JOURNAL OF BACTERIOLOGY, Apr. 1988, p. 1978–1979
0021-9193/88/041978-02$02.00/0

Vol. 170, No. 4

Homocitrate Cures the NifV⁻ Phenotype in *Klebsiella pneumoniae*

TIMOTHY R. HOOVER, JUAN IMPERIAL, PAUL W. LUDDEN,* AND VINOD K. SHAH

Department of Biochemistry and Center for the Study of Nitrogen Fixation, College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 30 November 1987/Accepted 23 January 1988

Dinitrogenase was isolated from a culture of a *Klebsiella pneumoniae* NifV⁻ strain derepressed for nitrogenase in the presence of homocitrate. The enzyme isolated from this culture was identical to the wild-type dinitrogenase. These data provide in vivo evidence that the absence of homocitrate is responsible for the NifV⁻ phenotype.

Nitrogenase catalyzes the reduction of N_2 to ammonium (1). Nitrogenase is composed of two proteins, dinitrogenase (component I, Mo-Fe protein) and dinitrogenase reductase (component II, Fe protein) (1, 3). Dinitrogenase contains a unique prosthetic group, the iron-molybdenum cofactor (FeMo-co), that comprises Fe, Mo, and S (15). Biochemical and genetic studies indicate that at least six *nif* (nitrogen fixation) gene products are involved in the biosynthesis of FeMo-co. *Klebsiella pneumoniae* strains containing mutations in *nifB*, *nifN*, or *nifE* fail to synthesize FeMo-co (12, 15). Strains with mutations in *nifQ* do not synthesize FeMo-co when derepressed for nitrogenase in medium containing low levels of molybdate (8). Certain strains of *K. pneumoniae* and *Azotobacter vinelandii* containing mutations in *nifH* (encodes dinitrogenase reductase) fail to accumulate FeMo-co (2, 13). Dinitrogenase isolated from *K. pneumoniae* strains containing mutations in *nifV* exhibited altered substrate affinity and inhibitor susceptibility (10). Further studies indicated that NifV⁻ mutants were defective in FeMo-co synthesis (4). Recently, a system for the in vitro synthesis of FeMo-co was described that required ATP, molybdate, the gene products of *nifB*, *nifN*, and *nifE* (17), dinitrogenase reductase (unpublished data), and homocitrate (6). Accumulation of homocitrate by *K. pneumoniae* is correlated to the presence of a functional *nifV* gene, which apparently encodes homocitrate synthase (7). Homocitrate was found to accumulate in the medium of *K. pneumoniae* cultures during derepression for nitrogenase (6). We report here that the addition of homocitrate to the medium of *K. pneumoniae* NifV⁻ mutants cures that phenotype.

K. pneumoniae UN is a wild-type strain reisolated from strain M5a1, which is originally from P. W. Wilson's collection. Strain UN1991 (*nifV4945*) is a stable NifV⁻ mutant with a reversion frequency of 3×10^{-10} (T. MacNeil, Ph.D. thesis, University of Wisconsin-Madison, 1978) and has been described previously (9). Growth and derepression of nitrogenase in mutants of *K. pneumoniae* have been described (8). (*R*)-2-Hydroxy-1,2,4-butanetricarboxylic acid (homocitric acid) was isolated from the derepression medium of cultures of *K. pneumoniae* (6). Homocitrate was added to a culture of UN1991 to a final concentration of approximately 83 mg · liter^{-1} (0.4 mM). Dinitrogenase was purified by DEAE-cellulose chromatography (14) from strains UN, UN1991, and UN1991 which had been derepressed for nitrogenase in the presence of homocitrate.

Acetylene- and N_2-reduction assays have been described (16). Proton-reduction activity from nitrogenase was assayed by monitoring H_2 evolution with a Gow-Mac thermal conductivity gas chromatograph equipped with a Porapak R column (0.62 by 150 cm; Waters Associates). Where indicated, CO at a final pressure of 1.1 kPa was included in the proton-reduction assays. HD formation from nitrogenase (5) was assayed at room temperature with a Varian MAT 250 isotope mass spectrometer equipped with a 1.6-ml membrane-leak reaction chamber (F. Simpson, Ph.D. thesis, University of Wisconsin-Madison, 1985). All nitrogenase assays were done with an excess of dinitrogenase reductase (6 to 10 mol · mol of dinitrogenase^{-1}).

Dinitrogenase was quantitated with an enzyme-linked immunosorbent assays with rabbit antiserum directed against *K. pneumoniae* dinitrogenase and anti-rabbit IgG–alkaline phosphatase conjugate. A standard curve for quantitating the amount of dinitrogenase in each sample was prepared with the strain UN dinitrogenase preparation. A specific activity of 2,500 nmol of C_2H_2 reduced · min^{-1} · mg^{-1} was assumed for wild-type dinitrogenase (14).

During the course of the 5-h derepression for nitrogenase, 80 to 90% of the total homocitrate produced by cultures of NifV⁺ strains of *K. pneumoniae* was found to accumulate in the medium. To determine whether homocitrate could be assimilated by *K. pneumoniae*, homocitrate was added to a culture of a NifV⁻ mutant (UN1991) during the derepression period and dinitrogenase was isolated from this culture and characterized.

Dinitrogenase from NifV⁻ mutants has been reported to effectively reduce C_2H_2 and protons but not N_2 (10). In addition, proton reduction from the NifV⁻ enzyme, unlike that from the wild-type dinitrogenase, is inhibited by CO (10). As reported previously (10), the NifV⁻ dinitrogenase (from strain UN1991) was effective at C_2H_2 and proton reduction but was less effective at N_2 reduction (Table 1). CO (at 1.1 kPa) inhibited H_2 evolution from the NifV⁻ enzyme by 46%. Our data demonstrated that, in addition to these differences, the NifV⁻ dinitrogenase was much less effective at catalyzing the formation of HD than was the wild-type enzyme (Table 1). The specific activity for HD formation by the NifV⁻ enzyme was approximately 10% that of the wild-type enzyme.

At the end of a 5-h derepression for nitrogenase, cultures of strains UN, UN1991, and UN1991 plus homocitrate had whole-cell C_2H_2-reduction activities of 13.4, 4.5, and 14.0 nmol of C_2H_2 formed · min^{-1} · ml^{-1}, respectively. Purification and characterization of dinitrogenase from each of these cultures revealed that addition of homocitrate to the medium

* Corresponding author.

TABLE 1. Substrate specificity of dinitrogenase

Dinitrogenase source	Substrate specificity (nmol reduced or formed · min^{-1} · mg of dinitrogenase^{-1})					
	C_2H_2-reduction activity[a]	Proton-reduction activity[b]		N_2-reduction activity[c]	HD formation[d]	C_2H_2-reduction activity/N_2-reduction activity
		−CO	+CO			
UN	2,500	3,530	3,590	460	240	5.4
UN1991	2,200	3,210	1,730	245	23	9.0
UN1991 + homocitrate	2,700	3,400	3,310	420	180	6.4

[a] 10 μg of dinitrogenase was assayed.
[b] 50 to 70 μg of dinitrogenase was assayed.
[c] 10 to 20 μg of dinitrogenase was assayed.
[d] 50 to 60 μg of dinitrogenase was assayed.

cured the $NifV^-$ phenotype (Table 1). Dinitrogenase from strain UN1991 derepressed in the presence of homocitrate reduced N_2, C_2H_2, and protons effectively, CO only slightly inhibited H_2 evolution from the enzyme (2.6%), and the enzyme effectively catalyzed the formation of HD.

Homocitrate is capable of forming complexes with both Fe^{3+} and MoO_4^{2-} (6). The physiological significance of the presence of homocitrate in the medium has yet to be ascertained (for example, is homocitrate involved in the transport of Fe^{3+} or MoO_4^{2-} into the cell?). It should be noted that homocitrate is required for in vitro synthesis of FeMo-co, indicating that homocitrate has a role beyond transport of Fe^{3+} or MoO_4^{2-} into the cell. In addition, unlike the levels of siderophores (low-molecular-weight, low-iron-inducible, microbial iron-binding agents) (11), the levels of homocitrate produced by *K. pneumoniae* cultures were unaltered by changes in Fe^{3+} or MoO_4^{2-} concentration in the medium.

These data provide the first in vivo evidence that the lack of homocitrate is responsible for the $NifV^-$ phenotype. Furthermore, homocitrate can be taken into the cell; this results in the curing of the $NifV^-$ phenotype. Given the differences in whole-cell C_2H_2-reduction activities observed for these cultures, it also seems likely that holo-dinitrogenase accumulates to higher levels in vivo in the presence of homocitrate.

We thank Jihong Liang for his assistance with the HD-formation experiments and Gary Roberts for helpful discussions.

This research was supported by the College of Agriculture and Life Sciences at the University of Wisconsin-Madison and by Public Health Service grant R01GM35332-01A1 from the National Institutes of Health. J.I. is on leave from the Consejo Superior de Investigaciones Científicas, Spain.

LITERATURE CITED

1. **Bulen, W. A., and J. R. LeComte.** 1966. The nitrogenase system from *Azotobacter*: two enzyme requirement for N_2 reduction, ATP-dependent hydrogen evolution and ATP hydrolysis. Proc. Natl. Acad. Sci. USA **56**:979–986.
2. **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.
3. **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.
4. **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.
5. **Hoch, G. E., K. C. Schneider, and R. H. Burris.** 1960. Hydrogen evolution and exchange, and conversion of N_2O to N_2 by soybean root nodules. Biochim. Biophys. Acta **37**:273–279.
6. **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.
7. **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.
8. **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.
9. **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.
10. **McLean, P. A., and R. A. Dixon.** 1981. Requirement of *nifV* gene for production of wild-type nitrogenase enzyme in *Klebsiella pneumoniae*. Nature (London) **292**:655–656.
11. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
12. **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.
13. **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.
14. **Shah, V. K.** 1986. Isolation and characterization of nitrogenase from *Klebsiella pneumoniae*. Methods Enzymol. **118**:511–519.
15. **Shah, V. K., and W. J. Brill.** 1977. Isolation of an iron-molybdenum cofactor from nitrogenase. Proc. Natl. Acad. Sci. USA **78**:3438–3440.
16. **Shah, V. K., L. C. Davis, and W. J. Brill.** 1972. Nitrogenase. I. Repression and derepression of the Fe-Mo and Fe-proteins of nitrogenase in *Azotobacter vinelandii*. Biochim. Biophys. Acta **256**:498–511.
17. **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.