Role of the H Domain of the Histidine Kinase-like Protein NifL in Signal Transmission*

Richard Little, Isabel Martinez-Argudo†, Susan Perry, and Ray Dixon

From the Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, United Kingdom

The NifL protein from Azotobacter vinelandii senses both the redox and fixed nitrogen status to regulate nitrogen fixation by controlling the activity of the transcriptional activator NifA. NifL has a domain architecture similar to that of the cytoplasmic histidine protein kinases. It contains two N-terminal PAS domains and a C-terminal transmitter region containing a conserved histidine residue (H domain) and a nucleotide binding GHKL domain corresponding to the catalytic core of the histidine kinases. Despite these similarities, NifL does not exhibit kinase activity and regulates its partner NifA by direct protein-protein interactions rather than phosphorylation. NifL senses the redox status via a FAD co-factor located within the PAS1 domain and responds to the nitrogen status by interaction with the signal transduction protein GlnK, which binds to the GHKL domain. The ability of NifL to inhibit NifA is antagonized by the binding of 2-oxoglutarate to the N-terminal GAF domain of NifA. In this study we have performed site-directed mutagenesis of the H domain of NifL to examine its role in signal transmission. Our results suggest that this domain plays a major role in transmission of signals perceived by the PAS1 and GHKL domains to ensure that NifL achieves the required conformation necessary to inhibit the 2-oxoglutarate-bound form of NifA. Some of the substitutions discriminate the redox and fixed nitrogen sensing functions of NifL implying that the conformational requirements and/or domain interactions necessary for NifA inhibition differ with respect to the signal input.

The Azotobacter vinelandii NifL regulatory protein is an evolutionary relative of the histidine protein kinases (HPKs)† (1, 2) that senses both the redox and fixed nitrogen status to control the expression of nitrogen fixation genes (3, 4). NifL has domain architecture similar to cytoplasmic HPKs such as NtrB and FixL. It contains two N-terminal PAS domains (5) and a C-terminal transmitter region containing a conserved H motif (H domain) and nucleotide binding domain belonging to the GHKL superfamily of ATPases (6) (see Fig. 1). Although the C-terminal region of NifL is homologous to HPKs, NifL does not exhibit kinase activity. In response to signals, NifL tightly controls the activity of its partner protein NifA, a ω34-dependent transcriptional activator, by direct protein-protein interaction rather than by phosphorylation (4). NifA is not a response regulator, and its ability to interact with NifL is controlled by a regulatory N-terminal GAF domain (7) that is responsive to the binding of 2-oxoglutarate, a potential metabolic signal of the carbon status (8, 9). It, therefore, appears that NifL has evolved from canonical HPKs to control the activity of its partner by protein-protein interactions rather than by phosphorylation.

Discrete domains of NifL are required for its redox and nitrogen sensing functions. The N-terminal PAS domain (PAS1) binds a FAD co-factor that is responsive to redox status (10, 11). Oxidation of the flavin cofactor results in activation of NifL and consequent inhibition of NifA activity, whereas reduction of the FAD moiety deactivates NifL (12). The C-terminal GHKL domain of NifL has a bipartite function. As in the case of bona fide HPKs, this domain binds adenosine nucleotides, although it exhibits neither ATPase nor transphosphorylation activities. The binding of nucleotide to the GHKL domain potentiates the inhibitory functions of NifL and its interaction with NifA (13–15). The GHKL domain is also the target for interaction with the signal transduction protein GlnK, which in its noncovalently modified form interacts with the GHKL domain to convey the fixed nitrogen status (16–18).

The H domain of NifL is predicted to have a similar secondary structure to that of the dimerization and phospho-transfer (HisKA) domains of the HPKs and contains an H box motif signifying an autophosphorylation site. However, the conserved histidine residue in NifL is not essential for signal transduction (19). Nevertheless it is likely that the H domain plays an important role in signal transmission and interactions with NifA. We have recently identified an arginine residue (Arg-306) adjacent to the conserved histidine in the H motif that is critical for signal transmission within NifL. Substitutions of Arg-306 cause NifL to constitutively inhibit NifA irrespective of environmental cues. We have demonstrated that the phenotype of one such mutation, R306C, is associated with a conformational change that apparently locks NifL in a form similar to the oxidized “on” state so that the mutant NifL protein inhibits NifA even under conditions appropriate for nitrogen fixation (20). We observe that some mutations in the GHKL domain of NifL that substantially reduce ADP binding suppress the phenotype of the R306C mutation. These observations suggest that nucleotide binding may influence the topological relationship of the H and GHKL domains to bring about the conformational
changes required to inhibit NifA (20). The key role of arginine 306 in signal transmission has prompted us to investigate the role of other residues of the H domain of NifL in signal propagation. In this study we have analyzed the properties of 12 amino acid substitutions in the predicted helical regions of the H domain. Our results suggest that this domain plays a major role in signal transmission to influence interactions with NifA.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The A288T and L292I mutations were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). Apal-BstEI fragments containing the nifL mutations were subcloned into pPR34 encoding wild-type NifL and NifA (13). Two further mutations were constructed by PCR using primers MS1 (5'-GGGAAAACCTCGCCCGCCCCC-3') and L307A reverse (5'-GTGGACCGGGCCCTTGCGCGATGATGGCAGCGT-3') for the L307A mutation and S315A forward (5'-CTGGAGCGCCCGTCAACCTGATCGCGGGCGGATGATGGCAGCGT-3') and L2 (5'-GTCGCTGTTCAGGTGGAGG-3') for the S315A mutation. The remainder of the site-directed mutagenesis was carried out by two-step PCR. The first step consisted of two PCR reactions; one carried out with primer MS1 and a reverse primer containing the desired mutation and primer L2. PCR products were purified and used as a template for the second step PCR using the above primers MS1 and L2. The resulting fragments were cut with NotI and Apal and cloned into plasmid pPR34. All mutations were confirmed by DNA sequencing.

**β-Galactosidase Assays**—In vivo activity of NifL and NifA was measured in *Escherichia coli* strain ET8000 using the reporter plasmid pRT22 which carries a *nifH lacZ* translational fusion. NifL and NifA were expressed on a second plasmid from a constitutive promoter (13). Cultures were grown in 4 ml of NFDM medium (27) supplemented with casein hydrolysate (200 μg/ml) for nitrogen limiting conditions or with (NH₄)₂SO₄ (1 mg/ml) for nitrogen excess conditions. Strains were grown in a plastic vial (internal volume, 7 ml) sealed with a rubber closure for anaerobic conditions. When conditions required aerobiosis, 5-ml cultures were grown with vigorous shaking in 25-ml conical flasks. Western Blotting—To obtain protein extracts, cultures containing pRT22 and the plasmid of interest were grown in the same conditions used for β-galactosidase assays. Cells were centrifuged (0.8 units at *A₅₀₀), and the pellet was resuspended in Laemmli loading buffer. Equivalent amounts of proteins were separated by electrophoresis on polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were probed with polyclonal antiserum against NifL. Primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit secondary antibodies. Detection was carried out by staining the membrane with 5-bromo-4-chloro-3-indolylphosphosphate and nitro blue tetrazolium.

**Protein Expression and Purification**—Plasmids pTJ45, pLM73, and pDB737 were used for overexpression of N₅₆₆-NifL, N₅₆₆-NifL-(147–519), and NifA, respectively (8, 20, 21). For overexpression of mutant derivatives of N₅₆₆-NifL, an Ndel-BamHI fragment encoding the corresponding mutation was cloned into plasmid pET28b (Novagen). For mutant derivatives of N₅₆₆-NifL-(147–519), an Ndel-BamHI fragment encoding the truncated NifL protein was generated using the forward primer NifL-147 and the reverse primer NifL2 (20). Cultures were grown aerobically in Luria-Bertani broth, and expression from the T7 promoter was induced by the addition of isopropyl-β-D-thiogalactopyranoside to 1 mM. Proteins were purified as described previously (8, 22). Concentrations of NifL and NifA were calculated on the basis that these proteins are tetrameric and dimeric, respectively.

**Size Exclusion Chromatography**—Gel permeation chromatography was performed over a Superdex 200 HR10/30 column (GE Healthcare) equilibrated with 50 mM Tris-Cl, pH 7.5, 150 mM sodium acetate, 200 mM NaCl, and 20 mM imidazole. Molecular weight standards used for calibration were cytochrome c, carbonic anhydrase, ovalbumin, bovine serum albumin, aldolase, catalase, ferritin and thyroglobulin. The void volume of the column was determined with blue dextran.

**Limited Trypsin Proteolysis**—Trypsin proteolysis of NifL was performed in TA buffer (50 mM Tris acetate, pH 7.0, 100 mM potassium acetate, 8 mM magnesium acetate, 1 mM dithiothreitol) at 25 °C. Samples were incubated for 5 min before initiating digestion with trypsin (Sigma, from bovine pancreas). The enzyme was diluted from a 0.5 mg/ml stock solution into the reaction at the trypsin:NifL weight ratio indicated in the legend to Fig. 4. At the times indicated, 15 μl of sample was withdrawn and added to microcentrifuge tubes containing 0.3 μg of trypsin inhibitor (Roche Applied Science). An equal volume of gel loading buffer (125 mM Tris-HCl, 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, 0.05% bromphenol blue, pH 8.6) was added, and samples were heated at 100 °C for 4 min before electrophoretic separation on 12% SDS-polyacrylamide gels. Densitometric analysis of bands was carried out using SynGene software (Synoptics Ltd).

**NifL: NifA Complexes**—All reactions and washing steps were carried out at room temperature in the following buffer: 50 mM Tris acetate, pH 8.0, 15% glycerol, 100 mM potassium acetate, 50 mM KSCN, 3.5 mM magnesium acetate, 20 mM imidazole, 200 mM NaCl, 50 μM ADP. Hexahistidine-tagged NifL (N₅₆₆-NifL) and mutant derivatives (final concentration, 400 nM) were incubated in 500 μl of buffer containing 50 μl of Ni-NTA magnetic-agarose bead suspension (Qiagen) for 45 min. Agitation was maintained throughout to prevent settlement of the beads. After this incubation the buffer was replaced and NifA (final concentration, 800 nM) added to a total volume of 500 μl. The samples were incubated for 1 h, after which four cycles of washing/buffer replacement involving complete re-suspension of the beads was performed. After final buffer removal, NifL was
E. coli (UniProt code Q9WZV7), alignment of the H domain with the HisKA domains of the histidine protein kinases: the NTA surface through injection of 20-Ni-NTA biosensor chip surfaces. Nickel was firstly bound to Hexahistidine-tagged derivatives of NifL were immobilized on performed using a Biacore X biosensor system (Biacore AB). 

were derived on the assumption that proteins and ligand were obtained with least squares estimates of the binding enthalpy, stoichiometry, and binding constant (23). Binding stoichiometries provided by MicroCal, using equations and curve-fitting analysis to obtain heat change of ligand binding to protein. The heat change for the dilution of the ligand in the absence of protein was measured for each experiment and was subtracted from the measured heat change of ligand binding to protein. Data analysis was performed with the Origin program, provided by MicroCal, using equations and curve-fitting analysis to obtain least squares estimates of the binding enthalpy, stoichiometry, and binding constant (23). Binding stoichiometries were derived on the assumption that proteins and ligand were fully active with respect to binding. 

Surface Plasmon Resonance (Biacore)—Experiments were performed using a Biacore X biosensor system (Biacore AB). Hexahistidine-tagged derivatives of NifL were immobilized on Ni-NTA biosensor chip surfaces. Nickel was firstly bound to the NTA surface through injection of 20-μl volumes of 500 μM nickel chloride. Proteins for immobilization were introduced to the protein-chip surface at a concentration of 40 nM. Experiments were performed at 25 °C in buffer containing 10 mM HEPES, 0.005% Tween 20, 150 mM NaCl, 25 mM MgCl₂, pH 7.4, at a flow rate of 20 μl/min. ATP and 2-oxoglutarate were present concentrations of 3.5 and 2 mM respectively. Sensorgrams were derived by injection of various concentrations of GlnK as indicated in the legend to Fig. 7. The control flow cell contained portion of NifL is not annotated as a dimerization and histidine phospho-transfer domain in some of the protein databases, secondary structure predictions using the PSIPRED server (26) suggest that this region comprises two α-helices connected by a turn, similar to that found in the structures of the cytoplasmic region of the Thermotoga maritima HK853 HKP (25) and the HisKA domain of E. coli EnvZ (24). Identical predictions were obtained with E. coli NtrB and E. coli PhoQ. Hereafter we refer to this region of NifL as the H domain. To shed light on the function of this domain, we made 12 amino acid substitutions (mostly to alanine) in residues corresponding to the helical regions of EnvZ and HK858 (Fig. 1).

The activity of the mutant proteins was measured in E. coli using a two plasmid system consisting of a nifHp-lacZ reporter and a second plasmid in which nifL and nifA are expressed from a constitutive promoter. Wild-type NifL inhibits NifA activity in response to oxygen (Fig. 2A, compare open and light gray bars) and fixed nitrogen (Fig. 2A, compare open and dark-gray bars). As shown previously in this system (22, 27), NifL inhibits NifA activity to a certain extent even under conditions appropriate for nitrogen fixation (anaerobic, nitrogen-limiting growth conditions) as the activity of the reporter is at least 4-fold lower in the presence of NifL compared with NifA alone (Fig. 2, compare open bars marked NifL with those marked as ΔNifL). Mutant proteins appeared to fall into three different classes, (a) a response similar to wild-type NifL to fixed nitrogen and oxygen (mutants F300A, M318A, and to a certain extent, S315A), (b) mutants deficient in the response to oxygen that retain a response to fixed nitrogen (N301A, M338A, and R339A), and (c) mutants substantially deficient in both the redox and fixed nitrogen response and lacking the ability to inhibit NifA activity under anaerobic, nitrogen-limiting conditions (A288T, L292I, M296A L307A, L349A, and L352A). 

All the mutant proteins were apparently stable under the appropriate for nitrogen fixation (anaerobic, nitrogen-limiting growth conditions) as the activity of the reporter is at least 4-fold lower in the presence of NifL compared with NifA alone (Fig. 2, compare open bars marked NifL with those marked as ΔNifL). Mutant proteins appeared to fall into three different classes, (a) a response similar to wild-type NifL to fixed nitrogen and oxygen (mutants F300A, M318A, and to a certain extent, S315A), (b) mutants deficient in the response to oxygen that retain a response to fixed nitrogen (N301A, M338A, and R339A), and (c) mutants substantially deficient in both the redox and fixed nitrogen response and lacking the ability to inhibit NifA activity under anaerobic, nitrogen-limiting conditions (A288T, L292I, M296A L307A, L349A, and L352A). 

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Redox-insensitive Mutants—The in vivo properties of the N301A, M338A, and R339A mutants suggest that they are

FIGURE 1. Domain structure of A. vinelandii NifL (UniProt code P30663) expanded to show a sequence alignment of the H domain with the HisKA domains of the histidine protein kinases: T. maritima HK853 (UniProt code Q9WZV7), E. coli EnvZ (UniProt code P0AEJ4), E. coli PhoQ (UniProt code P23837), and E. coli NtrB (UniProt code P0AFB5). The secondary structures of T. maritima HK853 (PDB code 2C2A) and E. coli EnvZ (PDB code 1JOY) are shown above the alignment with α-helices depicted as cylinders. Mutations characterized in this study are illustrated below the alignment.
defective in signaling the redox status but remain competent to inhibit NifA in response to fixed nitrogen. Whereas N301A and M338A were totally defective in redox sensing and partially defective in the nitrogen response, R339A retained some response to oxygen (~50%) but remained fully responsive to fixed nitrogen (Fig. 2A). N-terminal histidine tagged derivatives of the mutant proteins were overexpressed and purified and compared with the biochemical properties of wild-type NifL. Spectroscopic measurements revealed that all three mutant proteins had a similar FAD content to that of the wild-type NifL (~3 FAD/mol (12)) (Table 1) with an adsorption maxima at 445 nm that was bleached upon reduction with sodium dithionite (data not shown). These properties suggest that the N301A, M338A, and R339A mutants are not defective in flavin binding or perception of the redox signal but are apparently unable to couple signals sensed by the PAS1 domain to the inhibition of NifA activity.

In the presence of adenosine nucleotides, NifL is competent to inhibit NifA irrespective of the redox state of the PAS1 domain, and indeed truncated forms of NifL lacking the PAS1 domain retain the ability to inhibit NifA when ADP is present (12, 13). To examine whether N301A, M338A, and R339A mutant proteins exhibit this activity, we measured their ability to inhibit the formation of open promoter complexes by NifA. These assays quantify the formation of heparin-resistant RNA polymerase promoter complexes catalyzed by NifA, with GTP as the donor for nucleotide hydrolysis and ADP (50 μM) added as an effector of NifL. Surprisingly, N301A and R339A were more effective than wild type in this assay and exhibited greater inhibition of NifA activity at substoichiometric NifL: NifA ratios. M338A inhibited NifA activity to the same extent as wild-type NifL (Fig. 3A). Thus, the three

### Table 1

Flavin content and association state of mutant NifL proteins

| Protein             | Mol of FAD | Retention volume | Apparent molecular mass | Association state |
|---------------------|------------|------------------|-------------------------|-------------------|
| N6hisNifL           | 2.7        | 11.82            | 208,912                 | Tetramer          |
| N6hisNifL-N301A     | 3.1        | 11.80            | 211,084                 | Tetramer          |
| N6hisNifL-M338A     | 3.8        | 11.71            | 221,141                 | Tetramer          |
| N6hisNifL-R339A     | 2.9        | 11.70            | 222,288                 | Tetramer          |
| N6hisNifL-A288T     | 2.9        | ND               | ND                      | ND                |
| N6hisNifL-L292I     | 3.1        | ND               | ND                      | ND                |
| N6hisNifL-L307A     | 3.1        | ND               | ND                      | ND                |
| N6hisNifL-(147–519) | ND         | 13.13            | 83,428                  | Dimer             |
| N6hisNifL-(147–519)-N301A | ND     | 13.43            | 78,884                  | Dimer             |
| N6hisNifL-(147–519)-M338A | ND   | 13.24            | 86,199                  | Dimer             |
| N6hisNifL-(147–519)-R339A | ND   | 13.30            | 92,019                  | Dimer             |
| N6hisNifL-(147–519)-A288T | ND    | 13.33            | 82,653                  | Dimer             |
| N6hisNifL-(147–519)-L292I | ND   | 13.33            | 82,653                  | Dimer             |

*Expressed as mol of FAD/mol of tetramer using a molar absorption coefficient of 12,250 M⁻¹ cm⁻¹ for NifL-bound FAD (11).

**Gel permeation chromatography on Superdex 200 as described under "Experimental Procedures."

*Calculated from gel permeation chromatography.

*The subunit molecular masses of N6hisNifL and N6hisNifL-(147–519) are 59,991 and 43,514 respectively.
NifL and NifA were 100 and 250 nM, respectively. Data are plotted as the % of open promoter complexes formed by NifA in the absence of NifL.

2-oxoglutarate. Reaction conditions were identical to those for GlnK on the activities of oxidized mutant NifL proteins in the presence of 50 mM 2-oxoglutarate and the indicated concentration of GlnK on the inhibition of NifA (200 nM, dimer) to GAF domain (17). This interaction requires the ligands ATP and ADP. Reactions contained 4 mM GTP as the donor for nucleotide hydrolysis. Data on the extent of NifA activity in the absence of NifL with the concentration of NifL were determined by ITC. For M338A and R339A the calorimetric titration gave a best fit to single site model with a stoichiometry of 1 and a \( \Delta H \) similar to wild-type NifL (−20 to −25 \( \mu \)M) (Table 2).

Table 2: Thermodynamic parameters for ADP binding to NifL and mutant proteins determined by ITC

| Protein          | \( K_D \) (\( \mu \)M) | Stoichiometry | \( \Delta H \) (kJ/mol) |
|------------------|------------------------|---------------|------------------------|
| NifL             | 24.7 ± 2.5             | 0.95 ± 0.07   | −13,870 ± 1311         |
| NifL-N301A       | 10.8 ± 0.8             | 1.01 ± 0.016  | −10,130 ± 210          |
| NifL-M338A       | 23.0 ± 3.3             | 1.0 (fixed)   | −16,130 ± 874          |
| NifL-R339A       | 20.2 ± 0.7             | 0.98 ± 0.007  | −11,280 ± 116          |

Although the data in Fig. 3A imply that the N301A, M338A, and R339A mutants retain the response to ADP, we considered the possibility that mutations in the H domain could influence the affinity of this nucleotide for the GHKL domain. We, therefore, investigated the binding of ADP to the mutant proteins using isothermal ITC. For M338A and R339A the binding constant for ADP was lower than wild-type (−11 \( \mu \)M), perhaps explaining why this mutant form is more effective than NifL in inhibiting NifA in the presence of ADP.

The response of NifA to NifL is controlled by the binding of 2-oxoglutarate to the GAF domain of the activator. The ability of ADP to activate inhibition by the reduced form of NifL is antagonized by the binding of 2-oxoglutarate to the GAF domain of NifA, thus enabling the activator to escape inhibition by NifL under conditions appropriate for nitrogen fixation (8, 9). However, the 2-oxoglutarate-bound form of NifA is susceptible to inhibition by the oxidized form of NifL, thus ensuring that NifL inhibits transcriptional activation by NifA under oxidizing conditions (15). The ability of the oxidized forms of N301A, M338A, and R339A to inhibit NifA in the presence of 2-oxoglutarate was, therefore, examined using open promoter assays. As shown previously, the oxidized form of wild-type NifL inhibited NifA irrespective of the 2-oxoglutarate concentration. In contrast, the mutant proteins were defective in inhibiting the 2-oxoglutarate-bound form of NifA, with R339A exhibiting the most activity, and N301A exhibiting the least (Fig. 3B). Interestingly, the response of the mutant proteins in this assay parallels the response of the mutants in vivo (Fig. 2) as NifL-R339A retains some response to oxygen in vivo, whereas N301A does not.

Because the redox-insensitive mutants are responsive to nitrogen regulation in vivo, we anticipated that they might inhibit NifA activity in the presence of the non-modified form of the signal transduction protein GlnK, which conveys the nitrogen signal through direct interaction with the GHKL domain (17). This interaction requires the ligands ATP and 2-oxoglutarate, which bind to GlnK. The binding of GlnK to the GHKL domain enables the formation of a GlnK:NifL:NifA ternary complex that overrides the influence of 2-oxoglutarate on NifA. Because the oxidized form of wild-type NifL is competent to inhibit NifA in the presence of 2-oxoglutarate, no additional response is observed when GlnK is also added. However, because the oxidized forms of the N301A, M338A, and R339A mutants are defective in inhibition of NifA in the presence of...
H Domain of NifL

2-oxoglutarate, it was possible to assess the influence of GlkK on their activity. All three NifL mutants inhibited NifA at GlkK concentrations above 0.25 μM (Fig. 3C), demonstrating that the mutant proteins are responsive to nitrogen signaling in vitro, as expected from their in vivo phenotypes.

Because the H domain of the histidine protein kinases is likely to act as a dimerization interface, it is possible that the phenotype of the redox-insensitive mutants could arise from an alteration in oligomerization state. Both wild-type NifL and the isolated PAS1 domain sieve as tetramers on gel permeation chromatography, suggesting that PAS1 contains oligomerization determinants (12, 28). However, a truncated form of NifL lacking the PAS1 domain (residues 147–519) sieves as a dimer, whereas the isolated GHKL domain is monomeric, in accord with the proposed role of the H domain as a dimerization interface (13). We constructed mutant derivatives of Nhis6NifL—N301A, M338A, and R339A proteins revealed that the C-terminal transmitter region of the mutant proteins was cleaved more rapidly than wild-type NifL, but the rate of appearance and stability of the N-terminal PAS1 and PAS2 domains (band B) was similar to that of the wild-type (Fig. 4A). Densitometric analysis of the gels confirmed that these mutant proteins were more rapidly cleaved by trypsin than wild-type NifL (Fig. 4B). These observations suggest that the conformation of the transmitter region of these mutant proteins may be different to that of wild-type NifL as this region is more susceptible to trypsin proteolysis.

Mutants Defective in Both Redox and Fixed Nitrogen Signaling—Three mutants, A288T, L292I, and L307A, that were substantially deficient in the response to both oxygen and fixed nitrogen and failed to inhibit NifA in vivo were chosen for further biochemical analysis. As in the case of the redox signaling mutants, all three mutant proteins retained a normal complement of FAD (~3 FAD/mol) (Table 1), suggesting that the observed signaling defects cannot be attributed to the loss of this co-factor. Also, truncated derivatives of the mutant proteins, lacking the PAS1 domain, had identical elution profiles to Nhis6NifL-(147–519) on gel permeation chromatography (Table 1), implying that these mutations do not alter the oligomerization state. Limited trypsin proteolysis was used to probe for conformational changes in the A288T, L307A, and L292I proteins. We observed no difference in the rate of trypsin digestion compared with wild-type NifL (Fig. 4, C and D), implying that unlike the N301A, M338A, and R339 mutations, A288T, L307A, and L292I do not induce changes in conformation detectable by this assay.

To examine whether the A288T, L292I, and L307A mutant proteins retain the capacity to interact with NifA, we used a pulldown assay with histidine-tagged forms of NifL to detect the co-retention of NifA with NifL on NTA magnetic agarose beads. In these experiments we also compared the binding of NifA to the redox signaling mutants, N301A, M338A, and R339A (Fig. 5A). The amount of NifA retained with NifL after washing and elution from the beads was determined by densitometry of the protein bands visualized by SDS-PAGE (Fig. 5B). We have shown previously that the presence of adenosine nucleotides increases the stability of the NifL-NifA binary complex (14, 15). Accordingly, co-retention of NifA with wild-type NifL was stimulated by the inclusion of 50 μM ADP in the buffers (data not shown). All the mutant proteins retained the capacity to bind NifA, although complexes formed with the L292I, L307A, and A288T were notably less stable than those

![FIGURE 4. Limited trypsin proteolysis of NifL proteins.](image)
formed by the redox signaling mutants with L292I being the least stable (Fig. 5B).

To determine whether the L292I, L307A, and A288T proteins retained the ability to inhibit NifA, we performed open promoter complex assays in the presence of 50 μM ADP. The A288T and L307A proteins inhibited NifA activity under these conditions, although they were less competent than wild-type at protein concentrations lower than 100 nM (Fig. 6A). In contrast, L292I was substantially defective in inhibiting NifA activity in this assay (Fig. 6A).

We repeated the open complex assays at inhibitory concentrations of the oxidized forms of L307A and A288T proteins (150 nM) to determine whether these mutant proteins retain the ability to inhibit NifA activity in the presence of 2-oxoglutarate. In contrast to wild-type NifL, the addition of 2-oxoglutarate resulted in relief of NifL inhibition, and almost all NifA activity was recovered at 2-oxoglutarate concentrations above 500 μM (Fig. 6B). It should be noted that the relief of inhibition in response to 2-oxoglutarate with the A288T and L307A mutants was greater than that observed with the redox signaling mutants (Fig. 3B), commensurate with the higher level of NifA activity observed in vivo in the presence of A288T and L307A compared with N301A, M338A, and R339A (Fig. 2A). We titrated GlnK into reactions containing 2 mM 2-oxoglutarate to determine whether GlnK could activate the inhibitory function of the A288T, L307A, and L292I proteins. The A288T and L307A mutants were responsive to the addition of GlnK in this assay (Fig. 6C), but higher GlnK concentrations were required in comparison with N301A, M338A, and R339A (note that the NifL concentration in Fig. 6C was 150 nM compared with 100 nM in Fig. 3C). Complete inhibition of NifA activity was only
observed at GlnK concentrations above 0.5 μM. In contrast, L292I showed only a weak response to GlnK under these conditions (Fig. 6C), commensurate with the severe defect in NifA inhibition exhibited by this mutant protein. The decreased response of A288T and L307A mutations to GlnK could result either from a change in affinity in the interaction of the GHKL domain with GlnK or in a decreased ability to transmit the signal acquired by the GlnK-GHKL interaction. To investigate these possibilities we utilized Biacore surface plasmon resonance instrumentation to determine the affinity of GlnK for the mutant proteins. Sensorgrams obtained at ligand concentrations identical to those used in the open promoter complex assays with increasing concentrations of GlnK revealed that A288T, L307A, and L292I had similar affinities for GlnK to that of wild-type NifL (K_{D} ≈ 5 μM), and the association and dissociation rates were also similar (Fig. 7). Hence, the defects in the GlnK response are likely to be due to decreased ability to transmit the nitrogen signal rather than decreased affinity for GlnK itself.

**DISCUSSION**

The data reported here demonstrate that the H domain plays a crucial role in transmission of signals perceived by the PAS1 and GHKL domains of NifL. It is likely that the H domain provides not only a contact surface for interaction with NifA but is also a nexus for signal-dependent interdomain interactions. The two phenotypic classes of mutants we have isolated identify residues that are involved in transmission of the redox signal perceived by the PAS1 domain, whereas other residues influence inhibition of NifA activity irrespective of the environmental signal. With the exception of L292I, the mutant proteins characterized *in vitro* were not substantially defective in their interaction with NifA, at least when 2-oxoglutarate is absent, since the majority retained some capacity to bind and inhibit NifA activity in the presence of ADP. The ability of NifL to inhibit NifA in the absence of 2-oxoglutarate is a signal-independent function of NifL that does not require either the PAS1 domain or interaction with GlnK (13, 16). This function may involve a contact surface for NifA on the H domain, and it also requires the binding of adenosine nucleotide to the GHKL domain, which may alter H-GHKL interactions (20). It is possible, for example, that the L292I mutation prevents inhibition of NifA either directly, via a change in a NifA contact surface, or indirectly, by altering interactions between the H and GHKL domains.

All of the mutants characterized *in vitro* exhibited defects in their ability to inhibit NifA activity in the presence of 2-oxoglutarate. In the case of the three mutants that were primarily defective in redox rather than fixed nitrogen signaling (N301A, M338A, and R339A), this defect was nevertheless overcome by the addition of the GlnK signal transduction protein *in vitro*, explaining why the mutant proteins retain the ability to respond to the availability of fixed nitrogen *in vivo*. We have proposed that conformational changes elicited either by the oxidation of the FAD moiety in the PAS1 domain or by interaction of GlnK with the GHKL domain are required for NifL to inhibit the 2-oxoglutarate-bound form of NifA. We have previously suggested that the H domain is involved in relaying these conformational changes (20), and this is further suggested by the properties of the mutations analyzed in this study. The N301A, M338A, and R339A mutants appear to have an altered conformation, and our limited proteolysis studies suggest that this may influence the structure of the C-terminal transmitter region. We infer that this prevents communication of the redox signal from PAS1 to the transmitter region so that the oxidized forms of these proteins are defective in inhibiting the 2-oxoglutarate-bound form of NifA. In contrast, the A288T, L307A, and L292I mutations did not exhibit any differences in sensitivity to proteolysis, suggesting that these mutations do not promote significant changes in conformation. It is, therefore, possible that phenotypes of these mutations result from defects in protein-protein interactions rather than conformational changes.

The homology between the H domain and the Dhp (HisKA) domains of the histidine protein kinases (particularly with...
respects to the “H Box”) and the similarity in predicted secondary structure imply that the structure of the NifL H domain comprises two antiparallel helices that interact in the dimer to form a four helix bundle (24, 25). Site-directed mutagenesis studies of the HisKA domain reveal that mutations impair either autokinase or phosphatase activity (29–32). Cysteine scanning mutagenesis of the isolated HisKA domain of EnvZ identified two regions in the α helices that specifically are involved in phosphatase activity (32). Two of the NifL mutants defective in redox sensing, M338A and M339A, are located in the putative second helix of the H domain, referred to as the X-region in the HPks (31, 33, 34). Interestingly, mutations in this region of EnvZ, like those in NifL, influence protein conformation (31). Assuming that the H domain of NifL does constitute a helix bundle formed by dimerization of subunits, the mutations we have identified here do not appear to perturb the core structure. Truncated derivatives of the mutant proteins lacking the PAS1 domain behaved identically to equivalent forms of wild-type NifL on gel permeation chromatography and sieved as dimers. Likewise, full-length mutant proteins behaved like the wild-type and sieved as tetramers, indicating that the mutations do not alter the association state of NifL.

The recently determined structure of the complete cytoplasmic region of the HK853 HPk from T. maritima reveals a substantial interface between the HisKA and GHKL domains that is buried from solvent exposure. However, because the ATP binding site in the GHKL domain and the phosphoacceptor site are far apart in this structure, it is proposed that the HisKA-GHKL interface must be destabilized to allow the GHKL domain to swing around to perform the trans-histidine phosphorylation (25). An analogous model can be postulated for NifL, in which signals induce movement of the GHKL domain to allow NiFA to approach the H domain. Our previous studies demonstrating that the Arg-306 in the H domain is critical for signal transduction and that mutations in this residue lock NifL in the “on state” is consistent with such a model since this arginine residue may play a crucial role in controlling GHKL-H domain interactions (20). It is likely that some of the H domain mutations characterized in this study also influence GHKL-H domain interactions, thus preventing access of NifL to the 2-oxoglutarate-bound form of NiFA.

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