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Mutational Analysis of the Nucleotide-binding Domain of the Anti-activator NifL

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The NifL regulatory protein controls transcription of nitrogen fixation genes in *Azotobacter vinelandii* by modulating the activity of the transcriptional activator NifA through direct protein–protein interactions. The ability of NifL to integrate the antagonistic signals of redox and nitrogen status is achieved *via* the involvement of discrete domains in signalling specific environmental cues. NifL senses the redox status *via* an FAD cofactor located within the amino-terminal PAS domain and responds to the fixed nitrogen status by interaction with the signal transduction protein GlnK, which binds to the C-terminal GHKL domain of NifL. The GHKL domain binds adenosine nucleotides and is similar to the core catalytic domain of the histidine protein kinases. Binding of ADP to this domain increases the inhibitory activity of NifL and the formation of protein complexes with NifA. This inhibition is antagonised by the binding of 2-oxoglutarate, a key metabolic signal of the carbon status, to the amino-terminal GAF domain of NifA. In this study we have examined the properties of three mutations within conserved residues in the GHKL domain of NifL that impair signal transduction. All three mutations decrease the affinity of NifL for ADP significantly, but the mutant proteins exhibit discrete properties. The N419D mutation prevents inhibition of NifA activity by NifL both *in vivo* and *in vitro*. In contrast, the G455A and G480A mutations eliminate the redox response, but the mutant proteins retain some sensitivity to the fixed nitrogen status and the ability to interact with the GlnK signal transduction protein. Our data suggest that the absence of the redox switch in the G455A and G480A mutants is a consequence of their inability to override the allosteric effect of 2-oxoglutarate on NifA activity. Overall, these results demonstrate that the binding of adenosine nucleotides to the GHKL domain of NifL plays an important role in counteracting the response of NifA to 2-oxoglutarate, under conditions that are inappropriate for nitrogen fixation.

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Introduction

The NifL–NifA regulatory system controls transcription of the nitrogen fixation (*nif*) genes in *Azotobacter vinelandii via* protein–protein interactions in which the anti-activator protein NifL exclusively controls the activity of the transcriptional activator protein NifA in response to redox, nitrogen and possibly cellular energy status.[1,2] In order to activate transcription, NifA, a σ^{54}-dependent activator belonging to the AAA^{+} superfamily of ATPases, binds to the enhancer-like sequences and contacts the σ^{54}-RNA polymerase (RNAP) holoenzyme positioned at the promoter. NifA hydrolyses nucleoside triphosphates (GTP or ATP) thereby activating DNA melting by σ^{54}-RNAP to promote transcriptional initiation.[3] The ability of NifL to integrate the antagonistic signals of redox

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Abbreviations used: RNAP, RNA polymerase; ITC, isothermal titration calorimetry.

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and nitrogen status is achieved *via* the involvement of discrete domains in signalling specific environmental cues. NifL senses the redox status *via* an FAD cofactor located within an N-terminal PAS domain,[4,5] and deletion of this domain or removal of the FAD moiety renders NifL insensitive to redox.[6,7] Signalling in response to the fixed nitrogen status is achieved *via* interaction of the signal transduction protein GlnK (a member of the PII-like family of nitrogen regulatory proteins), which interacts with the C-terminal domain of NifL.[8,9]

Initially, on the basis of sequence homology, *A. vinelandii* NifL was thought to be a histidine protein kinase;[10] it contains conserved residues corresponding to the N, F, G1, G2 and G3 boxes in the catalytic domain of histidine kinases (also known as domain B) and a region that may correspond to the H box, a dimerisation domain containing the autophosphorylation site in *bona fide* histidine kinases (domain A).[11] Although its partner protein, NifA, is not a member of the response regulator family, there remained the possibility that NifL might control the activity of an "adaptor" protein that, in turn, could modulate NifA activity. However, there is now considerable evidence to indicate that NifL does not have histidine protein kinase activity. Firstly, the conserved histidine residue in the H box is not required for NifL-mediated regulation *in vivo*;[12] secondly, there is no evidence that NifL has either autophosphatase or kinase activities *in vitro*.[13,14] Moreover, signal transduction from NifL to NifA requires stoichiometric protein–protein interactions.[15,16]

The C-terminal domain of NifL, which extends from residues 360–519, possesses homology to the core catalytic domain of the histidine kinases and is a member of the GHKL (HATPase_c) superfamily of ATPases, which includes DNA gyrase B, Hsp90, histidine kinases, SpoIIAB and MutL.[17] The NifL C-terminal domain does not exhibit ATPase activity, possibly because it lacks the catalytic base for water activation in ATP hydrolysis. Nevertheless, this domain binds adenosine nucleotides in a Mg^{2+}-dependent manner with a tenfold higher affinity for ADP than for ATP.[7] Both ADP and ATP stimulate the inhibitory activity of NifL[7,18] by increasing the stability of the NifL–NifA interaction[15] and, in the presence of ADP, NifL is apparently able to interact with both the N-terminal and AAA^+ domains of NifA.[16,19] ATPγS (a non-hydrolysable ATP homologue) also stabilises NifL–NifA complexes and enhances the inhibitory activity of NifL *in vitro* in the presence of GTP,[20] suggesting that ATP hydrolysis is not required for NifL to inhibit NifA activity. Limited proteolysis experiments suggest that both ADP and ATP induce conformational changes in the C-terminal region of NifL.[7] Inhibition by the ADP-bound form of NifL is antagonised by the binding of 2-oxoglutarate to the amino-terminal GAF domain of NifA, enabling the NifL–NifA system to respond to a key metabolic signal of the carbon status.[20,21]

The response of the NifL-NifA system to nucleotides is reminiscent of the interaction between the *Bacillus* anti-sigma factor SpoIIAB (a member of the GHKL family) and the anti-anti-sigma factor SpoIIAA, which is responsive to the binding of ADP. However, this protein–protein interaction is controlled also by the serine kinase activity of SpoIIAB, which phosphorylates SpoIIAA.[22,23] The GHKL domain of *A. vinelandii* NifL is also the target for interaction with the signal transduction protein GlnK, which binds to NifL exclusively in its non-uridylylated form to signal nitrogen replete conditions.[20] This interaction, which requires the binding of 2-oxoglutarate and ATP to GlnK, also stimulates the inhibitory activity of NifL.[9] An analogous interaction occurs between the *Escherichia coli* PII paralogues GlnB and GlnK with the nitrogen regulatory histidine kinase NtrB, resulting in inhibition of autophosphorylation and activation of phosphatase activity.[24] As in the case of the *A. vinelandii* NifL–GlnK interaction, *E. coli* GlnB binds to the GHKL domain of NtrB.[25]

The function of adenosine nucleotide binding by NifL is not understood, nor is the physiological significance of the higher affinity of NifL for ADP as opposed to ATP. One possibility is that NifL is sensitive to the ATP:ADP ratio *in vivo* and senses the cellular energy levels. However, estimates of intracellular concentrations of nucleotide suggest that NifL would be saturated with ADP under all environmental conditions, given the relatively high binding constant of NifL for ADP ($\sim 10\ \mu M$). Although it is assumed that the binding of adenosine nucleotides to NifL favours the NifL–NifA interaction, we cannot entirely rule out the possibility that the increase in inhibitory activity of NifL is a consequence of nucleotide binding to the AAA^+ domain of NifA. Here, we have performed site-directed mutagenesis of conserved residues in the GHKL domain of NifL to investigate their influence on nucleotide binding and NifL activity. Mutations in conserved residues in the N, G1 and G2 boxes, decrease the affinity for ADP and severely impair inhibition by NifL *in vivo*. The ability of NifL to inhibit NifA in response to the redox status is eliminated by these mutations but the G box mutants show weak responses to the nitrogen status. Biochemical experiments demonstrate that the oxidised forms of the mutant proteins are unable to inhibit NifA when 2-oxoglutarate is present, but the G box mutations allow inhibition in response to the GlnK signal transduction protein.

Results

Site directed mutagenesis of NifL

In order to investigate the function of the kinase-like domain of NifL, we chose to mutate three residues in NifL, N419, G455 and G480, equivalent to conserved amino acids in the GHKL superfamily that are important for nucleotide binding (Figure 1). NifL N419 corresponds to a highly conserved

Figure 1. Sequence alignment of *A. vinelandii* NifL with the C-terminal GHKL domains of histidine protein kinases. The following Swiss-Prot sequences were used as input; NIFL_AZOVI (residues 340–519), NTRB_ECOLI (residues 174–349), ENVZ_ECOLI (residues 273–450), PHOQ_ECOLI (residues 310–485), CHEA_THEMA (residues 351–541). Sequences with corresponding structures are boxed; yellow highlights indicate α-helix and green highlights indicate β-sheets. Structural features correspond to the following PDB codes: NtrB, 1r62; EnvZ, 1bxd; PhoQ, 1ido; and CheA, 1b3q. Conserved residues mutated in this work are highlighted in red. The Figure was prepared using the Multalign Viewer extension from the UCSF Chimera molecular graphics program.[47]

residue in the N box, and mutation of this residue to aspartate in members of the histidine protein kinase family eliminates kinase activity and reduces greatly the affinity for ATP. Structures of histidine kinase and related GHKL domains identify the asparagine residue equivalent to N419 as a ligand for the Mg^{2+} cofactor, thereby demonstrating the importance of the N419 residue in Mg^{2+}ATP binding. Residues corresponding to NifL D451 G453 and G455 are highly conserved within the G1 box of the GHKL domain and are involved in direct or indirect (*via* interaction with water) contact with the adenine ring.[26–28] We choose to mutate NifL G455 to alanine as the equivalent mutation in NtrB reduces both kinase and phosphatase activity.[29] The G2 box residues in the GHKL superfamily forms part of the "ATP lid", a flexible loop that encloses the ATP phosphate groups. The conformation of this loop changes, dependent on nucleotide binding.[26,28] We chose to mutate NifL G480 to alanine, since mutation of this conserved residue in NtrB reduces kinase activity and alters the regulation of phosphatase activity.[29,30]

The GHKL domain substitutions disrupt NifL function *in vivo*

The effect of the NifL mutant proteins on transcriptional activation by NifA was measured *in vivo* in *E. coli* using a two-plasmid system consisting of a *nifHp-lacZ* reporter and a second plasmid expressing *nifL* and *nifA* from a constitutive promoter.[7,31–33] Activation of the reporter was measured under anaerobic or aerobic growth conditions either in the presence of excess fixed nitrogen or under nitrogen-limiting conditions.

Wild-type NifL inhibited NifA activity about tenfold in response to oxygen (Figure 2(a), compare open and cross-hatched bars) and ∼100-fold in response to fixed nitrogen (Figure 2(a), compare open and left-handed hatched bars), comparable to our previous results.[7] However, as reported previously,[31,32] NifL inhibited NifA activity even under anaerobic nitrogen-limiting growth conditions (conditions suitable for nitrogen fixation), since the level of expression of the reporter gene was tenfold lower than when NifA alone was present (Figure 2, compare open bar in (a) for plasmid pPR34 (∼2000 Miller units) with open bar in (b) for plasmid pPR39 (∼20,000 Miller units); note the change of scale). In contrast, the NifL-N419D and NifL-G480A proteins were unable to inhibit NifA activity under any of the growth conditions tested, and the level of reporter activation was similar to that of NifA in the absence of NifL (Figure 2(b)). Therefore, these two NifL mutant proteins are unable to respond to either the redox or nitrogen status *in vivo*. The NifL-G455A protein was also unable to inhibit NifA in response to oxygen, but an approximately two- to threefold increase in the level of inhibition was observed in the presence of fixed nitrogen, suggesting that the mutant protein retains some ability to inhibit NifA under these conditions (Figure 2(b)).

We have recently identified a mutation in the N-terminal GAF domain of NifA that renders the activator hypersensitive to inhibition by NifL.[33] The mutant NifA-F119S protein is sensitive to wild-type NifL even under anaerobic, nitrogen-limiting conditions, because this protein is unable to respond to 2-oxoglutarate, which normally binds to the GAF domain to prevent inhibition by NifL under conditions appropriate for nitrogen fixation.[21] It was therefore of interest to determine the properties of the NifL GHKL mutant proteins, when combined with the NifA-F119S substitution.

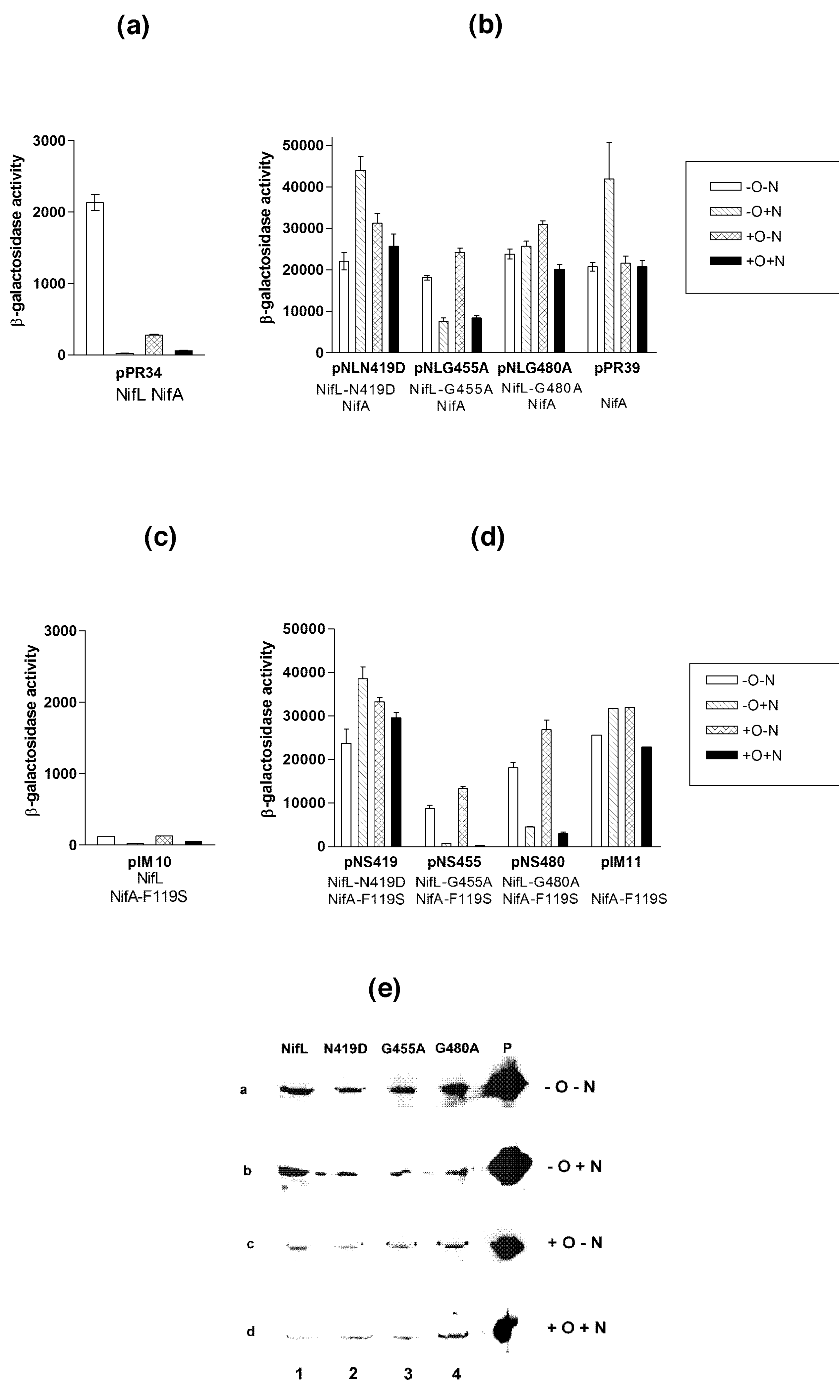


Figure 2. Influence of mutant NifL proteins on transcriptional activation by NifA *in vivo*. Cultures were grown under the following four conditions; anaerobically, under nitrogen-limiting conditions with casein hydrolysate as nitrogen source (–O–N, white bars), anaerobically with $(NH_4)_2SO_4$ as nitrogen source (–O+N, hatched bars), aerobically with casein hydrolysate as nitrogen source (+O–N, cross-hatched bars) aerobically with $(NH_4)_2SO_4$ as nitrogen source (+O+N, black bars). In (a)–(d) cultures were assayed for β-galactosidase activity as a reporter of NifA-mediated activation from the *nifH-lacZ* fusion on plasmid pRT22 in *E. coli* strain ET8000 as described in Materials and Methods. Values represent the means of at least three independent determinations. The following plasmids were present in addition to the reporter pRT22: (a) pPR34 (wild-type NifL and NifA); (b) pNLN419D, pNLG455A and pNLG480A (mutant NifL and wild-type NifA) or pPR39 (NifA only); (c) pIM10 (wild-type NifL and NifA-F119S); (d) pNS519, pNS455 and pNS480 (mutant NifL and NifA-F119S) or pIM11 (NifA-F119S only). (e) Western analysis of NifL expressed in cultures analysed in (a) and (b) using anti-NifL serum. P indicates purified NifL protein.

As demonstrated previously,[33] NifA-F119S is hypersensitive to NifL under all growth conditions (Figure 2(c)). However, NifA-F119S was not more responsive to NifL-N419D than wild-type NifA, indicating that the latter mutation completely inactivates the inhibitory properties of NifL (Figure 2, compare (b) and (d)). In contrast, NifA-F119S was more sensitive than wild-type NifA to inhibition by NifL-G455A, particularly when excess fixed nitrogen was present in the growth medium (Figure 2, compare (b) and (d)). Although the NifL-G480A mutant was not competent to inhibit wild-type NifA, inhibition of NifA-F119S activity was observed under conditions of fixed nitrogen excess (Figure 2(d)). Hence, these observations suggest that the NifL-G455A and NifL-G480A mutants are competent to inhibit the activity of NifA-F119S in response to the nitrogen source.

To determine the stability of the mutant proteins, Western blotting was carried out on whole cell extracts grown under the conditions of the *in vivo* assays and the level of NifL was detected using a polyclonal NifL antiserum. The mutant proteins were found to accumulate at levels similar to wild-type NifL when grown under the four conditions used for assaying NifL activity (Figure 2(e)). Therefore, it appears that the phenotypes of the NifL GHKL mutants *in vivo* are due to the intrinsic

properties of the mutant proteins *per se* rather than effects on protein stability.

The fixed nitrogen status is conveyed to NifL *via* interaction with the non-uridylylated form of the signal transduction protein GlnK, which binds to the GHKL domain of NifL.[9] We have observed previously that the *A. vinelandii* NifL-NifA system is responsive to regulation by PII-like proteins in *E. coli*, and that the nitrogen response of NifL is absent from *E. coli* mutants that fail to express both the GlnB and GlnK paralogues.[31] Since the nitrogen response of the NifL-G455A and NifL-G480A mutants in Figure 2 implicates interaction with the PII signal transduction proteins, we examined the activity of these mutants in the *glnB*, *ntrC* strain RT8000, which does not express GlnB or GlnK. The response of wild-type NifL to fixed nitrogen was severely impaired in this strain, as observed previously (Figure 3(a), compare open and right crossed hatched bars).[31] The ability of NifL-G455A to inhibit NifA in the presence of excess fixed nitrogen in the ET8000 strain (Figure 2(b)) was absent from the *glnB*, *ntrC* strain (Figure 3b), suggesting that NifL-G455A is responsive to PII signal transduction proteins *in vivo*. However, the nitrogen response of NifL-G480A in strain ET8000 was insufficient to determine whether this mutant is responsive to the PII-like proteins.

The NifA-F119S mutant remains responsive to wild-type NifL under both nitrogen-limiting and nitrogen-excess conditions, presumably because this form of NifA is unable to resist inhibition by NifL, even when the PII-like proteins are absent (Figure 3(c)).[33] However, the ability of the NifL-G480A mutation to inhibit NifA-F119S in the presence of excess fixed nitrogen in the ET8000 strain (Figure 2(d)), was not observed in the *glnB*, *ntrC* strain (Figure 3(d)). This phenotype suggests that the inhibition of NifA-F119S by NifL-G480A in response to fixed nitrogen requires the PII-like proteins. A similar phenotype was shown by the NifL-G455A, NifA-F119S double mutant in the *glnB*, *ntrC* strain. This implies that the PII paralogues are required for both of these NifL GHKL mutants to inhibit the activity of NifA-F119S.

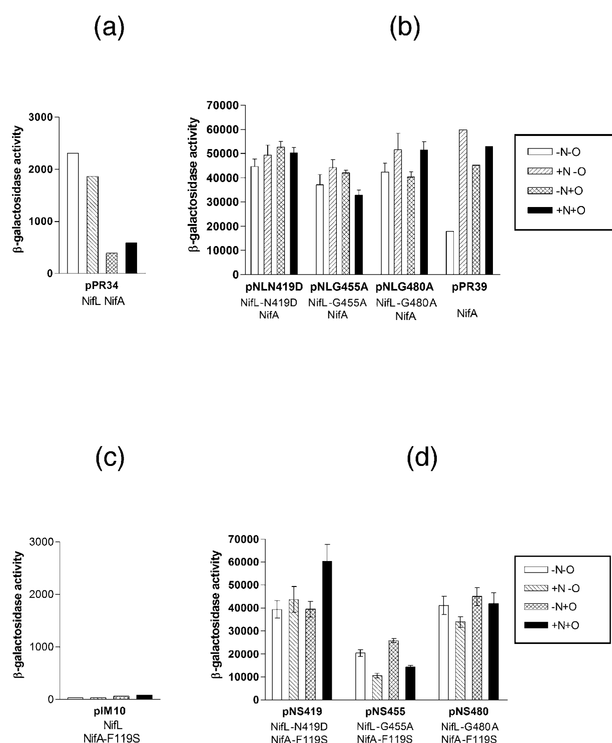


Figure 3. Influence of NifL mutations on transcriptional activation by NifA in the absence of GlnB and GlnK *in vivo*. Cultures were assayed for β-galactosidase activity as a reporter of NifA-mediated activation from the *nifH-lacZ* fusion on plasmid pRT22 in *E. coli* strain RT8000 (*glnB*, *ntrC*) as described in Materials and Methods. Growth conditions, legends and bar appearance are identical with those in Figure 2. The following plasmids were present in addition to the reporter pRT22: (a) pPR34 (wild-type NifL and NifA); (b) pNLN419D, pNLG455A and pNLG480A (mutant NifL and wild-type NifA) or pPR39 (NifA only); (c) pIM10 (wild-type NifL and NifA-F119S); and (d) pNS519, pNS455 and pNS480 (mutant NifL and NifA-F119S).

The NifL mutant proteins are not deficient in FAD binding

To further characterise the mutant proteins *in vitro*, hexahistidine-tagged versions of the NifL mutant proteins were purified using metal chelate affinity chromatography. All three mutant proteins co-purified with FAD-specific spectral features and appeared to bind 1 mol of FAD per NifL monomer (data not shown), as demonstrated previously for wild-type NifL.[4,6] As the FAD moiety is the means by which NifL senses the redox status, it is unlikely that these mutants are deficient in perception of the redox signal.

Influence of GHKL mutations on the binding of ADP

The binding of ADP to NifL and mutant derivatives was investigated by isothermal titration calorimetry in buffer containing a saturating concentration of Mg^{2+} (10 mM) (Figure 4). Titration of wild-type NifL (purified as an N-terminal hexahistidine fusion, N_{6his}NifL) with ADP gave a k_D of 13.6($\pm$0.7) μM. This dissociation constant is similar to that obtained previously (apparent $k_D \sim 13$ μM) using limited proteolysis as an indirect method to assay binding.[7] Data analysis gave a best fit to a single-site binding model with a stoichiometry of 0.97($\pm$0.01) mol/monomer (Figure 4, filled squares). In contrast, titration of N_{6his}NifL-N419D (78.5 μM) with ADP showed no interaction over a fourfold molar excess of this ligand (Figure 4, open circles) and no interaction was observed up to 13 fold molar excess (data not shown). Thus, this mutant protein is defective in nucleotide binding. Similarly, only small changes in enthalpy were observed upon titration of N_{6his}NifL-G455A and

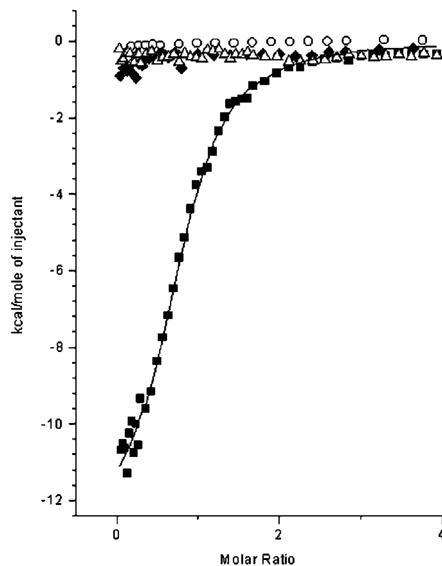


Figure 4. Calorimetric titration of the binding of ADP to wild-type and mutant NifL proteins. Binding isotherms obtained by injecting ADP into 55.5 μM N_{6his}NifL-wild-type (filled squares), 78.5 μM N_{6his}NifL-N419D (open circles), 29.8 μM N_{6his}NifL-G455A (open triangles) and 35.7 μM N_{6his}NifL-G480A (filled diamonds). Concentrations of protein were calculated as monomer values. The integrated heats of reaction are plotted against the molar ratio of total ligand concentration to total protein concentration. Concentrations of ligand before injection ranged from 1 mM to 5 mM, depending upon the concentration of protein. Titrations were carried out in buffer containing 3.5 mM magnesium acetate as described in Materials and Methods. For N_{6his}NifL-G455A, the concentration of glycerol in the buffer was increased to 20% (v/v) and potassium acetate was replaced by 200 mM sodium chloride. The continuous line shows the best fit to the data according to an equivalent site model, giving a stoichiometry of $n=0.97\pm0.1$ and $k_D=13.6(\pm0.7)$ μM for the interaction between N_{6his}NifL-wild-type and ADP.

N_{6his}NifL-G480A with ADP (Figure 4, triangles and diamonds), indicating that these mutant proteins also are substantially defective in the binding of this nucleotide.

NifL GHKL mutants do not undergo adenosine nucleotide-mediated conformational changes

Limited proteolysis experiments indicate that the conformation of wild-type NifL changes upon binding ADP and ATP.[7] In the absence of nucleotide, the major fragments generated upon partial trypsin digestion of $NifL_{6his}$ are a 53 kDa fragment (labelled A in Figure 5), which extends from the N terminus to a cleavage site within the GHKL domain, and a 33 kDa fragment (labelled B in Figure 5), which represents the N-terminal region of NifL [7] (Figure 5(a)). The presence of MgADP or MgATP decreases the rate of trypsin digestion of the full-length protein and apparently prevents proteolysis of the GHKL domain, since fragment A is no longer evident. An additional 18 kDa fragment (labelled D) is more prominent in the presence of adenosine nucleotides (Figure 5(a)). This fragment constitutes the GHKL domain. Thus, the binding of adenosine nucleotides to NifL protects the GHKL domain from trypsin digestion.[7]

The kinetics of trypsin digestion of NifL-$N419D_{6his}$ in the absence of nucleotide was apparently slower than that of wild-type NifL, and the proteolysis pattern was somewhat different (Figure 5(b)), perhaps indicative of an altered conformation. Nevertheless, the overall pattern of proteolysis was not altered significantly by the presence of adenosine nucleotides. Both NifL-G455A and NifL-G480A gave patterns of trypsin digestion similar to that of $NifL_{6his}$ in the absence of nucleotide. However, the kinetics of trypsin digestion did not change in the presence of either MgADP or MgATP. Fragment A remained prominent under these conditions, and protection of fragment D was not observed (Figure 5(c) and (d)). These results suggest that the conformation of the mutant proteins does not alter in the presence of adenosine nucleotides and support the conclusion from the isothermal titration calorimetry (ITC) experiments that the GHKL mutants are defective in nucleotide binding.

Influence of NifL mutations on complex formation with NifA

We showed previously that the presence of adenosine nucleotides increase the stability of complexes formed between NifL and NifA and the binary complex can be isolated from crude extracts when MgADP is included in chromatography buffers.[15,16] We anticipated that mutations in the GHKL domain could influence the level of complex formation with NifA. Histidine-tagged forms of the mutant NifL proteins were used in a "pull-down" assay to detect NifA binding in NTA microtitre plates at various concentrations of ADP. A 1 : 2 molar ratio of NifL/NifA was incubated with the matrix. The amount of NifA retained with NifL after washing and subsequent elution from the plates was quantified by densitometry of the protein bands identified by SDS-PAGE (Figure 6). As observed previously, the complex was not detectable with wild-type (N_{6his}NifL) in the absence of MgADP. Approximately 50% of NifA was retained with N_{6his}NifL in the range 5–50 μM ADP, which is expected, as the affinity of NifL for this ligand is within this range. However, binding of NifA increased to almost 100% at 2 mM ADP (Figure 6(a)). This suggests either the presence of a second, low-affinity binding site on NifL or it is a consequence of the binding of this nucleotide to NifA, which is known to alter the conformation of NifA,[16] and by analogy with other members of the sub-family of σ^{54}-dependent AAA^+ activators, the oligomerisation state.[34] In contrast to the wild-type protein, the N_{6his}NifL-N419D mutant protein was

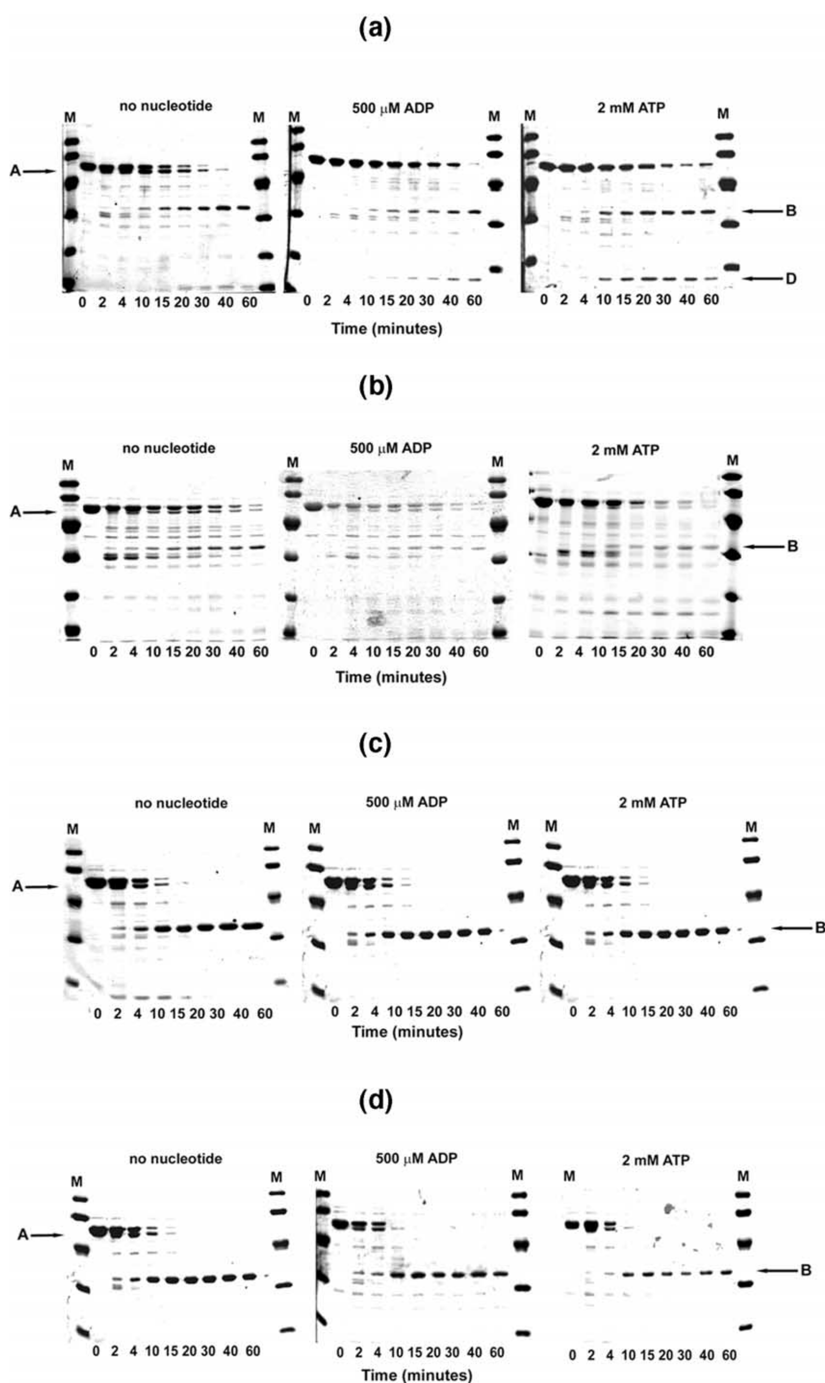
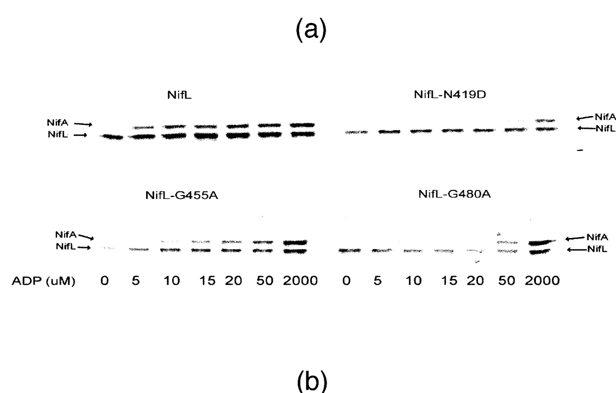


Figure 5. Limited trypsin proteolysis of NifL proteins and the influence of nucleotides on the digestion pattern. Proteins were incubated with trypsin (200 : 1, w/w) for the times indicated beneath each lane, in the presence or in the absence of nucleotides as indicated above each gel. Lanes marked M are molecular mass markers. The arrows indicate previously defined cleavage products discussed in the text.[7] (a) Wild-type NifL ($NifL_{6his}$); (b) NifL-$N419D_{6his}$; (c) NifL-$G455A_{6his}$; and (d) NifL-$G480A_{6his}$.

substantially deficient in binding NifA at low concentrations of ADP but was competent to form complexes with NifA at 2 mM MgADP (Figure 6(b)). In contrast, the N_{6his}NifL-G455A mutant protein bound NifA at low concentrations of ADP, although the level of NifA retained was approximately 50% lower than that of N_{6his}NifL at concentrations of 20 μM ADP or less. The N_{6his}NifL-G480A mutant protein had properties intermediate between that of N_{6his}NifL-N419D and N_{6his}NifL-G455A, being deficient in binding NifA at low concentrations of ADP, but gave substantial levels of binding at concentrations of ADP of 50 μM and above. These observations suggest that the GHKL domain substitutions influence binding to NifA at low concentrations of ADP, with the NifL-N419D substitution having a more severe effect than NifL-G480A or NifL-G455A.

Influence of the NifL mutations on NifA activity *in vitro*

In the presence of nucleoside triphosphates, NifA activates transcription initiation by σ^{54}-RNAP by catalysing the formation of heparin-resistant open promoter complexes in which the duplex DNA surrounding the transcription start site is locally denatured. We used a gel mobility-shift assay to



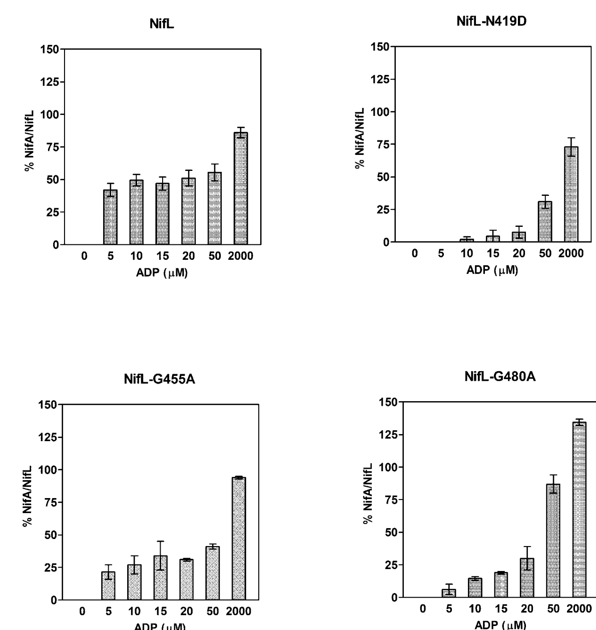
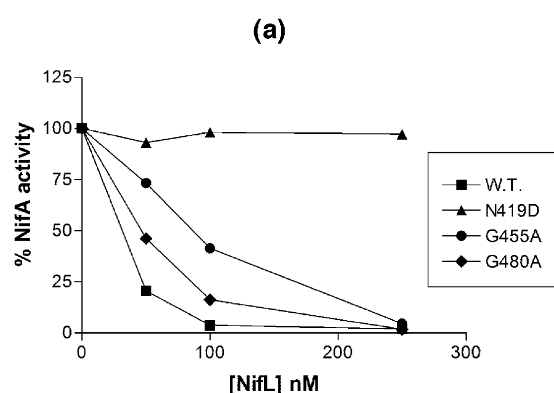


Figure 6. Influence of ADP concentration on the stability of binary complexes formed between oxidised NifL proteins and NifA. Hexahistidine-tagged forms of NifL were immobilised on NTA micro-titre plates and co-retention of NifA in response to the indicated concentration of ADP was analysed by SDS-PAGE as described in Materials and Methods. (a) An example of the primary data. (b) Densitometric quantification of the amount of NifA retained as a percentage of NifL. Results are the mean of two independent experiments.



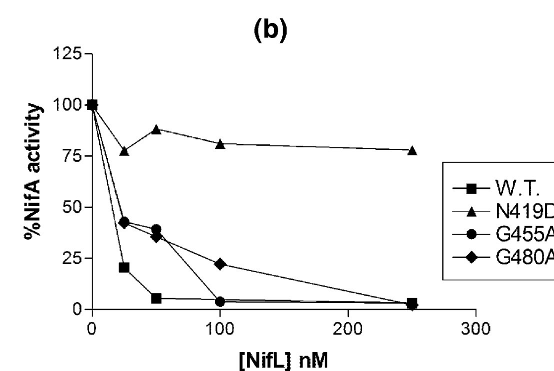


Figure 7. Influence of oxidised NifL proteins on the formation of open promoter complexes by NifA in the presence of adenosine nucleotides. The data are plotted relative to the extent of NifA activity in the absence of NifL on the *y* axis relative to the concentration of NifL (calculated as a tetramer). All reactions contained 125 nM NifA (tetramer) and the other components required for open complex formation listed in Materials and Methods. NifL proteins were: N_{6his}NifL (squares), N_{6his}NifL-N419D (triangles), N_{6his}NifL-G455A (circles), N_{6his}NifL-G480A (diamonds). (a) Response of NifA to oxidised NifL in the presence of 50 μM ADP. Reactions contained 4 mM GTP as donor for nucleotide hydrolysis. (b) Response of NifA to oxidised NifL in the presence of 3.5 mM ATP and 0.5 mM GTP. Reactions contained 12 mM creatine phosphate and 20 units/ml of creatine kinase to provide an ATP-regenerating system.

measure the ability of the NifL mutant proteins to inhibit open promoter complex formation by NifA. GTP was used to catalyse open complex formation by NifA at the *nifH* promoter and, as initiating nucleotide, stabilises the complexes to heparin challenge. We showed previously that the oxidised form of NifL inhibits transcription initiation and open complex formation by NifA, and that this inhibitory activity is increased by the addition of ADP.[6,18] As expected, in the presence of 50 μM ADP, oxidised wild-type NifL (N_{6his}NifL) inhibited NifA activity completely at stoichiometric concentrations and above (Figure 7(a), squares). In contrast, the oxidised form of the N_{6his}NifL-N419D mutant protein was unable to inhibit NifA activity at any of the concentrations tested (Figure 7(a), triangles). Surprisingly, the N_{6his}NifL-G480A mutant protein was competent to inhibit NifA activity under these conditions, although higher concentrations were required compared to wild-type NifL (Figure 7(a), diamonds). The N_{6his}NifL-G455A mutant protein was also competent to inhibit NifA activity but was less effective than N_{6his}NifL-G480A at lower concentrations of protein (Figure 7(a), circles). When present at high concentrations, ATP increases inhibition of NifA activity by NifL. Since this

inhibition is observed in the presence of an ATP-regenerating system, it is likely that ATP is the effector rather than ADP released *via* hydrolysis by NifA.[20] In the presence of 3.5 mM ATP and a regenerating system, the oxidised mutant proteins gave a pattern of inhibition similar to that observed in the presence of ADP, with N_{6his}NifL-N419D being ineffective as an inhibitor, whereas the N_{6his}-NifL-G455A and N_{6his}NifL-G480A proteins were able to inhibit NifA activity, although higher concentrations of these mutant proteins were required in comparison with wild-type NifL (Figure 7(b)).

While the *in vitro* data for N_{6his}NifL-N419D are in agreement with our *in vivo* observations (Figure 2), inhibition of NifA activity *in vitro* by the oxidised forms of N_{6his}NifL-G455A and N_{6his}NifL-G480A was unexpected. Since inhibition of NifA by these two mutants requires higher concentrations of protein than wild-type NifL, it is possible that the level of expression of the mutant proteins *in vivo* is insufficient to inactivate NifA. An alternative possibility is that inhibition of NifA activity by these mutant forms is more sensitive to the concentrations of nucleotide compared with the wild-type. The concentrations of adenosine nucleotide used in the open complex assays in Figure 7 were saturating with respect to the binding constants for wild-type NifL. However, since the mutant proteins are less competent to bind NifA than wild-type at low concentrations of ADP (Figure 6), we considered the possibility that N_{6his}NifL-G455A and N_{6his}NifL-G480A might be ineffective as inhibitors of NifA at lower concentrations of ligand. To investigate this, open complex assays were performed with 4 mM GTP at a 2 : 1 molar ratio of NifL/NifA, and inhibition of NifA activity was monitored in response to concentration of ADP. Under these conditions, oxidised wild-type N_{6his}NifL inhibited NifA at concentrations of ADP of 2.5 μM and above (Figure 8), consistent with the binding constant for ADP observed in the ITC experiments (Figure 4) and the influence of ADP concentration on formation of NifL–NifA complexes (Figure 6). In contrast, substantial inhibition by oxidised N_{6his}NifL-G455A and N_{6his}NifL-G480A was observed only at concentrations of ADP of 50 μM and above (Figure 8). Hence, the decreased affinity of these mutant proteins for ADP influences their ability to modulate NifA activity at relatively low concentrations of this nucleotide.

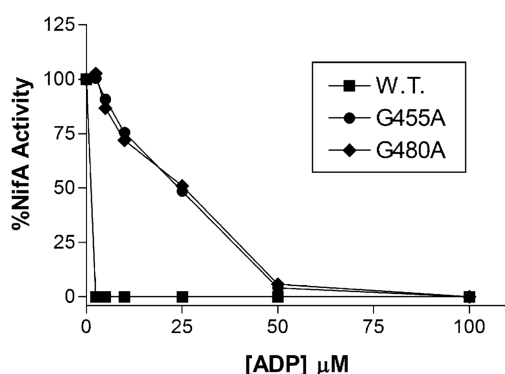


Figure 8. Influence of ADP concentration on inhibition of NifA activity by oxidised NifL. NifA activity was measured by the formation of open promoter complexes and plotted as the percentage of open promoter complexes formed in the absence of NifL. Reactions contained 4 mM GTP, 125 nM (tetramer) NifA and either 250 nM (tetramer) N_{6his}NifL (squares), N_{6his}NifL-G455A (circles) or N_{6his}NifL-G480A (diamonds). Concentrations of ADP are indicated on the *y* axis.

Effect of 2-oxoglutarate binding to NifA on NifL inhibition

We showed previously that the binding of 2-oxoglutarate to the GAF domain of NifA plays an important role in relieving inhibition by the ADP-bound form of NifL under conditions appropriate for nitrogen fixation.[20,21] Thus when NifL is in the reduced form and fixed nitrogen is limiting, the binding of 2-oxoglutarate to NifA prevents inhibition by NifL. However, the response to this ligand is overridden either when NifL is oxidised, or when the signal transduction protein GlnK interacts with NifL.[33] It was therefore of interest to determine whether the inhibitory activity of the NifL mutants was altered in the presence of 2-oxoglutarate. Since the oxidised form of wild-type NifL antagonises the 2-oxoglutarate response of NifA, we examined whether this was also the case with the GHKL mutants. As expected, in the presence of 50 μM ADP, oxidised NifL inhibited NifA irrespective of the concentration of 2-oxoglutarate (Figure 9, squares). Also, as anticipated, the N_{6his}NifL-N419D protein did not inhibit NifA activity under these conditions (data not shown). In contrast, inhibition of NifA by the oxidised forms of the N_{6his}NifL-G455A and N_{6his}NifL-G480A proteins was responsive to 2-oxoglutarate (Figure 9, circles and diamonds) within the range of concentration observed previously for truncated NifL proteins that cannot perceive the redox signal.[20,21,33] Thus, in contrast to wild-type NifL, oxidised N_{6his}NifL-G455A and N_{6his}NifL-G480A are defective in the inhibition of NifA activity in the presence of physiological concentrations of 2-oxoglutarate. This may explain why these mutants are unable to inhibit NifA under oxidising conditions *in vivo*.

Response of NifL mutants to the GlnK signal transduction protein

The results of previous studies have established that the nitrogen status is conveyed to NifL by the interaction of the non-modified form of the signal transduction protein GlnK with the nucleotide-binding GHKL domain.[9] Since the *in vivo* analysis suggested that NifL-G455A and NifL-G480 proteins are partially responsive to the nitrogen status, and this response is apparently dependent upon expression of the PII paralogues in *E. coli* (Figure 3),

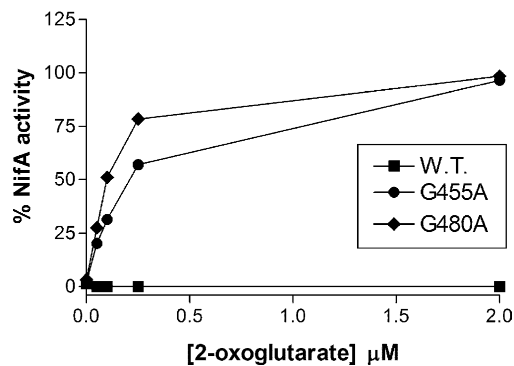


Figure 9. Influence of 2-oxoglutarate on the inhibition of NifA activity by oxidised NifL. NifA activity was measured by the formation of open promoter complexes and plotted as the percentage of open promoter complexes formed in the absence of NifL. Reactions contained 3.5 mM ATP, 0.5 mM GTP, 12 mM creatine phosphate, 20 units/ml of creatine kinase and the concentration of 2-oxoglutarate indicated on the *x*-axis. Reactions contained 125 nM (tetramer) NifA and either 250 nM (tetramer) N_{6his}NifL (squares), N_{6his}NifL-G455A (circles) or N_{6his}NifL-G480A (diamonds).

it was of interest to determine the influence of non-uridylylated GlnK on the activity of the mutant proteins *in vitro.* Truncated forms of NifL that lack the redox-sensing PAS domain are responsive to the interaction with GlnK, which increases inhibition by NifL. The ligands ATP and 2-oxoglutarate, which bind to GlnK, are required for the interaction with the GHKL domain of NifL.[9] Since the oxidised form of wild-type NifL is fully competent to inhibit NifA activity in the presence of these ligands, no additional increase in inhibition was expected if GlnK was added to reactions containing N_{6his}NifL. However, since the oxidised GHKL mutant proteins were defective in inhibiting NifA, particularly in the presence of 2-oxoglutarate, it was possible to examine the influence of GlnK on the activity of the mutant proteins. In these experiments, we used a saturating concentration of 2-oxoglutarate (2 mM) that allows substantial NifA activity in the presence of oxidised N_{6his}NifL-G455A and N_{6his}NifL-G480A (Figure 9), and then determined the level of inhibition in response to increasing concentrations of GlnK (Figure 10). The N_{6his}NifL-G455A and N_{6his}NifL-G480A proteins were clearly responsive to GlnK in this assay, resulting in complete inhibition of NifA activity at a GlnK concentration of 500 nM. Oxidised N_{6his}NifL-G455A was more effective at inhibiting NifA than N_{6his}NifL-G480A at lower GlnK concentrations (Figure 10). In contrast, N_{6his}NifL-N419D was not responsive to the presence of GlnK. Therefore, although N_{6his}NifL-G455A and N_{6his}NifL-G480A are defective in binding ADP, these proteins still retain the capacity to respond to GlnK. These results mirror the *in vivo* phenotype of the mutants (Figures 2 and 3), since the NifL-G455A mutant was more responsive to the nitrogen source than NifL-G480A, and NifL-N419D did not demonstrate a fixed nitrogen response.

Discussion

Our studies demonstrate that mutations in conserved residues in the C-terminal domain of NifL impair signal transduction to NifA, reinforcing the crucial role of nucleotide binding to the GHKL domain for NifL function *in vivo*. However, although the N419D, G455A and G480A substitutions disrupt NifL activity *in vivo* and influence binding to ADP significantly, it is apparent that these substitutions have discrete effects on NifL function. While all three substitutions inactivate signal transduction in response to the redox status, suggesting that nucleotide binding to the NifL C-terminal domain is essential for communication of the redox switch to NifA, the G455A mutant and G480A mutant proteins exhibited a partial response to the fixed nitrogen status *in vivo* and retained the capacity to interact with the GlnK signal transduction protein. Surprisingly, although all three substitutions reduce the affinity for ADP and the associated nucleotide-dependent conformational changes observed with the wild-type protein, the mutant proteins were able to form stable binary complexes with NifA *in vitro* at relatively high concentrations of MgADP (>50 μM ADP). Binary complexes formed between NifL-N419D and NifA were apparently inactive, in agreement with the *in vivo* data, whereas the oxidised forms of the NifL-G455A and NifL-G480A proteins inhibited NifA activity at ADP concentrations above 50 μM. This implies that the conformational change induced by the binding of ADP to the C-terminal domain of NifL is not absolutely required for inhibition of

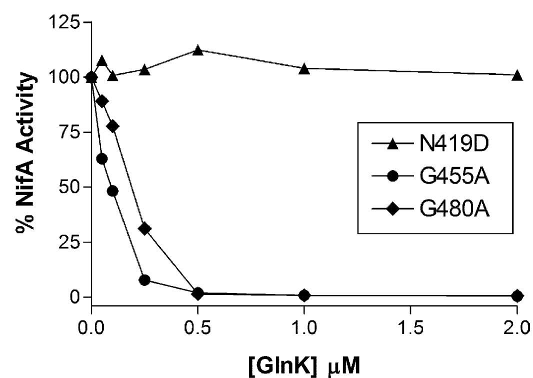


Figure 10. Influence of GlnK on the activities of oxidised mutant NifL proteins in the presence of 2-oxoglutarate. NifA activity is plotted on the *y* axis as a percentage of open promoter complexes formed by NifA in the absence of NifL and GlnK. Reactions contained 3.5 mM ATP, 0.5 mM GTP, 12 mM creatine phosphate, 20 units/ml of creatine kinase, 2 mM 2-oxoglutarate and the concentration of GlnK indicated on the *x* axis. Reactions contained 125 nM (tetramer) NifA and either 250 nM (tetramer) N_{6his}NifL-N419D (triangles), N_{6his}-NifL-G455A (circles) or N_{6his}NifL-G480A (diamonds).

NifA activity by the NifL-G455A and NifL-G480A proteins.

As the oxidised forms of the NifL-G455A and NifL-G480A proteins are competent to inhibit NifA activity *in vitro* at concentrations of ADP above 50 μM, why are they unable to inhibit NifA activity *in vivo*? One possibility is that the mutations reduce the affinity for nucleotide below the cellular concentration available to activate NifL under physiological conditions. Although the concentration of free (unbound) nucleotide is not known, we feel that this possibility is unlikely, since the total concentration of ADP in enteric cells is reported to be ∼800 μM,[35] which would be sufficient to activate the mutant proteins under our *in vitro* conditions. However, our data also demonstrate that oxidised NifL-G455A and NifL-G480A are unable to inhibit the 2-oxoglutarate-bound form of NifA. Hence, in contrast to oxidised wild-type NifL, which is competent to inhibit NifA even when 2-oxoglutarate is present, inhibition of NifA activity by oxidised NifL-G455A and NifL-G480A is sensitive to the addition of 2-oxoglutarate. Hence, it is likely that the inhibitory activity of the mutant proteins is abolished *in vivo* because these mutants are unable to interact productively with NifA when 2-oxoglutarate is bound to the GAF domain of the activator. Since, in contrast to wild-type NifL, the NifL-G455A and NifL-G480A mutants do not apparently undergo a change in conformation in response to high concentrations of ADP, it is feasible that such a conformational change is necessary for oxidised NifL to inhibit the 2-oxoglutarate-bound form of NifA.

The interaction of *A. vinelandii* GlnK with the C-terminal domain of NifL antagonises the influence of 2-oxoglutarate on NifA to promote the formation of an inhibitory GlnK-NifL-NifA ternary complex.[9,21] Our *in vivo* studies suggest that NifL-G455A and NifL-G480A retain some capacity to inhibit NifA when GlnK is present. In agreement with these results, we observed that NifL-G455A and NifL-G480A, but not NifL-N419D, can be activated by GlnK to inhibit NifA *in vitro* in the presence of 2-oxoglutarate. Thus, in contrast to the oxidised forms of the mutant proteins, the presence of GlnK enables NifL-G455A and NifL-G480A to override the influence of 2-oxoglutarate on NifA. This suggests that the mutations do not impair interactions with GlnK completely and, conceivably, nucleotide binding to the C-terminal domain of NifL may not be essential for the GlnK interaction.

Although NifL is not a *bona fide* histidine protein kinase, it is of interest to compare the results of this study with data obtained from equivalent mutations in well-characterised representatives of histidine protein kinases. A mutation in the N box in CheA equivalent to NifL-N419D, reduces the affinity for ATP by ∼100-fold and eliminates kinase activity, since a stabilising contact with the nucleotide is lost.[36] The equivalent mutation in EnvZ (N347D) also is severely defective in ATP binding and kinase activity but retains phosphatase activity that is antagonised by high concentrations of ADP.[37] In the absence of structural information, it is difficult to rationalise why the NifL-N419D mutant is more defective than the NifL-G455A and NifL-G480A mutants in inhibiting NifA activity. However, the NifL-N419D protein apparently exhibited a reduce rate of trypsin hydrolysis compared with the other mutants and wild-type NifL, suggestive of an altered conformation.

Structural studies on CheA suggest that mutation of the G1 box will disrupt the structure of the nucleotide-binding cavity,[28] in agreement with the observation that CheA G1 box mutations have severe effects on ATP binding.[36] The equivalent mutation to NifL-G455A, located in the G1 box of NtrB (G291A), is partially defective in both kinase and PII-regulated phosphatase activity.[29] Since there is no evidence to suggest that the G1 region of NtrB is involved in the interaction with PII,[38,39] it is possible that the phosphatase defect arises from decreased affinity for ATP. Evidence for the involvement of the G2 box in controlling phosphatase activity of different histidine protein kinases is contradictory. The NtrB mutant protein G313A, which contains a mutation in the G2 box at an equivalent position to NifL-G480A, exhibits phosphatase activity in the absence of PII.[29] However, the equivalent mutation in EnvZ (G403A) prevents activation of phosphatase activity by ADP and decreases the affinity for ATP significantly.[40]

Current evidence from various histidine kinases suggests that the "ATP lid", which includes the G2 box of the kinase catalytic domain, interacts with the homodimeric core domain or H box to modulate both kinase and phosphatase activity.[28,41] Although the central region of NifL is not strongly homologous to the H box of the histidine kinases, it is conceivable that nucleotide interactions alter the juxtaposition of the C-terminal and central domains, as suggested by the change in the pattern of proteolysis that occurs upon ADP binding. This possibility is supported by our recent observation that the phenotype of the NifL-G455A and NifL-G480A mutants is suppressed by a mutation in the H box region, R306C, which restores regulation of NifA activity *in vivo*.[42] Hence, communication between the H box region and the GHKL domain of NifL may be important for signal transduction. It is conceivable that the binding of GlnK to the C-terminal domain alters interactions between the ATP lid and the H box region, as has been proposed for the interaction of PII with NtrB.[40] The properties of the NifL-G455A and NifL-G480A mutants indicate that, although these substitutions decrease the affinity for ADP, productive interactions with NifA are still possible, provided that 2-oxoglutarate is absent. However, if the conformation of NifA is altered by the binding of 2-oxoglutarate, then the interaction with GlnK is necessary for inhibition of NifA activity by these mutant proteins. Hence, co-operative conformational changes induced by the binding of ADP and GlnK to the C-terminal GHKL

domain may activate wild-type NifL, resulting in the formation of the GlnK–NifL–NifA ternary complex, even at high concentrations of 2-oxoglutarate. Our data suggest that oxidation of the FAD co-factor in NifL does not bring about a conformational change equivalent to that promoted by the binding of GlnK, since the oxidised forms of NifL-G455A and NifL-G480A are not competent to inhibit NifA when 2-oxoglutarate is present. The possibility that signal transduction induces different conformational changes in NifL, in response to the redox and nitrogen status, is supported by the isolation of mutant forms of NifA that can discriminate between the oxidised binary complex and the GlnK-activated ternary complex.[32,33]

Materials and Methods

Strains and plasmids

All plasmids and bacterial strains used in this study are listed in Table 1.

Mutagenesis

Site-directed mutagenesis was carried out with the QuickChange site-directed mutagenesis kit (Stratagene). ApaI-BstEII fragments containing the *nifL* mutations were subcloned into pPR34 and single mutations were confirmed by DNA sequencing.

β-Galactosidase assays and growth conditions

To assay β-galactosidase activity, *Escherichia coli* strains were transformed with plasmid pRT22, which carries a *nifH-lacZ* translational fusion. NifA activity was measured by determining the level of expression from the *nifH* promoter. To monitor the ability of NifL to inhibit NifA activity, *E. coli* strains were transformed with plasmids pRT22 and either pPR34 or its mutant derivatives (Table 1). The activity of NifA alone was assayed by transforming *E. coli* strains with plasmids pRT22 and either pPR39 or its mutant derivatives.

For β-galactosidase assays, *E. coli* ET8000 and RT8000 strains were grown to late exponential phase in Luria-Bertani medium at 30 °C in the presence of appropriate antibiotics. Aliquots (50 μl) of these cultures were then inoculated into 4 ml of NFDM medium supplemented with casein hydrolysate (200 μg/ml) for nitrogen-limiting conditions or with $(NH_4)_2SO_4$ (1 mg/ml) for nitrogen-excess conditions. Cultures of the RT8000 strain were supplemented with glutamine (25 μg/ml). Strains were grown in a plastic vial (internal volume 7 ml) sealed with a rubber closure for anaerobic conditions. When conditions required aerobiosis, 5 ml cultures were grown with vigorous shaking in 25 ml conical flasks. β-Galactosidase activity was determined as described.[43] Activities were derived from the average of at least three independent determinations and assays on each culture were performed in duplicate.

Western blotting

To obtain protein extracts, cultures containing pRT22 and the plasmid of interest were grown in the conditions used for β-galactosidase assays. Cells were centrifuged (0.8 A_{600} unit) and the pellet was resuspended in Laemmli loading buffer. Equivalent amounts of proteins were separated by electrophoresis on polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were probed with polyclonal antisera against NifL. Primary antibodies were detected with the ECL chemiluminescence system (Amersham).

Protein expression and purification

In all cases, protein overexpression was carried out in *E. coli* strain BL21(DE3) pLysS. Plasmids used for over-expression of NifL mutant proteins are listed in Table 1. Cultures were grown aerobically in Luria–Bertani broth and expression from the T7 promoter was induced by addition of isopropyl-β-D-thiogalactopyranoside to 1 mM. NifL and NifA proteins were purified as described.[21,32]

Isothermal titration calorimetry (ITC)

Experiments were performed in a VP-ITC isothermal titration calorimeter (MicroCal, Inc.) at 28 °C in a cell volume of 1.35 ml as described.[9] Buffer conditions were 50 mM Tris-acetate (pH 8.0), 100 mM potassium acetate, 50 mM KSCN, 15% (v/v) glycerol, 3.5 mM magnesium acetate. NifL protein samples were dialyzed overnight at 4 °C prior to ITC, and protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. Ligands were titrated from a 250 μl injection syringe into the sample cell, which was stirred at 300 rpm. The heat change for the dilution of the ligand in the absence of protein was measured for each experiment and was subtracted from the measured heat change of ligand binding to protein. Data analysis was performed with the Origin program, provided by MicroCal, using equations and curve-fitting analysis to obtain least-squares estimates of the binding enthalpy, stoichiometry, and binding constant.[44] Binding stoichiometries were derived on the assumption that proteins and ligand were fully active with respect to binding.

Limited trypsin proteolysis

Limited proteolysis was performed at 20 °C in 50 mM Tris-acetate (pH 7.0), 100 mM potassium acetate, 8 mM magnesium acetate, 1 mM DTT. Reactions were incubated in the presence or in the absence of nucleotides for five minutes before initiating digestion. A trypsin/NifL ratio of 1 : 200 (w/w) was used. Samples were removed at the time-points indicated in the Figure legends to tubes containing a twofold weight excess of soybean trypsin/chymotrypsin inhibitor. An equal volume of gel loading buffer (125 mM Tris–HCl (pH 8.6), 4% (w/v) SDS, 20% glycerol, 10% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue) was added and samples were heated at 100 °C for four minutes before electrophoretic separation on SDS/12% polyacrylamide gels.

NifL–NifA complexes

All reactions and washing steps were carried out in the following buffer; 50 mM Tris–HCl (pH 8.0), 15% glycerol, 100 mM potassium acetate, 50 mM KSCN, 3.5 mM magnesium acetate, 20 mM imidazole, 200 mM NaCl. Reactions containing 1.6 μM NifA and 0.8 μM N_{6His}-NifL plus the appropriate concentration of ADP (100 μl final

Table 1. Strains and plasmids used in this work

Strain/or plasmid	Relevant genotype	Reference/or source
E. coli strains		
ET8000	*rbs lac*Z::IS*1 gyrA hutC*^{c} $_k$ (wild-type)	45
RT8000	*rbs lac*Z::IS*1 gyrA hutC*^{c} $_k$ Δ*glnB1 ntrC10*::Tn*5*	31
Plasmids		
pRT22	p*nifH-lac*Z in pACYC184	46
pPR34	*A. vinelandii nifLA* translated from the natural ribosome ribosome-binding site of *nifL* in pT7-7	7
pNLN419D	Derivative of pPR34 encoding mutant NifL-N419D	This work
pNLG455A	Derivative of pPR34 encoding mutant NifL-G455A	This work
pNLG480A	Derivative of pPR34 encoding mutant NifL-G480A	This work
pIM10	Derivative of pPR34 encoding wild-type NifL and mutant NifA-F119S	This work
pPR39	Derivative of pPR34 expressing NifL (454–519) and wild-type NifA	7
pNS419	Derivative of pPR34 expressing NifL-N419D, NifA-F119S	This work
pNS455	Derivative of pPR34 expressing NifL-G455A, NifA-F119S	This work
pNS480	Derivative of pPR34 expressing NifL-G455A, NifA-F119S	This work
pTJ45	Derivative of pET28a (+) vector expressing N_{6his} NifL	7
pRL15	Derivative of pET28a (+) vector expressing N_{6his} NifL-N419D	This work
pRL16	Derivative of pET28a (+) vector expressing N_{6his} NifL-G455A	This work
pRL17	Derivative of pET28a (+) vector expressing N_{6his} NifL-G480A	This work
pTJ40	Derivative of pT7-7 expression vector encoding $NifL_{his6}$	6
pTJ54	Derivative of pT7-7 expression vector encoding $NifL(147–519)_{his6}$	7
pTJXLN419D	Derivative of pT7-7 vector expressing $NifL\text{-}N419D_{his6}$	This work
pTJXLG455A	Derivative of pT7-7 vector expressing $NifL\text{-}G455A_{his6}$	This work
pTJXLG480A	Derivative of pT7-7 vector expressing $NifL\text{-}G480_{his6}$	This work
pIM28	Derivative of pRL10 encoding $NifA\text{-}F119S_{his6}$ (1–180)	This work

volume) were incubated at room temperature for 30 minutes and then transferred to the wells of a HIS-Select™ high-capacity, nickel-coated 96-well Plate (Sigma). Hexa-histidine-tagged NifL protein was bound to the plates by shaking for 2.5 hours at room temperature. The supernatant was then removed and the wells washed four times for 30 minutes with 250 μl of buffer containing the appropriate concentration of ADP. Bound protein was eluted from the wells by shaking for ten minutes in the presence of 20 μl of 1 M imidazole and the eluant was analysed by SDS-PAGE.

Open promoter complex assays

NifA-promoted catalysis of open promoter complexes by σ^{54}-RNA polymerase was used to assay NifA activity and its inhibition by NifL as described.[9,18] Linearised template DNA was provided by digesting plasmid pNH8 with EcoRI and BamHI to yield a 260 bp fragment, including the *Klebsiella pneumoniae nifH* promoter and upstream activator sequences, which was 3′ end-labelled with [α-^{32}P]dGTP at the BamHI site. Reactions (reaction mixture final volume 15 μl) were carried out in TAP buffer (50 mM Tris-acetate (pH 7.9), 100 mM potassium acetate, 8 mM magnesium acetate, 3.5% polyethylene glycol 8000, 1 mM DTT) containing 5 nM template DNA, 3.4 μg/ml of denatured salmon sperm DNA, 125 nM core RNA polymerase, 200 nM σ^{54} 100 nM IHF, and 4 mM GTP. The reaction components were incubated for two minutes at 30 °C, and reactions were then initiated by the addition of either NifA alone (250 nM dimer) or NifA (250 nM) plus NifL (at concentrations specified in the Figures). After incubation for 20 minutes, reaction mixtures were mixed with 3 μl of dye mixture containing 50% glycerol, 0.05% bromophenol blue, 0.1% (w/v) xylene cyanol, and 2 μg of heparin and loaded immediately onto a 4% (w/v) polyacrylamide gel (acrylamide/bisacrylamide ratio, 80 : 1, w/w) in 25 mM Tris-400 mM glycine (pH 8.6), which had been run at 180 V at room temperature down to a constant power of 2 W. Gels were run for 1–1.5 hours at 160 V and were dried; the percentages of radioactivity in open complexes were quantified with a Fujix BAS1000 phosphorimager.

Acknowledgements

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