

MINIREVIEW

The NifL-NifA System: a Multidomain Transcriptional Regulatory Complex That Integrates Environmental Signals

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The ability of bacteria to respond to a multitude of environmental signals and integrate these signals to trigger adaptive responses provides a successful strategy for survival in rapidly changing environments. In many cases integration can be achieved via the interlinking of different regulatory circuits in which various master regulators respond to different environmental cues. However, in some systems an economy of scale can be achieved if individual regulatory proteins are able to respond to more than one input signal. In this review we consider a particular example where multiple signals are integrated by a regulatory protein complex to finely tune transcriptional regulation of nitrogen fixation in free-living diazotrophic bacteria.

The ability to fix atmospheric nitrogen to ammonia by using the enzyme nitrogenase enables diazotrophs not only to survive but also to proliferate under conditions of extreme fixed-nitrogen deprivation. This strategy, however, incurs an energetic penalty since nitrogenase consumes 16 mol of ATP per mol of dinitrogen fixed *in vitro*, and the energy cost increases to up to 40 mol of ATP per mol *in vivo* (44). The irreversible inactivation of nitrogenase by oxygen also imposes physiological constraints on diazotrophs such that they may only be able to utilize the enzyme under anaerobic conditions or, alternatively, they may employ elegant physiological strategies to protect the enzyme from damage by oxygen under aerobic conditions. In addition to its oxygen sensitivity, nitrogenase is a kinetically slow enzyme (97). Growth in the absence of fixed nitrogen requires a high concentration of the enzyme, and some diazotrophs accumulate 10 to 20% of the total cell protein as nitrogenase under nitrogen-fixing conditions. Nitrogen fixation thus provides an opportunistic strategy to colonize nitrogen-deficient environments, but the cellular commitment in terms of protein synthesis and ATP consumption is appropriate only under specific environmental conditions. The oxygen concentration and the availability of fixed nitrogen are therefore important factors in the regulation of nitrogenase biosynthesis. These signals are integrated to provide transcriptional control of *nif* gene expression in free-living diazotrophs.

In diazotrophic representatives of the *Proteobacteria*, *nif* gene transcription is mostly dependent on the alternative sigma factor σ^{54} , which recognizes promoters with consensus

sequences located at positions –24 and –12 (9, 16). Transcription initiation by the σ^{54} -RNA polymerase holoenzyme requires a specific class of transcriptional activator, which binds to enhancer-like elements upstream of σ^{54} -dependent promoters and through ATP hydrolysis catalyzes conformational changes in σ^{54} which enable the holoenzyme to undergo the transition to the transcriptionally competent open promoter complex. σ^{54} -dependent activators, which are also known as enhancer binding proteins (EBPs) (72, 95), belong to the AAA+ superfamily of ATPases that transform energy into mechanical function to remodel their substrates (75, 103). Activation of σ^{54} -dependent *nif* gene transcription requires NifA, a conserved EBP that regulates genes necessary for the synthesis of molybdenum nitrogenase in proteobacteria.

Activation of transcription by NifA is regulated in response to oxygen and fixed nitrogen. In many diazotrophic members of the alpha and beta subgroups of the *Proteobacteria*, NifA activity is apparently directly responsive to the oxygen status, and conserved cysteine residues have been implicated in the response (32, 33). There is also evidence that the activity of these NifA proteins is regulated in response to fixed nitrogen. However, in some nitrogen-fixing organisms, particularly members of the gamma subgroup of the *Proteobacteria*, the activity of NifA is not intrinsically oxygen or nitrogen responsive, and a partner protein, NifL, is required to modulate NifA activity directly in response to oxygen and fixed nitrogen (24, 89).

Initial sequencing of the *nifL-nifA* operon suggested that these genes could encode a two-component regulatory system. Although NifA does not contain an archetypal response regulator receiver-like domain, the C-terminal region of NifL shows homology to the histidine protein kinases (HPKs) (12, 26). Moreover, *Azotobacter vinelandii* NifL contains a conserved histidine residue found in the transmitter domains of histidine kinases, suggesting that this NifL might employ a classical phosphoryl transfer mechanism to communicate environmental signals to NifA. However, replacement of this conserved histidine by a number of other amino acids does not disable signal transduction (101). Furthermore, NifL is competent to inhibit NifA *in vitro* in the absence of ATP, and signal transduction requires stoichiometric protein-protein interactions between the two regulatory proteins (6, 43, 57, 69, 87). Current evidence suggests that NifL controls the activity of NifA by a relatively stable protein-protein interaction that is modulated by redox changes, ligand binding, and interactions with other signal transduction proteins and membrane components.

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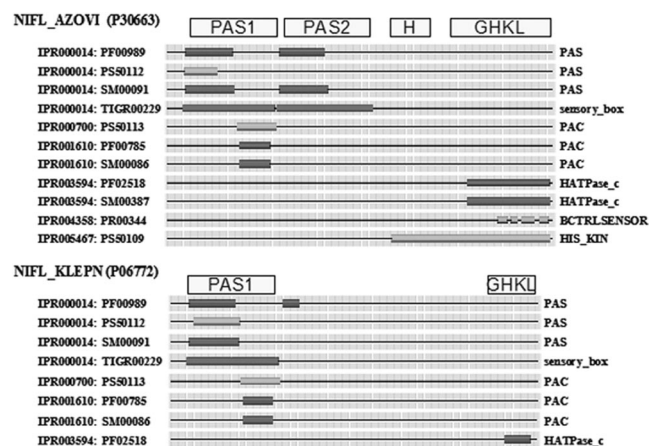


FIG. 1. Graphic view of the domain structure of Av NifL (NIFL_AZOVI) and Kp NifL (NIFL_KLEPN) from the INTERPRO server (<http://www.ebi.ac.uk/interpro/>). INTERPRO assignments and database cross-references are indicated on the left, and the corresponding domains are indicated on the right. Domain designations used in this paper are above the diagram. PAS domains are subdivided into PAS and PAC motifs in some databases (78), but both of these motifs form an integral part of a single structural domain. The term PAS domain in this paper refers to the single structural fold that encompasses both the PAS and PAC motifs.

NifL homologues are found in various enterobacteria, including *Klebsiella pneumoniae* (51), *Klebsiella oxytoca* (53), and *Pantoea agglomerans* (92), and in the aerobic diazotrophs *A. vinelandii* (12, 81) and *Pseudomonas stutzeri* (23). Database searches have suggested that the NifL-NifA system is also present in the plant pathogen *Erwinia chrysanthemi* and in the methane-oxidizing bacterium *Methylococcus capsulatus* strain Bath. A NifL-NifA system has also been reported in *Azoarcus* sp. strain BH72, a diazotrophic grass endophyte belonging to the beta subgroup of the *Proteobacteria* (29). A sequence (PY07698) encoding a NifL-NifA fusion protein in the genome of the rodent malaria parasite *Plasmodium yoelii yoelii* (18) is probably an artifact due to contamination with *Methylococcus* DNA sequences. In this review we focus on the NifL-NifA systems of *K. pneumoniae* and *A. vinelandii*, which are the most

well-studied systems and provide examples of sophisticated multidomain signaling interactions that are responsive to environmental cues. The NifL and NifA proteins of *K. pneumoniae* are designated Kp NifL and Kp NifA, respectively, and the NifL and NifA proteins of *A. vinelandii* are designated Av NifL and Av NifA, respectively.

DOMAIN STRUCTURES

Av NifL contains at least three discrete domains. The N-terminal region has a sensing function, and the C terminus exhibits homology to transmitter domains of the HPKs. Separating these regions is a glutamine-rich hydrophilic sequence representing a Q linker (102). Kp NifL has a domain structure that is more difficult to define, but it also has an N-terminal sensory region and a C-terminal region required for signal transmission. The domain annotation for Av NifL and Kp NifL is shown in Fig. 1. One or more PAS domains are found in the N-terminal region (104). Although limited homology has been observed among PAS domains at the sequence level, structural studies have demonstrated that various cofactors, including heme, flavin adenine dinucleotide (FAD), flavin mononucleotide, and 4-hydroxycinnamic acid, are retained by a common α/β fold (96). The NifL sequences of aerobic diazotrophs (e.g., *A. vinelandii*) possess two PAS domains, which we designate PAS1 and PAS2, whereas the NifL sequences of bacteria which fix nitrogen under anaerobic conditions (e.g., *K. pneumoniae*) have only a single PAS domain, PAS1 (Fig. 1). The PAS1 domains of both Kp NifL and Av NifL have been shown to contain FAD as a prosthetic group (45, 88) and are required for the redox-sensing functions of these proteins, as discussed below. Like wild-type Av NifL, the isolated PAS1 domain is tetrameric and contains ~1 mol of FAD per monomer (40–42). However, the function of the Av NifL PAS2 domain is unknown. Truncated derivatives of Av NifL lacking the PAS1 domain are not responsive to the redox status but are competent to signal the response to fixed nitrogen, demonstrating that redox and nitrogen sensing are discrete functions of the protein (Table 1) (93).

As noted above, the sequence of Av NifL suggested homology to the transmitter domains of members of the HPK family.

TABLE 1. Properties of truncated derivatives of Av NifL

Fragment	Domain(s)	FAD ^a	Association state ^b	ATP-ADP binding ^c	NifA interaction ^d	GlnK interaction ^e	ADP response ^f	Nitrogen response		Oxygen response	
								In vivo ^g	In vitro ^h	In vivo ^g	In vitro ^h
Wild-type NifL	PAS1, PAS2, H, GHKL	+	Tetramer	+	+	+	+	+	+	+	+
1–140	PAS1	+	Tetramer	ND ⁱ	ND	ND	ND	ND	ND	ND	ND
1–284	PAS1, PAS2	+	Tetramer	–	–	–	–	ND	–	ND	–
147–519	PAS2, H, GHKL	–	Mostly dimer	+	+	+	+	+	+	–	–
360–519	GHKL	–	Monomer	+	+	+	+	ND	–	ND	–

^a Determined from UV-visible absorption spectra (41, 45, 64, 93).

^b Determined by analytical gel filtration (42, 93).

^c Derived from limited proteolysis data (93).

^d Complex formation measured by coaffinity chromatography in the presence of Mg-ADP (69).

^e Ability to bind GlnK in the presence of 2-oxoglutarate and ATP, determined by pull down assays and surface plasmon resonance (60).

^f Ability to inhibit open promoter complex formation by NifA in vitro in the presence of ADP (31, 93).

^g Response of a *nifH-lacZ* reporter construct in *E. coli* (85, 93).

^h Inhibition of open promoter complex formation by NifA in vitro in the presence of ADP, 2-oxoglutarate, and GlnK (60, 63).

ⁱ Inhibition of open promoter complex formation by NifA in vitro under oxidizing conditions (45, 93).

^j ND, not determined.

TABLE 2. Properties of truncated derivatives of Kp NifL

Fragment	Domain(s)	FAD ^a	Association state ^b	ATP binding ^c	ATP hydrolysis ^d	Inhibition of NifA ^e
Wild-type NifL	PAS1, GHKL?	+	ND ^f	+	+	+
1–113		–	ND	+	ND	–
1–242	PAS1	+	ND	+	+	–
136–495	GHKL?	–	ND	ND	ND	+
266–495	GHKL?	–	Dimeric	ND	ND	+
306–495	GHKL?	ND	Dimeric	–	–	+

^a Determined from UV-visible absorption spectra (55, 88).

^b Wild-type Kp NifL is highly insoluble (7) and is highly aggregated even when it is expressed as an MBP fusion protein (74). The association states of truncated derivatives were measured by analytical gel filtration (74).

^c Measured by filter binding with labeled ATP and affinity chromatography on ATP-agarose. This activity was detected only when NifL was purified from cells grown under nitrogen-sufficient conditions (56).

^d Determined by hydrolysis of labeled ATP. This activity was detected only when NifL was purified from cells grown under nitrogen-sufficient conditions (56).

^e Wild-type Kp NifL is competent to inhibit Kp NifA in response to fixed nitrogen and oxygen in vivo (46, 66). When expressed from a multicopy vector, wild-type Kp NifL and some deletion derivatives are competent to inhibit Kp NifA even under anaerobic, nitrogen-limiting conditions in vivo. These deletion derivatives are also competent to inhibit transcriptional activation by NifA in vitro (74).

^f ND, not determined.

Structural studies of representatives of the HPK family have revealed a C-terminal catalytic domain, required for ATP binding and *trans* phosphorylation, and a dimerization domain (H domain) containing the conserved histidine residue required for phosphotransfer to the response regulator (94). The catalytic kinase domain belongs to the GHKL (HATPase_c) superfamily of ATPases, which includes DNA gyrase B, Hsp90, SpoIIAB, and MutL (27). Av NifL contains conserved residues corresponding to the N, G1, F, and G2 boxes that constitute the ATP binding domain of the GHKL superfamily, and this domain of Av NifL has been demonstrated to bind adenosine nucleotides (93) (see below). However, the corresponding region of Kp NifL is less homologous to the GHKL superfamily and apparently does not bind nucleotides (56). Structural predictions also indicate that the region of Av NifL located between the PAS2 and GHKL domains may constitute an antiparallel helix bundle, similar to the structures of the dimerization (H) domains of the HPKs EnvZ and CheA.

The domain architecture of diazotrophic NifA proteins is more uniform than that of NifL proteins. The N-terminal region of NifA proteins constitutes a GAF domain, a ubiquitous sensory module found in signaling proteins in all phyla (1). GAF domains have a three-dimensional structure similar to that of PAS domains (47) and are known to bind small molecules, including cyclic nucleotides (50) and formate (48). Truncated NifA proteins lacking the N-terminal GAF domain have altered regulatory properties and, in the case of Av NifA and Kp NifA, have altered responses to NifL (8, 25). The central domain of NifA proteins is highly conserved and comprises the AAA+ module required for nucleotide hydrolysis and σ^{54} interaction that is common to all EBPs (72, 95). AAA+ proteins are oligomeric and commonly form a hexameric ring-like structure in which nucleotide is bound between adjacent protomers (99). Oligomerization of EBPs promotes ATP hydrolysis, which in turn couples changes in protomer structure to binding interactions with σ^{54} (17, 19, 103). A conserved GAFTGA motif in the AAA+ domain that is unique to EBPs is critical for transcriptional activation (100). This region constitutes an interaction surface that binds σ^{54} and couples the energy of ATP hydrolysis to open complex formation (15). As is the case for all EBPs, the C-terminal domain of NifA contains a helix-turn-helix motif required for recognition of the

enhancer-like, upstream activator sequences (70). Mutations in the recognition helix of Kp NifA that influence transcriptional activation occur at residues that exhibit chemical shifts in the nuclear magnetic resonance spectra of the C-terminal domain upon DNA binding, directly demonstrating that the helix-turn-helix motif interacts with the enhancer-like sequences (82).

LIGAND BINDING AND THE NifL-NifA INTERACTION

Since NifL proteins have a domain structure similar to that of the HPKs, it was expected that these proteins might bind adenosine nucleotides. Av NifL apparently does not hydrolyze ATP, exhibit autophosphorylation, or phosphotransfer to NifA. However, the C-terminal GHKL domain of Av NifL binds ATP and ADP (93). The binding of these nucleotides influences the conformation of the C-terminal region, which is particularly susceptible to trypsin cleavage in the absence of nucleotide. The affinity of Av NifL for ADP (apparent K_d , ~13 μ M) is approximately 10-fold higher than that of ATP (93). The presence of ADP also influences the activity of NifL in vitro, strongly stimulating the ability of Av NifL to inhibit the activity of Av NifA (31, 45). The ability of ADP to act as an effector of NifL activity is reflected by the increased stability of the protein complexes formed between Av NifL and Av NifA in the presence of this ligand (69). Removal of the nucleotide causes dissociation of the complex. Neither the N-terminal region of Av NifL (69) nor the GAF domain of Av NifA (8, 58) is essential for the interaction. Protein footprinting experiments indicate that complex formation alters the protease sensitivity of the Q linker region in Av NifL and the GAF domain and linker region between the GAF and AAA+ domains of Av NifA (68). Overall, these experiments suggest that the binding of adenosine nucleotides to the GHKL domain of Av NifL influences the conformation of the transmitter region and facilitates interaction with Av NifA.

Expression of *K. pneumoniae* *nifL* and *nifA* genes is coupled at the translational level, and immunoprecipitation experiments have demonstrated that Kp NifL and Kp NifA form a protein-protein complex in vivo (34, 35, 43). However, the requirements for complex formation appear to be different from those of the *A. vinelandii* NifL-NifA system (Table 2). As

mentioned above, the C-terminal region of Kp NifL is less homologous to the HPKs than Av NifL is, and it lacks conserved residues required for nucleotide binding in the GHKL superfamily. In contrast to Av NifL, the presence of adenosine nucleotides is not specifically required for the inhibition of Kp NifA activity by Kp NifL (57). Although neither ATP hydrolysis (10) nor kinase activities (87) have been reported previously, Kp NifL has recently been shown to exhibit ATP binding and ATPase activity when it is purified from cultures grown under conditions of nitrogen sufficiency (56). In contrast, Kp NifL purified from cultures grown under conditions of nitrogen deficiency is catalytically inactive. Although both of these forms are competent to inhibit Kp NifA in vitro, an increase in inhibitory activity is observed in the presence of adenosine nucleotides. Surprisingly however, nucleotide binding to Kp NifL is apparently associated with the PAS1 domain and not with the C-terminal GKHL-like domain (56). The sequences required for the interaction between Kp NifL and Kp NifA also appear to be different than the sequences required in the *A. vinelandii* NifL-NifA system. Unlike the isolated C-terminal kinase-like domain of Av NifL, the C-terminal domain of Kp NifL is sufficient to inhibit Kp NifA both in vivo and in vitro (74) (Table 2).

Although the response of Av NifL to adenosine nucleotides might provide a mechanism for sensing the energy charge, the physiological relevance of nucleotide binding is unclear, since the intracellular concentrations of ATP and ADP are far greater than the association constants of Av NifL for these nucleotides. Potentially, the affinity of Av NifL for nucleotides could be altered in response to environmental cues, as postulated for Kp NifL (56). Alternatively, other ligands may influence the NifL-NifA interaction. It has recently been shown that 2-oxoglutarate is an allosteric effector of the *A. vinelandii* NifL-NifA system that counteracts the inhibitory function of the ADP-bound form of Av NifL (63). 2-Oxoglutarate has been implicated as a key metabolic signal of the carbon status, but the concentration of this metabolite also reflects the nitrogen status. The response of the *A. vinelandii* NifL-NifA system to 2-oxoglutarate in vitro is within the physiological range, which extends from ~ 100 μM under conditions of carbon limitation (excess of fixed nitrogen) to ~ 1 mM under conditions of nitrogen deficiency (excess of carbon) (85, 90). Thus, at relatively low 2-oxoglutarate concentrations, the ADP-bound form of NifL is competent to inhibit NifA activity, but at high 2-oxoglutarate levels, NifA is not responsive to NifL in the presence of ADP (63). Isothermal titration calorimetry experiments have demonstrated that 2-oxoglutarate binds to Av NifA but not to Av NifL (61). The GAF domain of Av NifA exhibits stoichiometric binding of 2-oxoglutarate with a dissociation constant of 60 μM , and binding is not observed with a truncated form of Av NifA lacking the GAF domain. The interaction of 2-oxoglutarate with the GAF domain may induce conformational changes in Av NifA which render it resistant to Av NifL, since the binding of 2-oxoglutarate alters the susceptibility of this domain to digestion with trypsin (61). In contrast, the GAF domain of *K. pneumoniae* NifA apparently does not bind 2-oxoglutarate, emphasizing the mechanistic differences between the *A. vinelandii* and *K. pneumoniae* NifL-NifA systems.

NITROGEN REGULATION

Nitrogen fixation is essential for growth only under conditions of fixed-nitrogen deficiency, and thus the activity of NifA is stringently controlled by NifL in response to the fixed-nitrogen status. Key information related to the intracellular nitrogen and the carbon status is communicated by the signal transduction protein PII, which integrates these signals and transmits the information globally to various receptors to control nitrogen assimilation (in this case the NifL-NifA system). PII-like proteins constitute a highly conserved family of signaling proteins found in all three kingdoms of life (2, 76). Many bacteria possess more than one PII-like protein; these proteins have common functions but perform discrete physiological roles. Enteric bacteria express two PII paralogues, designated GlnB and GlnK, that are both subject to reversible covalent modification by the uridylyltransferase/uridylyl-removing enzyme, the product of *glnD* (2). The activity of this enzyme is regulated by the intracellular level of glutamine, a key signal of the nitrogen status. Under conditions of nitrogen deprivation, when the intracellular level of glutamine is relatively low, GlnD uridylylates the PII proteins. Conversely, at high glutamine concentrations, the PII proteins are deuridylylated by GlnD. The site of covalent modification is a conserved tyrosine residue (Tyr 51) which is located at the tip of a surface-exposed loop (the T loop) required for the interaction of PII proteins with their receptors. The activity of PII is controlled not only by covalent modification but also by the binding of the ligands ATP and 2-oxoglutarate (76). As with Av NifA, the interaction with 2-oxoglutarate enables PII proteins to respond allosterically to a signal of central carbon metabolism. PII proteins are trimeric and contain three binding sites for 2-oxoglutarate and ATP.

The primary physiological role of GlnB in enteric bacteria is to control nitrogen assimilation under conditions of relative nitrogen sufficiency, whereas GlnK mediates nitrogen control under conditions of nitrogen starvation (5, 13). This enables nitrogen regulatory cascades in which the PII paralogues operate at different levels. Such a cascade mediates nitrogen regulation of nitrogen fixation in *K. pneumoniae*. By modulating the activity of the NtrB-NtrC two-component regulatory system, GlnB controls the phosphorylation state of the EBP, NtrC, a key activator of nitrogen-regulated genes. This first level of the cascade ensures that activation of the *glnK* and *nifLA* promoters is determined by the phosphorylation state of NtrC. Both of these promoters possess relatively weak enhancers, and a high level of NtrC-P is required for their activation (5, 67). The first level of nitrogen control, therefore, governs the expression of NifL, NifA, and GlnK, the major players at the second level of the cascade (39) (Fig. 2). Although *glnB* mutations influence the first step of the cascade by altering the level of NtrC-P, they do not influence the fixed-nitrogen response of the Kp NifL and Kp NifA proteins per se, which occurs at the second level of the cascade. However, in *glnK* mutants, Kp NifA activity is constitutively inactivated by Kp NifL, indicating that in the second step of the cascade, GlnK is required to prevent Kp NifL from inhibiting Kp NifA under conditions of nitrogen starvation (38, 49). Although there is no biochemical evidence, these results suggest that GlnK could bind to either Kp NifL or Kp NifA to prevent interaction

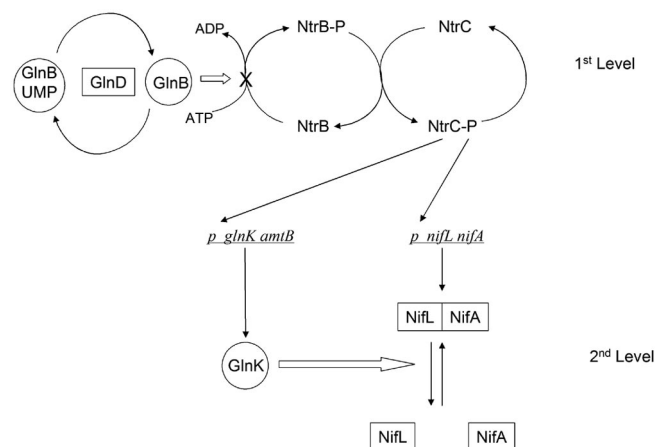


FIG. 2. Cascade regulation of *nif* genes in *K. pneumoniae* in response to the fixed nitrogen status. Under nitrogen-limiting conditions, GlnB is uridylylated and NtrB phosphorylates NtrC, leading to activation of transcription of the *glnK*, *ambB*, and *nifLA* operons. Expression of GlnK prevents Kp NifL from inhibiting Kp NifA, leading to activation of *nif* transcription. Under nitrogen-sufficient conditions, GlnD deuridylylates GlnB, which is then competent to activate the phosphatase activity of NtrB; this limits the availability of NtrC-P and prevents expression of *nifLA* and *glnK ambB*. Following ammonium upshift GlnK may be sequestered by AmtB, as discussed in the text.

between the partners under nitrogen-limiting conditions (Fig. 2). The ability of GlnK to relieve inhibition by Kp NifL under conditions of nitrogen starvation does not require covalent modification of GlnK, since normal regulation by NifL is observed in *glnD* mutants and with a mutant form of GlnK, GlnK-Y51N, which cannot be uridylylated by GlnD (28, 38). The specificity of the interaction between GlnK and the *K. pneumoniae* *nif*-specific regulatory proteins has been investigated by comparing the T-loop regions of *Escherichia coli* GlnB and GlnK. Substitution of residues 43 and 54 of GlnB by the analogous residues in GlnK enables GlnB to relieve NifL inhibition of Kp NifA (3). Expression of GlnB on a multicopy plasmid also relieves NifL inhibition (4), which is congruent with the finding that specific physiological roles played by GlnB and GlnK are dependent upon expression levels rather than discrete functions (13).

In *A. vinelandii* similar components required for nitrogen regulation are present, but the cascade appears to have become disconnected and the mechanism of regulation is somewhat different. Although this organism possesses a bona fide NtrB-NtrC two-component regulatory system which regulates nitrate assimilation, NtrC is not required to activate transcription from the *nifLA* promoter (98). This promoter is active under conditions of nitrogen excess and, surprisingly, is not dependent on *rpoN* even though there is a potential σ^{54} interaction site appropriately positioned upstream of the transcription start site (12, 81). Another unusual feature of *A. vinelandii* is the presence of only a single gene encoding a PII-like protein, designated *glnK*, which is located in an operon with *ambB* and is transcribed constitutively (20, 65). Therefore, both the *nifLA* and *glnK ambB* operons are transcribed under conditions of nitrogen sufficiency, and thus the first level of the nitrogen regulatory cascade present in enteric bacteria is absent in *A. vinelandii*. The *glnK* gene is apparently essential in *A. vinelandii*

(65), and GlnK is uridylylated by a homologue of GlnD previously known as NfrX (20). Mutants with mutations in *glnD* which decrease uridylyltransferase activity are unable to fix nitrogen. However, the Nif⁻ phenotype of these strains can be suppressed by secondary mutations that inactivate NifL (20, 21). This suggests that the uridylylation function of GlnD is necessary to prevent Av NifL from inhibiting Av NifA in *A. vinelandii*, in contrast to enteric bacteria, in which uridylylation of GlnK is not required. Since *A. vinelandii* *glnK* is an essential gene, it has not been possible to examine the phenotype of strains with *glnK* null mutations. However, a strain expressing a mutant form of GlnK with a mutation in the T loop, GlnK-Y51F, which prevents uridylylation by GlnD, is stable providing that the strain contains a secondary mutation (*gln-71*) that also prevents the adenylation of glutamine synthetase (86). The secondary mutation is necessary because the nonuridylylated form of GlnK results in constitutive adenylation of glutamine synthetase, thus preventing ammonia assimilation (20). Strains containing the *glnK-Y51F* and *gln-71* mutations are impaired for nitrogen fixation, and Av NifA is inactivated by Av NifL even under nitrogen-fixing conditions. As is the case for the *glnD* mutations, this phenotype is suppressed by insertion mutations in *nifL* (86). Thus, uridylylation of GlnK is necessary in order to prevent inhibition by Av NifL under conditions of fixed-nitrogen deficiency.

The *A. vinelandii* *nifLA* system responds to nitrogen regulation when it is introduced into *E. coli* (93), which allows comparisons between the responses of the *K. pneumoniae* and *A. vinelandii* NifL-NifA systems to well-characterized nitrogen-regulatory mutations. In contrast to the response of the *K. pneumoniae* system in *E. coli*, neither GlnK nor GlnB is required to relieve inhibition by Av NifL under nitrogen-limiting conditions. Moreover, in double *glnB ntrC* mutants or in triple *glnB glnK ntrC* mutants, which do not express the PII proteins, the activity of Av NifA is not regulated by Av NifL in response to the nitrogen source (85). This suggests that in contrast to the *K. pneumoniae* NifL-NifA system, the PII proteins are required to activate the inhibitory function of Av NifL rather than prevent inhibition (Fig. 3). This conclusion is strongly supported by the results of biochemical experiments performed with purified components. The inhibitory function of Av NifL is activated by the nonuridylylated form of *E. coli* GlnB but not by the fully uridylylated form (GlnB-UMP) (63). Likewise, the

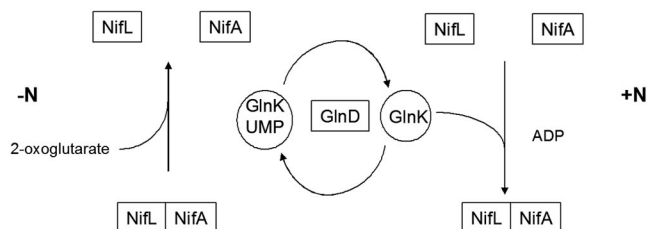


FIG. 3. Nitrogen source regulation of *nif* gene transcription in *A. vinelandii*. Under conditions of fixed-nitrogen limitation (-N), GlnK is mainly uridylylated and not competent to interact with NifL. Under these conditions, binding of 2-oxoglutarate to the GAF domain of Av NifA relieves inhibition by Av NifL, freeing NifA to activate transcription. Under conditions of fixed-nitrogen sufficiency (+N), GlnD deuridylylates GlnK, which interacts with NifL, promoting formation of the NifL-NifA complex.

nonmodified form of *A. vinelandii* GlnK (Av GlnK) is competent to activate the inhibitory function of Av NifL in the presence of 2-oxoglutarate and ATP. However, when fully uridylylated by the *A. vinelandii* GlnD protein, Av GlnK does not activate Av NifL (63). This is fully consistent with the *in vivo* data and demonstrates that covalent modification of Av GlnK by GlnD is necessary to prevent Av GlnK from activating the inhibitory function of Av NifL.

Potentially, Av GlnK could interact with either Av NifL or Av NifA to modulate their activities. Interactions between Av GlnK and Av NifL, but not between Av GlnK and Av NifA, have been detected by coaffinity assays, surface plasmon resonance experiments, and yeast two-hybrid assays (13, 86). The *in vitro* interaction of Av GlnK with Av NifL is abolished by a mutation, E44C, in the T loop of GlnK and also, as expected, when Av GlnK is fully uridylylated. Similar to the interactions of the *E. coli* PII proteins with their receptors, the binary interaction is dependent upon Mg^{2+} , ATP, and 2-oxoglutarate (60). The role of Av GlnK in communicating the nitrogen status to Av NifL may be analogous to the role of *E. coli* GlnB in signaling to the histidine kinase NtrB, since in both cases the interactions have been localized to the C-terminal GHKL domain of the receptors (60, 77). However, the response of the interaction to 2-oxoglutarate appears to be different in each case. This ligand is an allosteric effector of the *E. coli* GlnB-NtrB interaction but not the Av GlnK-Av NifL interaction (60). This difference may arise from the discrete ligand binding properties of *E. coli* GlnB and Av GlnK. As mentioned above, GlnB binds a single molecule of 2-oxoglutarate with high affinity, and the binding of subsequent molecules is inhibited by anticooperativity. However, isothermal titration calorimetry experiments have suggested that unlike *E. coli* GlnB, Av GlnK does not exhibit negative cooperativity in the binding of 2-oxoglutarate (60). The involvement of 2-oxoglutarate in the Av GlnK-Av NifL interaction is also complicated by the binding of this ligand to the GAF domain of Av NifA. Protease footprinting experiments have suggested that the interaction with Av GlnK promotes the formation of a GlnK-NifL-NifA ternary complex (61). Whereas the binding of 2-oxoglutarate to the GAF domain of NifA appears to favor dissociation of the binary *A. vinelandii* NifL-NifA complex, the presence of nonmodified GlnK favors the ternary interaction, even at high 2-oxoglutarate concentrations.

The mechanism by which GlnK communicates the nitrogen status to the *A. vinelandii* NifL-NifA system is thus clearly very different from that observed in *K. pneumoniae*. In *A. vinelandii*, Av GlnK is required to activate the inhibitory function of Av NifL, whereas in *K. pneumoniae* GlnK is required to prevent Kp NifL from inhibiting Kp NifA. *K. pneumoniae* GlnK could interact either with Kp NifL or Kp NifA to promote dissociation of the complex. In contrast, the nonmodified form of Av GlnK promotes association of a ternary complex. Covalent modification of Av GlnK by GlnD modulates the interaction with Av NifL in response to the N status, whereas in *K. pneumoniae* the signal for nitrogen starvation is provided by activation of *glnK* expression by NtrC-P, and modification of GlnK is not required to transmit the signal (compare Fig. 2 and 3).

REDOX-OXYGEN SENSING

The need to reconcile the oxygen sensitivity of nitrogenase with strictly aerobic metabolism in *A. vinelandii* has necessitated the evolution of various protection mechanisms to ensure that nitrogenase is not damaged by oxygen (44, 73, 79). However, when respiration is unable to cope with excess oxygen, it is necessary to ensure that synthesis of nitrogenase is prevented. Likewise in *K. pneumoniae*, which is capable of fixing nitrogen only under anaerobic conditions, it is important to prevent *nif* transcription in response to aerobiosis. It is well established that NifL inhibits NifA in response to the external oxygen concentration, but the first clue to a potential mechanism for redox sensing was the demonstration that Av NifL is a flavoprotein with FAD as a prosthetic group (45). Spectroscopy of purified Av NifL revealed a characteristic flavin spectrum with absorption maxima at 360 and 445 nm and shoulders at 420 and 470 nm indicative of a protein-bound flavin moiety. While the oxidized form of Av NifL is competent to inhibit transcriptional activation by Av NifA, this inhibition is reversed when the flavin is reduced with sodium dithionite (45). These observations demonstrate that Av NifL is a redox-sensitive regulatory protein. Av NifL binds FAD in the N-terminal PAS1 domain, which has sequence similarity with the corresponding PAS domain in the *E. coli* flavoprotein Aer, a signal transducer for aerotaxis (11, 83). The N terminus of Kp NifL also contains bound FAD (88), although in this case redox-mediated control of NifA activity has not been demonstrated *in vitro*, as truncated forms of NifL lacking the N-terminal domain inhibit NifA activity. However, the biochemical experiments to date with the *K. pneumoniae* system have not included GlnK, which may be required to promote dissociation of Kp NifL and Kp NifA under reducing conditions.

The FAD moiety in Av NifL can be reduced with a variety of enzymes as electron donors in a two-electron reduction with a redox potential of ~ 225 mV at pH 8 (64). Oxygen is a potential physiological oxidant as Av NifL is rapidly oxidized upon exposure to air, which yields hydrogen peroxide as a product (62). However, the physiological electron donor to Av NifL in *A. vinelandii* is unknown. The redox potential of the reduced FAD-oxidized FAD couple in Kp NifL is ~ 275 mV at pH 8, and the reduced form of the protein also oxidizes rapidly in the presence of air (55). Although there is no evidence that Kp NifL contains Fe or an iron-sulfur cluster, iron is required in the culture medium to prevent Kp NifL from inhibiting Kp NifA even under anaerobic, nitrogen-limiting conditions (87). This suggests that an iron-containing protein could be required to signal the redox status to NifL by acting as an electron donor. One candidate iron-containing protein that acts as a general oxygen sensor is the global regulator Fnr, which contains an oxygen-labile [4Fe-4S] cluster (52). Analysis of the *K. pneumoniae* NifL-NifA system in *fnr* mutants of *E. coli* and *K. pneumoniae* indicated that Fnr is required to maintain Kp NifL in a noninhibitory state under anaerobic, nitrogen-limiting conditions (36). Since Fnr is unlikely to be a direct electron donor to Kp NifL, the physiological electron donor is likely to be an electron transport component encoded by a gene(s) that is subject to transcriptional activation by Fnr under anaerobic conditions. *K. pneumoniae* strains having mutations in either *fdnG* (encoding formate dehydrogenase N) or *nuoCD* (encod-

ing NADH:ubiquinone oxidoreductase) show reduced *nif* gene activation, but the mutations do not influence the expression levels of Kp NifL and Kp NifA (37). This suggests that in the absence of these membrane-bound oxidoreductases, NifL is maintained in an inhibitory form under anaerobic conditions, although it is not obvious why both mutations apparently influence electron donation to Kp NifL. The *fdn* operon is subject to Fnr control in *E. coli* (59), whereas the *nuo* operon is regulated by ArcA (14). It will therefore be of interest to determine whether Fnr is involved in regulation of the corresponding *K. pneumoniae* operons. It has been proposed that Kp NifL is reduced by electrons from the reduced quinone pool since the quinone derivatives dimethyl naphthoquinol and menadiol are able to reduce the flavin moiety of Kp NifL in the absence of an electron mediator in vitro (37).

DYNAMIC LOCALIZATION

Translational coupling of the *nifLA* operon in *K. pneumoniae* ensures that the two encoded proteins are produced in stoichiometric amounts (34). Alterations in the ratio of Kp NifL to Kp NifA disrupt bona fide regulation, demonstrating that the stoichiometry is important for effective signal transduction (35). Under anaerobic nitrogen-limiting conditions, a high proportion of Kp NifL partitions to the membrane, whereas NifL is found primarily in the cytoplasm when cultures are grown aerobically or under conditions of nitrogen sufficiency (54). In contrast, Kp NifA remains primarily in the cytoplasm under all conditions tested. The spatial separation of NifL and NifA under derepressing conditions suggests that membrane association plays a significant role in releasing Kp NifA from inhibition by Kp NifL. It seems likely that reduction of Kp NifL promotes membrane association, since NifL is found mainly in the cytoplasm in *fnr*, *nuoCD*, and *fdnG* mutant strains in which redox sensing by Kp NifL is disabled (37, 54). Likewise, membrane association is not observed in *glnK* mutants which are unable to signal the nitrogen status (54). Thus, both the presence of GlnK and reduction of NifL appear to be necessary for membrane association. The sequestration of GlnK by AmtB upon ammonium upshift may be a factor in promoting the release of NifL from the membrane (22, 54). The oxidation of the flavin moiety may also release NifL to the cytoplasm. However, the precise factors involved in promoting association and dissociation of NifL with the membrane remain to be determined.

MECHANISM OF NIFL INHIBITION

There are several stages at which NifL could inhibit transcriptional activation by NifA through the formation of a protein-protein complex. These stages could potentially involve prevention of binding to enhancers by the C-terminal DNA binding domain, inhibition of the functions of the AAA+ domain, which include oligomerization, nucleotide binding hydrolysis, and interaction with σ^{54} (103), and remodeling of the GAF domain to induce intramolecular repression of the AAA+ domain (91). Since inhibition by NifL is specific to NifA and NifL apparently does not control transcriptional activation by other EBPs, it is likely that there are surface residues in NifA that are specific to the interaction with NifL.

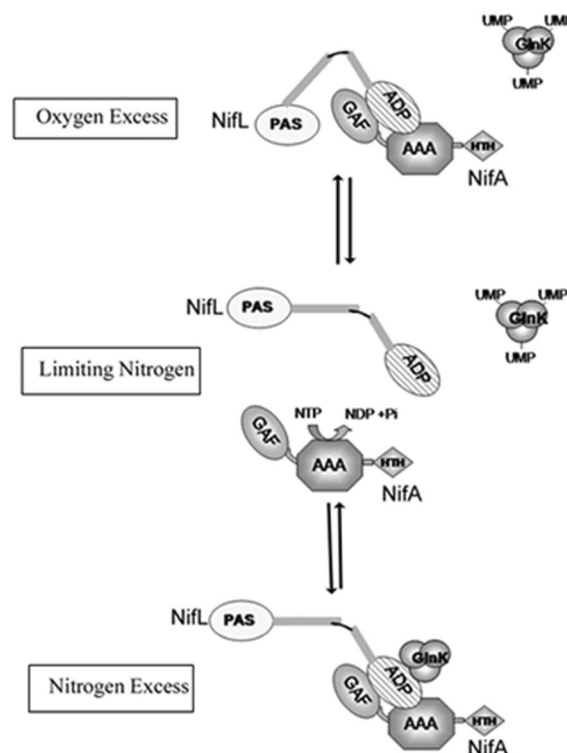


FIG. 4. Model showing potential interactions between Av NifL and Av NifA in response to environmental cues. Only the PAS1 and ADP binding domains of NifL are shown (open and cross-hatched ovals, respectively). The three domains of Av NifA are labeled GAF, AAA, and HTH. Under nitrogen-limiting conditions GlnK is uridylylated, and provided that the flavin moiety in Av NifL is reduced, Av NifA is free to activate transcription, catalyzed by ATP hydrolysis (center diagram). However, when Av NifL is oxidized, the NifL-NifA binary complex is formed, perhaps promoted by conformational changes mediated via the PAS domain. Formation of the complex sequesters Av NifA, preventing transcriptional activation. Under nitrogen-excess conditions, when GlnK is in the noncovalently modified form, it interacts with the C-terminal ADP binding domain of Av NifL to promote formation of a ternary complex in which the activity of Av NifA is also inhibited.

Mutations in Av NifA that confer resistance to inhibition by Av NifL have been isolated from a library of random PCR-generated *nifA* mutants. Mutations conferring resistance are located in both the GAF and AAA+ domains of Av NifA, implying that both of these domains are involved in the response (84). Some NifA mutants appear to discriminate between the forms of NifL present in response to different environmental conditions. One of the mutations in the AAA+ domain, NifA-Y254N, is sensitive to Av NifL under aerobic growth conditions but is resistant to inhibition under conditions of nitrogen excess. This mutation may disfavor formation of the ternary complex with GlnK but remains sensitive to the binary complex formed with oxidized NifL (Fig. 4).

As mentioned above, nucleotide hydrolysis catalyzed by the central domain of EBPs is necessary to drive conformational changes that enable σ^{54} -RNA polymerase to form the open promoter complex. The ATPase activity of Av NifA is inhibited by Av NifL (8, 31), suggesting that NifL inhibits steps required either for nucleotide binding (e.g., assembly of AAA+ domain

protomers) or for nucleotide hydrolysis. A major function of NifL is therefore to inhibit catalysis by NifA and thus prevent transcriptional activation. However, in a truncated form of Av NifA lacking the GAF domain, nucleotide hydrolysis is not strongly inhibited by Av NifL, even though the ability of NifA to activate open complex formation is inhibited (8). Similarly, transcriptional activation by the isolated central domain of Kp NifA is inhibited by Kp NifL in the absence of inhibition of the ATPase activity of Kp NifA (10). This suggests that NifL may inhibit access of σ^{54} to interaction surfaces in the AAA+ domain of NifA (e.g., the GAFTGA motif). Thus, NifL may be able to inhibit two discrete functions of the AAA+ domain, nucleotide hydrolysis and interaction with σ^{54} . Since inhibition of nucleotide hydrolysis apparently requires the GAF domain, it appears that this domain may control the ATPase activity of the AAA+ domain in response to NifL by interdomain repression (8). Thus, as suggested by the mutations conferring resistance to NifL, both the GAF and AAA+ domains of Av NifA may contact Av NifL. There is also evidence that NifL may inhibit the enhancer binding function of NifA (8, 71).

NifL may have inhibitory functions analogous to the functions of other adaptors of the EBP family, including PspA, which controls the activity of PspF in *E. coli* (30), and HrpV, which inactivates HrpR-HrpS in *Pseudomonas syringae* (80). However, PspF and HrpR-HrpS do not contain an N-terminal regulatory domain, and NifL, PspA, and HrpV do not exhibit detectable sequence homology. Like NifL, however, PspA appears to inhibit two functional states of the PspF AAA+ domain, productive interactions with σ^{54} and nucleotide interactions (30). However, PspA interacts directly with the AAA+ domain to inhibit the ATPase activity of PspF, whereas in the NifL-NifA interaction this function requires the GAF domain.

CONCLUDING REMARKS

The NifL and NifA proteins constitute a remarkably intricate multidomain regulatory complex in which complementary interactions between the partners are finely tuned to integrate signals of redox oxygen, carbon, and the fixed-nitrogen status. Although the complexities of the interactions are only just beginning to emerge, it is evident that at least some of the mechanisms for signal communication are different in *K. pneumoniae* and *A. vinelandii*. The NifL and NifA proteins from these organisms are 30 and 57% identical, respectively, and yet the two systems have evidently adapted to the physiologies of their hosts. Nitrogen regulation provides an obvious example. The two PII-like proteins in *K. pneumoniae* regulate the nitrogen response at both levels of the cascade. The uridylylation status of GlnB controls transcription of the *nifLA* operon via the level of NtrC-P. Under conditions of nitrogen deprivation this also allows activation of GlnK expression, which is absolutely required to prevent formation of the inhibitory complex between Kp NifL and Kp NifA. However the precise role of GlnK in the nitrogen response of the *K. pneumoniae* NifL-NifA system remains unclear since the uridylylation state of GlnK does not apparently influence its function in preventing NifL-mediated inhibition. In contrast, *A. vinelandii* contains only a single PII-like protein, which regulates the activity of Av NifL in response to uridylylation. The nonmodified form of GlnK activates NifL, thus favoring formation of the NifL-NifA com-

plex under conditions of nitrogen excess. These contrasting mechanisms nevertheless qualitatively provide similar nitrogen regulatory switches. However, the *K. pneumoniae* system is likely to be more stringent, only permitting nitrogen fixation under conditions of extreme nitrogen deprivation, perhaps reflecting the high energetic penalty for nitrogen fixation under anaerobic growth conditions.

Unlike many other members of the EBP family of transcriptional activators, Kp NifA and Av NifA constitutively activate transcription in the absence of their partner NifL proteins. In contrast, the activities of many transcriptional activators of the EBP family are regulated directly by the amino-terminal regulatory domain, and a partner protein is not required. Indeed, in many diazotrophic representatives of the α and β subgroups of the *Proteobacteria*, NifA directly integrates signals for fixed nitrogen and oxygen in the absence of a NifL-like protein. What, therefore, are the advantages of regulating nitrogen fixation by this unusual two-component regulatory system? Clearly, a two-component system is likely to be more sophisticated as it provides more opportunities for interdomain communication and signaling interactions and it permits regulation via spatial separation of the partners. The similarity between NifL and the HPKs suggests that the NifL-NifA system may have evolved from a conventional two-component system in which the original equivalent of NifA may have been a response regulator. Loss of the kinase activity of the ancient precursor of NifL and acquisition of a GAF domain at the N terminus of NifA, via a domain swap, may have facilitated evolution of this fascinating regulatory system.

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