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Involvement of a Low-Molecular-Weight Substance in In Vitro Activation of the Molybdoenzyme Respiratory Nitrate Reductase from a *chlB* Mutant of *Escherichia coli*

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The soluble subcellular fraction of a *chlB* mutant contains an inactive precursor form of the molybdoenzyme nitrate reductase, which can be activated by the addition to the soluble fraction of protein FA, which is thought to be the active product of the *chlB* locus. Dialysis or desalting of the *chlB* soluble fraction leads to the loss of nitrate reductase activation, indicating that some low-molecular-weight material is required for the activation. The protein FA-dependent activation of nitrate reductase can be restored to the desalted *chlB* soluble fraction by the addition of a clarified extract obtained after heating the *chlB* soluble fraction at 100°C for 8 min. The heat-stable substance present in this preparation has a molecular weight of approximately 1,000. This substance is distinct from the active molybdenum cofactor since its activity is unimpaired in heat-treated extracts prepared from the organism grown in the presence of tungstate, which leads to loss of cofactor activity. Mutations at the *chlA* or *chlE* locus, which are required for molybdenum cofactor biosynthesis, similarly do not affect the activity of the heat-treated extract in the in vitro activation process. Moreover, the active material can be separated from the molybdenum cofactor activity by gel filtration. None of the other known pleiotropic chlorate resistance loci (*chlD*, *chlG*) are required for the expression of its activity. Magnesium ATP appears to have a role in the formation of the active substance. We conclude that a low-molecular-weight substance, distinct from the active molybdenum cofactor, is required to bestow activity on the molybdoenzyme nitrate reductase during its biosynthesis.

The chlorate-resistant mutants (*chlA*, *B*, *D*, *E*, and *G*) are pleiotropically defective in the activity of molybdoenzymes (1, 2, 7, 9), the best characterized of which in *Escherichia coli* is the respiratory nitrate reductase (4, 5, 10, 22, 27). All known molybdoenzymes of *E. coli* are thought to contain the molybdenum cofactor (2, 8), and this cofactor is different from that present in nitrogenase (23, 28). It has been shown that *chlA* and *chlE* mutants are lacking in molybdenum cofactor activity, consistent with their pleiotropic effect on molybdoenzymes (21). These loci probably encode the enzymes required for molybdenum cofactor biosynthesis (14, 15). However, *chlD* and *chlG* strains, when grown under certain conditions, exhibit molybdenum cofactor activity (21), and it is likely that the *chlD* locus is responsible for molybdate transport (13). Molybdenum cofactor activity is present in *chlB* strains (21), so the pleiotropic loss of molybdoenzyme activity cannot be attributed to the simple lack of cofactor.

A route to the biochemical analysis of molybdoenzyme biosynthesis is provided by the observation of Azoulay and collaborators (3) that mixing of soluble subcellular fractions of the *chlA* and *chlB* mutants leads to the formation of nitrate reductase activity. Inactive nitrate reductase species which are probably intermediates in the normal pathway for nitrate reductase maturation are present in all pleiotropic, chlorate-resistant strains (7, 11, 19, 20). In the mixing experiment, nitrate reductase activity is bestowed on both the *chlA* and *chlB* nitrate reductase derivatives, although the activation of the latter accounts for the majority of the overall activity restored. A protein, termed protein FA, has been purified from the *chlA* soluble fraction which is able, when added to the soluble *chlB* fraction, to bring about the efficient activation of the nitrate reductase derivative present in the *chlB* extract (25). Similarly a protein P_A has been purified from the *chlB* soluble fraction which, when added to the *chlA* soluble fraction, can bring about some activation of the *chlA* nitrate reductase derivative (12). We have recently shown that the molybdenum cofactor and protein FA are required for the in vitro activation of nitrate reductase from a *chlB* strain which has been grown in the presence of tungstate (26). Similar treatment of extracts from wild-type strains grown with tungstate, however, failed to restore nitrate reductase activity (26).

In this report, we investigate the in vitro activation of the nitrate reductase species found in the soluble fraction of a *chlB* mutant. We demonstrate that the activation process requires along with protein FA, a heat-stable low-molecular-weight compound which is distinct from the active molybdenum cofactor.

MATERIALS AND METHODS

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

Growth of bacteria and preparation of subcellular fractions. The strains were grown in rich media containing the following additions to the previously described basal medium (6): glucose, 2 g/liter; yeast extract (Difco Laboratories), 2 g/liter; Bacto-Peptone (Difco), 2 g/liter; and 1 μM sodium

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TABLE 1. Strains used in this study

Strain	Genotype	Origin
AP601	F⁻ *thr leu his pro arg thi ade gal lacY malE xyl ara mtl str* T1^r λ^r	(3)
AP15	As AP601, but *chlA*	(3)
AP24	As 601, but *chlB*	(3)
RK4353	Δ*lacU169 araD139 rpsL gyrA non*	(29)
RK5200	As RK4353 *chlA*::Mu *cts*	(29)
RK5201	As RK4353 *chlE*::Mu *cts*	(29)
RK5208	As RK4353 *chlB*::Mu *cts*	(29)
RK5209	As RK4353 *chlD*::Mu *cts*	(29)
RK5231	As RK4353 *chlG*::Mu *cts*	(29)

molybdate. For expression of nitrate reductase, potassium nitrate (1 g/liter) was also included. When indicated, the medium contained 10 mM sodium tungstate, which replaced the sodium molybdate. Anaerobic growth was accomplished in closed vessels filled almost to the top with medium. The cultures were not agitated during growth. Growth was conducted at 37°C, except when bacteriophage Mu (Mu *cts*) lysogens (29) were used, when the temperature was kept at 30°C.

Cultures were harvested at mid to late exponential phase of growth and suspended after being washed in 50 mM Tris hydrochloride (pH 7.6)–1 mM benzamidine hydrochloride. The bacteria were either used immediately or stored as pellets at −80°C until required.

Bacteria were ruptured in a French press (7), and cellular debris was removed by centrifugation at 18,000 × *g* for 25 min. The broken cell preparation was centrifuged at 120,000 × *g* for 90 min to yield the soluble fraction and the membrane fraction, which was suspended in 50 mM Tris hydrochloride (pH 7.6)–1 mM benzamidine hydrochloride. The soluble fraction prepared from AP24 (*chlB*) was taken as the *chlB* soluble fraction.

The heat-treated extract was routinely prepared from strain AP24 (*chlB*) to yield the heat-treated *chlB* extract referred to in the text, essentially as described previously (26). Approximately 1-ml portions of the *chlB* soluble fraction (about 30 mg of protein per ml) were placed in a boiling water bath for 8 min. Initially, this was done under a nitrogen-flushed atmosphere, but satisfactory results were obtained by using capped tubes which entrapped a small volume of air. After heating, the coagulated suspension was centrifuged at 18,000 × *g* for 15 min to yield a clear supernatant. Less than 1% of the dry weight of the soluble fraction was still present in the heat-treated extract. The extract could be stored at −80°C for several weeks without significant loss of activity.

Activation of nitrate reductase in *chlB* preparations. The activation procedure was modified after that described by Riviere et al. (25). Portions of *chlB* preparations (about 1 mg of protein) made from strain AP24 grown anaerobically, in the presence of nitrate, were mixed with protein FA (50 μg of protein) in a total volume of 0.50 ml of 50 mM Tris hydrochloride, pH 7.6. The mixture was incubated for up to 90 min at 32°C under a N_2 atmosphere. The mixtures were kept on ice until the start of the activation, which was initiated by the addition of protein FA. After the incubation period, the tubes were opened to the air and returned to an ice bath. Portions were then removed for nitrate reductase assay. When heat-treated extracts were introduced into the activation mixture, the total volume of the mixture was kept constant by adjustment of the buffer addition. Protein FA was prepared as described previously (25).

Preparation of subfractions from the *chlB* soluble fraction in which nitrate reductase can be activated. Dialysis of the *chlB* soluble fraction was accomplished by dialyzing 4 ml of the soluble fraction in 1 liter of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.7)–1 mM benzamidine hydrochloride with rapid agitation for 4 h at 4°C. The contents of the dialysis bag were used directly in the activation experiments. Low-molecular-weight material was also removed from the *chlB* soluble fraction by gel filtration on Sephadex G25 (coarse grade). About 3 ml (100 mg of protein) of the *chlB* soluble fraction was applied to a Sephadex G25 column (15 by 1.6 cm, inside diameter), equilibrated, and eluted in 50 mM Tris hydrochloride (pH 7.6)–1 mM benzamidine hydrochloride. The protein-containing fractions were combined and used directly in the nitrate reductase activation experiments. The chromatography was done at 4°C.

In later experiments in which the effects of magnesium ATP and heat-treated *chlB* extracts were differentiated, the column medium was replaced with Sephadex G25 (fine grade). The fractionation was done exactly as described above.

Fractionation of the heat-treated *chlB* extract. Fractionation of the heat-treated *chlB* extract was achieved by gel filtration on a Sephadex G50 (fine grade) column (21.5 by 1.6 cm, inside diameter), equilibrated, and run in 20 mM Tris sulfuric acid (pH 7.6)–1 mM benzamidine hydrochloride. About 2.5 ml of extract, containing about 15 mg of protein, was applied to the column. This column was calibrated for molecular-weight estimation by using the following substances (molecular weight): α-chymotrypsinogen (25,000), cytochrome *c* (12,500), insulin B chain (3,200), flavin adenine dinucleotide (830), and ATP (507).

Analytical procedures. Nitrate reductase (benzyl viologen-nitrate oxidoreductase) activity was measured spectrophotometrically by monitoring the nitrate-dependent oxidation of reduced benzyl viologen at 600 nm as described previously (16). Alternatively, it was assayed by estimating nitrite production from nitrate in the presence of reduced benzyl viologen (17). One unit of nitrate reductase activity produces 1 μmol of nitrite per min. Protein was measured by the method of Lowry et al. (18).

The ability to restore nitrate reductase activity to a *chlB* soluble fraction prepared after anaerobic growth in the presence of nitrate and 10 mM sodium tungstate, in a protein FA-dependent manner, was taken as a measure of molybdenum cofactor content (26).

RESULTS

Involvement of material of low molecular weight in the in vitro activation of the nitrate reductase precursor of a *chlB* mutant. The mixing of the soluble fractions of a *chlA* and *chlB* mutant leads to the appearance of nitrate reductase activity. If, however the soluble fractions are dialyzed beforehand, nitrate reductase activity is not restored (data not shown). The nitrate reductase derivative present in the *chlB* soluble fraction can be activated by the direct addition to the preparation of protein FA, the probable active product of the *chlB* locus (25). However, prior dialysis of the *chlB* soluble fraction led to the loss of the ability of protein FA to bring about activation of nitrate reductase (Fig. 1). Clearly, some low-molecular-weight material in the *chlB* soluble fraction is essential for the in vitro activation of the *chlB* nitrate reductase derivative.

The addition of a heat-treated preparation of the *chlB* soluble fraction almost fully restored the activation of nitrate

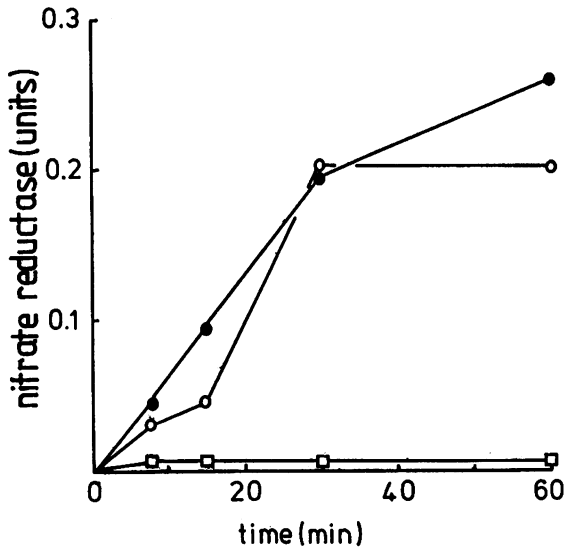

FIG. 1. Heat-treated *chlB* extract restores ability to activate nitrate reductase to dialyzed *chlB* soluble fraction. The soluble fraction, prepared as described in Materials and Methods from a washed culture of strain AP24 grown anaerobically in the presence of nitrate, was dialyzed against 20 mM HEPES (pH 7.7)–1 mM benzamidine hydrochloride exactly as detailed in Materials and Methods. Portions (200 μl) of the dialyzed (□) and undialyzed (●) *chlB* soluble fraction were incubated as indicated with protein FA (5 μl) in a total volume of 0.5 ml, as described in Materials and Methods. Samples of the resulting mixture were analyzed for nitrate reductase activity. Heat-treated (100°C for 8 min) *chlB* extract (200 μl) was added to dialyzed *chlB* soluble fraction (200 μl), protein FA (5 μl), and buffer to give a total volume of 0.5 ml, which was treated in an identical manner to those above. The data from this incubation (○) are corrected for the small change in protein concentration in the soluble *chlB* fraction brought about by dialysis. The data are expressed as units of nitrate reductase activity per equivalent amount of protein in the 200 μl of the *chlB* soluble fraction.

reductase activity (Fig. 1). Addition of protein FA alone to the heat-treated fraction yielded no nitrate reductase activity; indeed, nitrate reductase antigens were absent from this preparation. Dialysis of the heat-treated extract itself led to the complete loss of its ability to restore nitrate reductase activity. We conclude, therefore, that the activation of the nitrate reductase derivative in the *chlB* soluble fraction requires, in addition to protein FA, low-molecular-weight, heat-stable material which is present in the heat-treated *chlB* extract.

More reproducible results could be obtained by substituting for the dialysis treatment the rapid passage of the *chlB* soluble fraction through a Sephadex G25 desalting column. The major protein-containing fractions from such a column, which yielded nitrate reductase activity on incubation with the heat-treated extract and protein FA, were combined and used in the experiments described below. This material could be stored at −80°C for 1 week without significant loss of its ability to be activated. The addition of material from the G25 column, which elutes just before the totally included volume, to the combined activable fractions, in the place of the heat-treated *chlB* extract, also gave some activation of nitrate reductase (data not shown). This indicates that heat treatment per se is probably not necessary for the production of the active component in the heat-treated extract, but rather that material normally present in the *chlB* soluble fraction is responsible (see below). The level of activation, in relation to that obtained when the heat-treated *chlB* soluble fraction was used, was always low in such experiments. We attribute this to the inevitable dilution that occurred on passage through the desalting column.

Assay of the active material present in the heat-treated *chlB* soluble fraction. The addition of the heat-treated *chlB* fraction to the unfractionated *chlB* soluble fraction in the presence of protein FA led to a nonsignificant (no more than 20%) enhancement of activation of the nitrate reductase over that found in its absence. The factor which limits the level of nitrate reductase activation is, therefore, present in the *chlB* soluble fraction and is essentially absent from the heat-treated extract. It is possibly the concentration of the precursor nitrate reductase itself or of some other heat-labile material that is also required. Protein FA was always present in excess in these experiments. Analysis of the kinetics of the nitrate reductase activation revealed, however, that in the presence of the heat-treated *chlB* extract, the activation of the *chlB* nitrate reductase precursor was more rapid (Fig. 2). This is consistent with the involvement of the active material of the heat-treated *chlB* extract in a step of the activation process which normally limits the rate of the activation but not its extent.

We examined the effects of adding small amounts of the heat-treated *chlB* extract to the activable protein fraction in the presence of an excess of protein FA. If a prolonged incubation period is used, then, as anticipated, the level of nitrate reductase activation is limited by the active material in the heat-treated *chlB* extract. A linear relationship was obtained between the amount of the heat-treated extract added and the extent of nitrate reductase activation (Fig. 3). This system can, therefore, be used for the assay of the active material in the heat-treated extract. The proportionality, of course, breaks down when larger amounts of the heat-treated extracts are used, since under these conditions the level of activation observed is limited by material in the

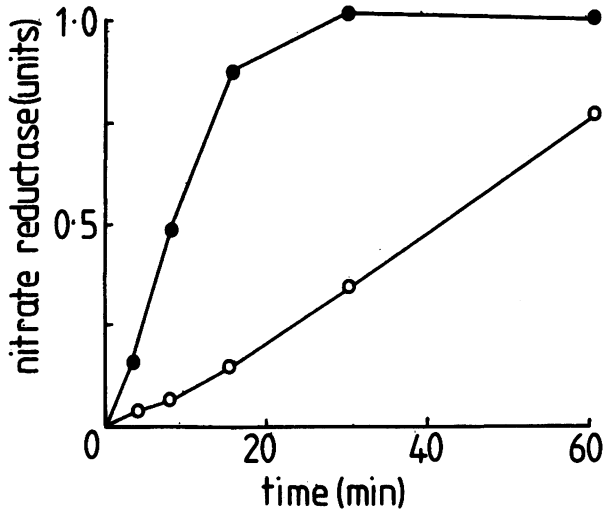

FIG. 2. Effect of heat-treated *chlB* extract on the ability of protein FA to activate nitrate reductase in a *chlB* soluble fraction. Soluble fraction (0.20 ml) of a *chlB* mutant (AP24) was mixed with protein FA (5 μl) in a total volume of 0.50 ml in either the absence (○) or the presence (●) of heat-treated *chlB* extract (0.25 ml). The mixtures were incubated for the times indicated, and nitrate reductase was assayed exactly as described in Materials and Methods. Nitrate reductase is expressed as units activated per incubated mixture.

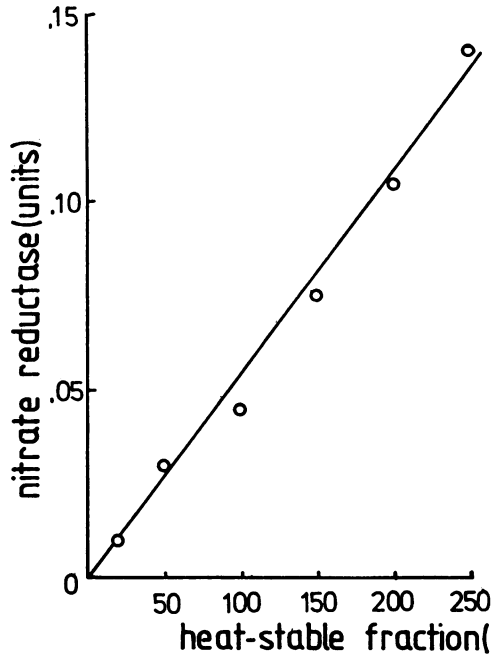

FIG. 3. Assay of active component in the heat-treated *chlB* extract. Samples (0.20 ml) of the combined protein-containing fractions obtained after passage of the soluble fraction of a *chlB* mutant (AP24) down the desalting Sephadex G25 column (see Materials and Methods) were mixed with protein FA (25 μl) and various amounts of the heat-treated *chlB* soluble fraction as indicated. The mixtures made up to a standard volume of 0.50 ml with buffer were incubated for 90 min as described in Materials and Methods. The nitrate reductase activity refers to the total units present in the incubation mixtures.

desalted *chlB* soluble fraction. This latter material must, therefore, always be present in excess in such assays. The absolute level of nitrate reductase activity formed for a given amount of heat-treated *chlB* extract was reasonably constant with different preparations of the desalted *chlB* soluble fraction.

Adenine nucleotides influence the activation of the *chlB* nitrate reductase derivative. Using the assay system described above, we tested the ability of a variety of compounds to substitute for the heat-treated *chlB* extract in the activation process. Of the substances examined, only ATP, ADP, UTP, and GTP were capable of participating in the process (Table 2). The level of activation achieved was greatest with ATP and ADP, but even in these cases it was only about 25% of that observed when the heat-treated *chlB* extract was used. However, the inclusion of a small amount of the heat-treated *chlB* extract, which alone did not lead to significant nitrate reductase activation, along with the substances examined, enhanced the activation observed with ATP and ADP to a level close to that found with an excess of heat-treated *chlB* extract (Table 2). No such enhancement was found with any of the other substances. This suggests not only an involvement of ATP, ADP, or both, but also that a further heat-stable substance is required for the activation process.

ATP appears to be inhibitory to the process at higher concentrations (more than 2 mM). The relative efficiencies of ATP and ADP in the process were examined. ATP was more effective than ADP at low concentrations. The latter was effective only at concentrations greater than 1 mM (data not shown). This behavior suggests that ATP and not ADP is involved in the process. The activation observed at higher ADP levels may result from ATP synthesis catalyzed by adenylate kinase, which would be present in the protein fraction obtained from the G25 desalting column. Consistent with the involvement of adenine nucleotides, the activation process requires Mg^{2+}. This is demonstrated by the inhibition of the activation process by EDTA and its restoration by the addition of $MgCl_2$ (data not shown). An involvement of Mg^{2+} in the activation process has been suggested earlier (24).

However, magnesium ATP alone is an insufficient replacement for the heat-treated *chlB* extract in the activation process. Analysis of the desalted *chlB* extract along with protein FA confirmed that ATP was absent from the preparation, and yet its nitrate reductase derivative can be efficiently activated with the heat-treated *chlB* extract along with protein FA, i.e., in the absence of ATP. Furthermore, the presence of small amounts of the heat-treated *chlB* extract greatly enhanced the activation found with ATP alone (Table 2). The use of a creatine phosphate-creatine kinase ATP-generating system did not significantly influence the activation process over that found when ATP itself was used, indicating that endogenous ATPase activity does not unduly influence the results.

Demonstration of the involvement of a compound distinct from ATP in the activation process. The *chlB* soluble fraction was fractionated to a greater resolution by gel filtration, and the activation of nitrate reductase in the eluate was analyzed, by using both magnesium ATP and the heat-treated *chlB* extract along with protein FA. We identified certain fractions, in which nitrate reductase could be activated by the addition of heat-treated *chlB* extract but not of magnesium ATP (Fig. 4). Magnesium ATP was able to bring about only a small part of the activation achieved by the heat-stable *chlB* extract. Indeed, the more highly resolving the fractionation by gel filtration of the *chlB*-soluble fraction, the less nitrate reductase activation was observed by using magnesium ATP, which is consistent with the involvement of a further component.

Further information was obtained by monitoring the fractionation of the heat-treated *chlB* extract by gel filtration on

TABLE 2. Ability of compounds to substitute for heat-treated *chlB* extract in activation of *chlB* nitrate reductase

Added substance[a]	Nitrate reductase activity[b] with other addition	
	None	Heat-stable fraction[c]
ATP	13	45
ATP (1 mM)	20	52
ADP	22	48
ADP (1 mM)	3	5
UTP	14	17
AMP	<1	<1
GTP	7	4
Others[d]	<1	<1

[a] Added at final concentration of 5 mM unless indicated otherwise.

[b] Expressed as nanomoles of nitrate reduced per minute per incubated mixture (see Materials and Methods).

[c] A limiting amount of heat-treated *chlB* extract was added which gave rise to some nitrate reductase activity (12 nmol of nitrate reduced per min per tube). This was subtracted from the activations observed. In these experiments, the presence of excess heat-treated *chlB* extract produced an activity of 70 nmol of nitrate reduced per min per tube.

[d] Other substances tested were NAD, NADH, NADP, NADPH, glutathione, Na_2S, dithiothreitol, Na_2MoO_4, and Na_2WO_4.

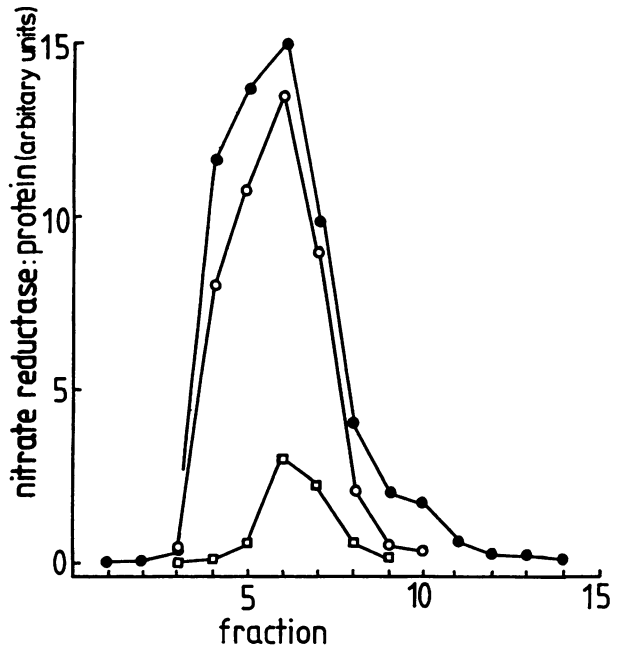

FIG. 4. Differential ability of ATP and heat-treated *chlB* extract to activate *chlB* nitrate reductase after gel filtration. The soluble fraction (120 mg of protein) of a *chlB* mutant (AP24) was applied to a Sephadex G25-fine column and eluted as described in Materials and Methods. Protein (●) and the ability of each fraction to yield nitrate reductase activity after incubation with protein FA and heat-treated *chlB* extract (○) was measured as indicated in Materials and Methods. Portions of each fraction were mixed with magnesium ATP (final concentration, 1 mM) and protein FA and incubated to promote nitrate reductase activation (□), as detailed in Materials and Methods. The nitrate reductase activities shown for the magnesium ATP- and heat-treated *chlB* extract-dependent activations, although only qualitatively indicated, are directly comparable.

Sephadex G50. We analyzed the ability of the eluate to bring about the activation of nitrate reductase in preparations which require the heat-treated *chlB* extract but not magnesium ATP (for instance, fraction 4 in Fig. 4). A single peak possessing such a property was identified in the eluate of the G50 column (Fig. 5a). The material eluted in a manner consistent with a molecular weight of approximately 1,000. Calibration of the column revealed that ATP itself would elute in a distinct position just after the active component (data not shown). A single peak of activity eluting at a position indistinguishable from that found for the heat-treated *chlB* extract was found for the untreated *chlB* extract (Fig. 5b).

The active compound in the heat-treated *chlB* extract is not the molybdenum cofactor. Strains carrying mutations in the *chlA* and *chlE* loci have been shown to lack molybdenum cofactor activity (14, 15, 21, 26). We prepared heat-treated extracts from a series of mutants possessing insertions at the chlorate resistance loci. We examined the ability of these extracts to participate in the process leading to the activation of the nitrate reductase derivative present in the *chlB* soluble fraction. Extracts from the *chlA*, *chlE*, *chlD*, and *chlG* mutants were as effective as that of the *chlB* extract used in the work described in the preceding sections (Table 3). The wild-type strains examined also contained the activating material. The activity of this compound therefore does not require the participation of any of the products of the known chlorate resistance loci. This is inconsistent with its being the active molybdenum cofactor. Furthermore growth of a wild-type strain in the presence of tungstate, which leads to a loss of molybdenum cofactor activity (17, 21, 26, 27) does not affect the ability of the heat-treated extract to participate in the activation (Table 3). Control experiments revealed that the wild-type strain grown (anaerobically with nitrate) in the presence of tungstate exhibited less than 1% of the nitrate reductase activity present after growth in its absence, consistent with the loss of molybdenum cofactor activity.

We have recently shown that the molybdenum cofactor is required for in vitro activation of nitrate reductase in the soluble fraction of the *chlB* mutant prepared after growth in the presence of tungstate (26). The heat-treated *chlB* extract was used in that study as the source of the molybdenum cofactor, so clearly the molybdenum cofactor is present in the extract used in this study. However, the material responsible for the activation of the nitrate reductase species in the tungstate-grown *chlB* strain (the molybdenum cofactor) is distinct from the compound identified in this report (Fig. 6). Two peaks, in the G50 column eluate of the heat-treated *chlB* extract, representing the protein-bound and free forms of the active molybdenum cofactor (26) restore, in the presence of protein FA, nitrate reductase activity to the complete soluble fraction of the *chlB* mutant prepared after growth with tungstate. Furthermore, the peak of activity identified here as being required for the activation of the nitrate reductase derivative of the *chlB* mutant grown in the absence of tungstate is distinct from both of these peaks. The compound identified in this report will also be required in the activation of the tungstate-grown *chlB* nitrate reductase derivative. It is present in the unfractionated tungstate-grown *chlB* soluble fraction used in that process. Unlike the results found for molybdenum cofactor activity (26), exposure of the heat-treated *chlB* extract to a mixture of proteinases (β-chymotrypsin, trypsin, and subtilisin) had no effect on the elution characteristics of the active compound on G50 gel filtration analysis (analysis not shown).

DISCUSSION

This report attempts to analyze in molecular terms the nature of the events that lead to the activation of the nitrate reductase species present in a *chlB* mutant. We have shown that a heat-stable compound with a molecular weight of about 1,000 is required for the activation process, in addition to the previously defined protein FA, which is the probable active product of the *chlB* locus (25). The compound is distinct from the active molybdenum cofactor which at present is the only known component common to all molybdoenzymes except nitrogenase (23, 28). Several lines of evidence indicate the lack of identity of this compound with the molybdenum cofactor. It is present in strains possessing lesions at the *chlA* and *chlE* loci which are defective in the biosynthesis of the cofactor (14, 15, 21, 26). Its activity is unimpaired in heat-treated extracts from the wild-type strain grown in the presence of tungstate, conditions which lead to the loss of molybdenum cofactor activity (21, 26, 28). Furthermore, it is resolved by gel filtration, from another component present in the heat-treated *chlB* extract, which is required for the activation of the *chlB* nitrate reductase species present in tungstate-grown cultures. We have previously shown this compound to be the active molybdenum cofactor (26). Since all known molybdoenzymes in *E. coli* are defective in *chlB* mutants and they require protein FA for their in vitro activation (8), the

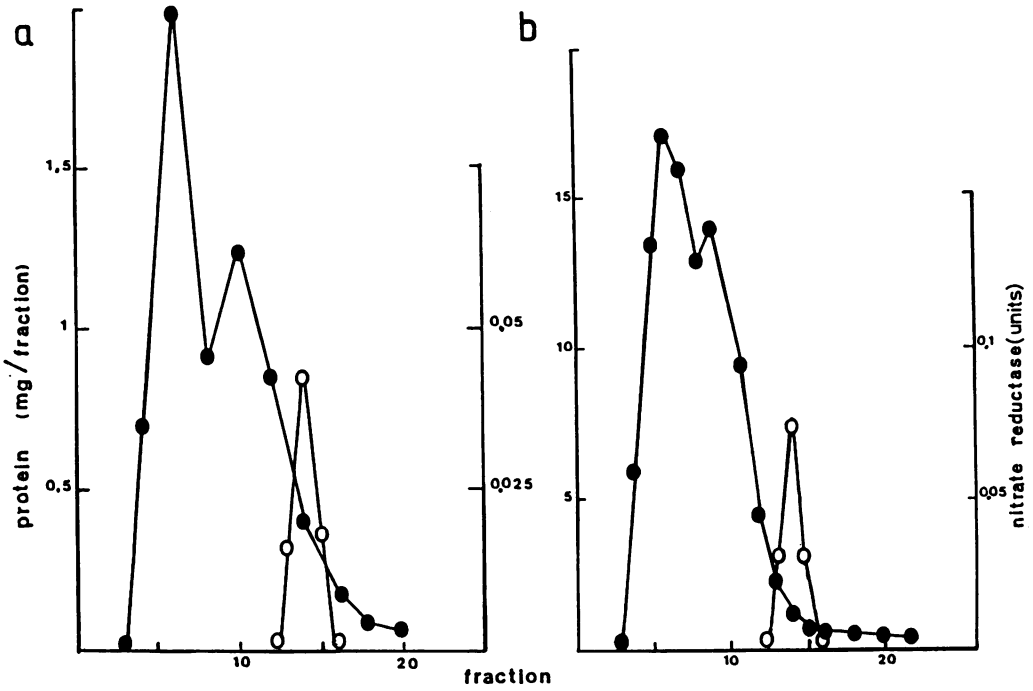

FIG. 5. Analysis by Sephadex G50 gel filtration of the active component in the heat-treated *chlB* extract. Conditions for the running of the column are described in Materials and Methods. (a) Heat-treated *chlB* extract (2.5 ml, 15 mg of protein) was applied to the column, and the eluate (2.5-ml fractions) was analyzed for protein (●) and its ability to promote activation of nitrate reductase when incubated with the protein fraction obtained after Sephadex G25 fractionation of the *chlB* soluble fraction, along with protein FA (○). (b) As in panel a, but untreated *chlB* soluble fraction (120 mg of protein) was applied to the same column. Activation of nitrate reductase was observed only in the fractions indicated in both cases.

compound that we describe here, therefore, is probably involved in the in vitro activation of other molybdoenzymes.

Since the molybdenum cofactor is present in both a free and protein-bound state in the soluble subcellular fraction (2, 26), it will be present in both the dialyzed and desalted *chlB* fractions. The molybdenum cofactor is, therefore, present in all the mixtures described in this work, that lead to activation of the *chlB* nitrate reductase species. We, therefore, cannot exclude the involvement of the molybdenum cofactor in the activation process. What is clear, however, is that protein FA does not catalyze the simple insertion of the cofactor into the *chlB* nitrate reductase derivative as was originally suggested (25).

The nature of this compound is obscure. Its biosynthesis does not require the participation of the gene products of any of the known pleiotropic chlorate resistance loci, since it is present in normal amounts in heat-treated extracts prepared from such strains. The compound is clearly distinct from ATP, since mixtures of appropriate preparations which we have established do not contain ATP can lead to the activation of the *chlB* nitrate reductase derivative. The compound has been distinguished from the active molybdenum cofactor, but we cannot directly exclude the possibility that it is an inactive precursor of the cofactor. If this is the case, then protein FA addition must bring about the synthesis of the active cofactor from this precursor. This is highly unlikely, since active molybdenum cofactor is present in *chlB* strains (21, 26) which lack protein FA activity. We have developed an assay for the compound, which we are presently using to further characterize the compound. Its concentration in the heat-treated *chlB* extract is likely to be low. On the basis of the data in Fig. 3, which indicate that 1 ml of the heat-treated extract can bring about the activation of sufficient nitrate reductase to catalyze the reduction of about 0.5 μmol of NO_3 per min, and the specific activity of purified nitrate reductase, about 75 mol of NO_3^- reduced per min per mg of protein (22), we estimate its minimum concentration to be about 35 nM. This assumes that one molecule of the compound is required for the activation of one molecule of nitrate reductase (minimum M_r, 200,000) (22). However, the presence of ATP in the crude extract may bring about significantly higher concentrations.

The role of ATP, or some substance that is readily synthesized from ATP in crude extracts, in the activation process clearly requires further investigation. Our findings suggest that the concentration of the active substance in the heat-treated extract is enhanced in the presence of ATP. The simplest interpretation of this is that ATP is required for

TABLE 3. Heat-stable factor content of chlorate-resistant mutants

Strain	Relevant genotype	Heat-stable factor content (%)[a]
RK4353	Parental	100
RK5200	*chlA*	100
RK5208	*chlB*	100
RK5209	*chlD*	105
RK5201	*chlE*	120
RK5231	*chlG*	95
AP601	Parental (grown with Na_2WO_4)[b]	100
AP24	*chlB*	115
AP24	*chlB* (grown with Na_2WO_4)[b]	100

[a] The amount of heat-stable factor (see Materials and Methods for assay) was set independently at 100% for each of the two parental strains.
[b] 10 mM Na_2WO_4 was added to the growth medium.

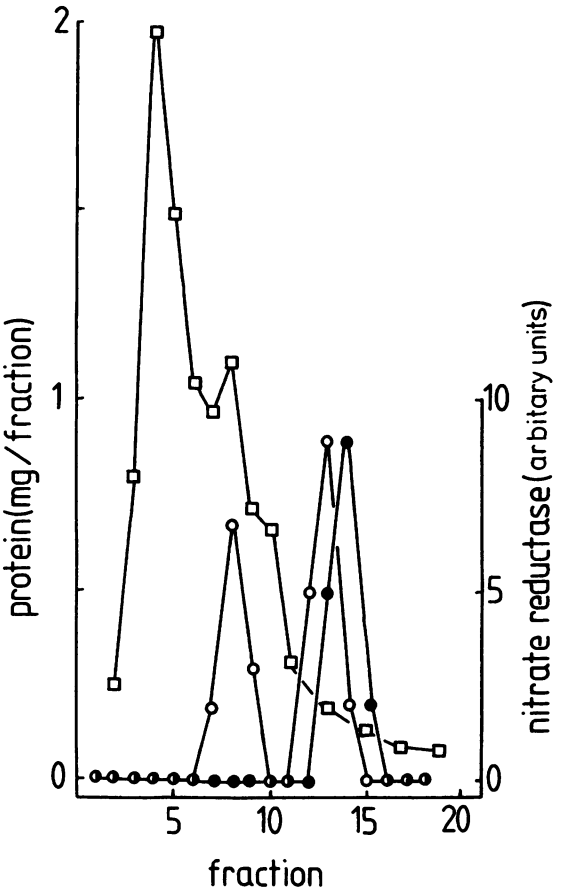

FIG. 6. Distinction between the active component in the heat-treated *chlB* extract and molybdenum cofactor activity. Heat-treated *chlB* extract was prepared from the soluble fraction (100 mg of protein) of mutant AP24 and applied to a Sephadex G50 column as described in the legend to Fig. 5 and Materials and Methods. Fractions (2.5 ml) of the eluate were assayed for protein (□), molydenum cofactor activity (○), and for their ability to function in the activation of *chlB* nitrate reductase (●) as described in Materials and Methods. The molybdenum cofactor and nitrate reductase activities, expressed in arbitrary units in the figure, have been adjusted to give equivalent activities for the most active fraction in each assay.

the synthesis of the compound from some other heat-stable low-molecular-weight material. Such a reaction could not be catalyzed by protein FA, since the active material is present in *chlB* mutants which are defective in protein FA activity. Presumably it is catalyzed by an enzyme present in the dialyzed or desalted *chlB* soluble fraction. The fractions from the G25 column eluate, which are activated by the heat-treated *chlB* extract but not by ATP alone, would not contain the precursor substance required for the synthesis of the active compound. The analysis of the mechanism of the in vitro activation of the *chlB* nitrate reductase derivative should lead to an understanding of the pathway for cofactor acquisition during molybdoenzyme biosynthesis. An important step in this process will be the molecular characterization of the active compound identified in this study.

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