JOURNAL OF BACTERIOLOGY, Aug. 1983, p. 793–801
0021-9193/83/080793-09$02.00/0

Vol. 155, No. 2

Molybdenum Cofactor in Chlorate-Resistant and Nitrate Reductase-Deficient Insertion Mutants of *Escherichia coli*

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Received 24 January 1983/Accepted 10 May 1983

We examined molybdenum cofactor activity in chlorate-resistant (*chl*) and nitrate reductase-deficient (*nar*) insertion mutants and wild-type strains of *Escherichia coli* K-12. The bacterial molybdenum cofactor was assayed by its ability to restore activity to the cofactor-deficient nitrate reductase found in the *nit-1* strain of *Neurospora crassa*. In the wild-type *E. coli* strains, molybdenum cofactor was synthesized constitutively and found in both cytoplasmic and membrane fractions. Cofactor was found in two forms: the demolybdo form required additional molybdate in the assay mix for detection, whereas the molybdenum-containing form was active without additional molybdate. The *chlA* and *chlE* mutants had no detectable cofactor. The *chlB* and the *narG*, *narI*, *narK*, and *narL* (previously designated *chlC*) strains had cofactor levels similar to those of the wild-type strains, except the *chlB* strains had two to threefold more membrane-bound cofactor. Cofactor levels in the *chlD* and *chlG* strains were sensitive to molybdate. When grown in 1 μM molybdate, the *chlD* strains had only 15 to 20% of the wild-type levels of the demolybdo and molybdenum-containing forms of the cofactor. In contrast, the *chlG* strains had near wild-type levels of demolybdo cofactor when grown in 1 μM molybdate, but none of the molybdenum-containing form of the cofactor. Near wild-type levels of both forms of the cofactor were restored to the *chlD* and *chlG* strains by growth in 1 mM molybdate.

Molybdoenzymes are found in all major phylogenetic groups where they catalyze several oxidation-reduction reactions (6). All molybdoenzymes, with the sole exception of nitrogenase (26), share a common molybdenum-containing cofactor. The molybdenum cofactor is of low molecular weight and includes a pterin (15), with an, as yet, incompletely determined structure (16).

The molybdoenzymes of *Escherichia coli* include the inducible, membrane-bound proteins nitrate reductase (6) and trimethylamine oxidase (33), the constitutively synthesized, soluble enzyme biotin sulfoxide reductase (8), and two or more forms of formate dehydrogenase which can be either soluble or membrane bound (7). The most well-known mutations affecting the molybdoenzymes are in the chlorate resistance (*chl*) loci of *E. coli*, although similar sets of mutations are found in *Aspergillus nidulans* and *Neurospora crassa* (25, 34).

The *chl* mutants of *E. coli* are in several classes, designated *chlA*, *-B*, *-C*, *-D*, *-E*, and *-G*, all of which are deficient in nitrate reductase activity or regulation. In addition, with the exception of the *chlC* mutants, *chl* mutants are generally deficient in the other molybdoenzymes. The pleiotropic nature of the *chlA*, *-B*, *-D*, *-E*, and *-G* mutants indicates that the products of these genes are involved in synthesis or regulation of the molybdenum cofactor (1, 8, 30, 32), which is the only known common component of the molybdoenzymes. Stewart and MacGregor (32) recently isolated a series of Mu and Tn*10* insertion mutants of the *chl* loci. Their results are consistent with the idea that the pleiotropic mutations affect the synthesis or use of the molybdenum cofactor. In addition, they found that the *chlC* locus is divisible into at least five phenotypic groups, affecting synthesis or regulation of the nitrate reductase. Accordingly, the *chlC* locus was renamed *nar* (a mnemonic for nitrate reductase) and includes the *G*, *H*, *I*, *K*, and *L* phenotypes (32). Insertion mutants are often especially useful for biochemical studies because the insertions typically destroy the activity of the affected gene.

The *chl* gene products and the molybdenum cofactor thus stand at the center of an interesting network of cellular functions catalyzed by molybdoenzymes. Under different growth conditions, the bacteria probably require widely dif-

fering amounts of molybdenum cofactor which must be distributed among the several membrane-bound and soluble enzymes. We wish to understand how the synthesis and cellular distribution of the molybdenum cofactor proceeds and how the availability of the cofactor affects the synthesis and localization of molybdoenzymes. As a step in this work, we have determined the activity and cellular location of the molybdenum cofactor in the well-defined *chl* and *nar* insertion mutants of Stewart and MacGregor (32). We grew each of the *chl* insertion mutants in several ways and found that each type of mutant had a characteristic pattern of cofactor activity which was, in some cases, significantly different from that of noninsertion *chl* mutants (1). These results can explain many previously reported observations of molybdoenzyme activities in *chl* mutants, and they provide information about the roles of the *chl* gene products in synthesis of the molybdenum cofactor.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 strains used in this study are listed in Table 1.

All bacteria were grown at 30°C on M9 minimal medium (20) with 0.4% glucose and other growth requirements at 40 μg/ml as required. Na_2MoO_4 was added as indicated. In the absence of added molybdate, M9 medium had a molybdenum concentration of approximately 1 μM, largely due to contaminating molybdenum in sodium phosphate stocks (unpublished data). Aerobic cultures were grown in 500-ml flasks with rotatory shaking at 200 rpm in a New Brunswick incubator. Anaerobic cultures were shaken similarly, but placed in anaerobic chambers (Gas-Pak; Becton, Dickinson & Co.). Bacteria were always harvested at an optical density at 550 nm of between 0.3 and 0.6 (i.e., during exponential growth) unless noted otherwise.

Growth of *N. crassa* and preparation of extracts. *N. crassa* mutant strain *nit-1* (allele 34547) was obtained from the Fungal Genetics Stock Center, Humboldt State University, Arcata, Calif. Mycelia were grown in Fries basal medium, and *nit-1* nitrate reductase apoenzyme was induced by a 4-h incubation in nitrate-containing medium as described earlier (11). Extracts of nitrate-induced *N. crassa nit-1* for use in the molybdenum cofactor assay were made fresh each day, as described previously (2), from *nit-1* mycelia that were stored at −70°C.

Assay for molybdenum cofactor. The assay for molybdenum cofactor is based on the ability of molybdenum cofactor from *E. coli* to reconstitute the NADPH-dependent nitrate reductase from the cofactor-less *N. crassa nit-1* mutant (17, 19). Complementation of the *nit-1* nitrate reductase with *E. coli* molybdenum cofactor was performed by mixing together equal volumes of 50 to 100 μl each of extracts of *E. coli* and *nit-1* and incubating them for 10 min at room temperature. Na_2MoO_4 was added to this complementation mixture as indicated. The assay for reconstituted NADPH nitrate reductase activity was performed as described previously (1). The reaction was stopped and the amount of nitrite produced was determined with the sulfanilic acid–*N*-1-naphthylethylenediamine colorimetric reaction described previously (11). All chemicals were reagent grade from Sigma Chemical Co. Molybdenum cofactor activity is expressed as units of nitrate reductase reconstituted during the 10-min complementation (1). One unit of nitrate reductase activity produces 1 nmol of nitrite per min.

TABLE 1. *E. coli* K-12 strains

Strain	Relevant markers[a]	Reference
PK27	Wild type	12
382 (CGSC 4442)[b]	*chlA*	27, 28
JRG97 (CGSC 4459)	*chlE*	28
RK4353	Wild type	32
RK5200	*chlA200*::Mu *cts*	32
RK5201	*chlE201*::Mu *cts*	32
RK5202	*chlD202*::Mu *cts*	32
RK5203	*chlA203*::Mu *cts*	32
RK5206	*chlG206*::Mu *cts*	32
RK5207	*chlD240*::Mu *cts*	32
RK5208	*chlB207*::Mu *cts*	32
RK5209	*chlD241*::Mu *cts*	32
RK5221	*chlA218*::Mu *cts*	32
RK5227	*chlB224*::Mu *cts*	32
RK5228	*chlE225*::Mu *cts*	32
RK5231	*chlG227*::Mu *cts*	32
RK5251	*chlB242*::Mu *cts*	32
RK5255	*chlE237*::Mu *cts*	32
RK5256	*chlG238*::Mu *cts*	32
RK5265	*nar-202*::Tn*10* ($NarG^-$)	32
RK5266	*nar-203*::Tn*10* ($NarK^-$)	32
RK5267	*nar-204*::Tn*10* ($NarI^-$)	32
RK5270	*nar-207*::Tn*10* ($NarK^-$)	32
RK5272	*nar-209*::Tn*10* ($NarG^-$)	32
RK5274	*nar-211*::Tn*10* ($NarI^-$)	32
RK5278	*nar-215*::Tn*10* ($NarL^-$)	32

[a] Markers relevant to this study are in chlorate resistance genes. Full genotypes are given in original references. RK strains with numbers 5200 or greater are derivatives of RK4353 and were provided by V. Stewart, Department of Biological Sciences, Stanford University, Stanford, Calif.

[b] CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.

Preparation of *E. coli* extracts. Extracts of *E. coli* for use in the cofactor assay were made in two ways. For some experiments, 50 ml of late log-phase cells were harvested by centrifugation, washed, resuspended in 5 ml of 100 mM potassium phosphate buffer (pH 7.4)–0.5 mM EDTA–1 mM 2-mercaptoethanol and passed through a French pressure cell at 20,000 lb/in^2. After centrifugation at 3,000 × *g* for 10 min, the resulting supernatant was assayed for cofactor. Alternatively, we adopted a faster method, in which protoplasts were made from 5 ml of log-phase bacteria with the lysozyme-EDTA method of Weiss (37). The washed and pelleted protoplasts were lysed in 100 μl of 1 mM $MgCl_2$ with traces of DNase and RNase. The lysed protoplasts were centrifuged for 1 min in an Eppendorf table-top centrifuge, and the supernatant fluid was used for cofactor assays.

To separate cytoplasmic and membrane fractions for cofactor localization, 175 μl of bacterial extracts made by either of the described methods was centrifuged for 30 min at 130,000 × *g* in a Beckman Airfuge A-100 rotor. All of the membrane-bound nitrate reductase activity was pelleted. The supernatant fluid was directly assayed as the cytoplasmic fraction. The small pellets representing the membrane fraction were resuspended in 100 μl of 100 mM potassium phosphate buffer (pH 7.4) with 0.5 mM EDTA, 1 mM 2-mercaptoethanol, and 2% Triton X-100 to release cofactor from the membrane. An equal volume of *nit-1* extract was added. After the assay for cofactor activity in the membrane fractions was completed, additional Triton X-100 was added to the assay mix to a final concentration of 1% to clarify the reaction mix for colorimetric readings. Extracts of wild-type bacteria made either from protoplasts or by French press gave similar cofactor activities.

The *nit-1* complementation assay used in this work to measure molybdenum cofactor levels must be performed with uniformly produced extracts to get quantitatively comparable measurements. Accordingly, the values for cofactor levels reported here were determined with extracts made from a single preparation of *N. crassa nit-1* mycelia. To measure the reproducibility of the cofactor assay, we measured cofactor activity on five different days, using samples of a single preparation of *E. coli* and freshly prepared extracts from the same batch of *nit-1* mycelia. Values of 5.2, 4.4, 5.2, 4.7, and 4.2 cofactor units/mg of protein were found, which is 4.7 ± 0.45 for average and standard deviation. Thus, a standard deviation of approximately ±10% should be kept in mind when comparing the cofactor levels reported here. The variation is even larger near the detection limit of the assay of 0.01 cofactor unit/mg of cell protein.

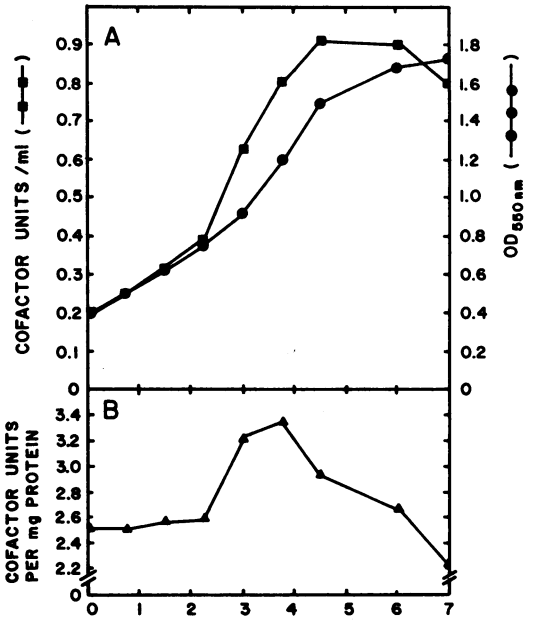

FIG. 1. Growth and molybdenum cofactor activity of *E. coli* wild-type strain RK4353. A 50-ml amount of bacteria was grown aerobically at 30°C on M9 medium plus 0.4% glucose. Samples (5 ml) of the culture were taken at the indicated times and assayed for total cofactor activity in the presence of 2 mM Na_2MoO_4 as described in the text. Symbols: (A) Turbidity (optical density at 550 nm [OD_{550nm}]) of the culture (●), and units of cofactor activity per milliliter of culture (■); (B) units of cofactor activity per milligram of cell protein (▲).

RESULTS

Molybdenum cofactor in wild-type *E. coli*. The amount of molybdenum cofactor activity found in the cells was dependent on the growth stage of the culture (Fig. 1). The total amount of cofactor activity in the culture increased at the same rate as the number of cells during early exponential growth, and the amount of cofactor per milligram of cell protein was nearly constant. At later stages of growth, total cofactor activity in the culture first increased more rapidly than cell number and then declined as the culture reached stationary phase. In the experiments that follow, therefore, bacteria were always harvested in early log phase (i.e., optical density at 550 nm = 0.3 to 0.6) to allow comparison of cofactor levels in different bacterial strains.

Molybdenum cofactor activity was found in both the cytoplasm and membranes of *E. coli* (Table 2), confirming previous results (1). We assayed molybdenum cofactor activity in the cytoplasmic and membrane fractions both with and without additional molybdate in the complementation mixture. This protocol measured both the demolybdo form of the cofactor, which lacked molybdenum and was detected only when additional molybdate was included in the complementation mixture, and the molybdenum-containing form of the cofactor, which required no additional molybdate in the complementation mixture for activity. The demolybdo form of the cofactor generally made up 80 to 90% of the total cofactor activity (Table 2). Only 10 to 20% of the cofactor was in the molybdenum-containing form when extracts were prepared with the methods used here. As noted below, the assay of both forms of the cofactor allowed differentiation of the otherwise similar *chlD* and *chlG* strains.

Less than 10% of the cofactor activity in wild-type cells was in the membrane fraction (Table 2). Unlike the cofactor in the cytoplasm, which was detectable without further treatment of the extract, the membrane-bound cofactor was detected only after treatment of the membranes with heat or Triton X-100. In a series of control experiments, the amount of cofactor released with the Triton treatment was always within 10 to 20% of the amount of cofactor released by the

TABLE 2. Effect of growth conditions on molybdenum cofactor activity

Strain	Growth conditions[a]	Cofactor activity (U/mg of cell protein) of:			
		Cytoplasm[b]		Membrane[b]	
		+Mo[c]	−Mo	+Mo	−Mo
RK4353	+Mo, $+O_2$	4.3 ± 0.4[d]	0.5 ± 0.2	0.16	0.05
	−Mo, $+O_2$	2.9 ± 0.4	0.3 ± 0.1	0.18	0.07
	+Mo, $-O_2$	4.4 ± 0.4	0.8 ± 0.3	0.20	0.10
	−Mo, $-O_2$	2.0 ± 0.2	0.3 ± 0.1	0.12	0.04
PK27	+Mo, $+O_2$	3.1	0.4	0.20	0.05
	−Mo, $+O_2$	2.0	0.2	0.17	0.05
	+Mo, $-O_2$	3.6	0.6	0.21	0.06
	−Mo, $-O_2$	1.8	0.3	0.08	0.04

[a] Bacteria (both strains were wild type in *chl* and *nar* genes) were grown in M9 minimal medium plus 0.4% glucose with or without 1 mM Na_2MoO_4 and with or without access to oxygen, as described in the text.

[b] Cytoplasmic and membrane fractions were prepared from *E. coli* protoplasts as described in the text.

[c] Complementation of *N. crassa nit-1* with *E. coli* extracts was performed with (+) or without (−) added 10 mM Na_2MoO_4. Final MoO_4 concentration in the assay mix was 2 mM.

[d] Average ± standard deviation (n = 3).

previously described heat treatment (1). Because the Triton X-100 treatment of membranes to release cofactor was faster and more easily reproducible, we adopted it as our standard method in the results reported here.

The presence or absence of oxygen and molybdenum during growth had relatively small effects on the amount of molybdenum cofactor found in wild-type cells. Bacteria grown aerobically without extra molybdate added to the medium had 60 to 70% of the total cofactor found in cells grown with 1 mM molybdate. The additional molybdate had a somewhat greater effect during anaerobic growth. Cells grown anaerobically without added molybdate had only 40 to 50% of the cofactor of cells grown with 1 mM molybdate. The distribution of cofactor between the cytoplasm and membrane and the relative proportions of demolybdo- and molybdenum-containing cofactor were relatively unchanged by these large changes in growth conditions.

Molybdenum cofactor in *chl* mutants. (i) *chlA* and *chlE*. A striking finding was that mutants carrying a Mu *cts* insertion in the *chlA* or *chlE* region had no detectable molybdenum cofactor activity (data not shown). Three independently isolated *chlA* strains (RK5200, RK5203, and RK5221) lacked cofactor activity under any of the growth conditions tested. Similarly, three *chlE* strains (RK5201, RK5228, and RK5255) had undetectable cofactor activity. These findings are in distinct contrast with those reported earlier (1) which showed that different, noninsertion *chlA* and *chlE* strains do make large amounts of cytoplasmic cofactor.

We assayed insertion and noninsertion *chlA* and *chlE* strains for molybdenum cofactor, using an earlier assay (1) and the protocols reported here. These experiments confirmed that some noninsertion *chlA* and *chlE* strains do have molybdenum cofactor under some conditions, but the insertion mutants at the *chlA* and *chlE* loci do not have molybdenum cofactor activity. Specifically, we grew strains 382 (*chlA*, CGSC 4442), JRG97 (*chlE*, CGSC 4459), RK5200 (*chlA*::Mu *cts*), and RK5201 (*chlE*::Mu *cts*) on tryptone soy-yeast broth medium (20) to approximately 10^9 cells per ml and passed these cells through a French pressure cell. The extracts were immediately assayed for cofactor activity in the presence of 50 mM Na_2MoO_4 as described by Amy (1). Under these conditions, strains 382 (*chlA*) and JRG97 (*chlE*) had near wild-type levels of molybdenum cofactor in the cytoplasm (2.2 and 2.8 U/mg of cell protein, respectively), whereas cofactor was still undetectable in RK5200 and RK5201. When extracts of these four strains were assayed using protoplasts as described above, it was necessary to use freshly made, unfrozen extracts to reproducibly assay cofactor in strains 382 and JRG97. Typically, frozen protoplasts made from wild-type cells could be stored for 1 to 3 days at −20°C before use in cofactor assays without significant loss of activity, but this was not the case with protoplasts of strains 382 and JRG97. The cofactor in these strains was apparently unstable to freezing compared with cofactor found in wild-type strains. Under no conditions, however, have we found cofactor activity in the Mu insertion strains of *chlA* and *chlE*.

(ii) *chlB*, *narG*, *narK*, and *narL*. The amount of molybdenum cofactor activity in the cytoplasm of the *chlB*::Mu *cts* strains was similar to that in the wild-type strains under all growth conditions, but the cofactor activity in the membranes of the *chlB* insertion mutants was in-

creased 1.5- to 3-fold above wild-type levels (Table 3). This effect was most marked when the cells were grown aerobically with 1 mM molybdate and was consistently observed under all growth conditions. In only 1 of 10 measurements performed were the cofactor levels in the *chlB* strain membranes not more than 50% greater than the levels in wild-type membranes. The increased cofactor level was found with either heat- or Triton-treated membrane fractions from *chlB* strains.

Insertion mutants at four *nar* loci had molybdenum cofactor activity that was generally similar to the levels found in wild-type cells grown under the same conditions (Table 4). The *narG* and *narI* strains lack one or more of the three nitrate reductase polypeptides, and the *narL* strain has altered regulation of nitrate reductase levels by nitrate (30, 32). The phenotypic alterations in these strains did not significantly affect molybdenum cofactor levels.

(iii) *chlD* and *chlG*. The molybdenum cofactor activity of *chlD*::Mu *cts* and *chlG*::Mu *cts* strains was highly dependent on the amount of molybdenum in the growth and assay media, but the two mutants differed from each other, and from other *chl* and wild-type strains, in their responses to added molybdenum (Table 4).

When cells carrying an insertion at the *chlD* locus were grown in minimal medium without added molybdenum, they had only 15 to 25% of the wild-type activity of both the molybdenum-containing and demolybdo forms of the cofactor. This large reduction in cofactor level compared with wild-type was seen in both the cytoplasm and membranes of the *chlD* strains grown without added molybdate. These results were similar whether the cells were grown under anaerobic or aerobic conditions. The amount of cofactor activity was, however, increased to near wild-type levels when *chlD* strains were grown with 1 mM molybdate. Thus, *chlD* strains had a very low molybdenum cofactor activity unless they were grown with additional molybdate.

The *chlG* strains had a different response to molybdenum than *chlD* strains (Table 4). When *chlG* strains were grown without added molybdate, no molybdenum-containing cofactor activ-

TABLE 3. Effect of growth and assay conditions on molybdenum cofactor activity in *chlB* and *nar* mutants[a]

Strain	Relevant genotype	Growth conditions	Cofactor activity (U/mg of cell protein) of:			
			Cytoplasm		Membrane	
			+Mo[b]	−Mo	+Mo	−Mo
RK5208[c]	*chlB*	+Mo, +O_2	2.7	0.52	0.99	0.28
		−Mo, +O_2	2.0	0.18	0.17	0.05
		+Mo, −O_2	3.8	0.66	0.26	0.13
		−Mo, −O_2	3.3	0.60	0.22	0.20
RK5265[d]	*narG*	+Mo, +O_2	4.3	0.32	0.25	0.06
		−Mo, +O_2	3.7	0.24	0.09	0.03
		+Mo, −O_2	4.4	0.36	0.09	0.03
		−Mo, −O_2	1.1	0.30	0.06	0.03
RK5267[e]	*narI*	+Mo, +O_2	4.8	0.42	0.10	0.03
		−Mo, +O_2	2.0	0.24	0.06	0.02
		+Mo, −O_2	5.9	0.96	0.12	0.02
		−Mo, −O_2	1.4	0.16	0.09	0.03
RK5266[f]	*narK*	+Mo, +O_2	3.8	0.50	0.10	0.03
		−Mo, +O_2	2.9	0.20	0.05	0.02
		+Mo, −O_2	4.5	0.60	0.15	0.03
		−Mo, −O_2	2.4	0.22	0.04	0.02
RK5278	*narL*	+Mo, +O_2	4.1	0.63	0.15	0.03
		−Mo, +O_2	3.0	0.21	0.06	0.04
		+Mo, −O_2	3.4	0.38	0.06	0.02
		−Mo, −O_2	1.6	0.13	0.10	0.03

[a] See footnotes *a* and *b* to Table 2 for growth and fractionation conditions.
[b] Complementation of *N. crassa nit-1* with *E. coli* extract was performed with (+) or without (−) added 10 mM Na_2MoO_4. Final MoO_4 concentration in the assay mix was 2 mM.
[c] Similar results were obtained with RK5227 and RK5251.
[d] Similar results were obtained with RK5272.
[e] Similar results were obtained with RK5274.
[f] Similar results were obtained with RK5270.

TABLE 4. Effect of growth and assay conditions on molybdenum cofactor activity in *chl* mutants[a]

Strain	Relevant genotype	Growth conditions	Cofactor activity (U/mg of cell protein) of:			
			Cytoplasm		Membrane	
			+Mo[b]	−Mo	+Mo	−Mo
RK5202[c]	*chlD*	+Mo, $+O_2$	3.5	0.57	0.08	0.04
		−Mo, $+O_2$	0.56	0.05	0.02	0.0[e]
		+Mo, $-O_2$	4.1	0.75	0.17	0.07
		−Mo, $-O_2$	0.50	0.06	0.05	0.02
RK5206[d]	*chlG*	+Mo, $+O_2$	2.5	0.32	0.08	0.03
		−Mo, $+O_2$	1.4	0.01	0.10	0.0[e]
		+Mo, $-O_2$	3.6	0.35	0.17	0.03
		−Mo, $-O_2$	2.1	0.0[e]	0.15	0.0[e]

[a] See footnotes *a* and *b* to Table 2 for growth and fractionation conditions.
[b] Complementation of *N. crassa nit-1* with *E. coli* extract was performed with (+) or without (−) 10 mM Na_2MoO_4. Final MoO_4 concentration in the assay mix was 2 mM.
[c] Similar results were obtained with RK5207 and RK5209.
[d] Similar results were obtained with RK5231 and RK5256.
[e] Measurements from four experiments gave 0.0 in each case.

ity was detected, but the amount of demolybdo cofactor was near wild-type levels. Both forms of the cofactor were, however, restored to near wild-type levels when *chlG* cells were grown with 1 mM molybdate. Thus, synthesis of the demolybdo cofactor was apparently normal when *chlG* strains were grown in medium with low molybdate, but these strains seemed unable to make the molybdenum-containing form of the cofactor unless molybdate was present at rather high concentration in the growth medium. Thus, the activities of the molybdenum-containing and demolybdo forms of the cofactor in Mu::*cts* *chlD* and *chlG* strains were distinctly different when grown with different amounts of molybdate.

DISCUSSION

Molybdenum cofactor in *chl* insertion mutants. The results presented here clarify the roles of the *E. coli chl* and *nar* loci in production of the molybdenum cofactor by showing that the different *chl* and *nar* insertion mutants had characteristic levels of molybdenum cofactor activity when grown under different conditions. The most striking finding was that the *chlA* and *chlE* strains had no detectable cofactor under any of the growth conditions tested. In contrast, the *chlB* and *nar* strains shared the wild-type pattern of high cofactor activity under all conditions, with the exception that the *chlB* strains had elevated cofactor activity in membranes compared with wild type. The cofactor activity in the *chlD* and *chlG* strains was much more sensitive to molybdate concentration than in the wild type or other *chl* strains. When *chlD* strains were grown in the low molybdenum concentration of unsupplemented medium, activities of both demolybdo and molybdenum-containing forms of the cofactor were reduced to 15 to 20% of the wild-type levels. When the *chlG* strains were similarly grown in low molybdenum, however, activity of the demolybdo cofactor was near wild type but there was essentially no activity in the molybdenum-containing form. Both the *chlD* and *chlG* strains had cofactor activities restored to near wild type by growth in 1 mM molybdate.

The finding that *chlA* and *chlE* insertion mutants did not have cofactor was surprising considering the previous report (1) that other strains with mutations in these loci do have cofactor. Thus, it appears that mutations in the *chlA* or *chlE* locus can lead to two different phenotypes: one in which cofactor activity is completely missing and one in which cofactor is present but defective in producing nitrate reductase activity.

Different possibilities may explain the finding of two phenotypes associated with the *chlA* and *chlE* sites. First, the results may be due to the difference between the Mu *cts* insertion mutants used here and the strains used previously, which arose spontaneously or after chemical mutagenesis (4, 14, 27, 28, 36) and may thus be leaky point mutations. An alternative explanation is that the *chlA* and *chlE* loci each contain two or more coding regions, a possibility supported by genetic (35) and biochemical studies (24). If the *chlA* and *chlE* loci were indeed complex and expressed as operons, then if the coding regions were properly ordered, the highly polar Mu insertions would all be expected to result in the phenotype that lacks cofactor activity. Nonpolar point mutations in such an operon could, however, produce a phenotype with defective cofactor. Fine-structure mapping or sequencing of these genetic regions may be required to distinguish these possibilities.

The only difference in cofactor activities

among the *chlB*, *nar*, and wild-type strains was the elevated cofactor activity in the membranes of *chlB* strains. Different, noninsertion *chlB* and *chlC* strains also have near wild-type cofactor activity (1), although the membrane cofactor activity is near wild type in this previously examined *chlB* strain. There is currently no information available on how the molybdenum cofactor is associated with the membrane or how it is released in a *nit-1* complementing form from the membrane by heat or Triton X-100. Thus, we do not know why membrane cofactor activities are elevated in the *chlB* insertion strains. It is clear, however, that the *chlB* gene product must not be required to partition the cofactor between membrane and cytoplasm.

The *chlD* and *chlG* insertion mutants were the only *chl* mutants in which molybdenum cofactor levels were markedly sensitive to molybdenum levels, but the two types of mutant had different responses to added molybdenum. The *chlG* strains appeared to make the demolybdo form of the cofactor normally, but failed to add molybdenum to the cofactor unless relatively large amounts of molybdenum were available. The *chlG* mutation may, therefore, be in transport or processing of molybdenum. The *chlD* strains, in contrast, appeared to successfully add molybdenum to low levels of the cofactor when grown without added molybdate, although cofactor activity was much reduced compared with wild-type strains. Perhaps the *chlD* strains produce a cofactor that is unstable in low concentrations of molybdenum. The *chlD* and *chlG* insertion strains had near wild-type levels of cofactor activity when grown with 1 mM molybdate, as do previously examined noninsertion mutants (1).

Molybdenum cofactor in wild-type strains. The variations in growth conditions tested here had relatively small effects on the cofactor activity in wild-type cells. Cofactor was synthesized constitutively, and cofactor activities varied two- to threefold when the bacteria were grown aerobically or anaerobically, with or without added 1 mM molybdate. In addition, cofactor activities varied somewhat with the growth phase of the culture (Fig. 1). A similar dependence of cofactor activity on culture phase is found during anaerobic growth of *Proteus mirabilis*, and wild-type cofactor activity in this other member of the *Enterobacteriaceae* is dependent on growth conditions in a manner highly similar to *E. coli* (5). Because the activities of some molybdoenzymes would be expected to vary greatly under the different growth conditions tested here, it appears that the molybdenum cofactor is synthesized relatively independently of some of the enzymes in which it functions.

It is clear from the results that our minimal medium without added molybdenum contains sufficient molybdenum (ca. 1 μM) for wild-type cells to make the active, molybdenum-containing form of the cofactor. At concentrations of molybdate in the growth medium of <1 μM, we would expect to find a larger dependence of cofactor activity on molybdenum concentration in wild-type strains, and this dependence would reflect the capacity of the cell to transport molybdenum and insert it into the cofactor. *E. coli* cells are apparently rather good scavengers of environmental molybdenum. Perhaps the cells have a molybdenum chelating and transport system similar to the siderophore system used for iron transport (21), a possibility suggested by work with other species (18, 22).

***chl* gene products, phenotype, and the cell economy.** The molybdoenzyme-deficient phenotypes associated with different *chl* mutants are often due to different underlying alterations in molybdenum cofactor activity. The *chlA* and *chlE* insertion mutants had no active cofactor; thus, despite the presence of the apoenzyme (32), the mutants had no nitrate reductase activity. The *chlA* and *chlE* insertion strains also lack biotin sulfoxide reductase activity (8), again due to the lack of molybdenum cofactor. It is not clear, however, why noninsertion *chlA* and *chlE* strains, which do have cofactor (1), fail to make active molybdoenzymes.

The *chlB* and *nar* strains apparently made normal molybdenum cofactor, as would be expected for mutations that affect use, but not synthesis, of the cofactor. The *chlB* gene product is an "association factor" (29) responsible for inserting cofactor into the appropriate apoenzyme. The *nar* locus (previously designated *chlC*) consists of at least four phenotypic classes with altered nitrate reductase activity (32). Among these, *narG* and *narI* strains lack one or more of the nitrate reductase polypeptides, and the *narL* strains are defective in regulation by nitrate. Thus, the molybdoenzyme-deficient phenotypes of the *chlB* and *nar* strains are due to impaired utilization of the normal cofactor, or to lack of synthesis or improper regulation of an apoenzyme, and not to defects in the cofactor.

The deficiencies of nitrate reductase (8, 12, 30, 32) and biotin sulfoxide reductase (8) found in *chlD* and *chlG* strains are repaired by growth on relatively high levels of molybdate. The finding that molybdenum cofactor activity is markedly increased by added molybdenum in the *chlD* and *chlG* growth media demonstrates that the repair of the molybdoenzyme-deficient phenotype in these strains by growth in high molybdenum is concomitant with the restoration of the cofactor activity required for molybdoenzyme function. The presence of some molybdenum-containing cofactor when the *chlD* and *chlG* are

grown in low molybdenum media probably explains why these strains retain small amounts of active nitrate reductase during growth in low molybdenum media. Mutants at the *chlD* and *chlG* loci usually (32), but not always (30), make the nitrate reductase apoenzyme when grown in low molybdenum medium. In some *chlD* strains, however, nitrate reductase apoenzyme synthesis is induced by added molybdate (23). The relative contributions of increased cofactor activity and increased apoenzyme synthesis to the restoration of nitrate reductase activity by molybdate in the *chlD* and *chlG* strains remains to be determined.

The results presented here allow us to deduce possible functions and a probable sequence of action of the *chl* gene products in synthesis of the molybdenum cofactor. Thus, the products of the *chlA* and *chlE* loci, both of which are likely to have more than one coding region, probably act early in the synthesis of the basic cofactor structure, because strains carrying insertions in these loci had no cofactor activity and the phenotypes of insertion mutants would be expected to reflect the complete loss of activity of the affected locus. Next in our hypothetical sequence would be the *chlD* gene product(s), which may modify the basic cofactor structure to a form that is stable in the absence of molybdenum. The *chlG* gene product might then act to insert molybdenum into the stable demolybdo form of the cofactor, and the *chlB* association factor would finally insert the molybdenum-containing cofactor into the apoenzymes synthesized by the molybdoenzyme structural genes.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AM30841 from the National Institutes of Health.

We thank Valley Stewart for providing strains.

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