Synthesis of the Iron-Molybdenum Cofactor of Nitrogenase Is Inhibited by a Low-Molecular-Weight Metabolite of *Klebsiella pneumoniae*

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The in vitro synthesis of the iron-molybdenum cofactor nitrogenase was inhibited by a low-molecular-weight factor. This inhibitory factor was present in the membrane extracts of wild-type and *nif* mutant strains of *Klebsiella pneumoniae* that were grown under conditions that either repressed or derepressed nitrogenase expression. In vitro, the inhibition was specific for the NifB protein. Addition of this factor to *K. pneumoniae* cells at various times during *nif* derepression decreased nitrogenase activity, presumably through inhibition of iron-molybdenum cofactor synthesis. The inhibitor was purified by solvent extraction and chromatography on DEAE-cellulose, silica gel, and aluminum oxide columns.

Nitrogen fixation is an important process for the assimilation of molecular nitrogen from the environment, and the enzyme which catalyzes the reduction of N₂ to ammonia is nitrogenase. This enzyme is also capable of reducing acetylene to ethylene, which is often used as a convenient way of measuring nitrogenase activity. Nitrogenase is a complex consisting of two proteins, dinitrogenase and dinitrogenase reductase (2, 4). The active site of dinitrogenase is thought to be composed of a unique cofactor, iron-molybdenum cofactor (FeMo-co), that contains Fe, Mo, S, and homocitrate (5–7, 9, 14, 18, 19, 24).

Previous work has implicated six *nif* (nitrogen fixation) gene products in the synthesis of FeMo-co in *Klebsiella pneumoniae*. These include the products of *nifQ* (10), *nifB*, *nifN*, and *nifE* (15, 26), *nifV* (5), and *nifH* (the structural gene for dinitrogenase reductase) (3, 16). In 1986, Shah et al. (22) described an in vitro system for the synthesis of FeMo-co, which has provided an excellent tool for studying FeMo-co synthesis by using combinations of extracts from well-defined mutants. Such analysis has allowed the determination that the *nifV* gene product is required for the synthesis of homocitrate (7, 8), which was subsequently shown to be an integral part of FeMo-co (6). The in vitro assay system has also been used to monitor the purification of the NifNE protein complex (13), as well as for partial purification of the NifB protein, which has been shown to be an integral membrane protein in *K. pneumoniae* (V. Shah, unpublished results). However, there is currently no biochemical information on the roles of these proteins in FeMo-co synthesis.

In this article we report the characterization of a low-molecular-weight compound found in *K. pneumoniae* membranes that inhibits FeMo-co synthesis in vitro and also affects nitrogenase activity in vivo. We suggest that this molecule is a normal metabolite of cellular metabolism, as we found no growth or strain specificity for its accumulation. This molecule specifically inhibits the *nifB* gene product and thus provides an opportunity to learn more about the biochemical function of this protein in FeMo-co biosynthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. pneumoniae* UN1217 (*nifN*4536), 1655 (*nifB*4691), 1222 (*nif*4541), and 1100 (*nifE*4420) (15) and *Azotobacter vinelandii* mutant strains UW45 (*nifB*) (21) and CA12 (Δ *nifHDK*) (1, 22) have been described, as have the growth and derepression of *A. vinelandii* (20, 22). K medium was used as an N-free minimal medium (12) for *K. pneumoniae*, and KN medium containing 25 mM ammonium acetate was used when nitrogenase expression was not required. *K. pneumoniae* strains were derepressed on a large scale as follows. Cultures were grown to full density on KN medium, and a 1:30 inoculum was made into K medium containing 0.6 g of serine per liter. These cultures were sparged sparingly with N₂ to maintain anaerobiosis and allowed to grow for 18 to 20 h. Cells were then harvested anoxically with a Pellicon filtration device (Millipore Corp.). Small-volume derepressions of *K. pneumoniae* were carried out as follows. Cultures were grown to full density in KN medium, cells were pelleted and suspended in an equal volume of K medium, and a 1:10 inoculum was made into K medium containing 0.3 g of serine per ml (final volume, <2 ml) in vials that were evacuated repeatedly and filled with N₂. Derepressions were carried out at 30°C for 5 h.

Preparation of extracts. *A. vinelandii* crude extracts were prepared by osmotic shock as described previously (20). Preparations containing the NifB protein were made from the membrane fraction of *K. pneumoniae* mutant strain UN1217 obtained by anoxic French pressure cell breakage (22). The membranes were then washed sequentially with 2% Triton X-100 and 1 M NaCl (both in 0.1 M Tris hydrochloride [pH 7.4]). The washed membranes were then solubilized with 1% *N*-lauryl sarcosine in 0.1 M Tris HCl (pH 7.4). This preparation of NifB is estimated to be 20% pure and contains no other Nif proteins (V. Shah, unpublished results). All manipulations were done anoxically in buffers containing 1.7 mM sodium dithionite.

In vitro FeMo-co synthesis and acetylene reduction assays. Assays for in vitro FeMo-co synthesis have been described before (8). Briefly, the cell extract of *A. vinelandii* mutant strain UW45 (*nifB*) was the source of all protein components (except the NifB protein) required for FeMo-co synthesis.

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UW45 extract and partially purified NifB protein (from *K. pneumoniae* UN1217) were incubated with homocitrate, molybdate, Mg-ATP, and dithionite for 30 min for the synthesis of FeMo-co and its insertion into the apodinitrogenase. Following this incubation, nitrogenase activity was determined by the acetylene reduction method (8). Where stated, purified inhibitor was added before ATP plus dithionite, before the incubation of the synthesis components. Two protocols were used for FeMo-co synthesis. In most cases, the protein components were provided by a mixture of UW45 (*nifB*) crude extract and a limiting amount of partially purified NifB protein. Alternatively, crude extracts of CA12 (Δ *nifHDK*) provided all proteins except dinitrogenase reductase (added as pure protein) and apodinitrogenase (supplied by UW45 extracts). Where noted, tetrathiomolybdate was used at a 20 nM concentration to prevent FeMo-co insertion into apodinitrogenase (23). Protein concentrations were measured by the bicinchoninic acid method (25). Dinitrogenase reductase and FeMo-co were purified as described previously (17, 18).

Purifications of inhibitor. The inhibitory factor was purified aerobically because no differences were observed between the aerobically and anaerobically purified product. *K. pneumoniae* cells were suspended in 4 volumes of methanol and stirred at room temperature for 2 h, and the supernatant was collected after centrifugation at $11,000 \times g$ for 15 min. The supernatant was concentrated to ca. 1/10 volume with a rotary evaporator under vacuum at 40 to 50°C. Four volumes of deionized water were added, and the suspension was again centrifuged at $11,000 \times g$ for 15 min. The supernatant was concentrated 20-fold and applied to a DEAE-cellulose column (2.5 by 15.5 cm) equilibrated in deionized water. The column flowthrough contained all of the inhibitory activity. This fraction was concentrated to a minimum volume and added dropwise with stirring to 8 volumes of methanol. The resulting suspension was centrifuged at $15,000 \times g$ for 15 min. The supernatant (which contained inhibitory activity) was vacuum dried and resuspended in a minimum volume of deionized water. This fraction was added dropwise to 10 volumes of acetone, and the resulting suspension was centrifuged at $15,000 \times g$ for 15 min. The pellet (containing the inhibitory activity) was suspended in a minimum volume of deionized water. This fraction was applied to a Florisil column (0.75 by 35 cm) equilibrated with methanol. The factor was eluted from the column by washing with a 9:1:1 mixture of methanol, ammonium hydroxide, and water. The fractions containing the factor were pooled and vacuum dried. The residue was suspended in a minimum volume of methanol and applied to an aluminum oxide column (0.75 by 15 cm) equilibrated with methanol. The column was washed with 2 bed volumes of methanol before the inhibitory activity was eluted with 2 bed volumes of a 9:1:1 ratio mixture of methanol, ammonium hydroxide, and water. This fraction, containing the inhibitory activity, was vacuum dried and suspended in a small volume of deionized water for use as the purified preparation of inhibitor. This preparation left three spots on a thin-layer chromatography plate as visualized by UV quenching and staining with iodine vapor. These three compounds were separated with a Sephadex G-10 gel filtration column, and only the slowest-migrating one had inhibitory activity.

Mass determination. Molecular mass was determined by high-resolution mass spectrometry.

Chemicals. ATP, phosphocreatine, glycerol, Tris base, and DNase I were obtained from Sigma Chemical Co., St. Louis, Mo. Creatine phosphokinase was obtained from

FIG. 1. Inhibition of the in vitro FeMo-co synthesis system. Reaction mixtures contained 0.2 ml of strain UW45 crude extract (3.6 mg of protein), 50 μ l of partially purified NifB protein (0.31 mg of protein), 0 to 100 μ l of purified inhibitor, and other components as described in Materials and Methods. Incubations were at 30°C for 35 min. Acetylene reduction assays were carried out for 15 min at 30°C.

Miles Laboratories, Elkhart, Ind. Ultrapure sodium molybdate was obtained from Research Organic/Inorganic Chemical Corp., Sun Valley, Calif. DEAE-cellulose (DE52) is a Whatman product. Diaflo membranes were from Amicon Corp., Lexington, Mass. Florisil (100-200 mesh) was a product of Fisher Scientific Co., Fair Lawn, N.J. MN-Aluminum oxide was a product of Macherey Nagel, Doren, Germany. Sephadex G-10 and G-25 were from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of ultrapure or analytical grade available commercially.

RESULTS

Initial observations. When extracts of *K. pneumoniae* membrane, solubilized with 1% *N*-lauryl-sarcosine, were used as a source of NifB protein in the in vitro FeMo-co synthesis system, the observed acetylene reduction activity was not proportional to the amount of membrane extract added. This was in contrast to experiments with partially purified NifB protein, in which activity is proportional to the volume added (when all other assay components are in excess) (V. Shah, unpublished results).

The same sarcosine extracts, after passage through a G-25 Sephadex gel filtration column, yielded activity proportional to added volume in the in vitro FeMo-co synthesis system. Furthermore, inhibition of the FeMo-co synthesis system was observed when a concentrated Diaflo filtrate (Amicon P-10 membrane) of these membrane extracts was added to the reaction mixture. These data suggested that a cell product was inhibiting the in vitro FeMo-co synthesis system. An inhibition profile made with a purified preparation (see Materials and Methods) of the inhibitory compound is shown in Fig. 1.

Localization and properties of inhibitory compound. The inhibitory effect of *K. pneumoniae* membrane extract was first observed in a Nif^{NE} strain (UN1217) which had been derepressed for nitrogenase activity as described in Materials and Methods. We have subsequently shown that this factor (based on activity) can be isolated from wild-type *K. pneumoniae* UN900 as well as a variety of other *nif* mutant strains (UN1655, UN1100, and UN1222). It is particularly interesting that this factor could be isolated from wild-type cells grown in the presence of excess ammonia, completely repressing expression of the *nif* genes (11). This result strongly suggests that the factor is not *nif* specific.

TABLE 1. Effect of inhibitor on in vitro FeMo-co synthesis

FeMo-co synthesis conditions ^a	Addition after synthesis	Ethylene formed (nmol/assay)	% of control activity
Complete system	None	212	180
Without apodinitrogenase (control)	Apodinitrogenase	118	100
Without apodinitrogenase, with inhibitor	Apodinitrogenase	47	40
Without apodinitrogenase	Apodinitrogenase + inhibitor	101	86
Without apodinitrogenase	Apodinitrogenase (inhibitor added after insertion)	101	86

^a The complete system contains 0.2 ml of CA12 ($\Delta nifHDK$) crude extract (2.8 mg of protein), 0.2 ml of UW45 (*nifB*) crude extract (as a source of apo-dinitrogenase) (3.6 mg of protein), 15 μ l of dinitrogenase reductase (25 μ g), and other components described in Materials and Methods. Purified inhibitor (30 μ l) was added where indicated. The assays were carried out as follows: (i) initial components (including UW45 [*nifB*] extract in the complete system and inhibitor as indicated) were combined and incubated at 30°C for 35 min; (ii) UW45 extract or inhibitor was added; (iii) the sample was incubated at room temperature for 5 min; (iv) 20 nM tetrathiomolybdate was added to each assay, followed by an additional 5 min of incubation; (v) 0.8 ml of ATP plus dithionite was added to all assays and inhibitor was added to one; and (vi) acetylene reduction assays were carried out at 30°C for 15 min.

This compound was found in the membranes. In the control experiment, when the soluble extracts of these strains were carried through the purification procedure, no similar activity was found (data not shown). The inhibitor could be recovered from the membrane by 1% Triton X-100 elution. However, it was not eluted from the membrane by a 1 M NaCl wash, suggesting that it interacts in a hydrophobic and not an ionic manner with the membrane.

The inhibitory factor was stable to O₂, strong alkali, and strong acid, and the activity did not bind to a cation- or anion-exchange matrix. The compound was highly soluble in water and methanol but not in acetone or other nonpolar solvents. No absorbance in the UV or visible range was found to correspond to the inhibitory activity. The molecular mass of the inhibitor was determined to be 367 DA by high-resolution mass spectrometry.

Synthesis of FeMo-co is the target of inhibition. The assay system used for in vitro FeMo-co synthesis is complex, requiring at least three stages for the complete assay. These stages are (i) FeMo-co synthesis, (ii) FeMo-co insertion into apodinitrogenase, and (iii) assay of nitrogenase activity. In order to determine at which stage the inhibition was occurring, each stage in the process was assayed individually as follows.

In order to assay FeMo-co synthesis separately from its insertion into apodinitrogenase, a modified assay system was used to which a source of apodinitrogenase could be added after FeMo-co synthesis was complete. The results of this experiment are presented in Table 1. This system contains a crude extract of *A. vinelandii* CA12 ($\Delta nifHDK$), which lacks dinitrogenase and dinitrogenase reductase. To this extract, dinitrogenase reductase, homocitrate, molybdate, and ATP plus dithionite were added as described in Materials and Methods and incubated for 35 min to allow FeMo-co synthesis to occur. A source of apodinitrogenase (UW45) was then added, and the assay mixture was incubated for an additional 5 min to allow insertion of FeMo-co into apodinitrogenase. During this short additional time of incubation, no significant FeMo-co synthesis occurs, but insertion of existing FeMo-co can be completed (23; Downs, unpublished data). We are thus assaying the FeMo-co which is synthesized and accumulated during the initial 35-min incubation. At this point, further FeMo-co insertion is blocked by the addition of 20 nM tetrathiomolybdate. Shah et al. (23) have previously shown that tetrathiomolybdate specifically binds apodinitrogenase and prevents FeMo-co insertion. Nitrogenase activity is then assayed by acetylene reduction.

Table 1 shows the control level of acetylene reduction when all components (including a source of apodinitrogenase) of the assay system were added at time zero. When

apodinitrogenase was added after FeMo-co synthesis, acetylene reduction dropped about 50%. Similar results were seen when purified apodinitrogenase (a gift from T. Paustian) was used instead of UW45 extract. This reduction in activity when a source of apodinitrogenase was added after synthesis is not surprising, since FeMo-co is known to be unstable in aqueous solution (18). Under standard assay conditions (i.e., with apodinitrogenase present), FeMo-co is probably inserted immediately after being synthesized, and thus the instability of free FeMo-co is not a factor. The activity presented is thus the control activity with which the assays containing inhibitor should be compared. When inhibitor was added prior to FeMo-co synthesis and was therefore available to inhibit each of the three stages of the assay system, 60% inhibition resulted. When inhibitor was added concurrently with apodinitrogenase, after synthesis of FeMo-co was complete, the inhibitor only had the opportunity to inhibit insertion of FeMo-co and the nitrogenase assay itself, and the inhibition was significantly less. When the inhibitor was only available to inhibit the nitrogenase assay (i.e., added after tetrathiomolybdate had quenched all apodinitrogenase), 15% inhibition resulted. These results show there was no inhibition of FeMo-co insertion into apodinitrogenase and that FeMo-co synthesis was the primary target of the inhibitor when the complete synthesis assay system was used. It is interesting that use of a much higher level of inhibitor also resulted in only ~15% inhibition of the nitrogenase assay (data not shown). This suggests a nonspecific inhibition of some aspect of the nitrogenase assay, versus a concentration-dependent inhibition of FeMo-co synthesis (Fig. 1).

The following experiment was done to verify that inhibition by this compound was occurring primarily during FeMo-co synthesis. Duplicate vials for the complete synthesis system were prepared with and without inhibitor. Prior to the assay of acetylene reduction, excess FeMo-co was added to one of each duplicate and incubated for 5 min to allow its insertion into apodinitrogenase. In each case, addition of FeMo-co significantly increased the acetylene reduction activity relative to the assay containing only in vitro-synthesized FeMo-co (>120 additional nmol of acetylene reduced per assay; data not shown). This allows the conclusion that neither apodinitrogenase nor dinitrogenase reductase is the limiting component in the assay when inhibitor is present in the complete FeMo-co synthesis system.

NifB protein is the apparent target of inhibition. As a test of the target site of inhibition in the synthesis assay, the system was divided into two major components: limiting amounts of partially purified NifB protein and UW45 crude extract (containing apodinitrogenase, NifNE protein, and dinitroge-

TABLE 2. Effect of inhibitor on NifB activity

Conditions ^a	Ethylene formed (nmol/assay)		% Inhibition
	Without inhibitor	With inhibitor	
Standard assay	258	195	76
Preincubation with NifB	241	51	21
Preincubation with UW45	225	158	70

^a Standard assay contained 0.2 ml of UW45 (*nifB*) crude extract (3.6 mg of protein), 50 μ l of partially purified NifB protein (0.31 mg of protein), and other components listed in Materials and Methods. Where noted, 30 μ l of the purified preparation of inhibitor was added. Percent inhibition is defined as [(activity with inhibitor)/(activity without inhibitor)] $\times$ 100 for each condition. Preincubation mixtures contained all assay components except UW45 (*nifB*) crude extract for preincubation with NifB or purified NifB protein for preincubation with UW45 extract. Preincubations were done at 30°C for 30 min, after which the missing component was added and the standard assay was carried out.

nase reductase). The results of incubation experiments with these two components are shown in Table 2. Incubation of the inhibitor with partially purified NifB protein significantly increased the inhibition of acetylene reduction activity. This enhanced inhibition did not depend on homocitrate, molybdate, or ATP plus dithionite (data not shown). Incubation of the inhibitor with the UW45 extract produced no significant change in inhibition compared with the control assay (Table 2). These data strongly suggest that the NifB protein is the target of inhibition.

In both cases, the assays without inhibitor had lower activity than the standard assay. We attribute this to instability of some of the components during the extra 30-min preincubation. This variation in control activities is accounted for by comparing only the inhibited and uninhibited assays of the same incubation conditions.

If NifB protein is the target of the inhibitor, one would expect increased FeMo-co synthesis activity with increasing NifB protein at a given concentration of inhibitor. The results of such an experiment are shown in Table 3. Since UW45 crude extract was always in excess of our assays, there was a linear increase in activity of the uninhibited assays with increasing NifB protein. In the assays containing inhibitor, inhibition could be completely overcome by a large excess of NifB protein (data not shown). These results corroborate the previous conclusion that NifB protein is the primary target of inhibition. They also suggest an equilibrium or competition type of inhibition, since the percent inhibition remained the same with increasing acetylene reduction activities.

In vivo inhibition of FeMo-co synthesis. Although the complexity of the in vivo system does not allow a direct test

TABLE 3. Effect of NifB/inhibitor ratio on activity^a

NifB (μ g) μ g NifB	Ethylene formed (nmol/assay)		% Inhibition
	Without inhibitor	With inhibitor	
62	57	26	54
124	120	67	44
186	180	104	42

^a Assays contained 0.2 ml of UW45 crude extract (3.6 mg of protein), the indicated amounts of purified NifB protein, and other components listed in Materials and Methods. Where indicated, 30 μ l of purified inhibitor was added.

FIG. 2. Inhibition of whole-cell acetylene reduction activity. Cultures of UN900 were derepressed in K medium plus serine (0.3 g/liter) containing the indicated volume of inhibitor (see Materials and Methods). Vials were repeatedly evacuated and filled with N_2 . The derepression was carried out for 5 h with the final OD_{600} of the cultures ranging from 1.0 to 1.1. Acetylene reduction assays were carried out for 15 min at 30°C.

of FeMo-co synthesis inhibition, in vivo inhibition of whole-cell acetylene reduction activity was tested to demonstrate that the inhibition was not an artifact of the in vitro assay system. Small-scale *nif* derepressions of wild-type *K. pneumoniae* were carried out as described in Materials and Methods. The appropriate amount of inhibitor was added to the medium prior to the start of the 5-h derepression. Whole-cell nitrogenase activity was measured by the acetylene reduction assay. A titration of inhibitor under these conditions is shown in Fig. 2.

It is clear that inhibition was occurring in vivo, and at a similar rate as in the in vitro synthesis system. The rate of inhibition both in vivo and in vitro (Fig. 1) was 2.6 percentage points per μ l of inhibitor. The similarity in inhibition under these two different conditions suggests that uptake of the inhibitor is not a limiting factor.

The possibility that ammonium ion, which represses nitrogenase synthesis, might be present in the inhibitor fraction was tested, because ammonium is in the column solvents used for purification of the factor. Ammonia was removed by raising the pH of the sample to 12 and vacuum drying, followed by suspension in water. The sample with the highest concentrations used in Fig. 2 was shown to have undetectable levels of ammonia with Nestler reagent but was still fully active as an inhibitor after this treatment. This rules out the possibility that the inhibition of acetylene reduction in vivo is due to repression of the *nif* genes by ammonia.

To test whether FeMo-co synthesis could be the target of inhibition in vivo, inhibitor was added at various times during the 5-h derepression (Table 4). This experiment demonstrated a time-dependent inhibition of nitrogenase activity during derepression and showed only a minimal (<15%) inhibition of preformed nitrogenase activity. While we cannot definitively conclude that FeMo-co synthesis is the target of inhibition in vivo, these data are consistent with that interpretation and with the in vitro results.

DISCUSSION

This article describes the isolation and functional characterization of a small molecule that specifically inhibits the synthesis of the FeMo-co of nitrogenase. This molecule has

TABLE 4. Effect of addition of inhibitor at various times during nitrogenase derepression^a

Time of inhibitor addition (h of incubation)	Ethylene formed (nmol/assay)
None added	249
0	88
2	143
3	164
4	188
5	218

^a Derepressions were carried out as described in Materials and Methods. All cultures were assayed for acetylene reduction after 5 h. Acetylene reduction assays were carried out for 15 min at 30°C at the end of the 5-h derepression. Purified inhibitor (30 μ l) was added at the indicated times.

been found in all *K. pneumoniae* mutant strains tested, in addition to the wild type. It is present in cells grown under conditions which either repress or derepress *nif* expression and is located specifically in the membrane.

A strong specificity of the inhibitor for the NifB protein was demonstrated *in vitro*. The inhibition appears to be nondestructive with respect to the inhibitor because a given amount of inhibitor produces a constant reduction of activity with increasing concentrations of NifB protein. If the inhibitor were being consumed during this inhibition, one would expect activity to be proportional to the concentration of the NifB protein minus the concentration of inhibitor.

The reversibility of inhibition with respect to the NifB protein has not been addressed directly. However, the data in Table 2 suggest that the NifB protein may be irreversibly inhibited. Incubation of the NifB protein with this inhibitor greatly increased the extent of inhibition, suggesting that the NifB protein is rendered nonfunctional and addition of the remaining components required for FeMo-co synthesis cannot rescue synthesis activity.

We assume that the *in vivo* inhibition of nitrogenase activity by this compound is due to inhibition of the NifB protein, preventing FeMo-co synthesis. However, this has not been confirmed. The fact that this inhibitor works *in vivo* is an interesting paradox if we consider that the inhibitor molecule has been isolated from the very strains it inhibits. Thus, it seems unlikely that its natural function in the cell is to inhibit FeMo-co biosynthesis. It can be argued that the concentration or location of this compound in the cell is tightly controlled. Our results could then be explained by the high concentrations of inhibitor added to our assays.

There are other plausible explanations for this paradox, none of which can be rigorously tested until the structure of the molecule is determined. One explanation is that this molecule never interacts with the NifB protein *in vivo*. For example, if this molecule is compartmentalized away from the FeMo-co biosynthetic machinery, its effect would never be observed. It is interesting, however, that this molecule and its putative target (NifB protein) are both located in the membrane. The fact that the NifB protein and the inhibitor require very different extraction methods makes it reasonable that they may not interact *in vivo*.

The specificity of this inhibitor for the NifB protein will allow its use as a tool for probing the biochemical function of this protein in FeMo-co biosynthesis. Once the structure of this molecule has been determined, we will be able to address more subtle aspects of the biochemistry of this inhibition.

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LITERATURE CITED

1. Bishop, P. E., R. Premakumar, D. Dean, M. Jacobson, J. Chisnell, T. Rizzo, and J. Kopczynski. 1986. Nitrogen fixation by *Azotobacter vinelandii* strains having deletions in structural genes for nitrogenase. *Science* **232**:92-94.
2. Bulen, W. A., and J. R. LeComte. 1966. The nitrogenase system from *Azotobacter*: two-enzyme requirement for N₂ reduction. ATP-dependent hydrogen evolution and ATP hydrolysis. *Proc. Natl. Acad. Sci. USA* **56**:979-986.
3. Filler, W. A., R. M. Kemp, J. C. Ng, T. R. Hawkes, R. A. Dixon, and B. E. Smith. 1986. The *nifH* gene product is required for the synthesis or stability of the iron-molybdenum cofactor of nitrogenase from *Klebsiella pneumoniae*. *Eur. J. Biochem.* **160**:371-377.
4. Hageman, R. V., and R. H. Burris. 1978. Nitrogenase and nitrogenase reductase associate and dissociate with each catalytic cycle. *Proc. Natl. Acad. Sci. USA* **75**:2699-2702.
5. Hawkes, T. R., P. A. McLean, and B. E. Smith. 1984. Nitrogenase from *nifV* mutants of *Klebsiella pneumoniae* contains an altered form of the iron-molybdenum cofactor. *Biochem. J.* **217**:317-321.
6. Hoover, T. R., J. Imperial, P. W. Ludden, and V. K. Shah. 1989. Homocitrate is a component of the iron-molybdenum cofactor of nitrogenase. *Biochemistry* **28**:2768-2771.
7. Hoover, T. R., A. D. Robertson, R. L. Cerny, R. N. Hayes, J. Imperial, V. K. Shah, and P. W. Ludden. 1987. Identification of the V-factor needed for synthesis of the iron-molybdenum cofactor of nitrogenase as homocitrate. *Nature (London)* **329**:855-857.
8. Hoover, T. R., V. K. Shah, G. P. Roberts, and P. W. Ludden. 1986. *nifV*-dependent, low-molecular-weight factor required for *in vitro* synthesis of iron-molybdenum cofactor of nitrogenase. *J. Bacteriol.* **167**:999-1003.
9. Imperial, J., T. R. Hoover, M. S. Madden, P. W. Ludden, and V. K. Shah. 1989. Substrate reduction properties of dinitrogenase activated *in vitro* are dependent upon the presence of homocitrate or its analogues during iron-molybdenum cofactor synthesis. *Biochemistry* **28**:7796-7799.
10. Imperial, J., R. A. Ugalde, V. K. Shah, and W. J. Brill. 1984. Role of the *nifQ* gene product in the incorporation of molybdenum into nitrogenase in *Klebsiella pneumoniae*. *J. Bacteriol.* **158**:187-194.
11. MacNeil, D., J. Zhu, and W. J. Brill. 1981. Regulation of nitrogen fixation in *Klebsiella pneumoniae*: isolation and characterization of strains with *nif-lac* fusions. *J. Bacteriol.* **145**:348-357.
12. MacNeil, T., W. J. Brill, and M. M. Howe. 1978. Bacteriophage Mu-induced deletions in a plasmid containing the *nif* (N₂ fixation) genes of *Klebsiella pneumoniae*. *J. Bacteriol.* **134**:821-829.
13. Paustian, T. D., V. K. Shah, and G. P. Roberts. 1989. Purification and characterization of the *nifN* and *nifE* gene products from *Azotobacter vinelandii* mutant UW45. *Proc. Natl. Acad. Sci. USA* **86**:6082-6086.
14. Rawlings, J., V. K. Shah, J. R. Chisnell, W. J. Brill, R. Zimmerman, E. Munck, and W. H. Orme-Johnson. 1978. Novel metal cluster in the iron-molybdenum cofactor of nitrogenase. *J. Biol. Chem.* **253**:1001-1004.
15. Roberts, G. P., T. MacNeil, D. MacNeil, and W. J. Brill. 1978. Regulation and characterization of protein products coded by *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. *J. Bacteriol.* **136**:267-269.

16. Robinson, A. C., D. R. Dean, and B. K. Burgess. 1987. Iron-molybdenum cofactor biosynthesis in *Azotobacter vinelandii* requires the iron protein of nitrogenase. *J. Biol. Chem.* **262**: 14327–14332.
17. 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.
18. Shah, V. K., and W. J. Brill. 1977. Isolation of molybdenum-iron cofactor from nitrogenase. *Proc. Natl. Acad. Sci. USA* **78**:3438–3440.
19. Shah, V. K., J. R. Chisnell, and W. J. Brill. 1978. Acetylene reduction by the iron-molybdenum cofactor from nitrogenase. *Biochem. Biophys. Res. Commun.* **81**:232–236.
20. Shah, V. K., L. C. Davis, and W. J. Brill. 1972. Nitrogenase. I. Repression and derepression of the FeMo and Fe-proteins of nitrogenase in *Azotobacter vinelandii*. *Biochim. Biophys. Acta* **256**:498–511.
21. Shah, V. K., L. C. Davis, J. K. Gordon, W. H. Orme-Johnson, and W. J. Brill. 1973. Nitrogenase. III. Nitrogenaseless mutants of *Azotobacter vinelandii*: activities, cross reactions and EPR spectra. *Biochim. Biophys. Acta* **292**:246–255.
22. Shah, V. K., J. Imperial, R. A. Ugalde, P. W. Ludden, and W. J. Brill. 1986. In vitro synthesis of the iron-molybdenum cofactor of nitrogenase. *Proc. Natl. Acad. Sci. USA* **83**:1636–1640.
23. Shah, V. K., R. A. Ugalde, J. Imperial, and W. J. Brill. 1985. Inhibition of iron-molybdenum cofactor binding to component I of nitrogenase. *J. Biol. Chem.* **260**:3891–3894.
24. Smith, B., P. Bishop, R. Dixon, R. Eady, W. Fuller, D. Lowe, A. Richards, A. Thomson, R. Thorneley, and J. Postgate. 1985. *In* H. Evans, P. Bottomley, and W. Newton, (ed.), Nitrogen fixation research progress, p. 597–603. Martinus Nijhoff Publishers, Boston.
25. Smith, P., R. Krohn, A. Hermanson, A. Mallia, F. Gartner, M. Provenzano, E. Fujimoto, N. Goeke, B. Olson, and D. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85.
26. Ugalde, R. A., J. Imperial, V. K. Shah, and W. J. Brill. 1984. Biosynthesis of iron-molybdenum cofactor in the absence of nitrogenase. *J. Bacteriol.* **159**:888–893.