MOLYBDENUM AND CHLORATE RESISTANT MUTANTS IN ESCHERICHIA COLI K12

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SUMMARY

Radioactive molybdenum is used to detect the existence of molybdo compounds in E. coli K12. Three membrane bound Mo-proteins are found, using sodium dodecyl sulfate. One of them is the nitrate reductase. The nature of the other two is discussed. The soluble fraction of the cellular extract contains a small Mo binding molecule which could be peptidic in nature (MW is about 1,500). Different chlorate resistant mutants are analyzed on the basis of these molybdo-compounds. None of the mutants is found to contain radioactivity bound to nitrate reductase protein. Defects in the biosynthesis of a molybdenum coenzyme is deduced for chlorate resistant pleiotropic mutants.

INTRODUCTION

Most of the chlorate mutants of Escherichia coli, which have lost nitrate reductase(1) activity, were found to be pleiotropic since they also lack formic dehydrogenase(1) activity. Several classes of such pleiotropic mutants have been described (1, 2, 3, 4, 5, 6, 17). In order to explain this effect it was first proposed that the mutation produces an alteration on the cytoplasmic membrane (7).

Recently, another type of explanation has been proposed. In Aspergillus and Neurospora some NR^- mutants are also pleiotropic since they lack xanthine dehydrogenase activity (8, 9). Both proteins affected by the mutation are molybdoproteins and it is supposed that the mutation altered the incorporation of a molybdo-cofactor into these proteins (10). E. coli nitrate reductase is a Mo-protein (11, 12, 13). The molybdenum hypothesis has already been used in E. coli to explain chl D mutations (14, 15) and the deficiency of nitrate reductase activity of some mutants (16).

The problem is now to decide how far can the molybdenum hypothesis explain the pleiotropic chlorate mutants. Very little is known about molybde-

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(1)Nitrate reductase and formate dehydrogenase will also be written NR and FDH respectively.

num processing in E. coli. A first set of questions would concern the wild type strain : does the molybdenum enter the cell before binding to membrane nitrate reductase ? What is the nature of the molybdenum cofactor and how is it biosynthetized ? Are there many molybdo proteins in E. coli ? In a second step it is necessary to determine whether the pleiotropic mutants present a normal Mo-processing.

The present report tries to answer some of these questions by directly labeling molybdenum binding cellular compounds with radioactive Mo and examining their presence in various chlorate mutants.

MATERIALS AND METHODS

The following strains were used : E. coli K12, PA601 (356 in Puig's collection) and its chlorate resistant mutants : chl A, B (1, 2, 3), chl D and F (unpublished results), chl E (17, 18). The chl C mutant was obtained from Puig's collection (18). None of the pleiotropic mutants contained nitrate reductase activity. chl C had about 2 % of the wild type activity.

Cells were grown anaerobically on minimal medium in the presence of Mo isotope (as molybdate). The medium (prepared with double distilled water in order to minimize the concentration of dissolved molybdenum) was supplemented with selenite (10^{-6} M) (19) and the requirements of strain 356 (1, 2). 50 µCi of radio-active molybdate were added to each 200 ml of medium (about 10^{-6} M). Induction by nitrate was done 3 to 6 hours before harvesting.

Cells were washed once and disrupted by sonication (3' in ice bath). Cells were sedimented from the extract by low speed centrifugation. Then the extract was separated into pellet (P) and supernatant (S) by ultracentrifugation (120 000 g during 2 hours). P (10 mg/ml) was resuspended in buffer Tris 0.02 M pH 8, containing sodium dodecyl sulfate (SDS) 1 %, β-mercaptoethanol 0.05 M and ethylene diamine tetraacetic acid (EDTA) 0.05 M. After one hour of incubation the extract was centrifuged at a low speed to eliminate undissolved material and an aliquot (50 µl) was then applied to the top of polyacrylamide gels. SDS acrylamide gel electrophoresis was performed according to Shapiro et al. (20), (phosphate buffer, pH 7, 0.1 M, SDS 0.1 %, 5 mA/tube) for 8 to 12 hours. Gels were run in duplicate. Both gels were treated with methyl viologen reduced by dithionite (gels are deep blue after 1/2 hours) and then with nitrate which produced the appearence of a clear band at the level of the nitrate reductase, which was marked by slightly cutting the gel. One of these gels was frozen, then cut in pieces 1.5 mm long. The radioactivity of these gel slices was measured. The other gel was stained for proteins using Amido Schwartz. S was passed through a G-50 Sephadex column (450 x 20 mm). 2.5 ml fractions were collected and counted. ^{99}Mo radioactivity was measured in a γ counter. The life of ^{99}Mo is about 3 days, and each determination had to be corrected for decay. Radioactive molybdate was bought from CEA (Paris, France).

RESULTS

A - The wild type cells, grown in presence of radioactive molybdenum incorporate approximately 10^{-9} atomgrams of Mo ion/mg of protein (1.2×10^6 counts/min/mg of protein).

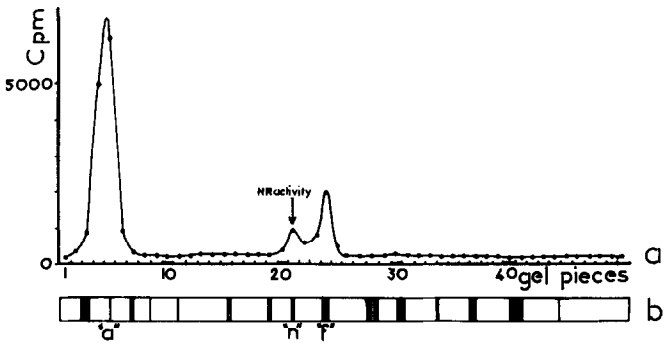

Fig. 1 - ^{99}Mo radioactivity profile in polyacrylamide gel electrophoresis of SDS solubilized particulate fraction.
(a) represents γ radioactivity throughout the gel. Gels are cut and counted as described in "Materials and Methods".
(b) represents a gel after amido black staining. Top of the gel is at the left. Bromophenol blue went out in about 5 hours of electrophoresis.

Four Mo binding molecules are identified, three solubilized from membrane by SDS and one in the soluble fraction S. Fig. 1 shows the radioactivity diagram obtained by polyacrylamide gel electrophoresis performed on a P aliquote. Three proteins associated radioactive peaks are well separated in 12 hours.

1) The first band ("a") is very narrow and poorly stained by amido black. It corresponds to a very high radioactive peak. "a" migrates very little and must be a high molecular weight molecule. Its nature is, at least partially, proteic. The ratio Mo/protein (Mo is evaluated from radioactivity of each peak and protein quantity is evaluated from color intensity of the stained protein band) is very high too (10 arbitrary units) and would support the idea of some sort of molybdenum storage molecule. The possibility of a nonspecific binding of molybdenum to a membrane particule cannot be discounted.

2) The second radioactive band ("n") migrates at the level of nitrate reductase, characterized by its activity on SDS gel (see Materials and Methods). The activity is associated to a stained protein band (Fig. 1). The ratio Mo/protein is about 0.2 arbitrary units.

3) The last radioactive peak corresponds to the protein band "f". It migrates a little faster than "n", indicating a lower molecular weight, and exhibits the same ratio Mo/protein than "n". Its function will be discussed later.

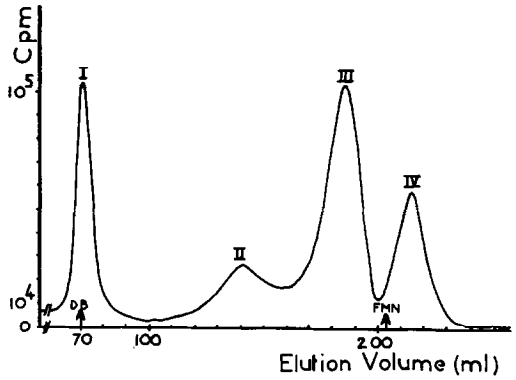

Fig. 2 - ^{99}Mo radioactivity elution diagram of soluble fraction in Sephadex G-50 column.
0.6 ml of S fraction (about 1 mg/ml of protein) was passed through the column. 2.5 ml fractions were recollected and counted. DB (dextran blue) and FMN (flavin mononucleotide) were used as standards. Dextran blue elution volume indicates column dead volume.

Radioactivity is found at the level of the tracking dye when shorter electrophoresis is performed. These small molybdo molecules can be produced by SDS denaturation of some molybdo proteins. It follows that the present short list of molybdomolecules may not be exhaustive.

S fraction is separated into four radioactive peaks by passing through a G-50 Sephadex column (fig. 2). Peak I corresponds to already described "a" and "n" proteins which are partially solubilized by sonication process (75 % of "a", 80 % of "n" and almost 100 % of "f" are in the particulate fraction P). Peak II has not been studied.

4) Peak III corresponds to a low molecular weight molecule associated to a large radioactive peak (fig. 2). Its molecular weight is estimated at 1,500 after two successive chromatographies on G-25 and G-15 Sephadex columns in presence of FMN and dextran blue as standards. The radioactivity associated to this molecule of MW 1500 is lost after treatment with a mixture of proteolytic enzymes and elutes as a sharp peak a little after FMN (MW < 450). Although more has to be done to decide if the nature of this molecule is peptidic, we shall call it Mo-"peptide" (its apparent hydrolysis could be due to esterase activity of trypsin).

The relative quantities of molybdenum bound to the four described molecules are 1,2, 5, 10 respectively for "n", "f", Mo-peptide and "a". It does not account for all the molybdenum present in the cells as much radioactivity is associated to very low molecular weight molecules ($\simeq$ 200), possibly molybdate and technetate.

TABLE I

	WT	chl A	chl E	chl F	chl B	chl D	chl C
^{99}Mo radioactivity bound to, or at the level of :							
"a"	+	+	+	+	+	++	+
"n"	+	-	-	-	-	-	-
"f"	+	-	-	-	-	-	+
Mo-"peptide"	+	-	-	-	+++	-	+

The proteins eluted in peak I of Fig. 2 ("a" and "n" proteins which have not been SDS treated) were treated at pH 2 and the resulting solution was passed through the previously described G-50 column. In this case the radioactivity does not remain bound to high molecular weight proteins but appears as two peaks of MW < 450 (one of them is about 200 and the other about 400). At that point it is important to recall that any Mo-protein treated at pH 2 is able to restore NADPH-nitrate reductase activity of an extract of nit-1 mutant from Neurospora crassa (10), which would suggest that Mo"peptide" is not the cofactor. But we cannot discard the possibility that the restoration pH used in the experiments of Nason et al. (about 8.0) may allow the spontaneous reassociation of the low molecular weight molybdo molecule with the peptide. We cannot yet decide if Mo-cofactor is the Mo-"peptide" or part of it.

B - When grown in presence of radioactive molybdenum, chlorate resistant mutants do not incorporate radioactivity in the same amount than the wild type (except for chl C) : chl A, E and F accumulate less (x 0.7), chl B two to three times more and chl D less only in the particulate fraction.

Each mutant has then been analyzed on the basis of the four Mo-compounds we have just described here. The results are summarized in Table I. The most important fact is that, while all the pleiotropic mutants have conserved "n" and "f" protein bands, none of these mutants exhibit radioactive peaks associated with such bands. This is the only characteristic common to all the pleiotropic mutants. The presence of "n" protein (and may be "f") in the particulate fraction of chl D and E mutants is in contradiction with previous studies (15, 21). Nevertheless, by immunological techniques, Forget also finds the presence of the NR protein in chl E (personnal communication).

These contradictions could be due to proteases activity which may vary according to the solubilization procedure used (22). Table I distinguishes three classes of pleiotropic mutants chl A, E and F have the same phenotype. They lack the Mo-"peptide" but still have Mo-"a" complex. chl D seems to differ from the first three mutants by the presence of twice more radioactivity bound to "a", which seems to indicate that Mo-"a" complex is not an artefact. chl B is the only pleiotropic mutant which can produce an apparently well-made Mo-"peptide" complex. All the chl B mutants tested synthetize two to three times more Mo-"peptide" than the wild type.

CONCLUSION

The non exhaustive analysis of molybdocompounds show that nitrate reductase is not the only molybdo protein in E. coli. The disappearance of molybdenum bound to both "n" and "f" bands in all the mutants lacking NR and FDH activities, and the disappearance of radioactivity associated to only "n" band in the NR specific mutant (chl C), suggest that "f" band may be the formate dehydrogenase protein. This is in agreement with the very recent purification of a molybdenum containing cytochrome b_1-FDH complex (23). It is then reasonnable to suppose that the lack of both activities in all the pleiotropic mutants is due to a defect in the incorporation of molybdenum into NR and FDH proteins. The pleiotropic effect would be due to the presence of a molybdenum cofactor common to both enzymes. The complexity of the cofactor biosynthesis could account for the large number of pleiotropic mutants. Some authors have already pointed out the possibility of a cofactor common to all molybdoenzymes (10). Such a cofactor, which has been characterized as being able to restore nitrate reductase activity of Neurospora nit-1 mutant (10) could be a low molecular weight peptide (1 000 < MW < 1 300) (24, 25, 26). This would be in favor of the here described Mo-"peptide" being the cofactor. But the poor restoration yields obtained only after acidic treatment of E. coli wild type soluble fractions by McGregor et al. (16) do not confirm this hypothesis.

As shown by chl B, the presence of Mo-"peptide" is not sufficient to get NR and FDH in their active form. We suggest that soluble Mo-"peptide" is a precursor of membrane bound Mo-cofactor, and that an enzyme (transferase ?) is necessary to bind Mo-cofactor to particulate proteins, using Mo-"peptide" as substrate. chl B would lack such enzyme. It is in agreement with other authors (16).

chl A, E and F mutants would lack some enzyme necessary to the biosynthesis of Mo-"peptide". It is interesting to note that these three genes map in the same region of E. coli chromosome (chl F being still uncertain) (6).

chl D mutant seems to be more complex to define. It is the only mutant which presents alteration in the quantity of radioactivity bound to "a" and the only one which can be restored by high molybdate concentration (14, 15). chl D gene product would have to deal with the formation of Mo-"peptide" complex but it is still impossible to decide whether "a" molecule is implied in the process. We have no evidence for a regulation role (15).

The present results support the idea that defects in the biosynthesis and transfer of a Mo-cofactor can explain the pleiotropic chlorate mutants. The alteration of the cytoplasmic membrane (27) would then be a secondary phenomenon.

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