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New Mechanism for Post-Translational Processing During Assembly of a Cytoplasmic Membrane Protein?

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Insertion of nitrate reductase into the *Escherichia coli* cytoplasmic membrane was examined by following the fate of pulse-labeled enzyme in both the membrane and cytoplasm during various times after the addition of an unlabeled chase. The polypeptide composition of this labeled enzyme was determined by autoradiography of immunoprecipitated material after separation on sodium dodecyl sulfate-polyacrylamide gels. The data presented here indicate that immediately after appropriate insertion of the enzyme into the membrane, a post-translational event occurs which converts the cytoplasmically synthesized form of subunit B (B') to the form found in the completely assembled enzyme (B). B' is distinguished from B by its more rapid electrophoretic mobility. B' was found in the cytoplasm of all strains tested, in the membrane of strains with defects in enzyme insertion (*hemA* and *chlE*), and as a transient component in the membrane of wild-type cells.

The mechanism of translocation of proteins in gram-negative bacteria either through the cytoplasmic membrane into the periplasmic space or into the outer membrane has been studied in considerable detail (25). These insertion or secretion processes require membrane-bound ribosomes and the transient existence of a sequence of approximately 20 hydrophobic amino acids, which is removed during translocation of the protein. Since the location of proteins in the cytoplasmic membrane, relative to their biosynthetic machinery, is rather different from that of periplasmic or outer membrane proteins, it is likely that their mechanism of synthesis is also different. The sparse amount of information available on such proteins (25) does indeed point to a different mechanism, since these proteins all lack leader sequences.

We have used the enzyme nitrate reductase from *Escherichia coli* as a model system to look at the biosynthesis of cytoplasmic membrane proteins. This enzyme consists of three subunits. Surface labeling studies (8, 17) have shown that the largest subunit (A; 142,000) is exposed to the cytoplasm, the smallest subunit (C; 19,500) is exposed to the periplasm, and the third subunit (B; 60,000) cannot be labeled and is presumed to be buried. Previous studies have shown that subunits A and B are synthesized in the cytoplasm and are inserted into the membrane only when at least one other protein is present (10). The cytochrome *b*-containing subunit (C) is known to be required for this insertion (16). A similar situation exists for succinic dehydrogenase in *Bacillus subtilis* (11). Here we present evidence that subunit B is synthesized in the cytoplasm in a precursor form which is modified to a more slowly migrating form after its insertion into the membrane.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* MC4100 is the wild-type parental strain of a *chlE* mutant selected by chlorate resistance (18), designated RK5201 (*non gyrA chlE201* Mucts). Both strains were obtained from R. Kadner, University of Virginia Medical School, Charlottesville. AN344 is defective in heme biosynthesis. It makes heme in the presence of δ-aminolevulinic acid (ALA). It is auxotropic for leucine and proline (16). All strains were grown on low-sulfate minimal salts medium containing all amino acids (except those omitted for labeling) and vitamins, with glucose as the carbon source (10). They were kept anaerobic by continuous bubbling with a mixture of 95% N_2–5% CO_2. MC4100 derivatives were labeled with $H_2{}^{35}SO_4$ in medium containing no cysteine or methionine and 13 μg of Na_2SO_4 per ml. This is sufficient sulfate to allow the culture to grow to maximum density. This amount of unlabeled Na_2SO_4 also permits uniform incorporation of ^{35}S throughout growth, even in continuously labeled cultures. AN344 was labeled with [^{14}C]leucine in medium containing both cysteine and methionine and 20 μg of leucine per ml with the same levels of Na_2SO_4.

Cell fractionation. Cells were harvested and converted to spheroplasts by the method of Weiss (24). They were spun at 3,000 × *g* for 5 min, and the pellet was frozen overnight. The thawed spheroplasts were suspended to approximately 20 times the volume of the pellet in 1 mM $MgCl_2$ containing a small amount of DNase and RNase and homogenized gently in a

glass homogenizer. This procedure lyses about 90% of the cells. Any unbroken cells were removed by centrifugation for 5 min at 3,000 × *g*. This crude extract was spun at 200,000 × *g* for 45 min. The supernatant material was considered to contain the cytoplasm, and the pellet contained the inner and outer membranes. The cytoplasmic membrane was solubilized by incubation with 2% Triton X-100 (at least 4 mg/mg of protein) in 0.1 M PO_4 buffer (pH 7.1). Insoluble material was removed by centrifugation at 200,000 × *g* for 30 min.

Antibody precipitation. Antiserum directed against pure nitrate reductase, containing only subunits A and B (15, 19), was used to precipitate the enzyme from cytoplasm or Triton-soluble fractions. The appropriate amount of antiserum was added, the sample was incubated for 30 min at room temperature, staphylococcal adsorbant (10) was added, and, after a second 30-min incubation, the pellet was collected by centrifugation. The pellet was washed once in phosphate-buffered saline, suspended in a small amount of solubilizing buffer (14), and boiled.

Gel electrophoresis. Sodium dodecyl sulfate (SDS) gels containing either 7.5 or 12.5% acrylamide were run using the Lugtenberg modification (14) of the Laemmli system. In all experiments, an attempt was made to add an equal number of counts to each gel sample. This was possible in all cases except in cytoplasmic fractions in which labeled material was being chased from the cytoplasm into the membrane. The gels were stained by the Fairbanks procedure (7), then dried and placed against Kodak XR5 film for autoradiography. Autoradiograms were scanned using an Ortec densitometer.

Assays. Nitrate reductase activity was determined colorimetrically by measuring nitrite production, using methyl viologen as the electron donor (19). Protein was determined by the method of Lowry et al. (13).

RESULTS

In attempting to follow the fate of the cytoplasmically synthesized nitrate reductase subunits A and B we began by labeling the enzyme with short pulses of $^{35}SO_4$. We found that at the shortest labeling times (30 s to 3 min) almost no labeled nitrate reductase could be found in the cytoplasmic membrane; however, by 5 to 8 min substantial amounts could be detected. An observation that we made during these studies was that the membrane-bound enzyme which was labeled during 5- to 10-min pulses contained two bands in the subunit B region, whereas pulses of 20 to 30 min or longer showed only one band at B, the one previously observed in cultures continuously labeled with $^{35}SO_4$ (Fig. 1a). To follow the fate of this more rapidly migrating peptide (B′) which appeared only in short pulses, we again labeled cells for 8 min with $^{35}SO_4$, then allowed this culture to grow for another 45 min in the presence of unlabeled Na_2SO_4, cysteine, and methionine. Figure 1b shows that B′ disappeared during this chase. Note in this figure that

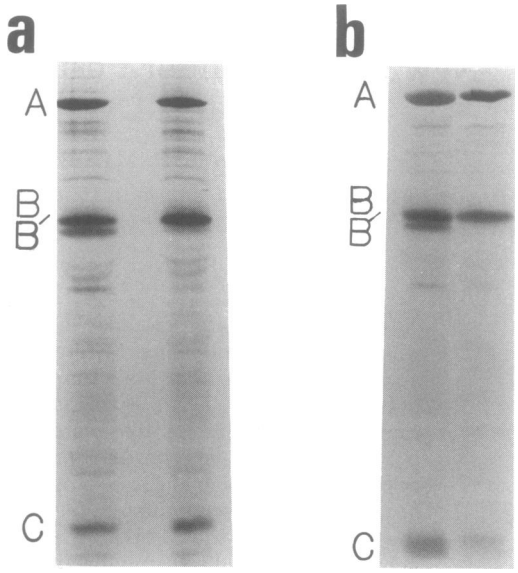

FIG. 1. *Autoradiograms of SDS gels of membrane-bound nitrate reductase labeled for short periods of time with $^{35}SO_4$. (a) MC4100 (wild type) was grown anaerobically to midlog phase. The culture was divided in half, and $^{35}SO_4$ was added at 10 μCi/ml to one half and 3 μCi/ml to the other. The sample with more isotope was incubated for 8 min, and the other was incubated for 30 min. The labeling was stopped by pouring the cells rapidly through ice. Cells were then converted to spheroplasts and lysed by osmotic shock, and the cytoplasmic membrane was isolated by extraction of the envelope fraction with Triton X-100 (see the text). Nitrate reductase was isolated by precipitation with antiserum, and the solubilized precipitates (see the text) were run on 12.5% SDS gels. The left lane shows the 8-min labeling, and the right shows the 30-min labeling. Subunits A, B and B′, and C are indicated. (b) Experimental conditions were as in (a) except that after an 8-min labeling a sample was removed, and cysteine and methionine were added at 100 μg/ml plus Na_2SO_4 at 300 μg/ml to the remaining culture, which was allowed to grow for 45 min.*

labeling of subunit C was also greater immediately after the pulse than after the chase.

Kinetics. To look at the kinetics of the disappearance of B′ and C, an experiment similar to that in Fig. 1b was done. After an 8-min pulse with $^{35}SO_4$, samples were taken at 0, 1, 5, 15, and 45 min after the addition of the unlabeled material. Figure 2a shows that B′ appeared in the membrane in greatest amounts at 1 min after the chase, then decreased gradually until it could not be detected at 45 min. SDS gels of the antibody-precipitable material found in the cytoplasm at these same time points contained mainly B′, and the amount of B did not increase. As demonstrated in Fig. 3, a small amount of the

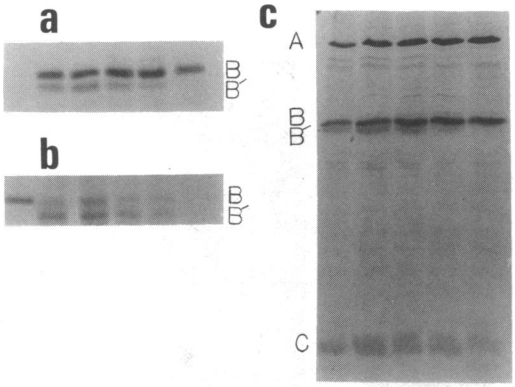

FIG. 2. *Autoradiograms of SDS gels of pulse-labeled nitrate reductase, chased for 1 to 45 min. MC4100 was labeled for 8 min and chased with unlabeled cysteine, methionine, and Na_2SO_4 as described for Fig. 1. Samples were removed immediately after the addition of chase (0 min) and at 1, 5, 15, and 45 min afterward. (a) and (b) show only the B subunit region of these samples run on 7.5% SDS gels. The upper samples are antibody precipitates from the membrane fraction (a), and the lower ones are from the cytoplasm (b). Time of chase increases from left to right. The 45-min sample from the membrane is seen at the left end of the cytoplasm samples. (c) The same samples from the membrane fraction were run on 12.5% gels to include the C subunit.*

B seen in the cytoplasm is probably due to slight contamination of the cytoplasm with small amounts of enzyme located on membrane vesicles too small to be pelleted. In Fig. 2b these same samples are shown run on 12.5% gels. Note that the amount of subunit C also decreased with time after the chase. To detect subunit C on these gels it must be bound to A and B, as the antibody is directed at only subunits A and B (15).

B' precursor. We have previously stated that subunits A and B accumulate in the cytoplasm of a mutant unable to make heme in the absence of ALA (*hemA*). (In these studies, a different gel electrophoresis system was used to analyze the polypeptides, and B was not distinguished from B'.) When the *hemA* strain which had been allowed to make nitrate reductase in the absence of ALA was switched to growth in the presence of ALA, it was found that the previously synthesized cytoplasmic enzyme became bound to the membrane as functional cytochrome *b* (subunit C) was made (16). If B' is in fact a precursor of B, it should accumulate in the cytoplasm of the *hemA* mutant in the absence of ALA and be modified to B when ALA is added and B' is incorporated into the membrane. Figure 3a (lanes 1 and 3) shows that in the absence of ALA, B' appeared in the cytoplasm, and mostly B' was found in the membrane. Upon the addition of ALA during the chase, B' disappeared from the membrane and B increased (Fig. 3a, lanes 2 and 4). During this time labeled enzyme decreased in the cytoplasm and increased in the membrane. No change in the relative proportions of B or B' was seen in the cytoplasm. Densitometer tracings showed that the ratio of subunit A to subunit B in the membrane just after the pulse was much greater than that of A to B after the chase. Thus B' must be converted to B after it binds to the membrane. Note that in this experiment [^{14}C]leucine was used instead of $^{35}SO_4$, since this mutant cannot grow without cysteine and methionine in the absence of ALA. The two ^{14}C-labeled bands seen in the *hemA* mutant corresponded exactly to B and B' labeled in the wild type with $^{35}SO_4$. Lanes 5 and 6 (Fig. 3a) show the cytoplasmic fraction from the same experiment in which part of the cells were broken in a French pressure cell. Previous experiments have shown that when cells are broken in this fashion, small membrane vesicles are formed which do not spin down, and the cytoplasmic fraction can contain as much as 60% of the total enzyme. This fraction also contains higher amounts of B. Osmotic lysis, however, gives much less cytoplasmic enzyme and thus contains only very small amounts of B. Thus we feel that the small traces of B found in the cytoplasmic fraction are a result of slight membrane contamination. Figure 3b shows that when the chase was performed in the absence of ALA, membrane-bound B' was not converted to B and there was no further incorporation of labeled enzyme into the membrane. This low level of enzyme found in the membrane is presumed to be due to "incorrect" binding of A and B to the membrane in the absence of heme-containing cytochrome *b* (16). This incorrect binding (perhaps to apocytochrome *b*) must not allow sufficient interaction between B' and the modifying enzyme to convert the majority of the B' to B. When ALA was added along with chloramphenicol during the chase, nearly all of the B' was converted to B. This conversion is presumably due to incorporation of heme into previously synthesized apocytochrome *b* (16) and subsequent binding of A and B' to this heme-containing cytochrome. The lack of complete conversion of B' to B is probably due to the presence of chloramphenicol. This inhibits the synthesis of subunit C, which is limiting. Without binding to functional, heme-containing C, B' cannot be converted to B. Figure 3c shows that during reconstitution of heme into apocytochrome *b*, binding of the A and B subunits to C was occurring.

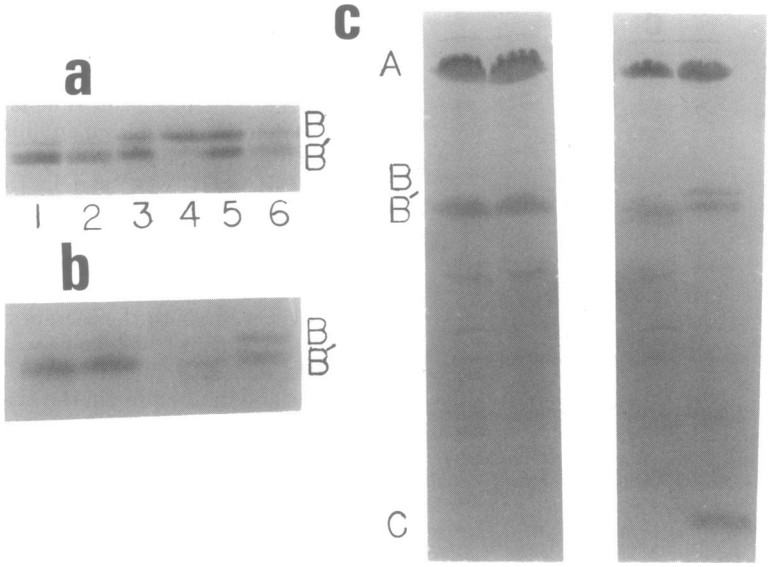

FIG. 3. *Autoradiograms of SDS gels of precursor nitrate reductase in the cytoplasm of the hemA mutant and its conversion to product form in the membrane. Strain AN344 was grown in 1.5 generations without ALA in the presence of 0.063 μCi of ^{14}C-labeled leucine per ml. A sample was removed, and 400 μg of unlabeled leucine per ml was added along with 40 μg of ALA per ml. After 45 min of growth, a second sample was taken. Each culture sample was divided in half; cells in one half were broken in a French pressure cell at 16,000 lb/in^2, and cells in the other were made into spheroplasts and osmotically lysed (see the text). (a) Nitrate reductase from the cytoplasm before (lane 1) and after (lane 2) the addition of ALA and from the membrane before (lane 3) and after (lane 4) the addition of ALA. These four samples were osmotically lysed. Lanes 5 and 6 contain cytoplasm of cells broken in a French pressure cell, before and after the addition of ALA, respectively. All samples were run on 7.5% gels, and only the B subunit region is shown. (b) Cytoplasmic and membrane-bound forms of subunit B after the unlabeled leucine chase in an experiment labeled as in (a) and chased either in the absence of ALA or in the presence of chloramphenicol plus ALA. Left to right, Chase minus ALA, cytoplasm (lane 1), and membrane (lane 2); chase plus ALA, chloramphenicol, cytoplasm (lane 3), and membrane (lane 4). All samples were run on 7.5% gels. (c) The same samples from (b) run on 12.5% gels. In the experiments described in (b) and (c), all samples were osmotically lysed.*

Figure 4 shows the kinetics of conversion of B′ to B in the membrane of the *hemA* mutant after the addition of ALA to the culture. Initially B′ was the major B component. Over 30 min, the B/B′ ratio gradually increased until only B was present in the membrane.

Further proof that B′ is a precursor form of B was demonstrated using a *chlE* mutant. This mutant lacks nitrate reductase and formate dehydrogenase activities; however, it makes polypeptides which cross-react with antibody to subunits A and B of nitrate reductase. SDS gels from continuously labeled cultures show equal amounts of B and B′ in the membrane. A pulse-chase experiment identical to that in Fig. 1b showed that only B′ appeared in the membrane during the pulse, whereas equal amounts of B and B′ appeared after the chase (Fig. 5). The reason for the incomplete conversion of B′ to B in these mutants is not known.

Inhibition. In an attempt to show the conversion of B′ to B in the presence of protein synthesis inhibition, the wild-type strain was pulsed with ^{35}S and chased as described for Fig. 1b with unlabeled cysteine, methionine, and Na_2SO_4, or the culture was allowed to continue to grow without the addition of unlabeled amino acids but in the presence of 100 μg of chloramphenicol or 25 μg of spectinomycin per ml. In each case, the addition of the chase or inhibitor inhibited the incorporation of ^{35}S into trichloroacetic acid-precipitable counts. Figure 6 shows that spectinomycin did not prevent the conversion of B′ to B, whereas chloramphenicol did. Puromycin (500 μg/ml) gave the same result as chloramphenicol (data not shown).

To determine the nature of the modification of B′ to B, the wild-type strain was once again pulsed for 8 min and then chased for 45 min in the presence of procaine, carbonylcyanide *m*-chlorophenylhydrazone, or arsenate. Procaine is a membrane perturbant which inhibits the cleavage of signal sequences of secreted proteins (12). Carbonylcyanide *m*-chlorophenylhydra-

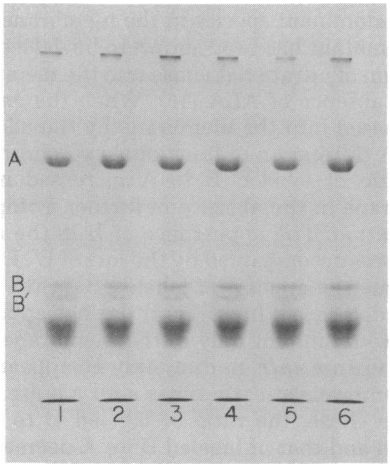

FIG. 4. *SDS gels of membrane-bound nitrate reductase from the hemA mutant before and after the addition of ALA. Strain AN344 was grown for three generations in the absence of ALA. A sample was removed before the addition of ALA (lane 1) and at 1, 2, 5, 15, and 30 min after the addition of ALA (lanes 2 through 6). Antibody-precipitable nitrate reductase from each sample was run on 7.5% gels and stained with Coomassie blue. The broad band nearest the bottom of the gel is immunoglobulin.*

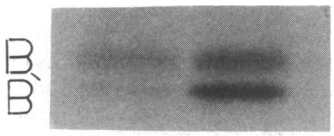

FIG. 5. *Autoradiogram of membrane-bound nitrate reductase from chlE labeled for 8 min with $^{35}SO_4$ and chased for 45 min with unlabeled cysteine, methionine, and Na_2SO_4. The experiment is identical to that shown in Fig. 1. Antibody precipitates were run on 7.5% gels. Only the B subunit region is shown: left, pulse; right, chase.*

zone is an energy poison which dissipates membrane potential and inhibits the cleavage of the signal sequences of the f1 coat protein (3). Arsenate is an inhibitor of ATP synthesis. It is known to inhibit transport systems which require ATP and cannot use energy produced by membrane potential (1). Table 1 shows that only arsenate inhibited the conversion of B' to B.

To see whether proteolysis was involved in the conversion of B' to B, MC4100 was pulse labeled with ^{35}S and then chased in the presence of unlabeled cysteine, methionine, and Na_2NO_4 alone or with the addition of various protease inhibitors. Table 1 shows that only benzamidine and *N*-α-*p*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK) inhibited conversion of B' to B. Protease inhibitors which did not inhibit

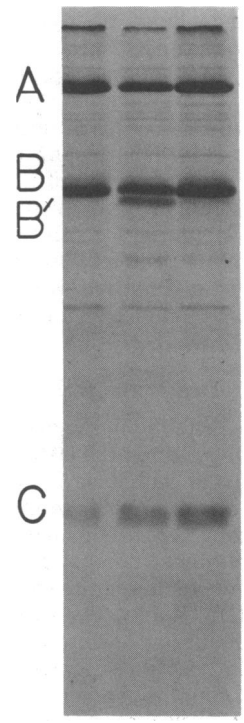

FIG. 6. *Autoradiogram of SDS gels of membrane-bound nitrate reductase pulsed with $^{35}SO_4$ and chased in the presence of inhibitors of protein synthesis. MC4100 was labeled as in Fig. 1 and then chased with, from left to right, cysteine-methionine-SO_4, chloramphenicol, or spectinomycin.*

TABLE 1. *Inhibition of processing of B' by various inhibitors*[a]

Addition[b] (concn)	Inhibition
None	−
Procaine (0.6%)	−
CCCP (60 μM)	−
Sodium arsenate (200 μM)	+
Benzamidine (35 mM)	+
ε-Amino caproic acid (10 mM)	−
TLCK (5 mM)	+
TPCK (1 mM)	−
PMSF (10 mM)	−

[a] MC4100 was pulsed with ^{35}S as described for Fig. 1. After a sample was removed, the remainder of the culture was divided into separate samples, and cysteine, methionine, and Na_2SO_4 were added to each, along with one of the potential inhibitors, for 45 min. Membrane-bound nitrate reductase was analyzed by autoradiography as for the figures, and the conversion of B' to B was determined. If no B' remained in the membrane, the result was recorded as no inhibition (−).

[b] Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride.

this conversion included phenylmethylsulfonyl fluoride, L-1-tosylamide-2-phenylethyl chloromethyl ketone, and ϵ-amino caproic acid.

When cells were grown in the presence of benzamidine, the same result was found: the membrane-bound form of the enzyme contained approximately equal amounts of B and B′ (data not shown). Moreover, growth in benzamidine appeared to preferentially inhibit the synthesis of nitrate reductase (Table 2). Membranes from cells grown in phenylmethylsulfonyl fluoride or L-1-tosylamide-2-phenylethylchloromethyl ketone contained only B.

DISCUSSION

Evidence presented here suggests that a more rapidly migrating form of the 60,000-dalton subunit (B) of nitrate reductase is synthesized in the cytoplasm and then binds to the membrane. After this binding occurs, this subunit is modified, presumably by some enzyme in the membrane, to a form which migrates more slowly on SDS-polyacrylamide gels. The evidence for the precursor-product relationship of B′ to B is the following. (i) In both pulse-labeled and continuously labeled (data not shown) cells, wild type or mutant, the cytoplasmic fraction contained mostly B′, not B. (ii) In pulse-chase experiments using wild-type cells, B′ appeared in the membrane at early times after the pulse, gradually disappeared during the chase, and was not present after chases of 45 min or longer or in cells labeled for many generations. (iii) In the *hemA* mutant grown without ALA, only enzyme with B′ accumulated in the cytoplasm, and B′ was the predominant species in the membrane. The *hemA* mutant has been shown to be defective in insertion of nitrate reductase into the membrane in the absence of ALA (16). When the enzyme was chased into the membrane by the addition of ALA (formation of functional cytochrome *b*), the ratio of labeled B to A increased in the membrane in the absence of further isotope incorporation. The appearance of B in the membrane was accompanied by the loss of B′. During this time the amount of labeled B′ in the cytoplasm decreased; however, B did not appear in the cytoplasm, but only in the membrane fraction. (iv) In a *chlE* mutant only B′ appeared in the membrane at early times after a pulse. During the chase, the ratio of labeled B to A increased and that of labeled B′ to A decreased.

TABLE 2. *Inhibition of nitrate reductase synthesis by benzamidine*

Fraction	10^6 cpm incorporated[a]		
	+ Benzamidine (A)	No addition (B)	A/B
Triton-soluble	43	37	1.16
Triton-insoluble	25	21	1.19
Cytoplasm	80	60	1.33
Nitrate reductase	0.38	2.4	0.16

[a] An overnight culture of MC4100 was divided in half, diluted, and allowed to grow to late log phase. To one, $^{35}SO_4$ was added to 5 μCi/ml, and to the other $^{35}SO_4$ was added to 10 μCi/ml along with benzamidine to 35 mM. Each culture was allowed to double. (Benzamidine increased the doubling time by about 2.5×.) Duplicate samples from Triton-soluble (cytoplasmic membrane), Triton-insoluble (outer membrane), and cytoplasm (after precipitation with cold trichloroacetic acid) fractions and antibody-precipitable nitrate reductase were counted. Counts were normalized to represent total counts per minute (cpm) incorporated in each fraction.

What is the nature of this modification of B′ to B? Two possibilities exist: (i) proteolytic cleavage of a hydrophobic "signal peptide," which would result in decreased SDS binding and thus retarded mobility; or (ii) the covalent addition of one or more small molecules which would decrease the SDS binding in some way and also retard mobility.

The first explanation seems unlikely. Although the protease inhibitors benzamidine and TLCK inhibit the conversion of B′ to B, similar compounds are known to deplete *E. coli* of ATP. Schecter et al. (22) showed that *p*-toluene sulfonyl fluoride, phenylmethylsulfonyl fluoride, and pentamidine isethionate caused a drastic decline in cellular ATP levels. Since arsenate also depletes cells of ATP, it is likely that benzamidine, TLCK, and arsenate all inhibit the processing of B′ by interfering with ATP synthesis. Thus ATP must be necessary for processing. Since carbonylcyanide *m*-chlorophenylhydrazone does not inhibit processing, the ATP requirement must be more specific than just a source of energy.

Further evidence against proteolytic conversion of B′ to B is provided by experiments by Rasul Chaudhry (unpublished data) in this laboratory. He has found that during purification of nitrate reductase, partially purified enzyme which originally contained mostly B and very little B′ can be converted to a form containing no B and only B′ by incubation at 37°C.

One might also argue that the disappearance of B′ from membrane fractions occurs during sample preparation and is due to a membrane-bound protease, since benzamidine and TLCK inhibit its disappearance. This argument is not valid for the following reasons. (i) If this were the case, all the membrane samples in Fig. 2 and 4 should look identical. They do not. (ii) B′ should be degraded equally well in the mem-

brane of the *hemA* mutant, regardless of the presence or absence of ALA. Figure 3b and c show that B′ was perfectly stable in the membrane when ALA was absent. (iii) The addition of benzamidine to all buffers during the entire preparation had no effect on the B/B′ ratio.

The second explanation is more likely. It is now known that changes in the charge of a single amino acid in a protein can cause drastic changes in its mobility on SDS-polyacrylamide gels. Studies on the interaction of SDS with free amino acids have shown that acidic and neutral hydrophilic amino acids bind little SDS and that basic and hydrophobic amino acids bind the most SDS (20). This is reflected in the altered mobilities on SDS gels of proteins with single amino acid differences. In α-crystallin chains, replacement of a hydrophilic amino acid by a hydrophobic one results in increased mobility (5). In mutant *hisT* proteins, mobility alterations in both directions are observed. Specifically, the substitution of a basic by a neutral amino acid causes a decrease in mobility (21). The addition of covalent negative groups (maleyl, succinyl, carboxymethyl) to proteins also decreases SDS binding and retards electrophoretic mobility (23). The elegant studies of DeFranco and Koshland (4), Chelsky and Dahlquist (2), and Engstrom and Hazelbauer (6) showed that when two membrane-bound chemotaxis proteins are methylated on glutamic acid residues, their migration on SDS gels is increased. This is presumably due to increased SDS binding as a result of the change from an acidic amino acid to a neutral one. In the case of nitrate reductase, a modification of a basic amino acid on B′ could result in the observed decreased electrophoretic mobility of B relative to B′. Since this B′-to-B change only occurs in the membrane, a membrane-bound enzyme is presumably responsible for this change.

We have noted previously (10) that chloramphenicol prevents the incorporation of cytoplasmic nitrate reductase into the membrane. The mechanism for this is thought to be by preventing the synthesis of subunit C, thereby preventing appropriate binding of B′. This, in turn, would prevent B′ from interacting with the processing enzyme. In the *hemA* mutant, some processing can occur because there is some apocytochrome *b* which can be reconstituted by the addition of heme (via ALA), but not enough to bind all the cytoplasmic subunits A and B′ (16). The lack of inhibition by spectinomycin could only be explained, then, by its failure to inhibit the synthesis of subunit C.

The lack of labeling of subunit B by either transglutaminase-dansyl cadaverine (17) or [^{125}I]lactoperoxidase (8) suggests that this subunit may be protected within the lipid bilayer. Thus it is likely to be involved in membrane binding and may be the subunit which interacts with the cytochrome *b* (subunit C). Evidence for this is found in Fig. 3c. When the *hemA* mutant is grown without ALA, the majority of the enzyme remains in the cytoplasm, and that which is membrane bound is thought to be bound "incorrectly" (16). That form of the enzyme contains mostly B′ and no associated cytochrome *b*. When the apocytochrome was reconstituted by the addition of ALA (heme) in the presence of chloramphenicol, interaction between subunits A and B and subunit C occurred (shown by coprecipitation of subunit C), and B′ was converted to B. The association between B′ and subunit C is apparently stronger than or different from that between B and subunit C, as the amount of subunit C which coprecipitated decreased as B′ was converted to B (Fig. 2c). This is probably the reason for the variable association of subunit C with A and B observed by this laboratory and others.

Giordano et al. (9) have also suggested the possibility of precursors to both the A and B subunits. They reported the existence of two polypeptides (α′ and β′) in the cytoplasm of a *chlA* mutant. After the *chlA* cytoplasm is mixed with *chlB* cytoplasm, these two polypeptides disappear and only subunits A and B are present in the "newly formed" particulate fraction. The β′ of Giordano et al. appears to be identical with our B′.

Thus it appears that nitrate reductase is assembled into the cytoplasmic membrane by a mechanism which differs drastically from that involved in translocation of proteins into the periplasmic space or the outer membrane. In contrast to the cleavage of a "signal peptide" upon translocation, there is a post-translational addition of one or more small molecules. The unmodified form appears to have a function similar to that of a signal peptide, i.e., it recognizes the membrane attachment site, the cytochrome; then, whereas signal peptides are proteolytically removed to generate mature proteins, the precursor form of nitrate reductase is modified by addition. Although this interpretation is compatible with the data presented here, final proof of this interpretation will require careful compositional analysis of both B and B′. Peptide mapping and partial sequencing of both B and B′ from purified nitrate reductase are now under way.

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