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Alkaline Phosphatase and OmpA Protein Can Be Translocated Posttranslationally into Membrane Vesicles of *Escherichia coli*†

LINGLING CHEN,[1] DAVID RHOADS,[1]‡ AND PHANG C. TAI[1,2]*

Bacterial Physiology Unit, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115[1]; and Department of Metabolic Regulation, Boston Biomedical Research Institute, Boston, Massachusetts 02114[2]

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We previously described a system for translocating the periplasmic enzyme alkaline phosphatase and the outer membrane protein OmpA into inverted membrane vesicles of *Escherichia coli*. We have now optimized and substantially improved the translocation system by including polyamines and by reducing the amount of membrane used. Under these conditions, efficient translocation was seen even posttranslationally, i.e., when vesicles were not added until after protein synthesis was stopped. This was the case not only with the OmpA protein, which is synthesized by free polysomes and hence is presumably exported posttranslationally in the cell, but also with alkaline phosphatase, which is synthesized only by membrane-bound polysomes and has been shown to be secreted cotranslationally in the cells. Prolonged incubation rendered the precursors inactive for subsequent translocation. Posttranslational translocation was impaired, like cotranslational translocation, by inhibitors of the proton motive force and by treatment of the vesicles with protease. Since it appears that *E. coli* can translocate the same proteins either cotranslationally or posttranslationally, the cotranslational mode may perhaps be more efficient, but not obligatory, for the secretion of bacterial proteins.

In recent years biochemical and genetic studies with bacteria have contributed greatly to our knowledge of the mechanisms of protein secretion and localization (reviews in references 4, 18, 23, and 26). A suitable in vitro translocation system from bacteria would be of considerable help in the further analysis of the molecular mechanisms, especially in conjunction with many mutants altered in the process. Despite several reported successes (3, 7, 10, 27), a reliable bacterial system has been elusive. It has thus not been possible to determine whether protein translocation into bacterial membranes resembles that of eucaryotic membranes in requiring a signal recognition particle for translational arrest (33, 34) and for interaction with the docking protein in the membrane (6, 17).

We have recently developed a system for translocating alkaline phosphatase and OmpA protein into a selected fraction of inverted *Escherichia coli* cytoplasmic membrane vesicles (25). In this paper we have further improved the system and have found that it can carry out posttranslational translocation, after protein synthesis has been stopped by chloramphenicol (or puromycin). This posttranslational translocation is observed not only with OmpA protein, which is made on free polysomes, but also with alkaline phosphatase, which is made on membrane-bound polysomes and can be secreted cotranslationally in the cell (29). Evidently, *E. coli* has the capacity to translocate the same protein either cotranslationally or posttranslationally.

MATERIALS AND METHODS

Bacterial strains and growth. The strains used in this work were all *E. coli* K-12. Strain MC1000(pHI-5) (9), which contains the *phoA* gene in its plasmid, was used for preparing mRNA for alkaline phosphatase and OmpA protein. The RNaseI-negative strain D10 and its OmpA$^-$ derivative (D10-773) were used for preparing membrane vesicles. Transduction of the *ompA* allele into D10 was by P_1 phage from JF773 (5) and was confirmed by the resistance of the transductant to phages K3H1 and TuII* (kindly provided by J. Foulds) and by the absence of OmpA protein in the membrane. S30 extracts were prepared from strain D10. All growth conditions and media have been described (25). Strains CW3747 and C600(pBR322), which were used for preparing polysomes, are described below.

RNA preparation. Total RNA, containing mRNA, was prepared as described previously (25), with modifications adapted from Legault-Demare and Chambliss (15). Cells from 2.5 liters were suspended in 70 ml of 10 mM Tris-hydrochloride (pH 7.6) containing 10 mM each KCl, $MgCl_2$, and NaN_3. Sodium dodecyl sulfate was added to 1%, and the suspension was mixed immediately with an equal volume of redistilled *m*-cresol. The mixture was passed through a French press at 10,000 lb/in^2 and centrifuged at 3,650 × *g* for 20 min. The upper, aqueous phase was removed and saved. The lower phase was mixed with 1/3 volume each of the same Tris-hydrochloride buffer containing 1% sodium dodecyl sulfate and of *m*-cresol and was passed through the French press again. After centrifugation, the aqueous phase solution was combined with the first aqueous solution and mixed with an equal volume of 4 M NaCl. The RNA was precipitated at −20°C overnight by the addition of 2 volumes of ethanol. After centrifugation, the pellet was dissolved in water and was shaken with an equal volume of redistilled phenol at room temperature, followed by the addition of chloroform. After centrifugation, the aqueous phase was removed and brought to 1 M NaCl, and ethanol was added to precipitate RNA as above. The pellet was dissolved in 20 ml of 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM [$Mg(OAc)_2$; the solution was treated with 60 μg of DNase and then mixed drop by drop with 20 ml of 4 M LiCl and set

* Corresponding author.

† Dedicated to Bernard D. Davis on the occasion of his retirement in June 1984.

‡ Present address: Department of Biology, University of Massachusetts, Boston, MA 02125.

on ice for 30 min. Pellets were collected by centrifugation, dissolved, and precipitated with LiCl. The pellet was again dissolved and precipitated with ethanol as above. The pellet was collected and dissolved in water, and portions were lyophilized and stored at −70°C.

Preparation of S30 extracts and membrane vesicles. Membrane vesicles active in translocation were prepared as described (25). S30 extracts were prepared (25) with the following modifications. To eliminate membrane vesicles, the crude S30 (19) was treated with 0.5% octylglucoside (Calbiochem) on ice for 15 min and dialyzed for 3.5 h against 100 volumes of 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM $Mg(OAc)_2$–1 mM dithiothreitol, with three changes of this buffer. After centrifugation at 30,000 × *g* for 30 min to remove insoluble membrane proteins, this pretreated S30 was stored in small portions at −70°C.

Protein synthesis and translocation. Protein synthesis, translocation, and electrophoretic analysis were as described previously (25), with the following modifications. Unless otherwise stated, protein synthesis mixtures (in 0.1 ml) contained 50 mM Tris-hydrochloride (pH 7.6), 40 mM KCl, 20 mM NH_4Cl, 1 mM Tris-ATP, 5 mM phosphoenolpyruvate-Tris, 3 μg of pyruvate kinase, 0.02 mM GTP-Tris, 6.5 mM $Mg(OAc)_2$, 1 mM spermidine-hydrochloride, 8 mM putrescine-hydrochloride, 2 units of absorbancy at 260 nm (A_{260}) of pretreated S30, 2 mM dithiothreitol, an optimal amount of mRNA (usually 5 A_{260} units of total RNA), 20 μCi of [^{35}S]methionine (1,000 Ci/mmol), and 19 other amino acids at 0.05 mM each. Protein synthesis was carried out at 40°C, which was best for protein synthesis and translocation (25). For cotranslational translocation, 90 μg of the reaction mixture was added at the indicated time to a tube containing 10 μl of membrane vesicles (0.15 A_{280} unit, measured in 2% sodium dodecyl sulfate) that had been warmed to 40°C, and incubation was continued for another 18 min, unless otherwise indicated. For posttranslational translocation, protein synthesis was stopped with 0.1 mg of chloramphenicol per ml (within 30 s) before the addition of membranes.

Samples of the reaction mixture (5 to 10 μg) were chilled and analyzed for total synthesized proteins by gel electrophoresis. To analyze translocation into membrane vesicles, samples were exposed to pronase (300 μg/ml, 15 min on ice) and centrifuged as described previously (25), with L-methionine present at 0.2 mg/ml to prevent nonspecific binding of [^{35}S]methionine. To improve the recovery of membrane vesicles, membrane fraction III (25) was added as a carrier (0.35 A_{280} unit) before centrifugation at 200,000 × *g* for 1 h, or the centrifugation was prolonged to 90 min without adding the carrier membrane. The two procedures gave comparable results. The pellets were directly dissolved and boiled in gel sample buffer containing 2% sodium dodecyl sulfate, followed by electrophoresis, as described previously (25). To visualize the radioactive polypeptides, gels were treated with Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to Kodak XR-5 film at −70°C.

The conditions for protein synthesis and translocation described above have been optimized. The ranges of variables tested were 0 to 100 mM KCl, 0 to 100 mM NH_4Cl, 2 to 13 mM $Mg(OAc)_2$, 0 to 3 mM spermidine, 0 to 16 mM putrescine, and 0.05 to 0.5 A_{280} unit of membrane.

Determination of site of specific protein synthesis in cells. Strain CW3747 (ATCC 27257), which is constitutive for alkaline phosphatase, and C600(pBR322), which harbors a plasmid coding for RTEM1 beta-lactamase, were used for preparing membrane-bound and free polysomes to determine the sites of synthesis of alkaline phosphatase and beta-lactamase, respectively. The preparation was as described previously, including L broth enrichment before harvesting (31). For C600(pBR322), polysomes were also prepared without L broth enrichment. Polysome runoff and immunoprecipitation were as described previously (31). Rabbit antisera against alkaline phosphatase, OmpA, matrix protein, and beta-lactamase were kindly provided by S. Lory, H. Wu, M. Inouye, and N. Citri, respectively.

Quantitation of proteins. Fluorograms of X-ray film were scanned at 580 nm with an Isco gel scanner model 1310 equipped with a UA-5 monitor. The areas of the protein bands of interest were weighed as described previously (8). This method proved more reliable than direct counting of excised protein bands. (We took into account that one methionine residue out of nine was removed from the precursor of alkaline phosphatase, and one out of six was removed from OmpA.)

Chemicals. Translational-grade [^{35}S]methionine was obtained from New England Nuclear Corp. or Amersham Corp. All other chemicals and enzymes were reagent grade, obtained from commercial sources.

RESULTS

Improvements of the in vitro translocation system. In our previously described system the synthesis and translocation were much less reliable for alkaline phosphatase than for the OmpA protein (25). We define the translocation as cleavage of the signal sequence from a precursor, protection from digestion by protease, and sedimentation of the product with the vesicles. Control experiments showed that upon solubilization of membranes with detergent Triton X-100 the proteins, which have been identified immunologically, are digested by the added protease. The active membrane fraction used (fraction I) was the least dense fraction in a sucrose gradient, farthest from the outer membrane fraction. Excess membrane was found to impair net protein synthesis, especially of alkaline phosphatase (data not shown), but when the amount of the membrane fraction was decreased from 0.5 A_{280} unit (per 0.1 ml of reaction mixture), previously used, to an optimized amount of 0.15 A_{280} unit, translocation of alkaline phosphatase could be consistently obtained. Under these conditions, the previously less active fraction II was found to be almost as active as fraction I. Moreover, we found that the amount and the reliability of translocation of alkaline phosphatase, which is sensitive to minor variations (25), and, to a lesser degree, that of OmpA protein was greatly improved by including 40 mM KCl, 1 mM spermidine, and 8 mM putrescine and by decreasing Mg^{2+} to 6.5 mM and NH_4Cl to 20 mM. All of the following experiments were carried out with these improvements.

Posttranslational translocation of alkaline phosphatase and OmpA protein. In our initial studies of the effect of time of membrane addition during protein synthesis for the translocation, substantial translocation still occurred even when membrane was added after 15 min, when total protein synthesis had virtually ceased, suggesting that alkaline phosphatase and OmpA protein can be translocated posttranslationally. This possibility can be tested directly by inhibiting protein synthesis before the addition of membrane vesicles. However, in experiments with the earlier, unimproved system we observed that apparent posttranslational translocation of alkaline phosphatase and OmpA protein occurred, but it turned out to be due to cotranslational uptake by vesicles contaminating the standard S30 preparation (data not shown). To eliminate this effect, we destroyed the endogenous vesicles by treating the S30 with 0.0125%

Triton X-100 or 0.57% octylglucoside. After 12-fold dilution into the reaction mixtures, detergent at these concentrations did not inhibit protein synthesis or interfere with subsequent translocation into added vesicles (data not shown). Accordingly, we have adopted as a standard procedure the addition of 0.5% octylglucoside during the preparation of S30 extracts, followed by dialysis to remove the detergent. All subsequent translocations with such pretreated S30 extracts are strictly dependent on added membrane (see Fig. 2 and 4).

Since we have found that normal membrane contains mRNA, which leads to synthesis of additional OmpA protein (unpublished data), we have used membrane prepared from an OmpA$^-$ strain to compare the efficiency of cotranslational and posttranslational translocation.

To examine posttranslational as well as cotranslational translocation of alkaline phosphatase and OmpA protein, membrane was added, with or without chloramphenicol (or puromycin), at various times after the initiation of protein synthesis, and incubation was continued for an additional

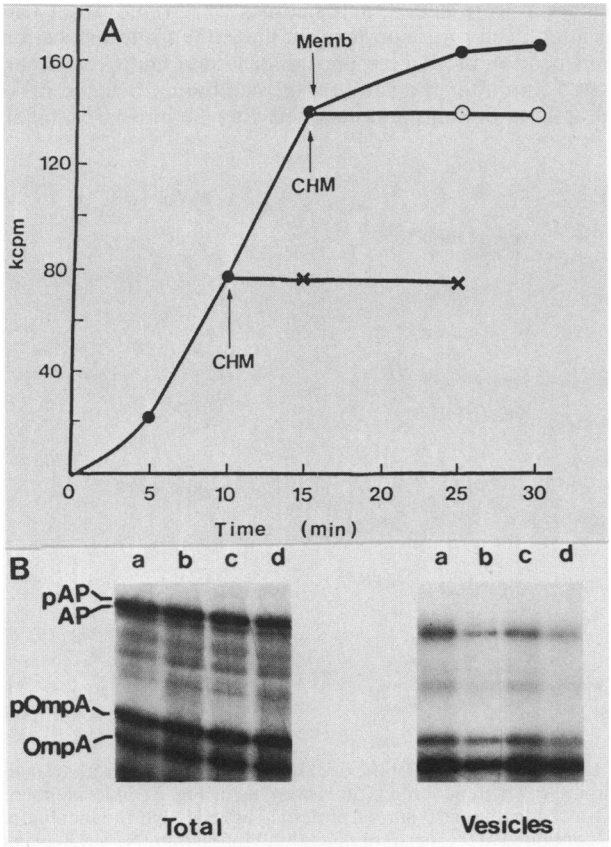

FIG. 1. Use of chloramphenicol to inhibit protein synthesis and to assay posttranslational translocation. (A) Protein synthesis reaction mixtures were incubated at 40°C as described in the text. At 10 min, chloramphenicol (CHM) was added to sets of samples (×) to a final concentration of 0.1 mg/ml. At 15 min, membrane (Memb) was added to sets of samples with (○) or without (●) CHM. Samples of 10 μl were assayed for acid-insoluble incorporation of [^{35}S]methionine. (B) After the reaction, mixtures were incubated as in (A) for 15 min, chloramphenicol (lanes b and d) or methionine (final concentration, 1.5 mg/ml; lanes c and d) was added to sets of samples. Membrane vesicles were added, and after 20 min samples were chilled and analyzed for total and for translocation into vesicles (pelleted without carrier membrane). Fluorograms were obtained after 16 h of exposure on Kodak XR-5 film.

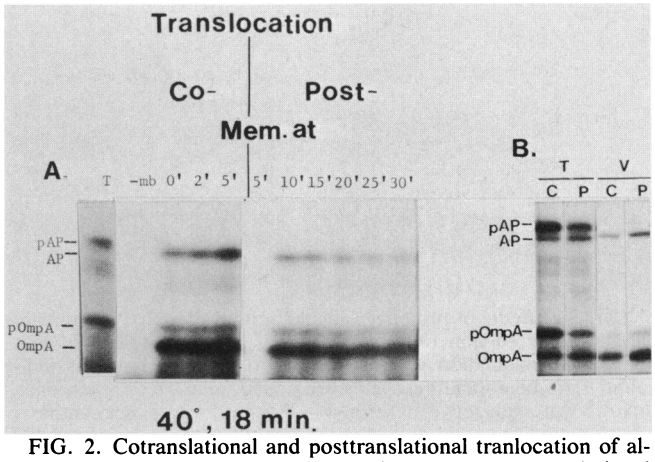

FIG. 2. Cotranslational and posttranslational tranlocation of alkaline phosphatase and OmpA protein. (A) For cotranslational translocation, reaction mixtures at 40°C were incubated with membrane vesicles added at various times. For posttranslational translocation, chloramphenicol (0.1 mg/ml) was added immediately before the addition of membrane. All reaction mixtures were incubated for 18 min after the addition of membranes to allow translocation. One sample without added membrane (−mb) was similarly incubated. The samples were chilled and treated with pronase. Carrier membrane was added, the mixtures were centrifuged, and the pelleted vesicles were electrophoresed and analyzed. The fluorograms were obtained after 8 h of exposure on Kodak XR-5 film. One sample (T), a protein synthesis mixture without membrane, provides markers for precursors. (B) The reaction mixtures were prepared and incubated as in A, except that for cotranslational translocation (C) membrane was added at 5 min, and for posttranslational translocation (P) chloramphenicol and membrane were added at 15 min. With each reaction mixture 10 μl was analyzed for total (T) net synthesis, without pronase treatment and centrifugation, and 90 μl was analyzed for translocation into vesicles (V). For clarity, the fluorogram for T was exposed for 7.5 h, and that for V was exposed for 4 h.

period (18 min) for translocation to take place. In the absence of added chloramphenicol, both cotranslational and posttranslational translocation could be measured, but in the presence of the drug, only posttranslational activity would be seen, provided inhibition of protein synthesis was complete. Figure 1A shows that the addition of chloramphenicol caused immediate and complete inhibition of protein synthesis, either in the absence or in the presence of membrane. Furthermore, the translocation after the addition of chloramphenicol was not affected by the addition of a 10,000-fold excess of unlabeled methionine to block any residual protein synthesis that may have contributed to the translocation (Fig. 1B). Similar results were obtained with puromycin as the inhibitor of protein synthesis.

Figure 2A demonstrates posttranslational translocation of alkaline phosphatase and OmpA protein and compares its efficiency with that of cotranslational translocation. At 5 min, when very little protein was completed, there was little posttranslational translocation of either protein (lane 6), but apparently cotranslational secretion, dependent on continued synthesis, could be seen (lane 5). After 10 min of synthesis, however, substantial amounts of the proteins were translocated after synthesis had been stopped, although less than with continued synthesis. Less translocation, especially of alkaline phosphatase, was observed when membrane was added at a later stage of incubation (compare lanes 11 and 6), even though more precursor should have been formed. This point is further addressed below.

In the experiments of Fig. 2A posttranslational translocation was about one-third as efficient for alkaline phosphatase

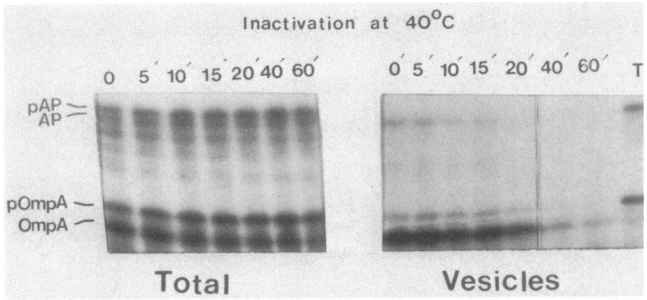

FIG. 3. Effects of prolonged incubation on capacity for translocation. After 20 min of protein synthesis, chloramphenicol was added, and incubation was continued. At various times, as indicated, D10 membrane vesicles were added, and after 18 min each mixture was analyzed as described in the text for total net synthesis and for translocation (vesicles). Lane T contains markers for precursors.

and one-half as efficient for OmpA protein as cotranslational translocation. The relative efficiency varied with the membrane preparations and the time of membrane addition. With some membrane preparations when protein synthesis was stopped at an earlier time posttranslational translocation of alkaline phosphatase and OmpA appeared even more efficient than cotranslational translocation (Fig. 2B). Under such conditions, the efficiency of cotranslational processing was about 26% for alkaline phosphatase and 50% for OmpA, but 46 and 70%, respectively, for posttranslational processing as judged by the conversion of precursor to mature protein. Full translocation, i.e, the recovery of the mature proteins resistant to pronase treatment and cosedimenting with membranes was 15 and 23% for alkaline phosphatase during cotranslation and posttranslational translocation, respectively, and 22 and 33% for OmpA protein.

We frequently observed with OmpA protein and sometimes with alkaline phosphatase, that a small fraction (10 to 20%) that electrophoresed at the precursor size survived pronase treatment and sedimented with the vesicles (Fig. 1B and 2B), although the concentration of pronase used (300 μg/ml) was 10-fold in excess of that needed to digest the precursors in the presence of Triton X-100 (data not shown). The nature of this molecular species is not clear. It may well be that some precursor is translocated, but not cleaved (see below), although it could also represent a different form of mature protein (36) or precursor that was protected from pronase by being bound to the membrane.

Loss of translocation capacity during prolonged incubation. To test whether the reduction of translocation when membrane was added at a late time was due to the loss of competence of already synthesized precursor molecules for translocation during prolonged incubation, the following experiment was carried out. Protein synthesis was stopped with chloramphenicol after 20 min, the mixtures were further incubated at 40°C, and membrane was added at various intervals, followed by incubation for a further 18 min. Incubation for 20 min or longer before the addition of membrane greatly reduced the subsequent translocation of both alkaline phosphatase and OmpA protein (Fig. 3). The reduction was not due to extensive proteolytic degradation of the precursors, since their total amount remained constant for at least 60 min (Fig. 3). The inactivation of some required factors in S30 seems unlikely, since fresh S30 (or ATP; data not shown) did not restore translocation (Fig. 4A).

The possibility that aged, denatured component(s) interfered with the translocation of competent ^{35}S-labeled precursor molecules was examined as follows. The denatured mixture was prepared by allowing protein synthesis to proceed in the presence of unlabeled methionine, followed by incubating for 60 min at 40°C in the presence of chloramphenicol. (A parallel control sample with [^{35}S]methionine was shown to be inactive for translocation [Fig. 4B, lane b].) The inactivated mixture was then combined in various proportions with competent ^{35}S-labeled precursors, and translocation was resumed (Fig. 4). Translocation was inhibited markedly by the presence of the inactivated components (Fig. 4, compare lanes c and e and lanes d and f), suggesting that competition was at some step of translocation.

Freeze-thawings also inactivated the mixtures. On the other hand, after 1 h (Fig. 4, lanes a) or even after several hours of incubation at 0°C translocation could still take place at 40°C (data not shown).

Kinetics of posttranslational translocation. To study the kinetics of posttranslational translocation, protein synthesis was allowed to proceed for 20 min, chloramphenicol and membrane were added, and samples were removed at various intervals for measurement of the extent of translocation. Translocation of alkaline phosphatase and OmpA was very slight 5 min after the addition of membrane, became maximal at 15 to 20 min, and declined after 30 min, presumably

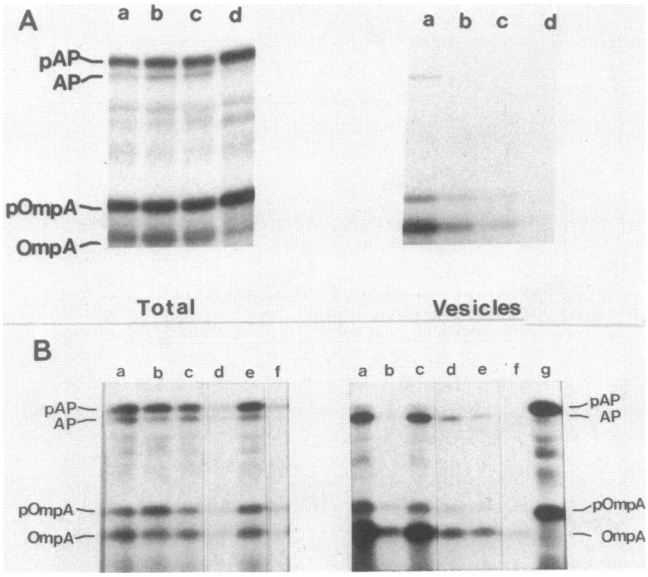

FIG. 4. Interference of the denatured component(s) with translocation. (A) The reaction mixtures were as in Fig. 3. Chloramphenicol was added after 20 min of protein synthesis, and the incubation was continued at 0°C (a, d) or 40°C (b, c) for 60 min. New S30 (same amount of A_{260} units as in the original reaction mixture) was added to one sample (c), and all samples were assayed for translocation (18 min). Also shown is a sample without added membrane (d). (B) The reaction mixtures II and III were as in A, but in reaction mixture II [^{35}S]methionine was replaced by 0.05 mM methionine. After 20 min at 40°C protein synthesis was stopped by chloramphenicol, and a portion of ^{35}S-labeled sample III was kept at 0°C. The other mixtures I and II were incubated further at 40°C for 60 min. Sample II and various portions of III alone or in combination with I were then incubated with membrane. Reaction mixtures were analyzed for total protein and for translocation protein (vesicles). Lanes: a, 100 μl of III; b, 100 μl of II; c, 75 μl of III; d, 25 μl of III; e, 75 μl of III and 25 μl of I; f, 25 μl of III and 75 μl of I; g, precursor markers.

due to proteolysis inside the vesicles or due to the membranes becoming leaky after long incubation (Fig. 5A).

Although the rate of translation of alkaline phoshatase and OmpA protein was markedly less at 37°C than at 40°C (25), translocation was only slightly slower at the lower temperature (data not shown). When translocation (for 18 min) at even lower temperatures was examined (Fig. 5B), it was found to be significantly less at 30°C and to drop off sharply at 15 to 20°C, which corresponds to the transition temperature where membrane fluidity changes drastically. Additional experiments showed that the full extent of translocation could be obtained at 20 to 30°C with longer incubation (data not shown). However, at 0°C virtually no translocation took place for up to several hours.

Effect of inhibitors on posttranslational translocation. We had found earlier (25) that cotranslational translocation of alkaline phosphatase and OmpA was inhibited by a compound (FCCP) that disrupts the membrane proton motive force and by agents (ethanol, phenylethyl alcohol) that perturb membrane fluidity and that may also act by abolishing proton motive force. These inhibitors had the same effects on posttranslational translocation in the experimental system described here (data not shown).

Treatment of membrane vesicles with proteinase K impaired their ability to posttranslationally translocate OmpA protein and alkaline phosphatase (Fig. 6), although the signal peptides were still cleaved (Fig. 6). (A similar effect was previously observed with cotranslational translocation of OmpA protein [25].) At various degrees of inactivation of membrane vesicles, the translocation of OmpA protein was less severely impaired than that of alkaline phosphatase, presumably reflecting the more competitive advantage or higher efficiency of the former for the limited sites of translocation in the membrane.

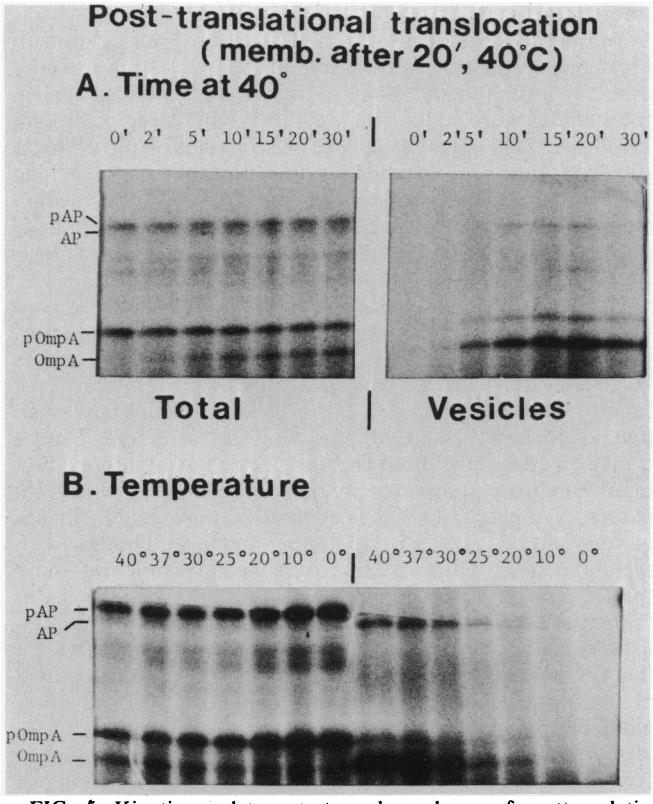

FIG. 5. Kinetics and temperature dependence of posttranslational translocation. (A) After 20 min of protein synthesis, chloramphenicol and membrane were added; after further incubation for various times, as indicated, portions were analyzed for total reaction and for translocation (vesicles). (B) Same as A, except that after protein synthesis the mixtures were incubated with membrane at various temperatures, as indicated, for 18 min before analysis.

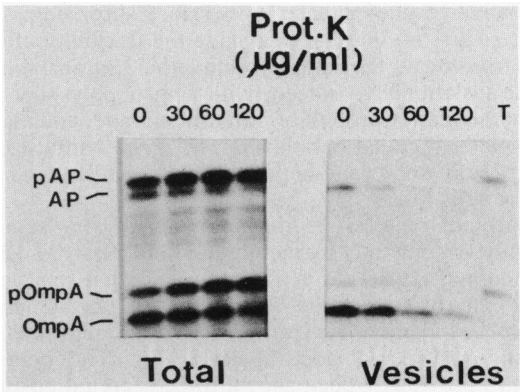

FIG. 6. Inactivation of membrane vesicles by mild protease treatment. Membrane vesicles were diluted to 15 A_{280} units per ml (about 3 mg of protein per ml) in 10 mM Tris-hydrochloride (pH 7.6)–50 mM KCl–10 mM $Mg(OAc)_2$ and were treated with various amounts of proteinase K, as indicated, on ice for 15 min. Phenylmethylsulfonyl fluoride was then added (2 mM) in all tubes to stop further protease action. The mixtures were each layered onto 0.85 ml of the above buffer containing 25% sucrose and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 200,000 × *g* for 90 min at 40°C. The pelleted membrane was suspended in the above buffer containing 0.5 mM phenylmethylsulfonyl fluoride. Equal amounts of the membrane were then added to assay posttranslational translocation as described in the text. Lane T contained precursor markers. Samples of 5 and 95 μl, respectively, were analyzed for synthesis (totals) and for translocation (vesicles). Fluorograms for totals and vesicles were exposed 16 and 3.5 h, respectively.

Synthesis on membrane-bound or free polysomes. Since posttranslational translocation would presumably be necessary for proteins that are synthesized on free polysomes, but not for those synthesized on membrane-bound polysomes, we determined the site of synthesis of the proteins under investigation. OmpA protein is synthesized mostly by free polysomes (Table 1), but alkaline phosphatase is synthesized only by membrane-bound polysomes (32). Moreover, the cotranslational secretion of alkaline phosphatase has earlier been directly demonstrated by extracellular labeling of nascent chains (29). The substantial posttranslational transloca-

TABLE 1. Site of synthesis of proteins[a]

Protein	% Synthesis by:		Protein location
	Membrane polysomes	Free polysomes	
OmpA	10	90	Outer membrane
Matrix	66	34	Outer membrane
Alkaline phosphatase	86	14	Periplasm
β-Lactamase			
With enrichment	90	10	Periplasm
Without enrichment	46	54	
EFTu, EFG	10	90	Cytoplasm

[a] Polysomes from CW3747 were run off and immunoprecipitated for OmpA, matrix protein, and alkaline phosphatase. Polysomes prepared from strain C600(pBR322) with or without L broth enrichment were assayed for β-lactamase synthesis. Elongation factors EFTu and EFG were also assayed in each set of preparations; the results were identical. The fluorograms were scanned and analyzed as described in the text.

tion of alkaline phosphatase is therefore surprising. These findings suggested that bacteria have the flexibility of being able to translocate the same protein either cotranslationally or posttranslationally. Indeed, with similar polysome preparations the matrix protein of outer membrane, and also the RTEM plasmid coded beta-lactamase, were synthesized by both free and membrane-bound polysomes recovered from the same cells (Table 1).

Of particular interest is the finding that beta-lactamase was synthesized mainly by membrane-bound polysomes in a rich medium (2), whereas in a poorer medium it was synthesized equally by membrane-bound and free polysomes (Table 1), suggesting that it is translocated in the same cell both cotranslationally and posttranslationally. The beta-lactamase has previously been shown to be secreted posttranslationally in UV-irradiated cells (13, 14).

DISCUSSION

We previously described an in vitro system for the translocation of a periplasmic secreted protein (alkaline phosphatase) and an outer membrane protein (OmpA) into *E. coli* inverted membrane vesicles in an operationally cotranslational manner (25). We have now optimized the system and markedly improved both the consistency and the amount of translocation by including polyamines and by reducing the amount of membrane vesicles (an excess of which drastically inhibits the net synthesis of alkaline phosphatase).

This improved system was found to carry out not only cotranslational, but also posttranslational, translocation, i.e., if vesicles were not added until after cessation of protein synthesis, both proteins could still be sequestered. In fact, under appropriate conditions the posttranslational translocation was as efficient as, if not more efficient than, cotranslational translocation (Fig. 2B). Both processes are impaired by treatment of membrane vesicles with protease and by compounds that perturb membrane fluidity and protonmotive force.

Although posttranslational translocation would be expected for OmpA protein that is synthesized on free polysomes, it was surprising for alkaline phosphatase, which in cells is made predominantly on membrane-bound polysomes (Table 1) (32). Moreover, its cotranslational secretion in vivo has been directly demonstrated by extracellular labeling of chains growing on membrane-bound polysomes (29). On the other hand, another secreted protein, beta-lactamase, and another outer membrane protein, matrix protein, are made by both free and membrane-bound polysomes (Table 1). It thus appears that the same protein can be secreted either cotranslationally or posttranslationally in the same cells, depending on how quickly the signal peptide of the nascent chain reaches the membrane. This finding would rule out a mechanism dependent on the energy of protein synthesis, which our earlier work already showed to be unlikely (4, 28). Wickner (35), and Koshland and Botstein (13, 14) have previously presented evidence for the posttranslational insertion of M13 phage coat protein and secretion of beta-lactamase in bacterial cells, respectively.

Josefsson and Randall (11, 12) found that the signal peptide of many exported protein precursors of *E. coli* can be cleaved both cotranslationally and posttranslationally in the cells. These findings have been interpreted as evidence for cotranslational and posttranslational translocation. Furthermore, Randall (24) showed that a large domain of secreted nascent chains accumulate on the inner surface of the cytoplasmic membrane before the chains reach the outer surface. It is possible that the cotranslational entry of signal peptide into membrane may be efficient, but not obligatory, for protein translocation and that bacteria, at least *E. coli*, have the flexibility of being able to translocate the same protein either cotranslationally or posttranslationally. A similar flexibility has been previously suggested for a mitochondrial protein by the finding that translatable mRNAs for imported mitochondrial proteins can be found on free as well as on mitochondrion-bound cytoplasmic polysomes (30).

In contrast to this flexibility, in eucaryotic systems translation and secretion are tightly coupled: a signal recognition particle causes translational arrest (33, 34), which is released on subsequent interaction with a receptor protein in the (dog pancreatic cells) membrane (6, 17). The insertion of certain eucaryotic membrane proteins (1) into dog pancreatic membranes still requires signal recognition particle. Although genetic evidence suggests a coupling of synthesis and secretion in the *E. coli* system (21, 22, 26), so far we have not been able to detect an analogous translational arrest. Indeed, such tight coupling may be a special property of the eucaryotic cell and its signal recognition particle, since the translational arrest and its relief by membrane were also observed with bacterial beta-lactamase in the wheat germ translational system (20). Whether bacterial membranes require the function of an analogous signal recognition particle for translocation remains to be determined. In this regard, protein complexes that appear to be involved in secretion have been detected in *Bacillus subtilis* (M. Caulfield, S. Horiuchi, P. Tai, and B. Davis, Proc. Natl. Acad. Sci. U.S.A., in press) and in *E. coli* (D. Oliver and J. Beckwith, mentioned in reference 26).

Smith (27) previously reported that the translocation of alkaline phosphatase into *E. coli* membrane vesicles occurs only cotranslationally, since it was severely reduced when the vesicles were added 30 min after the initiation of protein synthesis. This apparent lack of posttranslational translocation can now be explained by our finding that such long incubation rendered the precursors inactive for translocation (Fig. 3). Similarly, the inactivation of the precursor of phage coat protein by Mg^{2+} explains the apparent lack of posttranslational insertion into membrane vesicles (3, 7).

We have defined the successful in vitro protein translocation as cleavage of the signal peptide from a precursor, protection of the product from digestion by protease, and its sedimentation with the vesicles. However, a mutant prolipoprotein that is not cleaved in cells (16) and many such mutant precursors of LamB protein (26) of *E. coli* can be translocated even without cleavage. It is thus of interest that in our in vitro system an appreciable fraction (ca. 10 to 20%) of OmpA of precursor size is evidently translocated, since it is resistant to protease treatment and cosediments with membrane (Fig. 1 and 2). Moreover, we have similarly found that *E. coli* prolipoprotein can be translocated into membrane vesicles without cleavage of its signal peptide (unpublished data). If this is indeed the precursor, it would suggest that normal precursors can be translocated without removal of the signal peptide by a signal peptidase. On the other hand, precursors can still be processed when translocation is inhibited (Fig. 6) (25, 36). Thus it appears that processing of the precursors is neither a prerequisite for translocation nor a reliable indicator of cotranslational or posttranslational translocation (11, 12). It further suggests that the sites of signal peptide cleavage in membrane are distinct from those of protein translocation.

The ability to separate translocation from translation and the high efficiency of our in vitro system (Fig. 2) provide us a good handle to dissect the details of translocation. By

removing components necessary for protein synthesis, we are now analyzing the requirements for the translocation of alkaline phosphatase and OmpA.

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ADDENDUM IN PROOF

After this manuscript was accepted for publication, Pages et al. (Eur. J. Biochem. **143**:499–505, 1984) reported that the precursors of phosphate-binding protein accumulated in the cytoplasm of hyperproducing *E. coli* cells are not exported posttranslationally. This conclusion differs from that of our current work. One explanation is that pathways of secretion for proteins studied are different. The other more likely explanation is that the accumulated precursor was inactivated when export sites were saturated. Recently, Muller and Blobel (*Proc. Natl. Acad. Sci. U.S.A.* **81**:7421–7425, 1984) also reported in *in vitro* translocation system with *E. coli* membranes that could translocate protein posttranslationally.

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