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Synthesis of Nitrate Reductase Components in Chlorate-Resistant Mutants of *Escherichia coli*

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Specific antibody to purified nitrate reductase from *Escherichia coli* was used to identify enzyme components present in mutants which lack functional nitrate reductase. *chl*A and B mutants contained all three subunits present in the wild-type enzyme. Different peptides with a broad range of molecular weights could be precipitated from *chl*C mutants, and *chl*E mutants contained either slightly degraded enzyme subunits or no precipitable protein. No mutants produced significant amounts of cytoplasmic enzyme. The *chl*A and B loci are suggested to function in the synthesis and attachment of a molybdenum-containing factor. The *chl*C locus is suggested to be the structural gene for nitrate reductase subunit A and *chl*E is suggested to be involved in the synthesis of the cytochrome b_1 apoprotein.

In *Escherichia coli* there is a class of mutants with defects in anaerobic electron transport which are referred to as chlorate-resistant (*chl*) mutants. These have been mapped at seven different genetic loci (15). They are isolated by their ability to grow anaerobically in the presence of a compound which is lethal to wild-type organisms, $KClO_3$. This ability is a result of the loss of nitrate reductase activity in these mutants (1). Chlorate resistance mutations affect other cytoplasmic membrane proteins besides nitrate reductase. Formate dehydrogenase, cytochrome b_1, and other unidentified membrane proteins have been shown to be absent or nonfunctional in these mutants (9).

Nitrate reductase from wild-type *E. coli* was shown to contain three different subunits: subunit A (molecular weight 142,000) is thought to contain the catalytic site, subunit B (molecular weight 60,000) is thought to function in attachment of the enzyme to the membrane, and subunit C (molecular weight 19,500) is the cytochrome b_1 apoprotein (7, 8). The native enzyme probably consists of four A subunits and four B subunits (12), plus eight C subunits for a total molecular weight of almost 1,000,000 (8). To better understand the assembly and regulation of this complex enzyme system, nitrate reductase polypeptides from several different chlorate-resistant mutants were isolated by immune precipitation and compared to wild-type nitrate reductase.

MATERIALS AND METHODS

Strains. Chlorate-resistant strains were isolated from a spontaneous *gal*$^-$ mutant (RK 20) of RK 7 (12). *chl*D was not used in these experiments because, grown under conditions of maximum nitrate reductase induction, this mutant produced significant levels of enzyme. Mutants were checked for nitrate reductase activity by enzyme assay and by following the formation of nitrite in the growth medium (12). In all cases, enzyme activity was less than 0.5% of wild-type levels.

Growth medium. Strains were grown anaerobically both on minimal medium (7) and minimal medium supplemented with the following compounds (amounts are expressed as mg/liter): DL-alanine 400; L-asparate, 120; L-cysteine, 50; L-glutamate, 200; glycine, 100; histidine, 100; DL-isoleucine, 100; L-lysine, 200; L-leucine, 100; L-methionine, 50; proline, 100; phenylalanine, 100; L-serine, 50; L-arginine, 100; L-tyrosine, 50; DL-valine, 50; L-threonine, 50; L-tryptophan, 50; adenosine, 20; uracil, 20; thymine, 20; thiamine, 0.4; pantothenate, 0.4; nicotinic acid, 0.4; riboflavin, 0.4; and biotin, 0.4. In both cases, glucose was the carbon source. No difference was seen in the enzyme activity or protein profile between cells grown in the two media. The only difference was an increase in doubling time in the unsupplemented medium.

All other methods have been described in the previous papers (7, 8).

RESULTS

Components of the nitrate reductase system in the mutants were examined in two cell fractions: they were precipitated with antibody from Triton-solubilized cytoplasmic membrane (7) and they were precipitated with antibody from the cytoplasmic fraction, after removal of membrane fragments from the cytoplasm by incubation at 32 C (8, 11). Figure 1 shows the protein profile on sodium dodecyl sulfate (SDS)

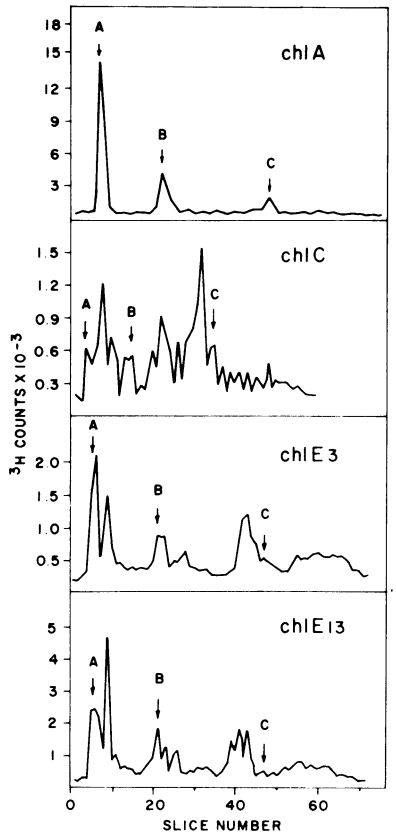

FIG. 1. *Protein profile on SDS gels of antibody-precipitable protein from the Triton-extracted cytoplasmic membrane of chlorate-resistant mutants. ^{3}H-labeled protein was precipitated from chlA and chlB extracts with both rabbit and goat serum (7). Only rabbit serum was used to precipitate proteins from chlC and chlE. Precipitates were mixed with a small amount of ^{14}C-labeled, Triton-solubilized nitrate reductase from strain RK 7 (7), then run on 7.5% gels. Subunits A, B, and C, as determined by the ^{14}C marker, are indicated by arrows. Protein profiles of chlA and chlB are identical, therefore only that of chlA is shown.*

gels of nitrate reductase protein found in the Triton-solubilized membrane fraction of mutants with defects which mapped at the *chl*A, *chl*C, and *chl*E loci (9). The membranes of *chl*A and B mutants contain nitrate reductase with all three subunits present. The mutant enzymes were identical on gels to that found in the wild type except that only 30% of subunit C was evident.

The membrane fraction of *chl*C also contains antibody-precipitable protein; however, this protein is composed of numerous peptides with a maximum molecular weight of 142,000. The membranes of three *chl*E mutants were examined for nitrate reductase protein. Two contained antibody-precipitable protein which appeared virtually identical on SDS gels. This enzyme consisted of fragments but they were less variable in size than those found in the *chl*C membrane (Fig. 1). The third *chl*E mutant contained no antibody-precipitable material.

Table 1 gives the percent of total cytoplasmic membrane protein which is precipitated by antibody against nitrate reductase. *chl*A and B have wild-type amounts of nitrate reductase protein; however, *chl*C and E have considerably less. Moreover, this protein is distributed in numerous peptides rather than in only three peptides (Fig. 1). For this reason, larger amounts of the precipitates from *chl*C and E had to be applied to SDS gels and consequently the immune co-precipitates (rabbit anti-nitrate reductase plus goat anti-rabbit gamma globulin) contained high background counts due to cross reaction of the goat serum with bacterial protein (7). Thus, these gels were difficult to interpret. When control precipitates with rabbit preimmune serum and goat anti-rabbit gamma globulins were run on gels and the nonspecific precipitate counts were subtracted out, the gel profiles of the *chl*C and E precipitates were identical to those of material precipitated with only the rabbit anti-nitrate reductase serum. Therefore only the gel patterns from direct

TABLE 1. *Distribution of nitrate reductase and cytochrome b_1 in mutant and wild-type (RK 7) strains*[a]

Strain	NR protein in cytoplasmic membrane (%)	Cytochrome b_1/mg of protein in cytoplasmic membrane	Cytochrome b_1 in precipitated NR
*chl*A-16	18	11	–
*chl*B-36	15	10.5	–
*chl*C-19	8	12	–
*chl*E-3	3	6.5	–
*chl*E-13	8	8	–
*chl*E-44	0	4	–
RK 7	15	24	+

[a] Protein in all strains was labeled with [^{3}H]leucine (7). The membrane fractions were isolated and solubilized with Triton ×-100 (7). Rabbit anti-nitrate reductase (NR) was added to 1 ml of each extract until no further precipitate appeared. The antibody mixtures were incubated (7) and the precipitated material was pelleted, washed, and suspended in 0.15 M NaCl. Difference spectra were run on both precipitates and Triton extracts (8). Cytochrome b_1 is expressed as Δ optical density at 558 nm per milliliter × 10^3. Nitrate reductase protein in the cytoplasmic membrane was determined by the amount of isotope present in the antibody precipitates. It is expressed as percent of total Triton-soluble protein.

precipitates with rabbit anti-nitrate reductase are shown in Fig. 1 for *chl*C and E.

Only very small amounts of material could be precipitated from the cytoplasmic fraction of any of the mutants. In the SDS gel profile of precipitates from *chl*A and B cytoplasm, only the A subunit was distinguishable. In *chl*C cytoplasm, a few high-molecular-weight peptides were found, and in *chl*E cytoplasm, fragments of all sizes were present. Thus, none of these mutants appears to be completely unable to attach the enzyme (or portions of the enzyme) to the membrane, as would be indicated by the presence of significant amounts of intact subunits in the cytoplasm. All except *chl*E-44 have precipitable material in the membrane.

Difference spectra (8) were run on both the Triton extract before immune precipitation and on the antibody precipitates from these extracts from all the mutants. It is evident from Table 1 that all the mutants had a reduced amount of cytochrome b_1 in their cytoplasmic membranes. Also, none of the mutants had any detectable absorbance at 558 nm (cytochrome b_1) in the precipitated nitrate reductase. This is somewhat surprising since *chl*A and B appear to have the cytochrome b_1 apoprotein bound to nitrate reductase, although it is present in reduced amounts. It was calculated that 30% of wild-type levels of cytochrome b_1 would have been easily detectable if it had been present in these precipitates.

DISCUSSION

Previous studies (9, 10, 11) have enabled us to draw some conclusions about the nature of the defect in these mutants. Reconstitution experiments with the various mutants showed that the formation of methyl viologen assayable nitrate reductase activity in vitro from mixtures of extracts required three things: (i) membrane-bound nitrate reductase protein subunits, (ii) a molybdenum-containing cofactor (13) (Mo-X), and (iii) a soluble association factor necessary for attachment of Mo-X to the enzyme. The exact nature of Mo-X and the association factor are unknown at this time. Table 2 shows the distribution of these various components in the particulate and soluble fractions of the mutants and the wild-type parent. We have shown that reconstitution of nitrate reductase in vitro occurs during the re-association at 32 C of non-sedimentable membrane fragments formed during cell breakage. The Mo-X cofactor and the association factor are considered soluble because they remain soluble when extracts are incubated at 32 C (11).

The data presented in this paper on the occurrence of the various subunits in the mutants support the observations summarized in Table 2 and allow some conclusions to be drawn about the nature of the genetic lesion in each of the classes of mutants. These are as follows.

ChlA. This mutant contains normal amounts of intact subunits A and B in the membrane. Subunit C (identified as the apoprotein of cytochrome b_1) is also present, but in reduced amounts. Since this mutant can provide membrane-bound nitrate reductase proteins in reconstitution experiments, the A and B subunits must be functional. Subunit C is not required for enzyme activity measured with methyl viologen as the electron donor.

Molybdenum is present in purified nitrate reductase (12) and it is known that this metal can, by itself, slowly reduce nitrate to nitrite (5). Since the essential polypeptides are normal in this mutant, the lack of activity must be due to a lack of molybdenum in the enzyme. Previous results (11) have shown that Mo-X is missing in both the cytoplasm and membrane of *chl*A, and it seems most likely that the lesion in this mutant is a defect in the synthesis of Mo-X.

ChlB. The polypeptide content of this mutant is identical to that of *chl*A, and membranes of this mutant are lacking Mo-X (11). Since Mo-X accumulates in the cytoplasm of this mutant, the defect must lie in the attachment or insertion of Mo-X into the enzyme.

ChlC. Membranes of this mutant contain a substantial amount of antibody-precipitable material. However, when this is analyzed on gels this consists of peptides ranging in size from nearly intact subunit A to very small peptides. The best explanation for this result is that this mutant makes a defective enzyme which is cleaved by proteolysis. Such a situation has been observed with other proteins in *E. coli* (2). For example, Goldschmidt (3) has studied the fate of β-galactosidase in a mutant which made a defective protein. The defective poly-

TABLE 2. *Distribution of nitrate reductase components in mutant and wild-type strains (10, 11)*

Strain	Nitrate reductase subunits		Mo-X cofactor		Association factor	
	Cyto-plasm	Mem-brane	Cyto-plasm	Mem-brane	Cyto-plasm	Mem-brane
Wild type	−	+	−	+	+	−
chl A	−	+	−	−	+	−
chl B	−	+	++	−	−	−
chl C	−	−	−	−	+	−
chl E	−	−	−	−	+	−

peptide, which was almost the same size as the wild-type enzyme, was synthesized and then subsequently degraded by proteolysis. Under the same conditions, the wild-type enzyme was stable.

It has been postulated that *chl*C is the locus for one of the structural genes for nitrate reductase (4), and that would be entirely consistent with the data presented here. Furthermore, it would seem most likely that *chl*C is the locus for the structural gene for subunit A. Two lines of evidence support this. First, it has been suggested by other experiments (7) that subunit B is involved in attachment of the enzyme to the membrane. If this is correct, one would predict that a mutation in the structural gene for subunit B would result in a defect in attachment of the enzyme, and subunit A should accumulate in the cytoplasm. Second, the gel profile of the antibody-precipitable protein from *chl*C indicates that subunit A is very extensively cleaved in this mutant, much more so than in heme-deficient mutants in which the B subunit is extensively cleaved (8).

ChlE. These mutants exhibit a variety of phenotypes. Two of the mutants examined made reduced amounts of antibody-precipitable protein, whereas one made none. In the mutants which made precipitable enzyme protein, the polypeptides were fragmented by proteolysis as was observed with *chl*C. The amount of fragmentation depended upon the age of the culture. In one experiment (data not shown), mutant *chl*E-13 membrane was harvested in both the log and stationary phase, and the immune-precipitable material was examined on gels. The immune precipitate from the log-phase culture was much more like that of the wild type, whereas the material from the stationary-phase sample was much more extensively cleaved. These results suggest that the defect in *chl*E also involves one of the structural genes of the enzyme. If the *chl*C locus is the structural gene for subunit A, then *chl*E must be the locus for the structural gene for either subunit B or C.

There is evidence to suggest that *chl*E might be the locus for the structural gene for subunit C, the apoprotein of cytochrome b_1. Previous studies of a *hem*A mutant (8) implicate cytochrome b_1 in the regulation of synthesis of the other nitrate reductase subunits. When the *hem*A mutant was grown under conditions where heme could not be synthesized, there was an overproduction of subunit A (and probably subunit B as well). If there were a defect in the structural gene for the cytochrome apoprotein, this might cause a reduction in the amount of the other polypeptides. It was also observed with the *hem*A mutant that when heme was not made, subunit C was not attached to the enzyme, and subunit B was extensively degraded by proteolysis. Thus, a defect in subunit C could lead to proteolysis of the remainder of the enzyme. Some of the differences between the *hem*A mutant and the *chl*E mutant (including the lack of methyl viologen assayable activity in the *chl*E mutants) could be explained by the total lack of cytochrome b_1 in the *chl*E mutants. The cytochrome apoproteins are made in the *hem*A mutant (6), although the b_1 apoprotein is not attached to nitrate reductase (8) and may not even be attached to the membrane. The presence of this cytochrome apoprotein may be necessary for the synthesis of Mo-X and thus be required for the enzyme to have methyl viologen assayable activity.

An earlier study on these mutants (9) showed that none of the *chl*E mutants made any nitrate-inducible cytochrome b_1, whereas in *chl*A, B, C, and D mutants the nitrate-inducible cytochrome b_1 was reduced but was still present in measurable amounts. This is also shown in Table 1, in which the level of cytochrome b_1 in the *chl*E mutants is reduced to approximately the level which is found in the wild-type grown without nitrate.

One fact which is difficult to explain is that the enzyme isolated from *chl*A and *chl*B contains what appears to be subunit C, although these immune precipitates did not contain any cytochrome b_1 which could be detected in difference spectra. One possible answer is that functional nitrate reductase must be made for cytochrome b_1 to be assembled normally. Cytochrome b_1 must bind to the enzyme near the electron transfer site, and this site may require the presence of molybdenum (14), to bind the cytochrome properly. In the mutants which make an enzyme lacking molybdenum, the cytochrome may be bound in such a way that heme or iron is not inserted, or that it is labile and is lost during the solubilization and antibody precipitation.

It seems clear that the regulation and assembly of nitrate reductase is quite complex, and that the regulation of the entire anaerobic electron transport system (nitrate reductase, cytochrome b_1, formate dehydrogenase) may be interrelated.

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LITERATURE CITED

1. Azoulay, E., J. Puig, and F. Pichinoty. 1967. Alteration of respiratory particles by mutation in *Escherichia coli*. Biochem. Biophys. Res. Commun. **27**:270-274.
2. Goldberg, A. L., E. M. Howell, J. B. Li, S. B. Martel, and W. F. Prouty. 1974. Physiological significance of protein degradation in animal and bacterial cells. Fed. Proc. **33**:1112-1120.
3. Goldschmidt, R. 1970. *In vivo* degradation of nonsense fragments in *E. coli*. Nature (London) **228**:1151-1154.
4. Guest, J. R. 1969. Biochemical and genetic studies with nitrate reductase C-gene mutants of *Escherichia coli*. Mol. Gen. Genet. **105**:285-297.
5. Guymon, E. D., and J. T. Spence. 1966. The reduction of nitrate by molybdenum (V). J. Phys. Chem. **70**:1964-1969.
6. Haddock, B. A., and H. U. Schairer. 1973. Electron transport chain in *E. coli*. Eur. J. Biochem. **33**:34-35.
7. MacGregor, C. H. 1975. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. **121**:1102-1110.
8. MacGregor, C. H. 1975. Anaerobic cytochrome b_1 in *Escherichia coli*: association with and regulation of nitrate reductase. J. Bacteriol. **121**:1111-1116.
9. MacGregor, C. H., and C. A. Schnaitman. 1971. Alterations in the cytoplasmic membrane proteins of various chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **108**:564-570.
10. MacGregor, C. H., and C. A. Schnaitman. 1972. Restoration of NADPH-nitrate reductase activity of a *Neurospora* mutant by extracts of various chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **112**:388-391.
11. MacGregor, C. H., and C. A. Schnaitman. 1973. Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **114**:1164-1176.
12. MacGregor, C. H., C. A. Schnaitman, D. E. Normansell, and M. G. Hodgins. 1974. Purification and properties of nitrate reductase from *Escherichia coli* K-12. J. Biol. Chem. **249**:5321-5327.
13. Nason, A., K. Lee, S. Pan, P. Ketchum, A. Lamberti, and J. DeVries. 1971. *In vitro* formation of assimilatory NADPH-nitrate reductase from a *Neurospora* mutant and a component of molybdenum enzymes. Proc. Natl. Acad. Sci. U.S.A. **68**:3242-3246.
14. Steifel, E. I. 1973. Proposed molecular mechanism for the action of molybdenum in enzymes: coupled proton and electron transfer. Proc. Natl. Acad. Sci. U.S.A. **70**:988-992.
15. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* strain K-12. Bacteriol. Rev. **36**:504-524.