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Accumulation on the Cytoplasmic Membrane of the Precursor to Dimethyl Sulfoxide Reductase in Molybdenum Cofactor-Deficient Mutants of *Rhodobacter sphaeroides* f. sp. *denitrificans*

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The role of the molybdenum cofactor (Mo cofactor) in the translocation of dimethyl sulfoxide (DMSO) reductase to the periplasmic space was studied in vivo by isolating chlorate-resistant mutants of *Rhodobacter sphaeroides* f. sp. *denitrificans*. More than 50% of the chlorate-resistant mutants isolated were defective in the biosynthesis of the Mo cofactor and all of these mutants accumulated the precursor form of the enzyme. About 45% of the mutants contained the same level of Mo cofactor as the parent strain and exhibited normal levels of DMSO reductase and nitrate reductase activities when chlorate was absent from the medium, but the activities of these enzymes were depressed when chlorate was present. Much of the accumulated precursor form of the enzyme in a Mo cofactor-deficient mutant was bound to the cytoplasmic membrane and was sensitive to treatment with proteinase K from the periplasmic side of the membrane, an indication that the precursor was exposed on the periplasmic surface of the membrane. The precursor accumulated on the membrane of the parent strain when molybdate was removed from the medium or upon addition of tungstate and this precursor was also sensitive to the treatment with proteinase K from the periplasmic side. These results suggest that the Mo cofactor is necessary for proteolytic processing of the precursor to the mature enzyme on the periplasmic side of the membrane, whereas binding of the precursor to the membrane and translocation across it can occur in the absence of the cofactor. Almost all of the Mo cofactor available for direct reconstitution in vitro of nitrate reductase activity from the *nit-1* mutant of *Neurospora crassa* was present in the cytoplasmic fractions.

Key words: Chlorate-resistant mutant — Dimethyl sulfoxide reductase — Molybdenum cofactor — Protein translocation — *Rhodobacter sphaeroides* f. sp. *denitrificans*.

Proteins located in the noncytoplasmic compartments of Gram-negative bacteria must be translocated across the cytoplasmic membrane after their synthesis in the cytoplasm. Mechanisms responsible for the translocation of proteins across the membrane have been elucidated to some extent by a combination of genetic and biochemical approaches. Almost all such researches have been conducted in *Escherichia coli* and involved outer membrane proteins and periplasmic enzymes (Benson et al. 1985, Wickner and Lodish 1985, Randall et al. 1987). However, translocation of proteins that contain prosthetic groups, such as those that participate in energy conservation, has been investigated to a lesser extent in bacteria. The question then arises, when we consider these proteins, as to how insertion of the prosthetic groups into apoproteins is coupled with translocation. In the case of mitochondria, it was reported that addition of heme is a prerequisite for the proteolytic processing of cytochrome c_1 that results in the production of mature-sized holocytochrome c_1 (Nicholson et al. 1989).

Rhodobacter sphaeroides f. sp. *denitrificans* (Satoh et al. 1976) is particularly suitable for investigations of the question mentioned above. The photodenitrifier possesses the enzyme that catalyze denitrification and DMSO respiration as anaerobic respiration systems. The terminal reductases in such respiratory pathways, namely, nitrate, nitrite, nitrous oxide, and DMSO reductases, are all located in the

Abbreviations: Mo cofactor, molybdenum cofactor; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

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periplasmic space as soluble proteins (Sawada and Satoh 1980, Urata et al. 1982). We studied the translocation of DMSO reductase and reported that the precursor to DMSO reductase, with a molecular mass of 84 kDa, was processed to the mature form with a molecular mass of 82 kDa during translocation. Furthermore, we demonstrated that the precursor accumulated in cells when molybdate was removed from the medium, a result that suggests that processing of the reductase is associated with the incorporation of the Mo cofactor into the apoprotein (Yoshida et al. 1991). In the present study, we examined this hypothesis by isolating mutants that lack the Mo cofactor.

In *Escherichia coli*, chlorate-resistant mutants can be isolated by virtue of their ability to grow anaerobically in the presence of chlorate ions, which are lethal to wild-type strains, and the mutations have been identified at loci *chlA* through *chlG*. Cells with mutations at the *chlA, chlB, chlD, chlE*, and *chlG* loci are pleiotropically defective with respect to the activity of molybdoenzymes. The loci *chlA* (recently resolved into *chlA* and *chlM*) and *chlE* (recently resolved into *chlE* and *chlN*) are involved in biosynthesis of the demolybdo cofactor (Miller and Amy 1983, Johnson and Rajagopalan 1987a, b). The product of the *chlB* gene is believed to be protein FA, but the reason for the pleiotropy of *chlB* is obscure (Miller and Amy 1983, Low et al. 1988). The product of the *chlD* gene catalyzes the transport of molybdate into cells (Miller and Amy 1983, Scott and Amy 1989). The product of the *chlG* gene is believed to catalyze the formation of the Mo cofactor from the demolybdo cofactor (Miller and Amy 1983). Thus, when chlorate-resistant mutants are isolated, many strains can be identified that lack the Mo cofactor.

The DMSO reductase of the photodenitrifier is a molybdoenzyme and has the ability to reduce chlorate. Therefore, DMSO respiration-deficient mutants can easily be isolated as chlorate-resistant mutants that grow anaerobically in the light, as the energy source, in the presence of DMSO and chlorate. It was believed that strains that lack the Mo cofactor could be isolated as *chl* mutants. In this report, we demonstrate that the *chl* mutants that lack the Mo cofactor are unable to process the precursor to DMSO reductase, with resultant accumulation of the enzyme on the periplasmic surface of the cytoplasmic membrane. We also demonstrate that the Mo cofactor that is available for direct reconstitution in vitro of nitrate reductase activity from the *nit-1* mutant of *Neurospora crassa* is located in the cytoplasm.

Materials and Methods

Bacteria and growth conditions—A green mutant strain of *R. sphaeroides* f. sp. *denitrificans* IL106 (Satoh et al. 1976) and its chlorate-resistant derivatives, isolated as described below, were used in this study. The medium and conditions for growth of the phototrophs were described previously (Yoshida et al. 1991).

Growth of Neurospora crassa and preparation of extracts—*N. crassa* mutant strain *nit-1* (allele 34547) was cultured as described earlier (Garret and Nason 1969). Preparation of extracts of nitrate-induced cells of *N. crassa nit-1* for use in the assay of the Mo cofactor was carried out essentially as described by Amy and Rajagopalan (1979).

Isolation of chlorate-resistant mutants—After mutagenesis with ethylmethane sulfonate of *R. sphaeroides* f. sp. *denitrificans*, cells were cultured in the light on plates that contained 0.2% (w/v) chlorate and 0.05% (v/v) DMSO under anaerobic conditions obtained by use of the GasPak systems (BBL Microbiology Systems). The colonies that developed on the plates were isolated as chlorate-resistant mutants.

Assay for the Mo cofactor—The assay for the Mo cofactor is based on the ability of the cofactor from the photodenitrifier to reconstitute the activity of NADPH-dependent nitrate reductase from cells of *N. crassa nit-1* which lack the cofactor. The assay was performed essentially as described by Miller and Amy (1983). Mo cofactor activity is expressed as units of nitrate reductase reconstituted during the 20-min complementation assay. One unit of nitrate reductase activity produces 1 nmol of nitrite per min under the conditions of the assay.

Preparation of extracts from the chl mutants for assays of total amounts of Mo cofactor—Cells were grown anaerobically in the light for 3 h in an 8-ml screw-capped tube filled with medium that contained 0.2% DMSO and an inoculum of 2 ml of an overnight culture. Cells from 2 ml of the resultant culture were suspended in 100 μl of sucrose-EDTA-Tris buffer and spheroplasts were prepared by the lysozyme-EDTA method (Yoshida et al. 1991). Then 100 μl of EDTA-Tris buffer, containing a trace of DNase, were added to the suspension of spheroplasts. The suspension was sonicated for 5 s and centrifuged at 15,000 $\times g$ for 5 min. The supernatant was heated at 80°C for 90 s in the presence of 24 mM β-mercaptoethanol, 2 mM ascorbic acid, and 25 mM sodium molybdate in order to release the Mo cofactor that was bound tightly to molybdoproteins.

Preparation of cytoplasmic, periplasmic, and membrane fractions—The procedure was carried out as described elsewhere (Yoshida et al. 1991).

Immunoblotting analysis—Immunoblotting analysis was carried out as described by Yoshida et al. (1991).

Assay of enzymatic activities—DMSO reductase was assayed as described by Satoh and Kurihara (1987). In this assay, one unit of DMSO reductase activity oxidizes 1 μmol of reduced benzyl viologen per min. The dissimilatory nitrate reductase activity of the photodenitrifier was assayed by quantitating the NO_2^- produced with reduced benzyl viologen as electron donor in the presence of 1 mM potassium cyanide, which inhibits nitrite reductase, as described

by Sawada and Satoh (1980). In this assay, one unit of the dissimilatory nitrate reductase activity produces 1 nmol of nitrite per min.

Results

Characterization of chlorate-resistant mutants—A total of 264 chlorate-resistant mutants were isolated from about 2×10^6 cells and they were tested for their ability to grow anaerobically in darkness with DMSO or NO_3^- as the electron acceptor in the presence or absence of chlorate. Their DMSO reductase or NO_3^- reductase activities were assayed in whole cells that had been grown anaerobically in the light with DMSO or NO_3^- in the absence of chlorate (Table 1). About 45% of the *chl* mutants isolated, for example, F-12, F-16, F-60, and F-80 in Table 1, were able to grow anaerobically in darkness with DMSO or NO_3^- when chlorate was not present in the medium, but they did not grow when chlorate was present (data not shown). They were, however, able to grow photosynthetically even when chlorate was present. Thus, their DMSO and NO_3^- reductase activities were almost the same as those of the parent strain when they were grown in the absence of chlorate, but these activities were undetectable in cells grown in its presence. Other mutants (more than 50% of the total), for example, F-35, F-98, F-145, F-182, and F-195 in Table 1, were unable to grow anaerobically in darkness by respiration of DMSO or NO_3^- irrespective of whether or not chlorate was present. DMSO and NO_3^- reductase activities were undetectable in these cells grown under either set of conditions. Strain F-55 was able to grow by NO_3^- respiration, but not by DMSO respiration.

Mo cofactor in chl mutants—Mutants F-35, F-98, F-145, F-182, and F-195 were pleiotropic in so far as neither DMSO reductase activity nor NO_3^- reductase activity was detectable. This observation suggests the absence of the Mo cofactor. The mutant strains listed in Table 1 were grown anaerobically with DMSO in the light in the absence of chlorate and their Mo cofactor activity was determined. Strains F-12, F-16, F-60, and F-80 shared the wild-type pattern of high cofactor activity, indicating that they are normal with respect to the synthesis of the cofactor. By contrast, strains F-35, F-98, F-145, F-182, and F-195 manifested low cofactor activity, as expected, suggesting that their mutations are in genes associated with the synthesis of the cofactor. Strain F-55 had high cofactor activity.

Processing of DMSO reductase in chl mutants—The relationship between Mo cofactor-deficiency and processing of DMSO reductase was examined using the *chl* mutants listed in Table 1. Exponentially grown cells of the various mutant strains were harvested 3 h after induction of DMSO reductase in the absence of chlorate and their DMSO reductase was analyzed by SDS-PAGE and immunoblotting (Fig. 1). Strains with high cofactor activity accumulated predominantly the mature form of the reductase, as did the parent strain. They generated single intense bands. By contrast, those strains with low cofactor activity accumulated the precursor form of the reductase protein as expected, although a small amount of the mature form was evident. These results are summarized in Table 1. The

Table 1 Activities of DMSO and nitrate reductases, the activity of the Mo cofactor and the nature of the accumulated form of DMSO reductase in *chl* mutants of *R. sphaeroides* f. sp. *denitrificans* grown in the absence of chlorate

Strain	Anaerobic growth[a] in darkness with		Enzymatic activity[b]		Mo cofactor activity[c]	Accumulated form
	DMSO	Nitrate	DMSO reductase	Nitrate reductase		
Parent (IL106)	+	+	10.8	3.5	597	Mature
F12	+	+	17.8	4.4	506	Mature
F16	+	+	17.2	2.3	665	Mature
F60	+	+	9.9	2.2	375	Mature
F80	+	+	18.3	3.7	492	Mature
F35	−	−	0.2	0.3	58	Precursor
F98	−	−	0.3	0.1	17	Precursor
F145	−	−	0.9	0.7	50	Precursor
F182	−	−	0.2	0.1	13	Precursor
F195	−	−	0.4	0.4	72	Precursor
F55	−	+	2.6	2.1	410	Mature

[a] +, wild-type growth; −, no growth.
[b] Units (mg cell dry weight)$^{-1}$.
[c] Units (mg protein)$^{-1}$.

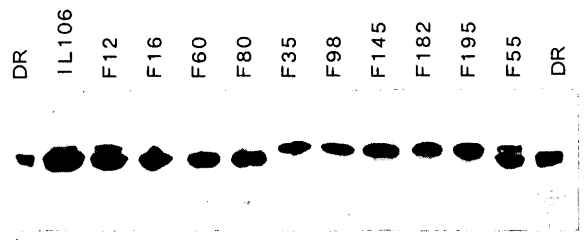

Fig. 1 The nature of the accumulated forms of DMSO reductase in chlorate-resistant mutants. Mutant strains listed in Table 1 were grown anaerobically in the light for 3 h in screw-capped tubes (8 ml) filled with medium that contained 0.2% DMSO with a 2-ml inoculum. Two ml of the culture were withdrawn and proteins in it were subjected to SDS-PAGE and immunoblotting analysis. DR refers to purified DMSO reductase.

results indicate that the Mo cofactor is essential for the processing of the precursor protein to the mature enzyme. Strain F-55 accumulated the mature form although the strain exhibited low DMSO reductase activity.

Localization of the accumulated precursor—Localization of the accumulated precursor in F-182, a typical mutant with low cofactor activity, was examined (Fig. 2). Exponentially grown cells of the parent strain and F-182 were harvested 3 h after induction of DMSO reductase, as described in the legend to Figure 1, and the cytoplasmic, membrane and periplasmic fractions were prepared. Little of the mature enzyme was found in the periplasmic fractions of F-182, whereas almost all the mature form was recovered in those fractions from the parent strain. Although the precursor to the enzyme in the parent strain was present in both the cytoplasmic and the membrane fractions, most was in the cytoplasm, as described earlier (Yoshida et al. 1991). The mature form found in the cytoplasmic fractions from the parent strain is probably the result of contamination from both the periplasmic fractions and the intact cells that escaped the action of lysozyme. By contrast, in F-182, much of the precursor seemed to be accumulated on the membranes, as judged from the fact that the amount of precursor in the membrane fractions appeared larger than that in the same fractions from the parent strain, whereas the amounts in the cytoplasmic fractions were similar for F-182 and the parent.

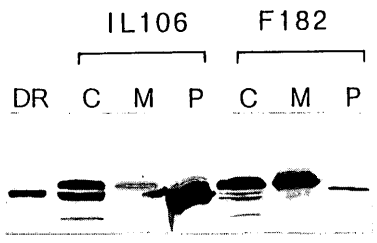

Fig. 2 Localization of the precursor and mature forms of DMSO reductase in the parent, IL-106, and in a mutant, F-182. DMSO reductase was induced for 3 h in a 120-ml culture as described in the legend to Fig. 1. The cells were then fractionated into cytoplasmic fractions (C), membrane fractions (M) and periplasmic fractions (P) as described previously (Yoshida et al. 1991) with the exception that the membranes were suspended in a volume equivalent to the periplasmic and cytoplasmic fractions. Proteins were then analyzed by SDS-PAGE and immunoblotting. DR refers to purified DMSO reductase.

Accessibility to proteinase K of the membrane-bound precursor—We reported previously that the membrane-bound precursor trapped during the course of the translocation in the wild type strain was exposed on both the cytoplasmic and periplasmic sides of the membrane (Yoshida et al. 1991). Therefore, we next examined the accumulation of the precursor on the membranes of F-182 cells. Spheroplasts of mutant F-182, which have right-side-out membranes, and chromatophores, which have inside-out membranes, were prepared, and the accessibility to proteinase K of the precursor was examined (Fig. 3). The spheroplasts treated with proteinase K were disrupted by sonication after the addition of PMSF and fractionated into cytoplasmic and membrane fractions. The precursor in the cytoplasmic fraction was recovered without digestion

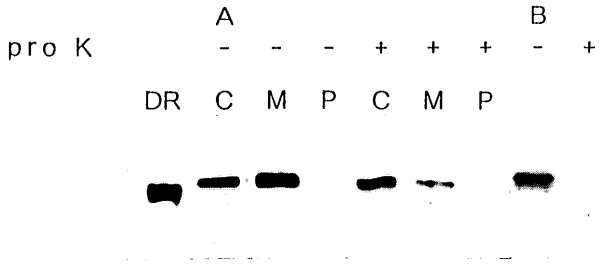

Fig. 3 Accessibility to proteinase K of the precursor form of the enzyme in spheroplasts and chromatophores of the mutant, F-182. A. DMSO reductase was induced for 3 h in a 120-ml culture and spheroplasts and a periplasmic fraction (P) were prepared. These fractions were then treated with proteinase K and PMSF as described elsewhere (Yoshida et al. 1991). The spheroplasts were further fractionated into cytoplasmic (C) and membrane (M) fractions in the presence of PMSF. The membrane fractions of the mutant F182 were suspended in a volume equal to 20% of the periplasmic and cytoplasmic fractions being 5 times more concentrated than two fractions. They were then subjected to SDS-PAGE and immunoblotting. DR and pro K refer to purified DMSO reductase and proteinase K, respectively. B. Effects of proteinase K on the precursor to the enzyme in the chromatophores. Chromatophores were obtained from cells, from 1,200-ml culture, which had been treated for induction of DMSO reductase for 3 h. The chromatophores were treated with proteinase K and PMSF and then proteins were subjected to SDS-PAGE and immunoblotting.

by the protease as anticipated, but that bound to the membranes had been digested completely. Furthermore, the precursor bound to the chromatophore membranes was digested completely by proteinase K. These results indicate that the precursor that accumulated on the membranes was exposed to both the cytoplasmic and periplasmic sides. This result is almost the same as that obtained with the parent strain (Yoshida et al. 1991) with the exception that the precursor was accumulated to a much greater extent on the membranes in the Mo cofactor-deficient mutant.

We reported previously that removal of molybdate from the medium and the addition of tungstate to the medium effectively inhibited the processing of the precursor, with resultant accumulation of the precursor (Yoshida et al. 1991). This phenomenon seems to resemble that observed in the Mo cofactor-deficient mutant. Therefore, the accessibility to proteinase K of the precursor was examined in the parent cells grown in molybdate-depleted medium (Fig. 4A) and also in molybdate-depleted medium to which tungstate had been added in order to generate a clear result (Fig. 4B). Figure 4B clearly shows that the formation of the mature form of the enzyme was inhibited and that the precursor accumulated on the membrane and was digested completely by proteinase K.

Subcellular distribution of the Mo cofactor—The Mo cofactor is thought to occur in two forms: one that is bound tightly to molybdoproteins and is able to support reconstitution of nitrate reductase activity from the *nit-1* mutant of *N. crassa* in vitro, but only after release of the cofactor by denaturing treatments, such as heat treatment or detergent extraction; and the other that is able to support reconstitution of the nitrate reductase activity without such treatments (Johnson 1980, Miller and Amy 1983). The latter form is believed to be the form that is incorporated into apoproteins to generate holomolybdoproteins.

Since it appeared that the Mo cofactor interacts with the precursor form of DMSO reductase on the periplasmic side of the membrane, we next examined the location of the Mo cofactor that is able directly to support the reconstitution in vitro of nitrate reductase activity from the *nit-1* mutant. Cells cultured with or without DMSO were fractionated into cytoplasm, membranes and periplasm and the activity of the Mo cofactor was assayed either without or after heat treatment of the respective fractions (Table 2). Almost all of the Mo cofactor activity that was detected in the absence of heat treatment was in the cytoplasmic fractions, whether or not cells had been cultured with DMSO. The cytoplasmic activity appeared to be unaffected by heat treatment. In the cytoplasmic fractions from cells cultured

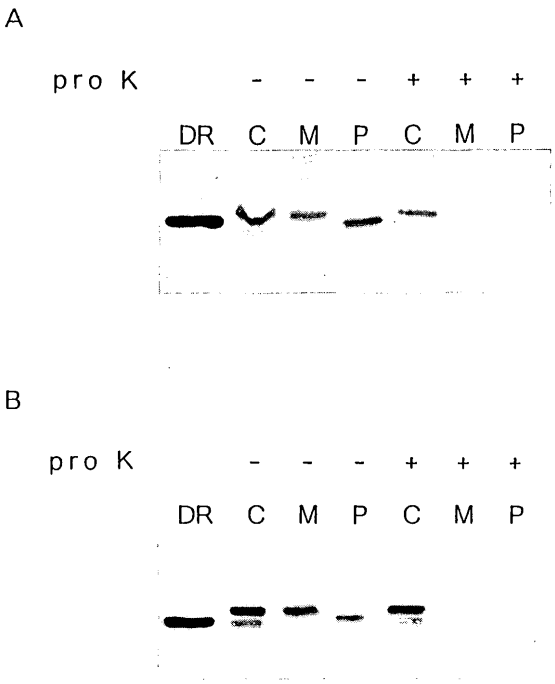

Fig. 4 Accessibility to proteinase K of the precursor to the enzyme in spheroplasts of the parent strain grown in molybdate-depleted medium (Fig. 4A) and in molybdate-depleted medium to which 10 mM tungstate (final concentration) had been added (Fig. 4B). Details of procedures and symbols can be found in the legend to Fig. 3A.

Table 2 Subcellular distribution of the Mo cofactor

Fraction	Mo cofactor activity[a]			
	Cells cultured without DMSO		Cells cultured with DMSO	
	Without heat	After heat	Without heat	After heat
Cytoplasm	64.1	59.0	80.1	184
Membrane	0.2	0.2	0.1	2.8
Periplasm	0.1	70.9	0.1	1,070

Cells (a 1,200-ml culture) were grown in medium with or without DMSO and fractionated into cytoplasmic, membrane and periplasmic fractions, and then the activity of the Mo cofactor in each fraction was assayed either without or after heat treatment.

[a] Total activity (units).

with DMSO, the activity of the Mo cofactor after heat treatment was about twice that in the absence of heat treatment. This result is probably due to contamination from the periplasmic fractions, in which the activity of the Mo cofactor was only detected after heat treatment and in which the activity of the cofactor increased considerably in cells cultured with DMSO. This observation indicates that most of the periplasmic activity of the cofactor was derived from mature DMSO reductase to which the Mo cofactor had been tightly bound. There was little detectable activity of the Mo cofactor on the membranes, irrespective of heat treatment. These results suggest that the Mo cofactor that is incorporated directly into apo-DMSO reductase to generate the holoenzyme is synthesized constitutively in the cytoplasm and stored there independently of induction of the enzyme by DMSO.

Discussion

The mechanism of translocation of proteins that contain prosthetic groups, such as those that participate in energy conservation, has not been fully investigated in bacteria. It is of interest to ask when and where the prosthetic group is incorporated into the apoprotein during the processing of the translated protein to the mature enzyme. We reported that molybdate, probably as the Mo cofactor, is necessary for processing of the precursor of DMSO reductase (Yoshida et al. 1991). In the present experiments, we isolated Mo cofactor-deficient strains as chlorate-resistant mutants and studied the role of the Mo cofactor in the processing of this precursor. More than 50% of the *chl* mutants isolated were blocked in the synthesis of the cofactor, as is the case for similar mutants of *E. coli* (Table 1). Five strains typical of such mutants all accumulated the precursor to the enzyme (Fig. 2), a result that strongly suggested the need for the Mo cofactor in the processing of DMSO reductase. Next we asked where the precursor accumulates. The precursor appeared to be trapped on the membrane and to be exposed on the outer surface of the membrane (Figs. 2 and 3). These results suggested that binding of the precursor and subsequent translocation across the membrane occur without the Mo cofactor and that the Mo cofactor is necessary on the periplasmic side for the proteolytic processing to the mature enzyme. This conclusion is consistent with the reports that removal of the amino-terminal signal sequence of secretory proteins by the processing protease in Gram-negative bacteria occurs on the periplasmic side (Randall et al. 1987), but where is the Mo cofactor incorporated into the apo-DMSO reductase? It seems likely that this incorporation occurs on the periplasmic side; a conformational change in the precursor, caused by interaction with the Mo cofactor on the periplasmic side, could be a prerequisite for susceptibility to processing by the signal peptidase. We presumed, therefore, that the Mo cofactor that was available for the interaction with the precursor would exist in the periplasm but, in fact, the cofactor was exclusively in the cytoplasm (Table 2) and did not accumulate in the periplasm or on the cytoplasmic membrane. It is, therefore, conceivable that translocation of the precursor is accompanied by transport of the Mo cofactor to the periplasmic side of membrane from the cytoplasm.

Four mutants, F-12, F-16, F-60, and F-80, contain the same levels of DMSO reductase and nitrate reductase as the parent strain when chlorate is not present in the medium, but their reductase activities are depressed when chlorate is present. This depression in activity explains why these mutants are resistant to chlorate. This type of mutant seems not to be isolated in *E. coli*. Since mutants of photosynthetic bacteria, even if they have no DMSO reductase or nitrate reductase activity in chlorate-containing medium, can grow anaerobically, with their demands for energy being met by photosynthesis, many such mutants may have been isolated.

Mutant F-55 is of particular interest. The level of the Mo cofactor is normal and the mature form of DMSO reductase is produced, but the mutant has low DMSO reductase activity and is unable to grow anaerobically by DMSO respiration. We have no explanation for this phenomenon at present.

The structure of the Mo cofactor of the DMSO reductase of this photodenitrifier was recently found to be unusual in that it contains a modified form of molybdopterin, namely, molybdopterin guanine dinucleotide (Johnson et al. 1990). However, the Mo cofactor extracted from DMSO reductase was able to support reconstitution in vitro of an active nitrate reductase from the *nit-1* mutant of *N. crassa*. This result suggests that addition of GMP to the molybdopterin is not essential for the functioning of the cofactor.

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