JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 574-583
0022-538X/79/01-0574/10$02.00/0

Vol. 137, No. 1

Localization of Proteolytic Activity in the Outer Membrane of *Escherichia coli*

C. H. MACGREGOR,* C. W. BISHOP,† AND J. E. BLECH

Georgetown University, Department of Microbiology, Schools of Medicine and Dentistry, Washington, D.C. 20007

Received for publication 30 October 1978

An enzyme in the cytoplasmic membrane, nitrate reductase, can be solubilized by heating membranes to 60°C for 10 min at alkaline pH. A protease in the cell envelope has been shown to be responsible for this solubilization. The localization of this protease in the outer membrane was demonstrated by separating the outer membrane from the cytoplasmic membrane, adding back various forms of outer membrane protein to the cytoplasmic membrane, and following the increase in nitrate reductase solubilization with increasing amounts of outer membrane proteins. This solubilization is accompanied by the cleavage of one of the subunits of nitrate reductase and is inhibited by the protease inhibitor *p*-aminobenzamidine. Analysis of membrane proteins synthesized by cells grown in the presence of various amounts of *p*-aminobenzamidine revealed that *p*-aminobenzamidine affects the synthesis of the major outer membrane proteins but has little effect on the synthesis of cytoplasmic membrane proteins. When outer membrane is reacted with the protease inhibitor [^{3}H]diisopropylfluorophosphate, a single protein in the outer membrane is labeled. Since the interaction with diisopropylfluorophosphate is inhibited by *p*-aminobenzamidine, it is suggested that this single outer membrane protein is responsible for the in vitro solubilization of nitrate reductase and the in vivo processing of the major outer membrane proteins.

Very little information is available on proteases that are located in and act on membranes. Such proteases should be present at sites within membranes where proteins are secreted. This is predicted by the signal hypothesis of Blobel and Dobberstein (3). Using detergent-solubilized microsomes for in vitro synthesis of immunoglobulin light chain, they found that the protein synthesized was larger than the secreted light chain. When protein-synthesizing systems containing membrane-bound ribosomes were used, the light chain synthesized was cleaved to its normal size (3). To explain these observations they proposed that secreted proteins are synthesized with extra amino acids attached and that these precursor proteins are cleaved to their final size during passage through the membrane. Examples of such precursor proteins have also been found in bacterial cells: penicillinase from *Bacillus licheniformis* (1), a major outer membrane protein in *Escherichia coli* (5), and *E. coli* alkaline phosphatase (7). The protease responsible for the cleavage of pro-alkaline phosphatase has been suggested to be in the outer membrane. In this paper we describe a protease located in the outer membrane of *E. coli*. Assayed in vitro, this protease will cleave a protein located in the cytoplasmic membrane, the respiratory enzyme nitrate reductase. This cleavage is accompanied by solubilization of the enzyme. Nitrate reductase is an intrinsic membrane protein which contains three subunits: A (molecular weight, 142,000), which is thought to be the active site of the enzyme; B (molecular weight, 60,000), function unknown; and C (molecular weight, 19,500), which is the apoprotein of cytochrome b_1 (12). Previous experiments have shown that nitrate reductase can be solubilized from the cytoplasmic membrane by heating envelope preparations in dilute alkaline buffer. The enzyme released from the membrane contains only subunits A and B, with considerable variation in the size of the B subunit, suggesting degradation. The addition of the protease inhibitor *p*-aminobenzamidine (PAB) during heating inhibits the solubilization of the enzyme (11). To look for the function of this protease in intact cells, PAB was added to growing cells. This resulted in the inhibition of synthesis of several of the major outer membrane proteins, but had only slight effect on the biosynthesis of cytoplasmic membrane proteins.

† Present address: Department of Human Nutrition, Virginia Polytechnic Institute, Blacksburg, VA 24060.

MATERIALS AND METHODS

Growth of cells. *E. coli* K-12 1485 (obtained from C. Schnaitman) was grown on minimal medium in the presence of nitrate. Growing cells were made anaerobic by slow bubbling of a mixture of 95% nitrogen and 5% carbon dioxide as previously described (11). Cells were grown aerobically in medium with the same salts but lacking nitrate, bicarbonate, molybdate, and selenite. Glucose was the carbon source in both cases.

Cells were continuously labeled with [^{3}H]leucine as previously described (11). For labeling with ^{35}S, cells were grown in medium in which chloride salts were substituted for sulfate salts. Three hundred milliliters of cells was pulsed with 150 μCi of $H_2{}^{35}SO_4$.

For comparison of cells grown in the presence and absence of PAB, the cells were first grown aerobically to log phase without PAB. Then 50 μCi of $H_2{}^{35}SO_4$ was added per 100 ml of culture, and the culture was immediately divided into three portions. To one portion, 6 mM PAB was added; 15 mM PAB was added to the second; and no PAB was added to the third. All three cultures were grown for another 45 min, and then each was harvested.

Isolation of cytoplasmic and outer membrane. Whole cells were suspended in 50 mM sodium phosphate buffer (pH 7.1) and subjected to 16,000 lb/in^2 in a French pressure cell at 4°C. After breakage, 2 mM $MgCl_2$ and a small amount of deoxyribonuclease and ribonuclease were added to the crude extract. The extract was spun at 5,000 × *g* for 5 min to remove whole cells. The supernatant from this centrifugation was spun at 200,000 × *g* for 45 min. The pellet from the second centrifugation is referred to as the envelope and contains cytoplasmic and outer membranes. This envelope was suspended in the same phosphate buffer, layered on a 20 to 55% (wt/wt) sucrose gradient, and spun for 16 h at 100,000 × *g*. The protein is distributed on this gradient in two main peaks, corresponding to cytoplasmic and outer membranes, as in Fig. 2.

To separate cytoplasmic from outer membrane by detergent extraction, the envelope (as prepared above) was suspended in the same phosphate buffer, and Triton X-100 was added to a final concentration of 2%, with at least 4 mg of Triton per mg of protein. This was incubated at room temperature for 15 min, then spun at 200,000 × *g* for 30 min. The cytoplasmic membrane is solubilized and the outer membrane remains in the pellet (15). The outer membrane can be partially solubilized by a similar extraction with Triton in the presence of 7.5 mM ethylenediaminetetraacetate (EDTA) (16).

Antibody precipitation. Nitrate reductase was precipitated from Triton-solubilized and heat-solubilized extracts by specific antibody as previously described (11).

Assays. Nitrate reductase activity was measured colorimetrically by following the production of nitrite (14). Methyl viologen was used as the electron donor. This permits the enzyme activity to be measured in the absence of cytochrome *b*.

To determine the amount of nitrate reductase solubilized, the appropriate samples were mixed to a protein concentration of 0.4 to 0.5 mg/ml in 7.5 mM sodium phosphate buffer (pH 8.3). The samples were heated to 60°C for 10 min, then placed immediately on ice. When cool, they were spun at 200,000 × *g* for 45 min. Nitrate reductase assays were performed on the supernatant fraction from this centrifugation and on the heated mixture prior to centrifugation. Percent nitrate reductase solubilized was calculated by dividing the amount of enzyme activity in the supernatant by that in the total mixture.

Protein was determined by the method of Lowry et al. (10).

DFP binding. Triton-insoluble outer membrane was isolated from aerobically grown cells as above. Two 1-mg samples of this protein were suspended in 0.5 ml of 7.5 mM phosphate buffer (pH 8.3). One also contained PAB at a final concentration of 40 mM. Then 8 μCi of ^{3}H-labeled diisopropylfluorophosphate ([^{3}H]DFP) (8 μM final concentration) was added to each sample, and they were incubated at 37°C for 20 min. Each was then diluted to 2 ml with cold buffer and spun at 200,000 × *g* for 30 min. The washed outer membrane was solubilized in sodium dodecyl sulfate (SDS), and equal amounts of protein from each sample were applied to an SDS gel.

Gels. Samples for gels were solubilized at 100°C, and 10-cm, 11.5% SDS gels were prepared according to Laemmli (9). They were stacked at 10 mA per gel and run at 150 V. Gels were prepared for photofluorography by soaking first in dimethyl sulfoxide, then in 23% 2,5-diphenyloxazole in dimethyl sulfoxide, and finally in 1% glycerol. The dried gels were then placed in contact with Kodak SB-5 film. Gels were stained for protein with Coomassie brilliant blue as previously described (6).

RESULTS

Localization of the nitrate reductase-solubilizing activity. Working on the assumption that the proteolytic activity responsible for the solubilization of nitrate reductase was located in the cytoplasmic membrane, we began a series of experiments to look for some association of nitrate reductase with the protease. Studies of Van Heerikhuizen et al. (19) showed that different enzymes in the cytoplasmic membrane could be localized on membrane particles of different densities. We attempted to see if nitrate reductase had such a specific distribution and, if so, whether the nitrate reductase-solubilizing activity had a similar distribution. Figure 1 shows the distribution on a sucrose gradient of nitrate reductase and protein from a crude preparation of cytoplasmic membrane. The enzyme activity appeared to be found in all portions of the cytoplasmic membrane, although specific activity was highest in the denser membrane particles. To test for the distribution of solubilizing activity, fractions from the gradient in Fig. 1 were pooled and collected by centrifugation. Each was suspended in alkaline buffer and heated to 60°C for 10 min, and then nitrate reductase activity was determined in the total mixture and in the

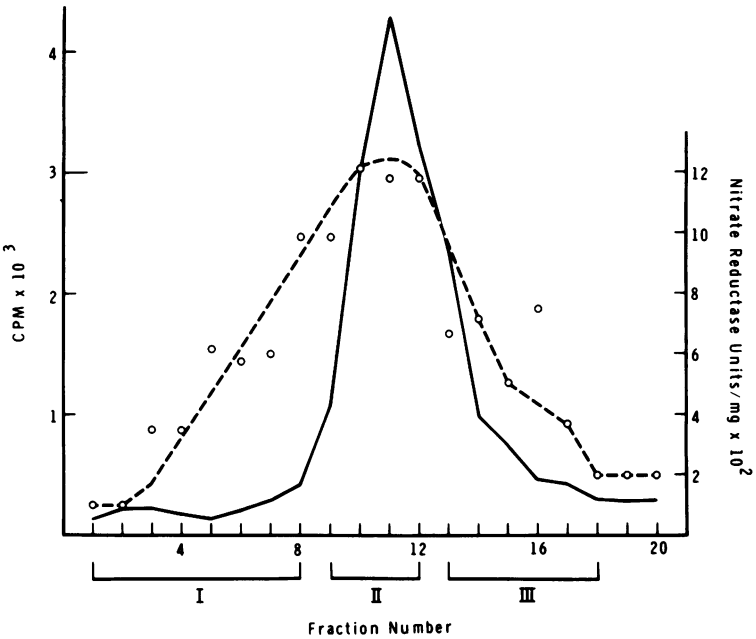

FIG. 1. *Distribution on a sucrose gradient of nitrate reductase activity and protein contained in a crude preparation of cytoplasmic membrane from anaerobically grown cells. ^{3}H-labeled cells were broken as described in the text, then spun at 36,000 × g for 30 min to remove whole cells and the bulk of the outer membrane. The supernatant was spun at 200,000 × g, and the pelleted membranes were suspended in buffer and run on a sucrose gradient as described in the text. Roman numerals correspond to the fractions pooled for heating (Table 1). Density decreases from left to right. The solid line corresponds to protein (counts per minute) and the dashed line to nitrate reductase activity per milligram of protein.*

supernatant after centrifugation. There was approximately a threefold difference in the percentage of nitrate reductase solubilized from the densest and lightest portions of the cytoplasmic membrane (Table 1), with solubilization decreasing with decreasing density of the fractions. This suggested the possibility that the solubilizing activity might be contained in the outer membrane and not in the cytoplasmic membrane. When cytoplasmic membrane isolated from such a sucrose gradient was analyzed by differential extraction with the detergent Triton X-100 (15), it was found to contain 81% Triton-soluble material and 19% Triton-insoluble material. SDS gel analysis of these two fractions showed that the Triton-insoluble material consisted mainly of the major outer membrane proteins. Thus, the results presented in Table 1 might be explained by the presence of increasing amounts of outer membrane in gradient fractions of increasing density. To test this hypothesis, we mixed and heated gradient-purified cytoplasmic membrane from anaerobic cells (containing nitrate reductase) with various gradient fractions of cytoplasmic and outer membrane from aerobic cells (lacking nitrate reductase) (Table 2; Fig. 2). The results of this experiment

TABLE 1. *Percent nitrate reductase solubilized from various membrane fractions from anaerobically grown cells*

Fraction heated[a]	% NR solubilized[b]
Whole envelope[c]	55
Gradient fraction I	80
Gradient fraction II	33
Gradient fraction III	17

[a] Each gradient fraction (Fig. 1) was heated separately at 60°C for 10 min at a protein concentration of 0.5 mg/ml.

[b] Percent nitrate reductase (NR) solubilized was calculated as described in the text.

[c] Refers to the material placed on the gradient, isolated as described in the text.

demonstrate that only outer membrane fractions are capable of increasing the solubilization of nitrate reductase above the background solubilization that occurs in the cytoplasmic membrane alone. The final proof that the nitrate reductase-solubilizing activity resides in the outer membrane was provided by mixing detergent-purified (15) fractions of cytoplasmic and outer membrane proteins with anaerobic cytoplasmic membrane containing nitrate reductase. These frac-

TABLE 2. *Percent nitrate reductase solubilized from cytoplasmic membrane by various membrane fractions from aerobically grown cells*

Mixtures heated[a] (anaerobic[b] + aerobic[c])	% NR solubilized[d]
Cytoplasmic membrane	31
Cytoplasmic membrane + gradient fraction I	77
Cytoplasmic membrane + gradient fraction II	60
Cytoplasmic membrane + gradient fraction III	39
Cytoplasmic membrane + gradient fraction IV	39

[a] Either 0.5 mg of anaerobic membrane was mixed with 0.5 mg of aerobic membrane in a volume of 2 ml, or anaerobic membrane alone was suspended to a concentration of 1 mg in 2 ml in the same buffer. Each sample was heated to 60°C for 10 min.

[b] Cytoplasmic membrane was isolated on a sucrose gradient from anaerobically grown cells.

[c] Envelope from aerobically grown cells underwent gradient fractionation (Fig. 2). After concentration of each pool of the aerobic membrane by centrifugation, each was suspended in 7.5 mM phosphate buffer (pH 8.3).

[d] Percent nitrate reductase (NR) solubilized was calculated as described in the text.

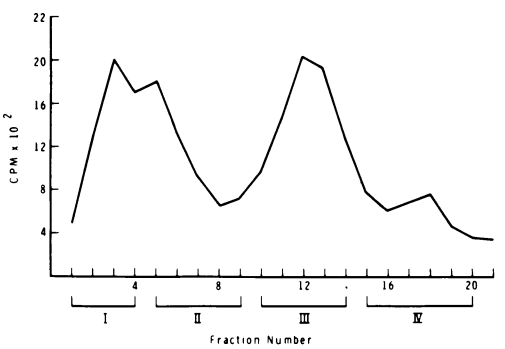

FIG. 2. *Distribution on a sucrose gradient of protein contained in an envelope preparation from aerobically grown cells. Whole envelope from ^{3}H-labeled cells was isolated and run on a sucrose gradient as described in the text. Roman numerals correspond to the fractions pooled for addition to anaerobic cytoplasmic membrane (Table 2). Fractions I and II contain mostly outer membrane; III and IV contain mostly cytoplasmic membrane. Density decreases from left to right.*

tions were isolated according to the diagram in Fig. 3. Outer membrane that was solubilized by incubation in Triton containing EDTA was also used. The detergent was removed from these solubilized fractions by the addition of cold ethanol prior to mixing with nitrate reductase-containing cytoplasmic membrane. Outer membrane that was isolated either on a gradient or by virtue of its insolubility in Triton was able to solubilize nitrate reductase significantly above the control value (Table 3). Further, Triton-solubilized cytoplasmic membrane did not significantly increase nitrate reductase solubilization, but Triton-EDTA-solubilized outer membrane did. This table demonstrates that the ability of the outer membrane to solubilize nitrate reductase increased after the removal of the cytoplasmic membrane (Triton insoluble) and after the solubilization of the outer membrane proteins

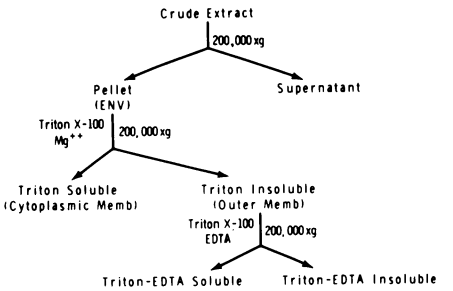

FIG. 3. *Detergent fractionation of cytoplasmic and outer membrane. Triton extractions were performed as described in the text.*

TABLE 3. *Percent nitrate reductase solubilized from cytoplasmic membrane by various forms of outer membrane*

Mixtures heated[a] (anaerobic[b] + aerobic[c])	% NR solubilized[d]
Cytoplasmic membrane	32
Cytoplasmic membrane + outer membrane (gradient)	77
Cytoplasmic membrane + Triton soluble	37
Cytoplasmic membrane + Triton insoluble	84
Cytoplasmic membrane + Triton-EDTA soluble	
(0.5 mg)	94
(0.1 mg)	63
(0.02 mg)	39

[a] A 0.5-mg sample of anaerobic membrane was mixed with 0.5 mg of aerobic membrane in a volume of 2 ml unless otherwise indicated. The samples were heated at 60°C for 10 min.

[b] Anaerobic cytoplasmic membrane was prepared as in Table 2.

[c] Aerobic membrane was isolated from a sucrose gradient (see the text) or by differential Triton extraction as shown in Fig. 3. Before adding to the anaerobic membranes, detergent was removed from all Triton-soluble fractions by precipitation of the protein from solution with two volumes of ethanol at 0°C. The precipitates were washed once with ethanol at 4°C and then suspended in 7.5 mM phosphate buffer (pH 8.3).

[d] Percent nitrate reductase (NR) solubilized was calculated as described in the text.

(Triton-EDTA soluble). Solubilization of nitrate reductase in all combinations of fractions (cytoplasmic membrane alone and cytoplasmic membrane plus outer membrane) was prevented by the presence of PAB during heating. Benzamidine also prevented solubilization, but higher concentrations were necessary. PAB at 20 mM decreased solubilization to control levels of 2 to 5%, whereas 60 mM benzamidine only decreased solubilization to 25%. Neither inhibited nitrate reductase activity. Both PAB and benzamidine are competitive inhibitors of trypsin-like proteases.

To insure that what we were measuring in these experiments was actually increasing amounts of enzyme being solubilized and not just the solubilization of a small but highly active fraction of nitrate reductase, the enzyme was also precipitated by antibody from the solubilized fractions after heating various mixtures from ^{3}H-labeled cells. These experiments (data not shown) indicated that as nitrate reductase activity increased in the supernatant increasing amounts of ^{3}H-labeled nitrate reductase could also be found in the antibody precipitates.

Kinetics of nitrate reductase solubilization. Further proof that an activity in the outer membrane is responsible for the solubilization of nitrate reductase is shown in Fig. 4. When increasing amounts of outer membrane (Triton insoluble) were added to gradient-isolated cytoplasmic membrane, solubilization of nitrate reductase was correspondingly increased. The enzymatic nature of the solubilization reaction is illustrated in Fig. 5 and 6. Figure 5 shows that the solubilization of nitrate reductase is time dependent. This is true in both the crude envelope fraction and the gradient-purified cytoplasmic membrane. The solubilization reaction is also temperature dependent, and solubilization increases logarithmically with temperature (Fig. 6).

Mechanism of solubilization. The mechanism by which nitrate reductase is solubilized appears to be by cleavage of a small piece from the B subunit (11) (Fig. 7). This cleavage of the B subunit was first observed during the purification of the enzyme (14) as a variability in the molecular weight of the B polypeptide.

Nature of the interaction between cytoplasmic and outer membrane. Since the solubilization reaction requires the interaction of two insoluble proteins and, in some cases, two distinct membranes (cytoplasmic membrane and outer membrane), the possibility existed that heating these fractions at 60°C might serve to denature the cytoplasmic membrane proteins, making nitrate reductase more susceptible to the protease. Thus, a series of experiments was done to determine how the heating process was affecting both the cytoplasmic and outer membranes (Table 4). The results demonstrate that heating either fraction by itself prior to mixing does not result in maximum solubilization of nitrate reductase, i.e., the elevated temperature must occur when both fractions are in contact. The same results were obtained using Triton-EDTA-solubilized outer membrane or gradient-isolated outer membrane.

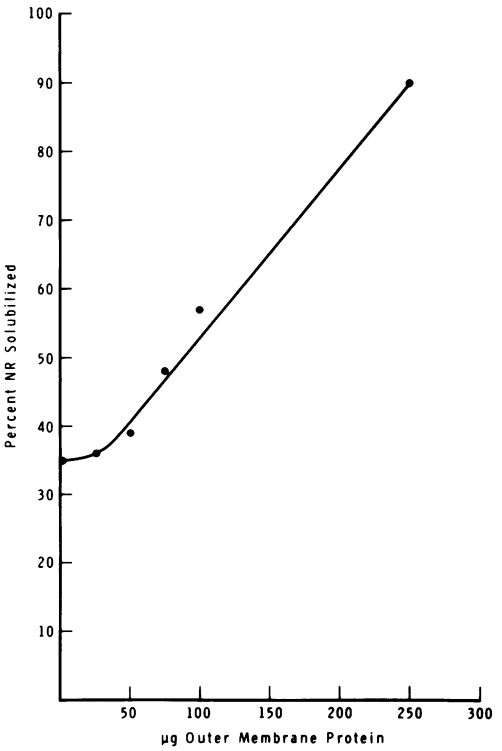

FIG. 4. *Effect of outer membrane protein on the solubilization of nitrate reductase. Cytoplasmic membrane from anaerobically grown cells was isolated from a sucrose gradient as described in the text. Outer membrane from aerobically grown cells was also isolated from a sucrose gradient, and then extracted with Triton (see the text) to remove all cytoplasmic membrane proteins. Increasing amounts of Triton-insoluble outer membrane protein were added to 500-µg samples of cytoplasmic membrane in a final volume of 2 ml. Each sample was incubated at 60°C for 10 min. Nitrate reductase solubilization was determined as in the text.*

Identification of the protease. We attempted to identify the protease or proteases by looking for outer membrane proteins that would interact with DFP. DFP is the strongest and most specific of the serine protease inhibitors. Because it reacts covalently with the serine at the enzyme active site, it has been used for affinity labeling of proteases (18). The Triton-

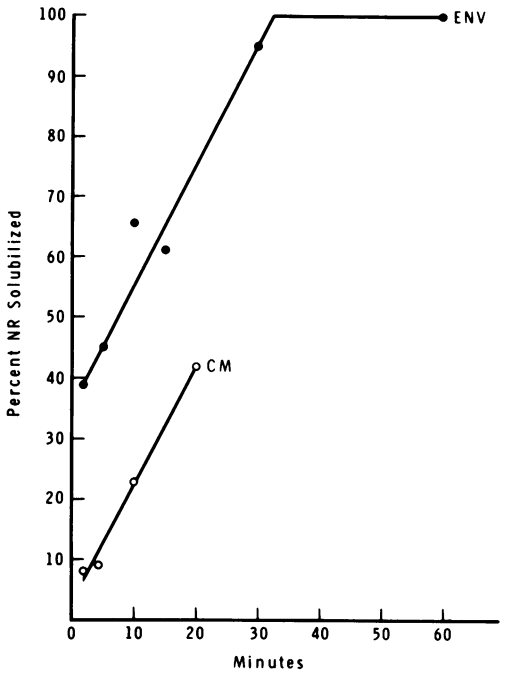

FIG. 5. *Effect of incubation time on the solubilization of nitrate reductase. Envelope (ENV) and cytoplasmic membrane (CM) from anaerobically grown cells were suspended (separately) to a protein concentration of 0.5 mg/ml, and samples were heated at 60°C for 2 to 60 min. Nitrate reductase solubilization and ENV and CM isolation were performed as described in the text.*

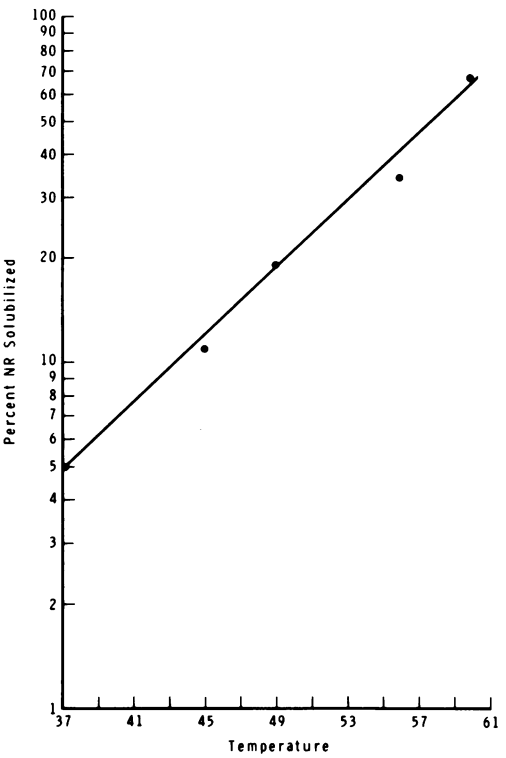

FIG. 6. *Effect of temperature on the solubilization of nitrate reductase. Envelope was isolated from anaerobically grown cells as described in the text. It was suspended to a protein concentration of 0.5 mg/ml, and samples were heated at the appropriate temperature for 10 min. Nitrate reductase solubilization was determined as described in the text.*

insoluble fraction of the outer membrane from aerobic cells was incubated with [^{3}H]DFP in the presence and absence of PAB. After washing, the incubated samples were solubilized in SDS and run on gels. Autoradiograms of these gels reveal a single band of DFP-labeled protein (Fig. 8). Densitometer tracings of these autoradiograms show that DFP binding is inhibited by 50% when PAB is added to the incubation mixture before DFP is.

Role of proteolytic activity in cell growth. It seemed unlikely that a proteolytic activity in the outer membrane would be responsible for solubilization of an enzyme in the cytoplasmic membrane in vivo. To see if we could find an in vivo function for this outer membrane protease, we grew cells aerobically in the presence of PAB and looked at the effect of inhibition of this protease on the biosynthesis of membrane proteins. To do this, a log-phase culture was divided into three parts and $H_2{}^{35}SO_4$ was added to each. At the same time, PAB was added to two of the three to final concentrations of 6 mM and 15 mM. After 45 min of growth, each culture was harvested, cytoplasmic and outer membranes were isolated, and each was run on SDS gels. An autoradiogram from such an experiment is shown in Fig. 9. Very few changes are visible in the cytoplasmic membrane; however, some very significant changes are observed in the outer membrane. There is a cessation of the biosynthesis of several of the major outer membrane proteins, with the most dramatic effect on the biosynthesis of protein Ia (6, 16). This loss of the major outer membrane proteins is also accompanied by the appearance of several high-molecular-weight proteins.

DISCUSSION

The proteolytic activity responsible for the cleavage and solubilization of nitrate reductase is present not in the cytoplasmic membrane but in the outer membrane. This is supported by the fact that neither gradient-isolated nor detergent-extracted cytoplasmic membrane can increase the release of nitrate reductase from the cytoplasmic membrane over background levels, but

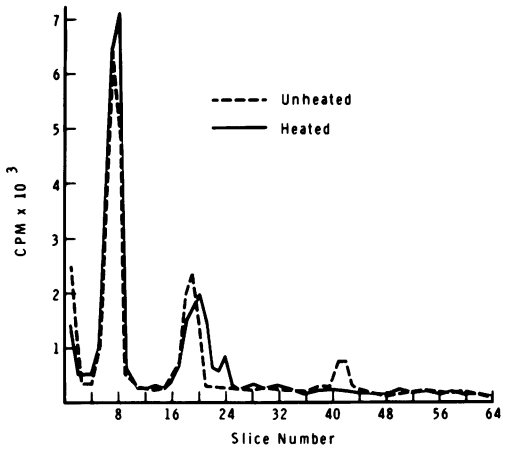

FIG. 7. *Comparison on SDS gels of antibody-precipitated nitrate reductase from heated and unheated envelope. ^{3}H-labeled envelope from anaerobic cells was suspended in 7.5 mM phosphate buffer (pH 8.3) containing 2% Triton X-100 and divided into two portions. One portion was incubated at 60°C for 10 min and the other at room temperature for 10 min. Each was then spun at 200,000 × g for 45 min, and nitrate reductase was precipitated by antibody from each supernatant fraction. Each precipitate was solubilized and run on a 7.5% SDS gel as previously described (11). The three peaks in the unheated sample correspond to (from left to right) subunits A, B, and C. Note the cleavage of subunit B in the heated sample.*

TABLE 4. *Percent nitrate reductase solubilized by Triton-EDTA-solubilized outer membrane at various temperatures*[a]

Separate incubation[b] (°C)		Combined incubation (°C)	Percent NR solubilized[c]
CM	TES		
4			0
60			24
		4	2
		37	6
		60	90
4	60	37	4
60	4	37	24
60	60	37	35

[a] Anaerobic cytoplasmic membrane (CM) and aerobic (Triton-EDTA-solubilized [TES]) samples were each suspended in 7.5 mM phosphate buffer (pH 8.3) at a protein concentration of 0.5 mg/ml. Either 2 ml of CM was incubated alone, 1 ml of CM was mixed with 1 ml of TES and they were incubated together, or each was incubated separately, then mixed and reincubated. Incubation temperatures are indicated for each combination.

[b] CM was prepared as in Table 2. TES was prepared by extraction of the outer membrane with Triton and EDTA as in Fig. 3. Triton was removed by ethanol precipitation as in Table 3.

[c] Percent nitrate reductase (NR) solubilized was calculated as described in the text.

outer membrane isolated by either procedure can increase solubilization to 100%. The low levels of solubilization which occur after heating gradient-isolated cytoplasmic membrane alone appear to be due to the presence of small amounts of contaminating outer membrane. This is suggested by the fact that nitrate reductase solubilization from cytoplasmic membrane with or without added outer membrane shows similar kinetics, and both are inhibited by PAB. This is supported further by the fact that 19% of the protein in gradient-isolated cytoplasmic membrane can be designated as outer membrane protein, both by virtue of its insolubility in Triton and by the fact that this Triton-insoluble fraction appears very similar to gradient-isolated outer membrane on SDS gels. When Triton-insoluble material obtained from gradient-isolated cytoplasmic membrane is added back to untreated cytoplasmic membrane and solubilization of nitrate reductase is measured (as in Table 3), it is always as effective as the Triton-insoluble material from gradient-isolated outer membrane, and often it is even more effective. It is possible that the outer membrane that remains in the cytoplasmic membrane fraction is actually attached to the cytoplasmic membrane at "fused regions." These regions have been postulated (2) to be areas of attachment between the outer and cytoplasmic membranes where proteins, DNA, and other macromolecules can be transported across both membranes in either direction. Thus, these regions might be required for protein secretion, and one would expect a processing protease to be rich in these regions. The possibility of this enrichment is presently being explored.

The mechanism of solubilization of nitrate reductase appears to be via cleavage of a small portion from the B subunit. This suggests that the B subunit contains a hydrophobic portion (4) which, when removed by proteolysis, allows both subunits A and B to become soluble. However, DeMoss (4) has suggested that proteolysis only enhances solubilization by heating. The heat treatment also causes loss of the C subunit. Since we do not have antibody to this subunit, it is impossible to determine whether the heating causes degradation of this protein or merely denaturation.

For this reaction to occur to a measurable extent over short periods of time, relatively high temperatures are required. One reason for this might be that at the elevated temperature the structure of the cytoplasmic membrane is somehow changed to allow the protease access to the B subunit of the enzyme. Since heating is nec-

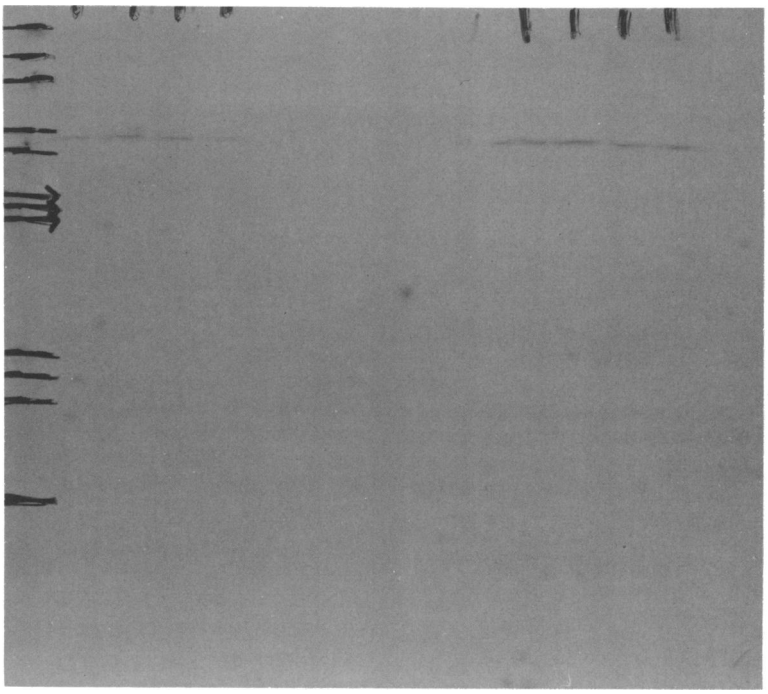

FIG. 8. *Autoradiogram of an SDS gel, showing binding of [^{3}H]DFP to outer membrane. Preparation of outer membrane fractions incubated with [^{3}H]DFP was carried out as described in the text. Contact autoradiograms of the SDS gel-fractionated samples were prepared after staining with Coomassie blue. The major bands were marked on the stained gel with radioactive ink prior to treatment for fluorography. The marks on the left correspond to the radioactive ink, with the three arrows indicating proteins Ia, Ib, and 3a or 2* (6, 16). The four samples on the left half of the gel correspond to the incubation with [^{3}H]DFP in the presence of PAB. They contain, from left to right, 20, 60, 40, and 20 μg of protein. The four on the right half correspond to the incubation with [^{3}H]DFP alone and contain, from left to right, 60, 40, 20, and 20 μg of protein. Areas under the peaks of densitometer tracings (40-μg samples) correspond to 300 for DFP alone and 167 for DFP plus PAB.*

essary even for Triton-EDTA-solubilized outer membrane to solubilize nitrate reductase, the effect of heat would appear not to be on the structure of the outer membrane. Nor is the effect of heat merely to denature or dissociate the cytochrome *b* from subunits A and B. If this were the case, previously heated cytoplasmic membrane should react with outer membrane at 37°C.

The data in Fig. 9 show that the addition of PAB to growing cells inhibits the biosynthesis of outer membrane proteins to a greater extent than the biosynthesis of cytoplasmic membrane proteins. During their synthesis, outer membrane proteins must go through the cytoplasmic membrane to the external surface of the outer membrane in a fashion analogous to proteins secreted from animal cells. Thus, one would expect that these proteins would initially contain a signal which would later be removed, whereas cytoplasmic membrane proteins might have a different mechanism of synthesis. Evidence for the biosynthesis of outer membrane proteins as pro-proteins has been presented by Halegoua et al. (5) and Sekizawa et al. (17). Inhibition of this activity which removes the signal might result in the inhibition of the synthesis of processed proteins. In a recent study (8), Ito showed that tosyl-lysine chloromethylketone, antapain, leupeptin, and DFP all inhibit the in vivo biosynthesis of an outer membrane protein, protein I. He finds differences in the proteins that are made after the addition of high-molecular-weight inhibitors in strains with different permeability properties. His interpretation of this result is that the inhibition of the biosynthesis of protein I is due to the action of the protease inhibitors at or near the cell surface. This interpretation supports our localization of the protease in the outer membrane. Whether the high-molecular-weight proteins which appear in PAB-grown cells are related to the missing proteins has yet to be determined. Sekizawa et al. (17) have identified a precursor to protein I; the

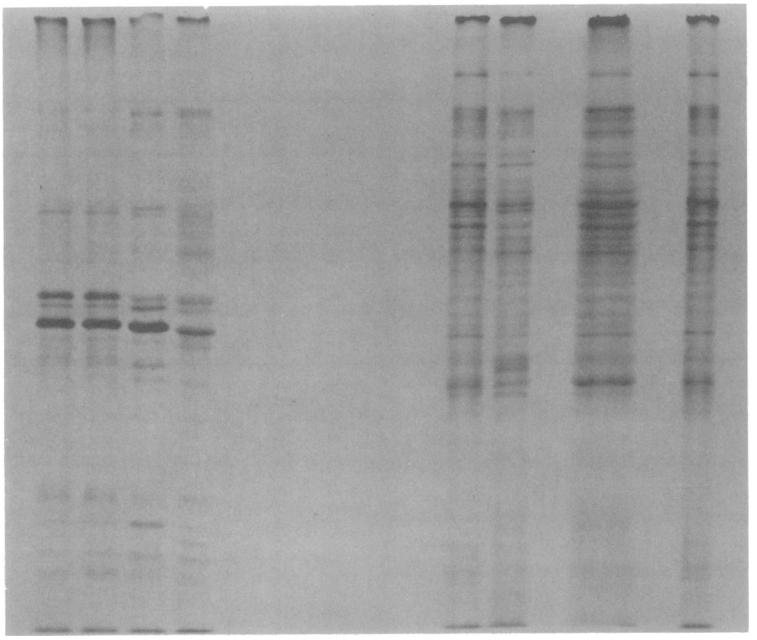

FIG. 9. *Autoradiogram of an SDS gel of cytoplasmic and outer membranes from cultures grown in the presence of PAB. Envelopes were isolated from each and fractionated by differential solubilization in Triton into cytoplasmic and outer membranes. A 2,500-cpm amount of each Triton-insoluble sample and 3,800 cpm of each Triton-soluble sample were applied to SDS gels, and contact autoradiograms were prepared. From left to right are shown outer membrane from cultures containing no PAB (lanes 1 and 2), 6 mM PAB (lane 3) and 15 mM PAB (lane 4), and cytoplasmic membrane from the cultures containing no PAB (lane 5), 6 mM PAB (lane 6), 15 mM PAB (lane 7), and no PAB (lane 8). All procedures were done as described in the text.*

high-molecular-weight proteins which appear during growth in PAB do not correspond in molecular weight to this precursor, however. The high-molecular-weight proteins that were found by Ito (8) after the addition of other protease inhibitors also do not correspond to this precursor.

Is the protease that cleaves the nitrate reductase the same one responsible for the processing of outer membrane proteins? The fact that solubilization of nitrate reductase and the biosynthesis of certain outer membrane proteins are both inhibited by PAB allows the assumption that a single protease or type of protease may be responsible for both. The binding of DFP to only one protein in the outer membrane suggests that there is a single serine protease. It is, however, possible that binding to other proteins might not have been detected. Since DFP binding to this protein is inhibited by PAB and since the biosynthesis of protein I is inhibited by both PAB and DFP (8), it is reasonable to assume that DFP and PAB could be acting on the same protease. Other evidence for the existence of a protease in the *E. coli* outer membrane has been provided by two laboratories. Inouye and Beckwith (7) have shown that crude outer membrane can cleave the precursor form of a secreted protein, alkaline phosphatase, to its in vivo size. Regnier et al. have shown that there is a proteolytic activity in the outer membrane which will hydrolyze ^{125}I-labeled casein (personal communication). No other investigators have been able to demonstrate proteolytic activity in the outer membrane. Although we have tried many different chromogenic protease substrates, we have never found any activity in the outer membrane that would hydrolyze any of these. This suggests that this protease acts on very specific sites within proteins. The data of Inouye and Beckwith (7) suggest that a hydrophobic region is cleaved from alkaline phosphatase. Similarly, a hydrophobic site is suggested by the signal hypothesis of Blobel and Dobberstein (3). Such a site would be provided by the B subunit of nitrate reductase (4). The use of nitrate reductase solubilization as an assay for proteolytic activity now allows us to be able to purify this unique protease and eventually to determine its relationship to the processing of membrane and secreted proteins.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants GM-23010 and GM-18006 from the National Institute of General Medical Sciences and Biomedical Research Sup-

port Grant RR-5360 from the Division of Research Resources, National Institutes of Health.

LITERATURE CITED

1. **Aiyappa, P. S. L. J. Traficante, and J. O. Lampen.** 1977. Penicillinase-releasing protease of *Bacillus licheniformis*: purification and general properties. J. Bacteriol. **129**:191-197.
2. **Bayer, M.** 1968. Areas of adhesion between wall and membrane of *E. coli*. J. Gen. Microbiol. **53**:395-404.
3. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835-851.
4. **DeMoss, J.** 1977. Limited proteolysis of nitrate reductase purified from membranes of *E. coli*. J. Biol. Chem. **252**: 1696-1701.
5. **Halegoua, S., J. Sekizawa, and M. Inouye.** 1977. A new form of structural lipoprotein of outer membrane of *E. coli*. J. Biol. Chem. **252**:2324-2330.
6. **Hindennach, I., and U. Henning.** 1975. The major protein of the *E. coli* outer cell envelope membrane. Preparative isolation of all major membrane proteins. Eur. J. Biochem. **59**:207-213.
7. **Inouye, H., and J. Beckwith.** 1977. Synthesis and processing of an *E. coli* alkaline phosphatase precursor *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **74**:1440-1444.
8. **Ito, K.** 1978. Protease inhibitors inhibit production of protein I. Biochem. Biophys. Res. Commun. **82**:99-107.
9. **Laemmli, U.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
11. **MacGregor, C. H.** 1975. Solubilization of *Escherichia coli* nitrate reductase by a membrane-bound protease. J. Bacteriol. **121**:1102-1110.
12. **MacGregor, C. H.** 1975. Anaerobic cytochrome b_1 in *Escherichia coli*: association with and regulation of nitrate reductase. J. Bacteriol. **121**:1111-1116.
13. **MacGregor, C. H.** 1976. Biosynthesis of membrane-bound nitrate reductase in *Escherichia coli*: evidence for a soluble precursor. J. Bacteriol. **126**:122-131.
14. **MacGregor, C. H., C. Schnaitman, D. Normansell, and M. Hodgins.** 1974. Purification and properties of nitrate reductase from *E. coli*. J. Biol. Chem. **249**: 5321-5327.
15. **Schnaitman, C. A.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. **108**:545-552.
16. **Schnaitman, C. A.** 1974. Outer membrane proteins of *E. coli*. III. Evidence that the major protein of *Escherichia coli* O111 outer membrane consists of four distinct peptide species. J. Bacteriol. 118:442-453.
17. **Sekizawa, T., S. Inouye, S. Halegoua, and M. Inouye.** 1977. Precursors of major outer membrane proteins of *E. coli*. Biochem. Biophys. Res. Commun. **77**: 1126-1133.
18. **Tokes, Z., and S. M. Chambers.** 1975. Proteolytic activity associated with human erythrocyte membranes. Biochim. Biophys. Acta **389**:325-338.
19. **Van Heerikhuizen, H., E. Kwak, E. VanBruggen, and B. Witholt.** 1975. Characterization of low density cytoplasmic membrane subfraction isolated from *E. coli*. Biochim. Biophys. Acta **413**:177-191.