Nitrogen fixation: key genetic regulatory mechanisms

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Abstract
The necessity to respond to the level of fixed nitrogen and external oxygen concentrations and to provide sufficient energy for nitrogen fixation imposes common regulatory principles amongst diazotrophs. The NifL–NifA system in Azotobacter vinelandii integrates the signals of redox, fixed-nitrogen and carbon status to regulate nif transcription. Multidomain signalling interactions between NifL and NifA are modulated by redox changes, ligand binding and interaction with the signal-transduction protein GlnK. Under adverse redox conditions (excess oxygen) or when fixed nitrogen is in excess, NifL forms a complex with NifA in which transcriptional activation is prevented. Oxidized NifL forms a binary complex with NifA to inhibit NifA activity. When fixed nitrogen is in excess, the non-covalently modified form of GlnK interacts with NifL to promote the formation of a GlnK–NifL–NifA ternary complex. When the cell re-encounters favourable conditions for nitrogen fixation, it is necessary to deactivate the signals to ensure that the NifL–NifA complex is dissociated so that NifA is free to activate transcription. This is achieved through interactions with 2-oxoglutarate, a key metabolic signal of the carbon status, which binds to the N-terminal GAF (cGMP-specific and stimulated phosphodiesterases, Anabaena adenylate cyclases and Escherichia coli FhlA) domain of NifA.

Introduction
Nitrogen fixation plays a crucial role in the nitrogen cycle by compensating for the loss of fixed nitrogen incurred on denitrification and thus replenishes the overall nitrogen content of the biosphere. However, the requirement of high energy for nitrogen fixation and the oxygen sensitivity of nitrogenase (which is the complex metalloenzyme that catalyses the reduction of atmospheric dinitrogen to ammonia [1]) impose considerable physiological constraints on nitrogen-fixing organisms. Diverse eubacteria and Archaea have the capacity to fix nitrogen, but the physiological requirements for nitrogenase activity necessitate the provision of an intracellular environment devoid of oxygen with ample supplies of ATP and an appropriate reductant. Consequently, in most environments, the capacity to fix nitrogen is only utilized by diazotrophs under conditions of fixed nitrogen limitation, with the proviso that a suitable carbon source is available and the enzyme can be protected from oxygen damage. Stentiment regulation is applied to control the transcription of nitrogen fixation (nif) genes in response to the fixed-nitrogen and oxygen status to ensure that the process only occurs when these physiological conditions are satisfied [2].

NifL and NifA proteins
In the aerobic nitrogen-fixing bacterium Azotobacter vinelandii, two specific regulatory proteins, NifL and NifA, encoded by the nifLA operon, control the expression of nif genes in response to redox, nitrogen and carbon status [3,4]. NifL is an anti-activator that tightly regulates the activity of its partner protein, NifA, by the formation of an inhibitory complex. NifA is a member of the family of σ⁵⁴-dependent transcriptional activators known as EBPs (enhancer-binding proteins) that use ATP hydrolysis to catalyse conformational changes in the sigma factor σ⁵⁴, thus enabling the σ⁵⁴-RNA polymerase holoenzyme to form transcriptionally competent open promoter complexes [5,6]. In common with other EBPs, NifA contains a central domain belonging to the AAA+ (ATPase associated with various cellular activities) superfamily of ATPases that function as mechanochemical machines to remodel their protein substrates (Figure 1) [7]. In the EBPs, this domain interacts with σ⁵⁴ and couples the energy of ATP hydrolysis with open complex formation [8]. NifA also contains a C-terminal DNA-binding domain with an FIS-like helix–turn–helix motif that binds to enhancer-like elements located upstream of σ⁵⁴-dependent nif promoters (Figure 1). The N-terminal domain of NifA is a sensory input domain belonging to the GAF (cGMP-specific and stimulated phosphodiesterases, Anabaena adenylate cyclases and Escherichia coli FhlA) family of ubiquitous signalling modules that bind small molecules such as cyclic nucleotide monophosphates, tetrapyrroles and formate [9,10]. Our studies have demonstrated that the A. vinelandii NifA GAF domain binds 2-oxoglutarate and this domain controls the response of NifA to NifL [11,12].

In response to environmental conditions, NifL functions to inhibit transcriptional activation by NifA, primarily by inhibiting nucleotide triphosphate hydrolysis by NifA and access to σ⁵⁴ [13]. The N-terminal sensory region of

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Abbreviations used: EBP, enhancer-binding protein; GAF, cGMP-specific and stimulated phosphodiesterases, Anabaena adenylate cyclases and Escherichia coli FhlA; PAS, PER, ARNT, SML, UIox/UR; uridylyltransferase/uridyly-removing enzyme.
A. vinelandii NifL contains two PAS (PER, ARNT, SIM) domains, which we designate as PAS1 and PAS2 (Figure 1). PAS domains are commonly found in proteins that sense redox, oxygen and light and have various cofactors enclosed by a common $alpha/beta$ fold [14,15]. For NifL, the PAS1 domain contains a FAD cofactor and functions in redox sensing [16]. Oxidation of the FAD moiety activates the inhibitory function of NifL so that NifA is inactivated by the oxidized form of the anti-activator. Inhibition is prevented when the FAD moiety in NifL has been fully reduced [17]. The function of the second PAS2 domain in A. vinelandii NifL is unknown and an equivalent domain is not observed in other NifL proteins. The C-terminal domain of NifL is homologous with the catalytic (HATPase-c) domain of the histidine protein kinases and contains conserved residues corresponding to the N, G1, F and G2 boxes that constitute the ATP-binding domain of these proteins [18]. This domain is also homologous with the ATP-binding domains of the GHKL superfamily of ATPases, which include MutL, GyrB and Hsp90 (heat-shock protein 90). Although A. vinelandii NifL does not exhibit ATPase or autophosphorylation activities, we have demonstrated that this domain binds adenosine nucleotides [19]. The binding of ADP to NifL stimulates its inhibitory function and stabilizes the complexes formed with NifA [20,21]. Limited proteolysis experiments suggest that nucleotide binding influences the conformation of the NifL GHKL domain [19]. Although the binding of adenosine nucleotides to NifL might provide a means of sensing the energy charge, the physiological relevance of this interaction is unclear since the dissociation constants for ADP (∼13 $mu$M) and ATP (∼130 $mu$M) are far below the intracellular concentrations of these nucleotides.

Sensing the nitrogen status

NifA activity is stringently regulated by NifL in response to the fixed-nitrogen status to ensure that nitrogenase synthesis only occurs under conditions of fixed-nitrogen deprivation. Truncated forms of NifL lacking the redox-sensing PAS1 domain are competent to inhibit NifA in response to nitrogen-replete conditions, demonstrating that the redox status and nitrogen status are sensed independently by A. vinelandii NifL [19]. The nitrogen status is communicated to NifL through direct interaction with PII-like signal-transduction proteins. These proteins are widely distributed in nature and serve to integrate signals of nitrogen and carbon status to regulate nitrogen metabolism [22,23]. Many bacteria express at least two PII-like proteins, which have common functions but perform discrete physiological roles. These proteins are commonly trimeric and are subject to covalent modification in response to the nitrogen status (Figure 2). In the proteobacteria, PII-like proteins are subject to reversible covalent modification by UTase/UR (uridylyltransferase/uridylyl-removing enzyme) encoded by the glnD gene. This enzyme catalyses the non-co-operative uridylylation of PII proteins with up to three UMP groups attached per trimer. The activity of this enzyme is regulated by the intracellular level of glutamine, a key signal of the nitrogen status. Under nitrogen-limiting conditions, when the concentration of glutamine is relatively low, UTase/UR uridylylates the PII proteins, altering interactions with their targets. Conversely, under conditions of fixed nitrogen sufficiency, when the intracellular concentration of glutamine is relatively high, binding of glutamine to UTase/UR causes the enzyme to switch activity in favour of deuridylylation of the PII proteins. The site of covalent modification is a conserved tyrosine residue located at the tip of a surface-exposed loop (the T-loop) required for the interaction of PII proteins with their targets. In addition to control through covalent modification, the activity of the PII proteins is also modulated by binding of the low-molecular-mass effectors ATP and 2-oxoglutarate.

A. vinelandii is unusual in that it encodes only a single PII protein termed GlnK [24] (formerly known as Av PII [25]). This protein appears to be essential for viability since knockout mutations in the glnK gene are apparently lethal. A. vinelandii GlnK is subject to covalent modification by a homologue of E. coli UTase/UR encoded by glnD (formerly designated the nfrX gene). Strains with mutations in glnD that decrease uridylyltransferase activity are unable to fix nitrogen. However, the Nif$^-$ phenotype of these strains can be suppressed by insertions that inactivate nifL [26]. This suggests that the uridylylation function of the UTase/UR is necessary to prevent NifL from inhibiting...
Nitrogen regulation of the NifL–NifA system

(a) Structure of the E. coli PII signal-transduction protein [PDB (Protein Data Bank) code 2PII] [33], showing the conserved tyrosine residues in the T-loop of each monomer that are subject to reversible covalent modification by UTase/UR. (b) A schematic diagram depicting the role of GlnK in NifL–NifA regulation. Under conditions of excess nitrogen (+N), when the intracellular glutamine concentration is relatively high, GlnK is de-uridylylated by UTase/UR and interacts with the reduced form of NifL (NifL red) to promote the formation of a GlnK–NifL–NifA ternary complex in which NifA is inactive. Under nitrogen-limiting conditions (−N), when glutamine concentrations are low, GlnK is uridylylated by UTase/UR, and is not competent to interact with NifL. Under such conditions, the binding of 2-oxoglutarate to NifA releases the activator from inhibition by NifL.

NifA in A. vinelandii. One interpretation of this result is that the uridylylation status of GlnK mediates the nitrogen regulation of nitrogen fixation through direct interaction with the NifL–NifA system. This is also suggested by the properties of a mutant form of GlnK, namely GlnK–Y51F, which inactivates the uridylylation site in the T-loop and results in constitutive inhibition of NifA activity by NifL [27]. We have investigated the interaction of GlnK with the NifL–NifA system using purified components. The non-covalently modified form of GlnK stimulates the ability of NifL to inhibit transcriptional activation by NifA in vitro; however, when fully uridylylated by A. vinelandii UTase/UR, GlnK does not activate the inhibitory function of NifL [25]. These results are therefore consistent with the in vivo results and demonstrate that uridylylation of GlnK is necessary to prevent inhibition by NifL under conditions of nitrogen deficiency. The biochemical results also indicate that the non-modified form of GlnK is required to signal the nitrogen status under conditions of nitrogen excess (Figure 2). Direct interaction between A. vinelandii GlnK and NifL has been demonstrated by ‘pull-down’ assays, surface plasmon resonance experiments [28] and the yeast two-hybrid system [27]. The in vitro interaction of GlnK with NifL is abolished by a mutation in the T-loop of GlnK and, as expected, could not be detected when the GlnK protein is fully uridylylated. The target of interaction on NifL is the C-terminal nucleotide-binding (GHKL) domain. As for E. coli PII-target interactions, both ATP and 2-oxoglutarate are required for the NifL–GlnK binary interaction [28]. However, the requirement of 2-oxoglutarate for this interaction is clearly different from that observed for the interaction of the E. coli PII protein with its targets. Using isothermal titration calorimetry, we have demonstrated that the A. vinelandii GlnK trimer binds 2–3 molecules of 2-oxoglutarate, in contrast with E. coli PII; under identical conditions, E. coli PII binds only a single molecule of the ligand per trimer and further binding of the ligand is inhibited by negative co-operativity [28]. As a consequence, the GlnK–NifL interaction is less responsive to 2-oxoglutarate within the physiological range when compared with, for example, the interaction of E. coli PII with the histidine protein kinase NtrB [29].

Role of 2-oxoglutarate in the NifL–NifA interaction

In addition to the requirement of 2-oxoglutarate for the formation of the GlnK–NifL binary complex, this effector has also been shown to play a crucial role in modulating interactions between NifL and NifA. As noted above, the binding of adenosine nucleotides to the C-terminal GHKL domain of NifL increases the stability of the NifL–NifA binary complex, thus favouring inhibition of NifA activity [21]. Our studies have revealed that 2-oxoglutarate is an allosteric effector of the NifL–NifA system which binds to the GAF domain of NifA and antagonizes the influence of nucleotides on the inhibitory function of NifL. 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 ADP-bound NifL [25]. However, the presence of 2-oxoglutarate does not apparently influence the activity of NifA in the absence of NifL. The response to 2-oxoglutarate in vitro is within the physiological range observed in E. coli, which varies
Figure 3 | Schematic diagram to illustrate the response of the NifL–NifA system to environmental cues

The PAS1 domain of NifL is shown in purple (reduced form, FADH$_2$) and yellow (oxidized form, FAD) respectively. The blue oval depicts the nucleotide-binding GKH domain of NifL. The GAF, AAA$^+$ and DNA-binding (HTH) domains of NifA are shown in yellow, red and orange respectively. The grey star represents the interaction of 2-oxoglutarate with the GAF domain of NifA. See text for further explanation.

from approx. 100 µM under conditions of nitrogen excess to approx. 1 mM under conditions of nitrogen limitation [30]. When expressed in *E. coli*, the *A. vinelandii* NifL–NifA system is also apparently responsive to the 2-oxoglutarate concentration *in vivo* [31]. 2-Oxoglutarate is an intermediate in the tricarboxylic acid cycle and has been implicated as a key metabolic signal of the carbon status, but the concentration of this metabolite also indirectly reflects the nitrogen status, since it provides the carbon skeleton for nitrogen assimilation. Isothermal titration calorimetry experiments demonstrate that 2-oxoglutarate binds to *A. vinelandii* NifA, but not to NifL. The GAF domain of NifA exhibits stoichiometric binding of 2-oxoglutarate with a dissociation constant of 60 µM, and binding is not observed with a truncated form of NifA lacking the GAF domain [11]. Limited proteolysis experiments suggest that the interaction of 2-oxoglutarate with the GAF domain induces a conformational change in NifA that renders it resistant to the ADP-bound form of NifL. The ability of 2-oxoglutarate to prevent inhibition by NifL is antagonized by the presence of the non-modified form of GlnK, which overrides the effect of 2-oxoglutarate on the GAF domain of NifA, and promotes the formation of the GlnK–NifL–NifA ternary complex [11]. We have therefore argued that the physiological consequences of the binding of 2-oxoglutarate to NifA are most apparent under nitrogen-limiting, reducing conditions, when this effector enables NifA to escape inhibition by NifL under conditions appropriate for nitrogen fixation. This is supported by the observation that some mutations in the GAF domain of NifA result in hypersensitivity to NifL under nitrogen-fixing conditions. One of these mutant proteins, NifA-F119S, which does not bind 2-oxoglutarate, is hypersensitive to NifL *in vitro* and is inhibited by NifL under nitrogen-fixing conditions *in vivo* [12]. These observations support the hypothesis that the binding of 2-oxoglutarate to the GAF domain has a physiologically significant role in decreasing the affinity of NifA for NifL under conditions appropriate for nitrogen fixation.

Integration of antagonistic signals

Our current model for regulation of the NifL–NifA system involves reciprocal conformational changes in which the binding of 2-oxoglutarate to the GAF domain of NifA enables the activator to discriminate between different conformational states of NifL (Figure 3). Binding of adenosine nucleotide to the C-terminal domain of NifL promotes inhibition of NifA activity even when the flavin cofactor in NifL is in the fully reduced form (Figure 3a). However, binding of 2-oxoglutarate to the GAF domain of NifA results in a conformational change that releases inhibition by the reduced form of NifL (Figure 3b). Since 2-oxoglutarate is a metabolic signal of the carbon status and provides an indirect ‘readout’ of the nitrogen status, the availability of this ligand, coupled with the reduced state of NifL, indicates conditions appropriate for nitrogen fixation and, hence, NifA is released to activate *nif* transcription. We propose that NifL undergoes a conformational change when the FAD moiety is
oxidized, since, under these conditions, the oxidized form of NifL is competent to inhibit NifA even in the presence of 2-oxoglutarate (Figure 3c). Hence, the oxygen signal overrides the carbon and nitrogen signals to form an oxidized NifL–NifA binary complex. Under nitrogen-limiting conditions, the GlnK protein is uridylylated and is not competent to interact with NifL. However, under conditions of excess fixed nitrogen, when the GlnK protein is in its non-covalently modified form, it interacts with the C-terminal domain of NifL, enabling the formation of a ternary complex even at high concentrations of 2-oxoglutarate and, thus, the nitrogen signal overrides the carbon signal (Figure 3d). We have evidence that the independent perception of discrete signals by NifL leads to different conformations and/or affinities of NifL for NifA, since we have isolated NifA mutants that are capable of discriminating between the oxidized form of NifL and the form of NifL activated by the interaction with GlnK [12,32]. Hence, the NifL–NifA system is exquisitely sensitive to multiple environmental signals and integrates these to stringently regulate *nif* transcription.

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References


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