

# A crucial arginine residue is required for a conformational switch in NifL to regulate nitrogen fixation in *Azotobacter vinelandii*

Isabel Martinez-Argudo\*, Richard Little, and Ray Dixon†

Department of Molecular Microbiology, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

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NifL is an antiactivator that tightly regulates transcription of genes required for nitrogen fixation in *Azotobacter vinelandii* by controlling the activity of its partner protein NifA, a member of the family of  $\sigma^{54}$ -dependent transcriptional activators. Although the C-terminal region of *A. vinelandii* NifL shows homology to the transmitter domains of histidine protein kinases, signal transduction between NifL and NifA is conveyed by means of protein–protein interactions rather than by phosphorylation. Binding of the ligand 2-oxoglutarate to NifA plays a crucial role in preventing inhibition by NifL under conditions appropriate for nitrogen fixation. We have used a suppressor screen to identify a critical arginine residue (R306) in NifL that is required to release NifA from inhibition under appropriate environmental conditions. Amino acid substitutions at position 306 result in constitutive inhibition of NifA activity by NifL, thus preventing nitrogen fixation. Biochemical studies with one of the mutant proteins demonstrate that the substitution alters the conformation of NifL significantly and prevents the response of NifA to 2-oxoglutarate. We propose that arginine 306 is critical for the propagation of signals perceived by *A. vinelandii* NifL in response to the redox and fixed-nitrogen status and is required for a conformational switch that inactivates the inhibitory function of NifL under conditions appropriate for nitrogen fixation.

2-oxoglutarate | signal transduction | antiactivator | redox control | nitrogen regulation

Structural rearrangements of sensor proteins in response to environmental cues provide a fundamental mechanism for signal propagation within cells. However, although conformational changes have been well characterized in isolated signaling domains, mechanisms for signal transmission by means of inter-domain interactions are frequently less well understood. For example, structural studies have identified ligand-induced conformational changes in the sensor domains of histidine protein kinases (HPKs), but it is not known how these changes are communicated to the kinase domain to control phosphoryl transfer.

The *Azotobacter vinelandii* NifL regulatory protein is a histidine kinase-like protein that controls the expression of the genes required for nitrogen fixation in response to the redox, nitrogen, and carbon status. NifL is an antiactivator that tightly regulates the activity of its partner protein NifA, a member of the family of  $\sigma^{54}$ -transcriptional activators (1, 2), by means of the formation of an inhibitory complex (3–5). The domain architecture of NifL is similar to that of some HPKs, with an N-terminal Per–Arnt–Sim (PAS) domain (6, 7) containing a flavin adenine dinucleotide (FAD) cofactor that senses the redox status (8, 9) and a C-terminal domain containing conserved residues corresponding to the N, G1, F, and G2 boxes that constitute the ATP-binding domain of the GHKL superfamily of ATPases (10–14) (Fig. 1). Unlike the HPKs, the GHKL domain of NifL does not exhibit ATP hydrolysis or transphosphorylation activity, but the binding of ADP to this domain strongly stimulates the inhibitory activity of NifL and the stability of the NifL–NifA complex (8,

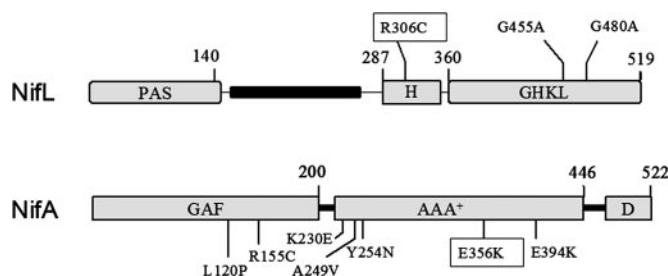


Fig. 1. Domain structure of *A. vinelandii* NifL and NifA showing the mutations analyzed in this study.

15, 16). Also, the GHKL domain is involved in sensing the nitrogen status because it is the site of interaction with the signal transduction protein GlnK (17–19), the PII-like protein of *A. vinelandii* (20–23).

Most HPKs contain an H-box motif located in a dimerization domain (also known as domain A) that contains the autophosphorylation site and interacts with the corresponding response regulator (24, 25). Although NifL contains an H-box motif (Fig. 1), the conserved histidine residue is not required for regulation of NifA activity (26) and neither autophosphorylation of NifL nor phosphotransfer to NifA has been detected (27, 28). These observations, together with the fact that NifA is not a member of the response-regulator family, suggest that NifL is not a bona fide HPK.

Whereas signals of the redox and fixed-nitrogen status are both perceived by NifL, the NifA protein is responsive to allosteric control by 2-oxoglutarate, a metabolic signal of the carbon status, which binds to the N-terminal GAF domain of this activator. Binding of 2-oxoglutarate to NifA antagonizes the influence of adenosine nucleotides on the NifL–NifA interaction, thus enabling NifA to escape from inhibition by NifL, under nitrogen-fixing conditions (29, 30). Our current model for activation of NifL implies that conformation changes caused either by changes in oxidation state of the PAS domain or the binding of GlnK to the GHKL domain are transmitted to the C-terminal region to enable NifL to inhibit NifA in the presence of 2-oxoglutarate. To identify residues that are involved in signal transmission, we have screened for NifL suppressor mutations that inhibit constitutive (NifL-resistant) forms of NifA. We have identified an arginine residue (R306) in the H motif of NifL that appears to be crucial for signal transmission because substitutions at this position prevent transcriptional activation by NifA

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Abbreviations: HPK, histidine protein kinase; PAS, Per–Arnt–Sim.

\*Present address: Department of Biochemistry, University of Bristol, Bristol BS8 1TD, United Kingdom.

†To whom correspondence should be addressed. E-mail: ray.dixon@bbsrc.ac.uk.

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under nitrogen-fixing conditions. We observe that the R306C mutation alters the conformation of NifL and that the mutant protein is competent to inhibit NifA in the presence of 2-oxoglutarate, thus preventing nitrogen fixation. The mutation also suppresses substitutions in conserved residues in the GHKL domain, suggesting that interactions between the nucleotide-binding domain and the H-motif region of NifL are important for signal transduction.

## Methods

**Mutagenesis and Isolation of Suppressor Mutations.** PCR mutagenesis was carried out with *Taq*DNA polymerase by using standard reaction conditions. Reaction mixtures contained 10 ng of template plasmid pPMA (31) (encoding NifL and NifA-E356K), 40 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate, 2 mM MgCl<sub>2</sub>, and 5 units of enzyme. Primers NifL1 (5'-CCGCCGCAAGGACAAGACC-3') and P240 (5'-CCTTGCCGGTACCGGACTC-3') were used to mutate the central and C-terminal domains of NifL. PCR products were purified, digested with *Mlu*I and *Mun*I, and recloned into plasmid pPMA digested with the same enzymes. To identify *nifL* mutants that are able to suppress the effect of the *nifA-E356K* mutation (which renders NifA insensitive to NifL; ref. 31), ligation mixtures were electroporated into strain DH5 $\alpha$ . The transformation mixture was incubated for 16 h, and plasmid DNA was then extracted and used to transform strain ET8000 containing the reporter plasmid pRT22, which carries a *nifH-lacZ* fusion (32). Transformants were selected on NFD medium containing 1 mg/ml (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as nitrogen source, 20  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside, 30  $\mu$ g/ml chloramphenicol, and 50  $\mu$ g/ml ampicillin, and incubated aerobically. Mutants that restored inhibition by NifL were identified as white or light-blue colonies able to suppress the resistant phenotype of *nifA-E356K*. Plasmid DNA was recovered from selected colonies and reintroduced into the host strain to recheck phenotypes. DNA from selected mutants was isolated and sequenced to identify the mutations.

Site-directed mutagenesis of *nifL* was done by two-step PCR. The first step consisted of two PCRs, one of which was carried out with primer MS1 (5'-GGGAAAACCTCGCCGCC-3') and a reverse primer containing the desired mutation, and the other of which was carried out with a forward primer containing the desired mutation and primer L2 (5'-GTCGCTGTTTCAGGTGGAGG-3'). PCR products were purified and used as a template for the second-step PCR, using the primers MS1 and L2 described above. The resulting fragment was cut with *Nor*I and *Apa*I and cloned into plasmid pPR34 encoding wild-type NifL and NifA (32).

**A. *vinelandii* Transformation and Analysis of Transformants.** Mutations were introduced into the *A. vinelandii* genome by using plasmid pIM32, which contains a 1-kb region upstream of *nifL*, a spectinomycin-resistance cassette in the *Sma*I site upstream of the *nifL* promoter (33), and wild-type *nifL* and *nifA* sequences. This plasmid also contains an engineered *Eco*RI site close to the *Sma*I site within *nifL* to facilitate identification of recombinants by PCR. *nifL* and *nifA* mutations were cloned into pIM32, and the resultant plasmids were linearized and then transformed into competent *A. vinelandii*, as described (34).

**The  $\beta$ -Galactosidase Assays.** *In vivo* activity of NifL and NifA was measured in *Escherichia coli* strain ET8000 by using the reporter plasmid pRT22, which carries a *nifH-lacZ* translational fusion. NifL, NifA, and their mutant forms were expressed on a second plasmid from a constitutive promoter (32). The  $\beta$ -galactosidase assays were carried out as described (29, 35)

**Protein Expression and Purification.** Plasmids pTJ45, pDB737, and pIM15 were used for overexpression of N<sub>his6</sub>NifL, NifA, and NifA-E356K (28–30). For overexpression of N<sub>his6</sub>NifL-R306C, an *Nde*I-*Bam*HI fragment encoding the corresponding mutation was cloned into plasmid pET28, resulting in plasmid pIM22. Plasmids pIM75 and pIM74, which overexpresses N<sub>his6</sub>NifL (147–519) and N<sub>his6</sub>NifL-R306C (147–519), were made by PCR amplification using primers NifL-147 (5'-GGGAATTC-CATATGAACAACCAGCGCTGATGATCG-3') and NifL2 (5'-CGAAGGATCCTCAGGTGGAGGCCGAGAAGGG-3'). After digestion with *Nde*I and *Bam*HI, the fragments were cloned into plasmid pET28. In all cases, protein overexpression was carried out in *E. coli* strain BL21(DE3) pLysS. Cultures were grown aerobically in Luria-Bertani broth, and expression from the T7 promoter was induced by addition of isopropyl  $\beta$ -D-thiogalactopyranoside to 1 mM. Proteins were purified as described (30, 31).

**Open-Promoter Complex Assays.** NifA-promoted catalysis of open-promoter complexes by  $\sigma^{54}$ -RNA polymerase was used to assay NifA activity and its inhibition by NifL as described (16, 18).

**Limited Trypsin Proteolysis.** Trypsin proteolysis was performed in TA buffer (50 mM Tris·acetate, pH 7.9/100 mM potassium acetate/8 mM magnesium acetate/1 mM DTT) at room temperature. A trypsin/NifL weight ratio of 1:100 was used. The proteins were incubated with nucleotides for 5 min before digestion was started. Samples of 12  $\mu$ l were removed at indicated time intervals to tubes on ice containing 12  $\mu$ l of Laemmli loading buffer, and samples were heated at 100°C for 5 min

**Table 1. Inhibition of NifA activity by NifL-R306C *in vivo***

Plasmid*	Proteins	$\beta$ -Galactosidase activity, Miller units			
		Anaerobic		Aerobic	
		–N <sup>†</sup>	+N <sup>‡</sup>	–N	+N
pPR34	NifL and NifA	3,138	31	245	75
pPMA	NifL and NifA-E356K	28,918	30,130	35,538	30,957
pIM17	NifL-R306C and NifA-E356K	859	52	290	199
pIM18	NifL-R306C and NifA	113	25	122	81
pPR54	NifL (147–519) and NifA	2305	49	2,522	471
pIM27	NifL-R306C (147–519) and NifA	116	19	119	118

\*In all cases, strain ET8000 (*rhs lacZ::IS1 gyrA hutC<sub>4</sub>*) contained the *nifH-lacZ* reporter plasmid pRT22 and the indicated plasmid expressing the listed Nif regulatory proteins.

<sup>†</sup>Cultures were grown in NFD medium (see text) with casein hydrolysate (200  $\mu$ g/ml) as nitrogen source (–N).

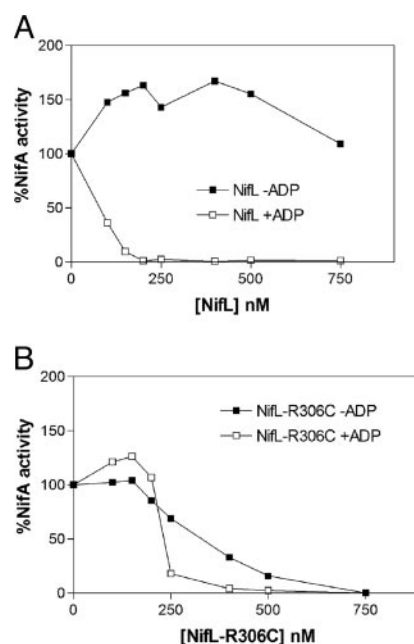
<sup>‡</sup>Cultures were grown in NFD medium (see text) with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1mg/ml) as nitrogen source (+N).

before electrophoresis in 4–12% gradient gels using MES buffer (50 mM MES/50 mM Tris/0.1% SDS/1 mM EDTA).

## Results

**Isolation of a *nifL* Mutation That Suppresses the Resistant Allele *nifA-E356K*.** The surfaces of *A. vinelandii* NifL that are required for the interaction with NifA are not well defined. Previous experiments have established that the N-terminal region of NifL is not essential for the interaction and, because the isolated C-terminal nucleotide-binding domain is not competent to bind NifA, a candidate region for the interaction is the central region located between residues 287 and 360 that contains the H-box motif (Fig. 1) (15, 32, 36). In an attempt to identify the NifL residues that are important for the NifA interaction, we used suppression mutagenesis, taking advantage of a well characterized mutant form of NifA that is resistant to inhibition by NifL (29, 31). We anticipated that the resistance phenotype of a NifA mutant unable to interact with NifL can be suppressed by a second-site suppressor mutation in *nifL*. The NifL-resistant mutant NifA-E356K (Fig. 1) was chosen to seek suppressor mutations because position 356 is conserved in NifA proteins but not in other members of the  $\sigma^{54}$ -dependent activator family. To carry out the screening, we used a previously described two-plasmid system in *E. coli*, consisting of a *nifHp-lacZ* reporter and a second plasmid expressing *nifL* and *nifA* from a constitutive promoter (29, 31, 32, 35). After random PCR mutagenesis of the region encoding the central and C-terminal domains of NifL and introduction of the mutant library into the *nifA-E356K* background, we screened for suppressors on 20  $\mu\text{g}/\text{ml}$  5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside indicator plates containing excess fixed nitrogen under aerobic conditions. Colonies expressing *nifL* and the resistant allele *nifA-E356K* are blue under these conditions, and we anticipated that suppressors would give rise to white or light-blue colonies. Only one suppressor with the appropriate phenotype that expressed wild-type levels of NifL and NifA survived the screen. This mutation generated an arginine-to-cysteine substitution at position 306 in the central H-box motif of NifL (Fig. 1), adjacent to the conserved histidine residue at position 305 that is autophosphorylated in bona fide HPKs.

**NifL-R306C Inhibits NifA Under Nitrogen-Fixing Conditions.** We have previously demonstrated that the activity of the *A. vinelandii* NifL-NifA system is regulated in response to nitrogen and oxygen status in *E. coli*. However, the activity of NifA-E356K is constitutive and insensitive to NifL (Table 1, compare first and second rows). NifA-E356K exhibits considerably higher activity than wild-type NifA under anaerobic nitrogen-limiting conditions because NifL retains some inhibitory activity under these conditions (31, 35). The *nifL-R306C* mutation effectively suppressed the constitutive activity of *nifA-E356K*, although a low level of NifA activity was detectable under anaerobic, nitrogen-limiting conditions. (Table 1, row 3). To determine whether *nifL-R306C* is an allele-specific suppressor of *nifA-E356K*, the *nifL-R306C* mutation was combined with wild-type



**Fig. 2.** Response of NifA to oxidized  $N_{\text{his6}}$ NifL and  $N_{\text{his6}}$ NifL-R306C. NifA activity was measured by the formation of open-promoter complexes, as described in *Methods*, and plotted relative to the extent of NifA activity in the absence of NifL. Each data point is the mean of at least two independent experiments. All assays contained 4 mM GTP as hydrolyzable nucleotide and 250 nM NifA (calculated as a dimer). (A) Response of NifA to the indicated concentration of  $N_{\text{his6}}$ NifL (calculated as a tetramer) in the absence (■) or presence (□) of 0.05 mM ADP. (B) Response of NifA to the indicated concentration of  $N_{\text{his6}}$ NifL-R306C (calculated as a tetramer) in the absence (■) or presence (□) of 0.05 mM ADP.

*nifA*. Surprisingly, NifA activity was also inhibited by NifL-R306C under conditions appropriate for nitrogen fixation (Table 1, row 4). Hence, *nifL-R306C* is not an allele-specific suppressor of *nifA-E356K*, and it has the ability to inhibit transcriptional activation by both mutant and wild-type forms of NifA. When introduced into *A. vinelandii*, the *nifL-R306C* mutant had a  $\text{Nif}^-$  phenotype and was unable to fix nitrogen on nitrogen-free medium containing molybdenum (see Fig. 6, which is published as supporting information on the PNAS web site). Therefore, the presence of the R306C substitution confers on NifL the ability to inhibit NifA under nitrogen-fixing conditions.

To determine whether the inhibitory effect of NifL-R306C requires the N-terminal PAS domain of NifL, we introduced the *nifL-R306C* mutation into a truncated version of NifL, NifL (147–519), that does not exhibit the redox response but is competent to inhibit NifA activity in response to the fixed-nitrogen status in *E. coli* (Table 1, row 5) (32). As in the case of NifL-R306C, the truncated mutant protein, NifL-R306C (147–519), was able to inhibit NifA activity under all of the conditions tested (Table 1, row

**Table 2. Suppression of mutations in the nucleotide-binding domain of NifL by NifL-R306C**

Plasmid	Proteins	$\beta$ -Galactosidase activity, Miller units			
		Anaerobic		Aerobic	
		–N	+N	–N	+N
pNLG455	NifL-G455A and NifA	19,623	11,441	31,054	14,491
pIM23	NifL-R306C, G455A, and NifA	1,619	17	486	106
pNLG480	NifL-G480A and NifA	25,538	20,165	31,335	12,977
pIM25	NifL-R306C, G480A, and NifA	6,315	18	1,417	28

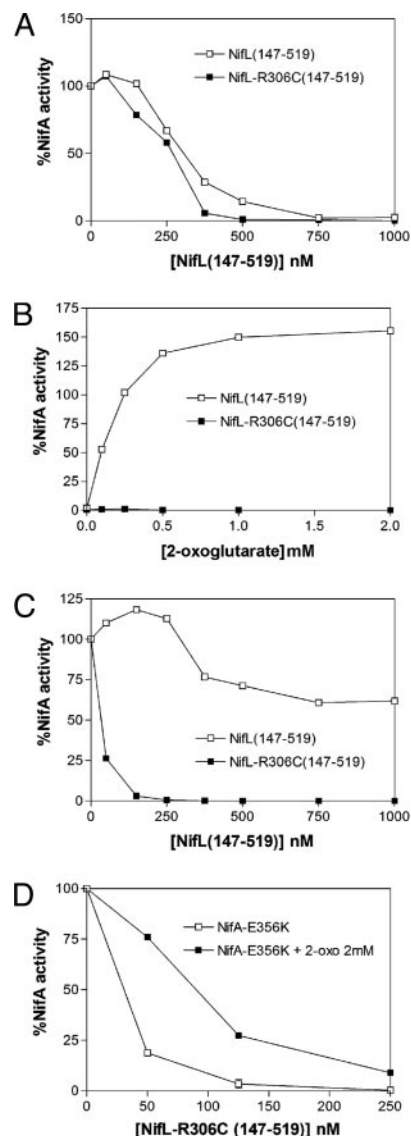
Assay conditions were identical to those shown in Table 1.

6). Hence, the N-terminal PAS domain of NifL is not required for constitutive inhibition of NifA by the NifL-R306C substitution. We also tested whether inhibition of NifA activity by the mutant NifL protein was influenced by the PII-like signal transduction proteins, by introducing appropriate plasmids into a *glnB*, *ntnC* background (*E. coli* strain RT8000) that does not express either PII or GlnK (35). Unlike wild-type NifL, NifL-R306C was competent to inhibit NifA activity under all conditions in this background (data not shown) implying that the PII-regulatory proteins are not required for inhibition by NifL-R306C.

**NifL-R306C Inhibits NifA Activity in the Absence of ADP.** To investigate the characteristics of the *nifL-R306C* mutation further, we purified the mutant protein as an N-terminal hexahistidine fusion and analyzed its *in vitro* properties. The ability of NifL to inhibit NifA was determined by measuring the formation of open-promoter complexes by NifA at the *nifH* promoter, in the presence of  $\sigma^{54}$ -RNA polymerase and integration host factor (IHF), with GTP as substrate for nucleotide hydrolysis by NifA. Gel-retardation assays were used to quantitate the formation of heparin-stable nucleoprotein complexes (16). The presence of adenosine nucleotides, particularly ADP, stabilizes complexes between NifL and NifA and increases the inhibitory activity of NifL (15, 16). Hence, wild-type  $N_{\text{his6}}$ NifL inhibits NifA in the presence of ADP, whereas in the absence of this nucleotide, NifA is resistant to  $N_{\text{his6}}$ NifL (Fig. 2A). By contrast,  $N_{\text{his6}}$ NifL-R306C was able to inhibit NifA in the absence of ADP, although its inhibitory influence was stimulated by ADP (Fig. 2B).

To determine whether the integrity of the nucleotide-binding (GHKL) domain is essential for NifL-R306C to inhibit NifA activity under nitrogen-fixing conditions, we used two substitutions of conserved residues in the GHKL domain of NifL (NifL-G455A and NifL-G480A) that reduce the binding of ADP substantially and, consequently, severely impair inhibition of NifA activity by NifL *in vivo* (S. Perry, N. Shearer, R. L., and R. D., unpublished data). Interestingly, the *nifL-R306C* mutation suppressed the regulatory defects exhibited by the mutations in the nucleotide-binding domain and, in particular, restored strong inhibition of NifA activity in response to the fixed-nitrogen status *in vivo* (Table 2). However, the double mutants apparently lost the ability conferred by NifL-R306C to inhibit NifA fully under nitrogen-fixing conditions (compare Tables 1 and 2). These results support the observation that ADP is not essential for NifL-R306C to inhibit NifA *in vitro*. They also suggest that the integrity of the NifL nucleotide-binding domain is more important for inhibition of NifA by NifL-R306C under nitrogen-limiting conditions.

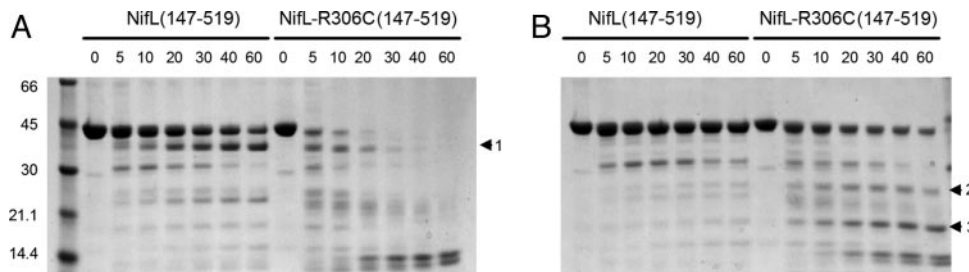
**NifL R306C Overrides the Effect of 2-Oxoglutarate on NifA Activity.** Binding of 2-oxoglutarate to the GAF domain of NifA relieves inhibition by the reduced ADP-bound form of NifL, enabling NifA to escape from inhibition by NifL under conditions appropriate for nitrogen fixation (29, 30). However, the allosteric effect of 2-oxoglutarate on NifA activity is overridden under oxidizing or excess fixed-nitrogen conditions, ensuring that NifA is inhibited under adverse environmental conditions. We considered the possibility that the *nifL-R306C* mutation locks NifL in a form that prevents the response of NifA to 2-oxoglutarate under conditions appropriate for nitrogen fixation. Because oxidized NifL overrides the effect of 2-oxoglutarate on NifA, we made use of the truncated version of NifL, NifL (147–519), which lacks the PAS domain and does not exhibit a redox response (32). Recall that *nifL-R306C* is competent to inhibit NifA under all conditions in the absence of the PAS domain *in vivo* (Table 1). In the presence of ADP, NifL (147–519) inhibits NifA, but this inhibition is relieved by the binding of 2-oxoglutarate to the GAF domain of NifA (19, 29, 30). As anticipated,  $N_{\text{his6}}$ NifL (147–519) and  $N_{\text{his6}}$ NifL-R306C (147–519) were both effective in inhibiting open-promoter complex formation by NifA in the presence of ADP (Fig. 3A). However, whereas



**Fig. 3.** Response of NifA proteins to  $N_{\text{his6}}$ NifL (147–519) and  $N_{\text{his6}}$ NifL-R306C (147–519). NifA activity was measured by the formation of open-promoter complexes and plotted relative to the extent of NifA activity in the absence of NifL. Each data point is the mean of at least two independent experiments. All assays contained 4 mM GTP as hydrolyzable nucleotide, 0.05 mM ADP, and 250 nM NifA (calculated as a dimer). (A) Response of NifA to the indicated concentration of  $N_{\text{his6}}$ NifL (147–519) and  $N_{\text{his6}}$ NifL-R306C (147–519) (calculated as a dimer). (B) Response of NifA to 2-oxoglutarate. NifA activity is plotted relative to the extent of NifA activity in the absence of NifL and 2-oxoglutarate. Reactions contained 750 nM  $N_{\text{his6}}$ NifL (147–519) (□) or 500 nM  $N_{\text{his6}}$ NifL-R306C (147–519) (■), and the concentration of 2-oxoglutarate is indicated on the x axis. (C) Response of NifA-E356K to the indicated concentration of  $N_{\text{his6}}$ NifL (147–519) and  $N_{\text{his6}}$ NifL-R306C (147–519) (calculated as a dimer). (D) Response of NifA-E356K to the indicated  $N_{\text{his6}}$ NifL-R306C (147–519) concentration in the absence (□) or presence (■) of 2 mM 2-oxoglutarate.

inhibition of NifA activity by  $N_{\text{his6}}$ NifL (147–519) was alleviated in response to 2-oxoglutarate as demonstrated previously, NifA activity was not responsive to 2-oxoglutarate in the presence of  $N_{\text{his6}}$ NifL-R306C (147–519), and inhibition was observed irrespective of the concentration of this ligand (Fig. 3B). Therefore, it seems likely that the ability of NifL-R306C to inhibit NifA *in vivo* under nitrogen-fixing conditions is due to its ability to override the influence of 2-oxoglutarate on NifA activity.

As anticipated from the *in vivo* suppression data,  $N_{\text{his6}}$ NifL-



**Fig. 4.** Limited trypsin proteolysis of  $N_{\text{his6}}\text{NifL}$  (147–519) and  $N_{\text{his6}}\text{NifL-R306C}$  (147–519) in the absence (A) or presence (B) of 2 mM ADP. We incubated 2  $\mu\text{M}$  protein with trypsin (weight ratio, 100:1) for the times (in min) indicated above each lane. Arrowheads indicate the proteolysis products described in the text.

R306C (147–519) was competent to inhibit NifA-E356K activity *in vitro* in the presence of ADP, in contrast to  $N_{\text{his6}}\text{NifL}$  (147–519), which was ineffective (Fig. 3C). NifA-E356K was apparently more sensitive to  $N_{\text{his6}}\text{NifL-R306C}$  (147–519) than wild-type NifA at equivalent protein concentrations (compare Fig. 3A and C). We were also interested in determining whether the NifA-E356K mutant was able to respond to 2-oxoglutarate in the presence of NifL-R306C. We observed that inhibition of NifA-E356K activity was responsive to 2-oxoglutarate at low  $N_{\text{his6}}\text{NifL-R306C}$  (147–519) concentrations (Fig. 3D), in contrast to wild-type NifA (Fig. 3B), which exhibited no response to this ligand, irrespective of the  $N_{\text{his6}}\text{NifL-R306C}$  (147–519) concentration (data not shown). These differences may account for the *in vivo* properties of the *nifL-R306C*, *nifA-E356K* double mutant, which retains some NifA activity under anaerobic nitrogen-limiting conditions in *E. coli* (Table 1, second and third rows). This property is apparent also in *A. vinelandii* because, in contrast to the single *nifL-R306C* mutant, which is unable to fix nitrogen, the *nifL-R306C*, *nifA-E356K* double mutant is Nif<sup>+</sup> (Fig. 6).

**NifL-R306C Exhibits a Conformational Change.** Because NifL-R306C is not an allele-specific suppressor of NifA-E356K and is a potent inhibitor of wild-type NifA, we rationalized that this NifL mutation may induce a conformational change that locks NifL in a form that is competent to inhibit wild-type NifA even in the presence of 2-oxoglutarate. Limited trypsin proteolysis was used to probe the conformations of  $N_{\text{his6}}\text{NifL}$  (147–519) and  $N_{\text{his6}}\text{NifL-R306C}$  (147–519). Binding of ADP to NifL protects the C-terminal domain from proteolytic digestion (32). In the absence of ADP,  $N_{\text{his6}}\text{NifL}$  (147–519) exhibited a major cleavage product (marked 1 in Fig. 4A) that results from trypsin cleavage in the C terminus of NifL (32). This band is not apparent in the presence of ADP, which protects the C-terminal domain from proteolysis and increases the stability of  $N_{\text{his6}}\text{NifL}$  (147–519) significantly (Fig. 4B). The proteolysis pattern of  $N_{\text{his6}}\text{NifL-R306C}$  (147–519) was surprisingly different from that of  $N_{\text{his6}}\text{NifL}$  (147–519) and was considerably more sensitive to trypsin digestion in the absence of ADP (Fig. 4A). Although ADP decreased the rate of digestion of  $N_{\text{his6}}\text{NifL-R306C}$  (147–519), enhanced sensitivity to trypsin was evident also under these conditions in comparison with  $N_{\text{his6}}\text{NifL}$  (147–519) (Fig. 4B). Two bands (marked 2 and 3) corresponding to C-terminal fragments (32) were more evident after cleavage of  $N_{\text{his6}}\text{NifL-R306C}$  (147–519) in the presence of ADP. These results suggest that the R306C protein remains responsive to the binding of ADP, and the mutation significantly alters the conformation of NifL.

**Influence of *nifL-R306C* on Other *nifA* Mutations.** Although *nifL-R306C* was isolated originally as a suppressor of the *nifL*-resistant *nifA* allele *nifA-E356K*, it seems likely from our experiments that the mutation influences the inhibitory properties of NifL rather than the specificity of the NifL–NifA interaction. Therefore, it was of interest to check whether *nifL-R306C* is able to suppress other mutations that render NifA resistant to NifL.

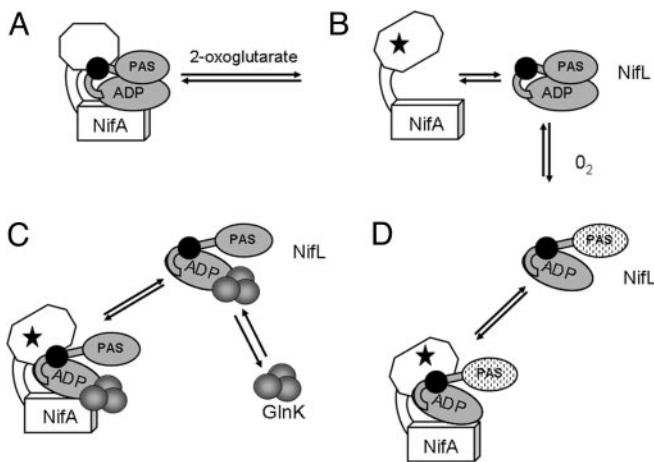
Such mutations have been isolated in both the N-terminal GAF and AAA<sup>+</sup> domains of NifA (Fig. 1) (31). NifL-R306C inhibited the AAA<sup>+</sup> domain substitutions NifA-A249V and NifA-Y254N, and it also partially suppressed the NifL-resistant phenotype of NifA-E394K in the *in vivo* reporter assay in *E. coli*. As in the case of wild-type NifA, NifL-R306C inhibited the activity of these mutant NifA proteins under conditions appropriate for nitrogen fixation. However, the GAF domain substitutions NifA-L120P and NifA-R155A and the AAA<sup>+</sup> domain substitution NifA-E230K were not suppressed by *nifL-R306C* *in vivo* (see Table 3, which is published as supporting information on the PNAS web site). Clearly, only some of the NifL-resistant NifA mutants are susceptible to inhibition as a consequence of the altered conformation of NifL-R306C.

**R306 Is Essential for the Normal Function of NifL.** To examine whether the phenotype of *nifL-R306C* is specific to the arginine-to-cysteine substitution, we constructed a series of substitutions at position 306 by site-directed mutagenesis. Strikingly, all tested substitutions had very similar *in vivo* phenotypes to that of the cysteine substitution, resulting in constitutive inhibition of NifA activity in *E. coli* irrespective of whether polar, hydrophobic, or charged side chains were introduced (see Fig. 7, which is published as supporting information on the PNAS web site). Western blotting indicated that the mutant proteins accumulated to the same extent as wild-type NifL under the conditions used for the *in vivo* assays. These results clearly demonstrate that the arginine residue at position 306 is essential for appropriate regulation of NifA activity by NifL.

## Discussion

The regulatory dialogue between the NifL and NifA proteins involves reciprocal conformational changes in which the binding of 2-oxoglutarate to the GAF domain of the transcriptional activator NifA plays a major role in modulating the response to the NifL antiactivator (29, 30) (Fig. 5). In this study, we have characterized a substitution in NifL that constitutively inhibits NifA, giving rise to a Nif<sup>−</sup> phenotype. The mutant protein is apparently locked in an antiactivation conformation so that NifL inhibits NifA under conditions that are appropriate for nitrogen fixation, even when 2-oxoglutarate is available. This substitution may enable NifL to interact with the altered conformation of NifA that is induced by the binding of 2-oxoglutarate, or alternatively, the mutant protein may lock NifA in an inactive form that is unable to undergo the conformational change normally induced by binding of this ligand.

The R306C substitution is located in the H-box region of NifL adjacent to a histidine residue that corresponds to the autophosphorylation site of the HPKs. However, this histidine residue is functionally redundant, and NifL does not seem to exhibit autokinase activity (26). Nevertheless, this region of NifL may have structural similarity to the dimerization and phosphotransfer domain (domain A) of the HPKs (24). Although, it is conceivable that the R306C substitution could influence dimer-



**Fig. 5.** Model for regulation of NifA activity by NifL. The PAS- and ADP-binding (GHKL) domains of NifL are shown in gray, and the H-box region is represented by a black circle. The GAF and AAA<sup>+</sup> domains of NifA are indicated by an open octagon and cube, respectively. For simplicity, the interacting partners are shown as monomers. (A) In the absence of 2-oxoglutarate, both the reduced and oxidized forms of NifL inhibit the activity of NifA, provided that adenosine nucleotide is bound to NifL (8, 15, 19). (B) Binding of 2-oxoglutarate to the GAF domain of NifA (indicated by stars) induces a conformational change that releases inhibition by the reduced form of NifL, enabling NifA to activate transcription (29, 30). (C) Oxidation of the flavin in the PAS domain of NifL (indicated by stippled ovals) causes a conformational change that enables NifL to inhibit NifA in the presence of 2-oxoglutarate. (D) Binding of the signal transduction protein GlnK (indicated by gray circles) to the C-terminal domain of NifL, enables the reduced form of NifL to interact with NifA, in the presence of 2-oxoglutarate. It is possible that the GlnK–NifL interaction promotes a conformational change similar to that induced by the oxidation of NifL. The GlnK–NifL–NifA ternary complex is formed under nitrogen-excess conditions when GlnK is primarily in the nonuridylylated form. Uridylylation of GlnK under nitrogen-limiting conditions prevents this interaction (17–19). We infer that the R306C substitution locks NifL in a conformation that is analogous to that shown in C and D, so that it is competent to inhibit NifA irrespective of other signals.

ization of NifL, both NifL (147–519) and NifL-R306C (147–519) chromatographed as dimers on gel filtration (data not shown), and therefore, we favor the hypothesis that this substitution influences protein conformation rather than oligomerization. Clearly, the arginine at position 306 is essential for the normal function of *A. vinelandii* NifL because other substitutions at this position give identical phenotypes to that of R306C. An arginine residue at this position is also observed in *Pseudomonas stutzeri* NifL but not in other NifL proteins, implying that the function

of this residue is context-dependent. We infer that arginine 306 is critical for the propagation of signals perceived by *A. vinelandii* NifL in response to the redox and fixed-nitrogen status and also that it is required for a conformational switch that normally inactivates the inhibitory function of NifL under conditions that are appropriate for nitrogen fixation (Fig. 5).

The altered behavior of R306C with respect to the ADP requirement for inhibition of NifA activity provides a potential clue to the nature of the conformational change elicited by this mutation. Unlike wild-type NifL, the mutant protein is able to inhibit NifA in the absence of ADP. This property implies that the NifL-R306C–NifA complex is more stable in the absence of adenosine nucleotides than the wild-type complex, congruent with the finding that the NifL-R306C mutation is competent to suppress substitutions in the nucleotide-binding GHKL domain *in vivo*. However, the presence of the GHKL domain is required for NifL-R306C to inhibit NifA, and we observe that ADP is necessary for inhibition of NifA activity by the oxidized form of NifL-R306C, when 2-oxoglutarate is present (data not shown). These results are consistent with the *in vivo* suppression data (Table 2), which demonstrates that NifL-R306C is less effective in suppressing nucleotide-binding mutants under nitrogen-limiting conditions when the intracellular concentration of 2-oxoglutarate increases. Overall these results suggest that the binding of ADP to the GHKL domain contributes to the conformational changes that are required to inhibit NifA in the presence of 2-oxoglutarate.

In the HPKs, interactions between the dimerization and phosphotransfer domain (domain A) and the catalytic GHKL domain (domain B) modulate kinase and phosphatase activity (10, 13). For example, suppressors of mutations in the G2 region of the nucleotide-binding domain of the histidine kinase EnvZ are located in domain A, suggesting a model in which residues in the phosphotransfer domain interact functionally with the ATP-binding face of the GHKL domain (domain B) to regulate enzymatic function (37, 38). By analogy, the R306C mutation may alter the topological relationship between the H-box region and the GHKL domain of NifL, thus enabling inhibition of NifA, even under conditions that are appropriate for nitrogen fixation, when 2-oxoglutarate levels are elevated. Thus, although signal transmission by NifL does not involve phosphoryl transfer reactions, the interdomain movements that are necessary to modulate the inhibitory function of NifL may be similar to those required to control the catalytic function of the HPKs.

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