene cluster, probably in each case, as in E. coli (X16181), as an operon of four cistrons.

Specimens of total RNA from cells grown aerobically and from those shifted to denitrifying conditions were analyzed by Northern hybridization. The transcripts of the other oxidoreductase genes. The size of the largest narG signal, 6.9 kb, corresponded to that expected from a narGHJI operon but would not be large enough to also include genes encoding the nitrate or nitrite transporters upstream, or other genes downstream of this operon. No narG transcripts were detected under denitrifying conditions in the MK21 (WT) and strain MRX119 (narX-). Detection was carried out with a protein A-peroxidase conjugate and chromophenol (28). The NarG levels obtained by nitrate or nitrite induction differ in this experiment by a factor of 2.3. Amounts of cell extracts used were 48 μg each for NarGH and NorB and 6 μg each for NirS and NosZ. Mass references (in kilodaltons) were derived from the SeeBlue standard (Novex).

FIG. 3. NarX functions as a sensory component with a preference for nitrate. Immunolabeling was done with polyclonal antisera raised against the purified nitrate reductase (NarGH; upper bands represent the NarG subunit, and lower bands represent the NarH subunit), cytochrome cd, nitrite reductase (NirS), the cytochrome b subunit of NO reductase (NorB), and NAD reductase (NosZ). Lanes 1 and 2, cells cultured for 8 h with nitrate and nitrite, respectively (see Materials and Methods). Each panel shows the results obtained with strain MK21 (WT) and strain MRX119 (narX-). Detection was carried out with a protein A-peroxidase conjugate and chromophenol (28). The NarG levels obtained by nitrate or nitrite induction differ in this experiment by a factor of 2.3. Amounts of cell extracts used were 48 μg each for NarGH and NorB and 6 μg each for NirS and NosZ. Mass references (in kilodaltons) were derived from the SeeBlue standard (Novex).

FIG. 4. NarL acts as a transcriptional activator for the narG operon. The DNA probe used for Northern blot analysis is given at the top of each panel. Total RNA was extracted from MK21 (lanes 1), MRL118 (ΔnarL) (lanes 2), and MRX119 (ΔnarX) (lanes 3). Cells were grown for 3 h with oxygen in the absence of nitrate (-O2) and then shifted to nitrate-denitrifying conditions and extracted 1 h after the shift (denitrifying). Transcripts from the nir operon are found as monocistronic nirS and polycistronic nirSTB messages (22). Size standards are the RNA molecular weight marker no. 1 (Boehringer GmbH, Mannheim, Germany) and the 16S and 23S rRNA species.

FIG. 5. Determination of the 5' ends of the narG and narK transcripts by primer extension analysis. Total RNA was obtained from wild-type cells (MK21) grown aerobically (lane 1), under O2 limitation (lane 2), or under nitrate-denitrifying conditions (O2 limitation in the presence of nitrate) (lanes 3). The right panel for narK shows the lack of extension products of RNA from MRL118 (ΔnarL) (lane 4), which had been induced for denitrification identical to that of the wild type. Primer extension was performed with oligonucleotides complementary to the 5' ends of the coding regions of narG and narK shown in Fig. 6. Lanes A, C, G, and T show the results of dideoxy sequencing reactions carried out with the same primers. For MRL118 only the dideoxyadenine reaction is shown.
activates the narG operon in response to oxygen withdrawal is still missing (41).

Figure 5 shows that the determination of the transcription start site of narK revealed two putative promoters. Under aerobic conditions no transcripts were detected, but both promoters were weakly activated under O\textsubscript{2}-limited conditions. Transcription was enhanced by the addition of nitrate, and promoter P1 showed the stronger response. Since the pattern of appearance of P2 followed that of P1, we cannot rule out the possibility that the RNA species generating P2 is a processing product from the RNA giving rise to P1. In the narL mutant MRL118, no transcripts were detected (Fig. 5). The promoter P1 of narK shows sequence motifs corresponding to recognition sites for NarL and FNR at typical distances from the transcription initiation site (Fig. 6A). Again, this is in conformity with the observed induction pattern for denitrification.

### DISCUSSION

We have argued that the denitrification process consists of three to four modules, i.e., partly independent respiratory systems utilizing nitrate, nitrite, nitric oxide, or N\textsubscript{2}O (44, 45). Only nitrite reduction is tightly coupled with the subsequent reaction, the reduction of NO to N\textsubscript{2}O; presumably to maintain NO at a low steady-state level and to limit the toxic effects of this radical. Our data show an autonomous element for regulating nitrate respiration in the form of the NarXL two-component system, distinct from regulators affecting nitrite denitrification, i.e., the reduction of nitrite to a gaseous product. The regulatory independence of denitrification in the strict sense from the initiator reaction supports our concept of a modular design.

A phylogenetic analysis by the CLUSTAL W program of the known NarL and NarP proteins, and putative homologs deduced from genomic sequencing projects, shows that the NarL proteins of the pseudomonads are most closely related to NarL\textsubscript{Ec} (data not shown). NarL of E. coli binds to cognate promoters via heptameric sequences whose consensus is TACYYMY (42). The location of the NarL site is typically variable with respect to distance from and orientation toward the start of transcription (13). In anaerobically, nitrate-regulated promoters, the NarL site is usually found at greater distances from the transcription start site than the FNR site for regulated promoters, the NarL site is usually found at greater distances from the transcription start site than the FNR site for regulated promoters. They show a remarkable degree of conservation among the five proteins compared in Fig. 2. DNA footprinting or mutational analysis is still required to attribute functionality to the heptameric motifs.

The P and C regions are specific for NarX-type sensor proteins. They show a remarkable degree of conservation among the five proteins compared in Fig. 2. The P region was shown in an elegant mutational study to be responsible for the binding of nitrate and nitrite and to harbor elements essential for the discrimination of these ions. Whereas NarX\textsubscript{Ps} is strongly biased towards nitrate (narG expression in a narQ null mutant is induced 100-fold by nitrate but only 4-fold by nitrite), no such bias is incorporated in NarX\textsubscript{Ec} (43). The preference of NarX\textsubscript{Ps} for nitrate is only about twofold. Extending a comparison of the P-box sequences of NarX and NarQ to include the sensor proteins of the pseudomonads lowers the identity score to 10

### TABLE 1. Heptameric sequence motifs in NarL-regulated promoters of P. stutzeri

<table>
<thead>
<tr>
<th>Gene</th>
<th>Recognition heptamer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Distance&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Orientation of inverted repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>narG</td>
<td>TACCTCT</td>
<td>−96</td>
<td>Divergent from −104 site</td>
</tr>
<tr>
<td>narK</td>
<td>TACTCAT</td>
<td>−104</td>
<td>Convergent with −99 site</td>
</tr>
<tr>
<td>dnrE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>TACCTCT</td>
<td>−199</td>
<td>Convergent with −56 site</td>
</tr>
<tr>
<td>narG of E. coli</td>
<td>TACYMY&lt;sup&gt;d&lt;/sup&gt;</td>
<td>−57 to −208</td>
<td>Isolated half-site</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotides deviating from consensus are lowercased.

<sup>b</sup> Number of nucleotides counted from −1 of the start of transcription to the middle position of the heptameric motif.

<sup>c</sup> Data from reference 41.

<sup>d</sup> Consensus sequence; Y represents T or C; M represents C or A.
(from 15) of 18 consecutive amino acids. The amino acids Ser43, His45, and Lys49 of NarX (E. coli sequence numbering), supposedly of discriminatory quality compared with the positionally equivalent residues of NarQ proteins, Asp, Glu, and Ile, lose their differentiating value, but it is still to be noted that the P-box of NarXps resembles that of NarQEs more closely than that of NarXEc. As suggested previously, elements outside of the P-box are also likely to participate in discriminating between nitrate and nitrite (43).

An important element of the P-box is the conserved Arg54, whose mutagenic results in a ligand-unresponsive protein (7, 43). When nitrate or nitrite interact with a protein usually they require a transition metal for binding and their catalytic transformation. We have previously drawn attention to structures of protein nitrate complexes, even though they are unrelated to nitrate sensing, suggesting a mode of nitrate binding for the sensory domain of NarX (45). In Limulus polyphemus, hemocyanin nitrate occupies the site of the allosteric effector chloride (23), whereas in the tyrosine phosphatase of cyanin nitrate occupies the site of the allosteric effector chlorite (24).

NarX is involved in the nitrate sensor (43). When nitrate or nitrite interact with a protein usually they interact with a transition metal in the nitrate sensor. Therefore, nitrate and nitrite are induced by anion binding. Thus, these models indicate that the binding of nitrate to the conserved arginine residue in the P region of NarX and transmembrane signaling may take place without a requirement for a transition metal in the nitrate sensor.

ACKNOWLEDGMENTS

We are indebted to John A. DeMoss and Valley Stewart for kindly providing plasmids and to the late I Rasheed for protein sequencing. We thank H. Körner for a gift of NarG protein, B. Schreckenberger for technical assistance, and D. Jahn for sharing sequence information prior to publication.

The generous financial support of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.

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