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Energy-Requiring Translocation of the OmpA Protein and Alkaline Phosphatase of *Escherichia coli* into Inner Membrane Vesicles

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In developing a reliable in vitro system for translocating bacterial proteins, we found that the least dense subfraction of the membrane of *Escherichia coli* was superior to the total inner membrane, both for a secreted protein (alkaline phosphatase) and for an outer membrane protein (OmpA). Compounds that eliminated the proton motive force inhibited translocation, as already observed in cells; since protein synthesis continued, the energy for translocation appears to be derived from the energized membrane and not simply from ATP. Treatment of the vesicles with protease, under conditions that did not interfere with subsequent protein synthesis, also inactivated them for subsequent translocation. We conclude that export of some proteins requires protein-containing machinery in the cytoplasmic membrane that derives energy from the proton motive force.

The study of protein translocation into or across membranes has been greatly advanced by the finding that many such proteins, in both procaryotic and eucaryotic cells, have an N-terminal signal sequence that is cleaved during export (6, 23). However, it is not certain whether translocation in bacteria requires a specific apparatus for active translocation in the cytoplasmic membrane (6, 23), a passive channel, or only conformational interactions with the membrane (42).

A biochemical approach to this problem has shown that ribosomes complexed with membrane in *Bacillus subtilis* cover tightly a protein of 64 kilodaltons (14), and antibodies to this protein can precipitate a complex with several other proteins (M. Caulfield, P. C. Tai, and B. D. Davis, manuscript in preparation). Moreover, mutations in *sec* genes in *Escherichia coli* (18, 26) reduce the ability of cells to process and export multiple proteins, and impairment of export of a specific protein by mutations in its signal sequence can be corrected by an extragenic suppressor mutation (8). These findings all suggested the presence of an apparatus in the membrane. On the other hand, the proteins in question might be located in a ribosome-attached signal recognition particle, which has recently been discovered in eucaryotic cells (40) and which attaches to a docking protein of a secretory apparatus in the eucaryotic membrane (12, 22).

If a more elaborate apparatus than a docking protein exists in the membrane, it might utilize energy from the membrane. Studies in intact bacterial cells have revealed a requirement for an energized membrane: dissipation of the proton motive force (PMF), by carbonyl cyanide-*m*-chlorophenyl hydrazone or by valinomycin, blocked processing (4, 9, 45), and translocation (45) of several outer membrane (OM) and periplasmic proteins. Moreover, carbonyl cyanide-*m*-chlorophenyl hydrazone inhibited processing of several precursors that had accumulated in the presence of phenethyl alcohol (28). However, a simpler system is necessary to distinguish between a direct and an indirect role of the PMF, and such a system would be useful for investigating other aspects of the export process in detail.

In the present work we have undertaken to develop a procedure for obtaining membrane vesicles of *E. coli* capable of translocating proteins, i.e., processing the precursor and sequestering the product from proteases. Vesicles with this capacity have been isolated from animal cells (2), but it has proved more difficult to obtain reproducible results with bacteria, perhaps because of the activity of their proteases or nucleases (or both). Of the several preparations from *E. coli* that have been described (3, 20, 35), we found that of Smith (35), based on the separation of inner membrane (IM) from OM by the procedure of Osborn and Munson (27), the most promising, and we have introduced modifications that improve activity and reproducibility.

In this paper we describe vesicles of *E. coli* that translocate the periplasmic protein alkaline phosphatase (which is secreted cotranslationally in cells [36]) and the OM protein OmpA in an operationally cotranslational manner. Moreover, as with intact cells, this translocation is inhibited by carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) or valinomycin. In a subsequent paper we will describe the posttranslational activity of our system.

MATERIALS AND METHODS

Bacterial strains. The strains used in this work are all *E. coli* K-12. MC1000(pHI-1) (15), which contains the *phoA* gene in its plasmid, produced about 10 times as much alkaline phosphatase as did the wild type when phosphate limited, and so it was a good source of mRNA (7) for this protein as well as of the stable mRNA for the OmpA protein. The RNase I^- strain D10 was used for preparation of membranes and S30 extracts.

Media. L broth was supplemented with A salts (38) and medium A (5). Medium P2, used for limiting phosphate, contained 0.1 M Tris-maleate (pH 7.0), 10 mM KCl, 0.5 mM $MgSO_4$, 2 mM K_2HPO_4, 1 μM each $ZnSO_4$ and $FeSO_4$, 1 mM Na_3 citrate, and 10 μg of thiamine per ml.

Membrane preparation. Strain D10 was grown with forced oxygenation in 2.5 liters of supplemented L broth containing 0.2% glucose; pH was maintained near neutrality by the periodic addition of 10 N NaOH. At 1.2×10^9 to 1.6×10^9 cells per ml (well before the stationary phase) the culture was poured over ice and centrifuged, and the cells were converted to spheroplasts by the method of Witholt et al. (43). Typically, cell pellets were quickly dispersed mechanically in 150 ml of 0.25 M sucrose containing 0.2 M Tris-hydrochloride (pH 8.0) and 50 μg of chloramphenicol per ml

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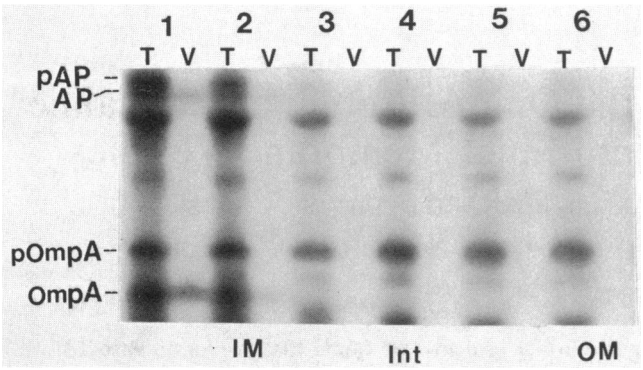

FIG. 1. Comparison of membrane fractions. Membranes were prepared as described in the text, except that one-fourth the amount of cells was used, and total membrane was collected by a 1-h centrifugation onto a cushion of 2.0 M sucrose in 20 mM EDTA–1 mM DTT, centrifuged overnight at 3°C in a linear gradient (1.0 to 2.0 M sucrose in 20 mM EDTA–1 mM DTT), and harvested in six fractions. Membrane from each fraction was collected by centrifugation, and equal amounts were assayed for translocation activity as described in the text. Fractions were numbered from top to bottom. Fractions 2, 4, and 6 contained the visible bands and were the major peaks of IM, intermediate density membrane (int), and OM, respectively. T, Total polypeptides synthesized in the presence of the indicated membrane fraction; V, polypeptides sedimenting with the vesicles after pronase treatment.

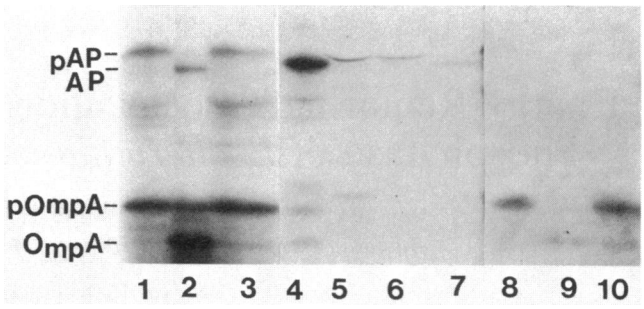

FIG. 2. Immunological identification of alkaline phosphatase and OmpA protein. Lanes 1 through 3, translocation assay: 1, total polypeptides synthesized in the absence of membrane vesicles; 2, polypeptides sedimenting with vesicles after pronase treatment; 3, total polypeptides synthesized in the presence of fraction I membrane vesicles. Lane 4, [^{3}H]leucine-labeled periplasmic protein enriched for alkaline phosphatase. Lanes 5 through 7, precipitation with antiserum to alkaline phosphatase: 5, from total polypeptides synthesized without vesicles; 6, from synthesis with vesicles; 7, from vesicles. Lanes 8 through 10, precipitation with antiserum to OmpA protein: 8, immunoprecipitate from total synthesis without vesicles; 9, immunoprecipitate from vesicles; 10, immunoprecipitate from total synthesis with vesicles.

at room temperature. The following additions were then made sequentially, each followed by gentle swirling to ensure complete mixing: 1.5 ml of 50 mM trisodium EDTA (pH 8.0), 1.5 ml of a lysozyme solution (6 mg/ml), and 150 ml of water. When spheroplast formation, monitored by phase-contrast microscopy, was about 95% complete (usually within 10 to 25 min), the suspension was chilled.

Spheroplasts were centrifuged at 4°C (5 min, 7,000 × *g*) and partially lysed by suspension in 60 ml of ice-cold 20 mM EDTA (brought to pH 7.2 with NaOH)–1 mM dithiothreitol (DTT). Subsequent steps were carried out at 0 to 4°C. The suspension was passed through a French pressure cell at 7,500 lb/in^2 to complete the lysis, shear the released DNA, disrupt membrane aggregates, and invert membrane vesicles. Large debris was removed by centrifugation (5 min, 7,000 × *g*). In the standard preparation (used in all experiments after that in Fig. 1) the lysate was layered over a two-step gradient (1 ml each 0.5 M and 1.4 M sucrose in 20 mM EDTA–1 mM DTT) and centrifuged in a Beckman SW41 rotor at 37,000 rpm for 3 h; much of the OM sedimented through the cushion. The visible interface band, consisting mostly of IM, was collected and centrifuged for 17 to 18 h on a five-step sucrose gradient (0.6 M to 1.4 M, also in 20 mM EDTA–1 mM DTT); it yielded two visible bands toward the bottom of the gradient. Material above these bands, as well as the visible bands themselves, were collected separately, diluted fourfold with 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM magnesium diacetate–1 mM dithiothreitol, and centrifuged for 75 min at 160,000 × *g*. The pelleted membrane was dispersed in a minimal volume of the same buffer and stored at −70°C. Membranes could be frozen and thawed several times without losing activity. Membrane concentration was measured by absorbance at 280 nm in 2% sodium dodecyl sulfate (SDS).

RNA preparation. Strain MC1000/pHI-1 was grown with forced oxygenation in 2.5 liters of P2 medium supplemented with 1% glucose, 0.5% vitamin-free low-phosphate Casamino Acids (ICN Pharmaceuticals, Cleveland, Ohio), 0.04% L-leucine, and 10 μg of tetracycline per ml. The culture was maintained near neutrality by addition of 2 M Tris. At 1 to 2 h after depletion of the phosphate in the medium began to induce alkaline phosphatase (qualitatively assayed by *p*-nitrophenylphosphate hydrolysis), chloramphenicol (50 ml, 2.5 mg/ml) was added, and the culture was quickly poured over ice. One-half of the culture was centrifuged, and then the other half was centrifuged in the same set of centrifuge tubes without removing the first pellets. The combined pellets were quickly frozen in an ethanol-dry ice bath, chipped out of the centrifuge tubes with a metal spatula and with gentle warming, weighed, and transferred to a mortar chilled on a bed of dry ice. An equal weight of levigated alumina, 10 to 20 mg of bentonite, and 0.01 ml of 2% (vol/wt) lithium heparin were added, and the mixture was ground with a pestle until the pellets were broken into 1- to 2-mm pieces. These were transferred gradually with continual mixing to 100 ml of 2% SDS–0.17 M sodium acetate (pH 5)–0.02% lithium heparin at 55 to 60°C. The suspension was cooled to room temperature and centrifuged (3 min, 3,000 × *g*), and the supernatant was transferred to glass centrifuge bottles. RNA was extracted by the method of Palmiter (29), with modifications. The first LiCl precipitate was dissolved in several milliliters of 1 mM Tris-hydrochloride (pH 7.6)–5 mM KCl–1 mM magnesium diacetate containing 10 μg of DNase (Worthington Diagnostics) per ml and was incubated at room temperature for 20 to 30 min to reduce the viscosity. The final ethanol precipitate was dried under a gentle stream of N_2 and dissolved in water. Samples were lyophilized and then stored at −70°C.

Protein synthesis and translocation. Protein synthesis was performed with an S30 extract of *E. coli* D10 (24) as previously described (39), with RNA added to an absorbancy at 260 nm of 5 and with 10 to 20 μCi of [^{35}S]methionine per 0.1 ml. The messenger was total RNA from strain MC1000/pHI-1; incubation was at 40°C (see below). After 5 min (to ensure initiation of protein synthesis without inhibition by membrane; few protein chains are completed at this time), 90 μl was added to 10 μl of membrane suspension containing 0.5 units of absorbancy at 280 nm (measured in 2% SDS). Incubation was continued for 25 or 35 min, followed by

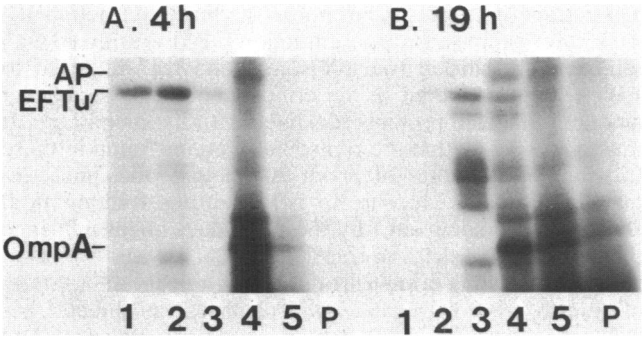

FIG. 3. Cosedimentation of translocated proteins with membrane vesicles. Translocation was performed as described in the text with the following exceptions. After pronase treatment diluted samples were loaded onto gradients containing layers of 0.5, 1.4, and 1.6 M sucrose in 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM magnesium acetate in Beckman SW50.1 tubes and centrifuged for either 4 or 19 h at 189,000 × *g* (average). Fractions of 1 ml each were collected by pipette, precipitated with trichloroacetic acid, and electrophoresed. Only the relevant portion of the fluorogram is shown. Lane 1 was from the top, and lane 2 was the sample–0.5 M sucrose interface. (They contain EFTu, which is not translocated.) Lane 4 contains the 0.5–1.4 M sucrose interface (and therefore the majority of membrane vesicles), lane 5 contains the 1.4–1.6 M sucrose interface (which also contains a fair amount of vesicles), and lane P contains the pellet.

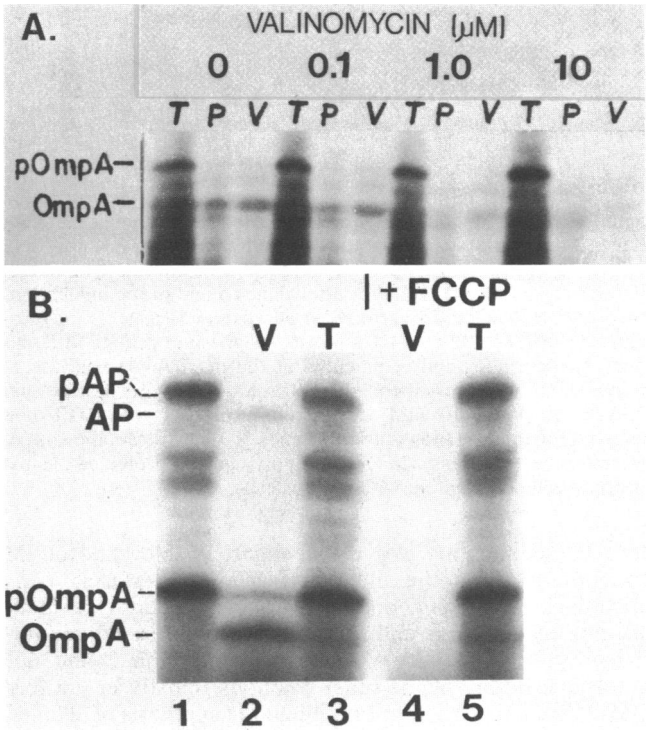

FIG. 4. Effect of valinomycin and FCCP on translocation of alkaline phosphatase and OmpA (A) Reaction mixtures for the translocation assay contained the indicated amount of valinomycin and 75 mM KCl instead of 75 mM NH_4Cl. T, Total polypeptides; P, total polypeptides after pronase treatment; V, vesicle-associated polypeptides after pronase treatment. (B) Effect of FCCP. Lanes 1, 3, and 5 were total polypeptides synthesized, and lanes 2 and 4 were vesicle-associated polypeptides: lane 1, no membrane added; lanes 1 through 3, no FCCP; lanes 4 and 5, 50 μM FCCP.

chilling on ice to terminate both protein synthesis and translocation.

For electrophoretic analysis of the total protein synthsized, a 10-μl sample of the incubation mixture was treated with 1 ml of 10% trichloroacetic acid and centrifuged; the pellet was washed with ice-cold 90% acetone, dried, and dissolved in gel sample buffer (25 mM Tris [pH 6.8], 2% SDS, 1% mercaptoethanol, 4% glycerol, 20 mg of bromophenol blue per ml). Alternatively, 5 μl of 5× gel sample buffer was added to the sample, followed by heating for 3 min at 90°C. All samples were electrophoresed on a 10% polyacrylamide gel (19), which was then impregnated with either En^{3}Hance (New England Nuclear Corp., Boston, Mass.) or Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to X-ray film to visualize the polypeptides.

To assess translocation, the remainder of the incubation mixture was treated with pronase (200 μg/ml) for 15 min on ice and then was diluted with 1 ml of 10 mM Tris-hydrochloride (pH 7.6)–0.5 M KCl–10 mM magnesium diacetate containing 0.5 mM each phenylmethylsulfonyl fluoride, 1,10-phenanthroline, and sodium nitriloacetate to reduce the pronase action. To collect the membrane the sample was layered over 2 ml of 0.25 M sucrose and 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM magnesium acetate–1 mM DTT and centrifuged at 200,000 × *g* for 1 h. The pellet, either with or without reprecipitation by 10% trichloroacetic acid, was dissolved in hot gel sample buffer and then electrophoresed as described above. When the direct dissolution method was used, L-methionine was present at 200 μg/ml during the pronase treatment and at 10 μg/ml in the sucrose cushion, to diminish binding of [^{35}S]methionine to proteins.

Immunoprecipitation. Immunoprecipitation was performed as described by Ito et al. (16). Rabbit antiserum against SDS-denatured *E. coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was prepared by S. Lory of this laboratory, and rabbit antiserum against *E. coli* OmpA protein was kindly provided by Henry Wu.

Chemicals. FCCP and valinomycin (Sigma) were dissolved in absolute ethanol. Appropriate amounts were dried to the bottom of tubes before the addition of membranes. Pronase was from Calbiochem-Behring, La Jolla, Calif.; proteinase K was from E.M. Science, Gibbstown, N.J.

RESULTS

Isolation of active membrane fraction. Since earlier work in this laboratory had separated two fractions of cytoplasmic membrane, complexed with ribosomes or free, a logical approach to the isolation of vesicles active in translocation would be the isolation of complexed membrane, followed by release of the attached ribosomes. However, this procedure proved unsatisfactory because of the similar density of the complexed membrane and the outer membrane. We therefore freed the total membrane of ribosomes by exposure to EDTA and compared the translocation activity of various fractions. The mRNA was the total RNA from cells containing a multicopy plasmid carrying the *phoA* gene. Synthesis was at 40°C (see below) with [^{35}S]methionine; it was allowed to proceed for 5 min before the addition of a membrane fraction.

In the initial fractionation the total membrane was centrifuged in a linear 1.0 to 2.0 M sucrose gradient and was collected as six samples, which were assayed at equal protein concentration (Fig. 1). The uppermost fraction (frac-

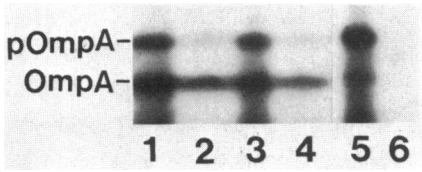

FIG. 5. Effect of protease treatment of vesicles on translocation activity. Membrane vesicles were incubated for 15 min at 0°C with (lanes 3 through 6) or without (lanes 1 and 2) proteinase K (0.25 mg/ml), after which 2 mM of phenylmethylsulfonyl fluoride was added, either before (lanes 3 and 4) or after (lanes 5 and 6) the incubation. Samples were layered over 0.25 M sucrose in 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM magnesium acetate in Beckman Airfuge tubes and sedimented at the maximum speed for 10 min. Pellets were resuspended in 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM magnesium acetate–1 mM DTT and assayed for translocation activity. Lanes 1, 3, and 5, Total polypeptides synthesized; lanes 2, 4, and 6, polypeptides protected against pronase and sedimenting with the vesicles.

tion 1), sedimenting above the major, visible band of IM (fraction 2), was the most active in sequestering (i.e., attaching, processing, and protecting from pronase) both alkaline phosphatase and the OmpA protein (identified initially by M_r). Activity was lower in fraction 2 and not detectable in any of the other fractions (mostly or entirely OM). Moreover, fraction 2 inhibited net synthesis of alkaline phosphatase, but not of OmpA, whereas the denser bands prevented it completely (lanes marked T; Fig. 1). In the preparation shown in Fig. 1, the yield of fraction 1 was about 25% that of fraction 2.

In isolating the most active fraction on a larger scale the limiting step is the linear sucrose gradient fractionation of IM and OM, since overloading here increases cross-contamination. Accordingly, in the standard preparation (see Materials and Methods), membrane from the cell lysate was first roughly fractionated by collection on a cushion of sufficiently low density (1.4 M sucrose) to allow most of the dense membrane to pellet. A larger amount of active membrane, from the interface, could then be fractionated on a multistep gradient (see above). There were three bands. The most active material (referred to as fraction I) was found at about 1.0 M sucrose and formed a diffuse, brownish band that scattered little light compared with the other two. Fraction II, a whitish band sedimenting at about 1.2 M, varied, but usually possessed no more than half the activity of fraction I per unit protein. The third band (fraction III), also whitish, but sedimenting at 1.4 M, was not active.

It is not certain why fraction I is more active than fraction II in sequestering exported proteins. Gel electrophoresis, followed by Coomassie blue staining, revealed no significant difference in protein pattern. Because overnight centrifugation did not result in density equilibrium, the more active fraction might be sedimenting at the top because the vesicles were smaller. However, in negatively stained micrographs, no gross difference in the distribution of vesicle sizes was seen (50 to 250 nm in diameter). On the other hand, when centrifuged to equilibrium (60 h) fraction II sedimented as a sharp band at 1.193 g/cm^3; fraction I sedimented as a diffuse band, and the half in the region of fraction II was less active than the less dense remainder, centering at 1.175 g/cm^3. It thus appears that the most active vesicles of the IM are the least dense. They would therefore be more completely separated from OM, which impairs net protein synthesis.

Interestingly, two-dimensional electrophoresis (11) revealed some ribosomal proteins (L1, L3, L11, S5, S6, and S9) in fractions I and II, but not in the inactive fraction III. To test for simple adsorption, fraction I or II was mixed with radioactively labeled free ribosomes and then exposed to EDTA and recovered as described above; no significant amount of labeled proteins remained with the membrane. It thus seems likely that the ribosomal proteins found in these fractions are residues of previously bound ribosomes, remaining after the exposure to EDTA during fractionation. Blockage of binding sites by these residues might influence the activity of vesicles in translocation.

Factors affecting protein synthesis and translocation. Study of translocation of alkaline phosphatase was difficult because the presence of various membrane preparations during protein synthesis impaired or even abolished its net production. OmpA, in contrast, showed no such sensitivity in the range of membrane concentration studied. It seems likely that the monomer of alkaline phosphatase (which normally is stabilized by dimerization after secretion into the periplasm) is very sensitive to proteases present in the OM, although inclusion of inhibitors of serine proteases (diisopropylphosphofluoridate, phenylmethylsulfonyl fluoride, and tosyl-lysyl chloromethyl ketone) did not alter the results. (After completion of this work it was found that production of alkaline phosphatase became much more reproducible when the concentration of membrane was decreased and the reaction mixture contained polyamines [L. Chen, P. C. Tai, and B. D. Davis, unpublished data].)

Net synthesis of both proteins (in the absence of membrane) was very dependent on temperature, increasing severalfold between 36 and 40°C. Moreover, translocation into vesicles was slight or undetectable at the lower temperature, but substantial at the higher one (data not shown). Subsequent experiments were therefore all carried out at 40°C.

Translocation in our system is defined by the following three criteria: processing of precursor into mature protein, sedimentation with the vesicles, and protection from added protease. To show that a protein was translocated, both sedimentation and pronase treatment were necessary; the sedimented vesicles contained a significant amount of newly synthesized polypeptide (adsorbed to the surface or incompletely inserted) that could be removed by pronase treatment, and some of the total pronase-resistant protein was found in the supernatant after centrifugation (data not shown).

Identification of precursors and mature proteins. To confirm the identity of the precursors and the sequestered mature proteins, initially inferred from their molecular weights, we employed rabbit antiserum (Fig. 2). After synthesis in the absence of membrane (Fig. 2, lane 1) immunoprecipitation yielded a major component, both for OmpA (lane 8) and for alkaline phosphatase (lane 5), that migrated more slowly than the mature form. These polypeptides were evidently the respective precursors. After synthesis in the presence of membrane (lane 3), the OmpA immunoprecipitate from the total reaction mixture also contained a second polypeptide (lane 10), which comigrated with OmpA extracted from cell envelopes (data not shown). In contrast, the efficiency of processing of alkaline phosphatase was low, and no mature form could be detected in the immunoprecipitation of the total protein at this exposure (lane 6). However, when the vesicles were treated with pronase and then isolated, they were found to contain labeled bands of OmpA and alkaline phosphatase (lane 2), precipitable by the respective antisera (lanes 9 and 7) and comigrating with authentic proteins (only alkaline phosphatase is shown). The efficiency of the translocation of the precursor formed varied from 1 to

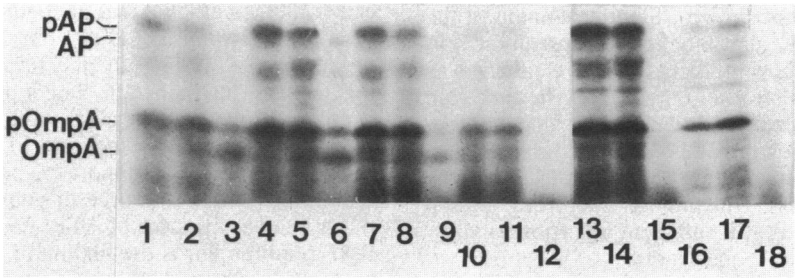

FIG. 6. Effect of ethanol and phenethyl alcohol on OmpA translocation. Translocation was performed with the addition of the amounts of ethanol or phenethyl alcohol indicated below. Lanes 1, 4, 7, 10, 13, and 16, Total synthesis without membranes; lanes 2, 5, 8, 11, 14, and 17, total synthesis in the presence of membranes; lanes 3, 6, 9, 12, 15, and 18, vesicle-associated polypeptides. Lanes 1 through 3, Control; lanes 4 through 6, 1% ethanol; lanes 7 through 9, 2% ethanol; lanes 10 through 12, 4% ethanol; lanes 13 through 15, 0.33% phenethyl alcohol; lanes 16 through 18, 0.67% phenethyl alcohol.

10% for alkaline phosphatase and from 5 to 30% for OmpA protein.

Translocated alkaline phosphatase and OmpA are firmly associated with membrane vesicles. The sedimentation of alkaline phosphatase and OmpA with membrane vesicles and their pronase resistance might have been due to the formation of aggregates rather than to sequestration into vesicles. However, treatment with Triton X-100 eliminated the resistance to pronase (data not shown), as would be expected of sequestered, but not necessarily of aggregated, proteins. As a further test we carried out a translocation experiment, including pronase treatment, and then centrifuged the reaction mixture over a 1.4 M sucrose cushion at 189,000 × *g* for 4 h, a time well in excess of that necessary to concentrate the membranes on the interface. Any putative protein aggregates without lipid would be expected to be too dense to float on the cushion. In fact, both labeled proteins cosedimented with the vesicles (Fig. 3A, lane 4), even when the centrifugation was carried out for 19 h (Fig. 3B). It thus appears that these proteins had indeed associated firmly with the vesicles and not merely formed pronase-resistant aggregates.

Effect of FCCP and valinomycin on translocation. To determine whether energy is required for translocation into vesicles, we tested the effect of agents known to dissipate the PMF. The proton conductor FCCP at 50 μM diminished translocation of both OmpA and alkaline phosphatase severalfold (Fig. 4B). The K^+ ionophore valinomycin at 1 μM had a similar effect on OmpA translocation (Fig. 4A); a 10-fold higher concentration caused further inhibition. (In this experiment production of alkaline phosphatase was too low to follow.)

Since the PMF and ATP are the interconvertible sources of energy in this system, via the H^+-ATPase, inhibition by these uncouplers might conceivably not be due to a direct involvement of the PMF in translocation, but might reflect ATP hydrolysis as a consequence of dissipating the PMF. However, protein synthesis, which requires ATP, was not inhibited by either uncoupler (Fig. 4).

Effect of protease treatment of vesicles. It has been reported that translocation by IM vesicles depends on one or more membrane proteins, since this activity was destroyed by pretreatment of the vesicles with pronase (33). However, in attempting to confirm this finding we found that sufficient pronase adheres to the vesicles, even after washing, to prevent recovery of complete polypeptides in the reaction mixture; hence the inferred effect of the enzyme on the vesicles had not been demonstrated. We therefore used proteinase K, which can be inactivated by phenylmethylsulfonyl fluoride. When vesicles were incubated on ice for 20 min with this protease (250 μg per ml), and the protease was then inactivated, the vesicles were no longer active in a subsequent translocation assay, in which protein synthesis was not impaired (Fig. 5).

The destruction of activity by proteinase K does not appear to be due to inactivation of leader peptidase; after this treatment the vesicles, solubilized with Triton X-100, could still process the OmpA precursor (data not shown; see below). We conclude that translocation involves at least one membrane protein, accessible on the surface, other than leader peptidase. Whether the inactivated protein is part of a specific apparatus of translocation or part of the energy-generating system is not certain; for although the protease treatment did not inactivate the H^+-ATPase or release its F_1 portion, as was demonstrated by direct assay (data not shown), it is conceivable that another protein required for a PMF was damaged.

Effect of membrane perturbants on translocation. To test further the similarity between in vitro translocation and export by the cell, we examined the effect of the following two membrane perturbants: ethanol, which has been shown to inhibit export of the TEM beta-lactamase of *E. coli* to the periplasm (30), and 2-phenethyl alcohol, which inhibits assembly of several proteins into the OM of cells (7). Although ethanol at 4% only slightly inhibited protein synthesis (Fig. 6; compare lanes 10 and 11 with lanes 1 and 2), it strongly inhibited translocation of OmpA (compare lane 12 with lane 3). Interestingly, a lower concentration of ethanol stimulated both protein synthesis and translocation (lanes 4 through 6). Phenethyl alcohol at 0.33% similarly prevented translocation (compare lane 15 with lane 3), whereas it stimulated protein synthesis severalfold (compare lanes 13 and 14 with lanes 1 and 2).

We were able to exclude the possibility that ethanol or phenethyl alcohol had this effect on sequestration by allowing pronase to reach the product in the vesicles, rather than by preventing translocation; when not added until just before pronase treatment, they did not decrease the amount of sequestered OmpA (data not shown). It thus appears that these membrane perturbants interfered directly with translocation.

Processing without translocation. In the experiments described above, cleavage of the signal peptide unexpectedly still occurred when translocation was blocked by PMF inhibitors. Substantial amounts of the mature alkaline phosphatase and OmpA protein were found in the total protein mixture (Fig. 4 and 6) and also in the soluble fraction (in parallel experiments), but not in control reaction mixtures

without membrane (data not shown). Since treatment of the vesicles with proteinase K also blocked sequestration, but not cleavage (Fig. 5), it seems unlikely that the cleavage was due to signal peptidase on the outside of the inverted membrane vesicles; moreover, studies of *E. coli* spheroplasts and inverted vesicles have shown that the peptidase can be inactivated by protease on the outer surface of the cytoplasmic membrane (44). Our findings therefore suggest that without a PMF there is still sufficient insertion to allow cleavage, but not complete sequestration.

DISCUSSION

We have isolated a fraction of the fragmented *E. coli* IM that can translocate both alkaline phosphatase and the OmpA protein synthesized in its presence; i.e., the vesicles process the precursors, and they retain the mature proteins in a location where they are protected from pronase. The most active fraction (fraction I) is recovered, after release of ribosomes by EDTA, from the upper region of a multistep sucrose gradient; it is less dense than the bulk IM (fraction II), as shown by density equilibration. The electrophoretic patterns of the proteins of these two fractions on SDS-polyacrylamide gels did not differ detectably, in contrast to the striking differences between the ribosome-associated and the free cytoplasmic membrane fractions (21). The greater activity of the less dense subfraction in translocation is not necessarily due to a higher concentration of effective sites for ribosome binding: this fraction might also simply be less contaminated with the denser OM, which inhibits net protein synthesis (probably by protease action).

It has been suggested that an exit site for nascent peptide on the ribosome may contact the membrane (1). It is intriguing that in the present study some ribosomal proteins were found associated with membrane after its treatment with EDTA. It seems possible that these proteins have not been adsorbed, but are residues left on some of the ribosome-binding sites after the disruption of the ribosomes by EDTA. This interpretation is supported by the finding that when membrane was mixed with labeled free ribosomes and then exposed to EDTA it failed to pick up ribosomal proteins. Since unencumbered ribosome-binding sites are presumably responsible for the observed translocation, the imperfect release of ribosomal proteins in our membrane preparations may contribute to their low efficiency (maximal 30% for OmpA) in converting precursors to sequestered proteins.

Pretreatment of vesicles by proteinase K destroyed their sequestering activity (Fig. 5). A similar effect of pronase had already been reported (35), but in our hands that procedure did not test for sequestration because the vesicles retained adsorbed enzyme, which would destroy the product. In our experiment, however, inactivation of the proteinase K after the treatment prevented that effect; hence the protease treatment could be shown to interfere with translocation. The interference was not due to inactivation of signal peptidase, since the treated vesicles still cleaved the signal sequence (without translocation), and after solubilization they also showed little impairment of cleavage. We conclude that translocation requires a specific membrane protein, other than leader peptidase, accessible to the cytoplasmic surface.

The translocation observed here was blocked, like translocation of various proteins to the periplasm and to the OM in cells (4, 9, 28, 45), by FCCP or valinomycin, which dissipate the PMF (Fig. 4). (Translocation was also sensitive, as in the cell [7, 30], to ethanol and phenethyl alcohol [Fig. 6], which perturb membrane fluidity; but since these agents also dissipate the PMF [9] they may be acting on translocation via this mechanism.) Since the uncouplers did not affect protein synthesis (Fig. 4), they clearly did not act by depleting the ATP. Accordingly, our findings, like those obtained with the same agents in cells, support the conclusion that the PMF is required as a direct source of energy for the translocation, rather than as a source of ATP. Also consistent with a direct PMF requirement is the finding of Enequist et al. (9) that in an ATPase mutant anaerobiosis reduced the PMF and inhibited export of the maltose-binding protein, while leaving ATP levels and protein synthesis relatively intact.

Wickner (42) has proposed that the PMF essentially electrophoreses a loop of a protein across the IM, thereby exposing the cleavage site to leader peptidase on the external surface; translocation would not require a proteinaceous pore or channel. His results with the small M13 phage coat protein present a paradox, since this protein requires a membrane potential for efficient insertion across the membrane in cells (25, 44), yet it can be sequestered (and processed) by liposomes that contain no protein besides leader peptidase and hence cannot generate a PMF (25). On the other hand, the liposomes cannot sequester the OmpA protein (which also requires a PMF in cells [4, 44]), although they can process its precursor (41). This result is consistent with our finding that vesicles require a PMF for sequestration, but can still cleave the signal peptide of OmpA when sequestration is impaired, either by inhibition of the PMF or by protease treatment of the vesicles (Fig. 4 through 6). (The cleavage might be due to the presence of some noninverted vesicles, with the peptidase exposed on the surface, but the protease treatment should inactivate such molecules.)

It thus seems likely that the initial insertion and the cleavage of signal peptide do not require (although they might be facilitated by) any proteins other than the peptidase, whereas the PMF acts on a machinery in the IM required for subsequent stages of translocation. Such a machinery might utilize energy obtained by direct coupling with proton cycling, as in the active transport of many small molecules. Alternatively, as suggested previously (33), it might function as a passive channel whose effective conformation requires an energized membrane. In principle these two possibilities might be distinguished by testing for increased proton flow associated with translocation, but detection of the difference would be difficult.

Translocation of proteins (although not necessarily of all proteins) requires a PMF not only in bacteria (4, 9, 28, 45, 46), but also in mitochondria (10) and chloroplasts (13). Furthermore, it has been observed to occur posttranslationally as well as cotranslationally in these three systems (17, 32, 34). In contrast, in the endoplasmic reticulum, which lacks the proton pumps required to generate a PMF, only cotranslational translocation has been observed (31). Since no other source of energy is evident in the endoplasmic reticulum, translocation there would presumably have to be tightly coupled to translation, and it has been suggested that the machinery of the signal recognition particle and its receptor has evolved to ensure this tight coupling (40). In contrast, the other three systems may have evolved the use of the PMF to provide a source of energy for the unwinding or the posttranslational transfer (or both) of already completed proteins. However, such systems might be expected also to begin their action even before translation is completed. Indeed, this flexibility has been demonstrated in mitochondria (37).

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