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Identification of the Molybdenum Cofactor in Chlorate-Resistant Mutants of *Escherichia coli*

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Experiments were performed to determine whether defects in molybdenum cofactor metabolism were responsible for the pleiotropic loss of the molybdoenzymes nitrate reductase and formate dehydrogenase in *chl* mutants of *Escherichia coli*. In wild-type *E. coli*, molybdenum cofactor activity was present in both the soluble and membrane-associated fractions when the cells were grown either aerobically or anaerobically, with and without nitrate. Molybdenum cofactor in the soluble fraction decreased when the membrane-bound nitrate reductase and formate dehydrogenase were induced. In the *chl* mutants, molybdenum cofactor activity was found in the soluble fraction of *chlA*, *chlB*, *chlC*, *chlD*, *chlE*, and *chlG*, but only *chlB*, *chlC*, *chlD*, and *chlG* expressed cofactor activity in the membrane fraction. The defect in the *chlA* mutants which prevented incorporation of the soluble cofactor into the membrane also caused the soluble cofactor to be defective in its ability to bind molybdenum. This cofactor was not active in the absence of molybdate, and it required at least threefold more molybdate than did the wild type in the *Neurospora crassa nit-1* complementation assay. However, the cofactor from the *chlA* strain mediated the dimerization of the *nit-1* subunits in the presence and absence of molybdate to yield the 7.9S dimer. Growth of *chlA* mutants in medium with increased molybdate did not repair the defect in the *chlA* cofactor nor restore the molybdoenzyme activities. Thus, molybdenum cofactor was synthesized in all of the *chl* mutants, but additional processing steps may be missing in *chlA* and *chlE* mutants for proper insertion of cofactor in the membrane.

Numerous biochemical and genetic studies have established that the molybdoenzymes nitrate reductase, formate dehydrogenase, xanthine dehydrogenase, aldehyde oxidase, and sulfite oxidase contain a molybdenum cofactor which can be released by acid treatment of these enzymes and complement in vitro the *Neurospora crassa nit-1* nitrate reductase (14, 20, 21). Recently this cofactor was isolated and shown to be a novel pterin (13). This cofactor differs from the cofactor of nitrogenase, which contains acid-labile sulfide and iron as well as molybdenum, and expresses catalytic and physical properties that are different from the molybdenum cofactor of other molybdoenzymes (23, 30).

In *Escherichia coli*, the molybdenum cofactor is present in a readily accessible pool in the soluble fraction (1) and in the membrane fraction as a tightly associated component of nitrate reductase and formate dehydrogenase. These molybdoenzymes, the syntheses of which are induced when *E. coli* is grown anaerobically with nitrate, function to use nitrate as the terminal electron acceptor in place of oxygen (31).

The synthesis of active molybdenum cofactor may be a complex process. In *Aspergillus nidulans*, five *cnx* loci (seven complementation groups) have been implicated in the synthesis of molybdenum cofactor. Defects in these genes result in pleiotropic mutants lacking both nitrate reductase and xanthine dehydrogenase activities (22). Four similar genes (six complementation groups) have been identified in *N. crassa* (32). As yet, the steps involved in the synthesis of the molybdenum cofactor are not known.

E. coli mutants lacking nitrate reductase, *chl* mutants, can be isolated by their ability to grow anaerobically on chlorate, which is lethal to wild-type strains. Seven genetic loci have been identified, *chlA* through *chlG*. Of these, *chlA*, *chlB*, *chlD*, and *chlE* are pleiotropic mutations involving not only the loss of formate-dependent nitrate reductase activity, but also the loss of formate dehydrogenase activity (31). In addition, *chlC* mutants lack nitrate reductase activity and display various amounts of formate de-

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hydrogenase ranging from 5 to 90% of the wild-type activity (3, 10). It was thus of interest to determine whether the *chl* loci could be involved in the synthesis or processing of the molybdenum cofactor in *E. coli*. To this end, wild-type *E. coli* and the *chl* mutants were examined for levels of storage cofactor as well as membrane-bound cofactor under various conditions of growth.

(A preliminary account of these results has appeared previously [N. K. Amy, Fed. Proc. **39**: 391, 1980].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *E. coli* used in this study are described in Table 1. The bacteria were routinely grown in complete medium composed of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 30 g/liter with 1 mM Na_2MoO_4 and 0.1 μM Na_2SeO_3. When indicated, 1% $NaNO_3$ was added. The M9 minimal medium was described previously (1). When required, the following supplements were added at a concentration of 40 mg/l to the minimal medium: adenine, arginine, guanine, histidine, tryptophan, tyrosine, proline, threonine, and leucine; thiamine was added at 5 mg/liter.

For growth studies on molybdenum-free medium, the molybdate was omitted for the minimal medium.

For aerobic growth, the bacteria were grown at 37°C with vigorous shaking on a rotary shaker until the late log phase. For anaerobic growth, the bacteria were grown in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) at 37°C. Cells were harvested by centrifugation, washed twice, and frozen as a pellet at −70°C until used.

Growth of *N. crassa*. *N. crassa* mutant strain *nit-1* (allele *34547*) was obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcatia, Calif. *Neurospora* mycelia were grown in liquid culture on Fries basal medium as described previously (6).

Assays. Crude extracts of *E. coli* and *N. crassa* were prepared as described previously (1).

The *E. coli* extracts were assayed for the presence of molybdenum cofactor by the complementation assay, using the *Neurospora nit-1* mutant. Typically, 5 to 50 μl of *E. coli* extracts, 100 μl of *nit-1* crude extract, and 8 mM Na_2MoO_4 was added to 10 mM phosphate buffer (pH 7.4) with 0.5 mM EDTA to a final volume of 250 μl and incubated for 10 min at room temperature; then samples were removed and assayed for NADPH-nitrate reductase (EC 1.6.6.3) activity as described by Garrett and Nason (7).

Molybdenum cofactor activity in the membrane fraction was determined as follows. The crude extract was centrifuged at 198,000 × *g* for 100 min. The pellet was washed in 50 mM phosphate buffer (pH 7.4) and then centrifuged again. This pellet was suspended at 20 to 50 mg of protein per ml in 0.1 M phosphate buffer (pH 7.4) with 0.5 mM EDTA and 0.1 mM dithiothreitol. A sample (5 to 50 μl) of this fraction was mixed with 200 μl of the resuspension buffer with 50 mM Na_2MoO_4 and heated in a boiling-water bath for 30 s. The sample was immediately centrifuged in an Eppendorf centrifuge (model 3200) for 15 s to pellet the denatured protein. The entire supernatant fluid was mixed with 100 μl of the *nit-1* extract for the complementation assay.

Nitrate reductase (EC 1.7.99.4) in *E. coli* extracts was assayed by the method of MacGregor et al. (19), using reduced methyl viologen as the electron donor and measuring the production of nitrite. Formate dehydrogenase activity was measured by the dichlorophenolindophenol reduction assay described by Lester and DeMoss (15).

Activity units for all other assays are reported as nanomoles of substrate consumed per min. Cofactor activity with the *nit-1* assay is defined as the amount of cofactor which will reconstitute 1 U of nitrate reductase activity per 10 min in the complementation incubation.

Protein concentration was measured by the method of Lowry et al. (16), using bovine serum albumin as the standard.

The gel filtration experiments with Sephadex G-25 were performed by using Pharmacia PD-10 prepoured columns. Each column had a bed volume of 9.1 ml and a bed height of 5 cm and was equilibrated and eluted with 10 mM phosphate buffer (pH 7.4) with 0.1 mM EDTA. Typically, a 2.5-ml sample volume was applied, the excluded volume fraction was 3.5 ml, and the included fraction was 7 ml.

The isokinetic gradients were prepared from 5 and

TABLE 1. *Strains of E. coli used*

Strain	Relevant marker	Source	Proposed gene function	Reference
PK27	Wild type	J. A. DeMoss		9
382	*chlA*	CGSC 4442[a] (J. Puig strain)	Synthesis or processing of Mo cofactor	24, 25
442	*chlB*	CGSC 4443 (F. Casse strain)	Association factor (F_A)	2, 24, 27
426	*chlC*	CGSC 4444 (J. Puig strain)	Structural gene for nitrate reductase	3, 18, 24, 26
C122	*chlD*	CGSC 4458 (W. Venables and J. Guest strain)	Processing of molybdenum	9, 33
C26	*chlE*	CGSC 4459 (J. Guest strain)	Cytochrome b_1 apoprotein	17, 33
JF1130	*chlG*	CGSC 5567 (J. Friesen strain)	Not known	11, 12

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

25.5% sucrose solutions, and the sedimentation coefficients were determined as described previously (1).

RESULTS

Induction of nitrate reductase and formate dehydrogenase. Molybdenum cofactor activity, as measured by the complementation of *N. crassa nit-1* extracts, was present in a soluble pool in aerobically grown *E. coli.* Under these conditions, the membrane-bound nitrate reductase and formate dehydrogenase were not fully induced. To determine whether the synthesis or subcellular distribution of the molybdenum cofactor was regulated by the factors which induce the synthesis of these molybdoenzymes (i.e., nitrate and anaerobic conditions), the molybdenum cofactor activity was assayed in both the soluble and membrane fractions of *E. coli.* Wild-type *E. coli* (PK27) was grown under anaerobic and aerobic conditions, with and without nitrate in minimal and complete media with 1 mM Na_2MoO_4. Extracts prepared from these cells were centrifuged for 100 min at 198,000 × *g*, and the supernatant (soluble fraction) and pellet (particulate fraction) were assayed for nitrate reductase, formate dehydrogenase, and molybdenum cofactor activities (Table 2). Nitrate reductase and formate dehydrogenase activities (in the particulate fraction) were highest in the cells induced under anaerobic conditions with nitrate. Molybdenum cofactor activity was present in both the soluble and particulate fractions of cells grown under all conditions. More molybdenum cofactor activity was found in the soluble fraction of cells grown under aerobic conditions than in cells grown anaerobically or aerobically with nitrate. Although care was taken to find optimal conditions for release of cofactor from the membrane, it is still not certain that all of the membrane-associated cofactor was released and retained activity. Hence, quantitative comparisons between the amounts of soluble and particulate cofactor are impossible.

In cell extracts, the molybdenum cofactor in the membrane fraction was not readily exchangeable with the cofactor in the soluble pool. When crude extracts were assayed and then centrifuged at 198,000 × *g* for 100 min, the molybdenum cofactor activity was recovered in the soluble fraction (Table 2). No molybdenum cofactor activity was detected in the particulate fraction until the latter was subjected to heat treatment (Table 3). Maximum expression of molybdenum cofactor activity occurred when the particulate fraction was heated for 30 s in a boiling-water bath as described above. Cofactor activity was enhanced two to threefold by including 50 mM Na_2MoO_4 with the extract during heat treatment. Cofactor activity was labile after release from the membrane, and 50% of the activity was lost after 5 min of incubation at 22°C. No additional cofactor activity was detected in the soluble fraction after heat treatment.

Effect of molybdate in the growth medium. To determine whether the amount of molybdate in the growth medium influenced the molybdenum cofactor activity, wild-type *E. coli* were grown in medium with 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M Na_2MoO_4. Crude extracts of these cells were assayed with 0, 8, and 50 mM molybdate in the complementation mixture (Table 4). Maximum cofactor activity was detected in cells grown with 10^{-3} molybdate. Less cofactor activity was present in cells grown in lower levels of molybdate, and even 50 mM molybdate added

TABLE 2. *Effect of growth conditions on molybdenum cofactor activity in wild-type E. coli*

Growth conditions[a]	Nitrate reductase[b]	Formate dehydrogenase[c]	Cofactor activity of:					
			Crude extract		Soluble fraction[d]		Particulate fraction[d]	
			U/ml	U/mg of protein	U/ml	U/mg of protein	U/ml	U/mg of protein
Complete medium								
Aerobic	0	30	1,134	91	1,426	176	230	27
Aerobic + NO_3	0	17	545	50	798	112	135	19
Anaerobic + NO_3	2.3	39	tr		tr		345	36
Minimal medium								
Aerobic	0	11	335	55	311	80	48	12
Aerobic + NO_3	0	10	262	50	300	81	41	8
Anaerobic + NO_3	3.7	44	98	20	103	32	65	17

[a] All cells were grown in medium containing 1 mM Na_2MoO_4 and 0.1 μM Na_2SeO_3 and were homogenized in 5 ml of buffer per g of cells.

[b] Micromoles of nitrite formed per milliliter in the crude extract.

[c] Micromoles of dichloroindophenol reduced per minute per optical density unit (660 nm) of cell suspension.

[d] Obtained by centrifuging the crude extract at 198,000 × *g*.

TABLE 3. *Factors affecting the activity of the molybdenum cofactor released from E. coli membranes*

Treatment[a]	Molybdenum cofactor activity[b]
Heat treatment (boiling-water bath) (s)	
0	0
10	3.9
20	4.8
30	4.9
60	3.0
120	0.3
Molybdate present during 30-s heat treatment (mM)	
0	1.8
1	2.8
25	4.3
50	6.1
Acid treatment[c]	0
Incubation of released cofactor at 22°C (min)[d]	
0	8.3
5	3.6
15	2.2

[a] An extract of cells grown aerobically in minimal medium with nitrate was centrifuged at 198,000 × *g*, and the pellet was used as described in the text.

[b] Units per milligrams of protein.

[c] As described previously (14).

[d] The particulate fraction was heated in boiling water for 30 s with 50 mM Na_2MoO_4 and centrifuged, and the supernatant fractions were incubated at 22°C.

to the complementation mixture of these cells did not increase their cofactor activity.

Molybdenum cofactor activity in the *chl* mutants. Extracts of the *chl* mutants of *E. coli* were assayed for cofactor activity to determine whether pleiotropic loss of nitrate reductase and formate dehydrogenase activities was caused by a defect in the molybdenum cofactor metabolism. The *chl* mutants were grown under the same conditions which affected the synthesis of the molybdoenzymes in the wild type, i.e., under aerobic and anaerobic conditions, with and without nitrate. Crude extracts were assayed for molybdenum cofactor activity (Table 5). Molybdenum cofactor activity was present in each of the *chl* mutants under all of the growth conditions tested in complete and minimal media. There was variation in the amount of cofactor detected in the extracts owing to the harvesting of the cells at different cell densities, and to the use of different preparations of *nit-1* mycelia.

The *chl* mutants incorporate the subunits of nitrate reductase and formate dehydrogenase into the membrane to various degrees (5, 11, 17). Thus, we assayed the membranes of the *chl* mutants to determine whether molybdenum cofactor was also incorporated into the membrane. The cultures were grown under anaerobic conditions with nitrate and 1 mM molybdate; then extracts were prepared and centrifuged at 198,000 × *g* for 100 min to yield soluble and particulate fractions. The cofactor activity in the crude extracts of the *chl* mutants was recovered in the soluble fraction after centrifugation (Table 6). When the membrane fractions were heat-treated and assayed, molybdenum cofactor activity was detected only in the *chlB*, *chlC*, *chlD*, and *chlG* mutants. No molybdenum cofactor activity was detected in membranes not subjected to the heat treatment. As was seen with the membrane fraction of the wild type, the presence of molybdate during the heat treatment enhanced the amount of cofactor activity recovered but was not essential for the detection of activity.

Sephadex G-25 gel filtration. Recent studies have shown that in *chlA* strains, the subunits of nitrate reductase are incorporated into the membrane (8). Thus, it was of interest to determine why molybdenum cofactor was present in the soluble extract of the *chlA* strain but was not incorporated into the membrane with the nitrate reductase subunits during anaerobic growth with nitrate. One explanation could be that cofactor from the *chlA* strain lacked the carrier molecule that was seen with the wild type (1).

When an extract of wild-type *E. coli* was passed through a Sephadex G-25 column, 75% of the cofactor activity eluted in the excluded volume of the column associated with a high-molecular-weight carrier molecule, and 25% of the cofactor activity was in the included volume, eluting with substances with molecular weights of less than 1,000, as seen previously (1). These

TABLE 4. *Effect of molybdate in the growth medium on molybdenum cofactor activity*

Growth conditions[a] (MoO_4^{2-})	Molybdenum cofactor activity[b]		
	0 mM MoO_4^{2-}	8 mM MoO_4^{2-}	50 mM MoO_4^{2-}
1 μM	2	11	10
10 μM	2.7	12	10.5
100 μM	4.3	17	14.3
1 mM	7.6	19.6	15.8

[a] *E. coli* PK27 was grown aerobically on minimal medium with the various amounts of sodium molybdate added.

[b] Units per milligram of protein. Molybdate was added to the complementation mixture.

TABLE 5. *Molybdenum cofactor activity in the chl mutants*

Growth condition[a]	Molybdenum cofactor activity in:[b]						
	PK27	*chlA*	*chlB*	*chlC*	*chlD*	*chlE*	*chlG*
Aerobic	76 ± 8	27 ± 7	26 ± 7	15 ± 4	36 ± 1	12 ± 2	22 ± 2
Aerobic + NO_3	41 ± 10	27 ± 7	27 ± 9	18 ± 4	38 ± 2	22 ± 5	23 ± 3
Anaerobic	34 ± 3	22 ± 8	31 ± 8	28 ± 7	39 ± 8	7 ± 2	26 ± 4
Anaerobic + NO_3	2 ± 2	21 ± 9	18 ± 4	21 ± 9	35 ± 10	11 ± 3	35 ± 2

[a] The cells were grown in complete medium with 1 mM Na_2MoO_4 and 0.1 μM Na_2SeO_3.

[b] Units per milligram of protein in crude homogenates (± standard error). Data are from at least four different experiments for each growth condition.

TABLE 6. *Subcellular distribution of cofactor activity in the chl mutants of E. coli*

Strain[a]	Molybdenum cofactor activity[b]	
	Soluble fraction	Particulate fraction
PK27	7.6	6.2
chlA	24	0
chlB	4.7	0.9
chlC	6.4	2.8
chlD	6.6	2.8
chlE	8.9	0
chlG	23	0.4

[a] Cells were grown in complete medium with 1% $NaNO_3$ and 1 mM Na_2MoO_4 under anaerobic conditions.

[b] Units per milligram of protein in supernatant fluid and pellet from crude extract centrifuged at 198,000 × *g*.

fractions displayed molybdenum cofactor activity when assayed in the presence and absence of molybdate in the complementation mixture, although there was less activity in the absence of molybdate (Table 7). When the *chlA* extract was fractionated on the same Sephadex G-25 column, molybdenum cofactor activity eluted in both the excluded and the included fractions, indicating that *chlA* cofactor, like wild-type cofactor, was apparently associated with a carrier molecule. In contrast to the wild type, the fractions from the chromatography of the *chlA* extract displayed cofactor activity only when molybdate was included in the complementation mixture. The molybdenum cofactor activity in the crude extract of the *chlA* strain before desalting on Sephadex G-25 could have been caused by free molybdate in the extract which was subsequently separated from the excluded fraction. Thus, unlike the wild-type cofactor, the molybdenum cofactor in *chlA* extracts requires molybdate in the complementation mixture for expression of activity. This result may be interpreted to mean that cofactor from the *chlA* mutant can bind molybdenum only when the level of molybdate is high; when the sample was desalted to remove free molybdate, the cofactor was not active. To test this hypothesis, *chlA* extracts were incubated with 10 mM Na_2MoO_4 for 2 h, desalted on the Sephadex G-25 to remove the unbound molybdate from the cofactor, and assayed with and without molybdate in the complementation mixture (Table 8). Again, cofactor in extracts of wild-type *E. coli* displayed cofactor activity when assayed in the absence of molybdate, but there was negligible cofactor activity in the *chlA* extract when molybdate was not included in the complementation. Thus, molybdate did not remain associated with cofactor in *chlA* extracts.

Molybdenum saturation of *chlA* extracts. Wild-type *E. coli* cells grown in molybdenum-free medium synthesize cofactor which lacks molybdenum. When extracts of these cells are incubated with molybdate, cofactor activity is restored (1). To determine whether cofactor in *chlA* extracts was less able to bind molybdenum than the wild-type cofactor, the kinetics of activation of empty cofactor by molybdate of the wild-type and *chlA* strains were compared at various molybdate concentrations (Fig. 1). When cultures of the wild-type and *chlA* strains were grown in minimal medium lacking molybdenum and extracts of these cells were assayed without molybdate in the complementation mixture, no molybdenum cofactor activity was detected in

TABLE 7. *Effect of molybdate on molybdenum cofactor activity in wild-type and chlA extracts after Sephadex G-25 gel filtration.*

Sample[a]	Presence of MoO_4^{2-} during complementation[b]	Total cofactor units		
		Applied	Excluded	Included
PK27	+	1,181	895	337
	−	663	468	52
chlA	+	808	697	84
	−	129	0	0

[a] Crude extracts of *E. coli* grown aerobically with 1 mM Na_2MoO_4 on complete medium were used.

[b] With or without 10 mM Na_2MoO_4 included in the complementation assay. +, Present, −, absent.

TABLE 8. *Incubation of wild-type and chlA extracts with molybdate before Sephadex G-25 gel filtration.*

Incubation temp[a]	Cofactor activity[b]	
	+ MoO_4^{2-}	− MoO_4^{2-c}
PK27		
Control (no incubation)	673	191
4°C	580	81
22°C	581	113
chlA		
Control (no incubation)	196	3
4°C	187	4
22°C	218	2

[a] Crude extracts of aerobically grown *E. coli* were incubated with 10 mM Na_2MoO_4 for 2 h at 4 or 22°C before Sephadex G-25 gel filtration. Na_2MoO_4 (10 mM) was added to the control samples immediately before gel filtration.

[b] Units of cofactor activity per milliliter in the excluded fraction after Sephadex G-25 gel filtration.

[c] With or without 10 mM Na_2MoO_4 added to the complementation assay.

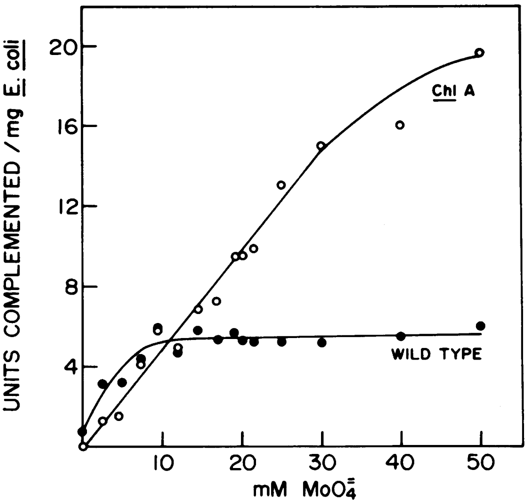

FIG. 1. *Effect of molybdate concentration in the complementation assay of the wild type (●) and chlA mutant (○). Extracts prepared from cells grown in molybdenum-free minimal medium were incubated with the nit-1 extract and the indicated concentration of molybdate for 10 min in the complementation buffer and then assayed for activity.*

chlA extracts, and only a trace of activity was seen in wild-type extracts. Increasing concentrations of molybdate added to the complementation mixture restored molybdenum cofactor activity in both the wild-type and *chlA* strains. However, at least 30 mM molybdate was necessary for maximum activity in the *chlA* strain, whereas only 10 mM molybdate was required for cofactor from the wild type. Identical results were seen with different amounts of cofactor and with either Na_2MoO_4 or K_2MoO_4 in the complementation buffer.

Growth of the *chlA* strain in high molybdate. The nitrate reductase activity of the *chlD* strain can be restored by growth of the cells in medium with 1 mM molybdate (9). To determine whether the defect in molybdenum binding in the *chlA* strain, too, could be repaired in vivo with high levels of molybdate in the growth medium, *chlA* was grown in medium containing 1, 10, or 100 mM Na_2MoO_4 and then assayed for nitrate reductase and molybdenum cofactor activity. Nitrate reductase activity was not restored by high molybdate levels in the growth medium. When molybdate was included in the complementation mixture, the same amount of cofactor activity was detected in each sample. When these samples were desalted on Sephadex G-25 and the cofactor in the excluded volume was assayed, cofactor activity was detected only when molybdate was included in the complementation. These results indicate that growth of the *chlA* strain with high concentrations of molybdate did not repair the defect in the cofactor in binding molybdenum.

Sucrose gradient centrifugation. In wild-type *E. coli*, molybdenum-free cofactor can mediate the dimerization of the subunits of *nit-1* (1). This dimerization was detected when the cofactor from *E. coli* grown in molybdenum-free medium was complemented with *nit-1* and centrifuged on a sucrose gradient. A peak of NADPH-cytochrome *c* reductase activity was generated which sedimented at 7.9S, which represented the nitrate reductase dimer. NADPH-nitrate reductase activity was restored when these fractions were incubated with molybdate. I was interested in determining whether the defect in the *chlA* strain which affects the association of the molybdenum with the cofactor would affect the ability of the *chlA* cofactor to cause the dimerization of the *nit-1* subunits in the absence of molybdate. To test this, the *nit-1* extract was complemented with the *chlA* extract, both with and without molybdate in the complementation, and then centrifuged in isokinetic sucrose gradients. The fractions were assayed for restoration of the *nit-1* NADPH-nitrate reductase activity, and the sedimentation coefficient of this enzyme was determined. In the gradient of *nit-1* complemented with *chlA* plus molybdate, a peak of NADPH-nitrate reductase activity sedimenting at 7.9S was generated (Fig. 2). In the gradient of *nit-1* complemented with *chlA* in the absence of molybdate, no NADPH-nitrate reductase activity was detected. However, when these fractions were incubated with 1 mM molybdate for 5 min, activity was generated in the fractions which sedimented at 7.9S. These results indicate that the defect in

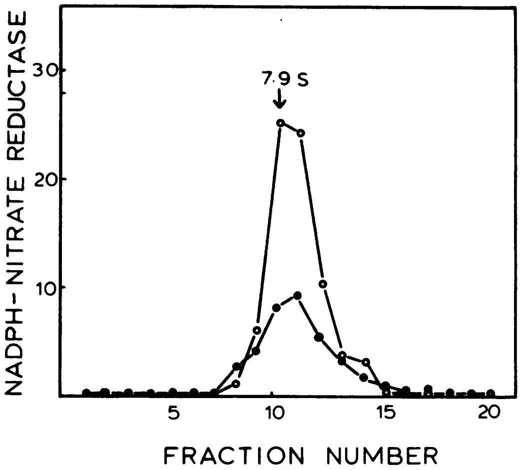

FIG. 2. *Sucrose density gradient centrifugation of nit-1 complemented with* (○) *chlA extract plus molybdate in the complementation or* (●) *chlA extract without molybdate in the complementation. Activity was detected only after the fractions from this gradient had been incubated for 10 min with 1 mM sodium molybdate and then assayed for NADPH-nitrate reductase.*

the cofactor in the *chlA* strain was in the molybdenum-binding capacity rather than in the structural elements essential for the cofactor binding to the *nit-1* protein.

DISCUSSION

Active molybdenum cofactor was present in wild-type *E. coli* grown aerobically with and without nitrate. Molybdenum cofactor activity was found in both the 198,000 × *g* supernatant and the pellet of cells grown under these conditions. The cofactor in the soluble fraction could be assayed directly, but the membrane fraction required heat treatment to release cofactor activity. Thus, molybdenum cofactor was present in both soluble and membrane-associated pools even when the molybdoenzymes nitrate reductase and formate dehydrogenase were not induced; yet the function of these pools is not known. Dubourdieu et al. (4) observed molybdenum-binding fractions of low molecular weight in the soluble and membrane fractions and suggest that they function as molybdenum storage forms. Alternatively, the pool of membrane-associated cofactor which is found when nitrate reductase and formate dehydrogenase are not induced may be a component of another, as yet unidentified, molybdoenzyme.

E. coli cells grown in molybdenum-free medium synthesize cofactor (1), but increased levels of cofactor activity were found when the cells were grown in medium with levels of molybdate up to 1 mM. Further experiments are needed to determine whether molybdate stimulated additional synthesis of cofactor or stabilized active cofactor in vivo or during cell disruption. However, the presence of molybdate in the homogenization buffer had no effect on the stability or recovery of cofactor activity (N. Amy, unpublished data).

Active molybdenum cofactor was found in the soluble fraction of the *chlA*, *chlB*, *chlC*, *chlD*, *chlE*, and *chlG* mutants, but in the membrane fraction of only the *chlB*, *chlC*, *chlD*, and *chlG* mutants. Thus, even though all of these *chl* mutants synthesized soluble molybdenum cofactor, defects in the *chlA* and *chlE* mutants prevented incorporation of active cofactor into the membrane. The *chl* mutants synthesize the subunits of nitrate reductase and formate dehydrogenase to various degrees (5, 8, 11, 17), and, as in the wild type, the synthesis of these subunits is induced when cultures are grown with nitrate under anaerobic conditions and repressed by oxygen (5).

The absence of membrane-associated molybdenum cofactor in the *chlA* mutant is puzzling. This mutant synthesizes the subunits for nitrate reductase, the F_A association factor (27), and soluble molybdenum cofactor. The experiments reported in this paper indicated that the *chlA* mutant exhibits a defect in the incorporation and binding of molybdenum into cofactor. Cofactor from *chlA* was unable to retain bound molybdenum during gel filtration, and it required more molybdate in the complementation mixture than did the wild type for maximum activity. Cofactor from the *chlA* mutant caused the dimerization of the nitrate reductase subunits in the *nit-1* strain even in the absence of molybdate, and molybdate was readily incorporated into the inactive enzyme as seen previously with wild-type molybdenum-free *E. coli* (1); however, the defect in the *chlA* strain was not repaired by growth in medium with a high molybdate concentration.

Thus, the *chlA* strain synthesizes cofactor, and I postulate that *chlA* lacks an additional process to insert molybdenum into cofactor in an active configuration. The experiments of Scott et al. (29) lend support to this hypothesis. They demonstrated that molybdate can activate nitrate reductase from tungsten-grown wild-type cells, even in the presence of chloramphenicol, in whole cells but not in cell extracts. Molybdenum was incorporated into the nitrate reductase in these cell extracts, but the protein was not active. They concluded that an additional factor or step was required for active enzyme formation that occurred in vivo but did not occur in cell extracts.

Active cofactor with bound molybdate may be essential for the processing of the subunits of nitrate reductase. Giordano et al. (8) have shown that the β and γ subunits of nitrate reductase are missing and that the α subunit is greatly diminished in the membranes of the *chlA* strain. However, two additional peptides, α' and β', were identified by the antiserum directed against nitrate reductase; these peptides disappear after reconstitution of *E. coli* nitrate reductase with *chlB* extract. The authors postulate that α' and β' accumulate in membranes in the absence of the *chlA* gene product. Thus, cofactor with bound molybdenum may be required.

The experiments of Scott and DeMoss (28) also suggest that an active molybdenum-containing cofactor may be essential for the processing of the subunits of nitrate reductase from wild-type *E. coli*. Cultures grown in the absence of molybdate, with tungstate, produced an altered form of nitrate reductase detected electrophoretically which was not active. Activation of this enzyme in vivo with molybdate resulted in an enzyme with the original electrophoretic properties.

It is not possible to speculate on the defect in the *chlE* strain in terms of molybdenum cofactor metabolism because different alleles of *chlE* display widely different phenotypes in that some alleles possess the subunits for nitrate reductase in the membrane, whereas other alleles have none. The *chlE* allele (*C26*) used in this study was shown to lack formate dehydrogenase activity, cross-reacting material, and nitrate reductase activity, but it contained 132% of the wild-type level of cross-reacting material to the nitrate reductase antiserum (11). Thus, even though the nitrate reductase protein was synthesized in the *chlE* mutant, no cofactor was incorporated, but other alleles must be tested before a generalization is possible.

In conclusion, the pleiotropic loss of nitrate reductase in the *chl* mutants is not due to the lack of synthesis of molybdenum cofactor, but rather may reflect more subtle defects in the processing and regulation of molybdenum metabolism.

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