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# Electron transport to nitrogenase in *Klebsiella pneumoniae*

(flavodoxin/*nif* genes)

D. NIEVA-GÓMEZ, GARY P. ROBERTS*, S. KLEVICKIS, AND WINSTON J. BRILL†

Department of Bacteriology and Center for Studies of Nitrogen Fixation, University of Wisconsin, Madison, Wisconsin 53706

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**ABSTRACT** **Cell-free extracts of *nifF* and *nifJ* mutants of *Klebsiella pneumoniae* are unable to couple acetylene reduction ($N_2$ fixation) by nitrogenase to the oxidation of organic metabolites. However, *nifF* and *nifJ* mutants can complement each other *in vitro* to establish the coupling. This indicates that the products of the *nifF* and *nifJ* genes constitute essential elements of the physiological electron pathway to nitrogenase. The electron-transfer-active product of the *nifF* gene, a flavoprotein, has been purified.**

It is generally believed that electron transport to nitrogenase involves the participation of ferredoxin, flavodoxin, or both (1). Cell-free extracts of *Klebsiella pneumoniae* fix $N_2$ (reduce acetylene) when electron transfer is coupled to the oxidation of pyruvate, malate, and NADPH (2). Electrons from photosynthetically driven ferredoxin-free spinach chloroplasts can be coupled to nitrogenase of an extract of *K. pneumoniae* that has been frozen for several months (2). In spite of difficulty in standardizing this treatment, it was reported that a flavoprotein as well as an uncharacterized protein each could independently couple electron transport to nitrogenase (2). The possible involvement of these proteins in electron flow to nitrogenase from organic metabolites was not investigated; therefore, the physiological roles of these electron transport proteins remained in question.

A gene, *nifF*, produces a product that seems to function in electron transport to nitrogenase, a conclusion based on the fact that NifF$^-$ strains have no nitrogenase activity *in vivo* but have activity *in vitro* (3, 4). The assay *in vitro* bypasses natural electron carriers, because when dithionite is added to the extracts in the presence of MgATP, it directly reduces component II of nitrogenase (5). Another gene, *nifJ*, also seems to be essential for electron transport to nitrogenase (4).

This paper reports the demonstration that mutations in *nifF* or *nifJ* impair physiological electron transport to nitrogenase, and it describes the purification and characterization of the product of the *nifF* gene.

## MATERIALS AND METHODS

**Strains.** *K. pneumoniae* M5a1 is the wild type. Mutant strains used were UN1777 (*nifF4746*), UN3409 (*nifF5520*), UN1691 (*nifJ4727*), and UN1137 (*nifJ4456*).

**Chemicals.** ATP, creatine phosphate, Hepes, sodium formate, malic acid, and creatine kinase were obtained from Sigma, DEAE-cellulose 52 from Whatman, and Sephadex G-50 from Pharmacia. Deazariboflavin was generously donated by V. Massey. All other reagents were of analytical grade commercially available.

**Derepression of Cells for Nitrogenase Synthesis.** Minimal medium, described previously (3), was modified to contain 12.5 g of $Na_2HPO_4$, 1.5 g of $KH_2PO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4.9 mg of $FeCl_3$, 0.25 mg of $NaMoO_4$, 2.0 g of NaCl, and 20 g of sucrose per liter. Bottles (200 ml) containing minimal medium and ammonium acetate (13 mM) were inoculated (0.5%) and incubated at 30°C without shaking for 12 hr. The cells were harvested and resuspended in minimal medium containing no fixed N. The cell suspension was incubated at 30°C for 1.5 hr, and then serine (0.5 mM, final concentration) was added. The cells were incubated for 4–5 hr and harvested under an argon atmosphere. The cell paste was stored frozen at −20°C under argon. For large-scale preparations, the same procedure was followed with 18-liter bottles and the cells were harvested with a Sharples centrifuge.

**Preparation of Crude Extract.** Cells were resuspended in 0.1 M Hepes (pH 7.5) at a concentration of 1 g of cell paste per 2 ml of buffer. The cells were broken with a French pressure cell, and the cell extract was kept under anaerobic conditions after this. The broken cells were centrifuged at 4300 × *g* for 30 min at 2–5°C, and the supernatant solution was collected with a syringe that had been flushed with argon.

**Acetylene-Reduction Assay.** The assay in the presence of dithionite has been described (6). When formate or malate was used, the assay was essentially as described by Yoch (2). The assay mixture contained 0.8 ml of ATP-generating solution (4.2 mM ATP, 50 mM creatine phosphate, 8.3 mM $MgCl_2$, and creatine kinase at 0.33 mg/ml in 0.1 M Hepes buffer, pH 7.5), plus 0.3 ml of substrate solution (180 mM sodium formate or 180 mM malate with 0.5 mM NAD). Between 50 and 100 μl of crude extract was used. The components of the assay system were placed, in the above order, in 8-ml vials previously evacuated and filled with argon and sealed with serum stoppers. Immediately after addition of the extracts, 0.5 ml of acetylene was injected to start the reaction and the vial was incubated at 30°C with shaking. After 10 min, the reaction was terminated by addition of 0.1 ml of trichloroacetic acid. Protein was determined by the method of Lowry *et al.* (7).

**Isolation of the *nifF* Gene Product.** A crude extract was prepared as described above, except that centrifugation was for 1 hr at 12,000 × *g*. All solutions referred to below were made in 0.1 M Hepes buffer (pH 7.5) and all operations were performed under strictly anaerobic conditions (however, dithionite was not added) at 4–10°C unless otherwise specified. The crude extract was applied to a DEAE-cellulose column (2.5 × 20 cm), and the column was washed with at least three bed volumes of 0.15 M NaCl. The *nifF* gene product was then eluted with 0.25 M NaCl. The fractions were assayed for activity in reconstituting electron flow to nitrogenase in an extract of a NifF$^-$ mutant with malate as electron source. The active fraction eluted near the front with 0.25 M NaCl. Active fractions were combined and concentrated in a Diaflo cell (Amicon, Lexing-

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Abbreviations: *nif*, nitrogen fixation gene; Nif, phenotype of the *nif* gene.

* Current address: Developmental Microbiology, Merck, Sharp & Dohme Research Laboratories, Rahway, NJ 07065.
† To whom reprint requests should be addressed.

ton, MA) with a PM10 ultrafiltration membrane. One milliliter of this concentrate was applied to a Sephadex G-50 column (1 cm × 75 cm) and eluted at a rate of about 12 ml/hr. The fraction active in reconstituting a NifF$^-$ mutant eluted as a single peak. Active material again was concentrated in a Diaflo cell and a 1.0- to 1.5-ml sample was fractionated by preparative polyacrylamide gel electrophoresis (8). The gel was run for 4 hr at 80 V, 100 pulses per sec, and then was changed to 150 V, 200 pulses per sec. A constant flow of $O_2$-free $N_2$ was maintained at all of the open points of the system. Samples were collected from the elution chamber with a syringe.

In a second method for purifying the *nifF* gene product, anaerobic conditions were not maintained. A crude extract was prepared as described above and then 1-butanol, cooled to dry ice/acetone temperature, was added to a final concentration of 30% by volume. The mixture was stirred in the cold for about 30 min and then was centrifuged at 8000 × *g* for 45 min. Both supernatant layers were added together and put on a DEAE-cellulose column (2.5 × 15 cm). The column was washed with buffer until no butanol odor was detected in the fractions. The active material and most of the protein then was eluted with 0.5 M NaCl. The NaCl was adjusted to 0.08 M by reduction of the sample volume in a PM10-containing Diaflo cell followed by addition of buffer. The sample then was loaded onto another DEAE-cellulose column (2.5 × 15 cm) and was purified in the same manner as in the first method, except that no attempt was made to exclude $O_2$.

## RESULTS

The NifF$^-$ and NifJ$^-$ mutants had the properties previously described (4). Intact cells failed to reduce acetylene *in vivo*, but their cell-free extracts catalyzed acetylene reduction *in vitro* with dithionite as the electron donor. Extracts of these mutants without dithionite did not reduce acetylene. Whereas a crude extract from the wild type can couple the oxidation of formate or malate to nitrogenase activity, neither the NifF$^-$ nor the NifJ$^-$ mutants were able to establish this coupling (Table 1). However, electron flow was established if a small amount of extract from a NifJ$^-$ mutant was added to the extract of a NifF$^-$ mutant and vice versa. Not all of the combinations are shown in Table 1, but all yielded similar results. Mixed extracts from two different NifF$^-$ mutants or from two different NifJ$^-$ mutants did not support electron transport to nitrogenase. The activities in Table 1 are much lower than they would be if dithionite were the electron donor.

Table 1. Formate- and malate-supported acetylene reduction by extracts of NifF$^-$ and NifJ$^-$ mutants

| Genotype of strain(s)* | Activity,† nmol ethylene formed per assay | |
|---|---|---|
| | Formate | Malate |
| Wild type (15.2) | 34.0 | 21.3 |
| *nifF4746* (10.6) | 0.0 | 0.0 |
| *nifF5520* (12.0) | 0.3 | 0.2 |
| *nifJ4727* (12.8) | 0.0 | 0.0 |
| *nifJ4456* (8.2) | 0.0 | 0.2 |
| *nifF4746* (8.8) + *nifJ4727* (2.1) | 83.0 | 68.8 |
| *nifF4746* (8.8) + *nifJ4456* (1.4) | 71.1 | 48.3 |
| *nifJ4727* (10.7) + *nifF4746* (1.8) | 54.9 | 82.5 |
| *nifJ4727* (10.7) + *nifF5520* (2.0) | 42.2 | 48.1 |
| *nifF4746* (8.8) + *nifF5520* (2.0) | 0.0 | Not done |
| *nifJ4727* (10.7) + *nifJ4456* (1.4) | 0.0 | Not done |

* Numbers in parentheses are the milligrams of extract protein used in the assay.

† The assay mixture contained 0.8 ml of ATP-regenerating solution, 0.3 ml of the electron donor (formate or malate), and 25–150 μl of extract.

The *nifF* gene product was purified from an extract of the wild type or a NifJ$^-$ mutant. During purification, fractions were assayed for their ability to reconstitute electron flow to nitrogenase in a crude cell-free extract of a NifF$^-$ mutant. NifJ$^-$ mutants produce more of the *nifF* gene product than does the wild type, a fact that has been corroborated by two-dimensional polyacrylamide gel electrophoresis of extracts from mutants (unpublished data). Hence, a NifJ$^-$ mutant, rather than the wild type, was used as the starting material for large-scale preparation of the *nifF* gene product.

The first of the two isolation procedures was anaerobic. The fraction containing the NifF-reconstituting activity was eluted together with nitrogenase from the DEAE-cellulose column with 0.25 M NaCl. No additional NifF-reconstituting activity was eluted with 0.35 M NaCl. After gel filtration chromatography and concentration of the active fractions, the material was fractionated further by preparative polyacrylamide gel electrophoresis. All of the activity was associated with a yellow fraction.

Table 2 shows the yields and specific activities of fractions during the purification procedure. At every step, the activity was associated with one fraction, or group of fractions in a peak, and there was no evidence of activity being associated with more than one chemical species. The yield and specific activity of the final product listed in Table 2 probably were underestimated because the assay was nonlinear with low concentrations of the active component.

The second purification method was not anaerobic. On occasions, butanol extraction led to the loss of most of the NifF-reconstituting activity, but we were unable to define the conditions responsible for the loss.

Fig. 1 reproduces a two-dimensional polyacrylamide gel of the final NifF-reconstituting protein (after preparative gel electrophoresis). The protein spot in the gel is elongated because of intentional overloading to emphasize possible low-level contaminant proteins. Both purification methods yielded the same protein. The purified protein was stable in $O_2$ and could be kept frozen without significant loss of activity. This protein has the same electrophoretic properties as the protein that is coded by *nifF* (unpublished data).

The purified protein exhibits the spectrum recorded in Fig. 2. It has the characteristic absorption of an oxidized flavoprotein with peaks at 360 and 452 nm. The protein after photoreduction to the semiquinone has a characteristic long-wave absorption. Thin-layer chromatography (11) of FAD and FMN standards and the flavoprotein that had been boiled for 5 min showed that FMN was the cofactor in the *nifF* gene product.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis indicated that the protein is composed of one polypeptide with

Table 2. Purification of the flavoprotein active in reconstituting NifF activity*

| Fraction | Recovery, % | Specific activity, nmol ethylene formed/(min × mg)† |
|---|---|---|
| Crude extract | 100 | 4.5 |
| DEAE-cellulose | 78 | 16.2 |
| Diaflo | 66 | 18.8 |
| Sephadex | 33 | 110 |
| Preparative gel | 18 | 192 |

* Strain UN1777 (NifF$^-$) was used to assay for NifF activity. The anaerobic purification procedure was used.

† Amount of protein in the fraction.

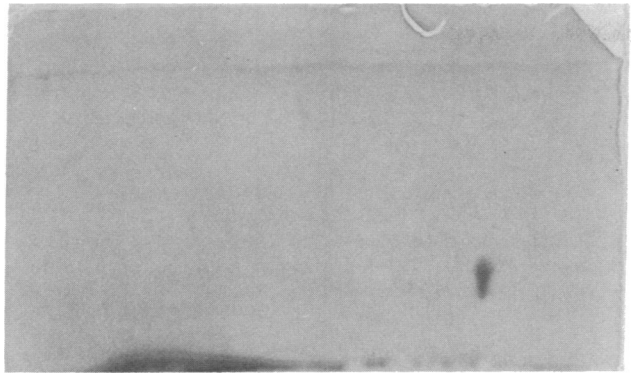

FIG. 1. Two-dimensional polyacrylamide gel of the purified NifF-reconstituting protein. The technique used has been described (9). The direction of isoelectric focusing is from right to left (basic end) and the direction of electrophoresis in sodium dodecyl sulfate is from top to bottom.

a molecular weight of 22,000. The elution profile on Sephadex G-50 indicated that the native protein has a molecular weight of approximately 20,000. Thus, the active protein appears to be a monomer.

## DISCUSSION

The data demonstrate that point mutations in the *nifF* and *nifJ* genes impair physiological electron flow to nitrogenase. Crude extracts from NifF$^-$ and NifJ$^-$ mutants can complement each other and reconstitute electron flow. With an assay based on this complementation, an isolation procedure was developed for the purification of a flavoprotein that is essential in the physiological electron transport chain coupling the oxidation of metabolic intermediates to nitrogenase.

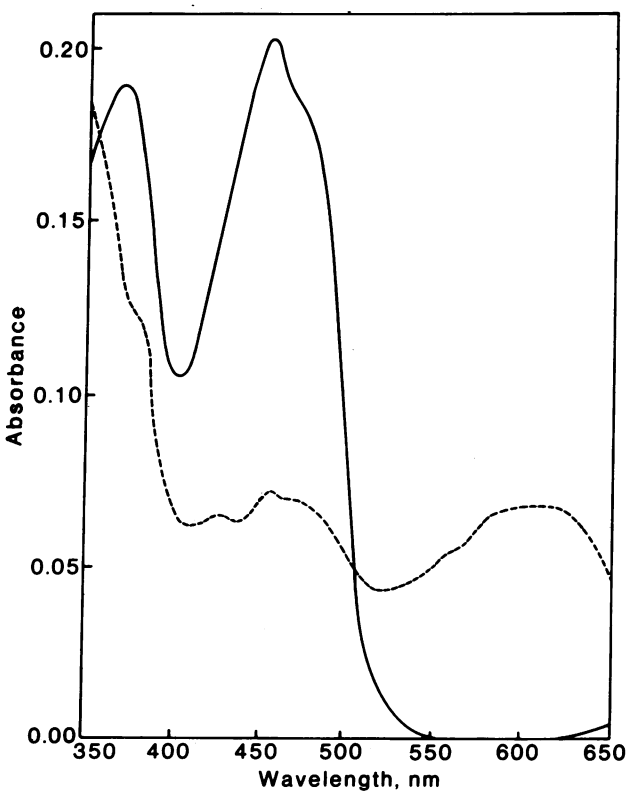


FIG. 2. Absorption spectra of oxidized (—) and photoreduced (- - -) NifF-reconstituting protein. The protein concentration was 0.22 μg/ml, and the optical path length was 1 cm. Photoreduction was done essentially as described (10). Deazariboflavin (3 μM) was added to the cuvette containing the protein and the material was kept under an atmosphere of argon. The sample was exposed to a slide projector lamp for 5 min.

In one of the two isolation methods, the material always was kept anaerobic to prevent the possibility of inactivation by $O_2$ of any other NifF-complementing species present. No such $O_2$-labile species was found.

That synthesis of the *nifF* gene product is controlled by the presence of fixed nitrogen through the *nifA* gene product (ref. 4 and unpublished data) furnishes further evidence that the NifF-reconstituting protein is specific for electron transport to nitrogenase.

By analogy with the low-potential flavodoxins from other organisms (1), the flavoprotein should be named *Klebsiella* flavodoxin. However, another flavoprotein has been so named (2), and the question arises as to whether the two proteins are the same. Several lines of evidence show that they are different. The flavoprotein isolated by Yoch (2) and the flavoprotein described here behave very differently during DEAE-cellulose chromatography.

In the photosynthetically driven system, an electron carrier from *Escherichia coli* also was capable of coupling electron transport to *Klebsiella* nitrogenase (2). *E. coli* with a plasmid containing the entire *nif* regulon from *Klebsiella* is able to fix $N_2$ anaerobically *in vivo* (12). When the *nif*-containing plasmid has a *nifF* mutation, there is no $N_2$ fixation. The NifF$^-$ plasmid-containing *E. coli* can be complemented *in vivo* with λ-*nif* specialized transducing phage containing *nifF* (unpublished results). This indicates that *E. coli* has no NifF-reconstituting activity. In the report by Yoch (2), each of two different electron carriers in *K. pneumoniae* can couple photosynthetically driven electron transport to nitrogenase; one of these is a flavoprotein. Only one protein (a flavoprotein) in a *K. pneumoniae* extract is capable of physiological electron transport in the assay that we use with extracts from a NifF$^-$ mutant. It seems, therefore, that the previously described flavoprotein (2) does not play a specific role for reducing nitrogenase *in vivo*. On the other hand, the flavoprotein described here specifically transfers electrons to nitrogenase and to our knowledge has no other physiological role. We were unable to obtain Yoch's flavodoxin for direct electrophoretic and spectrophotometric comparisons. We propose to call the *nifF*-coded flavoprotein "nitrogenase flavodoxin (*K. pneumoniae*)."

A protein, azotoflavin, has been reported to couple electron transport to nitrogenase in extracts of *Azotobacter vinelandii* (13, 14). However, our results raise the question as to whether azotoflavin actually is the electron carrier to nitrogenase *in vivo*, because azotoflavin is found both in $NH_4^+$- and $N_2$-grown *A. vinelandii* (13). Azotoflavin, unlike *Klebsiella nifF* product, does not exhibit regulatory specificity with nitrogenase.

FMN is the cofactor in the NifF-reconstituting protein, as it is in the flavodoxins from *Clostridium* (15, 16), *Desulfovibrio* (17), *Rhodospirillum rubrum* (18), and *Azotobacter* (19).

The precise role of the *nifJ* gene product, a protein of molecular mass 120,000 daltons in the monomer form (4), remains to be established, although it is clear that it forms a part (together with the *nifF* product) of an unbranched section of a specific electron-transport pathway to nitrogenase. If the role of the NifF-reconstituting protein is to directly reduce component II of nitrogenase (14, 20), then the *nifJ* encoded protein must have a role on the reducing side of the *nifF*-encoded protein. Perhaps the function of the *nifJ* product is to directly reduce the *nifF* product. Instability of the *nifJ* product has hampered our attempts to purify this protein.

**Note Added in Proof.** After this paper was submitted, other workers (21) showed that *Azotobacter* flavodoxin could reconstitute activity *in vitro* in a NifF mutant of *K. pneumoniae*.

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