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Azotobacter vinelandii NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch

(redox-responsive regulation/two-component regulatory system/NIFL–NIFA)

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ABSTRACT **The NIFL regulatory protein controls transcriptional activation of nitrogen fixation (*nif*) genes in *Azotobacter vinelandii* by direct interaction with the enhancer binding protein NIFA. Modulation of NIFA activity by NIFL *in vivo* occurs in response to external oxygen concentration or the level of fixed nitrogen. Spectral features of purified NIFL and chromatographic analysis indicate that it is a flavoprotein with FAD as the prosthetic group, which undergoes reduction in the presence of sodium dithionite. Under anaerobic conditions, the oxidized form of NIFL inhibits transcriptional activation by NIFA *in vitro*, and this inhibition is reversed when NIFL is in the reduced form. Hence NIFL is a redox-sensitive regulatory protein and may represent a type of flavoprotein in which electron transfer is not coupled to an obvious catalytic activity. In addition to its ability to act as a redox sensor, the activity of NIFL is also responsive to adenosine nucleotides, particularly ADP. This response overrides the influence of redox status on NIFL and is also observed with refolded NIFL apoprotein, which lacks the flavin moiety. These observations suggest that both energy and redox status are important determinants of *nif* gene regulation *in vivo*.**

The high energetic requirements for nitrogen fixation and the extreme oxygen sensitivity of the nitrogenase enzyme impose physiological constraints on diazotrophy, which necessitate stringent control of nitrogen fixation (*nif*) gene expression at the transcriptional level (1). In both *Azotobacter vinelandii* and *Klebsiella pneumoniae*, the NIFL protein regulates *nif* gene transcription in response to environmental oxygen and fixed nitrogen (2, 3). This control by NIFL is achieved through modulation of the activity of the transcriptional activator NIFA, an enhancer binding protein that catalyzes the formation of open promoter complexes by the alternative holoenzyme form of RNA polymerase containing the sigma factor σ^N ($E\sigma^N$) (4). Stimulation of open promoter complex formation by NIFA requires nucleoside triphosphate hydrolysis catalyzed by the central domain of this activator (5).

Sequence analysis of NIFL indicates that this protein is composed of two domains separated by a glutamine-rich flexible linker. The amino-terminal domain shows homology to the *bat* gene product from *Halobacterium halobium*, which potentially has an oxygen-sensing function and also to the rhizobial FixL family of heme-based oxygen sensors, although the significance of these homologies is at present unknown (2). The carboxyl-terminal domain of NIFL shares characteristic features with the histidine protein kinase family of two-component regulatory proteins, and in the case of the *A. vinelandii* protein possesses all five of the conserved regions found in other transmitter domains. However, although *A. vinelandii* NIFL contains a conserved histidine residue known to be the site of autophosphorylation in other members of this family, a number of substitutions of this residue do not impair function, implying that sensory transduction by NIFL does not involve phosphorylation of this residue (6). Moreover, neither autophosphorylation of NIFL nor phosphotransfer to NIFA has so far been detected *in vitro* (7, 8). Inhibition of NIFA activity by NIFL apparently requires stoichiometric amounts of the two proteins, implying direct protein–protein interaction rather than catalytic modification of NIFA activity. Since the nucleoside triphosphatase activity of *A. vinelandii* NIFA decreases when the inhibitory complex between NIFL and NIFA is formed, NIFL may block NIFA activity by inhibiting its catalytic function. Moreover, inhibition by *A. vinelandii* NIFL is stimulated by the presence of adenosine nucleotides, particularly ADP, suggesting that formation of the inhibitory complex might be regulated by the ATP/ADP ratio (9).

When NIFL is overexpressed aerobically in nitrogen-rich medium and purified under aerobic conditions, it is competent to inhibit NIFA activity *in vitro* (5, 8). The inhibitory activity of renatured *K. pneumoniae* NIFL is retained under anaerobic conditions, suggesting that this protein does not sense molecular oxygen directly, at least after refolding (7). Here we show that native *A. vinelandii* NIFL is a flavoprotein with FAD as the prosthetic group. The ability of NIFL to inhibit NIFA activity is not influenced by oxygen but is responsive to the oxidation state of the chromophore, indicating that NIFL is a redox-sensitive regulator. NIFL may represent a type of flavoprotein in which electron transfer is not coupled to catalytic activity.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Plasmid pTJ40, which encodes a modified form of *A. vinelandii* NIFL with an additional tryptophan and six adjacent histidine residues at the carboxyl terminus ($NIFL_{-6his}$), was derived from plasmid pPW53, which expresses NIFL from the T7 promoter and contains an *Eco*RI site adjacent to the *nifL* stop codon (8). The latter was digested with *Eco*RI, and the 5′ extensions were trimmed by incubation with mung bean nuclease. The plasmid was then digested with *Bam*HI (a site located downstream from the *Eco*RI site) and then ligated to a synthetic double-stranded sequence derived by annealing the oligonucleotides 5′-GCATCACCATCACCATCACTGAG-3′ and 5′-GATC-CTCAGTGATGGTGATGGTGATGC-3′. The DNA sequence in the vicinity of the inserted DNA was then confirmed. Overproduction of both NIFL and $NIFL_{-6his}$ was achieved by intro-

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Abbreviation: IHF, integration host factor.

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ducing pPW53 and pTJ40, respectively, into *Echerichia coli* strain BL21(DE3). Cultures were grown aerobically in Luria broth, and expression from the T7 promoter was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside.

Plasmid pNH8 carries the *K. pneumoniae nifH* promoter and upstream NIFA-binding site (*nifH* UAS) on a 240-bp *Eco*RI–*Bam*HI fragment (9). Plasmid pJES409 carries the *nifH* promoter regulatory region with a binding site for the regulatory protein NTRC precisely replacing the *nifH* UAS (10).

Protein Purification. The native form of the *A. vinelandii* NIFL protein was purified by a modification of the method described previously (8). After the ammonium sulfate fractionation step, the protein was chromatographed on a butyl-Sepharose column (Pharmacia) equilibrated in 1 M ammonium sulfate. The column was developed with a descending salt gradient, and the NIFL was eluted in TGED buffer (10 mM Tris Cl, pH 8/5% glycerol/0.1 mM EDTA/1 mM dithiothreitol) without ammonium sulfate. The NIFL-containing fractions were chromatographed on a HiTrap Q ion-exchange column (Pharmacia), where NIFL was eluted at 0.23 M NaCl. This was followed by gel filtration on Superose 12 (Pharmacia) in TGED buffer containing 150 mM NaCl. NIFL was eluted from this column as a single peak with an apparent molecular mass of 245 kDa, indicating that it is a tetramer in solution. The subunit molecular mass was determined by laser desorption mass spectrometry to be 57,875 ± 50, which is consistent with the predicted molecular mass of 57,827.

$NIFL_{\text{-6his}}$ was purified from extracts of induced cells, which were lysed in buffer A (50 mM Tris Cl, pH 8.5/300 mM NaCl/20 mM imidazole) containing lysozyme (130 μg/ml) and 1 mM phenylmethylsulfonyl fluoride and applied to either a 1- or 5-ml chelating Superose column (Pharmacia) charged with $NiCl_2$ and equilibrated in buffer A. The column was developed with an increasing imidazole gradient, and the NIFL was eluted with 0.25 M imidazole. $NIFL_{\text{-6his}}$ was further purified by chromatography on Superose 12 or Superdex 200 in TGED plus 150 mM NaCl. The elution profile of the protein was identical to that of the native form of NIFL.

A. vinelandii NIFA, core RNA polymerase, and σ^N from *K. pneumoniae* were all purified as described (8). *E. coli* integration host factor (IHF) was the kind gift of Howard Nash.

Denaturation and Refolding of Histidine-Tagged NIFL. Crude cell extract containing $NIFL_{\text{-6his}}$ was applied to a 5-ml chelating Superose column charged with $NiCl_2$ in buffer A. Contaminating proteins were eluted by washing the column in the same buffer, and the purified NIFL remained attached to the column. The column was then washed with 8 M urea in buffer A, which removed the flavin from NIFL, and the denatured NIFL was then eluted from the column with 0.25 M imidazole in buffer A containing 8 M urea. The protein was refolded by dialysis into 10 mM Tris Cl, pH 8/0.1 mM EDTA/1 mM dithiothreitol/5% glycerol/50 mM NaCl overnight at 4°C, and the material was further purified by gel filtration on Superose 12 to eliminate high molecular weight aggregates.

Flavin Analysis. Free flavin was isolated from NIFL protein by chromatography on Sep-Pak C_{18} columns (Waters) equilibrated in 25 mM ammonium acetate buffer at pH 8. Purified NIFL protein was applied to the column, and the protein fraction was eluted from the bound flavin by washing with the same buffer. The released flavin was then eluted from the column with 20% acetonitrile in ammonium acetate buffer and concentrated by lyophilization. For TLC analysis, the dried flavin was dissolved in 10 mM sodium phosphate buffer at pH 7 and applied to a Kieselgel $60F_{254}$ plate (Merck) in parallel with standards of FMN and FAD. After drying, the plate was developed with a 1-butanol/acetic acid/water mixture in the ratio of 12:3:5 (vol/vol). The spots were visualized under a UV light (336 nm). The R_f values for this development were 0.1 ± 0.01 for FAD and 0.2 ± 0.04 for FMN.

Assay of Open Complex Formation Under Anaerobic Conditions. Reaction mixtures containing 5 nM template DNA (normally the 240-bp *Eco*RI–*Bam*HI fragment from pNH8, 3′-end-labeled with [^{32}P]dGTP at the *Bam*HI site), denatured salmon sperm DNA at 3.4 μg/ml, 75 nM core RNA polymerase, 200 nM σ^N, 50 nM IHF, and 4 mM GTP (or other nucleotide combination as indicated in the figure legends) were degassed in TAP buffer (50 mM Tris acetate, pH 7.9/100 mM potassium acetate/1 mM dithiothreitol/3.5% polyethylene glycol 8000/8 mM magnesium acetate) and introduced into an anaerobic glove box. Sodium dithionite solutions in TAP buffer were prepared separately in the glove box and added to the above components where indicated. The above components were then preincubated at 30°C for 2 min prior to the addition (defined as time zero) of either NIFA or NIFA plus NIFL (final concentrations indicated in the figure legends), which were also degassed in TAP buffer and introduced separately into the glove box. Aliquots from reactions were removed at the indicated time intervals into 1/5th volume of a degassed dye mix containing 50% glycerol, 0.1% xylene cylanol, 0.05% bromophenol blue, and 2 μg of heparin. Control experiments indicated that open complexes formed in the presence of GTP were stable under these conditions for at least 1 hr. After the final time point, all the heparin-challenged samples were removed from the glove box and loaded onto a 4% polyacrylamide gel (acrylamide/*N,N′*-methylenebisacrylamide ratio, 80:1) in 25 mM Tris/400 mM glycine, pH 8.6, which had been pre-run at 180 V at room temperature down to a constant power of 2 W. Gels were run for 2.5–3 hr at 100 V. In lanes derived from reactions containing sodium dithionite, the loading dyes remained bleached throughout the time of the gel run. Gels were dried down, and the percentage of radioactivity in open complexes was quantitated with a Fujix BAS1000 phosphoimager as described (9).

RESULTS

***A. vinelandii* NIFL Is a Flavoprotein with FAD as the Prosthetic Group.** We have utilized two different purification procedures for NIFL; for native NIFL, we used a modification of the previously published procedure (8) with the addition of gel-filtration chromatography on Superose 12 as the final step. For purification by nickel affinity chromatography, NIFL was modified by the addition of six histidine residues at the carboxyl terminus ($NIFL_{\text{-6his}}$). In both preparations, which were carried out under aerobic conditions, we noticed that the protein was yellow in color throughout all steps of the purification, indicative of a chromophore that copurifies with NIFL. Visible absorption spectra of impure NIFL fractions, as well as highly purified material, revealed an absorption maximum of 445 nm, suggestive of a flavin species. Additional shoulders at 420 nm and 470 nm were observed, indicative of a protein-bound moiety (Fig. 1). These spectral features were retained throughout both purification procedures (compare *A* and *B* of Fig. 1), and the ratio of adsorption maximum to NIFL protein concentration was maintained in each case (Table 1). With both the native and $NIFL_{\text{-6his}}$ proteins, the absorbance at 445 nm was bleached upon reduction with sodium dithionite under anaerobic conditions (Fig. 1). After denaturation of NIFL and analysis of the supernatent solution by TLC, a predominant fluorescent species with an R_f characteristic of FAD was identified. The properties of NIFL therefore suggest that it is a flavoprotein based on the following criteria: (*i*) the protein shows a characteristic absorption maximum at 445 nm with shoulders at 420 and 470 nm indicative of a protein-bound moeity, (*ii*) the absorption maximum is proportional to NIFL concentration throughout all stages of purification, (*iii*) identical spectra are obtained independent of purification method, and (*iv*) denaturation of the protein releases primarily FAD. Assuming that FAD bound to NIFL has the same extinction

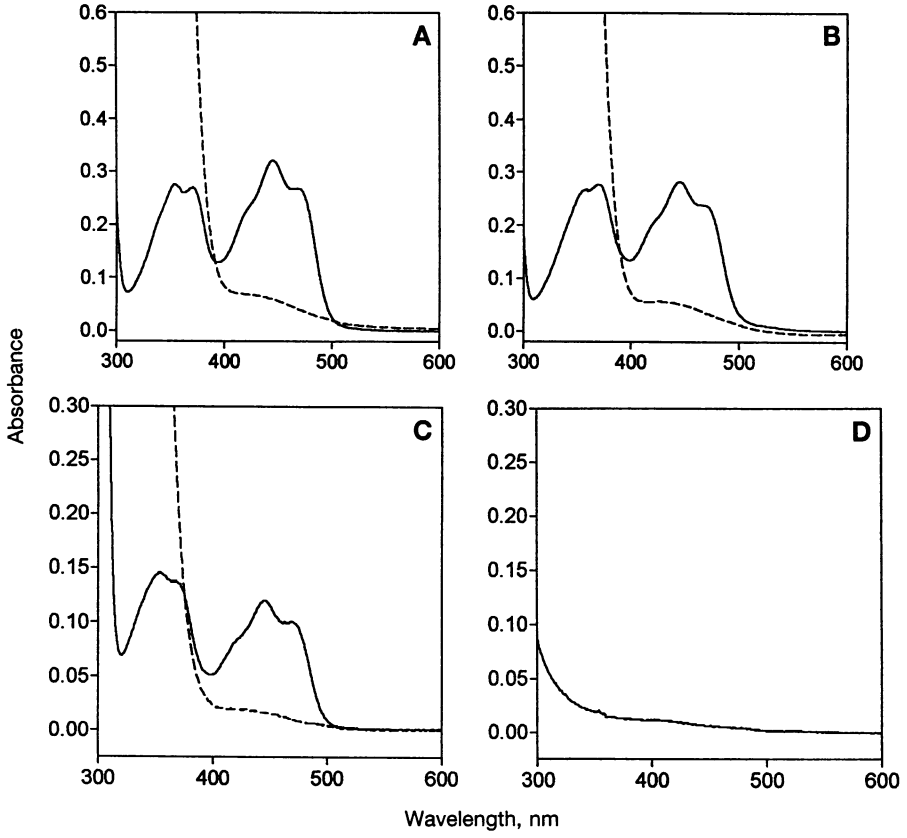

FIG. 1. Absorbance spectra of oxidized (solid lines) and dithionite-reduced NIFL (dashed lines). Spectra were recorded using a Shimadzu MP2000 spectrophotometer with a 1-cm light path and 2-nm slit width. With the exception of *C*, proteins were in storage buffer (TGED buffer containing 50% glycerol and 50 mM NaCl). All proteins were analyzed under an argon atmosphere (except for *D*) in the presence or absence of sodium dithionite (6 mM). (*A*) Native NIFL (18.3 μM). (*B*) $NIFL_{-6his}$ (18.3 μM). (*C*) $NIFL_{-6his}$ (6 μM) in TAP buffer containing 3.8 mM GTP and 0.2 mM ADP. (*D*) Refolded apo-$NIFL_{-6his}$ (1.2 μM) incubated under air.

coefficient as free FAD (11,300) and that it is a tetramer (see *Materials and Methods*), the FAD content is ≈3 per mol.

NIFL Modulates NIFA Activity via a Redox-Sensitive Switch. Since the NIFL chromophore is reduced by sodium dithionite, we anticipated that it might act as a redox sensor and consequently modulate transcriptional activation by NIFA. Because NIFL does not have any known catalytic function, we monitored its ability to inhibit NIFA activity. Transcriptional activation by NIFA can be measured by determining the rate of open promoter complex formation in a reaction that requires $E\sigma^N$, IHF, and an appropriate nucleoside triphosphate (11). Promoter complexes that have undergone the transition to the open promoter form are resistant to heparin challenge and can be quantitated on linear DNA templates using a gel-retardation assay (9).

Table 1. Comparison of the flavin content of native and histidine-tagged NIFL

Purification stage	A_{445} per mg of protein	NIFL as % of total protein	Molar ratio of flavin to NIFL
	Native NIFL		
Hydrophobic interaction	0.043	37.1	2.52
Ion exchange	0.089	62.5	3.10
Gel filtration	0.140	99.25	3.06
	$NIFL_{-6his}$		
Metal affinity	0.136	98.5	2.99
Gel filtration	0.154	100	3.34

At different stages of purification, the percentage of NIFL present in the total protein (determined by Bradford assay) was estimated by densitometric scanning of Coomassie-stained polyacrylamide gels. The flavin content was estimated by absorption at 445 nm. The molar ratio of flavin to NIFL was calculated using an extinction coefficient of 11,300 for FAD and a molecular mass of 245 kDa for NIFL.

As the spectral properties of the histidine-tagged form ($NIFL_{-6his}$) and the native form of NIFL are almost identical, we decided to utilize $NIFL_{-6his}$ for these experiments, since we could obtain more highly purified preparations of this protein using a two-step procedure that minimizes any loss of activity. Initial spectroscopic measurements showed that the absence of air was necessary to maintain NIFL in the dithionite-reduced form during the course of the assay. When NIFL was maintained under anaerobic conditions in a glove box in the absence of sodium dithionite, the flavin moiety remained in the oxidized form. Measurements of open promoter complex formation were therefore carried out using reactions that were incubated under these anaerobic conditions (less than 1 ppm O_2) in the presence or absence of sodium dithionite, using GTP to promote the formation of open promoter complexes. In the absence of NIFL, dithionite had little affect on the activity of NIFA, indicating that it did not influence the transcription activation assay (Fig. 2). However, when $NIFL_{-6his}$ was present in the oxidized form (dithionite absent), the accumulation of open promoter complexes by NIFA was strongly inhibited. NIFL probably inhibits the formation of open promoter complexes rather than their dissociation, since it had no effect on the stability of preformed open complexes (data not shown). In contrast, when $NIFL_{-6his}$ was reduced with dithionite, it failed to inhibit the formation of open complexes, and activity was stimulated ≈2-fold during the later stage of the time course (Fig. 2). We presume that the "lag phase" of the curve is due to the relatively slow rate of reduction of NIFL by sodium dithionite (data not shown). The stimulation of transcriptional activation by the reduced form of NIFL was unexpected, and it implies that

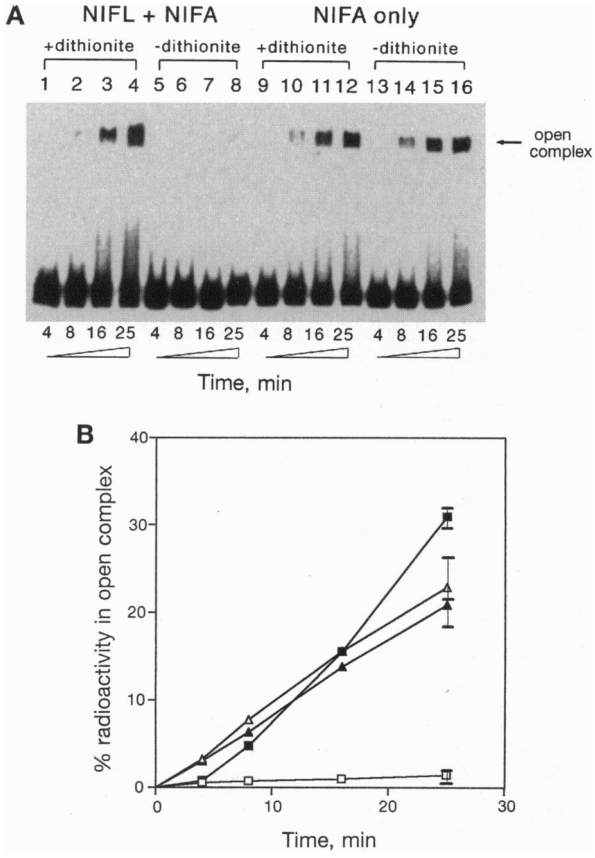

FIG. 2. Response of NIFL to redox status *in vitro*. The ability of $NIFL_{-6his}$ to modulate open complex formation by NIFA at the *K. pneumoniae nifH* promoter (240-bp *Eco*RI–*Bam*HI fragment from pNH8) was assessed by quantitating the formation of heparin-stable complexes in gel retardation assays as described in *Materials and Methods* using 4 mM GTP to promote formation of open complexes. Reactions were incubated in an anaerobic glove box (>1 ppm O_2) in either the presence or absence of 2 mM sodium dithionite and were initiated at time zero by the addition of either NIFA alone (200 nM, final concentration) or NIFA plus $NIFL_{-6his}$ (both at 200 nM). Samples were removed at the times indicated and challenged with heparin prior to electrophoresis (see *Materials and Methods*). (*A*) An example of the primary data after autoradiography. The relevant reaction components are indicated above the lanes, and the time of incubation is indicated below each lane. (*B*) Quantitation of the data from the phosphoimager. Each point is the mean from two independent experiments. Open symbols indicate sodium dithionite was absent, and closed symbols indicate dithionite was present in the reaction mixture. Reactions containing NIFA alone are represented by triangles and those containing NIFA plus $NIFL_{-6his}$ are indicated by squares.

NIFL may have a positive as well as a negative role in modulating NIFA activity. Thus the redox state of FAD in NIFL acts as a switch to regulate its activity. This switch is apparently specific to the NIFL–NIFA interaction since, although $NIFL_{-6his}$ slightly increased open complex formation by NTRC, in this case there was no modulation of transcriptional activation in response to redox status (Fig. 3).

ADP Stimulates the Inhibitory Activity of NIFL, Even When the Flavin Moiety Is in the Reduced Form. We have previously demonstrated that the inhibition of NIFA activity by NIFL under aerobic conditions is strongly stimulated *in vitro* by the presence of adenosine nucleotides, particularly ADP, and we have suggested that NIFL may be responsive to the ATP/ADP ratio *in vivo* (9). When ATP or ADP was added to anaerobic reactions in addition to GTP, $NIFL_{-6his}$ inhibited open complex formation, whereas little inhibition was observed in the presence of AMP or GDP (Fig. 4*A*). The specificity of inhibition under anaerobic conditions is therefore similar to that observed previously in aerobic conditions. Inhibition in response to ATP is presumably a consequence of the formation of ADP by the catalytic activity of NIFA, since we showed previously that this inhibition could be prevented by the addition of an ATP-regenerating system to the reaction mixture (9). The presence of ADP in anaerobic reactions resulted in strong inhibition of NIFA activity by NIFL (>98%) irrespective of whether sodium dithionite was present (Fig. 4*B*). Control experiments in the absence of NIFL showed that this concentration of ADP inhibited open complex formation to a much lower extent (maximum of 34%; Fig. 4). The presence of ADP does not prevent reduction of the flavin moiety in NIFL, since spectral analysis indicated that preincubation of NIFL with ADP does not prevent bleaching of the 445-nm signal by sodium dithionite (Fig. 1*C*). Addition of exogenous FAD did not prevent inhibition by ADP (data not shown).

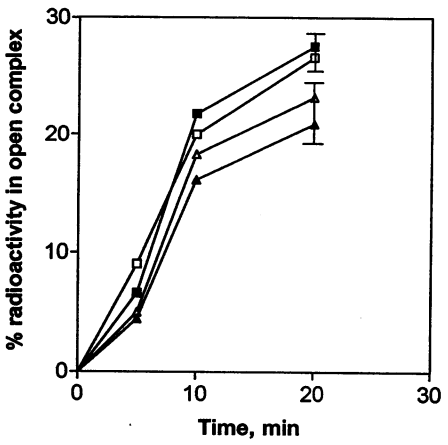

FIG. 3. Influence of NIFL on open complex formation by phosphorylated NTRC under anaerobic conditions. Reactions were incubated and analyzed as described in the legend to Fig. 2 with the exception that NIFA was replaced by NTRC (400 nM), each reaction also contained carbamoyl phosphate (10 mM), and the final concentration of $NIFL_{-6his}$ was 400 nM. Template DNA was the *Eco*RI–*Bam*HI *K. pneumoniae nifH* promoter fragment from pJES409 (5 nM). Each point is the mean from two independent experiments. Closed and open symbols indicate that dithionite was present or absent, respectively. Reactions containing NTRC alone are represented by triangles and those containing NTRC plus $NIFL_{-6his}$ are indicated by squares.

Refolding of the Apoprotein Eliminates Redox Sensing by NIFL but Not Its Response to ADP. To remove the flavin moiety, purified $NIFL_{-6his}$ was denatured in the presence of urea and then purified by metal chelate affinity chromatography to separate the apoprotein from the prosthetic group. After refolding of the protein and further purification by gel filtration, the absorption spectrum indicated that renatured $NIFL_{-6his}$ was substantially deflavinated, lacking a well-defined 445-nm signal (Fig. 1*D*). Hence the flavin moiety is apparently noncovalently bound to NIFL. In contrast to the flavoprotein, the apoprotein did not inhibit NIFA activity under anaerobic conditions, and NIFA was active whether or not dithionite was present (Fig. 5). Thus the apoprotein does not apparently modulate NIFA activity in response to redox status. However, like the holoenzyme, the refolded apoprotein strongly inhibited NIFA activity when ADP was present (Fig. 5), indicating that the prosthetic group may not be required for the response to adenosine nucleotides. It would therefore appear that the adenosine nucleotide switch is a discrete activity of NIFL that functions independently of its response to redox status.

DISCUSSION

One of the major questions concerning regulation of nitrogen fixation in free-living diazotrophs is the mechanism whereby

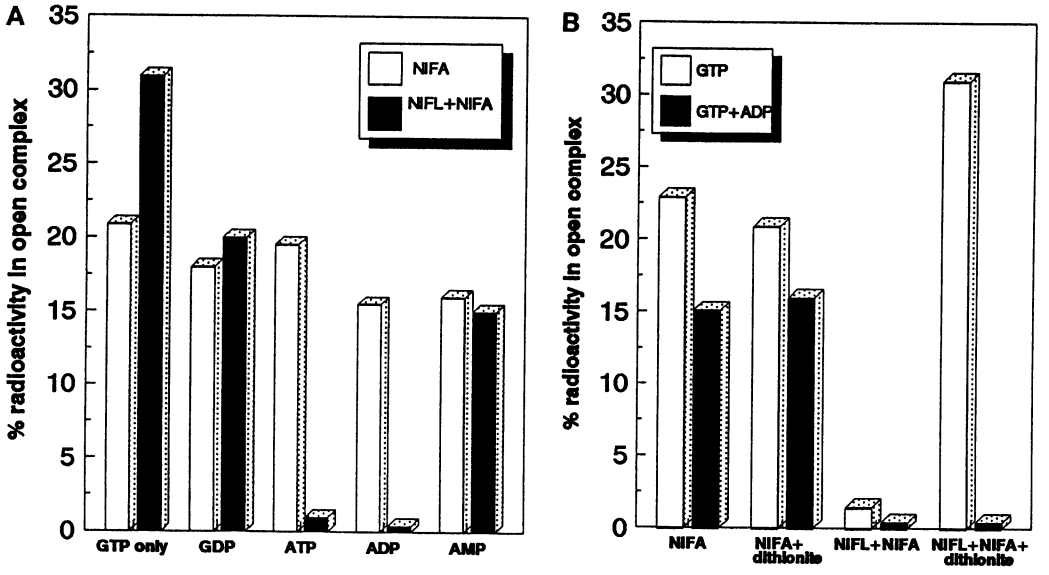

FIG. 4. Influence of ADP on the redox response of NIFL$_{\text{-6his}}$. (*A*) Assays for open complex formation under anaerobic conditions were carried out in the presence of sodium dithionite as described in the legend to Fig. 2 with the exception that the incubation time was 25 min in each case. Reactions contained either NIFA alone (open bars) or NIFA plus NIFL$_{\text{-6his}}$ (closed bars). The final concentration of each protein was 200 nM, and reaction mixtures contained either GTP (4 mM) or GTP (3.95 mM) plus an additional nucleotide (0.05 mM) as indicated on the *x* axis. (*B*) Reactions were carried out as in *A* in either the presence or absence of sodium dithionite and contained either 4 mM GTP (open bars) or 3.95 mM GTP plus 0.05 mM ADP (closed bars). Other relevant reaction components are indicated on the horizontal axis.

changes in extracellular oxygen concentration are communicated to the transcriptional activator NIFA by the NIFL regulatory protein. The finding that NIFL is a flavoprotein with FAD as the prosthetic group suggests that NIFL is susceptible to changes in redox status in accord with the major switch in activity observed when oxidized NIFL is converted to the reduced form. The switch between active and inactive forms (when the flavin changes from the oxidized state to the fully reduced form) is clearly redox driven, since in the absence of dithionite the flavin remains oxidized and the protein is active as an antiactivator when the oxygen concentration is lowered to 1 ppm. Preliminary experiments suggest that NIFL may undergo auto-oxidation in the presence of air but it is not yet known whether molecular oxygen is the physiological electron acceptor.

Although the flavin moiety confers redox properties upon NIFL, we cannot at this stage entirely rule out the possibility that this protein contains additional redox-active groups such as heme, an iron sulfur cluster, or a redox-active disulfide. However, metal analysis indicates that NIFL does not contain significant amounts of Fe, and EPR spectra of NIFL preparations are not indicative of an iron–sulphur center (S.A., S.H., S. Fairhurst, and D. Lowe, unpublished results). Similarity to the sensory domain of FIXL could implicate the presence of heme (13, 14), although staining of native gels did not reveal a heme moiety (S.A. and S.H., unpublished data). Therefore, unlike FIXL (15), NIFL is not apparently an oxygen-binding protein and is thus an additional representative of the redox-responsive class of transcriptional regulators, which include FNR, SoxR, and OxyR. Each of these regulators belongs to a different protein family, and the mechanism of redox sensing also appears to be different in each case. FNR appears to contain a relatively loosely bound Fe–S center, which in its reduced form probably stabilizes the protein in the active dimeric state (16, 17), whereas SoxR contains a more tightly bound iron–sulphur center, which activates the protein in the

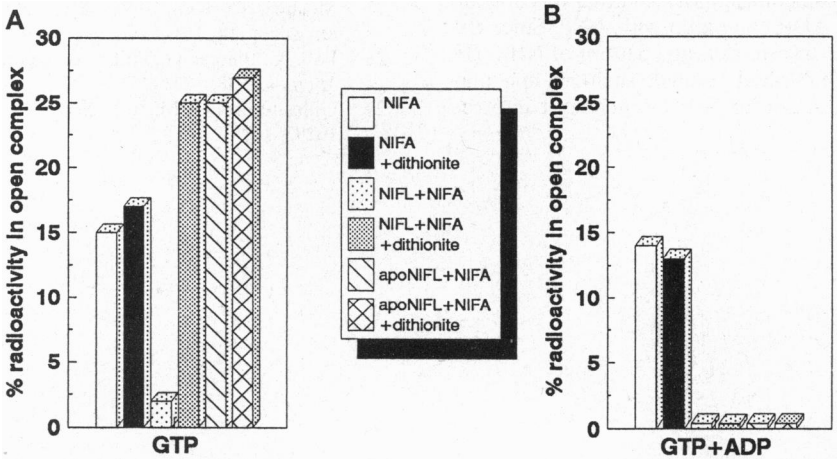

FIG. 5. Properties of refolded NIFL$_{\text{-6his}}$ apoprotein. Reactions were carried out under anaerobic conditions as described in the legend to Fig. 4 and contained NIFA alone (open bars), NIFA plus sodium dithionite (solid bars), NIFL$_{\text{-6his}}$ plus NIFA (lightly stippled bars), NIFL$_{\text{-6his}}$ plus NIFA plus dithionite (densely stippled bars), apoNIFL$_{\text{-6his}}$ plus NIFA (hatched bars), or apoNIFL$_{\text{-6his}}$ plus NIFA plus dithionite (crosshatched bars). Final protein concentrations were 200 nM NIFA and 200 nM NIFL in each case. Open complexes were formed in the presence of either 4 mM GTP (*A*) or 3.95 mM GTP plus 0.05 mM ADP (*B*).

oxidized form (18). The redox-active center of OxyR has not been fully characterized, but it contains a critical cysteine residue, which apparently does not bind metal ions but activates the protein when oxidized to sulfenic acid (19). Since we have not detected a catalytic activity for NIFL, it represents a rather unusual type of flavoprotein in which oxidation and reduction of the flavin acts as a molecular switch to control gene expression.

What are the natural electron donors to NIFL *in vivo*? Since the redox potential required to reduce the oxygen-sensitive nitrogenase Fe protein is around −400 mV (20) and oxygen inactivation of nitrogenase in *Azotobacter* is protected by "respiratory," "conformational," and "auto" protection (1), we would expect NIFL to respond to a considerably higher redox potential to ensure that NIFA activity is only inhibited when nitrogenase is susceptible to oxygen inhibition and damage. Potential electron donors to NIFL could be various dehydrogenases associated with the respiratory chain. Alternatively, the redox status of NIFL could be linked either to a nitrogenase-specific electron transport pathway or to the Shethna protein (21, 22) or perhaps even to the Fe protein. However, the latter seems unlikely because according to our model the reduced form of NIFL would be required for synthesis of the Fe protein.

NIFL is also required *in vivo* for regulation of *nif* transcription in response to the level of fixed nitrogen (2, 3, 6, 23). However, it would appear that NIFL does not sense fixed nitrogen directly since its activity under reducing conditions is not influenced by the presence of glutamine, ammonia, or glutamate (data not shown). Therefore the response to fixed nitrogen may involve interaction with another sensory protein or effector. One potential component of this sensing pathway is the Azotobacter *nfrX* gene product, which encodes a functional homologue of enteric uridylyltransferase encoded by *glnD* (12). We have shown previously that NIFL activity is modulated by the presence of adenosine nucleotides and that ADP in particular potentiates the form of NIFL that inactivates NIFA (9). We have shown here that ADP is a potent effector of NIFL activity and moreover it switches NIFL into the active (inhibitory) form even when the flavin moeity is either fully reduced or not present. Thus in addition to redox and nitrogen status, energy charge is likely to be an important factor in determining whether or not nitrogenase is synthesized. A similar phenomenon seems to occur in symbiotic diazotrophs since autophosphorylation of FIXL and consequent phosphorylation of FIXJ is very sensitive to the ATP/ADP ratio (24). Our working hypothesis is that the carboxyl-terminal domain of NIFL binds adenosine nucleotides and that this domain has a greater affinity for ADP compared with ATP. Since the addition of ADP alters the trypsin cleavage pattern of NIFL (E. Söderbäck and R.D., unpublished results), such binding may induce a conformational change in NIFL switching it into the inhibitory form.

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