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Molybdate reduction by *Escherichia coli* K-12 and its *chl* mutants

(nitrate reduction/biotin sulfoxide reductase/*chlD* mutants/phosphomolybdate/anaerobiosis)

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ABSTRACT **During anaerobic growth, *Escherichia coli* can reduce phosphomolybdate. The reduction can also be carried out by washed cells suspended in buffer at pH 5.7. Phosphate, molybdate, glucose, cells, and anaerobic conditions are required. Reduction is inhibited by 200 μM chromate, 290 μM nitrite, 10 mM tungstate, or 20 mM cysteine. Wild-type (*chl*$^+$) cells are inhibited by addition of 200 μM nitrate, but *chlA*, *chlB*, and *chlE* mutants are not. The inhibition of *chl*$^+$ cells results from reduction of nitrate to nitrite. This nitrate reduction is not catalyzed by nitrate reductase. Wild-type cells are more sensitive than *chl* mutants to inhibition by nitrite and cysteine but more resistant to chromate. Pregrowth of *chlD* cells in 1 mM Na_2MoO_4 increases their sensitivity to nitrite and cysteine, and pregrowth of *chl*$^+$ cells in 1 mM Na_2MoO_4 increases their resistance to these agents. Assays of biotin sulfoxide reductase show that the tightness of the *chlD* block depends on growth conditions; *chlD* cells grown aerobically in tryptone broth make about 50% as much active enzyme as *chl*$^+$ cells, whereas *chlD* cells grown anaerobically with tryptone plus glucose make less than 10%. The effect of anaerobic pregrowth on the inhibition of molybdate reduction by added nitrate indicates that *in vivo* nitrate reduction responds to growth conditions in the same manner as biotin sulfoxide reductase does.**

In studying the molybdoenzyme biotin sulfoxide reductase, we have sometimes grown *Escherichia coli* on media with elevated concentrations of molybdate. We noticed that cultures grown anaerobically with 10 mM molybdate turn deep blue and are devoid of biotin sulfoxide reductase activity. We later found that the blue color is also produced by washed cells suspended in buffer with molybdate, glucose, and phosphate.

We assume that the blue substance is a reduction product of molybdate, perhaps identical to that resulting from phosphomolybdate reduction at acid pH by strong reductants such as thiosulfate or ascorbate (1, 2). Our reaction resembles the spontaneous reaction in its phosphate dependence, but it takes place at pH 5–6 and requires the presence of cells.

The known metabolic function of molybdenum in *E. coli* is as a component of a cofactor for various redox enzymes such as nitrate reductase and biotin sulfoxide reductase. The products of five genes (*chlA*, *chlB*, *chlD*, *chlE*, *chlG*) are needed for molybdate assimilation and/or cofactor biogenesis. We investigated the effects of *chl* mutations on molybdate reduction.

Our results indicate that none of the *chl* genes is needed for molybdate reduction. Nevertheless, *chl* mutations affect molybdate reduction under some conditions, because cofactor-dependent pathways generate or remove inhibitors of molybdate reduction. A study of these effects has indicated some conclusions about reactions requiring molybdenum cofactors in *E. coli*.

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MATERIALS AND METHODS

Bacteria. We used the *chl*$^+$ strain RK4353 (which is F$^-$ *araD139* Δ*lacU169 non rpsL gyrA thi*) and some mutants derived from it: RK5200 [*chlA200*::Mu *c* (Ts)], RK5206 [*chlG206*::Mu *c* (Ts)], RK5207 [*chlD240*::Mu *c* (Ts)], RK5208 [*chlB207*::Mu *c* (Ts)], RK52228 [*chlE225*::Mu *c* (Ts)], and RK5265 (*narG202*::Tn*10*). These strains, kindly provided by Valley Stewart, have been described (3). All of the results reported in this paper were confirmed with other *chl* mutants from our own collection. Biotin sulfoxide reductase assays were performed on strain SA302 [F$^-$ *his* (λ *cI857*)] and its *chlD*-deleted derivative SA322 (4).

Growth Conditions. Tryptone broth was 1% tryptone/0.5% NaCl/0.0001% thiamine hydrochloride. Cultures were grown at 30°C. For aerobic growth, 200 ml of culture was shaken in 1000-ml flasks. For anaerobic growth, tryptone broth was supplemented with 1% glucose, and cultures were incubated in filled, stoppered bottles. Cells were centrifuged, washed, resuspended in water, and stored at 4°C. Molybdate-reducing activity was stable for at least 1 week.

Biotin Sulfoxide Reductase Assays. Sonic extracts were prepared as described (4). Lysozyme extraction followed the procedure of MacGregor *et al.* (5). We reported previously that enzyme from phage lysates differed from that in sonic extracts; enzyme from lysates was more heat stable and did not require added thioredoxin-like factors (4). Lysozyme extracts resemble those made by phage lysis. Apparently the important variable is the manner of breaking the cell, not any side effect of λ development.

Measurement of Molybdate Reduction. The absorption spectrum of the blue supernatant showed a broad peak with a maximum of 820 nm and a shoulder between 600 nm and 700 nm. The amount of reduction was routinely measured as absorbance at 820 nm in a Unicam spectrophotometer. Under standard assay conditions (Table 1), the ratio A_{820}/cell density in Klett units was about 0.03.

RESULTS

Conditions for Molybdate Reduction. To establish minimal conditions for reduction, washed cells were suspended in buffer plus molybdate, incubated 24 hr at 30°C, and centrifuged. The absorbance of the blue supernatant at 820 nm was then read.

Table 1 shows the standard assay conditions and their bases. Adequate anaerobiosis was achieved by filling 2-ml Microfuge tubes and stoppering them. If, instead, the suspension was aerated by shaking 2 ml in an open 18-mm test tube, little reduction was observed. Aeration inhibits reduction rather than preventing the synthesis of proteins needed for the reaction. Cells grown aerobically and assayed in the presence of chloramphenicol are fully active.

Glucose is required. It cannot be replaced by formate, succinate, glycerol, or ethanol. Reduction is stimulated several-

Table 1. Requirements for molybdate reduction by washed cells

Addition or deletion	Relative activity
Complete	(1)
+ Aeration	0.08
− Glucose	0.04
− Glucose + 1% glycerol	0.05
− Glucose + 1% EtOH	0.06
− Glucose + 80 mM formate	0.10
− Phosphate, 20 mM molybdate	0.02
5 mM phosphate, 20 mM molybdate	0.53
10 mM phosphate, 20 mM molybdate	0.13
20 mM phosphate, 20 mM molybdate	0.01
0.5 mM phosphate (40 mM molybdate)	0.89
5 mM phosphate (40 mM molybdate)	0.89
10 mM phosphate (40 mM molybdate)	0.69
20 mM phosphate (40 mM molybdate)	0.21
40 mM phosphate (40 mM molybdate)	0.01
5 mM phosphate, 80 mM molybdate	1.4
20 mM phosphate, 80 mM molybdate	0.68
40 mM phosphate, 80 mM molybdate	0.12
80 mM phosphate, 80 mM molybdate	0.01
− Chloramphenicol	1.3

The complete assay mixture contained washed cells plus 1% glucose, 2 mM KH_2PO_4, 40 mM sodium molybdate, 0.01% chloramphenicol, 100 mM NH_4OAc (pH 5.7), and 200 mM NaCl. After incubation for 24 hr at 30°C in closed Microfuge tubes, cells were pelleted, and the absorbance of the supernate was read at 820 nm. Activities, calculated by dividing A_{820} by the cell density in Klett units, were divided by the activity for the complete assay.

fold by 200 mM NaCl. Phosphate is required. At 40 mM molybdate, the optimal phosphate concentration is 0.5–5 mM. Higher concentrations inhibit. Experiments at different molybdate concentrations show that the amount of phosphate needed to inhibit increases with increasing molybdate concentration, as though the ratio of phosphate to molybdate were critical.

The optimal pH is between 5 and 6, and the optimal temperature is 30–36°C (data not shown). The amount of reduction is affected only slightly by chloramphenicol (Table 1). However, chloramphenicol affects the response to some of the inhibitors we will discuss later. The amount of reduction is proportional to cell density and to time of incubation. We chose 24 hr as our standard incubation time so as to have high readings; but shorter times or lower densities can be used.

Under standard conditions, no reduction was seen in the absence of cells. Some color developed spontaneously when the pH was adjusted to 4 or less but not above pH 4.5. Nor was any color detected when cells were incubated 24 hr with all components except molybdate, then removed by centrifugation before molybdate was added to the supernate, and incubated an additional 24 hr. Reduction was observed only when cells were in contact with molybdate.

Effect of *chl* Mutations and Inhibitors. Molybdate reduction was observed both with chl^+ cells and with *chl* mutants. The activities of wild-type and mutant suspensions were comparable and responded in the same manner to the variables studied in Table 1. However, mutants and wild-type differed in their response to certain inhibitors (Table 2).

Inhibition by Nitrate. Some inhibitors are more effective against chl^+ cells than against *chl* mutants. Nitrate is a good example. Table 3 shows that *chl* mutants were resistant to concentrations that completely inhibited molybdate reduction in chl^+ cells.

We believe that the inhibition by added nitrate results from its reduction to nitrite, which occurs in chl^+ cells but not in *chl* mutants. Nitrite is present in the chl^+ tubes at the end of incubation, and added nitrite inhibits both chl^+ and chl^- cells.

Table 2. Inhibitors of molybdate reduction

Compounds that preferentially inhibit chl^+	Compounds that preferentially inhibit *chl* mutants	Compounds that inhibit chl^+ and *chl* cells equally
KNO_3, 100 mM	K_2CrO_4, 100 μM	Na_2WO_4, 10 mM
$KClO_3$, 160 μM		Na_2SO_3, 8 mM
$NaNO_2$, 70 μM		
Cysteine, 10 mM		

Inhibitors were added to the standard assay described in Table 1. Concentrations given are the lowest concentrations tested that inhibited more than 50%.

The result is surprising in that nitrate reductase, the enzyme responsible for most nitrate reduction in *E. coli*, should not be present. Nitrate reductase is induced by nitrate during anaerobic growth. In our experiments, chl^+ cells that had been grown aerobically in the absence of nitrate reduce nitrate to nitrite in the presence of chloramphenicol. Furthermore, molybdate reduction in strain RK5265, which carries a mutation in the structural gene for nitrate reductase, was sensitive to added nitrate (data not shown). As molybdate reduction by *chl* mutants is insensitive to nitrate, the nitrate reduction by chl^+ cells must require molybdenum cofactor, but not nitrate reductase.

Inhibition by Chlorate. Like nitrate, chlorate appears to be reduced by one or more molybdoenzymes, perhaps including nitrate reductase (6). Like nitrate, chlorate inhibits molybdate reduction, and the minimal inhibitory concentration of chlorate is higher in *chl* mutants than in chl^+ cells. Chlorate differs from nitrate in that *chl* mutants are inhibited by high concentrations of chlorate (data not shown). We assume that the *chl*-dependent inhibition at low chlorate concentrations results from reduction to chlorite but have not investigated the matter further.

Biotin Sulfoxide Reduction and Molybdate Reduction in Wild-Type and Mutant Phenocopies. The nitrate case illustrates that one way *chl* mutations may influence molybdate reduction is by preventing conversion of an added substance to some product that is either more or less inhibitory than the substance itself. Such a mechanism is possible if the enzyme catalyzing the conversion requires a molybdenum cofactor. A formal alternative (which could, in principle, apply to some of the inhibitors in Table 2) is that the *chl* proteins interact with the molybdate reduction system directly.

One way to obtain evidence that the *chl* mutations act by preventing cofactor-dependent reactions is to convert chl^+ cells to mutant phenocopies by growth on 1 mM tungstate or to grow *chlD* or *chlG* cells in 1 mM molybdate, which con-

Table 3. Inhibition of molybdate reduction by nitrate and chlorate

Strain (*chl* genotype)	Residual activity in presence of inhibitor					
	KNO_3			$KClO_3$		
	200 μM	400 μM	4 mM	180 μM	320 μM	3.2 mM
RK4353 (chl^+)	0.04	0.04	0.0004	0.27	0.06	0.03
RK5200 (*chlA*)	1.0	0.97	1.1	1.1	1.3	1.4
RK5208 (*chlB*)		1.1				
RK5228 (*chlE*)		1.1				

Inhibitors were added to the standard assay system described in Table 1. The residual activity is the ratio of the specific activity in the presence of inhibitor to the specific activity of the same culture in the absence of inhibitor.

Table 4. Effect of molybdate supplementation on biotin sulfoxide reductase activity

Strain (*chl* genotype)	Method of extraction*	Molybdate, mM	Reductase activity under culture conditions: Aerobic (no glucose)	Aerobic (1% glucose)	Anaerobic (1% glucose)
SA302 (*chl*$^+$)	Lysozyme (5)	0		16	4.5
		1		9.4	4.1
SA322 (*chlD*)	Lysozyme	0	9.7	2.1	0.67
		1	35	30	7.0
RK4353 (*chl*$^+$)	Sonic (4)	0	31		
RK5207 (*chlD*)	Sonic	0	17	2.5	
		1	38	26	
RK206 (*chlG*)	Sonic	0	0.08		
		0.1	0.85		
		1	5.3		
		10	6.6		
	Lysozyme	0	0.13		
		1	1.0		
		10	2.8		

Specific activities are expressed as milliunits per milligram of protein, where units are defined as micrograms of biotin formed per 15 minutes (4).
*References in parentheses.

verts these (only partially in the case of *chlG*) to wild-type phenocopies. We tested the method with nitrate and later applied it to other inhibitors. If the nitrate resistance of molybdate reduction by *chl* mutants results from absence of active cofactor, as we presume it does, then growth on tungstate should make *chl*$^+$ cells more nitrate-resistant, and growth on molybdate should make *chlD* cells more nitrate-sensitive.

Before proceeding to such experiments, we should clear up one loose end from previous work on *chlD*. The effect of a *chlD* mutation on nitrate reductase and biotin sulfoxide reductase is qualitatively the same. The amount of enzyme in the *chlD* mutant is lower than in *chl*$^+$ when cells are grown on unsupplemented media, whereas the amount of enzyme in *chlD* mutants grown on media supplemented with 1 mM molybdate equals or exceeds that in a *chl*$^+$ strain. However, there was a quantitative discrepancy between the two results, in that the nitrate reductase activity in unsupplemented medium was less than 5% of the *chl*$^+$ level (7), whereas the biotin sulfoxide reductase level was about 50% (4). This is surprising if the *chlD* mutation affects both enzymes solely by reducing the amount of active cofactor. It turns out that the apparent discrepancy results from the conditions under which the cells used in assaying the two enzymes were grown.

In the biotin sulfoxide reductase studies, cells were grown aerobically on tryptone. Under those conditions, nitrate reductase is not formed. Nitrate reductase is induced by nitrate during anaerobic growth. Anaerobic growth requires a fermentable carbon source such as glucose. When we measured biotin sulfoxide reductase in anaerobic *chlD* cultures (with or without nitrate), the molybdate dependence was much stronger than in aerobic cultures. Even aerobically, glucose increased the dependence on molybdate supplementation (Table 4). These results suggest that cells grown aerobically without glucose have a *chlD*-independent pathway for cofactor synthesis.

In agreement with previous work on another *chlG* mutant (4), molybdate supplementation increased enzyme activity in the *chlG*::Mu strain RK5206, but not up to the wild-type level (Table 4).

Pregrowth on tungstate makes molybdate reduction by *chl*$^+$ cells more resistant to added nitrate, whereas pregrowth on molybdate increased the nitrate sensitivity of molybdate reduction by *chlD*, the increase being greater for *chlD* cells grown anaerobically with glucose than for aerobically grown cells (Table 5). This is the expected result if nitrate reduction *in vivo* (by cells in which nitrate reductase has not been induced) responds to these growth conditions in the same manner that biotin sulfoxide reductase does. Aerobically grown *chlG* cells were more resistant than *chlD* cells, also as expected. Although molybdate supplementation had no large effect on *chlG* in the experiment of Table 5, colorimetric assays for nitrite accumulation showed that *chlG* cells grown with 1 mM molybdate reduce nitrate more rapidly than *chlG* cells grown without added molybdate and that the rate of nitrite formation was unaffected by the presence of 20 mM molybdate in the assay tube (data not shown).

Inhibition by Nitrite. Inhibition of molybdate reduction by added nitrate seems to result from reduction to nitrite. At high enough concentrations (>1 mM), nitrite inhibited both *chl*$^+$ and *chl*$^-$ cells. However, at concentrations around 500 μM, nitrite inhibited *chl*$^+$ cells more strongly than *chl* mutants or *chl*$^+$ cells grown with tungstate, and *chlD* cells grown without molybdate supplementation were more nitrite-resistant than those grown with 1 mM molybdate. The inhibition of *chl*$^+$ cells by low concentrations of nitrite could be reversed by the addition of 40–80 mM formate (data not shown). These results indicate that inhibition of molybdate reduction by nitrite is conditioned by some molybdoenzyme(s), perhaps including the formate-dependent nitrite reductase (8).

Inhibition by Sulfur Compounds. Molybdate reduction was inhibited by 8 mM sulfite and 5–20 mM cysteine or other sulfhydryls, such as sodium thioglycollate or dithiothreitol, but was unaffected by 50 mM sulfate. Wild-type cells were more sensitive to cysteine inhibition than *chl* mutants were. Tungstate pregrowth decreased the cysteine resistance of *chlD* mutants (data not shown). These results suggest that inhibition results from a cofactor-dependent reaction involving cysteine.

Inhibition by Chromate and Tungstate. Tungsten and chromium belong to the same chemical group as molybdenum. Tungstate has been used extensively as a molybdate analog. Chromate has been used less, probably because it is toxic.

Tungstate at 10 mM strongly inhibited molybdate reduction. Wild-type cells and *chl* mutants were equally sensitive. Chromate concentrations above 200 μM (which are lethal to growing cells) inhibited molybdate reduction completely. Sublethal concentrations (50–200 μM) were more inhibitory to *chl* mutants than to *chl*$^+$ cells; and pregrowth with tungstate increased the sensitivity of *chl*$^+$ cells (data not shown).

Table 5. Effect of growth conditions on nitrate or chlorate sensitivity

Strain (*chl* genotype)	Growth conditions	Residual activity in presence of inhibitor			
		KNO_3		$KClO_3$	
		200 μM	400 μM	160 μM	320 μM
RK4353 (*chl*$^+$)	Aerobic	0.04	0.02	0.09	0.006
	Aerobic, 1 mM Na_2WO_4	0.02	0.12	0.42	0.18
	Anaerobic	0.25	0.01		
	Anaerobic, 1 mM Na_2WO_4	1.8	1.0		
	Anaerobic, 1 mM Na_2MoO_4	0.10	0.03		
RK5207 (*chlD*)	Aerobic	0.14	0.10	0.19	0.11
	Aerobic, 1 mM Na_2MoO_4	0.03	0.02	0.01	0.002
	Anaerobic	0.94	0.88		
	Anaerobic, 1 mM Na_2MoO_4	0.04	0.02		
RK5206 (*chlG*)	Aerobic	0.79	0.78	0.44	0.28
	Aerobic, 1 mM Na_2MoO_4	0.81	0.63	0.32	0.17

Cells grown under the indicated conditions were assayed for molybdate reduction as described in Table 1. See text for growth conditions. Residual activities were calculated as described in Table 3.

The results suggest that a molybdoenzyme might destroy an inhibitor, which could be either chromate itself or some compound generated in its presence.

DISCUSSION

We have reported here some observations on a new phenomenon, molybdate reduction by *E. coli*. Neither the mechanism of the reduction nor its biological significance is yet known. However, its study, in conjunction with other experiments, has suggested some properties of the molybdenum cofactor system in *E. coli*. Two conclusions are firm. Some others are quite speculative.

The first firm conclusion is that the effect of *chlD* mutations depends strongly on growth conditions. The molybdate reduction results indicate that this is true for nitrate reduction, at least for that nitrate reduction that does not depend on nitrate reductase. Note the large difference in nitrate sensitivity depending on whether growth in the absence of added molybdate is aerobic or anaerobic (Table 5). The biotin sulfoxide reductase assays show the same dependence on growth conditions for that enzyme. Effects of *chlD* mutations on cofactor synthesis (9) can be compared to effects on enzyme activity only when both assays have been done under similar growth conditions.

The second firm conclusion is that some nitrate reduction takes place *in vivo* in the absence of nitrate reductase. The extreme sensitivity of molybdate reduction to the presence of nitrite makes this easy to see. The nitrate reductase pathway is demonstrably the major pathway whereby growing cells reduce nitrate (6). Our *in vivo* results are concordant with published *in vitro* results (3), where a small amount of *narG*-independent nitrate reduction requires the integrity of the molybdenum cofactor genes and is independent of the state of aerobiosis during growth.

Of the inhibitors tested, chlorate and chromate are toxic at concentrations close to those we have used, and their effects on molybdate reduction might easily be effects on general metabolism. This does not invalidate our conclusions regarding the possible existence of molybdoenzymes that catalyze reactions involving chromate but may make the inhibition uninteresting in the study of molybdate reduction itself.

It is possible that nitrate, nitrite, chlorate, and O_2 all exert at least part of their inhibitory effect simply by acting as biological electron acceptors. Studies of the effect of nitrate concentration on molybdate reduction by growing cultures suggest that part of the inhibition results from the reduction itself rather than from the nitrite generated (data not shown). The fact that nitrate sensitivity depends completely on the *chl* genes, whereas nitrite sensitivity is affected only quantitatively, fits with the existence in *E. coli* and *Klebsiella pneumoniae* of more than one pathway for nitrite reduction, only one of which is *chl*-dependent (8, 10). The fact that 70 μM nitrite prevents reduction of 40 mM molybdate indicates that nitrite does not act stoichiometrically either to reoxidize reduced molybdate or to oxidize a cellular reductant that itself reacts stoichiometrically with molybdate. Under our conditions, nitrate is rapidly reduced to nitrite, but most of the nitrite is not further reduced (data not shown).

As to the mechanism of molybdate reduction, the only relevant fact is that reduction is seen only when cells and molybdate are in contact. We tentatively conclude that the reduction takes place either within cells or on the cell surface. Secretion of a reductant that reduces molybdate extracellularly is technically possible, but no such reductant accumulates when cells are suspended in the absence of molybdate. Some of our findings, such as the stimulation by 200 mM NaCl and the dependence on the ratio of phosphate to molybdate, are easier to understand if the concentrations of these substances in the medium are present at the reaction site, as would happen if electrons were being transferred to phosphomolybdate from the cell surface. If this is true, the ability of formate-dependent nitrite reductase to generate a membrane potential (8) may be relevant to the mechanism of inhibition by nitrite.

When *E. coli* cells are streaked on L agar plates containing 1% glucose plus 20 mM molybdate and incubated anaerobically, the colonies are dark blue. White mutants, which reduce molybdate very poorly in cell suspension, have been isolated and await characterization.

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