JOURNAL OF BACTERIOLOGY, Aug. 1993, p. 4907–4910
0021-9193/93/154907-04$02.00/0

Vol. 175, No. 15

The *nifY* Product of *Klebsiella pneumoniae* Is Associated with Apodinitrogenase and Dissociates upon Activation with the Iron-Molybdenum Cofactor

MARY J. HOMER,[1,2] TIMOTHY D. PAUSTIAN,[1,2] VINOD K. SHAH,[2,3] AND GARY P. ROBERTS[1,2]*

Departments of Bacteriology[1] and Biochemistry[3] and Center for the Study of Nitrogen Fixation,[2] University of Wisconsin—Madison, Madison, Wisconsin 53706

Received 6 April 1993/Accepted 21 May 1993

Apodinitrogenase, which lacks the iron-molybdenum cofactor at its active site, is an oligomer that contains an additional protein not found in the active dinitrogenase tetramer. This associated protein in *Klebsiella pneumoniae* is shown to be the product of the *nifY* gene. When apodinitrogenase is activated by the addition of the iron-molybdenum cofactor, NifY dissociates from the apodinitrogenase complex. The conditions for this dissociation are described. Finally, there are aspects of the dissociation and insertion process in *K. pneumoniae* that are different from that in *Azotobacter vinelandii*.

The enzyme nitrogenase is composed of two components. Component I (also known as dinitrogenase, or the MoFe protein) is the enzyme that catalyzes the reduction of N_2 and other substrates. It is an $\alpha_2\beta_2$ tetramer of 240 kDa encoded by the *nifK* and *nifD* genes (18). Component II (also known as dinitrogenase reductase, or the Fe protein) transfers electrons to dinitrogenase. It is an α_2 dimer of about 60 kDa and is encoded by the *nifH* gene (18).

The active site of component I, the iron-molybdenum cofactor (FeMo-co), is synthesized by the *nif* gene products, including those of *nifQ*, *-B*, *-V*, *-N*, *-E*, and *-H* (18). Many mutations in *nifB* and *nifNE* result in strains that are unable to fix N_2 (13) and accumulate a form of component I without the active site. Addition of purified FeMo-co to this apo-component I (Apo I) in vitro yields an enzyme that is catalytically active (15).

When Apo I was purified from a *nifB Azotobacter vinelandii* mutant, another protein of approximately 20 kDa copurified with it (11). A variety of efforts to dissociate this protein without destroying Apo I proved unsuccessful, demonstrating that the complex was very tight (11).

Lacking either a good genetic or biochemical perspective on this associated protein from *A. vinelandii*, we examined the Apo I from *Klebsiella pneumoniae*, reasoning that if it was important to the biochemistry of nitrogenase, a similar factor might be detected in that organism also. In fact, a previous publication describing the purification of Apo I from *K. pneumoniae* had already reported the presence of a 20-kDa contaminant (5). Very recently, NifY has been detected in partially purified samples of Apo I from *K. pneumoniae*, but the nature and role of this association were unclear (19). In this report, we show that the protein associated with pure Apo I from *K. pneumoniae* is the product of *nifY*. We further characterize the nature and role of the proteins associated with Apo I from both *K. pneumoniae* and *A. vinelandii*.

The initial indication that the associated factor in *K. pneumoniae* is NifY came from sequence analysis of the purified protein. To isolate the protein, we purified Apo I from *K. pneumoniae* UN1217 (*nifN4536*) through the hydroxylapatite column step as outlined previously (11), except that *K. pneumoniae* Apo I was eluted from the DEAE-cellulose column at 0.25 M NaCl. The reactivatible fractions were then analyzed on an alkyl superose fast protein liquid chromatography column and eluted at 0.63 M $(NH_4)_2SO_4$ in 0.025 M MOPS (morpholinepropanesulfonic acid) (pH 7.4) containing 1.7 mM $Na_2S_2O_4$. At this point, the Apo I protein is homogeneously pure. To isolate the associated protein from Apo I, the resulting protein preparation was separated on a sodium dodecyl sulfate–13% polyacrylamide gel electrophoresis (SDS-PAGE) gel prerun with 0.25% (wt/vol) thioglycolate in the buffer and transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.) at 0.5 A for 2 h. The N terminus of the protein was blocked, so an internal fragment generated by cyanogen bromide (CNBr) digestion was sequenced. The protocols used for the digestion of the protein by CNBr and the separation of the peptide fragments for sequencing are those recommended by Promega (Madison, Wis.) for their Probe Design Peptide Separation System. The resulting blot showed bands at 24 kDa (uncut protein) and fragments of 12, 5.5, and 4.7 kDa. The analyzed sequence of the N terminus of the 12-kDa fragment was MAALQGALPAALRIVRPAQ and matched the appropriate-sized fragment predicted for the CNBr digestion of the protein encoded by the *nifY* sequence in *K. pneumoniae* (MASLQGALPAHLRIVRPAQ) with one mismatch at the third and one at the eleventh position. There was also a contaminating sequence that was partially analyzed and that had a serine and a histidine at the third and eleventh positions, respectively, which if substituted into the first sequence would give a perfect match. Further, the partial sequence of the contaminating band matches the section of the *nifY* sequence starting at amino acid 178. Both the molecular mass of the associated protein (24 kDa) and its pI (7.9), as found on two-dimensional denaturing gels (9, 12), matched the values for NifY predicted from the gene sequence.

Antibodies were raised to the SDS-PAGE-purified protein that had been associated with purified Apo I. Figure 1 shows the results of a Western blot (immunoblot) of an SDS-PAGE gel using antibodies raised to the associated protein. Samples were separated by one-dimensional SDS-PAGE as previously described (9). The protocols used for Western

* Corresponding author.

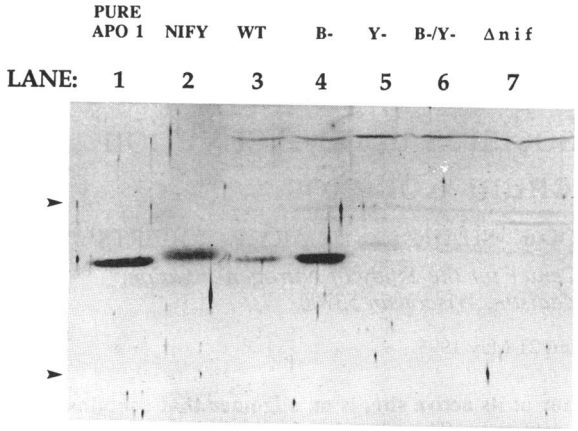

FIG. 1. Presence of *K. pneumoniae* NifY antigen in various genetic backgrounds. The Western blot of the SDS-PAGE gel shown was developed with NifY antibody. Lane 1, substantially purified *K. pneumoniae* Apo I; lane 2, partially purified, overexpressed NifY (Note: the slight mobility difference was due to the different loading buffer used to solubilize overexpressed protein); lane 3, UN (*nif*$^+$) extract; lane 4, UN1655 (*nifB*) extract; lane 5, UN5360 (*nifY*) extract; lane 6, UN5400 (*nifB nifY*) extract; lane 7, UN1978 (*nif*Δ) extract. Arrowheads indicate the positions of molecular mass markers at 29 and 18 kDa.

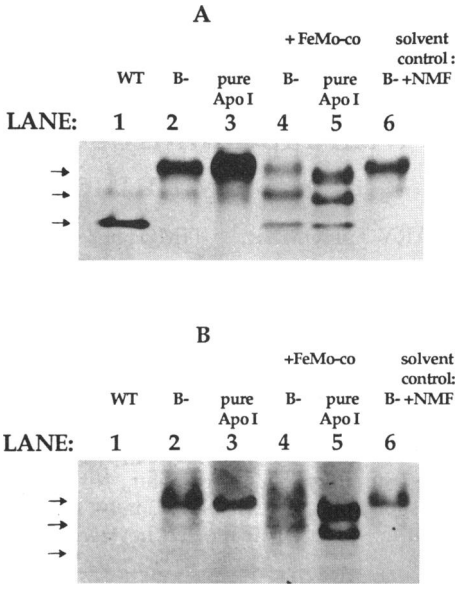

FIG. 2. Native gel showing the various electrophoretically distinct species of component I antigen from *K. pneumoniae* and the effects of FeMo-co addition on component I and NifY electrophoretic mobility. Western blots of an anaerobic native gel were developed with antibodies to component I (A) and NifY (B). Lane 1, extract from the wild type (WT), UN (*nif*$^+$); lane 2, extract from UN1655 (*nifB*); lane 3, pure Apo I from UN1655; lane 4, extract from UN1655 plus FeMo-co; lane 5, pure Apo I from UN1655 plus FeMo-co; lane 6, extract from UN1655 (*nifB*) plus *N*-methylformamide (the solvent that the FeMo-co is solubilized in). Arrows indicate the positions of the upper, middle, and lower species of component I.

blotting and developing (1) with modifications by Brandner et al. (2) have been described. Lanes 1 and 2 contain samples of purified Apo I from *K. pneumoniae* and partially purified overexpressed NifY, respectively. The antibodies raised to the associated protein cross-react with the *nifY* gene product that has been overproduced from a plasmid expression vector and partially purified. These results further demonstrate that the protein purified with Apo I from *K. pneumoniae* is the product of *nifY*. The antibodies raised to this protein are hereafter referred to as NifY antibodies.

Figure 1 also shows the cross-reactivity of extracts from various strains, and these results further confirm the identity of the protein. Cells were derepressed overnight in K medium with 0.6 μg of L-serine per ml (8). Extracts were prepared (16) and mixed with an equal volume of 40% glycerol in 25 mM MOPS (pH 7.4) containing 1.7 mM $Na_2S_2O_4$, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mg of leupeptin per liter and stored at −80°C. Equivalent amounts of protein, as determined by a bicinchoninic acid assay (17), were applied to the gel. Lanes 3 and 4 demonstrate that extracts from the wild type and from a *nifB* strain which accumulates activatable Apo I have cross-reactive material that comigrates with the NifY band in lane 2. On the other hand, extracts from strains UN5360 (*nifY* [Fig. 1, lane 5]) and UN5400 (*nifB nifY* [lane 6]) show no cross-reactive band in the region where NifY migrates. Finally, lane 7 (strain UN1978, a complete *nif* deletion) shows that the other cross-reactive bands are not *nif* encoded. The latter bands were also present in response to the preimmune serum (data not shown). It is interesting that there seems to be more cross-reactive material present in the *nifB* mutant than in the wild type. Levels of NifY may be related to the amount of unprocessed component I that is present in each extract, but this is as yet unclear.

To address whether NifY is associated with Apo I in crude extracts, as has been suggested previously (19), we examined extracts by using Western analysis of native gels. Proteins were separated on anaerobic native gels with 7 to 14% acrylamide and 0 to 20% sucrose gradients with 400 mM Tris-HCl buffer (pH 9.0). The electrophoresis buffer was N_2-sparged 65 mM Tris-borate buffer containing 1.7 mM $Na_2S_2O_4$. Gels were prerun for 1 h at 100 V for initial reduction and run for 17 h at 70 V in fresh, sparged 65 mM Tris-borate buffer containing 1.7 mM $Na_2S_2O_4$ (14). Equivalent amounts of protein from each extract were loaded. The procedure for Western blots was performed as described above except that the native gels were allowed to equilibrate in the transfer buffer for 30 min at 4°C prior to blotting. Figure 2 shows two Western blots from an anaerobically run native gel with identical samples; one panel (Fig. 2A) was developed with antibodies to component I, and the other (Fig. 2B) was developed with antibodies to NifY. The multiple protein species that cross-react with the component I antibodies will be referred to generally as component I antigens. Lane 2 in Fig. 2A shows the position of accumulated component I antigens in extract from strain UN1655 (*nifB*). There is one predominant protein species in this strain that coelectrophoreses with purified Apo I (lane 3), and this strain has FeMo-co-activatable material (Table 1); therefore, this predominant band likely represents Apo I from this strain, which is in agreement with previous reports (3, 4). The corresponding regions in Fig. 2B (lanes 2 and 3) show distinct bands which are cross-reactive to the NifY antibodies at the same positions, suggesting that NifY is complexed with Apo I in crude extracts and that the complex remains intact during electrophoresis.

Our previous study of Apo I purification utilized *A. vinelandii* as the source of protein, and a variety of methods

TABLE 1. Activities of *K. pneumoniae* extract

Strain	Description	Source or reference	Sp act[a] in:	
			Extract alone	Extract + FeMo-co
UN	Wild type	P. W. Wilson	54	54
UN1655	*nifB4691*	8	0	24
UN5360	*nifY6290::aphA*	M. Gosink	32	32
UN5400	*nifB4691 nifY6290::aphA*	M. Gosink	0	1.7
UN1978	*nif-4932*	8	0	0

[a] One unit corresponds to 1 nmol of acetylene reduced per min per mg of protein at 30°C.

were unsuccessfully employed to remove the associated protein from the Apo I complex (11). We repeated these experiments with both extracts and purified Apo I from *K. pneumoniae*, using Western analysis to monitor dissociation. A comparison of lanes 2 and 4 (Fig. 2) shows the effect of FeMo-co addition to Apo I in crude extract. Without FeMo-co addition (lane 2), the detectable component I antigens run as the single upper band (Fig. 2A) and comigrate with detectable NifY antigens (Fig. 2B). Addition of FeMo-co causes the component I antigen to shift predominantly to two lower positions (Fig. 2A, lane 4). The lower of these bands shows no associated NifY and comigrates with active component I from the wild type (Fig. 2, lane 1; for activity, see Table 1), while the middle band has some associated NifY (Fig. 2B). The small amount of component I antigen remaining at the Apo I position is also associated with NifY. These mobility shifts correlate with the production of functional component I in a FeMo-co insertion acetylene reduction assay (Table 1). Similarly, addition of FeMo-co to the purified Apo I from *K. pneumoniae* also causes a mobility shift and dissociation of NifY (Fig. 2A and B, lanes 3 and 5). There are slight differences in migration between the pure Apo I (lanes 3 and 5) and the Apo I in crude extracts (lanes 2 and 4) which are due to the slightly different salt concentrations in the samples. While it is certainly possible that other proteins may be involved in NifY dissociation in vivo, these results indicate that the in vitro dissociation of NifY from Apo I of *K. pneumoniae* requires only the addition of FeMo-co.

On the basis of the observation that the lowest band is the predominant species of component I antigen in the wild type and does not have NifY associated with it (Fig. 2), it is tempting to speculate that this band is active component I and the middle band is an incompletely processed form, as has been suggested previously (3, 4). As postulated previously, this species might have a FeMo-co at one site and one associated NifY molecule (3, 19). Such a hypothesis could predict that this band possesses component I activity, since the two FeMo-co sites may be functionally independent. To directly test this, we activated pure *K. pneumoniae* Apo I in vitro by addition of FeMo-co, separated the species on native gels, excised all three species from the gels, and assayed for acetylene reduction activity. Activities were normalized to the amount of the Coomassie-stained bands detected by a densitometry scan because determination of the amount of protein in milligrams would be technically very difficult and we were primarily interested in the relative activity of the species. The results of three independent experiments indicate that the middle species has substantial activity, an average of 46% of that seen with the lower band. This suggests that the middle band does reflect a component I species with a single functional active site and that the FeMo-co sites are functionally independent.

Given the apparently critical role of NifY in Apo I maturation, it is surprising that a *nifY* (UN5360) strain has 60 to 70% of the wild-type levels of acetylene reduction activity. In contrast, the *nifB nifY* double mutant (strain UN5400) accumulates an Apo I that is much less FeMo-co activatable than the Apo I found in a *nifB* mutant (Table 1). To rationalize these results, we favor a model in which the primary role of NifY is the stabilization of an activatable conformation of Apo I and in the absence of NifY this role may be provided by other factors in the cell, albeit less well. In *nifY* strains, the problem of an unstable Apo I in the absence of NifY is mitigated by the ready availability of FeMo-co as the protein is made, allowing a majority of the Apo I to be rapidly processed to the stable, active form. In a *nifB nifY* strain, however, the Apo I has to accumulate without NifY, leading to a form that has substantially less ability to be activated in vitro (Table 1). In the absence of NifY, however, a small population of the Apo I could be in the proper conformation for FeMo-co insertion, which may account for the low level of FeMo-co-activatable Apo I that is detected.

The precise role of NifY in component I maturation might be to protect, or to provide access to, the site of FeMo-co binding. The crystal structure of component I indicates that FeMo-co is positioned well within the mature protein (7) so that NifY might be necessary to hold and stabilize Apo I in a conformation that allows FeMo-co insertion.

These results create a paradox in that activation by FeMo-co causes NifY to dissociate from Apo I in *K. pneumoniae*, but the Apo I-associated protein in *A. vinelandii* does not dissociate from purified Apo I (11). Our preliminary results indicate that the associated protein in *A. vinelandii* would dissociate upon FeMo-co activation in crude extracts, but not from the purified Apo I, indicating that the processing of component I in this organism is different from that in *K. pneumoniae* (data not shown).

The identity of the Apo I-associated protein in *A. vinelandii* remains unknown but does not seem to be the product of the designated *nifY* gene (6), since the Apo I-associated protein from *A. vinelandii* has an acidic pI (10) and the predicted pI of NifY of that organism is extremely basic (6). Finally, antibodies raised to *K. pneumoniae* NifY failed to cross-react with *A. vinelandii* Apo I-associated protein and antibodies to the *A. vinelandii* Apo I-associated protein failed to cross-react with NifY (data not shown).

Summary. In conclusion, (i) NifY is associated with Apo I in crude extracts and pure Apo I from *K. pneumoniae*; (ii) NifY can be dissociated from Apo I in vitro by FeMo-co addition in the absence of other factors; (iii) NifY is associated with more than one, but not all, species of component I antigens present in wild-type and mutant extracts; and (iv) the requirements for dissociation of the Apo I-associated

protein in *K. pneumoniae* (NifY) are different from those in *A. vinelandii*.

We thank Mark Gosink for the construction of strains UN5360 and UN5400, Nancy Franklin for the production and partial purification of the overexpressed NifY, and Marc Rott for helpful discussions and assistance with the Western protocol. The blot was sent to the University of California—Riverside Biotech facility for peptide sequence analysis. Antibodies to *A. vinelandii* component I were a gift from P. W. Ludden.

This work was supported by the College of Agricultural and Life Sciences, by grants GM35163 to G. P. Roberts and GM35332 to P. W. Ludden from the Public Health Sciences (for V.K.S.), and by NIH training grant T32GM07215 (for M.J.H.).

REFERENCES

1. **Blake, M. S., K. H. Johnson, G. J. Russel-Jones, and E. C. Gotchlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136**:175–179.
2. **Brandner, J. P., A. G. McEwan, S. Kaplan, and T. J. Donohue.** 1989. Expression of the *Rhodobacter sphaeroides* cytochrome c_2 structural gene. J. Bacteriol. **171**:360–368.
3. **Govezensky, D., and A. Zamir.** 1989. Structure-function relationships in the α subunit of *Klebsiella pneumoniae* nitrogenase MoFe protein from analysis of *nifD* mutants. J. Bacteriol. **171**:5729–5735.
4. **Harris, G. S., T. C. White, J. E. Flory, and W. H. Orme-Johnson.** 1990. Genes required for formation of the apoMoFe protein of *Klebsiella pneumoniae* nitrogenase in *Escherichia coli*. J. Biol. Chem. **265**:15909–15919.
5. **Hawkes, T. R., and B. E. Smith.** 1983. Purification and characterization of the inactive MoFe protein of the nitrogenase from *nifB* mutants of *Klebsiella pneumoniae*. Biochem. J. **209**:43–50.
6. **Jacobson, M. R., K. E. Brigle, L. T. Bennett, R. A. Setterquist, M. S. Wilson, V. L. Cash, J. Beynon, W. E. Newton, and D. R. Dean.** 1989. Physical and genetic map of the major *nif* gene cluster from *Azotobacter vinelandii*. J. Bacteriol. **171**:1017–1027.
7. **Kim, J., and D. C. Rees.** 1992. Crystallographic structure and functional implications of the nitrogenase molybdenum-iron protein from *Azotobacter vinelandii*. Nature (London) **360**:553–560.
8. **MacNeil, T., D. MacNeil, G. P. Roberts, M. A. Supiano, and W. J. Brill.** 1978. Fine-structure mapping and complementation analysis of *nif* (nitrogen fixation) genes in *Klebsiella pneumoniae*. J. Bacteriol. **136**:253–266.
9. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
10. **Paustian, T. D., and G. P. Roberts.** Unpublished data.
11. **Paustian, T. D., V. K. Shah, and G. P. Roberts.** 1990. Apodinitrogenase: purification, association with a 20kD protein, and activation by FeMoco in the absence of dinitrogenase reductase. Biochemistry **29**:3515–3522.
12. **Roberts, G. P., and W. J. Brill.** 1980. Gene-product relationships of the *nif* regulon of *Klebsiella pneumoniae*. J. Bacteriol. **144**:210–216.
13. **Roberts, G. P., T. MacNeil, D. MacNeil, and W. J. Brill.** 1978. Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. J. Bacteriol. **136**:267–279.
14. **Shah, V. K., and W. J. Brill.** 1973. Nitrogenase. IV. Simple method of purification to homogeneity of nitrogenase components from *Azotobacter vinelandii*. Biochim. Biophys. Acta **305**:445–454.
15. **Shah, V. K., and W. J. Brill.** 1977. Isolation of an iron-molybdenum cofactor from nitrogenase. Proc. Natl. Acad. Sci. USA **74**:3249–3253.
16. **Shah, V. K., G. Stacey, and W. J. Brill.** 1983. Electron transport to nitrogenase: purification and characterization of pyruvate-flavodoxin oxidoreductase, the *nifJ* gene product. J. Biol. Chem. **258**:12064–12068.
17. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
18. **Triplett, E. W., G. P. Roberts, P. W. Ludden, and J. Handelsman.** 1989. What's new in nitrogen fixation. ASM News **55**:15–21.
19. **White, T. C., G. S. Harris, and W. H. Orme-Johnson.** 1992. Electrophoretic studies on the assembly of the nitrogenase molybdenum-iron protein from the *Klebsiella pneumoniae nifD* and *nifK* gene products. J. Biol. Chem. **267**:24007–24016.