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NITRATE REDUCTASE IN E. COLI: PROPERTIES OF THE ENZYME AND IN VITRO RECONSTITUTION FROM ENZYME-DEFICIENT MUTANTS

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The nitrate reductase of E. coli is an inducible membrane protein with a molecular weight of about 800,000. The enzyme consists of four subunits of 60,000 molecular weight, four subunits of 142,000 molecular weight, four molecules of molybdenum, and nonheme iron. The enzyme may be solubilized by heat extraction, which results from limited digestion by a membrane-bound protease, or by Triton X-100. When the enzyme is isolated from Triton-solubilized cytoplasmic membrane by immune precipitation, it contains a third protein of 20,000 molecular weight which may be a cytochrome.

Chlorate resistant (chl) mutants of E. coli lack functional nitrate reductase. Mutants of the classes chlA and chlB have all of the enzyme polypeptides present in the membrane in intact form, while in classes chlC and chlE the membrane contains degraded fragments of the polypeptides, suggesting proteolysis of a defective enzyme. Reconstitution of nitrate reductase activity occurs when soluble extracts of various classes of mutants are mixed and incubated at 32°C. This reconstitution requires three things: (a) intact enzyme polypeptides in the form of small soluble lipoprotein fragments resulting from fragmentation of the cytoplasmic membrane during cell breakage; (b) a molybdenum factor which is present in the wild-type membrane and which accumulates in the cytoplasm of chlB mutants in soluble form; and (c) a soluble factor or enzyme, presumably the chlB gene product, which adds the molybdenum factor to the enzyme

Two conclusions may be drawn from these observations. First, the enzyme is bound to the membrane by small, hydrophobic regions on one or more of the subunits. Second, the process of reconstitution from mutant extracts is different from the process involved in de novo synthesis of the enzyme in wild-type E. coli.

In 1967 Piechaud et al. (1) isolated a class of mutants in E. coli which they called chlorate-resistant because they were not killed by anaerobic growth in the presence of

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chlorate. This chlorate-resistance is due to the absence in all these mutants of the enzyme nitrate reductase, which will reduce chlorate to a compound which is lethal to the organism. During anaerobic growth, nitrate acts as a terminal electron acceptor. Formic dehydrogenase is apparently the main electron donor in this system, transferring electrons to nitrate reductase via cytochrome b (2). All of these components are tightly associated with the cytoplasmic membrane, and in most of these mutants they are absent or lowered in amount.

Since the initial isolation of these mutants, seven distinct genetic loci have been found, all of which were selected on the basis of the absence of nitrate reductase or formic dehydrogenase (3). Only one of these mutants has been characterized in regard to its defect. The chlD locus has been shown to be involved in the processing of molybdenum (a component of nitrate reductase), and these mutants can be restored to wild-type levels of the enzyme by growth in high amounts of molybdenum (4). It has been speculated that the chlC locus is the structural gene for nitrate reductase, since this mutant has the highest amount of formic dehydrogenase (5).

The most interesting fact about these mutants is that when the cytoplasmic extracts (i.e., without membranes) of two particular genetic types, chlA and chlB, are mixed, membranous particles are formed which show restored nitrate reductase activity (6). It was proposed that this in vitro reconstitution of active, membrane-bound enzyme mimics the in vivo assembly of the enzyme in wild-type cells.

RESULTS AND DISCUSSION

Nitrate Reductase: General Properties

In order to look at the assembly of functional nitrate reductase from nonfunctional enzyme parts, we should first consider the structure of the enzyme. To solubilize the enzyme, the envelope fraction was heated to 60°C in dilute alkaline buffer at a very low protein concentration. Released from the membrane in this fashion, the enzyme is soluble. It then was purified to homogeneity. The purified enzyme is an extremely large spherical molecule with a molecular weight of approximately 800,000. It is composed of two different subunits with molecular weights of 142,000 and 58,000 which are present in a 1:1 ratio. The structure we have proposed from this data is that of a double tetrahedron with four large and four small subunits. This octameric enzyme contains three to four molecules of molybdenum and an undetermined amount of nonheme iron (7).

Maximal amounts of this enzyme are made when E. coli is grown anaerobically in the presence of nitrate with trace amounts of molybdenum and selenium (8). Under these conditions, nitrate reductase makes up at least 15% of the total protein of the cytoplasmic membrane.

Chlorate-Resistant Mutants: General Properties

Since the defect in these mutants involves the functional loss of one or more membrane-associated enzymes, we examined the protein composition of their cytoplasmic membranes on SDS (sodium dodecyl sulfate)-polyacrylamide gels. The proteins of the cytoplasmic membrane are easily separated from those of the outer membrane by ex-

traction with Triton X-100 in the presence of Mg^{++} (9). When Triton-soluble proteins from a nitrate-induced culture are compared to those from an uninduced culture on gels, a striking difference is observed. In the membrane of the nitrate-induced culture, the larger subunit of the enzyme appears as a major band at the top of the gel. This band is barely detectable in membranes of uninduced cultures. Thus one can easily detect the presence or absence of enzyme in the cytoplasmic membrane by examining it on gels (10).

Gels of the cytoplasmic membranes of the mutants, grown in the presence of nitrate, showed that the membranes of chlC, chlD, and chlE completely lacked the larger enzyme subunit, while in chlA and chlB this protein was present in the membrane in normal amounts. Four of these mutants also lacked other lower-molecular-weight proteins, present in the membrane of the wild-type. A protein with a molecular weight of approximately 60,000 was missing in the chlC, chlD, and chlE mutants and probably corresponds to the smaller of the two enzyme subunits. ChlA lacked two other smaller proteins which are as yet unidentified. The membrane of chlB was similar to that of the wild-type. All five of these mutants contain no detectable enzyme activity when assayed with methyl viologen as the electron donor (10).

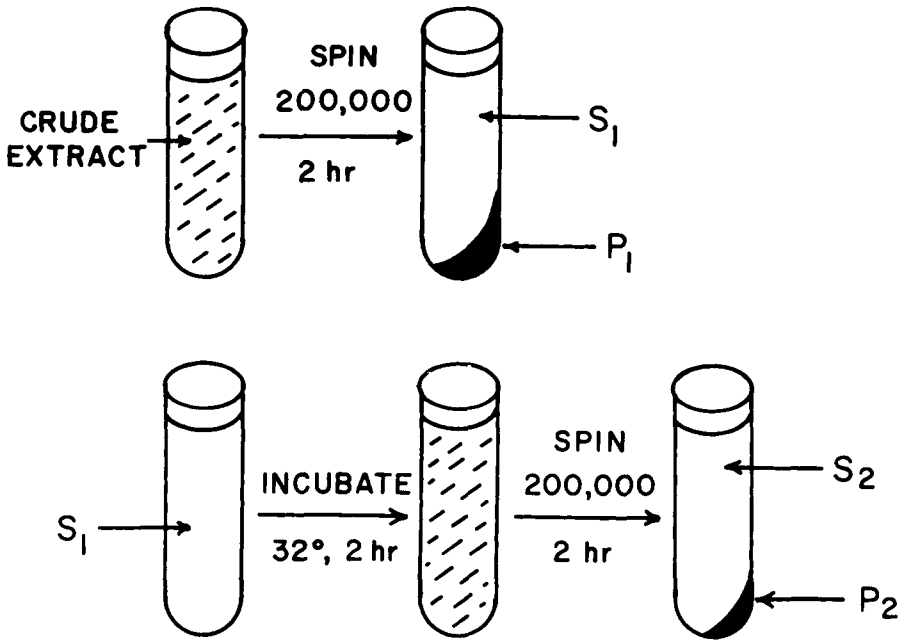
The amount of membrane-bound formic dehydrogenase was also measured in all the mutants and was found only in chlC at about 2% of the wild-type levels. All the mutants had lowered amounts of nitrate-induced cytochrome b. The highest levels of this cytochrome were found in chlA and chlB, which contained, respectively, 35% and 43% of that found in the wild type (10).

The cytoplasm of chlB contained very high levels of a molybdenum factor, shown by Nason et al. (11) to restore NADPH-nitrate reductase activity to a Neurospora mutant lacking this activity. This factor was present in the cytoplasm of the wild type in lower amounts than in the chlB and was present in the membrane as well. The membrane of the chlB did not contain this factor and neither did cytoplasm or membrane fractions of any of the other mutants (12).

Therefore, although none of these mutants possesses a functional nitrate reductase, two of them make normal levels of at least one of the enzyme subunits. It will become evident that the presence of these proteins is essential for reconstitution of functional nitrate reductase from various combinations of mutant extracts.

Membrane Formation

In the original experiments of Azoulay and Puig (6) reconstitution of active nitrate reductase was accomplished by first removing particulate proteins (cell envelope) from crude extracts of chlA and chlB by centrifugation. The supernatants from each were mixed and incubated at 32°C, resulting in the formation of sedimentable membranous particles which contained restored nitrate reductase activity. This formation of particles from soluble extracts was interpreted as being directly related to the reconstitution of active enzyme. Actually, one can observe the formation of these particles using wild-type E. coli extracts, and this occurs independently of enzyme reconstitution. This is shown in Fig. 1. Wild-type E. coli is broken in a French pressure cell and the crude extract is centrifuged at 200,000 × g, resulting in a particulate or envelope fraction (P_1) and a soluble or cytoplasmic fraction (S_1). When the clear soluble fraction is incubated at 32°C for two hours, the extract becomes turbid and, when centrifuged as before, a second particulate



	NR	SDH	MDH
S_1	5.5	165	855
P_1	118	1,358	> 0.5
S_2	> 0.1	43	873
P_2	87	1,647	> 0.5

Fig. 1. Fractionation scheme. After breakage in a French pressure cell, the crude extract is centrifuged for 2 hr at 200,000 × g. The particulate material (P_1) is separated from the soluble mateiral (S_1) and the clear S_1 fraction is incubated at 32°C. At the end of 2 hr the S_1 becomes turbid and, when recentrifuged for 2 hr at 200,000 × g, a second particulate fraction (P_2) is obtained. In the table below is shown the specific activity of two membrane-bound enzymes, nitrate reductase and succinic dehydrogenase, and a soluble enzyme, malic dehydrogenase. The membrane-bound enzyme in the S_1 fraction accounts for 20–30% of the total enzyme activity in the crude extract.

fraction (P_2) is obtained. If the first soluble fraction (S_1) is incubated at 4°C, no P_2 fraction is formed. Looking at the distribution of soluble and membrane-bound enzymes in these fractions (Fig. 1), we found that the removal of the P_2 fraction, after incubation, also removed the majority of the membrane-bound enzymes in the S_1 fraction, nitrate reductase, and succinic dehydrogenase. The soluble enzyme malic dehydrogenase was not

affected. This indicates that there are membrane proteins present in the soluble (S_1) fraction which can be removed by incubation at 32°C followed by centrifugation (13).

To interpret our reconstitution results, we had to determine the origin of these membrane proteins in the soluble fraction. It is possible that these proteins might be newly synthesized cytoplasmic membrane precursors present in the cytoplasm in a soluble pool. Another possibility is that these proteins are derived from the cytoplasmic membrane during breakage and are lipoprotein fragments too small to be sedimented. If the first explanation were correct, one would expect a precursor-product relationship between the protein which becomes particulate (P_2) and the already assembled membrane (P_1). Two lines of evidence show that this relationship does not exist. By measuring nitrate reductase levels in the wild-type P_1 and P_2 fractions at short intervals after enzyme induction, we found that new enzyme activity appeared at the same rate in both fractions. If the P_2 fraction contained membrane precursors, the enzyme should have appeared first in the P_2 and later in the P_1. Similarly, kinetics of incorporation of radioactive leucine into protein showed that radioactive leucine was incorporated into the P_1 and P_2 fractions at the same rate. We attempted to find evidence for a pulse of incorporated radioactivity being chased from the soluble membrane proteins (P_2) into the membrane (P_1) in prelabeled cells, but radioactivity was diluted from both fractions at identical rates (13). Therefore, the soluble membrane proteins which form the P_2 fraction must be lipoprotein fragments broken from the cytoplasmic membrane. The P_2 fraction has been shown to have a lipid-to-protein ratio similar to that of the cytoplasmic membrane (14).

There is still the question of whether these particles (P_2) result from reaggregation of specific membrane fragments or they represent random fragmentation of the membrane. To answer this, we compared on SDS gels the cytoplasmic membrane proteins from chlA and chlB to the Triton-soluble proteins in the P_2 fraction, produced by incubating chlA and chlB cytoplasmic (S_1) fractions together. The resulting P_2 fraction contained all the cytoplasmic membrane proteins of the chlA and chlB membranes in proportional amounts (13).

Contributions of chlA, chlB, chlC, and chlE During Reconstitution

Although we now know that the membrane proteins in the cytoplasmic (S_1) fraction are actually lipoprotein fragments which originated in the cytoplasmic membrane, we would like to be able to determine whether any truly soluble or cytoplasmic factors interact with these lipoprotein fragments to form the active nitrate reductase associated with the P_2 fraction after reconstitution. As illustrated in Fig. 1 we do have a means of removing 90% of the membrane lipoprotein fragments from the cytoplasm. We can then use the resulting S_2 fraction to look for soluble components that participate in reconstitution.

To test for reconstitution, each desired combination of mutant extracts was mixed together and incubated at 32°C for two hours. The resulting mixtures were assayed for nitrate reductase activity, centrifuged at 200,000 × g, and the P_2 fractions removed and assayed for nitrate reductase activity. From many experiments, involving all possible combinations of mutant extracts (Table I), the following observations were made:

(1) Any combination of S_1 or S_2 fractions which did not contain chlB produced no active enzyme. Since chlB is the only mutant which contains the molybdenum factor,

TABLE I. Reconstitution Combinations

Reconstitution +		Reconstitution −	
(1)	All combinations including chlB S_1	(1)	Any combination without chlB
(2)	chlB S_2 + chlA S_1	(2)	chlB S_2 + chlC or E S_1
(3)	Wild-type S_2 + chlB S_1	(3)	Wild-type S_2 + chlA, C, or E S_1
		(4)	Any combination of two S_2 fractions
		(5)	Any combination of two "preincubated" S_1 fractions
		(6)	chlB S_2 + chlE S_1 + soluble chlA enzyme
		(7)	chlB S_2 + chlA S_2 + chlA P_1

reaction between the lipoprotein fragments and the molybdenum factor must be necessary for reconstitution of active enzyme.

(2) Mixing chlB S_2 with the S_1 from chlC or chlE produced no active enzyme. Mixing chlB S_1 with the S_2 from chlC or chlE did produce active enzyme. Both of the combinations, chlB S_1 plus chlA S_2 and chlB S_2 plus chlA S_1, produced active enzyme. This tells us that the S_1 fraction, which donates the lipoprotein fragments to the mixture, must come from a mutant which has enzyme subunits already in its cytoplasmic membrane. Because the chlB S_2 fraction will still form active enzyme, the molybdenum factor must be a cytoplasmic (soluble) factor which can react with the particulate fragments supplied by chlA S_1 or chlB S_1. Formation of active enzyme also requires another soluble component (the chlB gene product) which is supplied equally well by the S_2 fraction of chlA, chlC, or chlE. This can react with the chlB S_1 which supplies both the molybdenum factor and the particulate enzyme subunits.

(3) The S_2 fraction from the wild type (which contains almost no measurable enzyme activity) also reacted with the chlB S_1 to produce active enzyme, but it did not react with any fraction of any other mutant. Therefore, the wild-type cytoplasm must contain the chlB gene product but not the molybdenum factor.

(4) No combination of S_2 fractions produced active enzyme. This indicates that enzyme subunits on lipoprotein fragments must be present. Further, if S_1 fractions are incubated alone at 32°C before mixing but the P_2 fraction is *not* removed, no active enzyme is produced when these preincubated S_1 fractions are mixed. It appears that aggregation of the soluble lipoprotein fragments prevents reconstitution.

(5) Using ^{14}C-labeled chlA and ^{3}H-labeled chlB in the combinations chlA S_1 plus chlB S_2 and chlA S_2 plus chlB S_1, we looked on gels for evidence of specific proteins donated to the P_2 fraction by either of the S_2 fractions. We found that very little radioactivity and no specific proteins were donated by either S_2. Therefore the cytoplasmic factors are (a) functioning in a catalytic fashion, (b) too small to be detected, or (c) not proteins.

(6) Using chlA S_2 plus chlB S_2 or chlB S_2 plus chlE S_1, no reconstitution could be achieved if we attempted to use chlA heat-solubilized enzyme, chlA Triton-solubilized

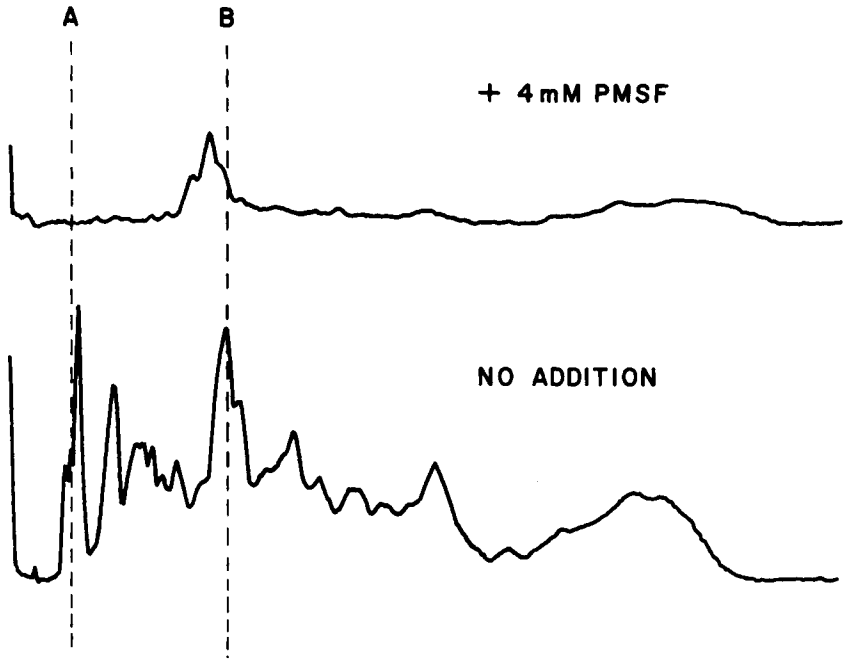


Fig. 2. Comparison of membrane protein solubilized in the presence and absence of PMSF. The washed cell envelope was suspended at 0.4 mg of protein per ml in 5 mM phosphate buffer, pH 8.3. After dividing this material in half, 4 mM PMSF was added to one half and both were heated at 60°C for 10 min. All particulate material was removed by centrifugation and the supernatants were concentrated and run on SDS gels. The figures are tracings of gels stained with Coomassie Blue.

enzyme, or mechanically fragmented chlA envelope (P_1) as a source of the enzyme subunits.

In summary, active nitrate reductase is reconstituted in these mutants by adding a molybdenum factor to enzyme subunits which exist in lipoprotein fragments, and this reaction is probably enzyme mediated (13).

Immune Precipitation of Enzyme Components

When nitrate reductase was purified to homogeneity after initial solubilization by heat extraction, different preparations showed a variability in their subunit composition. Although a single high-molecular-weight subunit (142,000) was always present, the number of lower-molecular-weight subunits varied from one to three. These smaller subunits all had a molecular weight of very close to 58,000 (7). This variability in subunit size led us to believe that solubilization might be the result of a protease which was activated by the alkaline heat treatment. We tested this idea by heating the cell envelope in the presence of the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF). When the heat extraction buffer contained 4 mM PMSF, all the enzyme activity remained associated with the envelope. A comparison on SDS-polyacrylamide gels (Fig. 2) of the material solubilized from the envelope in the presence and absence of PMSF shows that neither of the enzyme subunits (A and B) appears in the material solubilized in the

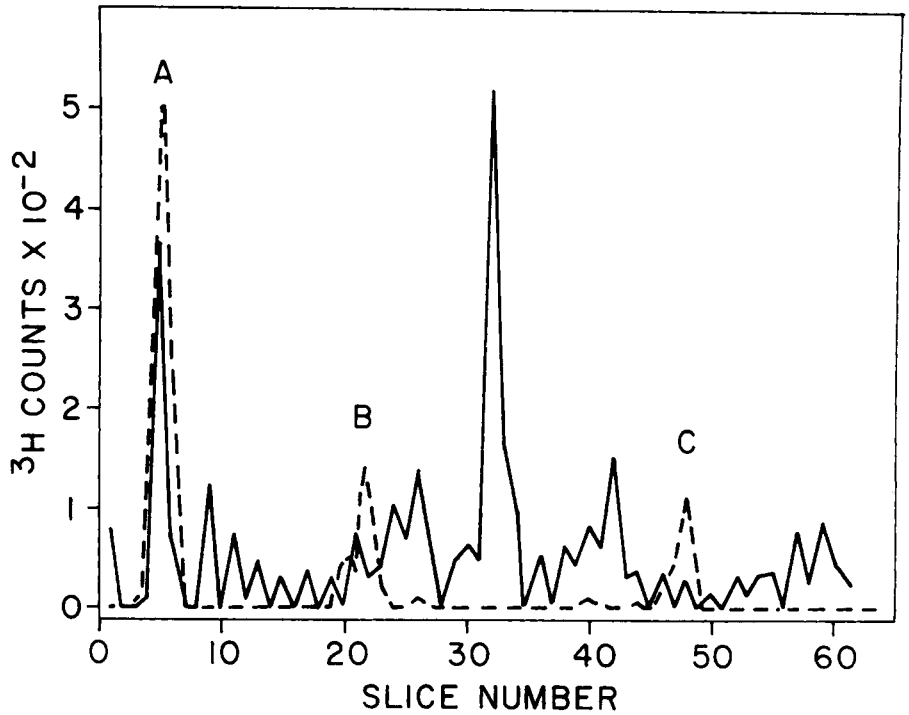


Fig. 3. Comparison of heat-extracted enzyme with Triton-extracted enzyme on SDS gels. ^{3}H-labeled proteins were solubilized from the cell envelope by heating as in Fig. 2 (no PMSF) or by extraction at room temperature with 2% Triton X-100. Each soluble extract was incubated at 37°C for one hour with rabbit anti-nitrate reductase serum. Goat anti-rabbit gamma globulin serum was used in precipitating the enzyme-antibody complex by incubation of the goat-rabbit-enzyme mixture at 37°C, then overnight in the cold. The washed precipitate was solubilized and run on SDS gels which were sliced and counted. The dashed lines indicate the heat-extracted protein; the solid lines represent the Triton-solubilized protein. The top of the gel is on the left.

presence of PMSF. Thus, the heat-solubilization procedure requires the action of a protease to release nitrate reductase from the membrane.

Further evidence for protease action was provided by experiments using immune precipitation of the enzyme. Rabbits were immunized with nitrate reductase purified to homogeneity after heat-solubilization. The serum from these rabbits specifically precipitated nitrate reductase proteins from a mixture of soluble proteins, and this was used to compare heat-solubilized enzyme to enzyme solubilized at room temperature with the detergent Triton X-100. Immune precipitation was done by incubating the heat-solubilized envelope and the Triton-solubilized envelope first with rabbit serum and then with goat anti-rabbit γG to precipitate the antigen-antibody complex. The envelope preparation was from a culture labeled with ^{3}H-leucine. The immune precipitate was washed, solubilized, and run on SDS gels. These were subsequently sliced and counted. Figure 3 shows a comparison of nitrate reductase proteins from heat-soluble and Triton-soluble extracts. The heat-solubilized enzyme contains the 142,000 and the 58,000 peaks plus fragments of various sizes. The Triton-solubilized enzyme contains a 142,000 peak

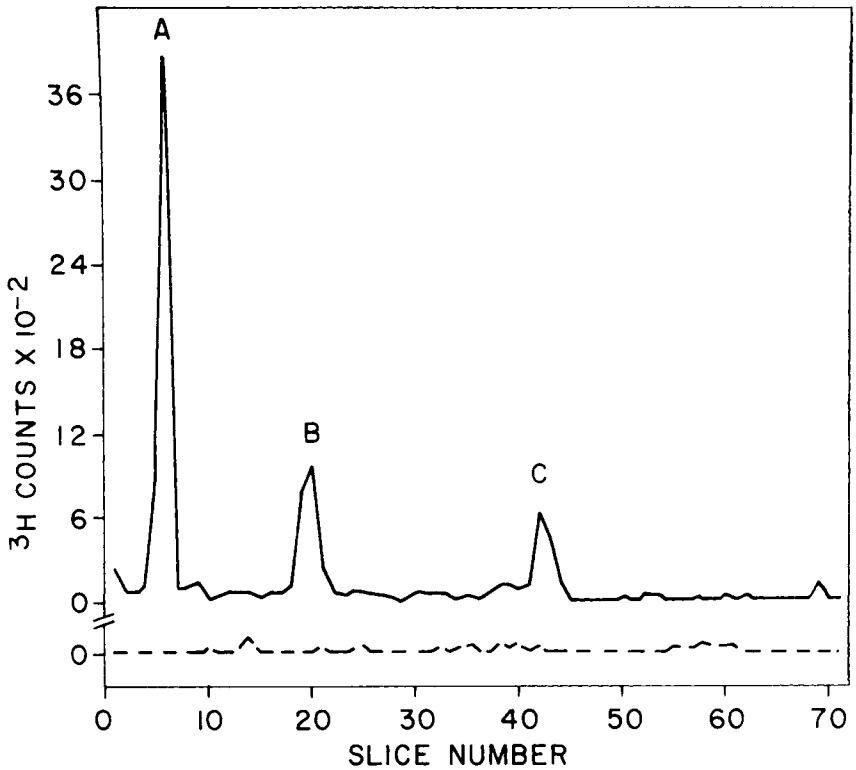


Fig. 4. Comparison of enzyme polypeptides precipitated with rabbit anti-nitrate reductase serum to the control immunoprecipitate observed with preimmune rabbit serum. Samples were prepared and run as in Fig. 3. The solid line is the material precipitated with the anti-nitrate reductase serum.

(A), a 60,000 peak (B), and no fragments. These fragments in the heat-solubilized preparation must correspond to pieces of the A and B subunits which have been degraded by protease action. Such fragments are also evident in the material solubilized in the absence of PMSF (Fig. 2).

As seen in Fig. 4, a third protein (C) is also precipitated from Triton extracts. Polypeptide C is not necessary for enzyme activity but must be tightly bound to the A and B subunits because it is precipitated by an antibody produced against A and B only. The molecular weight of C is 20,000, and polypeptides A, B, and C are present in the wild-type cytoplasmic membrane in a ratio of 1:1:2.

In the search for components A, B, and C in various fractions of the mutants, each one was grown with ^{3}H-leucine under conditions of maximal nitrate reductase induction, and S_2, P_1, and P_2 fractions were prepared as in Fig. 4. The proteins in the P_1 and P_2 fractions were solubilized by extraction with Triton X-100 at room temperature. Therefore, no proteolytic action was involved in their solubilization. The solubilized fractions were then precipitated with antibody and run on SDS gels as previously described.

The cytoplasmic membranes (P_1) of both chlA and chlB contained polypeptides A, B, and C (Fig. 5). However, in proportion to the A subunit, the amount of B subunit

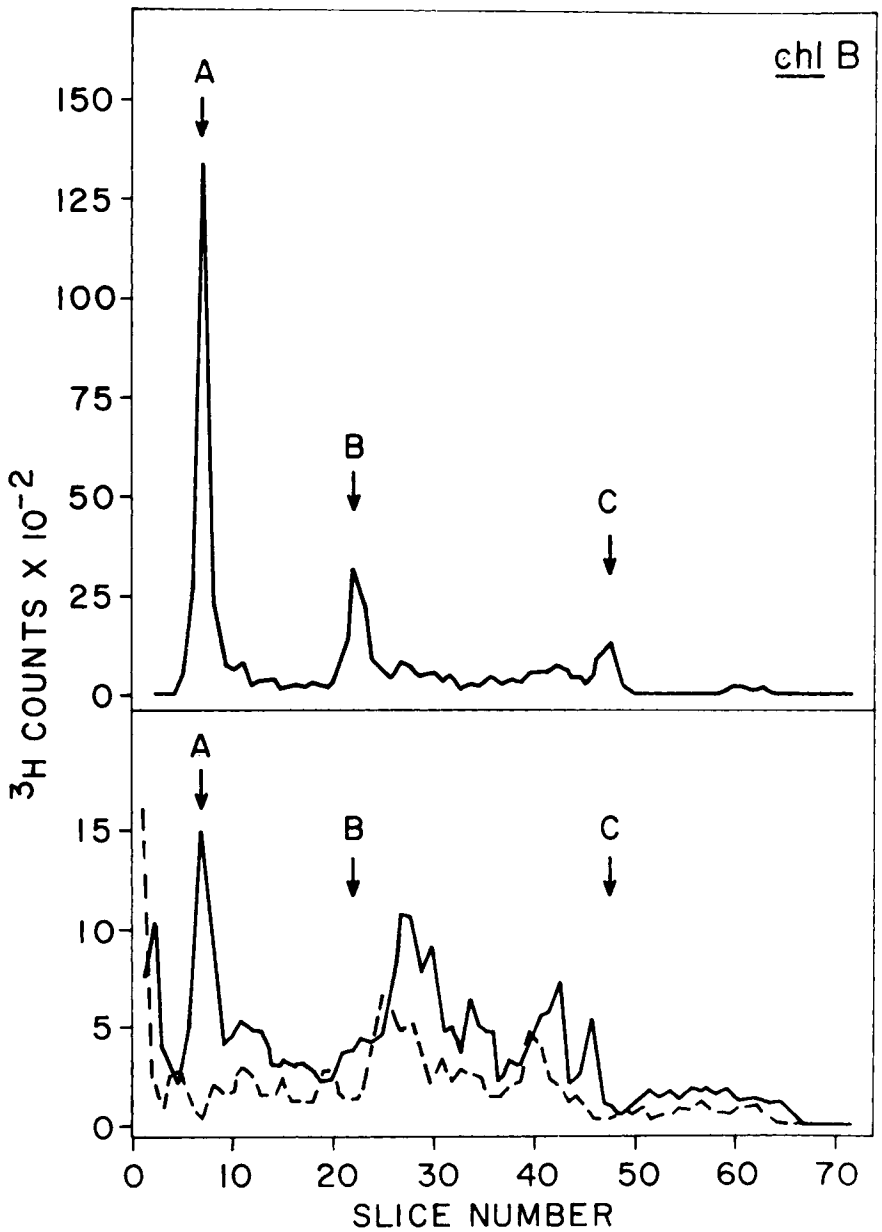


Fig. 5. SDS gel pattern of nitrate reductase polypeptides precipitated from the Triton-soluble membrane (P_1) protein of chlB (upper figure). The lower figure shows material precipitated from the chlB S_2 fraction. The dashed line is a control using preimmune rabbit serum. Samples were precipitated and run as in Fig. 3.

was slightly reduced and the amount of C was only 30% of that found in the wild type. From earlier experiments, we predicted that the P_1 fractions from chlC and chlE would contain no nitrate reductase proteins at all; however, immune precipitates from these mutants contained as much precipitable radioactivity as was found in the wild-type P_1. Gels of this precipitate revealed that this radioactivity was not in intact proteins A, B, and C but in many peaks spread throughout the gel (Fig. 6). This indicates that enzyme proteins capable of reacting with antibody were made, but these were probably defective and therefore were degraded by a protease after insertion into the membrane. Goldschmit (15) has described a similar degradation of a defective β-galactosidase subunit produced in mutant strains of E. coli. He showed by pulse-chase labeling that this defective subunit was made, then degraded, whereas the wild-type subunit was not degraded.

The proteins in the P_2 fractions of all the mutants were identical to those found in the P_1 fractions. S_2 fractions of all mutants except chlC contained virtually no precipitable radioactivity. ChlC contained significant amounts of the largest subunit (A) in its S_2 fraction (Fig. 6).

The localization of nitrate reductase components in these mutants allows us to better define the actual defects in these mutants. None of them appear to be defective in the attachment of the enzyme to the membrane. Such a defect would probably have resulted in elevated levels of the enzyme in the S_2 fraction and nothing in the membrane. All the mutants appear to make some form of the enzyme subunits. In chlC and chlE these are probably defective and are degraded. Since chlA has normal amounts of subunits A and B and some C, it must lack only the molybdenum factor. Similarly, chlB appears only to lack a means of reacting its soluble molybdenum factor with its particulate enzyme.

Although the function of the C compoent of the enzyme is as yet unknown, one possible identity for it is the cytochrome b apoprotein. Cytochrome b appears to be the direct link to nitrate reductase and therefore it would be found in close association with the enzyme. In some organisms (e.g., *Neurospora*) cytochrome b is an integral part of the nitrate reductase enzyme (16). The fact that this C protein is considerably reduced in chlA and chlB coincides with our spectral data showing reduced amounts of nitrate-induced cytochrome b in the mutants. Alternatively, one might speculate that this is a binding protein which holds the enzyme to the membrane. This is rather unlikely since in chlA and chlB, which have normal amounts of A and B but only 30% normal amount of C, the A and B subunits are still firmly membrane-bound.

GENERAL CONCLUSIONS

The results from the reconstitution experiments as well as those from the immune precipitations point to the conclusion that the enzyme is an integral protein and suggest that it is attached to the membrane by small hydrophobic regions on the B and perhaps on the A subunits. Niether the protease-cleaved, heat-extracted enzyme from the wild type or that from the mutants (chlA and chlB) will rebind to the membrane and precipitate with the P_2 fraction. The B subunit is smaller in the heat-solubilized enzyme than in the Triton-solubilized enzyme, suggesting that a small fragment (the hydrophobic region) was cleaved off when the enzyme was released. In addition, we found that pieces of the

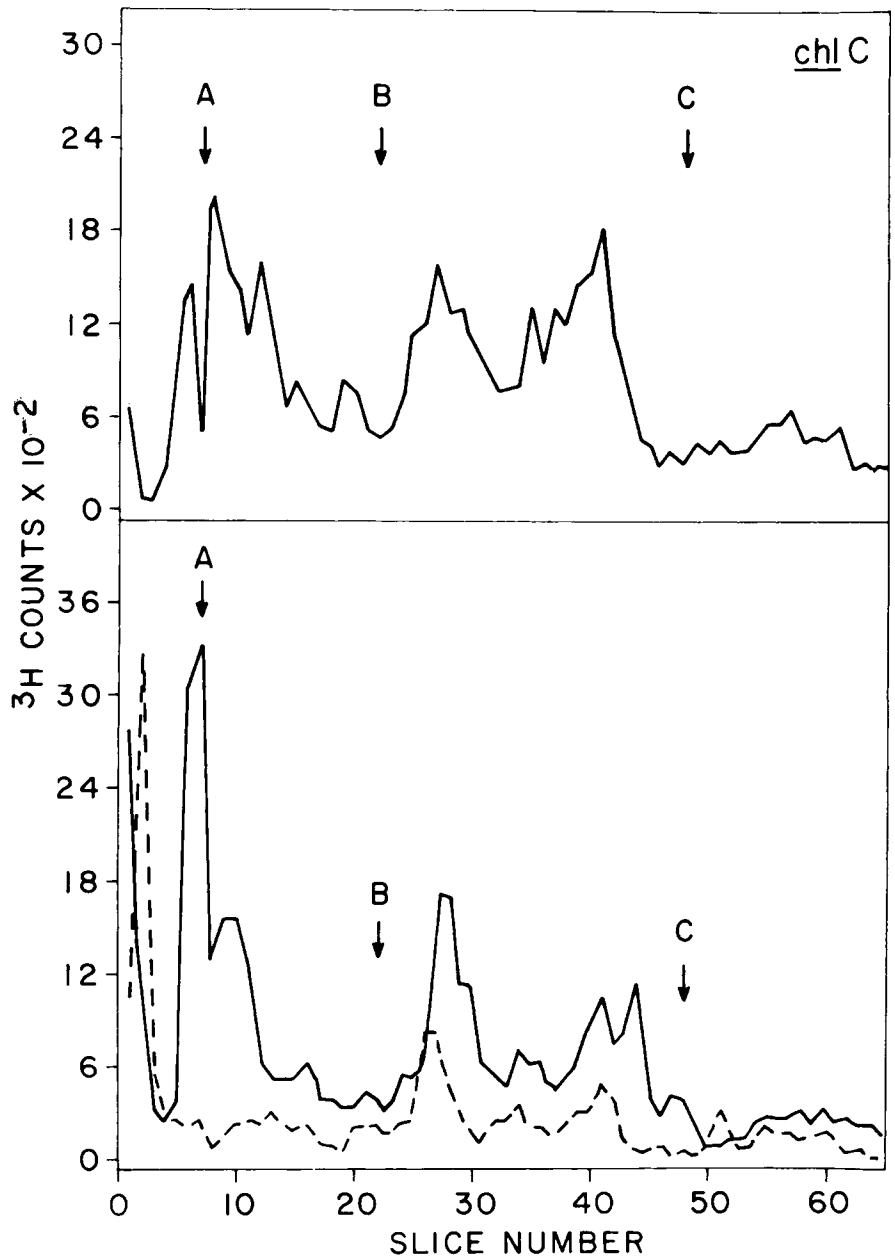


Fig. 6. SDS gel pattern of nitrate reductase polypeptides precipitated from the Triton-soluble membrane (P_1) protein of chlC (upper figure). The lower figure shows material precipitated from the chlC S_2 fraction. The dashed line is the control using preimmune serum. Samples were precipitated and run as in Fig. 3.

defective enzyme subunits made by chlC and chlE are still associated with the membrane even though they have been degraded. Presumably it is these hydrophobic regions, which cannot be attacked by protease, that bind the degraded fragments to the membrane.

These experiments have also demonstrated that in vitro reconstitution with mutant extracts involves the attachment of a molybdenumum factor to enzyme subunits already bound to lipoprotein fragments, in a reaction mediated by the chlB gene product. This is supported by the fact that chlB S_2 plus any other S_2 fraction must be present, as well as the S_1 fraction of a mutant containing intact enzyme in its membrane. Only enzyme bound to "soluble" lipoprotein fragments will react in reconstitution, and it is only below the transition temperature of the membrane lipids that these fragments are small enough so that the site on the enzyme which reacts with the molybdenum factor is exposed. This is illustrated by the inability of the heat-solubilized and Triton-solubilized enzymes or mechanically disrupted envelope fractions from chlA or chlB to substitute for the S_1 fraction of chlA or chlB. Further, if lipoprotein fragments in the S_1 fractions of these mutants coalesce as a result of being heated above the lipid transition temperature, reconstitution cannot occur. Finally, we have no evidence for an assembly factor which joins the individual nitrate reductase components with membrane components to form de novo enzymatically active membrane particles.

This leads us to conclude that in growing wild-type cells the enzyme attached to a lipoprotein membrane would be inaccessible to the molybdenum factor, so its attachment must occur in vivo as the enzyme is being inserted onto the membrane. Therefore, reconstitution with chlorate-resistant mutants, although a valuable tool for examining the system, does not mimic in vivo assembly of the nitrate reductase system.

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