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Effect of Molybdenum Starvation and Tungsten on the Synthesis of Nitrogenase Components in *Klebsiella pneumoniae*

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Klebsiella pneumoniae M5a1 grows well in the presence or absence of molybdenum in media containing excess NH_4^+. However, growth on N_2 is completely dependent on the presence of molybdenum in the medium. Tungstate competes with the molybdate requirement during growth on N_2. In molybdenum-depleted medium, neither protein component of nitrogenase is active and neither component can be detected antigenically. These data provide evidence that molybdenum is an inducer of nitrogenase synthesis.

Molybdenum has long been known to be an essential element for biological nitrogen fixation (3). Bulen and LeComte (4) separated nitrogenase of *Azotobacter vinelandii* into two fractions, one containing component I, the other containing component II.

Studies (4-6, 9) with purified component I from various organisms have revealed that molybdenum is an integral part of component I. The competitive inhibition by tungsten against the molybdenum requirement in N_2-fixing *Azotobacter* (13), as well as other molybdenum-containing enzymes such as xanthine and sulfite oxidases (J. C. Johnson, H. J. Cohen, and K. V. Rajogopalan, Fed. Proc. **32**:508, 1973) and nitrate reductase (12), has been seen in various biological systems. We have investigated the effect of molybdenum starvation and addition of tungsten on the derepression of nitrogenase components in *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Organism used and media. *K. pneumoniae* strain M5a1 was obtained from P. W. Wilson. The medium described by Yoch and Pengra (17), modified by omitting molybdate, was used as the basal medium. When required, sodium molybdate and sodium tungstate were added to the medium, yielding a final concentration of 5 μM.

Chemicals. Adenosine 5'-triphosphate (ATP), creatine phosphokinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2), creatine phosphate, deoxyribonuclease I (deoxyribonucleate 5'-oligonucleotidohydrolase, EC 3.1.4.5), and Tris base were obtained from Sigma Chemical Co., St. Louis, Mo. Ultrapure sucrose (special enzyme grade) and magnesium sulfate were obtained from Schwarz/Mann, Orangeburg, N.Y. Dibasic sodium phosphate, monobasic potassium phosphate, sodium molybdate, and ferric chloride were Baker analyzed reagents. Sodium chloride (Matheson, Coleman and Bell, Los Angeles, Calif.), sodium tungstate, analytical reagent (Mallinckrodt, St. Louis), and other chemicals of analytical grade available commercially were used. The water used was twice glass-distilled.

Growth of organism and preparation of extracts. Cells were grown anaerobically to mid-exponential phase at 30 C in 250 ml of medium containing 400 μg of nitrogen as ammonium acetate per ml. Cells were then centrifuged and suspended in 250 ml of nitrogen-free medium. After incubation for 1.5 h anaerobically, 10 mg of L-serine was added, and the cells were incubated for an additional 4.5 h. Cells then were centrifuged at 10,000 $\times$ *g* for 10 min at 4 C and resuspended in 0.025 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4, followed by centrifugation.

The pellet was suspended in 10 volumes (based on wet weight of pellet) of degassed 25% sucrose in 0.1 M Tris-hydrochloride buffer, pH 8.0, containing 0.0025 M ethylenediaminetetraacetic acid and 1 mg of lysozyme per ml. This cell suspension was incubated at 30 C for 45 min under helium atmosphere. The cell suspension was then centrifuged at 10,000 $\times$ *g* for 10 min, and the supernatant solution was discarded. The spheroplasts formed by lysozyme treatment were anaerobically lysed by shaking with 4 volumes (based on wet weight of cells) of degassed 0.025 M Tris-hydrochloride, pH 7.4, containing 100 μg of deoxyribonuclease and 0.3 mg of $Na_2S_2O_4$ per ml. The lysed cell suspension was incubated at 30 C for 30 min and then was centrifuged at 10,000 $\times$ *g* for 1 h to remove cell debris. The extract was transferred anaerobically to a stoppered serum vial containing helium atmosphere.

Specific activity of nitrogenase components. Specific activity of nitrogenase is defined as micro-

moles of ethylene formed per minute per milligram of protein. Component activity is obtained upon titration (16) of a cell-free extract with the complementary, purified component (V. K. Shah, R. T. St. John, and W. J. Brill, Bacteriol. Proc., p. 168, 1973).

Serological assay. Quantitation of antibody cross-reacting material corresponding to each component in crude extract was performed by a modification (11) of the Laurell electroimmunodiffusion technique (14). Antiserum to components was prepared as described by Davis et al. (7) by using purified components. Peak heights of precipitin cones were compared with standard dilutions of cell-free extract of known specific activity from cells derepressed in the presence of molybdenum.

RESULTS

Effect of molybdenum starvation. The absence or presence of molybdenum or tungsten has no effect on the growth rate of *K. pneumoniae* as long as excess NH_4^+ is present. However, when such cells were washed free of NH_4^+ and suspended in fresh medium without NH_4^+ and containing 0.38 mM L-serine, only cells in media containing molybdenum grew well (Fig. 1). The slight growth in a molybdenum-free medium presumably is due to low contamination of molybdenum in the chemicals or glassware. The cells did not grow at all in a medium in which tungsten replaced molybdenum. Addition of molybdenum readily overcomes this inhibitory effect. Slight initial growth in molybdenum-free and tungsten-containing media is due to the L-serine that was added.

Effect of molybdenum starvation on nitrogenase activity. Crude extracts of cells grown in these different media were assayed for acetylene reduction, a known indicator of nitrogenase activity (8). As can be seen from Table 1, only extracts of cells derepressed in a medium containing molybdenum have substantial nitrogenase activity. Only a trace of nitrogenase activity is seen in extracts from cells derepressed in molybdenum-free medium, and this activity is abolished upon addition of tungsten to the medium.

These extracts were titrated with purified nitrogenase components that had been isolated from cells grown in molybdenum-containing medium to determine whether one or both of the components were inactive. When cells were derepressed in molybdenum-free medium, only very slight activity was detected for each component (Table 2). This activity was depressed even further in molybdenum-free medium containing tungsten.

Did these cells, therefore, produce normal amounts of inactive components or does molybdenum starvation suppress the synthesis of both components? To answer this question, each component was assayed serologically (11) with antiserum prepared against purified components from *K. pneumoniae* grown in a medium containing molybdenum. The amount of antigenic cross-reacting material against the components in the cells grown in a medium containing molybdenum is defined as 100% (Table 2). Only very small amounts of antigenic cross-reacting components are found in cells derepressed in the absence of molybdenum. Therefore, it seems that molybdenum starvation

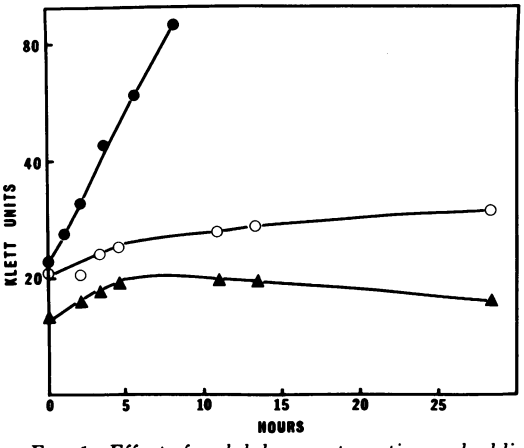

FIG. 1. *Effect of molybdenum starvation and addition of molybdenum or tungsten on growth.* ●, *N-free medium containing molybdenum;* ○, *N-free medium deficient in molybdenum;* ▲, *N-free medium containing W but no molybdenum.*

TABLE 1. *Effect of molybdenum starvation and tungsten addition during depression*

Additions to molybdenum-free medium	Sp act (milliunits/mg of protein)
Molybdenum	40.9
None	0.1
Tungsten	0.0

TABLE 2. *Component synthesis and activity under molybdenum-limiting conditions during derepression*

Additions to molybdenum-free medium	Sp act (milliunits/mg of protein)		Cross-reacting material[a]	
	I	II	I	II
Molybdenum	55.8	39.5	100	100
None	1.1	0.8	5.0	6.5
Tungsten	0.1	0.1	2.5	3.5

[a] Values are expressed as percentage of antigenic cross-reaction of cells grown on media containing molybdenum.

actually prevents the synthesis of both nitrogenase components.

Cells derepressed in molybdenum-free medium containing tungsten always have lower component specific activities as well as lower cross-reacting material than cells derepressed in molybdenum-free medium (Table 2). Increasing concentrations of tungsten depresses these levels even further (Fig. 2). Both component activity and component cross-reacting material were coordinately lowered by increasing tungsten concentration.

DISCUSSION

There are many cases in natural environments where molybdenum is a limiting factor for N_2 fixation. These include growth of the *Rhizobium*-clover symbionts in pastures of South Australia (1) and New Zealand (15) as well as growth of N_2-fixing alfalfa in certain New Jersey soils (10). If a N_2-fixing organism is in a situation whereby N_2 fixation would occur except for the molybdenum limitation, it might be advantageous to such a cell to be able to regulate the synthesis of nitrogenase by molybdenum. The data presented support the hypothesis that a form of molybdenum is an inducer for the synthesis of both nitrogenase components in *K. pneumoniae*. These cells, therefore, do not waste energy synthesizing nitrogenase components under a condition whereby reduction of N_2 is unable to occur, namely, molybdenum limitation. If the cells did not have this control, inactive component I and an active component II would be synthesized that would have no function for the cell.

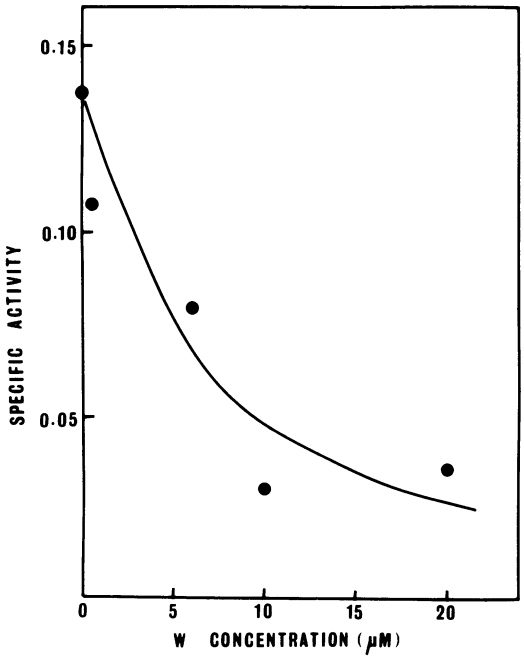

FIG. 2. *Effect of increasing tungsten concentration on acetylene reduction.*

The trace amount of nitrogenase activity in cells derepressed in molybdenum-free medium indicates that there is a low level of molybdenum in this medium. It is difficult to explain why there is enough contaminating molybdenum to support a low activity level, but not enough to fully induce the synthesis of components I and II. However, if the K_m for molybdenum is greater on a hypothesized repressor protein than on component I, the hypothesis of molybdenum induction still holds. Proof that molybdenum is an inducer for nitrogenase should come from studies with control mutants of *K. pneumoniae*.

Another explanation for these results is that an inactive component I is produced in the absence of molybdenum, and this inactive structure is rapidly degraded by intracellular proteolytic activity. The absence of component II may be explained by hypothesizing that component I is necessary for component II synthesis. Results with mutant strains of *K. pneumoniae*, however, show that it is possible for the cell to have normal amounts of active component II even though no component I is being synthesized.

Tungsten has a specific antagonistic effect on N_2 fixation. This is seen by the decreased growth rate in the absence of added molybdenum by cells in media containing tungsten. Increased amounts of tungsten decreased the basal activity of both components. The inhibitory action of tungsten might be at the level of molybdenum permeation, molybdenum mobilization to component I, or regulation by molybdenum.

It is interesting to compare the effect of molybdenum starvation in *K. pneumoniae* and *A. vinelandii*. In the latter organism, component I is not synthesized, but active component II is made (H. H. Nagatani and W. J. Brill, Biochim. Biophys. Acta, in press; H. H. Nagatani, P. W. Wilson, and W. J. Brill, Bacteriol. Proc., p. 168, 1973). When *A. vinelandii* is derepressed in media containing W, an inactive component I is produced (2), which then can be activated in vivo by the addition of sodium molybdate (H. H. Nagatani and W. J. Brill, Biochim. Biophys. Acta, in press).

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