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Characterization of Molybdenum Cofactor from *Escherichia coli*

NANCY KLEIN AMY* AND K. V. RAJAGOPALAN

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Molybdenum cofactor activity was found in the soluble fraction of cell-free extracts of *Escherichia coli* grown aerobically in media supplemented with molybdate. Cofactor was detected by its ability to complement the nitrate reductase-deficient mutant of *Neurospora crassa*, *nit-1*, resulting in the in vitro formation of nitrate reductase activity. Acid treatment of *E. coli* extracts was not required for release of cofactor activity. Cofactor was able to diffuse through a membrane of nominal 2,000-molecular-weight cutoff and was insensitive to trypsin. The cofactor was associated with a carrier molecule (approximately 40,000 daltons) during gel filtration and sucrose gradient centrifugation, but was easily removed from the carrier by dialysis. The carrier molecule protected the cofactor from inactivation by heat or oxygen. *E. coli* grown in molybdenum-free media, without and with tungsten, synthesized a metal-free "empty" cofactor and its tungsten analog, respectively, both of which were subsequently activated by the addition of molybdate. Empty and tungsten-containing cofactor complemented the nitrate reductase subunits in the *nit-1* extract, forming inactive, but intact, 7.9S nitrate reductase. Addition of molybdate to the enzyme complemented in this manner restored nitrate reductase activity.

Molybdoproteins from diverse phylogenetic sources contain an identical structural and functional component, a molybdenum cofactor, which binds the metal in a catalytically active configuration. Much genetic (1, 20) and biochemical evidence indicates that the cofactor is a component of all molybdoenzymes, with the single exception of nitrogenase. This enzyme possesses a cofactor containing iron, as well as molybdenum, i.e., FeMoco (21, 22).

Even though progress has been made on the isolation and structural characterization of the molybdenum cofactor (21; B. E. Hainline, J. L. Johnson, and K. V. Rajagopalan, Fed. Proc. **38:** 314, 1979), little is known about the biosynthetic events preceding its insertion into the apoprotein. It is known, however, that a number of genes regulate cofactor synthesis; for example, five *cnx* genes in *Aspergillus nidulans* (20) and five similar genes in *Neurospora crassa* (A. B. Tomsett and R. H. Garrett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, H62, p. 129) participate in the synthesis of molybdenum cofactor.

The most widely used system for assaying the molybdenum cofactor is the complementation of the *N. crassa* mutant *nit-1* (7, 10, 11, 17, 18). This mutant lacks cofactor, but retains the structural gene for nitrate reductase and synthesizes a protein with NADPH-cytochrome *c* reductase activity. This protein sediments on sucrose gradients at 4.5S (23), in contrast to the wild-type nitrate reductase from *N. crassa* which sediments at 7.9S and is composed of two similar subunits (2, 19). When a cell-free extract of *nit-1* is mixed with a source of molybdenum cofactor, the *nit-1* protein and the cofactor associate into the 7.9S species which expresses both NADPH-nitrate reductase and NADPH cytochrome *c* reductase activities (7, 18). The addition of molybdate to the complementation mixture stimulates the nitrate reductase activity (11). The nitrate reductase so formed is identical to native enzyme in molecular weight, sedimentation coefficient, and substrate affinities (7, 18).

Once incorporated into a molybdoenzyme, the molybdenum cofactor forms a stable association with the protein. Methods of effecting its release from various molybdoenzymes include treatment of the molybdoprotein with acid (7), organic solvents (21), or sodium dodecyl sulfate (B. E. Hainline, J. L. Johnson, and K. V. Rajagopalan, Fed. Proc. **38:**313, 1979). Most studies on the cofactor have utilized the material released from molybdoproteins. However, a few reports have dealt with molybdenum cofactor found in the free form in cell extracts from various sources. Nason et al. (17) initially used crude extracts of *Neurospora* lacking nitrate

reductase. Ketchum and co-workers (8, 9) detected cofactor in extracts from a number of different bacteria and found that it was a small molecule, since it could diffuse across a dialysis membrane. Such extracts did not require prior acid treatment to release the cofactor. Johnson et al. (6) used a cell-free extract of *Escherichia coli* to complement apo-sulfite oxidase of rat liver.

In this paper, we report on the state of the molybdenum cofactor in *E. coli*. In this work, cell-free extracts were used as the source of the cofactor for complementation of the nitrate reductase subunits in *nit-1* extracts of *N. crassa*. These studies have revealed the presence of free cofactor in extracts of *E. coli*, which appears to be loosely associated with a large-molecular-weight carrier molecule from which it is easily removed by dialysis. Cells grown on molybdenum-deficient media synthesized a cofactor lacking molybdenum, i.e., empty cofactor. Inclusion of tungsten in the molybdenum-deficient media led to the accumulation of tungsten-containing cofactor. The empty cofactor or the tungsten-containing cofactor analog could complement with *nit-1* to form an intact, but inactive, nitrate reductase. The activity of this enzyme could subsequently be restored by the addition of molybdate.

A preliminary account of these results has appeared (N. K. Amy and K. V. Rajagopalan, 1979, Abstr. Int. Congr. Biochem. 03-1-S152).

MATERIALS AND METHODS

Growth of *E. coli*. The *E. coli* culture (ATCC 9723e) was routinely grown under aerobic conditions in media composed of 30 g of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) per liter, 5 g of yeast extract per liter, and 1 mM sodium molybdate. The minimal medium used for several experiments contained (g/liter): $Na_2HPO_4 \cdot 7H_2O$, 11.3; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1.0; glycine or serine, 0.04; and $Na_2MoO_4 \cdot 2H_2O$, 0.24. The following components of the minimal medium were autoclaved separately and added aseptically (per liter): $CaCl_2$, 11 mg; $MgSO_4 \cdot 7H_2O$, 0.24 g; and glucose, 2 g. For growth studies on molybdenum-free medium, the molybdate was omitted from minimal medium. Tungsten-containing medium was prepared by adding 1 mM Na_2WO_4 to molybdenum-free minimal medium. Cultures were maintained on agar slants.

Cells were grown at 37°C with vigorous shaking on a rotary shaker until late log phase; they were then harvested, washed twice with 0.25 M sucrose, and frozen as a pellet at −70°C until used.

Growth of *Neurospora*. *N. crassa* wild type (STA-4) and mutant strain *nit-1* (allele *34547*) were obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, Calif. *Neurospora* mycelia were grown in liquid culture on Fries basal medium essentially as described previously (2). The *nit-1* mycelia were grown for 40 h in medium containing 80 mM ammonium chloride, washed with distilled water, and then transferred to fresh medium containing 20 mM sodium nitrate as the sole nitrogen source for 4 h. The mycelia were collected by filtration, washed, pressed to dryness, frozen in liquid nitrogen, and stored at −70°C until used.

Preparation of extracts. The *E. coli* cell pellet was suspended in 10 mM phosphate buffer (pH 7.4) with 0.5 mM EDTA; 10 ml of buffer was used per g of cell pellet. Cells were disrupted by passage through a French pressure cell at 20,000 lb/in^2; then the extract was centrifuged at 12,000 × *g* for 10 min and the supernatant was used.

Crude extracts of *Neurospora* were prepared by homogenizing mycelia in 2 volumes of cold 0.1 M phosphate buffer (pH 7.4) with 0.5 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride in a cold Duall tissue grinder. The homogenate was centrifuged for 15 min at 27,000 × *g*, and the supernatant, designated the crude extract, was used.

Assays. *E. coli* extracts were assayed for the presence of molybdenum cofactor by the complementation assay using the *Neurospora* mutant *nit-1*. Typically, 5 to 50 μl of *E. coli* extract and 100 μl of *nit-1* crude extract were added to 10 mM phosphate buffer (pH 7.4) with 0.5 mM EDTA and 20 mM $NaMoO_4$ in a final volume of 250 μl. This mixture was incubated for 10 min at room temperature; then samples were removed and assayed for NADPH-nitrate reductase activity.

The NADPH-nitrate reductase activity (EC 1.6.6.3) of *Neurospora* extracts was assayed as described by Garrett and Nason (4) by measuring the production of nitrite by the diazo-coupling colorimetric assay. A solution of freshly prepared Na_2SO_3 was added to this assay mixture, to a final concentration of 5 mM, to inhibit nitrite reductase activity in crude extracts (26). The NADPH-cytochrome *c* reductase activity was assayed as described previously (4). Kinetic data were obtained with a Gilford model 2000 recording spectrophotometer. The nitrate reductase activity (EC 1.7.99.4) in *E. coli* extracts was assayed by the method of MacGregor et al. (16), using reduced methyl viologen as the electron donor and measuring the production of nitrite.

Activity units for all assays are reported as nanomoles of substrate transformed per minute. Cofactor activity unit is defined as the amount of cofactor that will reconstitute 1 U of nitrate reductase activity per 10 min of complementation incubation. Protein concentration was measured by the Coomassie brilliant blue G-250 BioRad protein assay, using gamma globulin as the standard.

Subcellular fractionation. For the subcellular localization experiments, the *E. coli* cells were disrupted with a French pressure cell, and the extract was clarified by centrifugation at 5,000 × *g* for 10 min (crude homogenate). Samples were centrifuged at 198,000 × *g* for 2 h. The resulting pellet was washed with homogenization buffer; then both the 198,000 × *g* supernatant and the resuspended pellet were centrifuged at 198,000 × *g* for an additional 1 h. The pellet was resuspended in homogenization buffer to the original volume, and all samples were assayed for cofactor

activity and for reduced methyl viologen-nitrate reductase activity.

Dialysis experiments. The dialysis experiments were performed with dialysis tubing (flat width, 1 cm) which had been boiled in an EDTA solution and then washed. The dialysis sac containing the *nit-1* extract was placed in a test tube containing *E. coli* extract and incubated at room temperature with stirring. Samples were removed from inside the sac at intervals and assayed for NADPH-nitrate reductase activity.

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

Sucrose density gradient centrifugation. Isokinetic sucrose gradients were prepared, and the sedimentation coefficients were calculated as described by McCarty et al. (12, 13). The gradients were prepared from 5 and 25.5% sucrose solutions in 0.1 M phosphate buffer (pH 7.4) with 0.1 mM EDTA and 1 mM dithiothreitol. The gradients were prepared and maintained at 4°C and were allowed to equilibrate for several hours before use. Samples of 100 to 300 μl were layered onto the gradients, which were centrifuged in a Beckman SW41 swinging-bucket rotor for 22 h at 41,000 rpm at 4°C. The gradients were fractionated by puncturing the bottom of the tubes and collecting the fractions by gravity. Catalase, nitrate reductase from wild-type *Neurospora*, and the NADPH-cytochrome *c* reductase from *nit-1* extracts were used as standards for the calculation of sedimentation coefficients.

Tungsten-185 incorporation studies. The experiments for the incorporation of ^{185}W into nitrate reductase formed by the complementation of $^{185}WO_4$-grown *E. coli* and *nit-1* were performed as follows. The *E. coli* cultures were grown in 100 ml of molybdenum-free media to which 1 mCi of $^{185}WO_4$ (specific activity, 11.8 mCi/mg) was added. The cells were grown for 17 h at 37°C; they were then harvested and lysed as usual. A 6-ml amount of the *E. coli* extract was applied to Sephadex G-25 columns to remove unbound isotope, and the excluded fractions were used. Complementation of these fractions with 10 ml of *nit-1* extract was allowed to proceed for 30 min at room temperature. No molybdate was added to the complementation mixture.

The NADPH-nitrate reductase formed by the complementation of the *nit-1* with the ^{185}W-containing *E. coli* extract was partially purified and concentrated by precipitation with 33 to 50% ammonium sulfate. The resulting pellet was desalted on Sephadex G-25, applied to six identical sucrose gradients, and then centrifuged at 41,000 rpm for 22 h. The fractions containing the ^{185}W-nitrate reductase were pooled from each of the six gradients, concentrated by precipitation with 50% ammonium sulfate, and then desalted on Sephadex G-25. This sample was subjected to a subsequent sucrose gradient centrifugation and assayed. Samples of 0.1 to 0.25 ml from each fraction of the sucrose gradient were added to 5 ml of Aquasol-2 (New England Nuclear Corp.) and counted in a refrigerated Intertechnique SL-30 liquid scintillation counter.

Analysis of molybdenum. Samples were assayed for molybdenum on a Perkin-Elmer model 107 atomic absorption spectrophotometer fitted with a graphite atomizer (HGA model 2000).

Materials. NADPH, cytochrome *c*, methyl viologen, trypsin (type III), and chloramphenicol were purchased from Sigma Chemical Co. Pepsin and chymotrypsin were from Worthington Biochemicals Corp. Thermolysin was from Calbiochem. Sucrose was analytical reagent grade from Mallinckrodt. The dye reagents for the protein assay were from Bio-Rad Laboratories. All other chemicals were reagent grade. The radioactive ^{185}W was purchased from New England Nuclear as sodium orthotungstate in 0.94 N sodium carbonate with 99% radionuclidic purity. The 2,000-molecular-weight cutoff dialysis tubing was Spectrapor membrane tubing no. 6 from Spectrum Medical Industries.

RESULTS

Characterization of *E. coli* cofactor activity. To optimize the complementation of *E. coli* extracts with *nit-1*, we examined the reaction conditions. As can be seen in Fig. 1, the complementation activity increased linearly with in-

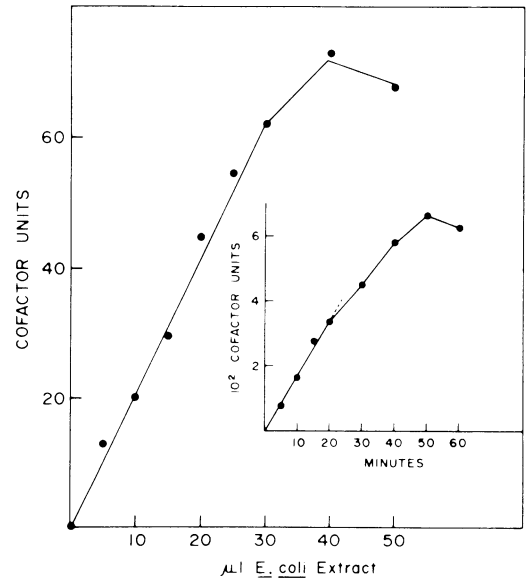

FIG. 1. *Effect of increasing amounts of E. coli extract on complementation activity, using a 10-min complementation incubation. Inset: Increase in complementation activity with time of incubation. A 60-μl amount of E. coli extract (6.3 mg/ml) was incubated with 300 μl of nit-1 extract plus 390 μl of 10 mM potassium phosphate buffer, pH 7.4, with 0.1 mM EDTA and 20 mM sodium molybdate. Samples were removed at intervals and assayed for NADPH-nitrate reductase activity.*

creasing amounts of *E. coli* extract added to 50 μl of *nit-1*, until a saturation point was reached. All subsequent assays were carried out with an excess of the *nit-1* extract so that a quantitative determination of the amount of cofactor in the *E. coli* extract could be made. Complementation of *nit-1* with *E. coli* extracts increased linearly with time of incubation at room temperature for at least 20 min (Fig. 1, inset). Complementation was inhibited by high concentrations of phosphate. No complementation activity was detected when either *nit-1* or *E. coli* extract was excluded from the complementation mixture.

Complementation activity was observed when *E. coli* extracts were incubated with *nit-1* without additional molybdate in the complementation mixture, but activity was stimulated severalfold by the addition of molybdate (Fig. 2). No inhibition of activity was detected when 5 mM sodium tungstate was added in the presence of 1 mM molybdate to the complementation mixture.

The optimal temperature of the complementation assay was 23 to 30°C, with greatly decreased activity at 0°C (Table 1). Lack of complementation at 37°C reflects the heat lability of the *nit-1* extract. The cofactor activity in crude extracts of *E. coli* was stable at 37°C for 30 min.

No stimulation of complementing activity was observed when 80 to 400 μM NADPH was added to the complementation mixture. The addition of 4 μM flavin adenine dinucleotide (FAD), either with the NADPH or alone, had no effect.

To rule out the possibility that the *nit-1* homogenate was supplying an additional unidentified low-molecular-weight component which could stimulate complementation with *E. coli*, the *nit-1* extract was precipitated by 35 to 50% ammonium sulfate fractionation, chromatographed on Sephadex G-50, and then assayed. The *nit-1* extract was also fractionated by sucrose density centrifugation, and the 4.5S peak of nitrate reductase monomer was isolated and assayed. In both cases, the *E. coli* extract was still able to complement with the *nit-1*.

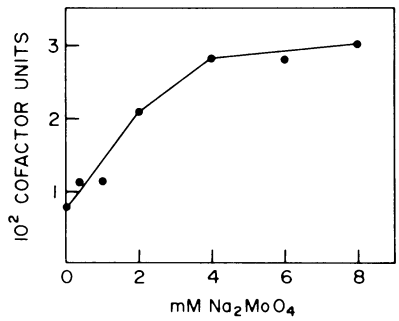

FIG. 2. *Effect of molybdenum concentration in the complementation assay. The E. coli extract was incubated for 10 min with nit-1 extract and the indicated amounts of sodium molybdate.*

TABLE 1. *Effect of temperature on complementation*

Temp (°C)[a]	Cofactor units/ml
0	50
23	217
30	223
37	0

[a] The *E. coli* extract was incubated with the *nit-1* for 10 min at the indicated temperature; then samples were removed and assayed for NADPH-nitrate reductase activity at room temperature.

Based on the above findings, complementation assays with *nit-1* and *E. coli* extracts were routinely performed by mixing 5 to 20 μl of *E. coli* extract with 100 μl of *nit-1* extract for 10 min at room temperature with 8 mM sodium molybdate added to the reaction mixture. Samples of 20 to 50 μl were removed and assayed for NADPH-nitrate reductase activity.

To estimate the efficiency of the complementation of *E. coli* cofactor with *nit-1*, we mixed excess *E. coli* extract with *nit-1* extract and allowed the complementation to approach completion. Under these conditions, the specific activity of the reconstituted nitrate reductase was 13 nmol of NO_2^- formed/min per mg of *nit-1* protein. This value compares favorably with the specific activity of wild-type *Neurospora* nitrate reductase, which is 15 nmol of NO_2^- formed/min per mg of protein in crude extracts (2).

Subcellular localization of cofactor. The nitrate reductase in *E. coli* is a membrane-bound molybdoenzyme which functions under anaerobic conditions to use nitrate as the terminal electron acceptor in place of oxygen (24). To determine whether the *E. coli* nitrate reductase was the source of the molybdenum cofactor activity in the extracts, we performed a subcellular fractionation. The extract was centrifuged at 198,000 × *g* for 2 h to pellet the membrane fraction. The resulting supernatant and pellet were each assayed for nitrate reductase activity and for cofactor activity (Table 2). All of the cofactor activity that was initially found in the crude homogenate before centrifugation was removed in the 198,000 × *g* supernatant fraction. No cofactor activity was found in the washed pellet fraction, whereas 91% of the *E. coli* nitrate reductase activity was found in the pellet fraction. The *E. coli* nitrate reductase activity in extracts does not interfere with the assay for the NADPH-nitrate reductase which is the basis for the complementation since the former is unable to use NADPH as an electron donor.

TABLE 2. *Subcellular fractionation of E. coli extract*

Fraction	Cofactor units/ml	MVH-NR/ml[a]
Crude homogenate	234	451
198,000 × *g* supernatant	262	36
198,000 × *g* pellet[b]	0	394

[a] Reduced methyl viologen-nitrate reductase (MVH-NR) activity.

[b] The 198,000 × *g* pellet was resuspended in buffer to the original volume of crude homogenate.

When the crude homogenate and the 198,000 × *g* supernatant and pellet were each acid-treated by the method used to release cofactor from molybdoproteins (7), cofactor activity decreased in the crude homogenate and 198,000 × *g* supernatant. A small, but variable, amount of cofactor activity was observed in the acid-treated pellet.

When the 198,000 × *g* supernatant fraction was heated at 30°C for 2 h and then centrifuged at 198,000 × g for an additional 2 h, all of the *E. coli* nitrate reductase activity was found in the pellet, as reported previously (15). Cofactor activity was still exclusively in the supernatant (not shown). Thus, the nitrate reductase in the *E. coli* extract does not appear to be the source of the molybdenum cofactor since it is possible to separate these two activities and yet retain full activity of each.

Dialysis of cofactor. Ketchum and Swarin (9) reported that the molybdenum cofactor activity found in several species of bacteria was dialyzable and insensitive to trypsin. These experiments were repeated with *E. coli* extracts. As can be seen in Fig. 3, the cofactor was able to pass through a dialysis membrane to complement the *nit-1* extract in the dialysis bag. Trypsin (1 mg/ml) added to the *E. coli* extract caused a greater rate of dialysis of complementation activity. The control experiment in which bovine serum albumin was added to the *E. coli* extract did not show an increased rate of complementation. This dialysis experiment was also performed with chymotrypsin, pepsin, or thermolysin added to the *E. coli* extract during the dialysis against the *nit-1* extract. Chymotrypsin appeared to stimulate the rate of complementation to a lesser extent; pepsin and thermolysin had no effect. When dialysis experiments were performed with the *nit-1* extract in dialysis tubing of 2,000-molecular-weight exclusion, the cofactor from the *E. coli* extract was still able to traverse this membrane to complement *nit-1*. These dialysis experiments show that the molybdenum cofactor is not sensitive to proteolytic enzymes and exists either free or loosely bound in the *E. coli* extracts.

Sephadex G-25 gel filtration. Further experiments were performed to determine whether all the *E. coli* cofactor was present exclusively in the low-molecular-weight fraction in a dialyzable form. The *E. coli* extract was applied to a Sephadex G-25 column (PD-10), and the fractions from this column were assayed for cofactor activity. Cofactor activity was found in both the included and excluded fractions, but predominantly in the fractions corresponding to the excluded volume of the column (Table 3). This result could indicate that there are two pools of cofactor in the *E. coli* extract, a pool of dialyzable, low-molecular-weight cofactor and a pool of cofactor bound to a large-molecular-weight species which was excluded from the G-25. To test this hypothesis, a portion of the cofactor which eluted in the excluded volume of the G-25 column was either chromatographed on a second G-25 column or dialyzed against *nit-1*. The cofactor activity from the fraction which had been excluded on the first G-25 column again was found to partition in both the excluded and included fractions of the column (Table 3). In addition, the cofactor from this fraction was able

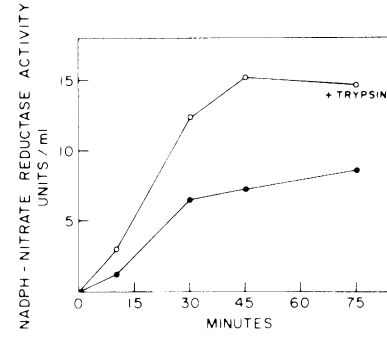

FIG. 3. *Time course of formation of nitrate reductase during the dialysis of E. coli against nit-1. Samples of the nit-1 extract were removed from the dialysis sac at intervals and assayed for NADPH-nitrate reductase activity. Symbols: ●, control; ○, 1 mg of trypsin per ml added to the E. coli extract.*

TABLE 3. *Fractionation of cofactor activity on successive Sephadex G-25 columns*

Column	Cofactor units		
	Applied	Excluded	Included
First	571	426 (74)[a]	217 (38)
Second	284[b]	214 (75)	51 (18)

[a] Numbers in parentheses are percentages recovered.

[b] From excluded fraction of first column.

to dialyze through a membrane to complement *nit-1*. These results indicate that the molybdenum cofactor is loosely associated with a macromolecule, but the association is easily disrupted by gel filtration or dialysis.

Since the dialysis of the cofactor across the membrane was stimulated by trypsin added to the *E. coli* extract, it was necessary to test whether the partition of the cofactor during G-25 gel filtration was due to a proteolytic enzyme in the crude extract which stimulated release of the cofactor from the carrier. To test this possibility, *E. coli* cells were homogenized in 10 mM phosphate buffer (pH 7.4) with 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor. The extract was then applied to a G-25 column, as described previously. The partition of cofactor activity in the phenylmethylsulfonyl fluoride-treated extract was the same as in the control extract, indicating that proteolysis was probably not responsible for the separation of the cofactor from the carrier molecule.

Acid treatment. Ketchum et al. (7) found that it was necessary to acid-treat molybdoenzymes to release the molybdenum cofactor for reconstitution with *nit-1*. *E. coli* extracts display significant cofactor activity without prior acid treatment, but extracts were acid-treated to determine whether additional cofactor activity could be released. The *E. coli* extracts were acidified with 0.1 N NaCl adjusted to pH 2 with HCl, incubated in ice for 2.5 min, and assayed. As can be seen in Table 4, no additional cofactor activity was released with acid treatment of extracts; in fact, substantial activity was lost. When the acid-treated extract was neutralized with NaOH and then applied to a G-25 column, cofactor activity again was found partitioned in both the excluded and included fractions, indicating that the release of molybdenum cofactor in *E. coli* did not require acid treatment, as is required with molybdoenzymes, and that the association between cofactor and carrier molecule was not disrupted by acid treatment. There was, however, a small increase in the proportion of the cofactor activity found in the included fraction. Possibly, some carrier protein was denatured by the acid treatment.

Further experiments in which *E. coli* extracts were incubated for 2 h with 0.1 N NaCl adjusted to various pH values with HCl or NaOH indicated that the cofactor activity in *E. coli* extracts was relatively stable from pH 3 to 11. Activity was sharply reduced below pH 3.

Estimation of size of active cofactor. In an attempt to estimate the size of the cofactor and the molecule it is associated with, *E. coli* extracts were subjected to sucrose density gradient centrifugation, and each fraction from the gradient was assayed with *nit-1* extract for complementing activity. Cofactor activity was found in a broad region, with a peak in the region of 3.5 to 4.3S.

When *E. coli* extracts were fractionated by Sephadex G-100 gel filtration chromatography, two peaks of cofactor activity were present (Fig. 4). There was a peak of cofactor activity in the large-molecular-weight fraction which eluted in the same region as ovalbumin (molecular weight, 40,000). The large-molecular-weight fraction of cofactor activity was polydisperse in both sucrose gradient centrifugation and gel filtration, which may indicate that cofactor was associated with carriers of different sizes or may reflect the fact that cofactor constantly comes off the carrier during fractionation. The second peak of cofactor activity was not associated with the major protein peak. When fractions from this peak were rechromatographed on a Sephadex G-25 column, cofactor activity was found exclusively in the included volume. When similar fractions were chromatographed on a Sephadex G-10 column, cofactor activity was eluted exclusively in the excluded volume. The molecular weight of the free cofactor was estimated to be

TABLE 4. *Sephadex G-25 fractionation of acid-treated E. coli extracts*

Fraction	Cofactor units
Crude extract	147
Extract after acid treatment	88
Units of acid-treated extract applied to G-25 column	197
Excluded	81
Included	116

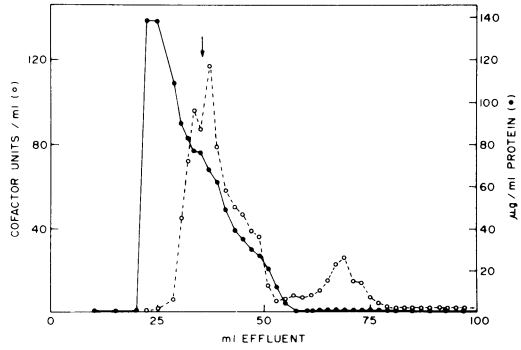

FIG. 4. *Sephadex G-100 gel filtration of E. coli extract. A 1-ml amount of E. coli extract was applied to a column of Sephadex G-100 (1.5 by 47 cm) equilibrated in 10 mM phosphate buffer, pH 7.4, with 0.5 mM EDTA and 10 mM ascorbic acid. The arrow indicates the peak of elution of the ovalbumin marker.*

between 700 and 5,000 from the exclusion limits of Sephadex G-10 and Sephadex G-25.

Stability of cofactor. Pienkos et al. (21) reported the isolation of molybdenum cofactor from acid-treated xanthine oxidase and found that the isolated cofactor was oxygen labile. We compared the stability of the *E. coli* cofactor under aerobic and anaerobic conditions, using both the cofactor found in crude extracts, which would be associated with the carrier molecule, and the cofactor from the included fraction on gel filtration, which would be free from the carrier molecule. As can be seen in Table 5, the cofactor from the crude extract was more stable under all conditions than the cofactor which was free from protein. The cofactor from the crude extract was stable at 0°C when exposed to either aerobic or anaerobic conditions for 2 h. However, 42% of the cofactor activity was lost by incubation of the crude extract at 37°C in air, and anaerobic conditions offered some protection. On the other hand, the included fraction lost 40% of its cofactor activity after 2 h at 0°C under either aerobic or anaerobic conditions. At 37°C, however, anaerobic conditions offered significant protection from inactivation. From these data we conclude that the cofactor from *E. coli* is somewhat oxygen labile and that the carrier molecule stabilizes the cofactor from inactivation by heat or oxygen.

Effect of molybdenum-free or tungsten-containing media on cofactor activity of *E. coli*. It has been shown previously that *Neurospora* wild-type mycelia grown on tungsten-containing media can incorporate this metal into a cofactor which, when added to *nit-1* extracts, led to the formation of an inactive, 7.9S species of nitrate reductase which contained tungsten (10). In the present studies, *E. coli* cultures were grown in various media to determine the conditions required for the formation of cofactor in the absence of molybdate. For these experiments, *E. coli* cells were grown in molybdenum-free media, transferred to fresh media with the various additions for 4 h, harvested, and assayed in either the presence or absence of molybdate in the complementation mixture (Table 6). Extracts from cells which were transferred to molybdenum-free or tungsten-containing media showed no cofactor activity when molybdate was omitted from the complementation mixture. However, the presence of molybdate in the complementation mixture led to the appearance of nitrate reductase activity. Even the *E. coli* cells which had been transferred to molybdenum-containing media showed a two- to threefold increase in cofactor activity when molybdate was added to the complementation mixture. Apparently cofactor synthesis occurred even when *E. coli* cells were grown in molybdenum-free or tungsten-supplemented media, and this molybdenum-free cofactor was subsequently activated by the addition of molybdate to the complementation mixture.

To determine whether molybdate could activate cofactor in vivo in the absence of protein synthesis, we transferred cells from molybdenum-free media to media with 1 mM molybdate plus chloramphenicol (200 μg/ml) to inhibit new protein synthesis. As can be seen in Table 6, no cofactor activity was detected in the sample exposed to molybdate in the presence of chloramphenicol when assayed without molybdate. However, when molybdate was added to the complementation assay, cofactor activity was detected. To determine whether chloramphenicol prevented the molybdate uptake, we assayed the molybdenum content of the extracts. Similar amounts of molybdenum were detected in extracts of cells exposed to molybdate alone or to molybdate plus chloramphenicol. In four similar experiments in which *E. coli* cultures were transferred to media with molybdate plus chloram-

TABLE 5. *Stability of E. coli molybdenum cofactor*

Incubation conditions[a]	Cofactor units/ml	
	Crude extract	Included fraction[b]
Aerobic, 0°C, initial time	686	19.3
Aerobic, 0°C	668	11.1
Anaerobic, 0°C	658	11.7
Aerobic, 37°C	393	0
Anaerobic, 37°C	504	8.8

[a] The fractions were incubated for 2 h and then assayed for cofactor activity. The samples were made anaerobic by repeated purging with oxygen-free N_2 and then were sealed.

[b] The included fraction was obtained from G-100 gel filtration and was free from protein.

TABLE 6. *Molybdenum cofactor activity of E. coli exposed to various media*

Growth conditions[a]	Cofactor units/mg of protein		Mo in extract (ng/mg of protein)
	$-MoO_4$[b]	$+MoO_4$[b]	
Mo-free	0	0.62	0.8
Na_2WO_4 (1 mM)	0	1.73	0.1
Na_2MoO_4 (1 mM)	0.68	1.65	34
Na_2MoO_4 (1 mM) + chloramphenicol (200 μg/ml)	0	0.88	40

[a] *E. coli* cells were grown aerobically on molybdenum-free minimal media until late log phase; then the culture was divided into four aliquots and transferred to fresh minimal media with the various additions for 4 h.

[b] Assay conditions.

phenicol, no cofactor activity was detected in three experiments when the samples were assayed without molybdate; in one experiment, activity of less than 10% of the level in cells grown in medium containing no chloramphenicol was seen.

To investigate further whether the *E. coli* cofactor from extracts of cells grown in molybdenum-free or tungsten-containing media was associated with a carrier, cell-free extracts from *E. coli* exposed to the various media were chromatographed on Sephadex G-25 and assayed for cofactor activity (Table 7). The molybdenum cofactor activity from molybdenum-grown *E. coli* was found in both fractions on Sephadex G-25 chromatography; the molybdenum cofactor activity from *E. coli* grown in molybdenum-free media, tungsten-containing media, or media containing molybdate plus chloramphenicol was eluted in both the excluded and the included fractions. These samples were assayed with molybdate in the complementation mixture. These results indicate that the cofactor without molybdenum was not localized in a different type of cellular association than the molybdenum-containing cofactor.

Sucrose gradient centrifugation. It has been established that sucrose gradient centrifugation of *nit-1* extract generates two peaks of NADPH-cytochrome *c* reductase activity: a 4.5S peak, which is the enzyme induced by nitrate and is FAD dependent, and an 11S peak which is constitutive, does not require FAD, and is unrelated to the nitrate reductase activity (23). Complementation of *nit-1* with a source of molybdenum cofactor leads to a diminution of the 4.5S peak of NADPH-cytochrome *c* reductase activity and the concomitant appearance of a 7.9S peak displaying both NADPH-cytochrome *c* reductase and NADPH-nitrate reductase activities (17). The 7.9S peak of nitrate reductase was also formed by *nit-1* complemented with *E. coli* extract, as reported previously (18).

To determine whether the presence of molybdenum or tungsten in the cofactor was a prerequisite for its ability to cause the dimerization of the 4.5S species to form the 7.9S nitrate reductase species, *nit-1* was complemented with an extract of *E. coli* grown in molybdenum-free medium and then centrifuged on a 5 to 25.5% sucrose gradient. No molybdate was added to the complementation mixture. As can be seen in Fig. 5, two peaks of FAD-dependent NADPH-cytochrome *c* reductase activity were formed, a 4.5S and a 7.9S species, but no NADPH-nitrate reductase activity was detected. However, when the gradient fractions were incubated with 1 mM molybdate, the peak of nitrate reductase activity so generated corresponded to the 7.9S peak of NADPH-cytochrome *c* reductase activity. Thus, empty cofactor from molybdenum-free *E. coli* can function to cause the formation of the 7.9S species of nitrate reductase.

When *nit-1* extracts were complemented with extracts from tungsten-grown *E. coli* and then subjected to sucrose gradient centrifugation (Fig. 6A), two peaks of FAD-dependent NADPH-cytochrome *c* reductase activity were observed, corresponding to the 4.5S and the 7.9S species, though the latter was devoid of NADPH-nitrate reductase activity. However, when 1 mM molybdate was added to each of the fractions from this gradient, nitrate reductase activity was generated in the fractions comprising the 7.9S region. When *nit-1* was complemented with extracts from tungsten-grown *E. coli* in the presence of molybdate and examined by sucrose gradient centrifugation (Fig. 6B), again two peaks of FAD-dependent NADPH-cytochrome *c* reductase activity were formed, and NADPH-nitrate reductase activity was detected in the 7.9S region without the need for additional molybdate in these fractions. It was

TABLE 7. *Fractionation of E. coli crude extracts on Sephadex G-25*

Sample	Total cofactor units		
	Applied	Excluded	Included
Mo-grown *E. coli*	1,760	1,290 (75)[a]	426 (25)
Mo-free	590	349 (88)	44 (12)
W-grown	1,578	664 (41)	960 (59)
Mo + chloramphenicol	111	73 (54)	62 (46)

[a] Numbers in parentheses are percentages recovered.

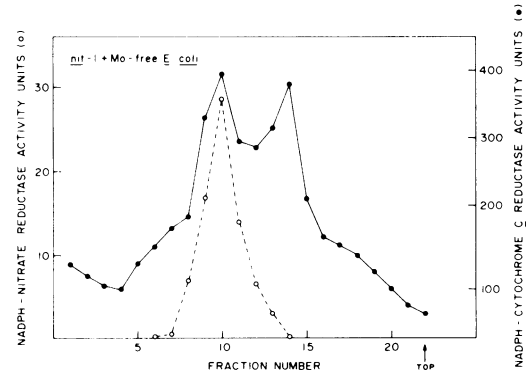

FIG. 5. *Sucrose density gradient centrifugation of extracts of molybdenum-free E. coli complemented with nit-1. The NADPH-nitrate reductase activity was detected only after the fractions from the gradient had been incubated for 5 min with 1 mM sodium molybdate.*

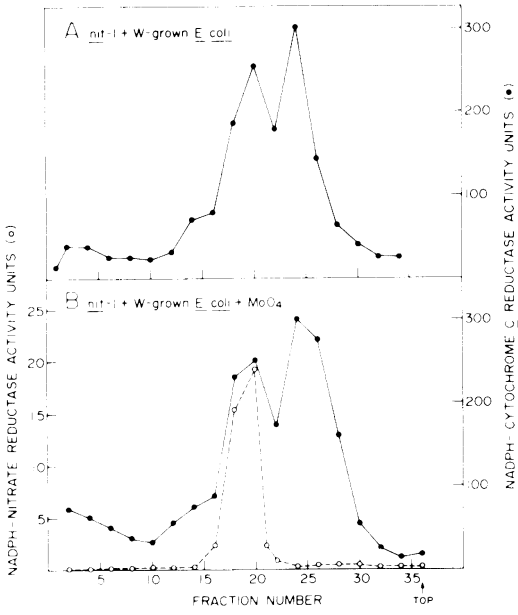

FIG. 6. *Sucrose density gradient centrifugation of nit-1 complemented with (A) tungsten-grown E. coli or (B) tungsten-grown E. coli plus 10 mM molybdate.*

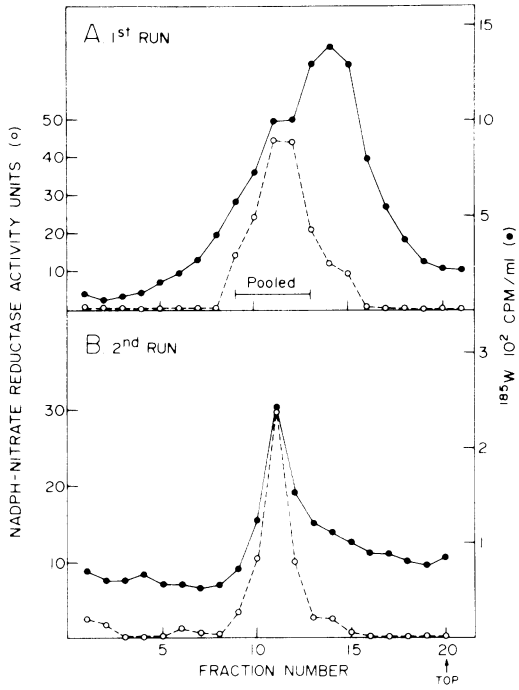

FIG. 7. *In vitro incorporation of tungsten-185 into nitrate reductase, as shown by sucrose density gradient centrifugation of a complementation mixture of nit-1 plus E. coli which had been grown in media containing $^{185}WO_4$. The samples were prepared and centrifuged as described in Materials and Methods. Samples from each fraction were counted or were incubated with 1 mM sodium molybdate for 5 min and then assayed for NADPH-nitrate reductase activity. The fractions from the peak tubes of nitrate reductase activity were pooled and subjected to a second sucrose gradient. The profiles for the first and second sucrose gradient centrifugation runs are shown in A and B, respectively.*

apparent from these data that the cofactor from tungsten-grown *E. coli* can function to cause the dimerization of the 4.5S species of NADPH-cytochrome *c* reductase of *nit-1* to a 7.9S form which still displays that activity but has no nitrate reductase activity. The latter species, however, display NADPH-nitrate reductase activity after the addition of molybdate.

The question remained whether the cofactor present in extracts of tungsten-grown *E. coli* was devoid of metal or contained tungsten. To answer this, extracts from *E. coli* grown in media with $^{185}WO_4$ were used for complementation with *nit-1*, and a partially purified mixture was applied to sucrose gradients (Fig. 7). When the fractions from the gradient were assayed for nitrate reductase activity in the presence of molybdate, the peak of activity corresponded with some of the radioactivity (Fig. 7A). The fractions which corresponded to the nitrate reductase were pooled, concentrated, and centrifuged on another sucrose gradient. As can be seen in Fig. 7B, there was a single peak of radioactivity. When molybdate was added to the fractions from the gradient, the peak of NADPH-nitrate reductase activity coincided with the peak of ^{185}W. In addition, when the above experiment was repeated including molybdate in the initial incubation of $^{185}WO_4$-grown *E. coli* extract with *nit-1*, radioactivity from tungsten was not associated with the nitrate reductase activity (not shown). These data show that the cofactor can incorporate tungsten and these tungsten-containing cofactor molecules can function to cause the dimerization of the nitrate reductase subunits in *nit-1*. However, the tungsten is loosely bound in this cofactor, as evidenced by the facts that (i) the incorporation of the ^{185}W was very low compared to the amount of nitrate reductase present and (ii) the tungsten was easily replaced by molybdenum, as shown by the restoration of nitrate reductase when molybdate was added to the fractions from the gradients.

DISCUSSION

The molybdenum cofactor of *E. coli* was found in the soluble fraction of the cell extract. Acid treatment was not required for release of

complementing activity. In cell-free extracts, regardless of growth and cell disruption conditions, the cofactor was associated with a large-molecular-weight carrier, which stabilized it considerably and from which it could be removed by dialysis or gel filtration. Although the nature of this carrier molecule is not yet known, the cofactor is clearly associated with a species of approximately 40,000 molecular weight during gel filtration on Sephadex G-100 and on sucrose gradient centrifugation. All attempts to purify the carrier molecule by conventional techniques failed because of the ease with which the cofactor dissociates from it. Whether or not the cofactor is specifically associated with a carrier molecule in vivo remains to be determined. Treatment of cell-free extracts with trypsin stimulated the appearance of dialyzable cofactor. Possibly trypsin, by hydrolyzing the carrier molecule, facilitates dissociation of the cofactor.

Nason et al. (18) and MacGregor and Schnaitman (14) tested extracts of *E. coli* for cofactor activity and found none in the soluble fraction, but did find some in extracts which had been acid-treated. These results differ from the data reported here for several reasons. Nason et al. used *E. coli* which had been grown in the absence of added molybdenum and did not add molybdate to the complementation mixture. Consequently, the pool of free molybdenum-containing cofactor may have been low, with the result that activity in the supernatant fraction was not detectable. Activity found after acid treatment correlated with the proportion of formate-nitrate reductase in the fractions assayed. Acid treatment of this membrane-associated enzyme would have released the molybdenum cofactor.

MacGregor and Schnaitman (14) assayed extracts of *E. coli* which had been grown anaerobically with nitrate. Under these conditions, the formate-nitrate reductase activity is maximally induced. Cofactor activity was detected only after acid treatment (molybdate was not added to the complementation mixture). This may reflect the high level of nitrate reductase formed under the growth conditions and depletion of the pool of free cofactor due to its incorporation into the enzyme.

Under the optimal growth and assay conditions reported here, we found 30-fold more complementing activity than reported previously in *E. coli*. A small amount of formate-nitrate reductase was present in our preparations. Although acid treatment made cofactor from that enzyme available, such treatment inactivated the large pool of soluble cofactor.

We found that the addition of molybdate to the complementation mixture increased the amount of nitrate reductase activity obtained. Lee et al. (11) also reported that the addition of molybdate to the complementation mixtures containing *nit-1* and acid-treated molybdoenzymes stimulated the complementation. They suggested that the added molybdate stabilized the cofactor and compensated for the metal lost from the cofactor on aging. However, even when *E. coli* cells were grown in media containing 1 mM molybdate, freshly prepared extracts were stimulated by the addition of molybdate to the complementation mixture. The molybdenum may indeed be in a labile association with the cofactor and require additional molybdate for stabilization. Alternatively, a pool of molybdenum-free cofactor may exist in *E. coli* in vivo.

Previously reported results have shown that *Neurospora*, grown in molybdenum-deficient or tungsten-containing media, synthesized an inactive nitrate reductase that sedimented at 7.8S (25). Lee et al. (10), using extracts of tungsten-grown wild-type *Neurospora* to complement *nit-1*, also observed an inactive, 7.9S nitrate reductase. Our results with empty cofactor and tungsten-containing cofactor correlate with these results and, in addition, show that the cofactor alone is the functional element which unites the subunits supplied by *nit-1*. Neither molybdenum nor tungsten is necessary for the cofactor to supply that function.

Our results, however, differ from those mentioned above in one aspect. The inactive nitrate reductase formed in vivo by growth of *Neurospora* on molybdenum-deficient media could not be activated by addition of molybdate to cell-free extracts (25). We found that the 7.9S species of nitrate reductase, formed by complementation using empty or tungsten-containing cofactor, could be activated by the addition of molybdate.

It is important to note that the cofactor in *E. coli* is synthesized constitutively, as was reported for the cofactor from *Neurospora* (17) and *Aspergillus* (3). It is synthesized under conditions in which the formate-nitrate reductase of *E. coli* is not induced and is also synthesized in the absence of molybdenum. This stands in contrast to the regulation of the molybdenum cofactor (FeMoco) of nitrogenase, which has been found only in acid-treated extracts of nitrogenase and whose synthesis is repressed by ammonia (21).

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