Mutational Analysis of a Conserved Signal-Transducing Element: the HAMP Linker of the *Escherichia coli* Nitrate Sensor NarX

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The HAMP linker, a predicted structural element observed in sensor proteins from all domains of life, is proposed to transmit signals between extracellular sensory input domains and cytoplasmic output domains. HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein, and phosphatase) linkers are located just inside the cytoplasmic membrane and are projected to form two short amphipathic α -helices (AS-1 and AS-2) joined by an unstructured connector. The presumed helices are comprised of hydrophobic residues in heptad repeats, with only three positions exhibiting strong conservation. We generated missense mutations at these three positions and throughout the HAMP linker in the *Escherichia coli* nitrate sensor kinase NarX and screened the resulting mutants for defective responses to nitrate. Most missense mutations in this region resulted in a constitutive phenotype mimicking the ligand-bound state, and only one residue (a conserved Glu before AS-2) was essential for HAMP linker function. We also scanned the *narX* HAMP linker with an overlapping set of seven-residue deletions. Deletions in AS-1 and the connector resulted in constitutive phenotypes. Two deletions in AS-2 resulted in a novel reversed response phenotype in which the response to ligand was the opposite of that seen for the *narX*⁺ strain. These observations are consistent with the proposed HAMP linker structure, show that the HAMP linker plays an active role in transmembrane signal transduction, and indicate that the two amphipathic α -helices have different roles in signal transduction.

Two-component regulatory systems are used by all domains of life to sense and respond to environmental changes (37). These systems stereotypically consist of a sensor kinase and a cytoplasmic response regulator. One prototypical class of sensor kinases shares the structure of methyl-accepting chemotaxis proteins (MCPs), which are homodimers located in the cytoplasmic membrane. This structure consists of a short amino-terminal cytoplasmic segment, a transmembrane α -helix (TM-1), an external ligand-binding sensory input domain, a second transmembrane α -helix (TM-2), and a cytoplasmic output module (13).

Sequence comparisons and mutational analysis of sensor kinases and MCPs identified a common structural element hypothesized to transmit signals between external input domains and cytoplasmic output modules (2, 17, 42). This element is called the HAMP (histidine kinase, adenylyl cyclase, MCP, and phosphatase) linker, or the P-type linker. More than 95% of HAMP linkers identified by similarity to the *Escherichia coli* EnvZ HAMP linker are from sensor kinases or MCPs; all five MCPs and 15 of the 30 known sensor kinases of *E. coli* contain HAMP linkers (Fig. 1) (2).

The HAMP linker comprises the approximately 50 amino acyl residues distal to TM-2. This region includes two sequences of hydrophobic amino acids in a heptameric arrangement, with phased spacing of hydrophobic residues characteristic of amphipathic α -helices. These sequences have been named amphipathic sequence (AS)-1 and AS-2. The complete HAMP linker is predicted to consist of these two amphipathic α -helices joined by an unstructured connector (Fig. 1). Com-

puter modeling suggests that the α -helices in a dimeric sensor kinase's HAMP linker may be in a coiled-coil conformation (32). No physical structure has been determined for any HAMP linker, and so the precise boundaries of these predicted secondary structures are unknown, and the tertiary structure may be dynamic.

Biochemical studies on cysteinyl-substituted HAMP linkers from the *Salmonella enterica* Tar MCP support the proposed structure of two amphipathic α -helices. Solvent accessibility measurements indicate that the phased hydrophobic residues are protected from the aqueous environment (6), and cross-linking studies suggest that the two AS-2s in a dimer may be adjacent, whereas the AS-1s are further apart (6). Interestingly, some missense mutations and deletions in the hydrophobic face of AS-1 of the *E. coli* MCP Tsr result in proteins that are modestly impaired for proper cytoplasmic membrane insertion (31). Together, these observations suggested the hypothesis that the conserved Pro (see Fig. 1) gives AS-1 helices a kink or flexibility that allows them to bury their hydrophobic surfaces in the cytoplasmic membrane (42).

Previous genetic analyses of HAMP linkers from the *E. coli* nitrate sensor NarX, osmosensor EnvZ, and serine-sensing MCP Tsr have yielded missense mutations at the conserved residues (Fig. 1) (1, 8, 16, 38). A few other missense changes at nonconserved residues in the HAMP linker have also been isolated. Most HAMP linker mutations studied to date result in a phenotype biased towards the ligand-bound signaling state. These mutations affect neither the sensory input domain nor the conserved output domain yet result in regulatory phenotypes, underscoring the importance of the HAMP linker as an intramolecular signal transducer.

Current understanding of the HAMP linker focuses on its structural rather than functional properties. Our goal in this study has been to better characterize the function of the

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TABLE 1. Strains and plasmids^a

Strain or plasmid	Relevant genotype	Source or reference
<i>E. coli</i> strains		
VJS632	Prototroph	34
VJS676	As VJS632 but $\Delta(\arg F-lac)U169$	Lab collection
VJS1945	As VJS676 but $\lambda\Phi(fdnG-lacZ)121_{UCA}$	3
VJS2197	As VJS676 but $\lambda\Phi(narG-lacZ)250$	29
VJS3057	As VJS676 but $\lambda\Phi(frdA-lacZ)402$	29
VJS4342	As VJS1945 but $\Delta narX242 narQ251::Tn10d(Tc) narP253::Tn10d(Cm)$	29
VJS5054	As VJS2197 but $\Delta narX242 narQ251::Tn10d(Tc) penB1 zad-981::Tn10d(Tc) penB1 zad-981::Tn10d(Km)$	43
VJS5720	As VJS2197 but $\Delta narX242 narL505(V88A) zch-2084::\Omega-Cm narQ251::Tn10d(Tc) penB1 zad-981::Tn10d(Km)$	43
VJS5768	As VJS3057 but $\Delta narX242 narQ251::Tn10d(Tc) penB1 zad-981::Tn10d(Km)$	43
VJS8024	As VJS4342 but $penB1 zad-981::Tn10d(Km)$	This work
Plasmids		
pHG165	Ap ^r ; pUC8 polylinker	33
pVJS2474	pHG165 with modified <i>narX</i> allele inserted between <i>EcoRI</i> and <i>HindIII</i> sites	This work
pVJS3440	pVJS2474 with silent <i>SpeI</i> site in sequence coding for <i>narX</i> TM-2	This work
pVJS3442	pVJS2474 with silent <i>NcoI</i> site in sequence coding for <i>narX</i> AS-1	This work

^a Strain and plasmid constructions are described in Materials and Methods.

HAMP linker by using the *E. coli* NarX sensor kinase as a model. We wished to determine whether the HAMP linker is essential for intramolecular signal transduction, whether there are functional differences between the different structural elements in the HAMP linker, and whether the conserved residues in the HAMP linker are essential for signal transduction. We probed these questions by using random, directed, and deletion mutagenesis in the NarX HAMP linker. Our evidence indicates that the HAMP domain's crucial features are the amphipathic α -helices rather than any particular amino acids, with the exception of the conserved Glu before AS-2. Also, deletion mutations in AS-1 conferred radically different phenotypes than deletion mutations in AS-2. This suggests that AS-1 and AS-2 play different roles in mediating transmembrane signal transduction.

MATERIALS AND METHODS

Strains, plasmids, and general methodology. *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. For mutagenesis, we used two plasmids which carried the *narX* gene modified by the insertion of several silent restriction sites. This allowed us to subclone small, easily sequenced DNA segments to reconstruct an intact *narX* gene following mutagenesis, thereby reducing the likelihood of unanticipated second-site mutations.

Plasmid pVJS2474 was used for generating six-, seven-, ten-, and eleven-codon deletions in the *narX* HAMP linker-coding sequence. This plasmid is a derivative of plasmid pVJS1241 (43), modified by deletion of *narK* sequences upstream of the *AgeI* site in *narK'* and deletion of *narL* sequences downstream of the *BglII* site in the *narL'* gene. The resulting modified *narX* allele carries sequence coding for the *narX* HAMP linker on a 402-bp *XhoI-PstI* restriction fragment. Plasmid pVJS3440 was used for generating missense mutations. This plasmid was constructed by site-directed mutagenesis (see below) of plasmid pVJS2474. Plasmid pVJS3440 carries a silent *SpeI* restriction site in sequences coding for *narX* TM-2 Leu-166 to Val-168 (TTA CTG GTG changed to TTA CTA GTG). This allows sequences coding for the NarX HAMP linker and part of TM-2 to be mutagenized and subcloned on a 170-bp DNA fragment. Plasmid pVJS3442 was

used to generate a deletion of almost all of the HAMP linker; it is a derivative of plasmid pVJS2474, in which site-directed mutagenesis was used to generate a silent *NcoI* restriction site in *narX* AS-1 Pro-181 to Trp-182 (CCG TGG changed to CCA TGG).

Standard methods were used for restriction endonuclease digestion, ligation, and transformation of DNA. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc (Beverly, Mass.). DNA for sequencing was isolated from purified plasmid minipreps (Qiagen, Valencia, Calif.) or amplified by PCR from boiled lysates of mutant colonies (4, 21, 30). Mutated *narX* DNA was sequenced either manually by using Sequenase (USB, Cleveland, Ohio) or automatically by the Department of Biological Sciences Automated DNA Sequencing Facility (University of California, Davis).

Media, culture conditions, and β -galactosidase assays. All cultures for β -galactosidase assays were grown in a mixture of defined minimal medium and specific complex medium, which was found to produce a favorable combination of rapid growth and robust response to nitrate by NarX. Equal amounts of glucose-supplemented, MOPS (3-[N-morpholino]propanesulfonic acid)-buffered minimal medium (24) and tryptone-yeast extract (TY) (21) were mixed and supplemented with ampicillin (200 μ g/ml). Cultures were grown anaerobically in the presence or absence of 40 mM NaNO₃ at 37°C in screw-cap tubes as previously described (35). Culture density was monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a number 66 (red) filter. Cultures were placed on ice upon reaching mid-exponential growth (between 30 and 40 Klett units).

β -Galactosidase activity was measured in CHCl₃-sodium dodecyl sulfate-permeabilized cells as described by Miller (22), except that the reactions took place at room temperature (approximately 21°C). Slight variation in the absolute level of β -galactosidase activity from individual strains was seen from day to day; however, the relative β -galactosidase activities between different strains were nearly constant. Each culture was assayed in duplicate, and the reported values are averaged from at least two independent experiments.

Directed mutagenesis. Deletions in the *narX* gene were generated by a modification of the Quickchange PCR protocol (Stratagene, La Jolla, Calif.), using high-fidelity thermostable DNA polymerase (Accuzyme, Bioline USA, Reno, Nev.). PCR products were generated from one mutagenic primer and another primer which annealed to the cDNA strand some 100 to 200 bp distant. The resulting double-stranded DNA fragment, carrying the desired mutation, served as the primer pair for a reaction in which the entire plasmid was replicated by high-fidelity thermostable DNA polymerase (PfuTurbo; Stratagene, La Jolla, Calif.). The resulting newly synthesized nicked plasmid DNA was used to transform *E. coli* strain DH5 α . Plasmid DNA was isolated from a transformant and digested with restriction endonucleases, and a 400-bp *XhoI-PstI* fragment (for derivatives of plasmid pVJS2474) or 170-bp *SpeI-PstI* fragment (for derivatives of plasmid pVJS3440) was subcloned into the unmutagenized parent plasmid. This ligation mixture was used to transform the appropriate reporter strain. The subcloned region of the resulting plasmid was then sequenced.

Random mutations were made at specified codons in the *narX* gene by a similar technique, except that the initial mutagenic primer was synthesized with a mixture of all four deoxynucleoside triphosphates at each position in the codon of interest. When different codons specifying the same amino acid residue were identified by sequencing, the codon used more frequently in *E. coli* was chosen (15). Deletion of the HAMP linker to form plasmid pVJS3449 was accomplished by digestion of plasmid pVJS3442 with restriction enzymes *NcoI* and *PstI*, followed by ligation with an octonucleotide (5'-CATGTGCA-3') which was complementary to the resulting cohesive ends. This replaced the intervening sequence with a single, in-frame Cys codon, forming the deletion Pro (AS-1 position +1)-Cys-Ala (AS-2 position +13).

Random mutagenesis and screening. Plasmid pVJS3440, carrying the *narX* gene, was subjected to random mutagenesis by two different techniques. First, plasmid purified from the *mutD mutS mutT* strain XL1-Red (Stratagene, La Jolla, Calif.) was digested with restriction enzymes *SpeI* and *PstI*, and the resulting 170-bp fragment was subcloned into unmutagenized plasmid pVJS3440. In the second method, error-prone PCR (7) was performed with primers flanking the HAMP linker coding sequence; the resulting PCR product was ligated into plasmid pCR-Blunt II-TOPO (Invitrogen, Carlsbad, Calif.) with this plasmid's attached ligase moiety. This product was used to transform strain DH5 α , and plasmid DNA was purified from the collected transformants. The pooled plasmid DNA was digested with restriction enzymes *SpeI* and *PstI*, and the resulting fragments were subcloned into unmutagenized plasmid pVJS3440. With both mutator-mediated and PCR-mediated mutagenesis, the resulting mixed plasmid population was used to transform strain DH5 α , and the resulting transformants were pooled and their plasmid DNA was extracted.

These pooled plasmid populations were screened for mutants following trans-

formation into strain VJS5054, a $\Delta(narX) narQ::Tn10 penB1$ reporter strain with a monocopy $\lambda\Phi(narG-lacZ)$ fusion (43). Complementation by $narX^+$ restores nitrate-inducible $\Phi(narG-lacZ)$ expression. Transformants were plated on MacConkey medium supplemented with 200 μ g of ampicillin per ml and 40 mM $NaNO_3$ and replica plated onto medium without $NaNO_3$ for screening. Mutants with induction phenotypes (white or pink on MacConkey plates with nitrate) or constitutive phenotypes (red on MacConkey plates without nitrate) were chosen for further analysis. Linkage to the plasmid was confirmed by retransforming the plasmids into strain VJS5054. All mutants from the first two mutagenized pools with background-level activity in β -galactosidase assays were found to carry $narX$ nonsense mutations; in subsequent screens, all such presumptive null mutants were discarded. Those plasmids conferring phenotypes different from plasmids pVJS3440 and pHG165 (the plasmid backbone of pVJS3440) were retained, and the sequence between the *SpeI* and *PstI* sites was determined.

Immunoblotting and detection. The accumulation of NarX protein in the envelope fraction of *E. coli* was documented by Western blotting. Cultures were grown under the same conditions as used for β -galactosidase assays, and crude membrane preparations were obtained essentially as described before (43). For immunoblotting (14), proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, Calif.), and probed with 1:9,000 diluted rabbit polyclonal antiserum raised against maltose-binding protein-NarX (14). Antibody-bound proteins were visualized with the Amersham enhanced chemofluorescence (ECF) Western blotting kit (Amersham Pharmacia, Arlington Heights, Ill.) and a Storm imaging system (Molecular Dynamics, Sunnyvale, Calif.).

RESULTS

HAMP linker structure. Figure 1 shows an alignment of the 22 known HAMP linkers in *E. coli*. Except for three positions, there is very little sequence conservation among HAMP linkers beyond the phased arrangement of hydrophobic residues (2, 42). Biochemical and sequence comparison analyses of selected bacterial sensor proteins suggest a model for HAMP linker structure in which two amphipathic α -helices are joined by an unstructured connector (Fig. 1).

The first amphipathic α -helix (AS-1) has been modeled as an extension of TM-2 (13), notwithstanding the conserved Pro residue just distal to the presumed carboxy-terminal end of TM-2, which may introduce a break or bend in the helix. We identify positions in AS-1 relative to this conserved Pro residue, which is assigned as position +1 in AS-1 (Fig. 1). For example, AS-1 of NarX extends from Arg-175 (position -6) through Val-192 (position +12; Fig. 1). AS-1 is joined to the second amphipathic α -helix, AS-2, by an unstructured connector at least 14 residues long. A highly conserved Glu residue is assigned here to the carboxy-terminal end of the connector rather than AS-2, in part because of the phenotypes of deletion mutations around and including this residue (see below). However, this is primarily an operational definition. We identify positions in AS-2 relative to this conserved Glu residue. For example, AS-2 of NarX extends from Met-209 (position +1) to Leu-223 (position +15; Fig. 1). Position +11 in AS-2 is usually occupied by Met or Leu (Fig. 1). Note, however, that none of the three conserved positions is invariant among projected HAMP linkers.

Rationale for random mutagenesis of the NarX HAMP linker. Aside from the biochemical studies summarized in the introduction, there is relatively little experimental basis from which to evaluate this model or to understand how these structures transmit a signal from input to output domains. To approach questions of HAMP linker structure and function, we used random mutagenesis to probe the HAMP linker of the *E.*

coli nitrate-responsive sensor kinase NarX, which provides a sensitive assay for perturbations of sensor kinase function. Interaction between NarX and its ligand, nitrate, results in phosphorylation of the response regulator NarL. Phospho-NarL controls expression of several operons, causing activation (e.g., approximately 100-fold activation at the *narGHJI* and *fdnGHI* operons) or repression (e.g., up to 10-fold repression at the *frdABCD* operon) (10, 36). (Expression of the *narG* operon is much more sensitive than expression of the *fdnG* and *frdA* operons to relatively minor perturbations of the Nar regulatory system.) In the $\Delta narX$ reporter strain VJS5054, the $\Phi(narG-lacZ)$ operon fusion is induced approximately 100-fold by $narX^+$ on a pBR322-based plasmid (the *penB1* mutation in this strain reduces plasmid copy number to approximately one [20]). This allows a simple and sensitive assay of the function of *narX* alleles with altered HAMP linkers.

We employed two general approaches to generating missense alterations within the *narX* HAMP linker coding region. First, we generated four independent pools of randomly generated mutants (see Materials and Methods). After subcloning mutagenized DNA into pVJS3440 to generate an intact *narX* gene, plasmids carrying the mutant *narX* alleles were used to transform the $\Phi(narG-lacZ)$ reporter strain VJS5054. Transformants were screened for phenotypes different from the parent $narX^+$ allele and from the plasmid vector (see Materials and Methods). Second, we used oligonucleotide-directed mutagenesis to generate a range of missense mutations at four specific codons. Three of these encode the conserved residues Pro-181 (position +1 in AS-1), Glu-208, and Met-219 (position +11 in AS-2; Fig. 1). The fourth codon, encoding Trp-182, was chosen because the NarX HAMP linker is unique among *E. coli* HAMP linkers in having a Trp at position +2 in AS-1, rather than the more common Leu or Ile (Fig. 1).

Phenotypes of *narX* HAMP missense mutations. We evaluated the phenotypes conferred by 40 different missense alterations in the *narX* HAMP linker coding region. Fourteen of these were isolated by screening randomly generated mutants for altered $\Phi(narG-lacZ)$ expression, and 26 were isolated by directed mutagenesis. Seven of the directed changes conferred essentially wild-type phenotypes (data not shown); with $narX^+$, uninduced basal expression of the $\Phi(narG-lacZ)$ operon fusion was low, whereas nitrate-induced expression was elevated by approximately 100-fold. These seven had changes at the conserved residue Pro-181 at AS-1 position +1 (changes to Gly, Ala, and Ser), Trp-182 (changes to Leu, Phe, and Met), or the conserved residue Met-219 at AS-2 position +11 (change to Tyr). Two of these (Pro-181 to Ser and Met-219 to Tyr) expressed $\Phi(narG-lacZ)$ at slightly elevated levels in uninduced cultures (40 and 70 LacZ units, respectively, versus 15 in $narX^+$). However, both were indistinguishable from $narX^+$ on MacConkey agar plates and in assays for expression of the *fdnG* and *frdA* operons (data not shown).

Six *narX* HAMP linker mutants exhibited impaired induction phenotypes (Table 2). For example, with the Ala-189 to Val mutant (position +9 in AS-1), uninduced basal expression was unaltered, whereas nitrate-induced expression was only about one-tenth of the $narX^+$ level (Table 2). Mutations causing similar phenotypes were in the coding region for the connector (Arg-195 to Cys) and AS-2 (Glu-222 to Lys at position +14). Three additional mutations resulting in this phenotype

TABLE 2. Representative missense mutations in the NarX HAMP linker

Phenotype and amino acid change ^a	Method of isolation ^b	β -Galactosidase sp act ^c (Miller units)	
		–NO ₃ [–]	+NO ₃ [–]
Vector only		10	10
Wild-type <i>narX</i>		15	2,250
Impaired induction			
R195C	Random	10	740
W182I	Directed	10	670
A189V	Random	10	170
W182V	Directed	10	150
E222K	Random	10	130
W182A	Directed	10	120
Constitutive			
L212F	Random	160	1,510
M219L	Directed	400	2,280
P181K	Directed	900	1,020
P181L	Random	1,100	1,880
E208D	Random	2,190	1,560
L185P	Random	2,300	2,170

^a Amino acid changes are represented by the one-letter code for the wild-type amino acid, followed by its position in NarX, followed by the one-letter code for the substituted amino acid. Groupings are based the phenotypic classes explained in Results.

^b Mutants were isolated either by screening randomly generated mutations in the HAMP linker for phenotypes different from *narX* (random) or by using oligonucleotide-directed mutagenesis to randomize selected codons (directed; see Materials and Methods).

^c β -Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units. Mutant *narX* alleles were expressed from derivatives of plasmid pHG165 transformed into strain VJS5054 Φ (*narG-lacZ*) *narX narPQ pcnB*. Cultures were grown anaerobically in the absence or presence of 40 mM NaNO₃.

(Trp-182 changed to Ala, Val, or Ile) were isolated by directed mutagenesis. Therefore, only three impaired-induction mutants were identified from the four pools of randomly mutagenized *narX* plasmid DNA. Note that none of the impaired-induction mutations alters the three highly conserved HAMP linker residues.

The remaining 27 missense mutations exhibited a continuum of constitutive phenotypes characterized by elevated basal-level Φ (*narG-lacZ*) expression (Fig. 2; data from representative constitutive-phenotype mutants are shown in Table 2). Eleven of these constitutive mutants were isolated by screening only two of the four mutagenized pools of *narX* HAMP linker DNA; four of these mutations alter conserved residues. The remaining 16 mutants came from directed mutagenesis of the three conserved residues. Some (e.g., Leu-212 to Phe) exhibited over fivefold induction in response to nitrate (Table 2). Others exhibited approximately the same level of Φ (*narG-lacZ*) expression irrespective of nitrate. Of these, some, e.g., Pro-181 to Lys at position +1 in AS-1, exhibited approximately one-half the Φ (*narG-lacZ*) expression of nitrate-induced *narX*⁺ (Table 2). This mutant was found to confer a similar constitutive phenotype in assays for expression of the *fdnG* and *frdA* operons (data not shown). Others, e.g., Leu-185 to Pro at +5 in AS-1, exhibited constitutive Φ (*narG-lacZ*) expression comparable to that of induced *narX*⁺ (Table 2). This mutant was found to confer a similar constitutive phenotype in assays for expression of the *fdnG* operon. By contrast, this mutant was impaired with respect to *frdA* operon repression (data not shown); we do not understand this observation.

As with mutations resulting in impaired induction phenotypes, mutations resulting in constitutive phenotypes were found in AS-1, the connector, and AS-2, and several were at the phased hydrophobic residues in AS-1 and AS-2. In contrast to the impaired-induction mutations, however, these constitutive mutations were found at both conserved and nonconserved residues in the HAMP linker.

Scanning deletion mutagenesis of the NarX HAMP linker.

We next analyzed the NarX HAMP linker by constructing a set of mutant *narX* alleles with deletions of seven codons. These deletions, which overlapped by three or four codons, scanned the *narX* gene from the end of TM-2 to the beginning of the central domain. Since seven amino acyl residues can form approximately two α -helical turns, these deletions were designed to abbreviate the HAMP linker's proposed amphipathic α -helices while preserving their rotational orientation. Although the connector is presumed to be unstructured, we also made deletions of seven codons in this region.

The phenotypic classes represented by these *narX* deletions correlate with the amphipathic sequences and predicted structural elements in the HAMP domain (Fig. 2). The five deletions scanning AS-1 exhibited an elevated level of Φ (*narG-lacZ*) expression (approximately 75% of the *narX*⁺ induced level) irrespective of added nitrate. This set of deletions includes one encompassing six residues, from positions –6 to –1 in AS-1. The three deletions scanning the connector from Arg-195 through Glu-208 conferred high-level Φ (*narG-lacZ*) expression (approximately 100% of the *narX*⁺ induced level) irrespective of added nitrate.

By contrast, strikingly unique phenotypes were conferred by seven-codon deletions within AS-2. Two of these (Met-209 through Ala-215 and Leu-212 through Asn-218) exhibited a reversed-response phenotype: Φ (*narG-lacZ*) expression was six- to ten-fold higher in the absence of nitrate than in its presence (Fig. 2). Conversely, the third deletion (Leu-212 through Glu-218) resulted in basal-level Φ (*narG-lacZ*) expression irrespective of nitrate (Fig. 2). These results indicate that AS-2 plays a unique role in HAMP linker function.

The two deletions that span the junctions between AS-2 and its flanking elements (Gly-205 through Met-211, and Met-219 through Glu-226; Fig. 2) both conferred similar phenotypes distinct from the others: elevated Φ (*narG-lacZ*) expression that was induced two- to fourfold by nitrate (Fig. 2).

Larger deletions in the NarX HAMP linker. We also constructed larger deletions to remove most of AS-1 and AS-2. The 11-codon deletion in AS-1 (Pro-181 through Ala-191) exhibited intermediate-level Φ (*narG-lacZ*) expression (approximately 50% of the *narX*⁺ induced level) irrespective of added nitrate (Fig. 2). This phenotype was not strikingly different from the elevated-level constitutive phenotypes conferred by the seven-codon deletions in AS-1.

The 10- and 11-codon deletions in AS-2 (Met-209 through Asn-218 and Met-209 through Met-219) also exhibited intermediate-level Φ (*narG-lacZ*) expression (approximately 50% of the *narX*⁺ induced level) irrespective of added nitrate (Fig. 2). This phenotype was quite distinct from that conferred by the seven-codon deletions in AS-2 (see above).

Finally, we constructed a deletion from an introduced *NcoI* site overlapping codon Trp-182 (position +2 in AS-1) to the native *PstI* site overlapping codon Ser-220 (position +12 in

FIG. 2. Missense mutations and deletions in the NarX HAMP linker. NarX amino acid sequence, heptad repeats, and the positions of AS-1 and AS-2 are displayed as in Fig. 1. Missense mutations are indicated above the NarX amino acid sequence; impaired induction and constitutive phenotypic classifications are described in the text. Positions indicated with an asterisk are loci of multiple substitutions, isolated by both directed and random mutagenesis. Deletions in the NarX HAMP linker are represented by solid boxes under the wild-type NarX amino acid sequence; the deleted residues are indicated in the solid boxes. The deletion Pro-181 through Ser-220 replaces the indicated residues with a Cys residue. β-Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units. Mutant narX alleles were expressed from derivatives of plasmid pHG165 transformed into strain VJS5054 (narG-lacZ) narX narPQ penB. Cultures were grown anaerobically in the absence (−NO₃[−]) or presence (+NO₃[−]) of 40 mM NaNO₃. Activity for narX[−] was 20 Miller units in the absence of nitrate and approximately 2,000 Miller units with added nitrate; activity for the vector only was about 10 Miller units irrespective of added nitrate.

TABLE 3. $\Phi(narG-lacZ)$ expression in VJS5720 derivatives carrying *narX* HAMP linker mutants^a

<i>narX</i> allele	β -Galactosidase sp act ^b (Miller units)	
	–NO ₃ [–]	+NO ₃ [–]
Wild type	780	3,260
Vector only	2,780	2,750
W182A	210	3,790
A189V	650	3,990
E222K	660	3,700
R195C	430	3,900
Δ (L216-E222)	4,730	4,050
Δ (W182-S220)	4,230	3,980

^a *narX* alleles with amino acid changes are represented by the one-letter code for the wild-type amino acid, followed by its position in NarX, followed by the one-letter code for the substituted amino acid. Deletions are represented by Δ (amino acids deleted, inclusive).

^b β -Galactosidase specific activity was measured as described in Materials and Methods and is expressed in Miller units. Mutant *narX* alleles were expressed from derivatives of plasmid pHG165 transformed into strain VJS5720 $\Phi(narG-lacZ)$ $\Delta narX narL505 narQ pcnB1$. Cultures were grown anaerobically in the absence or presence of 40 mM NaNO₃.

AS-2). Ligation with an octanucleotide placed a Cys codon at the junction (see Materials and Methods). This deletion conferred low-level $\Phi(narG-lacZ)$ expression that only weakly responded to nitrate addition (Fig. 2). Perhaps this large deletion imposes an improper dimeric association between the output domains, or perhaps output domain function is impeded by close association with the cytoplasmic membrane.

Further analysis of impaired-induction mutants. A few of the HAMP linker mutants were impaired for induction of $\Phi(narG-lacZ)$ expression. We used two assays to determine if these mutant proteins were inactive for trivial reasons (e.g., instability or mislocalization). The first assay was for negative regulation *in vivo*, and the second assay directly examined protein accumulation.

In addition to its role as a positive regulator (protein kinase), the NarX protein is also a negative regulator (phosphoprotein phosphatase) of target operon expression. Previously isolated missense mutants lacking positive response to nitrate retain negative control in its absence (41), whereas null mutants lack both activities. We therefore examined a subset of impaired-induction mutants to determine whether they retained negative regulation. Our assay for negative regulation exploits the Val-88 to Ala missense change in the structural gene for the NarL response regulator (*narL505* allele). The NarL(V88A) protein is phosphorylated *in vivo* by a NarX-independent mechanism and therefore causes constitutive target operon expression (11, 19). In *narX* null strains grown in the absence of nitrate, this allele confers high-level $\Phi(narG-lacZ)$ expression, whereas in *narX*⁺ strains, the level of $\Phi(narG-lacZ)$ expression is about fourfold lower as a consequence of NarX negative regulation (phosphoprotein phosphatase; Table 3).

We tested four representative impaired-induction missense mutants (Trp-182 to Val, Ala-189 to Val, Glu-222 to Lys, and Arg-195 to Cys; see Table 2) for negative regulation. All were at least as effective as *narX*⁺ in effecting decreased $\Phi(narG-lacZ)$ expression in the absence of nitrate (Table 3). By contrast, the two deletions tested (Leu-212 through Glu-218 in AS-2 and Trp-182 through Ser-220) mimicked the *narX* null strain with respect to negative regulation (Table 3).

We also used Western blot analysis to probe envelope fractions from the same mutants for NarX protein. All were present at approximately the wild-type level (data not shown).

DISCUSSION

We used random, directed, and deletion mutagenesis to probe the function, sequence, and structural requirements of the NarX HAMP linker. We employed a $\Phi(narG-lacZ)$ reporter to screen for and assay the activity of *narX* mutants. Because $\Phi(narG-lacZ)$ depends on phospho-NarL for expression, and because NarL is phosphorylated only by the induced kinase activity of NarX in the reporter strain used, we regard the resulting β -galactosidase activity as an indicator of NarX kinase activity. Hence, constitutive $\Phi(narG-lacZ)$ expression results from *narX* constitutive-kinase mutants that simulate the ligand-bound state. Conversely, poorly inducible $\Phi(narG-lacZ)$ expression results from *narX* impaired-induction mutants that simulate the ligand-unbound state.

Sequence and structure in the HAMP linker. Only three positions in the HAMP linker exhibit significant conservation, so we used directed mutagenesis to randomize these codons in the *narX* gene. These positions had previously been identified in mutant screens with NarX, Tsr, and EnvZ, and all three were also identified by our random mutagenesis reported in this paper. Our directed mutagenesis sought to identify any allowed substitutions at these positions.

Essentially wild-type function was retained when the conserved Pro residue at position +1 of AS-1 was replaced by Gly, Ala, or Ser (data not shown). These small side chains may allow distortions in AS-1 similar to the one associated with Pro (generally considered a helix-breaking residue) (5). By contrast, substitution at this position with Leu or Val, both of which are compatible with α -helical structure, caused constitutive kinase phenotypes. A Pro-to-Leu change at this position in the osmosensor EnvZ also causes constitutive kinase activity (38).

Position +11 in AS-2 is usually occupied by Met or Leu (Fig. 1). We found that substitution of this position (Met-219) in NarX with Ile or Leu resulted in constitutive phenotypes which retained a significant residual induction by nitrate, whereas substitution with other residues resulted in constitutive phenotypes insensitive to nitrate (Table 2 and data not shown). Changes of Met to Ile were previously identified in Tsr and NarX (1, 8). Therefore, this position seems to be relatively refractory to substitution.

We isolated 11 different substitutions at the conserved Glu residue immediately preceding AS-2 (Glu-208 in NarX), all of which resulted in constitutive phenotypes. Most substitutions, including Asp, resulted in NarX proteins which did not respond to nitrate (Table 2 and data not shown), although a few, such as Ile, conferred partially constitutive phenotypes which retained some inducibility by nitrate (data not shown). Changes of Glu to Lys were previously isolated in Tsr, NarX, and EnvZ (1, 8, 28). Clearly, this position plays a key role in HAMP linker function.

Some of the NarX changes at these conserved positions introduced residues that occur in other HAMP linkers, such as Pro to Ala (YfiN), Met to Leu (several), and Glu to Leu (Aer; Fig. 1 and 2). Some substitutions conferred an essentially wild-

type response (e.g., Pro to Ala), some conferred a constitutive phenotype with a residual response to nitrate (e.g., Met to Leu), and some conferred a constitutive phenotype with no additional induction by nitrate (e.g., Glu to Leu). These observations indicate that individual variations in different HAMP sequences probably evolved to function in that specific context.

We conclude that structure rather than sequence is the key feature of the HAMP linker. None of the conserved residues is invariant among *E. coli* HAMP linkers, and phenotypes caused by changes at these residues were indistinguishable from phenotypes caused by alterations at other residues throughout AS-1, the connector, and AS-2 (Table 2). There was no correlation between the position of missense mutations in the HAMP linker and either impaired-induction or constitutive phenotype. However, mutations which could disrupt α -helical structure, such as Pro substitutions at Leu-185 (position +5 in AS-1) and Ser-220 (position 12 in AS-2), conferred nitrate-nonresponsive constitutive phenotypes. Likewise, all deletions involving AS-1 or the connector, as well as the two larger deletions in AS-2, also resulted in nitrate-unresponsive constitutive phenotypes (Fig. 2). These mutations, which probably produce relatively severe structural perturbations, confer phenotypes that reflect loss of signaling function. Therefore, it seems likely that the HAMP linker is a negative effector of sensor output activity and that a signal from the periplasmic domain relieves the negative interaction between the HAMP linker and output.

By contrast, constitutive mutants in which one hydrophobic residue was substituted for another (e.g., Trp-182 at +2 in AS-1 to Leu, Phe, or Met, or Leu-212 to Phe at position +4 in AS-2) generally exhibited appreciable induction by nitrate. Therefore, these changes seem to diminish rather than eliminate HAMP linker function.

Special signaling role for AS-2. By contrast with deletions involving AS-1 or the connector, seven-residue deletions within or beyond AS-2 conferred a variety of phenotypes. One that extended past the carboxyl terminus of AS-2 caused a nitrate-responsive constitutive phenotype, whereas another within the carboxyl-terminal end of AS-2 caused an impaired-induction phenotype (Fig. 2).

The other two deletions in AS-2 resulted in a striking reversed-response phenotype, in which NarX kinase activity was decreased rather than increased by nitrate (Fig. 2). Both of these deletions bring the conserved residues Glu-208 in the connector and Met-219 at position 11 in AS-2 within one α -helical turn of each other. Their phenotype, in which the sensor's sensitivity to ligand is retained while the sign of the response is reversed, is unique among known sensor mutations and suggests that AS-2 has a critical role in signal transduction.

Reversed phenotypes have previously been reported for MCP Tar- and Tsr-mediated responses to membrane-permeating weak acids, oxygen tension, and changes in temperature (9, 23, 25, 26, 39). Mutations causing these phenotypes have been found in TM-2, the junction between the HAMP linker AS-2 and the adaptation domains, and the adaptation domains of these MCPs. In all cases, however, the response of these proteins to extracytoplasmic ligands remains normal. This leads us to view the MCPs as responding to multiple sensory inputs: ligand binding in the periplasm (with a signal that is transmitted by the HAMP linker to the output domain) and

structural alterations in the transmembrane and cytoplasmic regions caused by temperature, membrane-permeating acids, and others (with a signal that may or may not be transmitted by the HAMP linker). The MCP mutants with reversed responses to these nonperiplasmic inputs are thus defective in sensing or adaptation rather than signal transduction. In fact, similar phenotypes can be generated by mutations in CheB, a methyl-erase which modifies the adaptation domains of MCPs (9).

Models of HAMP linker structure and signal transduction.

One general model implied by the corpus of literature concerning MCP-mediated signal transduction depicts the HAMP domain as a rigid pushrod connecting the input domain to the output domain (12, 18). Ligand binding to the periplasmic input domain causes a small (1 to 2 Å) sliding displacement between the TM-2 helices in a dimer (27). The inference is that this displacement results in an identical displacement at the distal end of the HAMP linker, which abuts the output domain. Our results are inconsistent with this notion. If the HAMP linker is a rigid pushrod extension of TM-2, it would be immaterial whether a deletion shortening the HAMP linker is in AS-1 or AS-2. However, we found that radically different phenotypes were caused by deletions in AS-1 and AS-2.

An explicit hypothesis has been advanced that postulates an active, dynamic role for the HAMP linker in signal transduction (42). It posits that AS-2 acts as a negative regulator of the output domain, whereas AS-1 can interact with the inner face of the cytoplasmic membrane. In this hypothesis, ligand binding in the periplasm displaces TM-2 toward the cytoplasm, releasing AS-1 from the cytoplasmic membrane. Liberated AS-1 then interacts with AS-2, releasing its inhibition of the output domain. This hypothesis predicts that deletions that reduce or eliminate AS-1 should confer uninducible phenotypes because AS-2 interaction with the output domain would persist. However, all AS-1 deletions resulted in constitutive kinase activity. This hypothesis likewise predicts that deletions in AS-2 should confer constitutive output activity. However, some AS-2 deletions conferred reversed-response or uninducible phenotypes. Therefore, our results from HAMP linker deletion analysis effectively falsify this hypothesis (42).

Our results support a model of the HAMP linker with four significant elements: two amphipathic α -helices, an unstructured connector, and a Glu residue at the junction between the connector and AS-2. Additionally, AS-1 may have a discontinuity near its junction with TM-2. Our data do not yet provide definite boundaries for AS-1 and AS-2 or a clear model for HAMP linker function. However, these data indicate that AS-1 and AS-2 have quite different roles in signal transduction and that AS-2 is not functionally homogenous. As a whole, however, it seems clear that the HAMP linker is a negative regulator of output domain activity.

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