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Molybdenum Accumulation in *chlD* Mutants of *Escherichia coli*

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The content of molybdenum in wild-type and *chlD* cells was measured under a variety of growth conditions to determine if cells with a defective *chlD* gene were able to accumulate molybdenum. The *chlD* cells accumulated less molybdenum than wild-type cells did but concentrated molybdenum to a level at least 20-fold higher than the concentration in the culture medium. Molybdenum was present within spheroplasts of *chlD* cells and was not dialyzable. The *chlD* cells accumulated as much molybdenum as wild-type cells did when grown in medium containing 0.1 mM molybdate; thus, the capability of incorporation of molybdenum into cellular component(s) was equivalent to that of the wild type under these conditions.

The availability of molybdenum regulates a number of cellular processes, such as the accumulation of nitrate reductase subunits (6, 19); the expression of the *chlI* gene, which codes for a cytochrome for nitrate reductase (16); the accumulation of active Mo cofactor (2, 13); the activity of the *chlA* gene product, which is essential for the synthesis of Mo cofactor (7); and nitrate repression of the *frd* gene for the synthesis of fumarate reductase (10). The disruption of these cellular functions by lack of molybdenum is seen only in *chlD* strains because wild-type *Escherichia coli* are very efficient in scavenging trace levels of molybdenum from contaminants of purified culture media. Wild-type *E. coli* can synthesize appreciable amounts of active nitrate reductase even when cells are grown in media without added molybdenum. However, cells lacking a functional *chlD* gene require much higher levels of molybdenum (8). In the absence of high levels of molybdenum, *chlD* cells have low levels of Mo cofactor (13) and lack the activities of all the molybdoenzymes (5, 8, 17). The activity of the molybdoenzymes is restored when cells are grown in media with 0.1 mM molybdate, a level 1,000-fold higher than that required by wild-type cells.

Expression of the *chlD* gene is regulated by the concentration of molybdenum in growth media (14). In *chlD-lacZ* operon fusions, the fusion is highly expressed when the cells are grown with less than 10 nM molybdate, with less than 5% of the activity remaining when the cells are grown with 500 nM molybdate (14). Expression of *chlD-lacZ* is increased two- to threefold when cells are grown anaerobically with nitrate. Under these conditions, nitrate reductase and formate dehydrogenase are induced, which may increase the demand for molybdenum.

Glaser and DeMoss (8) found that the concentration of molybdenum in *chlD* mutant cells did not differ significantly from that of wild-type cells grown in medium supplemented with either 1 μM or 0.1 mM molybdate. The molybdenum within the cell was tightly bound in a nondialyzable form, and the *chlD* cells contained as much nondialyzable molybdenum as wild-type cells did. However, the subcellular distribution of molybdenum differed in that the concentration of molybdenum in the particulate fraction of *chlD* cells was less than that in the particulate fraction of wild-type cells. When the cells were grown with 0.1 mM molybdate, the molybdenum content in the particulate fraction of the *chlD* mutant was restored to about 50% of wild-type levels.

Johann and Hinton (11) proposed that the *chlD* locus is an operon involved in the active transport of molybdenum. The nucleotide sequence of the restriction fragment containing the cloned gene revealed two open reading frames which potentially code for proteins with sequence homology to those of bacterial active transport systems. The sequence of the *chlD* product showed regions of homology with nucleotide-binding proteins and hydrophilic inner membrane proteins. The sequence of the other open reading frame encoded a protein with properties of a hydrophobic inner membrane protein.

To determine if the defect in the *chlD* cells was the inability to accumulate molybdenum when the cells are grown in medium with very low levels of molybdenum, we measured the internal concentration of molybdenum in wild-type and *chlD* cells grown under conditions in which the cells require very different levels of molybdenum.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli chl*$^+$ SA302 F− *his* (λ *c*I837) and its derivative (SA322) which carries a deletion from *chlD* to *pgl* of approximately 4.5 kilobases (5) were used.

Cells were grown in low-phosphate minimal medium (21) containing 10 μg of thiamine per ml, 20 μg of L-histidine per ml, and 0.2% glucose. Where indicated, 20 mM sodium nitrate or 0.1 mM sodium molybdate was included. The total phosphate concentration was lowered to 10 μg/ml to reduce the molybdenum contamination from the phosphate stocks. The growth medium with no added molybdenum contained less than 10 nM molybdenum contamination and is referred to as low-molybdenum medium. We took great care to reduce the molybdenum contamination in the medium. All reagents were the purest obtainable commercially. Disposable plasticware was used whenever possible. The glassware was soaked in Radiacwash (Atomic Products Corp., New York, N.Y.) to remove residual molybdenum.

Anaerobic cultures were grown in sealed jars equipped with a palladium catalyst and gas-generating envelopes (GasPak; Becton Dickinson and Co., Paramus, N.J.). Bacteria were grown at 30°C and harvested at an optical density at 600 nm of 0.3 to 0.7.

The cultures of SA302 and SA322 used for comparison of the inductively coupled plasma (ICP), atomic absorption, and colorimetric determinations of molybdenum were grown

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in low-phosphate minimal medium in stainless steel fermentors. In order to supply a sufficient sample so that the three methods of determination of molybdenum could be performed on the same batch of cells, 100 liters of cells were grown in low-molybdenum medium and 12 liters of cells were grown in medium with 0.1 mM molybdate. The cells were grown to stationary phase, harvested by centrifugation, and washed twice with 50 mM Tris hydrochloride, pH 8.0. The cells were lyophilized, wet ashed until colorless (4), dried on a hot plate, suspended in 5% HCl, and assayed for molybdenum.

Sample preparation. Cells to be used for the determination of molybdenum by atomic absorption spectrometry were grown to mid-log phase in minimal medium, centrifuged at 10,000 × *g* for 15 min, and washed twice with a 0.25 volume of 10 mM Tris hydrochloride, pH 8.0. The cells were suspended in water, lyophilized, and weighed to determine dry weight. The cells were ashed in a low-temperature asher (IPC model S4075-11020; Branson International Power Corp., Hayward, Calif.) for 14 to 16 h. If necessary to completely ash the sample, a portion of 0.5 N HNO_3 was added and the sample was put on a hot plate to boil off the acid and returned to the asher for 12 h. The ash was suspended in 0.1 N nitric acid and assayed for molybdenum.

Measurement of molybdenum. Molybdenum levels were measured by electrothermal atomic absorption spectrometry in a spectrometer (model 380; The Perkin-Elmer Corp., Norwalk, Conn.) equipped with an HGA 2200 graphic furnace and an AS-1 auto sampling system. Pyrolytically coated graphite tubes were used. $A_{313.3}$ was measured with a spectral band of 0.7 nm by using integrated peak area. The maximum power control was used for all measurements. Argon was used as the purge gas, with a 7-s gas interruption during the atomization period. Samples (10 μl each) were dried at 110°C for 10 s, charred at 1,900°C for 50 s, and atomized at 2,800°C for 15 s. Optimal charring conditions were established from a charring curve of *E. coli* samples.

The molybdenum standard was an atomic absorption standard from Fisher Scientific Co., Pittsburgh, Pa., containing 1,000 ppm (1,000 μg/liter) of molybdic anhydride in dilute nitric acid. The molybdenum content of the *E. coli* samples was determined by the standard additions method, in which an internal standard curve is generated by two or three additions of a standard molybdenum solution to the sample. The internally generated slope was used to compensate for the loss of absorbance by molybdenum due to the number of firings of the tube. The data are expressed as micrograms of molybdenum per gram [dry weight] of the sample.

Molybdenum was measured by ICP spectrometry (spectrometer model 3510; Applied Research Laboratories). A mass flowmeter-flow controller (FM4575; Linde Union Carbide Corp., New York, N.Y.) regulated argon carrier gas flow at 1.000 liter/min. A sample was introduced into the carrier gas at 3.2 to 3.3 ml/min with a flowmeter (Gilson). Molybdenum was measured at 202.0200 ± 0.0028 nm, with an integration time of 1 s.

Molybdenum was also measured by the colorimetric method of Johnson and Arkley (12). This method is based on the formation of a molybdenum thiocyanate complex.

RESULTS

Method of measurement. The concentration of molybdenum in wild-type and *chlD* cells was measured by electrothermal atomic absorption spectrometry. This technique is very sensitive, and ashed samples can be used without prior extraction or separation of molybdenum. It is susceptible to a number of problems from the salt matrix of the sample, the limited lifetime of the graphite tube, and the refractory nature of molybdenum to volatilization. Verification of the method was by measurement of molybdenum of a standard reference material. Bovine liver standard reference material 1577a has a certified value for molybdenum of 3.5 ± 0.5 μg of molybdenum per g. The average value for molybdenum obtained in seven separate analysis of this material was 3.4 ± 0.2 μg of molybdenum per g of sample, which is within the experimental error of the certified value.

TABLE 1. Comparison of the molybdenum content of cells as determined by ICP, atomic absorption, and colorimetric methods

Strain	Addition of Mo[a]	Result (μg of Mo/g [dry weight]) of:		
		ICP spectrometry	Atomic absorption	Colorimetric assay
SA302	−	0.1	0.1	ND[b]
SA322	−	0.3	0.4	ND
SA302	+	14.1	21.0	17.2
SA322	+	10.7	12.3	13.5

[a] Cells were grown aerobically to stationary phase without (−) or with (+) the addition of 0.1 mM Na_2MoO_4.
[b] ND, Not detectable.

To determine if the matrix of the ashed *E. coli* interfered with the measurement of molybdenum in cells, the molybdenum content of wild-type and *chlD* cells was measured by atomic absorption, ICP, and the colorimetric assay of Johnson and Arkley (12). Both ICP and atomic absorption were sufficiently sensitive to measure the molybdenum concentrations of cells grown in low-molybdenum medium, and the concentration of molybdenum measured by both methods was similar (Table 1). Similar values for the molybdenum contents of the cells grown with 0.1 mM molybdate were obtained by all three methods. ICP was not used routinely, because it requires a much larger sample size than atomic absorption does.

Molybdenum concentration in cells. The concentration of molybdenum in wild-type (SA302) and *chlD* (SA322) cells was measured under a variety of growth conditions which affect *chlD* gene expression (Table 2).

When the cells were grown in low-molybdenum medium, either aerobically without nitrate or anaerobically with ni-

TABLE 2. Concentration of molybdenum in wild-type and *chlD* cells

Growth conditions[a]	Mo concn in strain:			
	SA302		SA322	
	μg/g (dry wt)[b]	Molarity[c] (μM)	μg/g (dry wt)	Molarity (μM)
Aerobic				
−Mo	0.54 ± 0.19	1.0	0.12 ± 0.04	0.2
+Mo	3.02 ± 0.52	5.6	2.47 ± 0.51	4.6
Anaerobic (+NO_3)				
−Mo	1.30 ± 0.40	2.4	0.12 ± 0.09	0.2
+Mo	6.87 ± 1.14	12.7	5.00 ± 0.02	9.2

[a] Cells were grown with (+Mo) or without (−Mo) 0.1 mM Na_2MoO_4; anaerobic growth conditions included 20 mM $NaNO_3$.
[b] Values are means ± standard deviations.
[c] Molarity of molybdenum in whole cells was calculated by estimating cell volume as 1.6×10^{-15} liter (18) and dry weight of one cell as 2.8×10^{-13} g (15).

trate, significantly more molybdenum was present in wild-type than in *chlD* cells. Wild-type cells grown anaerobically with nitrate contained twice as much molybdenum as those grown aerobically. In contrast, *chlD* cells did not display an increased concentration of molybdenum under these conditions. When the cells were grown in the presence of 0.1 mM molybdenum, wild-type and *chlD* cells accumulated approximately the same amount of molybdenum.

When the cellular concentration of molybdenum was calculated as molarity, the concentration of molybdenum in cells grown aerobically in low-molybdenum medium was 1.0 μM for wild-type and 0.2 μM for *chlD* cells. The estimated maximal concentration of molybdenum in the minimal medium is 10 nM molybdenum. Thus, wild-type cells concentrated molybdenum to a level at least 100-fold greater than the level in the culture medium, and *chlD* concentrated molybdenum to a level at least 20-fold greater. When wild-type and *chlD* cells were grown in medium with 0.1 mM molybdate, the concentration of molybdenum in these cells was 5 to 12 μM, which is well below that of the growth medium. Thus, the cells maintained an internal concentration of molybdenum within a narrow range, even though the concentration of molybdenum in the culture media varied 10,000-fold.

The concentration of molybdenum was measured in cells harvested in various cell densities. Both wild-type and *chlD* cells contained the most molybdenum per g of dry weight in early-log-phase cells and had approximately half as much when the cells reached the stationary phase.

Molybdenum content of spheroplasts. The molybdenum content of spheroplasts was compared with that of whole cells to determine whether molybdenum was incorporated into the cells or accumulated in the periplasmic space. The spheroplasting procedure (22) removes the periplasm and free molybdenum within this region. Molybdenum was present in spheroplasts of wild-type cells grown under all the conditions tested. Molybdenum was present in the spheroplasts of *chlD* when the cells were grown in low-molybdenum medium, indicating that the *chlD* cells were capable of incorporating some molybdenum into the cell interior, albeit much less than is present in spheroplasts of wild-type cells. When the cells were grown with added molybdate, the spheroplasts of *chlD* accumulated as much molybdenum as was present in the spheroplasts of the wild-type cells. This experiment indicates that even though the *chlD* did not accumulate as much molybdenum as wild-type cells did, these cells transfered molybdenum into the interior of the cell.

The molybdenum contents of dialyzed cytoplasmic and membrane fractions of *chlD* cells were measured. The sum of the molybdenum contents of the dialyzed cytoplasmic and membrane fractions was roughly equivalent to the molybdenum content of the portion of cells that had not been dialyzed. Even when the cells were grown in medium with 0.1 mM molybdenum, most of the cellular molybdenum was in a nondialyzable form. This suggests that most of the molybdenum in both wild-type and *chlD* cells was tightly bound to a cellular component.

DISCUSSION

The concentration of molybdenum in *chlD* mutants was measured to determine if the defect in these cells was an inability to accumulate molybdenum from medium containing a very low level of molybdenum. In growth medium with less than 10 nM molybdenum, wild-type cells, but not *chlD* cells, synthesize appreciable levels of molybdenum cofactor and nitrate reductase (13, 17). At low concentrations of molybdenum, the *chlD* gene product is highly expressed in the wild-type cells (14), and the differences between the wild type and the mutant would be most apparent.

The *chlD* mutants accumulated significantly less molybdenum than wild-type cells when grown in low-molybdenum medium. Less than 20% of the molybdenum present in wild-type cells was present in *chlD* grown aerobically. Thus, the defect in *chlD* mutants which resulted in less accumulation of molybdenum was apparent even when nitrate reductase and formate dehydrogenase were not induced. Less than 10% of the molybdenum present in wild-type cells was present in *chlD* mutants when the cells were grown anaerobically with nitrate which induced production of nitrate reductase and formate dehydrogenase.

The *chlD* mutants grown in medium with 0.1 mM molybdate contained approximately the same amount of molybdenum as wild-type cells, which was 5 to 12 μM, well below the concentration in the culture medium. Under these growth conditions, *chlD* cells displayed a phenotype similar to wild-type cells. The restoration of the activity of the molybdoenzymes and the accumulation of molybdenum to wild-type levels when the cells were grown in 0.1 mM molybdate could not be due to the *chlD* gene product, because these effects occurred in mutants that were deleted from the *chlD* gene. It is not known if high concentrations of molybdate overcome the lack of the *chlD* gene product because the *chlD* mediated process can occur nonenzymatically or because the process can be catalyzed by an alternative enzymatic system with a lower affinity for molybdenum.

The molybdenum in *chlD* mutants was present in spheroplasts, indicating that it was in the interior of the cells, not just in the periplasmic space or nonspecifically bound to the cell wall. Molybdenum was present in both the soluble and membrane fractions, but the form of molybdenum in the cell is not known. Some of the molybdenum is associated with the molybdenum cofactor, which is present in both the soluble and membrane fractions (1). The molybdenum in the cells was not dialyzable, even when the cells were grown with 0.1 mM molybdate. This indicates that free molybdenum is not present in the cells and that molybdenum is bound to cellular components during or immediately after transport.

Glaser and DeMoss (8) found that when the cells were grown in medium with 1 μM molybdate, *chlD* and wild-type cells accumulated the same amount of molybdenum, but the level of nitrate reductase activity was 100-fold higher in wild-type than in *chlD* cells. They concluded that the *chlD* gene product must have a role in the processing of molybdate to a form necessary for the electron transport systems of nitrate reductase and formate hydrogenlyase.

The *mol* mutants of *Klebsiella pneumoniae* have elevated requirements for molybdenum, are repaired by high concentrations of molybdenum in the culture medium, and appear to be genetically equivalent to *chlD* in *E. coli* (9, 20). The accumulation of molybdenum by *mol* mutants into the nonexchangeable pool was defective at low molybdenum concentrations, but molybdenum uptake was not impaired. Increasing the molybdenum concentrations resulted in parallel restoration of enzyme activity and molybdenum accumulation. Those authors hypothesize that the *mol* gene product is not involved in the transport of molybdenum but that cellular molybdenum is transformed by the *mol* gene product to yield an intermediate (Mo-X) common to both Mo cofactor and FeMo cofactor pathways.

Several lines of evidence show that the effect of the *chlD* mutation depends on the growth conditions. Campbell et al. (3, 5) have found that the molybdenum dependence of biotin sulfoxide reductase activity in *chlD* mutants is much more sensitive to molybdenum when the cells are grown with a fermentable carbon source such as glucose. Also, there was a large difference in the nitrate sensitivity of molybdenum reduction depending on whether growth in the absence of added molybdenum was aerobic or anaerobic. Ugalde (20) found that growth of the cells in cystine increased by 100- to 1,000-fold the requirement for molybdenum for nitrate reductase activity in both *mol* and *chlD* mutants, but not in wild-type cells.

In conclusion, the accumulation of molybdenum is impaired in *chlD* mutants, and the low level of internal molybdenum seems responsible for the variety of defects observed in *chlD* mutants, such as low level of Mo cofactor and lack of activity of the molybdoenzymes. Our experiments provide information on the size of the internal pool of molybdenum as a basis for further studies on transport but do not elucidate the mechanism of molybdenum transport in *E. coli*. Further experiments are necessary to elucidate the process that is catalyzed by the *chlD* gene product.

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