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ATP is essential for protein translocation into *Escherichia coli* membrane vesicles

(energetics/H^+-ATPase/protonmotive force/alkaline phosphatase/OmpA protein)

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ABSTRACT **The energy requirement for translocation of alkaline phosphatase and the outer membrane protein OmpA into *Escherichia coli* membrane vesicles was studied under conditions that permit posttranslational translocation and, hence, prior removal of various components necessary for protein synthesis. Translocation could be supported by an ATP-generating system or, less well, by the protonmotive force generated by D-lactate oxidation; the latter might act by generating ATP from residual bound nucleotides. However, when protonmotive force inhibitors were used or when ATP was further depleted by *E. coli* glycerol kinase, D-lactate no longer supported the translocation. Furthermore, ATP could still support protein translocation in the presence of proton uncouplers or with membranes defective in the F_1 fraction of the H^+-ATPase. We conclude that ATP is required for protein translocation in this posttranslational system (and probably also in cotranslational translocation); the protonmotive force may contribute but does not appear to be essential.**

In eukaryotic cells, secretion of proteins across membranes could well derive energy from protein synthesis, since a tight coupling of translation and translocation (1) and firm attachment of the secreting ribosomes to membranes (2) have been reported. However, in bacterial systems neither the attachment of ribosomes (3) nor the tight coupling of translocation and translation (4–8) has been found. Thus, the source of energy to translocate proteins across bacterial membranes remains a central problem (4, 7, 8). Studies in intact bacterial cells have provided evidence for a requirement for an energized membrane: dissipation of the protonmotive force (pmf) by carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or by valinomycin blocked processing and translocation of several outer membrane and periplasmic proteins (9–12). Moreover, CCCP inhibited processing of several precursors that had accumulated in the presence of membrane perturbants (13, 14). Similarly, in an *in vitro* system, compounds that eliminated pmf inhibited translocation into *Escherichia coli* membrane vesicles (15). However, in this *in vitro* system, ATP must be present for protein synthesis; hence it is difficult to distinguish between a direct role of pmf and an indirect role, generating ATP.

An opportunity to investigate this problem in greater detail arose from our recent finding (5) that *E. coli* can translocate the same protein either cotranslationally or posttranslationally *in vivo* and that under proper conditions, a normally cotranslationally secreted protein (alkaline phosphatase) and an outer membrane protein (OmpA) can be posttranslationally translocated into *E. coli* membrane vesicles. The energy requirement can thus be tested directly after removing components that are necessary for protein synthesis. The results presented here show that translocation requires ATP but does not appear to require either a functional H^+-ATPase or a pmf.

MATERIALS AND METHODS

Bacterial Strains and Media. The strains used in this work are all *E. coli* K-12. MC1000/pHI-5 (16), which contains the *phoA* gene in a plasmid, was a source of mRNA for alkaline phosphatase as well as a source of stable mRNA for OmpA protein (15). The RNase I^- strain D10 was used for preparation of S30 extracts (supernatants after centrifugation of homogenates at 30,000 × *g*) and of inverted membrane vesicles. The media and conditions for the growth of these strains are as described (15). For comparing membranes with or without functional ATPase, AN120 (*uncA410 arg*) and AN180 (*arg*), obtained from F. Gibson (17) via T. Wilson (Harvard University), and JSH79 (*uncA thiA met asn*) and JSH2 (*thiA asn*), obtained from J. S. Hong (Boston Biomedical Research Institute), were subcultured and maintained in minimal medium supplemented with the required amino acid and with glucose. The $ATPase^-$ phenotype of AN120 and JSH79 was confirmed by their inability to grow with succinate as carbon source.

Preparation of Various Fractions. Inverted membrane vesicles, mRNA, and S30 were prepared as described (5). For preparing membranes from strains AN120 and JSH79, a small portion of each culture just before harvesting was checked for $ATPase^-$ phenotype by seeding on medium containing glucose or succinate. Each preparation of AN120 and JSH79 formed $<10^{-5}$ as many colonies on succinate as on glucose. All membranes were resuspended in 50 mM potassium phosphate buffer, pH 7.6/5 mM $MgSO_4$.

Protein Synthesis and Posttranslational Translocation. *In vitro* protein synthesis was at 40°C for 15 min as described (5), with an S30 extract treated with octylglucoside, the optimal amount of RNA as message, and 20 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per 0.1 ml of reaction mixture. After incubation, chloramphenicol (0.1 mg/ml) was added and the reaction mixtures were cooled on ice. A Sephadex column was used to remove small molecules in the mixture. Unless otherwise stated, 3 ml of Sephadex G-50-150 (Sigma) equilibrated in 50 mM potassium phosphate, pH 7.6/6.5 mM $MgSO_4$ was packed in a plastic syringe and placed in a conical plastic tube in a swinging bucket. Just before use, 1 ml of 0.5% bovine serum albumin (product no. A 9647, Sigma) in the buffer was layered onto the column and centrifuged in an International Equipment model CL clinical centrifuge at the top speed for 2.5 min. A portion (0.3 ml) of reaction mixture was then layered onto the Sephadex column, with another conical plastic tube as a receptacle, and centrifuged for 2.5

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.
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min. The centrifugal eluate was centrifuged through a second column to remove residual small molecules. The second eluates, which contained precursors of alkaline phosphatase and OmpA protein, were pooled and used for translocation.

Translocation mixtures (0.1 ml) contained, unless otherwise stated, 50 mM potassium phosphate (pH 7.6), 5 mM $MgSO_4$, 1 mM spermidine phosphate, 8 mM putrescine phosphate, chloramphenicol at 0.10 mg/ml, 0.15 A_{280} units of membrane vesicles, 80 μl of the Sephadex-centrifuged precursor preparation, and 5 mM ATP. Translocation was allowed to occur at 40°C for 15 min. Samples then were exposed to Pronase (0.3 mg/ml, 15 min on ice), and membrane vesicles were isolated and analyzed by $NaDodSO_4$/PAGE as described (5, 15).

Quantitation of Fluorograms. Densitometric scanning of fluorograms for quantitation of protein translocation was as described (15).

Assay for ATPase. ATPase activity was assayed in 50 mM potassium phosphate, pH 7.6/10 mM $MgSO_4$/[γ-^{32}P]ATP (5 mM, 2 μCi/0.1 ml)/ membrane (0.6 mg of protein/ml) at 25°C as described by Reenstra and Kaback (18). The reaction was terminated by taking 45 μl of reaction mixture into 1 ml of 2.5% ammonium molybdate in 1 M HCl. Inorganic phosphate was extracted with 1.8 ml of H_2O-saturated isobutyl alcohol/benzene (1:1, vol/vol); radioactivity in 1 ml of the upper, aqueous phase was measured with a liquid scintillation counter. Results were corrected for background hydrolysis of ATP without membrane.

Chemicals. Translational grade [^{35}S]methionine was obtained from New England Nuclear. Glycerol kinase was obtained from Boehringer Mannheim. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Energy-Dependent, Posttranslational Translocation. Employing the posttranslational translocation of alkaline phosphatase and OmpA protein into *E. coli* membrane vesicles (5), we developed a system in which translocation was stringently dependent on the addition of an exogenous energy source. In this system, the translocated products are defined as those (precursor and mature proteins) that are resistant to Pronase digestion and that sediment with membrane vesicles. To this end, after synthesis of the protein precursors in the absence of membrane, high-energy-phosphate sources (ATP, GTP, phospho*enol*pyruvate) were removed by Sephadex gel centrifugation through a short column. Several kinds of Sephadex gel bead and various centrifugation times were assessed for their effectiveness in removing [γ-^{32}P]ATP with little loss of the precursor proteins. In the most satisfactory procedure, which was adopted for routine use, centrifugation through a Sephadex G-50-150 column for 2.5 min removed >98% of ATP while allowing the recovery of 70–95% of the precursors.

After centrifugation through a second column, the mixture containing the precursors was completely inactive for subsequent translocation with membranes (Fig. 1, compare lane 7 with lane 1), unless a mixture of high-energy phosphates was supplied (lane 4). Polyamine improved translocation in this reconstituted system (lane 3), just as in crude reaction mixtures (5), whereas addition of S100 cytoplasmic fraction (lane 6) or polyamine without energy source (lane 5) had no stimulatory effect.

The buffer systems tested (Tris Cl, Tris acetate, and potassium phosphate, all at pH 7.6) did not differ significantly in the translocation test (data not shown, but compare lanes 1 and 3). Neither an exogenous thiol-reducing agent (e.g., dithiothreitol) nor Mg^{2+} was necessary, as long as the polyamines were present. All subsequent experiments were carried out with polyamines in buffer containing 50 mM

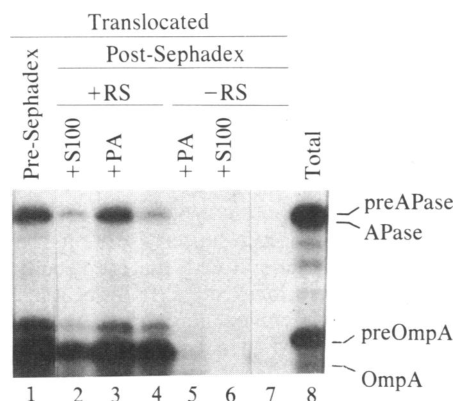

FIG. 1. Energy-dependent protein translocation of alkaline phosphatase (APase), OmpA protein, and their precursors. After protein synthesis, a portion of the reaction mixture (in Tris Cl buffer, lane 8) was assayed for translocation into membrane vesicles (lane 1), and the rest was centrifuged through a Sephadex column. Equal portions of the eluate were assayed for translocation into membrane vesicles, either in the presence of an ATP-regenerating system (RS, containing 5 mM phospho*enol*pyruvate, 3 μg of pyruvate kinase, 1 mM ATP Tris, and 0.02 mM GTP Tris) and 5 μl of S100 (lane 2), polyamines (PA, 1 mM spermidine and 8 mM putrescine) (lane 3), or no other addition (lane 4); or in the absence of RS but with polyamines (lane 5) or 5 μl of S100 (lane 6); or with no other addition (lane 7).

K_2HPO_4 (pH 7.6) and 5 mM $MgSO_4$, which has been used widely in studies on membrane energetics and active amino acid transport (*cf.* ref. 18).

ATP Can Drive Protein Translocation. To determine the active component(s) in the energy-source mixture that supported protein translocation in the assays of Fig. 1, each component was tested for its activity in supporting translocation. As Fig. 2 shows, the active compound was ATP (lanes 7 and 8): maximal activity was obtained at 3–5 mM. The strong stimulatory effect of phospho*enol*pyruvate (lanes 4 and 5) can be attributed to the regeneration of ATP, in conjunction with pyruvate kinase (which was present in the protein synthesis reaction mixture and would not have been removed by Sephadex filtration) and adenine nucleotides, presumably bound tightly, either to enzymes (e.g., aminoacyl-tRNA synthetases) or to membranes (19). At 1

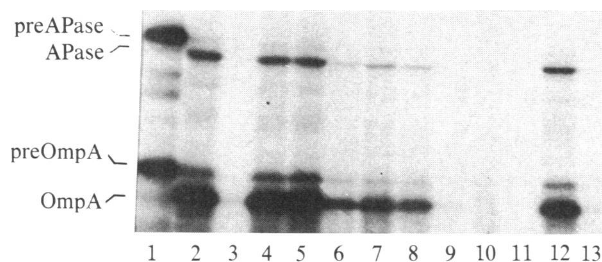

FIG. 2. ATP-driven protein translocation. After protein synthesis and centrifugation through Sephadex, samples were assayed for translocation as described in *Materials and Methods*, with the following additions: ATP-regenerating mixture, described in the legend to Fig. 1 (lane 2), no addition (lane 3), 5 mM phospho*enol*pyruvate and 3 μg of pyruvate kinase (lane 4), 5 mM phospho*enol*pyruvate (lane 5), 1 mM phospho*enol*pyruvate (lane 6), 5 mM ATP (lane 7), 1 mM ATP (lane 8), 20 mM D-lactate (lane 9), 20 mM succinate (lane 10), 20 mM ascorbate plus 10 μM phenazine methosulfate (lane 11), 5 mM creatine phosphate plus 3 μg of creatine kinase (lane 12), or 5 mM creatine phosphate (lane 13). An aliquot of the original protein-synthesis reaction mixture was also electrophoresed (lane 1) to provide markers. APase, alkaline phosphatase; preAPase and preOmpA, APase and OmpA precursor polypeptides, respectively.

mM, phospho*enol*pyruvate was only about as active as ATP in supporting translocation (compare lane 6 to lane 8). Elevating the ATP concentration increased the activity (lane 7), though not to the same extent as an elevated concentration of phospho*enol*pyruvate—a characteristic effect of an ATP-regenerating system. Furthermore, another ATP-regenerating system, creatine phosphate and its kinase, was as effective as the phospho*enol*pyruvate system (lane 12). It is thus evident that ATP can support protein translocation: either directly or, by generating a pmf, indirectly.

pmf Is Not Sufficient for Protein Translocation. To determine whether ATP might be acting by generating a pmf, oxidative substrates that also could generate a pmf were tested. D-Lactate, succinate, ascorbate/phenazine methosulfate (Fig. 2, lanes 9–11), or NADH could not substitute for ATP in the same membrane preparation described above. It thus appears that pmf alone, without ATP, is insufficient to drive protein translocation.

ATP Is Essential for Protein Translocation. In contrast to the membrane preparations described above, about half of our membrane preparations could utilize D-lactate or other oxidative substrates to translocate proteins, although at only about 15–30% the efficiency of ATP (Table 1). This activity could be attributed to the synthesis of ATP by H^+-ATPase from pmf (20). Thus cyanide (which inhibits respiration) or dinitrophenol, CCCP, or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (all of which dissipate a proton gradient) blocked D-lactate-driven protein translocation (Table 1). The translocation of OmpA precursor was inhibited only partially by CCCP or FCCP but more completely by cyanide; the reason is not clear. Moreover, D-lactate did not serve to provide energy via pyruvate and D-lactate dehydrogenase: though acetylphosphate could replace ATP for protein translocation (>95% relative translocation for all three proteins), pyruvate was completely inactive in this system.

In a more direct test, using the same preparation, ATP was drained off by hexokinase plus glucose or by glycerol kinase plus glycerol. The D-lactate-driven translocation was completely abolished (Table 1). The products of the kinases, glucose 6-phosphate or *sn*-glycerol 3-phosphate, each had no inhibitory effect. Since glycerol kinase is highly specific in utilizing only ATP and not other nucleotides (21, 22) and since it blocks translocation only in the presence of glycerol, ATP is clearly essential for protein translocation.

Neither pmf nor Functional H^+-ATPase Is Essential for Protein Translocation. Although the above findings show that ATP is necessary and cannot be replaced by a pmf, they do not exclude the possibility that its contribution to translocation includes generation of pmf, via the H^+-ATPase. Accordingly, uncouplers of a proton gradient were tested for their effect on the ATP-driven translocation. Cyanide or dinitrophenol had no effect on the ATP-driven translocation, whereas CCCP or FCCP caused partial inhibition that reached a limiting value at higher concentrations (Table 1). In contrast, as described above, D-lactate-driven translocation was completely blocked by cyanide, dinitrophenol, FCCP, or CCCP (Table 1). These data suggest that pmf may contribute to protein translocation but is not essential.

To demonstrate directly that the generation of pmf by H^+-ATPase is not essential, we used membrane vesicles from "uncoupled" (unc) mutants that can utilize D-lactate but not ATP to generate pmf. Membrane vesicles from two mutants defective in the F_1 portion of the ATPase, AN120 and JSH79, were active in protein translocation driven by ATP but not by D-lactate, whereas vesicles from their parent strain could utilize D-lactate for translocation, as expected (Table 2). These results provide strong evidence that pmf is not obligatory for posttranslational translocation of alkaline phosphatase and OmpA protein into *E. coli* membrane vesicles.

Nucleotide Specificity. To determine whether the requirement for ATP involves its hydrolysis, nonhydrolyzable analogs of ATP were each tested for the ability to support translocation. Adenosine 5'[γ-thio]triphosphate, adenosine 5'-[β,γ-methylene]triphosphate, adenosine 5'-[α,β-methylene]triphosphate, or adenosine 5'-[β,γ-imido]triphosphate could not replace ATP (Table 3).

Table 3 further shows that other nucleotides tested, including deoxynucleotides and ADP, could replace ATP for translocation but were not as effective, with one exception: dATP consistently supported higher amounts of protein translocation than did ATP. With the possible exception of

Table 1. Protein translocation supported by ATP and by D-lactate

	Protein translocation activity, %					
	20 mM D-lactate			5 mM ATP		
Addition	APase	preOmpA	OmpA	APase	preOmpA	OmpA
None	100 (15)	100 (20)	100 (29)	100	100	100
NaCN						
5 mM	0	14	5	122	115	102
10 mM	0	7	0	90	107	95
CCCP						
1 μM	0	77	28	82	50	81
5 μM	0	47	0	69	44	68
50 μM	ND	ND	ND	39	63	79
FCCP						
1 μM	0	86	0	46	54	69
10 μM	ND	ND	ND	21	50	51
50 μM	0	59	0	25	65	73
Dinitrophenol (1 mM)	0	45	0	98	95	97
Hexokinase (2 μg)						
+ glucose (10 mM)	0	0	0	0	0	0
Glycerol kinase (2 μg)						
+ glycerol (10 mM)	0	0	0	0	0	0
Glucose 6-phosphate (5 mM)	ND	ND	ND	104	157	97
sn-Glycerol 3-phosphate (5 mM)	ND	ND	ND	91	96	98

Translocation of alkaline phosphatase (APase), preOmpA, and OmpA was assayed; activity in the presence of D-lactate or ATP alone was taken as 100%. Numbers in parentheses give activities in the presence of D-lactate relative to those in the presence of ATP. ND, not done.

Table 2. ATP-driven protein translocation into H^+-ATPase-defective membrane vesicles

Membrane and energy source	Relative protein translocation activity, %		
	APase	preOmpA	OmpA
JSH2 (H^+-ATPase$^+$)			
+ ATP (5 mM)	100	100	100
JSH79 (H^+-ATPase$^-$)			
+ ATP (5 mM)	67	105	95
+ D-lactate (20 mM)	0	0	0
AN180 (H^+-ATPase$^+$)			
+ ATP (5 mM)	100	100	100
AN120 (H^+-ATPase$^-$)			
+ ATP (5 mM)	77	67	100
+ D-lactate (20 mM)	0	0	0

Translocation with membranes from various strains was assayed, and translocation with each respective wild-type membrane was taken as 100% activity. APase, alkaline phosphatase.

dATP, the activity of the others was evidently due to conversion to ATP, since the presence of hexokinase (plus glucose) or glycerol kinase (plus glycerol) abolished the translocation supported by these compounds but only partially inhibited dATP-driven translocation. It should be noted that extracts of the type used contain a highly active nucleoside diphosphokinase (23). In contrast, AMP could not substitute for ATP but inhibited ATP-dependent translocation (Table 3). These data indicate that the hydrolysis of ATP (or dATP) is required for protein translocation.

DISCUSSION

Building on our recent finding that translocation can be separated from translation in an *in vitro* system (5), we now have found that translocation of alkaline phosphatase and OmpA protein into *E. coli* membrane vesicles requires ATP or an ATP-regenerating system (Figs. 1 and 2), rather than pmf alone. This conclusion is based on several lines of evidence. First, D-lactate-driven translocation is evidently due to the generation of pmf, which allows the synthesis of ATP via H^+-ATPase, since the translocation is inhibited by inhibitors of respiration or proton uncouplers as well as by *E. coli* glycerol kinase (plus glycerol); the latter is highly specific for ATP as substrate (21, 22) and has been used to deplete ATP to demonstrate the specificity of DNA polymerase (24), but it should have no effect on pmf. Second, the ATP-driven protein translocation can still take place in the presence of compounds that dissipate pmf and that inhibit D-lactate-driven translocation. Third, with mutants defective in the F_1 portion of the ATPase, which cannot hydrolyze ATP to generate pmf, membrane vesicles could not utilize D-lactate but could still use ATP, even in the presence of a proton uncoupler, to translocate proteins. We conclude that ATP is essential for the posttranslational translocation of alkaline phosphatase and OmpA protein into inverted *E. coli* cytoplasmic membrane vesicles.

Since *E. coli* can translocate the same protein (e.g., alkaline phosphatase or β-lactamase) either co- or post-translationally (5), ATP is probably also necessary for cotranslational translocation in bacteria. However, such a requirement would be difficult to demonstrate because of the involvement of ATP in protein synthesis.

Although pmf is not essential for translocation, our data indicate that it contributes to the process, since ATP-driven translocation was reduced 50–70% in the presence of a proton uncoupler (CCCP or FCCP, Table 1). One role of pmf may be to maintain overall topology and organization of membrane proteins that are involved in translocation of the precursor, ensuring its proper interaction with signal peptidase to cleave the signal peptide and for the release of the mature protein. Another role of pmf may be to replenish ATP, as is clearly the case in the D-lactate-driven translocation (Table 1). It is well known that an ATP-regenerating system is more effective than ATP alone for other processes that require ATP (see also Fig. 2).

This interpretation differs somewhat from the generally held view that the electrical potential or pmf is required for protein translocation in bacteria. This view is based primarily on the finding that inhibitors of pmf prevent the maturation of precursor proteins in *E. coli* cells (9–14). However, it is now clear that translocation and maturation of a precursor are separable events, that a precursor can be translocated without cleavage, and that maturation is not necessarily a good indicator of translocation (5, 6, 25, 26). Moreover, it has been reported that pmf is not obligatory for cell growth, since *E. coli* cells can grow on medium containing CCCP (27); hence pmf may not be essential for translocation of the proteins that are necessary for cell growth. Another possibility is that some proteins utilize pmf for translocation and others utilize ATP. Finally, pmf may contribute to the faster kinetics of protein translocation and processing of precursors in the cells.

In our earlier, less active translocation system, in which translocation and translation were not uncoupled, protein translocation (but not synthesis of precursors) was prevented by inhibitors of pmf (FCCP and valinomycin). It had therefore been suggested that the energy source appears to be energized membrane and not ATP (15). However, in that system, ATP was also consumed in protein synthesis and was probably also depleted faster by the larger amount of ATPase-containing membranes used than in the current reconstituted ATP-replenished system. pmf may thus have been required for the regeneration of ATP. This was verified directly by the observation that addition of excess phospho*enol*pyruvate during protein synthesis in that system relieved the inhibition (data not shown). Depletion of ATP could also explain the puzzling observation that early addition of membrane vesicles, during protein synthesis, or the presence of an excessive amount of membranes not only

Table 3. Nucleotide specificity for protein translocation

	Relative translocation activity, %		
	APase	preOmpA	OmpA
ATP	100	100	100
GTP	71	96	94
CTP	46	59	71
UTP	64	97	72
ITP	46	74	89
NTP + hexokinase + Glc	0	0	0
NTP + glycerol kinase + Gro	0	0	0
dATP	204	177	165
dATP + glycerol kinase + Gro	12*	17*	25*
dATP + hexokinase + Glc	28*	88*	34*
dITP	44	98	89
dITP + hexokinase + Glc	0	0	0
ADP	24	12	38
ADP + glycerol kinase + Gro	0	0	0
AMP	0	0	0
Nonhydrolyzable ATP analog†	0	0	0

Activity with 5 mM ATP was taken as 100%. Each nucleotide was tested at 5 mM; where indicated 2 μg of either hexokinase or glycerol kinase was added; glucose (Glc) or glycerol (Gro) were used at 10 mM. APase, alkaline phosphatase.

*Expressed as percent activity of dATP.

†Adenosine 5′-[γ-thio]triphosphate, adenosine 5′-[α,β-methylene]triphosphate, adenosine 5′-[β,γ-methylene]triphosphate, or adenosine 5′-[β,γ-imido]triphosphate.

failed to enhance but actually decreased translocation (5, 15). Indeed, protein translocation under these conditions was increased by the further addition of ATP after protein synthesis (unpublished data).

A similar *in vitro* protein translocation system from *E. coli*, which can translocate proteins posttranslationally across inverted membranes, has recently been described and reported to depend on the retention of the F_1 portion of H^+-ATPase (6). This contrasts with our observation that membrane vesicles prepared from mutants defective in this fraction could still translocate proteins (Table 2); moreover, our most active membrane preparation was obtained in EDTA and low ionic strength buffer, conditions which release the F_1 portion (15). Indeed, these membrane preparations were low in H^+-ATPase activity, measured directly as *N,N′*-dicyclohexylcarbodiimide-sensitive hydrolysis (data not shown), and some could not utilize D-lactate for translocation; yet all preparations could utilize ATP to translocate proteins. Thus it seems possible that the requirement for F_1 of H^+-ATPase in that system (6) might be for regeneration of ATP. On the other hand, our data do not rule out the involvement of other subunit components (e.g., F_0) of H^+-ATPase, not directly involved in ATP hydrolysis, in protein translocation.

A requirement for ATP for posttranslational translocation of protein has also been reported for chloroplasts (28). The translocation of proteins into mitochondria, in the opposite direction to that in the bacterial membrane, has been reported to require pmf (29, 30); whether the pmf provides energy directly or acts by generating ATP is not known. The endoplasmic reticulum of eukaryotic cells does not have a pmf, and even though theoretical calculations have suggested that the energy of peptide chain elongation or peptide folding could be adequate (31, 32), it may well be that ATP is required. This mechanism would eliminate an apparently major difference in the source of energy for protein translocation in eukaryotic and prokaryotic cells.

The exact role of ATP in supporting protein translocation is not known. Moreover, dATP appears to serve better than ATP for this activity. Further work is needed to define the functions of these nucleotides and interacting proteins.

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