Electron donation to the flavoprotein NifL, a redox-sensing transcriptional regulator

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Transcriptional control of the nitrogen fixation (*nif*) genes in response to oxygen in *Azotobacter vinelandii* is mediated by nitrogen fixation regulatory protein L (NifL), a regulatory flavoprotein that modulates the activity of the transcriptional activator nitrogen fixation regulatory protein A (NifA). CD spectra of purified NifL indicate that FAD is bound to NifL in an asymmetric environment and the protein is predominantly α -helical. The redox potential of NifL is -226 mV at pH 8 as determined by the enzymic reduction of NifL by xanthine oxidase/xanthine in the presence of appropriate mediators. The reduction of NifL by xanthine oxidase prevented NifL from acting as an inhibitor of NifA. In the absence of electron mediators NifL could also be reduced by *Escherichia coli* flavohaemoprotein (Hmp) with NADH as reductant. Hmp

contains a globin-like domain with haem B as prosthetic group and an FAD-containing oxidoreductase module. The carboxy-ferrohaem form of Hmp was competent to reduce NifL, suggesting that electron donation to NifL originates from the flavin in Hmp rather than by direct electron transfer from the haem. Spinach ferredoxin:NAD(P) oxidoreductase, which adopts a folding similar to the FAD- and NAD-binding domains of Hmp, also reduced NifL with NADH as reductant. Re-oxidation of NifL occurs rapidly in the presence of air, raising the possibility that NifL might sense intracellular oxygen. We propose a physiological redox cycle in which the oxidation of NifL by oxygen and hence the activation of its inhibitory properties occurs rapidly, in contrast with the switch from the active to the reduced form of NifL, which occurs more slowly.

INTRODUCTION

Nitrogen fixation is an oxygen-sensitive and energy-intensive process occurring in a wide range of prokaryotes, including obligate anaerobes, facultative anaerobes and obligate aerobes. The enzymes responsible, the nitrogenases, all require anaerobic conditions, a source of low-potential electrons and a large amount of ATP to reduce N_2 to NH_3 [1,2]. Consistent with the oxygen-sensitivity of these enzymes, their synthesis is repressed under aerobic regimes where nitrogen fixation is inhibited [3]. The regulation of expression of the nitrogen fixation (*nif*) genes in most cases involves a common set of regulatory genes. Nitrogen fixation regulatory protein A (NifA) is a ubiquitous transcriptional activator of *nif* genes in proteobacteria. Either regulation of NifA expression or modulation of its activity occurs in response to environmental oxygen [4]. In many of the proteobacteria the NifA protein is apparently sensitive to oxygen, but in the gamma subdivision the activity of NifA is modulated by the anti-activator nitrogen fixation regulatory protein L (NifL).

The NifL protein occurs in the facultative anaerobe *Klebsiella pneumoniae* [5] and in the obligate aerobe *Azotobacter vinelandii* [6]. These proteins have many common features, even though the catabolic activities associated with nitrogen fixation differ in the two organisms; in *K. pneumoniae* the fermentative and micro-aerobic modes are used, whereas in *A. vinelandii* the activities of the respiratory chain provide considerable flexibility to cope with varying oxygen supply [3]. Both NifL proteins modulate the

activity of the transcriptional activator, NifA, an enhancer-binding protein [7] that catalyses open complex formation with RNA polymerase and the alternative sigma factor, (σ^N , also known as σ^{54}) [8–10]. Analyses of mutant phenotypes indicate that these NifL proteins inhibit the activity of NifA in response to elevated levels of environmental fixed nitrogen and oxygen *in vivo* [5,6,11,12].

We have recently shown that *A. vinelandii* NifL is a flavoprotein [13] and this has also been reported for the *K. pneumoniae* protein [14]. *A. vinelandii* NifL contains FAD as prosthetic group; the ability of NifL to inhibit NifA activity is affected by the redox state of the bound FAD. In its oxidized state, NifL inhibits the transcriptional activity of NifA *in vitro*. The reduction of FAD bound to NifL by sodium dithionite relieves this inhibition, indicating that NifL is a redox-sensitive regulator [13]. A somewhat similar redox switch is mediated by the multi-functional PutA protein to autoregulate transcription of the *put* operon. However in this case the regulatory properties of the PutA flavoprotein are determined by its proline dehydrogenase activity [15–17]. A recently identified signal transducer for aerotaxis, Aer, has a sensory domain similar to the N-terminus of NifL and is also a flavoprotein, raising the possibility that both proteins have a common FAD-binding fold [18,19]. Within this domain there are highly conserved motifs termed S-boxes, which are present in PAS domains and in a large family of proteins from all kingdoms that sense redox status, oxygen and light [20].

Abbreviations used: Hmp, flavohaemoprotein (*hmp* product); NifA, nitrogen fixation regulatory protein A (*nifA* product); NifL, nitrogen fixation regulatory protein L (*nifL* product).

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Although NifL clearly modulates NifA activity in response to redox status, physiological electron donors to NifL have not so far been identified. As a first step towards the identification of cellular components that might transfer electrons to this redox sensor we have determined the redox potential of NifL and analysed the environment of the FAD bound to NifL on the basis of CD spectra. Further, we demonstrate that NifL can accept electrons from two different, but structurally related, flavoproteins, *Escherichia coli* flavohaemoprotein (Hmp) [21] and spinach ferredoxin NAD(P) oxidoreductase [22]. In all cases the reduced form of NifL is rapidly oxidized on exposure to air, suggesting a fast response by NifL to oxygen *in vivo*, thus preventing biosynthesis of nitrogenase *de novo* under unfavourable conditions.

EXPERIMENTAL

Chemicals

Benzyl Viologen, Indigo Carmine, phenosafranin, xanthine and NADH were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Anthraquinone-2,6-disulphonic acid (disodium salt) was purchased from Aldrich Chemical Company (Gillingham, Dorset, U.K.).

Proteins

Xanthine oxidase was generously provided by Professor R. C. Bray (School of Molecular Sciences, University of Sussex, Brighton, U.K.). Superoxide dismutase and spinach ferredoxin NAD(P) oxidoreductase were purchased from Sigma. *A. vinelandii* NifL and *E. coli* Hmp were purified as described previously [13,23].

Determination of the redox potential

The redox potential of NifL was determined by the method described by Massey [24]. The cuvette contained 24 μ M NifL, 46 μ M anthraquinone-2,6-disulphonate ($E_0' -238$ mV at pH 8), 2 mM benzyl viologen ($E_0' -360$ mV) and 500 μ M xanthine in 1 ml of 50 mM Tris buffer/0.1 mM EDTA (pH 8) at 25 °C. After anaerobiosis had been established, a catalytic amount of xanthine oxidase was added to this mixture from a side arm of the cuvette and the ensuing reduction of NifL-bound FAD and the dye was followed spectrophotometrically. For each time point, the concentration of oxidized and reduced FAD as well as oxidized and reduced dye was calculated and $\log(\text{FAD}_{\text{ox}}/\text{FAD}_{\text{red}})$ was plotted against $\log(\text{dye}_{\text{ox}}/\text{dye}_{\text{red}})$ (see Figure 5).

CD spectroscopy

CD spectra were recorded with a Jasco J-710 spectropolarimeter at 20 °C. The pathlength of the cuvette was 0.2 cm; 25 spectra were accumulated for each sample and corrected by subtracting contributions from the buffer.

Secondary structure prediction based on protein CD spectra

CD spectra were recorded in the range 178–260 nm and the measured ellipticity was converted to molar CD by using the following equation: molar CD = $113 \times 3 \times 10^{-6}/cl$, where 113 is the average molecular mass of an amino acid, 3×10^{-6} is the factor to convert observed ellipticity to ΔA , c mg/ml is the concentration of the protein and l cm is the pathlength of the cuvette. The contribution of α -helix, β -sheets, turns and

loops and undefined structures was determined with the program SELCON [25].

Assay for open complex formation under anaerobic conditions

Assays for open complex formation were done in an anaerobic glove box as described previously [13] except that, where appropriate, NifL was introduced into the glove box in the reduced form (see the legend to Figure 3 for the reducing conditions).

RESULTS

Release of FAD from NifL

Treatment of NifL with trichloroacetic acid led to a yellow precipitate and FAD was not released from the protein. Unfolding of the protein in guanidinium chloride resulted in the loss of the 358 nm peak and the shoulders to either side of the 446 nm band (Figure 1; compare solid and dashed-dotted lines). After precipitation of the denatured protein with trichloroacetic acid and subsequent adjustment of the pH to neutrality (Figure 1, broken line), 89 % of the FAD was recovered in the supernatant (assuming that this material has the same molar absorption coefficient as the FAD in the unfolding buffer, i.e. in guanidinium chloride) (Figure 1, dashed line). On the basis of a molar absorption coefficient of $11400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for free FAD, the molar absorption coefficient of NifL-bound FAD of was calculated to be $12250 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

UV/visible CD spectroscopy of NifL

In comparison with free FAD, which shows only weak CD peaks at 330 and 380 nm [26], NifL-bound FAD showed a negative peak at 450 ($\Delta\epsilon 7 \text{ M}^{-1} \cdot \text{cm}^{-1}$) with a shoulder at 472 nm and a positive peak at 350 nm ($\Delta\epsilon 8 \text{ M}^{-1} \cdot \text{cm}^{-1}$), indicating an asymmetric flavin environment (Figure 2). A very similar CD spectrum was reported for *p*-hydroxybenzoate hydroxylase [27] and a

Figure 1 UV/visible absorbance spectrum of NifL (19 μ M) in 10 mM Tris/HCl buffer/10% (v/v) glycerol (pH 8) (solid line) and in 6 M guanidinium chloride, pH 6.8 (dotted line)

The dotted-broken line represents the UV/visible absorbance spectrum of FAD released after removal of the protein by trichloroacetic acid precipitation and centrifugation.

Figure 2 Far-UV (left panel) and visible (right panel) CD spectrum of NifL

The far-UV CD spectrum was recorded at a protein concentration of $2.1 \mu\text{M}$ in 10 mM potassium phosphate buffer, pH 7.5, and the visible CD spectrum at a protein concentration of $68 \mu\text{M}$ in 10 mM Tris/HCl buffer, pH 8, containing 10% (v/v) glycerol. All spectra were recorded at 20°C .

number of FMN-binding proteins of the dehydrogenase family. Despite the similarity of the CD spectra, a sequence alignment of NifL and *p*-hydroxybenzoate hydroxylase did not reveal obvious common structural motifs. The far-UV spectrum of NifL showed no detectable change on the addition of MgADP, indicating that binding of this nucleotide does not result in a major structural change in the protein, although the addition of ADP alters the trypsin cleavage pattern of the C-terminal domain of NifL [28].

Secondary structure predictions for NifL

The far-UV CD spectrum shown in Figure 2 (left panel) was used as a basis for secondary structure prediction with the program SELCON [25]. This revealed a predominantly α -helical protein with α -helix accounting for 36% of the total, β -structure for 17.5%, turns and loops for 24.5% and other structures for 22%. Secondary structure predictions based on sequence analysis with SSPRED [29], with the input as a single sequence, were in good agreement with the prediction for α -helix (37%) and β -sheet (20%). Secondary structure probabilities based on state-space modelling [30] were also in good agreement with the CD-based prediction. After input of the N- and C-terminal domains into the program as discrete sequences to allow more extensive analysis, both fragments were predicted to form α - β domains with a central β -sheet.

Enzymic reduction of NifL by xanthine oxidase

Because the redox state of NifL influences transcriptional activation by NifA it is important to characterize the factors involved in the reduction and oxidation of the NifL-bound FAD. We have previously reported [13] that NifL is reduced by sodium dithionite, although the rate of reduction is low.

The redox potential of the xanthine/urate couple is sufficiently low ($E_0' - 350 \text{ mV}$ at pH 7.0) to reduce most flavoproteins. As shown in Figure 3, NifL is efficiently reduced by this enzymic system in the presence of the electron mediator Benzyl Viologen.

Figure 3 Enzymic reduction of NifL with xanthine oxidase and xanthine

The cuvette contained $24 \mu\text{M}$ NifL, $400 \mu\text{M}$ xanthine and $1.5 \mu\text{M}$ Benzyl Viologen in 10 mM Tris buffer/0.1 mM EDTA (pH 8). After anaerobiosis had been established by repeated cycles of evacuation and flushing with N_2 , a catalytic amount of xanthine oxidase was added from a side arm of the cuvette (trace 1). Reduction of NifL was monitored with time and the spectra shown were recorded after 20 min (trace 2), 35 min (trace 3), 45 min (trace 4), 56 min (trace 5) and 62 min (trace 6). Reoxidation by air gave rise to the absorbance spectrum represented by trace 7.

In the absence of this dye, no reduction of NifL was observed (results not shown). No differences in reduction rates were detectable when superoxide dismutase was also present in the reaction, indicating that NifL is not reduced as a consequence of superoxide production by xanthine oxidase. Reduction proceeded from the oxidized to the fully reduced flavin without the detectable accumulation of either a red or a blue semiquinone. The final spectrum of the reduced FAD had a UV/visible absorbance maximum at 360 nm and a shoulder at approx. 425 nm. The addition of NADH did not increase the rate of NifL reduction; NADH was also not consumed during this process, indicating that electrons were derived solely from the xanthine/urate reaction catalysed by xanthine oxidase. The reoxidation of reduced NifL by molecular oxygen was rapid and essentially complete within a few seconds of mixing the solution with air (Figure 3, trace 7).

To confirm that the reduction of NifL by xanthine oxidase influenced its ability to modulate transcriptional activation by NifA, as shown previously with sodium dithionite, we monitored NifA activity in the presence of NifL by measuring the formation of open promoter complexes in reactions containing σ^{54} RNA polymerase, integration host factor and GTP under anaerobic conditions [9,13]. In the absence of xanthine oxidase, oxidized NifL inhibited the formation of open promoter complexes by NifA, whereas NifL reduced by xanthine oxidase had little influence on NifA activity (Figure 4).

Redox potential of NifL

The enzymic reduction of NifL with xanthine oxidase also provides a convenient method of determining the redox potential of NifL [24]. This method is based on the equilibration of catalytically generated reducing equivalents between the flavin moiety of NifL and a dye of known redox potential. Reduction of NifL occurs after reduction of Indigo Carmine ($E_0' - 125 \text{ mV}$

Figure 4 Response of NifL to reduction by xanthine oxidase

The ability of NifL (400 nM) to inhibit open complex formation by NifA (100 nM) at the *K. pneumoniae* *nifH* promoter was assessed by quantifying the formation of heparin-stable open promoter complexes. Reactions were incubated in the anaerobic glove box and were initiated at zero time by the addition of the reaction mixture (containing DNA, $E\sigma^{54}$ polymerase, integration host factor and GTP) to a mixture of NifA, and, where appropriate, either oxidized or reduced NifL in TAP buffer [50 mM Tris-acetate (pH 7.9)/100 mM potassium acetate/1 mM dithiothreitol/8 mM magnesium acetate/3.5% polyethylene glycol 8000] containing BSA (1.2 μ M) and the other components specified below. Samples were removed at the times indicated and challenged with heparin before electrophoresis. Reactions contained the following: NifA plus reduced NifL with xanthine (280 μ M), xanthine oxidase (40 nM) and Benzyl Viologen (800 nM) (◇), NifA alone with xanthine, xanthine oxidase and Benzyl Viologen (△) and NifA plus oxidized NifL with xanthine (280 μ M) (□).

Figure 5 Nernst plot of the log of the ratio of the concentration of oxidized and reduced anthraquinone-2,6-disulphonate (aq-2.6) against the log of the ratio of the concentration of oxidized and reduced FAD bound to NifL

The potential of the FAD can be calculated from the x-axis intercept where $\log(dye_{ox}/dye_{red})$ equals zero, as indicated by the arrow. From the midpoint potential of anthraquinone-2,6-disulphonate ($E_0' = -238$ mV at pH 8.0), the potential of NifL is -238 mV + (0.4×30) mV = -226 mV.

at pH 7) but before reduction of phenosafranin ($E_0' = -252$ mV at pH 7), indicating that the redox potential is in the range of -125 to -252 mV. An accurate value for the NifL redox potential was obtained by using anthraquinone-2,6-disulphonate as reference dye ($E_0' = -184$ mV at pH 7) (Figure 5). From three independent

measurements, an E_0' of -226 ± 3 mV at pH 8 was obtained. This compares with an E_0' of -219 mV for free FAD at pH 7 [31].

Enzymic reduction of NifL by Hmp from *E. coli*

Hmp is an NAD(P)H-dependent oxidoreductase that reduces Fe(III) [32], Fe(III) hydroxamate K (a siderophore) [33], cytochrome *c* [33] and oxygen [23]. The NADH oxidase activity of Hmp generates superoxide [32]. Hmp consists of a haem-containing globin-like N-terminal domain and an FAD-containing C-terminal domain that closely resembles the ferredoxin:NAD(P) oxidoreductase family. Fe has been implicated in the redox response of NifL *in vivo* [34,35] and we therefore considered the possibility that Hmp might have a role as a physiological electron donor to NifL. As shown in Figure 6(a), NifL is reduced by Hmp under anaerobic conditions in the presence of NADH. To eliminate the potential reduction of NifL by the superoxide anion, reaction mixtures also contained superoxide dismutase, although no differences in reduction rates were detected in the absence of this enzyme. The complete reduction of NifL (21 μ M) was accompanied by the consumption of a slight excess of NADH (26 μ M). This finding clearly indicates that the full reduction of the flavin ring moiety requires two electrons and that no other reducible cofactor or disulphide bridge is in equilibrium with the isalloxazine ring system. The reduction of FAD proceeds without the formation of a radical species. As with xanthine oxidase, reoxidation of the reduced FAD by O_2 occurs within seconds of mixing the sample with air (Figure 6).

Electron transfer from Hmp to NifL

It has been proposed that Hmp could have a general regulatory role as a signal-transducing oxygen sensor, because the binding of O_2 to the haem moiety influences the redox state of the bound FAD and might regulate electron transfer to appropriate acceptors [36,37]. We find that the reduction of NifL by Hmp apparently coincides with the formation of the deoxy form of the haem (results not shown). The combination of two redox centres within Hmp raises questions about the intramolecular route of electron transfer within Hmp and the subsequent routing of electrons to NifL. Previous studies have shown that CO binds rapidly to the reduced haem B, forming a low-spin complex [22] that does not inhibit electron donation to cytochrome *c* as acceptor [33]. Consistent with these observations, in the presence of CO the haem B rapidly formed an intense peak of the carbonmonoxy complex at 421 nm as well as α and β peaks at 545 and 560 nm on the addition of NADH (Figure 7, upper panel). The absorbance between 440 and 500 nm declined rapidly within 0.5 min of adding NADH, consistent with the reduction of the flavin moiety of Hmp (Figure 7, upper panel). With NifL present (Figure 7, lower panel), the continued decline in absorbance between 440 and 500 nm after the addition of NADH indicated that the FAD of NifL was being reduced even though haem B of Hmp was in the carbonmonoxy form. The presence of CO did not influence the rate of reduction of NifL and, as when CO was absent, re-oxidation of NifL occurred rapidly after the introduction of air. Thus electron transfer from Hmp to NifL and subsequent re-oxidation of NifL seems not to be strongly influenced by the occupancy of CO at the haem B. These results also indicate that because the ferrous haem is completely blocked by CO, electron donation to NifL originates from the FAD of Hmp rather than involving direct electron transfer from the haem.

Figure 6 Enzymic reduction of NifL by Hmp and spinach spinach ferredoxin:NAD(P) oxidoreductase

(a) Reduction of NifL with Hmp and NADH. The cuvette contained 21 μ M NifL, catalytic amounts of Hmp (200 nM) and superoxide dismutase (approx. 400 units) in 10 mM Tris buffer/0.1 mM EDTA (pH 8). After anaerobiosis had been established by repeated cycles of evacuation and flushing with N_2 (trace 1), 100 μ M NADH was added from a side arm of the cuvette (trace 2). Reduction of NifL was monitored over time and the spectra shown were recorded after 15 min (trace 3) and 85 min (trace 4). Reoxidation by air gave rise to the absorbance spectrum represented by trace 5 (dotted line). (b) Reduction of NifL by spinach ferredoxin:NAD(P) oxidoreductase. The cuvette contained 5 μ M NifL and approx. 0.06 unit of the enzyme in 10 mM Tris/HCl/1 mM EDTA (pH 8). After the addition of NADH (100 μ M),

Figure 7 Influence of CO on the reduction of NifL by Hmp

Reactions were set up as described in the legend to Figure 6 except that the Hmp and NifL concentrations were 500 nM and 8 μ M respectively. After a flushing with dinitrogen for 15 min, a stream of CO was passed over the liquid in the cuvettes for 30 s. The cuvette contents were shaken before the oxidized spectrum was recorded (trace 1) and then NADH (100 μ M) was injected. Spectra were recorded in the absence (upper panel) or presence (lower panel) of NifL at the following time intervals: (upper panel) 0.5 min (trace 2), 6.75 min (trace 3), 15 min (trace 4); (lower panel) 1 min (trace 2), 2 min (trace 3), 3 min (trace 4), 8 min (trace 5) and 14 min (trace 6). Reoxidation by air gave rise to the absorbance spectrum represented by trace 7 in the lower panel.

Enzymic reduction by spinach ferredoxin:NAD(P) oxidoreductase

Because electron transfer to NifL probably occurs via the FAD moiety within Hmp it is possible that only the C-terminal flavin-binding domain of Hmp is required to reduce NifL. The FAD- and NAD(P)-binding domains within Hmp show strong structural similarity to the ferredoxin:NADP oxidoreductase family

spectra were recorded at the following intervals: 1 min (trace 1), 6 min (trace 2), 12 min (trace 3), 30 min (trace 4) and 77 min (trace 5). Re-exposure to air for 5 min gave rise to the spectrum represented in trace 6. (c) Comparison of the reduction and re-oxidation rates of NifL in the presence of Hmp ($\square$, solid line) and spinach ferredoxin:NAD(P) oxidoreductase ($\triangle$, dotted line). Arrows indicate the introduction of air into the cuvettes.

of flavoproteins [22,38]. We were therefore interested to determine whether NifL could be reduced by spinach ferredoxin: NAD(P) oxidoreductase, a well-characterized enzyme of the ferredoxin oxidoreductase family whose structure has been determined [39]. The spectral changes observed on the addition of NADH to NifL in the presence of a catalytic amount of this enzyme indicated that NifL was being reduced under nitrogen (Figure 6). During this reduction the increase in absorbance around 600 nm was attributed to the semiquinone form of NifL, as there were no spectral changes in the absence of NifL (Figure 6b).

DISCUSSION

The enzymic reduction of NifL by xanthine oxidase in the presence of electron mediators has facilitated the analysis of the environment of FAD bound to NifL and determination of the redox potential, which is -226 mV at pH 8. Assuming that the pH dependence of the FAD_{red}/FAD_{ox} couple has a slope of -30 mV, the redox potential of NifL at pH 7 is approx. -196 mV, slightly more positive than that of free FAD_{red}/FAD_{ox} . This translates into a 6-fold tighter binding of the reduced form over the oxidized form of FAD. When reduced by xanthine oxidase, NifL shows an absorbance peak at 360 nm that is suggestive of monoanionic reduced FAD. This suggests that the pKa for the N(1) proton is in the same range as that determined for free FAD, i.e. $pK_a = 6.7$. These spectral features are also found in flavoprotein oxidases such as lactate mono-oxygenase [39] and glycolate oxidase [40], which stabilize the monoanionic form of the flavin. However, the protein environment within NifL does not seem to alter the redox potential of the FAD_{red}/FAD_{ox} couple significantly, whereas in the oxidase family the redox potential of the flavin is adapted to the nature of the enzyme reaction, i.e. the redox potential of the substrate/product couple. Thus flavoprotein oxidases have much more positive redox potentials than NifL: -65 mV for D-amino acid oxidase from *Rhodotorula gracilis* and -149 mV for L-lactate mono-oxygenase [41]. CD spectra also indicate that the FAD bound to NifL is located in an asymmetric environment similar to that found in flavodoxin and *p*-hydroxybenzoate hydroxylase ($E'_0 = -152$ mV at pH 7 in the free, uncomplexed form) and substantially different from the flavin environment in the oxidase family [27,42].

The redox state of NifL influences its ability to modulate the activity of the transcriptional activator NifA, but physiological electron donors to NifL have not yet been identified. Although NifL apparently does not contain iron, signal transduction to NifL might involve an Fe-containing protein [34,35]. A potential candidate Fe-containing electron donor is a flavohaemoglobin such as Hmp, which might act as a global intracellular oxygen sensor. It has been proposed that the binding of oxygen to the haem domain of Hmp might control the steady-state reduction of the flavin moiety and subsequent electron transfer to physiological electron acceptors such as redox-responsive transcriptional regulators [36]. NifL is clearly able to act as an electron acceptor for Hmp; reduction of the NifL-bound FAD proceeds with stoichiometric consumption of NADH without the formation of a flavin radical intermediate, indicative of a two-electron reduction. This is in contrast with the proposed electron-transfer pathway to haem through the flavin domain of Hmp, which proceeds via the formation of the anionic flavin semiquinone. When the haem of Hmp was converted to the low-spin complex by the addition of CO, reduction of NifL still occurred. This suggests that the two-electron reduction of NifL proceeds through the flavin domain of Hmp and is superoxide-

independent, because reduction was not influenced by the presence of superoxide dismutase. A similar route was proposed for the reduction of cytochrome *c* by Hmp [33]. The ability of the carbonmonoxyferrohaem form of Hmp to reduce NifL also indicates that this reduction is apparently not dependent on the unligated state of the ferrohaem moiety of Hmp.

The crystal structure of Hmp from *Alcaligenes eutrophus* reveals that the FAD oxidoreductase module adopts a very similar folding to that of the flavin- and NADH-binding domains of the ferredoxin oxidoreductase-like family of proteins [22]. Spinach ferredoxin:NAD(P)H oxidoreductase was also capable of reducing NifL, but in contrast with Hmp the reduction resulted in some formation of the anionic semiquinone radical. The appearance of the radical was probably due to a kinetic rather than a thermodynamic stabilization because the concentration of the oxidoreductase enzyme was catalytic. The ability of both Hmp and ferredoxin oxidoreductase to reduce NifL suggests that the physiological electron donor to NifL in *A. vinelandii* might be a member of this family of oxidoreductases. The redox potentials of the flavin in ferredoxin:NAD(P) oxidoreductase, -320 mV (one-electron reduction to the semiquinone) and -400 mV (two-electron reduction to the fully reduced flavin) [43], are near those associated with electron transfer to dinitrogenase. Redox EPR titrations of the flavin in Hmp indicate a redox potential of not less than -230 mV at pH 7 [44], which is thermodynamically appropriate for the reduction of NifL. However, the functional and physiological relevance of the reduction of NifL by Hmp and ferredoxin:NAD(P) oxidoreductase must await further studies with appropriate mutant strains lacking these proteins. The relatively high redox potential of NifL (-196 mV at pH 7) suggests that if conditions become favourable for nitrogen fixation then a number of different electron donors and NAD(P)H-dependent enzymes could potentially reduce NifL *in vivo*. Thus, rather than a specific electron donor being required, the reduction of NifL could provide a means of sensing the availability of reducing equivalents within the cell.

NifL is reduced relatively slowly by xanthine oxidase, by Hmp and by ferredoxin:NAD(P) oxidoreductase, but in each case re-oxidation (i.e. activation of NifL) occurs rapidly in the presence of oxygen. This provides an appropriate redox switch, allowing electron transfer to oxygen as acceptor at a faster rate than electron donation to NifL. A similar model has been proposed for the oxygen-responsive transcriptional regulatory protein FNR, which in contrast with NifL contains an Fe-S cluster. In this case oxygen is also implicated as a direct oxidant and it has been suggested that reduction occurs more slowly than oxidation, thus allowing oxygen to switch the activity of FNR rapidly even in the presence of cellular reductants [45]. A low rate of reduction of NifL would also be consistent with the requirement for sustainable reducing conditions to support dinitrogenase activity. Because the synthesis of nitrogenase necessitates a large investment in biosynthetic capacity, a gradual switching on of *nif* transcription would be economic. In contrast, a fast switching off as a result of the oxidation of NifL by oxygen would prevent the wasteful synthesis of proteins that are otherwise destined for inactivation. *A. vinelandii* nitrogenase can form an inactive complex with a constitutively produced redox protein, called the FeS II or Shethna protein [46,47]. This protein seems to stabilize nitrogenase during oxygen stress, and complex formation apparently depends on the controlled oxidation of the component proteins of the nitrogenase. The redox potential of the Shethna protein is -226 mV, close to that of NifL [48]. Because the mid-point potential of both proteins is similar, it is tempting to speculate that under hyperoxygenic conditions the oxidation of

both NifL and the FeS II protein would be coincident, thus allowing the simultaneous protection of nitrogenase from oxygen inactivation and the prevention of further enzyme synthesis under detrimental conditions.

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