

## *chlD* Gene Function in Molybdate Activation of Nitrate Reductase

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Received for publication 20 January 1975

*chlD* mutants of *Escherichia coli* lack active nitrate reductase but form normal levels of this enzyme when the medium is supplemented with  $10^{-3}$  M molybdate. When *chlD* mutants were grown in unsupplemented medium and then incubated with molybdate in the presence of chloramphenicol, they formed about 5% the normal level of nitrate reductase. Some *chlD* mutants or the wild type grown in medium supplemented with tungstate accumulated an inactive protein which was electrophoretically identical to active nitrate reductase. Addition of molybdate to those cells in the presence of chloramphenicol resulted in the formation of fully induced levels of nitrate reductase. Two *chlD* mutants, including a deletion mutant, failed to accumulate the inactive protein and to form active enzyme under the same conditions. Insertion of  $^{99}\text{Mo}$  into the enzyme protein paralleled activation;  $^{185}\text{W}$  could not be demonstrated to be associated with the accumulated inactive protein. The rates of activation of nitrate reductase at varying molybdate concentrations indicated that the *chlD* gene product facilitates the activation of nitrate reductase at concentrations of molybdate found in normal growth media. At high concentrations, molybdate circumvented this function in *chlD* mutants and appeared to activate nitrate reductase by a mass action process. We conclude that the *chlD* gene plays two distinguishable roles in the formation of nitrate reductase in *E. coli*. It is involved in the accumulation of fully induced levels of the nitrate reductase protein in the cell membrane and it facilitates the insertion of molybdenum to form the active enzyme.

Nitrate reduction in *Escherichia coli* is catalyzed under anaerobic conditions by a membrane-bound, multienzyme complex which includes formate dehydrogenase, cytochrome *b*<sub>1</sub>, and nitrate reductase (20). Although the formation of this enzyme complex is known to be under the control of at least seven genes, *chlA-G* (2, 5), the specific role of each of the genes has not been clearly established. One mutant class, *chlD*, lacks all the components of the complex but forms wild-type levels if the growth medium is supplemented with  $10^{-3}$  M molybdate (4). The *chlD* gene is not a structural gene for any of the components of the nitrate reductase complex since a *chlD* deletion mutant forms wild-type levels of the complex upon molybdate supplementation (4). Furthermore, *chlD* mutants do not appear to be affected in their ability to take up molybdate at normal concentrations (4). These results suggested that the *chlD* gene product is involved in some way in the processing or insertion of molybdenum into the active enzyme structure, and that high levels of molybdate circumvent the role of the *chlD* gene product.

The terminal enzyme, nitrate reductase, has been purified and characterized as a non-heme iron, molybdoprotein (22). We have initiated a study of the activation of this enzyme by molybdate in an attempt to clarify the role of the *chlD* gene in the formation of the active complex. In preliminary studies it was not possible to demonstrate a consistent activation of nitrate reductase by molybdate in extracts of *chlD* mutants. Therefore, we have studied the activation process in vivo under conditions where protein synthesis is inhibited.

### MATERIALS AND METHODS

**Cultures.** The wild-type and mutant strains used in this study are shown in Table 1. Cultures were maintained on L agar (9) and grown in a mineral salts medium containing (g/liter):  $\text{K}_2\text{HPO}_4$ , 7.0;  $(\text{NH}_4)_2\text{SO}_4$ , 1.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.22;  $\text{KH}_2\text{PO}_4$ , 2.0. This medium was supplemented with 0.8% (wt/vol) nutrient broth, 1.0% (wt/vol)  $\text{KNO}_3$ , and 1.0% (wt/vol) glucose.

**Enzyme assay.** Nitrate reductase was assayed using reduced methylviologen as the electron donor in frozen-thawed cells as previously described (21). One

TABLE 1. *Bacterial strains used in this study*

Mutant strain	Genotype	Source	Reference
RB205-2	Hfr, <i>chlD</i> , <i>thi</i> <sup>-</sup> , <i>str</i> <sup>+</sup>	Glaser	5
RB120-1	Hfr, <i>chlD</i> , <i>thi</i> <sup>-</sup> , <i>str</i> <sup>+</sup>	Glaser	5
RB127-1	Hfr, <i>chlD</i> , <i>thi</i> <sup>-</sup> , <i>str</i> <sup>+</sup>	Glaser	5
RB133-1	Hfr, <i>chlD</i> , <i>thi</i> <sup>-</sup> , <i>str</i> <sup>+</sup>	Glaser	5
SA242	$\Delta$ ( <i>gal</i> → $\lambda_{att}$ ), <i>str</i> <sup>-</sup> , F <sup>-</sup>	Campbell	1
PK27	Hfr, <i>thi</i> <sup>-</sup> , <i>str</i> <sup>+</sup>	Helinski	8

unit of enzyme is defined as the amount of enzyme necessary to produce 1  $\mu\text{mol}$  of nitrite per min.

**Protein determination.** Whole cell protein was determined by conversion of cell turbidity to micrograms of protein per milliliter, as in Lester and DeMoss (10). Other protein determinations were made by the method of Lowry et al. (11).

**Enzyme activation experiments.** Activation of nitrate reductase was performed in whole cells. Cells were grown in 100 ml of medium in a 200-ml tube fitted with a side arm for optical density measurements. The cultures were incubated at 37 C and constantly sparged with 95%  $\text{N}_2$ -5%  $\text{CO}_2$ . Growth was followed turbidimetrically using a Klett-Summerson colorimeter with a green filter. Uninoculated medium was used to zero the colorimeter. The cultures were grown to mid-log phase (60 to 80 Klett units) and chloramphenicol (50  $\mu\text{g}/\text{ml}$ ) was added. After 10 to 15 min, molybdate was added to a final concentration of  $10^{-3}$  M. Two-milliliter samples were withdrawn at various times and cooled to 0 C, and the cells were collected by centrifugation at  $12,000 \times g$  for 5 min. The cells were resuspended in 2 ml of mineral salts without chloramphenicol and frozen at -20 C overnight. The frozen cell suspensions were thawed and kept at 4 C, and 0.1-ml samples were assayed for nitrate reductase. Another portion (10 to 100  $\mu\text{l}$ ) was assayed directly for nitrite and was used as a correction factor in the enzyme assays.

Experiments using cells grown on tungstate were performed similarly. A 10% (vol/vol) inoculum was introduced into 400 ml of medium with sodium tungstate ( $10^{-3}$  M, final concentration). The cells were grown as above in a 500-ml glass bottle and, after the addition of chloramphenicol, divided into eight 50-ml aliquots, sparged with 95%  $\text{N}_2$ -5%  $\text{CO}_2$  at 37 C for 5 min to equilibrate them, and then varying levels of molybdate were added. Two-milliliter samples were withdrawn, processed as above, and assayed for nitrate reductase.

**Polyacrylamide gel electrophoresis.** Native 7% polyacrylamide gels were prepared by the method of Davis (3). A stacking gel was used and the samples, in a sucrose solution, were layered on the gels. Samples were prepared as follows. Cells grown under the appropriate conditions were harvested by centrifugation, frozen as a pellet, and stored at -20 C overnight. The cells were then thawed and suspended (suspension 30 to 40%, wt/vol) in 50 mM potassium phosphate buffer, pH 7.0, and broken in an Aminco French pressure cell at 15,000 lb/in<sup>2</sup>. The broken cell suspension was centrifuged ( $12,000 \times g$ , 5 min) to remove unbroken cells and cell wall debris. The supernatant solution was then centrifuged at  $90,000 \times g$  for 90 min

to pellet the membranes. The membrane pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.0, using a glass homogenizer, and washed by pelleting them in the ultracentrifuge at  $90,000 \times g$  for 90 min. The membrane pellet from the wash was resuspended as above in a small volume (1 to 5 ml) of 100 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.3. This membrane suspension was then subjected to a heat treatment of 60 C for 20 min. The suspension of heat-treated membranes was centrifuged at  $135,000 \times g$  for 60 min. The clear supernatant solution or "heat released" fraction was analyzed for protein content and applied to polyacrylamide gels. The gels were subjected to electrophoresis at 5 mA per gel in tris(hydroxymethyl)aminomethane-glycine buffer. Bromphenol blue was used as a tracking dye, and the protein bands were stained with Coomassie brilliant blue. Nitrate reductase activity on the gels was determined by the procedure of Sharp (K. Sharp, Ph.D. thesis, University of California at San Diego, 1974). Gels were added to tubes with a mixture of 100 mM potassium phosphate buffer, pH 7.0, 1.0% (wt/vol)  $\text{KNO}_3$ , and  $10^{-3}$  M oxidized methylviologen. The tubes were made anaerobic by sparging for 2 min with argon. This mixture was allowed to equilibrate for 15 min at room temperature. At this point excess sodium dithionite was added and the gels were incubated at 37 C for 10 to 15 min. The observation of a colorless band of oxidized methylviologen in the blue background of the gels indicated enzyme activity and the position was marked with a small piece of wire. The gels were either stained for protein as above or, using a Gilson gel crusher, fractionated for determination of radioactivity.

**Materials.** Tryptone, nutrient broth, yeast extract, and agar were obtained from Difco Co., Detroit, Mich. All chemicals were of highest purity commercially available and used without further purification. Sephadex G-25 beads were processed by the instructions of the manufacturer (Pharmacia, Upsala, Sweden). The isotopes [<sup>185</sup>W]sodium tungstate and [<sup>99</sup>Mo]ammonium molybdate were obtained from Amersham/Searle Corp., Arlington Heights, Ill., in 1- and 10-mCi lots, respectively.

## RESULTS

Although nitrate reductase activity can be restored to wild-type levels in *chlD* mutants by addition of  $10^{-3}$  M molybdate to the growth medium (4), it has not been possible to activate nitrate reductase with molybdate in cell-free preparations of the same mutants. However, the

results shown in Fig. 1 and Table 2 establish that the activation of nitrate reductase by molybdate in *chlD* mutants can occur in vivo in the presence of chloramphenicol. Grown in the absence of molybdate, the *chlD* mutants were devoid of activity. In the presence of chloramphenicol these cells formed nitrate reductase with a lag of 30 to 40 min after the addition of  $10^{-3}$  M molybdate giving final specific activities

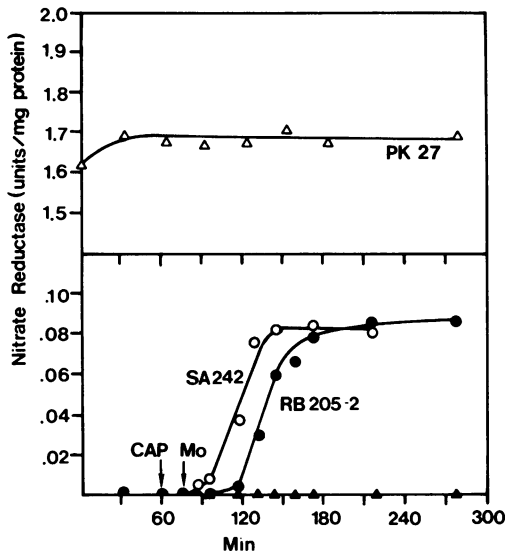


FIG. 1. Activation of nitrate reductase by molybdate. Cultures were grown in medium to 60 to 80 Klett units. Chloramphenicol (CAP, 50  $\mu$ g/ml) and molybdate (Mo,  $10^{-3}$  M) were added at the points indicated by the arrows to all the cultures. Samples were withdrawn and assayed as described in the text. Strains are indicated by the symbols:  $\Delta$ , PK27;  $\circ$ , SA242;  $\bullet$ , RB205-2; and  $\blacktriangle$ , SA242 and RB205-2 to which no molybdate was added.

TABLE 2. Activation of nitrate reductase by molybdate in the absence of protein synthesis in cells of *Escherichia coli*<sup>a</sup>

Strain	Nitrate reductase	
	U/mg of protein	% of wild type
RB205-2	0.083	4.9
RB127-1	0.105	6.3
RB120-1	0.110	6.6
RB133-1	0.082	4.9
SA242	0.079	4.8
PK27 (control)	1.680	100

<sup>a</sup> Cells were grown in medium without supplementation to 60 to 80 Klett units, harvested, and resuspended in medium containing 50  $\mu$ g of chloramphenicol per ml and  $10^{-3}$  M molybdate. Enzyme levels were determined 2 h after the addition of molybdate to the activation mixture at 37 C.

of about 0.1 U per mg of protein, or 5 to 7% that of the wild-type cells tested in the same way (Table 2). The addition of molybdate to wild-type cells under the same conditions did not significantly alter the levels of nitrate reductase (Fig. 1).

Tungstate has been shown to lead to the accumulation of several inactive molybdoproteins (7, 13, 15, 16). Therefore, cells were grown in the presence of tungstate in an attempt to increase the amount of inactive nitrate reductase that could be activated by molybdate (Fig. 2). Grown in the presence of  $10^{-3}$  M tungstate, the wild-type strain PK27 had significantly reduced levels of nitrate reductase, and the *chlD* mutants were completely devoid of activity. In other experiments it was shown that  $10^{-2}$  M tungstate could completely inhibit the formation of active nitrate reductase in the wild type; therefore, in subsequent experiments, the wild type was usually grown in  $10^{-2}$  M tungstate whereas the mutants were grown in  $10^{-3}$  M tungstate. Upon the removal of tungstate and the addition of chloramphenicol and  $10^{-3}$  M molybdate, active nitrate reductase was formed without a lag in wild-type and mutant RB205-2.

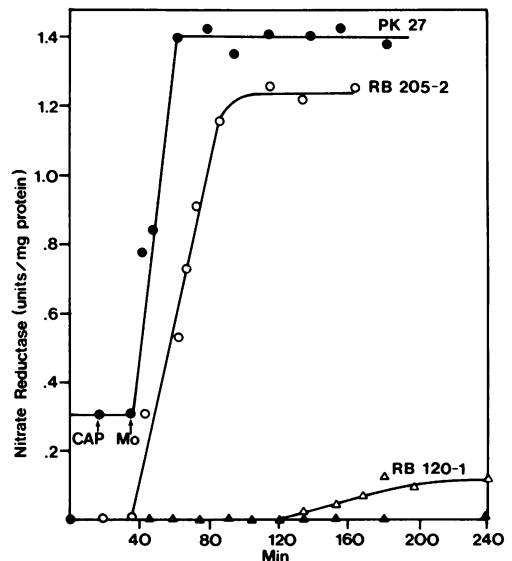


FIG. 2. Activation of nitrate reductase by molybdate in tungstate-grown cells. Cells were grown in medium containing  $10^{-3}$  M tungstate. The cells were washed and resuspended in medium without tungstate. The arrows indicate the points of addition of chloramphenicol (CAP, 50  $\mu$ g/ml) and molybdate (Mo,  $10^{-3}$  M). Enzyme activity was assayed as described in the text. Strains are indicated by the symbols:  $\bullet$ , PK27;  $\circ$ , RB205-2;  $\Delta$ , RB120-1; and  $\blacktriangle$ , SA242. Control cultures to which no molybdate was added are also shown ( $\blacktriangle$ ).

In each case the specific activity reached was equivalent to full wild-type levels. Two other *chlD* mutants, RB133-1 and RB127-1, behaved in a similar manner. In contrast, the *chlD* deletion mutant and *chlD* mutant RB120-1 formed very little active nitrate reductase (Fig. 2). Thus, in tungstate medium, some but not all *chlD* mutants accumulated large amounts of an inactive form of nitrate reductase which could be activated by molybdate in the presence of chloramphenicol.

Nitrate reductase was released from membrane fragments of *E. coli* by heating for 20 min at 60 C. When the heat-released fraction was subjected to gel electrophoresis, a major protein band could be detected by staining, which corresponds to the band of nitrate reductase activity detected by the procedure of Sharp (Ph.D. thesis, University of California at San Diego, 1974) outlined above. When PK27 or *chlD* mutants SA242 and RB205-2 were grown in the presence of  $10^{-3}$  M molybdate, electrophoresis of the heat-released fractions on poly-

acrylamide disc gels revealed essentially equivalent amounts of the nitrate reductase-associated protein band in each case (Fig. 3A). When the same strains were grown in medium without the supplemental molybdate, only the wild type gave this pattern (Fig. 3B); the mutants yielded little or none of the corresponding protein or activity bands. When the medium was supplemented with  $10^{-3}$  M tungstate, the heat-released fraction from both wild-type and mutant RB205-2 contained an inactive protein band corresponding to nitrate reductase, whereas the *chlD* deletion mutant, SA242, did not (Fig. 3C). These results show that an inactive protein, electrophoretically identical to nitrate reductase, accumulated during growth on tungstate only in strains which can be activated by molybdate. The *chlD* deletion mutant failed to accumulate the inactive protein in tungstate medium and was not activated by molybdate.

To obtain direct evidence that the activation of nitrate reductase proceeds by the insertion of

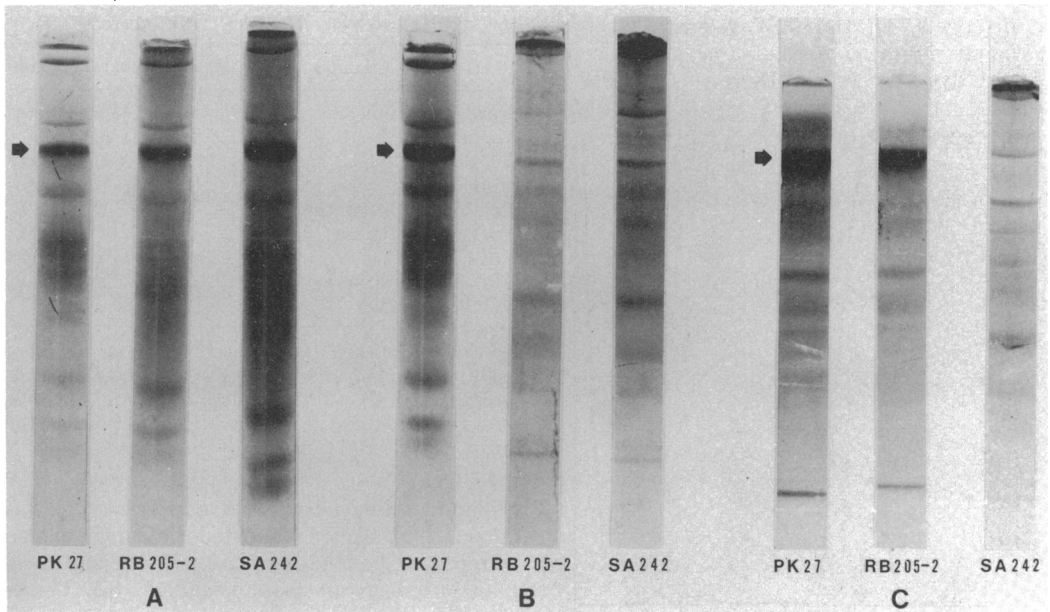


FIG. 3. Polyacrylamide gel electrophoresis of heat-released fractions from membranes of strains grown under varying conditions. (A) Strains PK27, RB205-2, and SA242 grown in medium supplemented with  $10^{-3}$  M molybdate. The arrow indicates the protein band exhibiting nitrate reductase activity determined as described in the text. Levels of protein applied to the gels were: 85  $\mu\text{g}$  (PK27), 102  $\mu\text{g}$  (RB205-2), and 165  $\mu\text{g}$  (SA242). Samples were subjected to electrophoresis at 5 mA/gel for 90 min. (B) Strains PK27, RB205-2, and SA242 grown in medium without supplementation. The arrow indicates the active band in PK27 only determined as described in the text. Levels of protein applied to the gels were: 128  $\mu\text{g}$  (PK27), 63  $\mu\text{g}$  (RB205-2), and 98  $\mu\text{g}$  (SA242). Samples were subjected to electrophoresis at 5 mA/gel for 90 min. (C) Strains PK27, RB205-2, and SA242 grown in medium supplemented with  $10^{-2}$  M tungstate (PK27) and  $10^{-3}$  M tungstate (RB205-2 and SA242). The arrow indicates the inactive protein band in strains PK27 and RB205-2 only, corresponding to nitrate reductase. Protein levels applied to the gels were: 135  $\mu\text{g}$  (PK27), 105  $\mu\text{g}$  (RB205-2), and 63  $\mu\text{g}$  (SA242). The samples were subjected to electrophoresis at 5 mA/gel for 65 min.

molybdenum into the enzyme, [ $^{99}\text{Mo}$ ]molybdate was used in the activation. If tungsten replaces molybdenum in the enzyme, stabilizing it in an inactive form, and activation proceeds by molybdenum replacing tungsten, then activation using  $^{99}\text{Mo}$  should be accompanied by the formation of an active enzyme containing  $^{99}\text{Mo}$ . This was indeed the case in both wild-type (Fig. 4) and mutant RB205-2 (Fig. 5). The activation of nitrate reductase and  $^{99}\text{Mo}$  incorporation into the enzyme band proceeded in both strains at both 4 and 37 C, although better activation was obtained in both strains at 37 C.

The hypothesis that tungsten stabilizes the enzyme in an inactive form by forming a tungsten-enzyme complex was tested using [ $^{185}\text{W}$ ]tungstate. Cells of mutant RB205-2 were grown with [ $^{185}\text{W}$ ]tungstate. Table 3 shows the fractionation of these cells. The membrane fraction contained 10,570 counts/min which could not be removed by washing and 2,550 counts/min were released by heat treatment. However, upon electrophoresis on polyacrylamide disc gels, all the  $^{185}\text{W}$  released by heat treatment migrated to the same point as the tracking dye (Fig. 6) and there was no  $^{185}\text{W}$  at

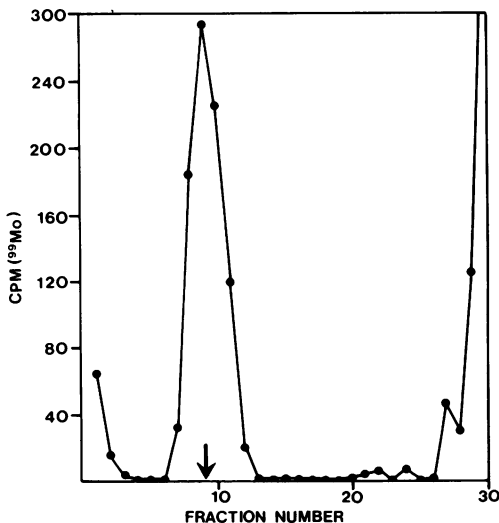


FIG. 4. Incorporation of  $^{99}\text{Mo}$  during nitrate reductase activation in strain PK27. Cells were grown in medium supplemented with  $10^{-2}$  M tungstate, harvested, and washed three times to remove the tungstate. Activation by molybdate in the presence of chloramphenicol was performed as described in the text. The heat-released fraction from membranes was subjected to electrophoresis at 5 mA/gel on 7% polyacrylamide gels, crushed in 2-mm slices, and counted for  $^{99}\text{Mo}$ . The point of enzyme activity (arrow) was determined as described in the text.

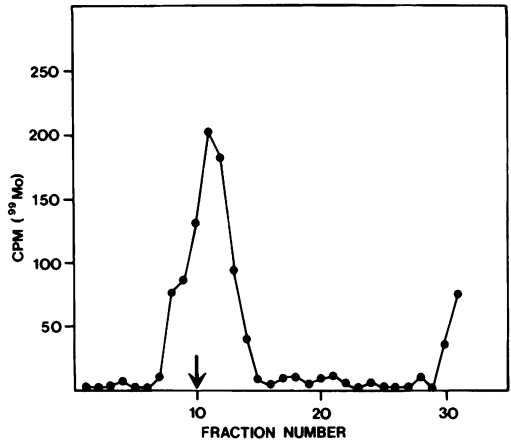


FIG. 5. Incorporation of  $^{99}\text{Mo}$  during nitrate reductase activation in *chd* mutant RB205-2. Cells were grown in medium supplemented with  $10^{-3}$  M tungstate and washed to remove the tungstate. Activation was performed as described in Fig. 4.

TABLE 3. Fractionation of *Escherichia coli* RB205-2 cells grown on  $10^{-4}$  M [ $^{185}\text{W}$ ]tungstate<sup>a</sup>

Sample	Total counts/min
Growth medium	438,000,000
Washed cells	3,420,000
Cell debris	1,523,000
Crude extract	1,856,000
Membrane supernatant	1,390,000
Washed membranes	10,570
Wash	408
Heat-released fraction	2,550
Membrane pellet	10,900

<sup>a</sup> Cells of mutant RB205-2 were grown on 500 ml of medium supplemented with  $10^{-4}$  M tungstate containing 0.25 mCi of [ $^{185}\text{W}$ ]sodium tungstate. The cells were harvested and fractionated as described in the text.

the point where active, cold, carrier enzyme was detected. It has been reported that tungsten can be removed from a tungsten-nitrate reductase of plant origin by electrophoresis (15, 16). Therefore, the heat-released material was passed through a Sephadex G-25 column to determine if the  $^{185}\text{W}$  was in the form of small molecules, as the electrophoresis data implied, or indeed associated with protein. All the  $^{185}\text{W}$  eluted from the column with the fractions containing small molecules (Fig. 7).

These findings imply that tungsten is lost from the enzyme at or before the heat-release step, if it is ever associated with the enzyme. Over 90% of the  $^{185}\text{W}$  was released from the membranes by three 1-min sonic treatments,

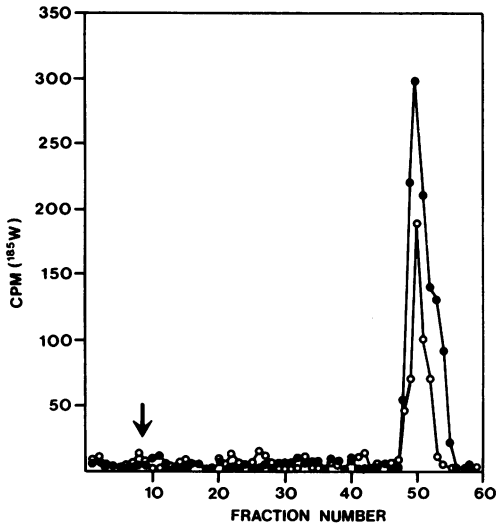


FIG. 6. Polyacrylamide gel electrophoresis of the heat-released fraction from membranes of *chID* mutant RB205-2. The cells were grown in 500 ml of medium supplemented with [<sup>185</sup>W]tungstate (0.25 mCi in 10<sup>-4</sup> M tungstate). The gels were crushed (1-mm slices) and counted for <sup>185</sup>W. The gel (●) contains only heat-released material. The other gel (○) contained both heat-released material and active carrier enzyme. The arrow indicates the point of migration of the active carrier as determined by the procedure outlined in the text. The tracking dye was detected in fractions 49-52 in both gels.

whereas only 7% of the protein was solubilized. This treatment neither inactivated nor released active nitrate reductase to a significant degree. These data suggest that the tungsten-enzyme association is either extremely labile, or that the radioactive tungsten in the membranes is trapped in the membranes between layers or in vesicles.

Since high levels of molybdate circumvent the defect in *chID* mutants, it is clear that none of the above experiments, using 10<sup>-3</sup> M molybdate, have tested whether the *chID* gene product is involved in the activation of the tungsten-stabilized nitrate reductase protein by molybdate. The possible participation of the *chID* gene product in this activation can only be tested at concentrations of molybdate which are restrictive to these mutants (i.e., <10<sup>-5</sup> M) but not to the wild-type strain. Therefore the kinetics of nitrate reductase activation in both the wild-type and a *chID* mutant were followed at various molybdate concentrations. The responses of the two strains to lower concentrations of molybdate were quite distinct. The wild-type cells gave the maximum rate of activation with 10<sup>-5</sup> M molybdate (Fig. 8), whereas the *chID* mutant formed active nitrate reductase at a

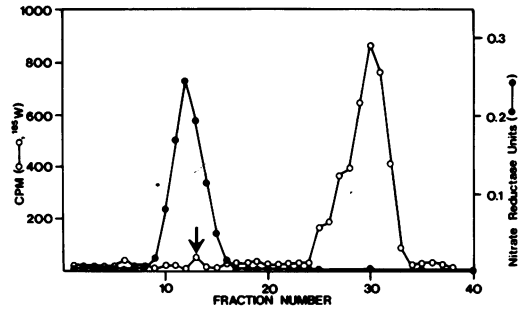


FIG. 7. Sephadex G-25 column chromatography of the heat-released fraction from membranes of *chID* mutant RB205-2 cells grown in 500 ml of medium supplemented with [<sup>185</sup>W]tungstate (0.5 mCi, final tungstate concentration of 10<sup>-4</sup> M). The column was calibrated with active enzyme (●), blue dextran 2000, K<sub>3</sub>Fe(CN)<sub>6</sub> (a yellow compound), and [<sup>185</sup>W]tungstate in separate applications. The open circles indicate the elution profile of the heat-released material. The arrow indicates the peak fraction of the elution of blue dextran 2000 and active nitrate reductase. [<sup>185</sup>W]tungstate and K<sub>3</sub>Fe(CN)<sub>6</sub> were detected in fractions 30 and 31, respectively.

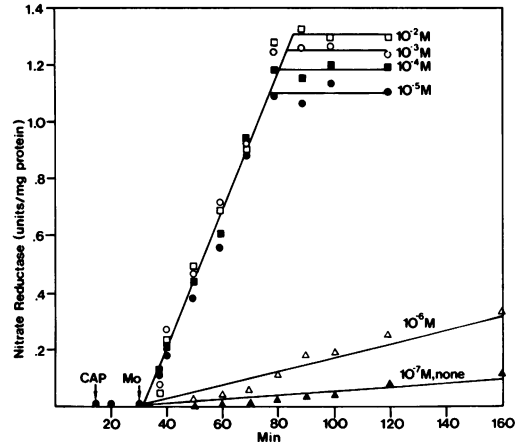


FIG. 8. Activation of nitrate reductase in tungstate-grown cells of strain PK27 with varying concentrations of molybdate. Cells were grown in medium with 10<sup>-2</sup> M tungstate. The first arrow (CAP) indicates the point of addition of chloramphenicol (50 μg/ml). The cells were harvested and washed three times to remove tungstate and resuspended in medium containing chloramphenicol. Molybdate concentrations of 10<sup>-2</sup> M (□), 10<sup>-3</sup> M (○), 10<sup>-4</sup> M (■), 10<sup>-5</sup> M (●), 10<sup>-6</sup> M (△), and 10<sup>-7</sup> M or none (▲) were added to separate aliquots of the same cell suspension at the point indicated by the second arrow (Mo). Enzyme activity was determined as described in the text.

rate which continued to increase with the molybdate concentrations up to  $10^{-3}$  M (Fig. 9). The rate of activation in the *chlD* mutant was directly proportional to the molybdate concentration over the whole range of concentrations tested (Table 4), suggesting that activation proceeds by a mass action process in this case. In contrast, the rate of activation did not remain proportional to molybdate concentration in the case of the wild type. Instead nitrate reductase in the wild type was activated at significantly greater rates than in the mutant at low concentrations of molybdate and the process was saturated at  $10^{-5}$  M, indicating that the *chlD* gene product plays an active role at molybdate concentrations found in the usual bacteriological media (see Table 4).

### DISCUSSION

The *chlD* gene product appears to play two distinguishable roles in the formation of nitrate reductase: (i) as shown by the kinetic experiments, at low molybdate concentrations formation of nitrate reductase in tungstate-grown cells depends on a functional *chlD* gene, and (ii) the *chlD* gene product also appears to be involved in the accumulation of fully induced levels of the inactive protein in the membrane. During growth on tungstate the wild-type and some *chlD* mutants accumulated large amounts of an inactive protein, electrophoretically identical to nitrate reductase, which could be activated by molybdate in the presence of chloram-

phenicol. However, under the same conditions, two *chlD* mutants, including the deletion mutant SA242, did not accumulate the inactive protein and did not form active nitrate reductase. These observations indicate that the accumulation of the inactive protein depends on some function of the *chlD* gene which is not

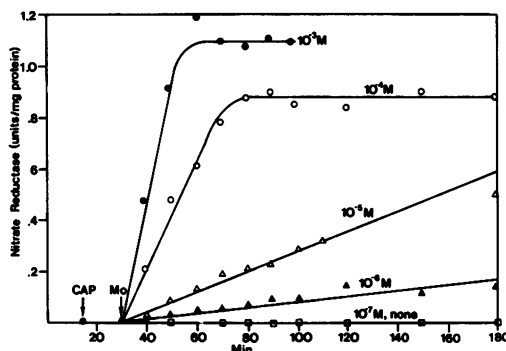


FIG. 9. Activation of nitrate reductase in tungstate-grown cells of *chlD* mutant RB205-2 with varying concentrations of molybdate. Cells were grown in medium containing  $10^{-3}$  M tungstate. The first arrow indicates the point of addition of chloramphenicol (50  $\mu$ g/ml). The cells were harvested and washed three times to remove the tungstate and resuspended in medium containing chloramphenicol. Molybdate concentrations of  $10^{-3}$  M ( $\bullet$ ),  $10^{-4}$  M ( $\circ$ ),  $10^{-5}$  M ( $\Delta$ ),  $10^{-6}$  M ( $\blacktriangle$ ), and  $10^{-7}$  M or no added molybdate ( $\square$ ) were added to separate aliquots of the same cell suspension at the point indicated by the second arrow (Mo). Enzyme activity was assayed as described in the text.

TABLE 4. Kinetics of activation of nitrate reductase by varying molybdate concentrations in tungstate-grown cells of *Escherichia coli*

Molybdate concn (M)	PK27		RB205-2	
	Rate <sup>a</sup>	V/[molybdate] <sup>b</sup>	Rate <sup>a</sup>	V/[molybdate] <sup>b</sup>
$10^{-2}$	0.0203	$1.02 \times 10^{-7}$		
$10^{-3}$	0.0197	$9.85 \times 10^{-7}$	0.0655	$3.28 \times 10^{-6}$
$10^{-4}$	0.0208	$1.04 \times 10^{-6}$	0.0160	$8.00 \times 10^{-6}$
$10^{-5}$	0.0213	$1.07 \times 10^{-4}$	0.0031	$15.0 \times 10^{-6}$
$10^{-6}$	0.0083	$4.15 \times 10^{-4}$	0.0004	$20.0 \times 10^{-6}$
$10^{-7}$	0.0013	$6.77 \times 10^{-3}$	0	0
None <sup>c</sup>	0.0011	$6.49 \times 10^{-3}$	0	0

<sup>a</sup> Rates are taken from Fig. 8 and 9 and are expressed as units of enzyme formed per minute per milligram of protein.

<sup>b</sup> Assuming a mass action process,  $V = k[\text{enzyme}][\text{molybdate}]$ , where  $V$  is the rate of enzyme formation in moles per minute per milligram of protein,  $k$  is a constant,  $[\text{enzyme}]$  is the molar concentration of inactive enzyme, and  $[\text{molybdate}]$  is the molar concentration of molybdate.  $[\text{Enzyme}]$  was taken to be constant because each of the molybdate concentrations were tested on different aliquots of the same cell suspension. Therefore,  $V/[\text{molybdate}]$  should be constant for a mass action process. Rates are converted into  $V$  by assuming 1 mol of enzyme has a molecular weight of  $200,000 \times g$  and pure enzyme has 100 U of activity per mg of protein (K. Sharp, personal communication).

<sup>c</sup> A value of  $3.67 \times 10^{-7}$  M molybdate was used for suspending medium (from atomic absorption data; G. Sperl, unpublished data).

affected by some point mutations. Both of these functions can be circumvented by high levels of molybdate since the deletion mutant, as well as the other *chlD* mutants, formed normal levels of nitrate reductase when grown in the presence of  $10^{-3}$  M molybdate.

In contrast to the tungstate grown cells, *chlD* mutants grown in unsupplemented medium formed only 5% the wild-type level of nitrate reductase when molybdate was added in the presence of chloramphenicol. The 30- to 40-min lag in the appearance of active enzyme suggests that molybdate activation in this case is more complex than that in tungstate grown cells.

On the basis of a study of pleiotropic mutants in *Aspergillus* which lack both nitrate reductase and xanthine dehydrogenase, Pateman et al. hypothesized that a common cofactor, CNX, was essential for the activities of these two enzymes and that CNX was a repressor of nitrate reductase synthesis (18). Although no direct evidence was presented for the regulatory role, later studies (19) showed that high levels of molybdate will restore these two enzymes to normal levels in the *cnxE* mutants. This suggests that the *cnxE* locus is analogous to *chlD* in *E. coli*.

The accumulation of inactive forms of molybdoproteins in the presence of tungstate is generally believed to result from tungsten replacing molybdenum in the enzyme structures (13, 15, 16). Although molybdenum incorporation paralleled the activation of the tungstate-accumulated inactive nitrate reductase, the presence of tungsten in the inactive protein could not be demonstrated. The failure to detect tungsten in the enzyme may be the result of an unstable tungsten-enzyme association or tungstate may promote the formation of inactive enzyme by a process not involving its association with the protein. Tungsten has been demonstrated to be associated with other inactive molybdoproteins which were formed in the presence of tungstate, including sulfite oxidase (7), xanthine oxidase (7) and nitrate reductase from spinach (15) and *Chlorella* (17). In the case of spinach, tungsten dissociated from the inactive protein during electrophoresis. If tungsten is associated with inactive nitrate reductase in *E. coli*, it is lost during heat treatment or sonic treatment of the membrane fraction.

The results represented here are analogous to those obtained with sulfite oxidase and xanthine oxidase from rat liver by Johnson and Rajagopalan (7), with nitrogenase component I from *Azotobacter* by Nagatani et al. (13), and with nitrate reductase in tobacco cells by Heimer and Filner (6). In each case, protein

synthesis was not required for activation of tungstate-accumulated inactive enzyme. However, in no case was evidence reported which implicated a gene function analogous to *chlD* in *E. coli*.

Attempts to obtain activation of the tungstate-accumulated inactive nitrate reductase in vitro have failed. Variations in molybdate levels, in supplements, or in methods of extract preparation have not produced consistent activation in vitro although low levels of activation can be obtained in some cases. Recently, Nagatani et al. (13) reported that the tungstate-inactivated form of nitrogenase component I from *Azotobacter* could be activated by acid-treated preparations of molybdenum containing component I although molybdate itself was inactive. Nason et al. (14) reported that acid treatment of various molybdoproteins generated a cofactor which, by insertion of molybdenum, activated the inactive form of nitrate reductase produced by *nit-1* mutants of *Neurospora crassa*. Again, free molybdate was not effective and no genetic locus analogous to the *chlD* gene was implicated in the process.

Insertion of molybdenum and activation of nitrate reductase may or may not be the last step in the assembly of this enzyme system. However, our failure to obtain consistent activation in cell-free extracts points out at least one difficulty in studying in vitro assembly of nitrate reductase. Reported attempts to study the formation of nitrate reductase in vitro by mixing extracts from mutants of *E. coli* have generally resulted in the formation of only very small amounts of nitrate reductase activity (2, 12). Insertion of molybdenum may be one of the major obstacles to overcome in obtaining consistent and significant in vitro assembly.

#### ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant GM19511 from the National Institute of General Medical Sciences and grant AU-570 from the Robert A. Welch Foundation. George T. Sperl was the recipient of a postdoctoral fellowship from the Robert A. Welch Foundation.

#### LITERATURE CITED

- Adhya, S., P. Cleary, and A. Campbell. 1968. A deletion analysis of prophage lambda and adjacent genetic regions. *Proc. Natl. Acad. Sci. U.S.A.* 61:956-962.
- Azoulay, E. 1970. The genetic and biochemical analysis of structured polyezyme system in the membrane of *Escherichia coli* K-12, p. 209-217. In A. Perez-Miravet (ed.), *Recent advances in microbiology*. Tenth International Congress for Microbiology, Mexico City.
- Davis, B. J. 1964. Disc electrophoresis. Methods and applications to human serum proteins. *Ann. N.Y. Acad. Sci.* 121:404-427.
- Glaser, J. H., and J. A. DeMoss. 1971. Phenotypic restoration by molybdate of nitrate reductase activity



- in *chiD* mutants of *Escherichia coli*. *J. Bacteriol.* **108**:854-860.
5. Glaser, J. H., and J. A. DeMoss. 1972. Comparison of nitrate reductase mutants of *Escherichia coli* selected by alternative procedures. *Mole. Gen. Genet.* **116**:1-10.
  6. Heimer, Y. M., and P. Filner. 1971. Regulation of the nitrate assimilation pathway in cultured tobacco cells. *Biochim. Biophys. Acta* **230**:362-372.
  7. Johnson, J. L., and K. V. Rajagopalan. 1974. Molecular basis of the biological function of molybdenum. Effect of tungsten on xanthine oxidase and sulfite oxidase in the rat. *J. Biol. Chem.* **249**:859-866.
  8. Kahn, P. L. 1968. Isolation of high-frequency recombinating strains from *Escherichia coli* containing the V colicinogenic factor. *J. Bacteriol.* **96**:205-214.
  9. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**:190-206.
  10. Lester, R. L., and J. A. DeMoss. 1971. Effects of molybdate and selenite on formate and nitrate metabolism in *Escherichia coli*. *J. Bacteriol.* **105**:1006-1014.
  11. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1961. Protein determination with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
  12. MacGregor, C. H., and C. A. Schnaitman. 1973. Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutants of *Escherichia coli*. *J. Bacteriol.* **114**:1164-1176.
  13. Nagatani, H. H., V. K. Shah, and W. J. Brill. 1974. Activation of inactive nitrogenase by acid treated component I. *J. Bacteriol.* **120**:697-701.
  14. Nason, A., K. Lee, S. Pan, P. A. Ketchum, A. Lamberti, and J. DeVries. 1971. *In vitro* formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductase from a *Neurospora* mutant and a component of molybdenum-enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **68**:3242-3246.
  15. Notton, B. A., and E. J. Hewitt. 1971. The role of tungsten in the inhibition of nitrate reductase in spinach (*Spinacea oleracea* L.) leaves. *Biochem. Biophys. Res. Commun.* **44**:702-710.
  16. Notton, B. A., and E. J. Hewitt. 1972. Comparative aspects of incorporation of vanadium, tungsten or molybdenum into protein of nitrate reductase of *Spinacea oleracea* L. leaves. *Biochim. Biophys. Acta* **275**:355-357.
  17. Paneque, A., J. M. Vega, J. Cardenas, J. Herrera, P. J. Aparicio, and M. Losada. 1972. <sup>185</sup>W-labeled nitrate reductase from *Chlorella*. *Plant Cell Physiol.* **13**:175-178.
  18. Pateman, J. A., D. J. Cove, B. M. Rever, and D. B. Roberts. 1964. A common cofactor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. *Nature (London)* **201**:58-60.
  19. Pateman, J. A., B. M. Rever, and D. J. Cove. 1967. Genetic and biochemical studies of nitrate reduction in *Aspergillus nidulans*. *Biochem. J.* **104**:103-111.
  20. Ruiz-Herrera, J., and J. A. DeMoss. 1969. Nitrate reductase complex of *Escherichia coli* K-12: participation of specific formate dehydrogenase and cytochrome B<sub>1</sub> components in nitrate reduction. *J. Bacteriol.* **99**:720-729.
  21. Showe, M. K., and J. A. DeMoss. 1968. Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. *J. Bacteriol.* **95**:1305-1313.
  22. Taniguchi, S., and E. Itagaki. 1960. Nitrate reductase of nitrate respiration type from *Escherichia coli*. I. Solubilization and purification from the particulate system with molecular characterization as a metalloprotein. *Biochim. Biophys. Acta* **44**:263-279.