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Effects of Nucleotides on ATP-Dependent Protein Translocation into *Escherichia coli* Membrane Vesicles

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We have shown previously that *Escherichia coli* can translocate the same protein either co- or posttranslationally and that ATP hydrolysis is essential for the posttranslational translocation of the precursors of alkaline phosphatase and OmpA protein into inverted *E. coli* membrane vesicles. ATP-dependent protein translocation has now been further characterized. In the absence of exogenous Mg^{2+}, dATP, formycin A-5′-triphosphate, ATP-α-S, and N^1-oxide-ATP could replace ATP, but many other nucleotides were not only ineffective but inhibited ATP-dependent translocation. The inhibitors included nonhydrolyzable ATP analogs, ATP-γ-S, 8-azido-ATP, AMP, ADP, cyclic AMP, PP_i, and tripolyphosphate. On the other hand, adenosine, adenosine 5′-tetraphosphate, and N^1,N^6-etheno-ATP neither supported nor inhibited translocation. Moreover, photoaffinity labeling of azido-adenine nucleotides rendered membranes inactive for subsequent ATP-dependent protein translocation. These results suggest that protein translocation involves at least an ATP-binding site in the membrane and hydrolysis of ATP and that both the adenosine and phosphate moieties of ATP play a role.

The source of energy for protein translocation across bacterial membranes has been the focus of intensive studies (reviewed in references 1 and 3). Since in vitro translocation can be accomplished posttranslationally (2, 11), we could examine the energy requirement for translocation after removal of the components necessary for protein synthesis; we found that translocation of both precursors of alkaline phosphatase and OmpA protein into *Escherichia coli* membrane vesicles requires ATP or an ATP-generating system (3). The requirement of ATP for protein translocation appears to be specific: many nucleotides can partially replace ATP but, except for dATP, their activity is abolished by the presence of glycerol kinase (plus glycerol), a highly specific scavenger of ATP (14). Several nonhydrolyzable analogs of ATP are not active in supporting translocation, suggesting that the hydrolysis of ATP is necessary (3); on the other hand, proton motive force is not obligatorily required (3, 4). In this paper, the effects of nucleotides on ATP-dependent protein translocation are further characterized.

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MATERIALS AND METHODS

Bacterial strains and media. The strains used in this work were all derivatives of *E. coli* K-12. MC1000(pHI-5), 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 (13). The RNase⁻ strain D10 was used for the preparation of S30 extracts and of inverted membrane vesicles. The media and conditions for the growth of these strains were described earlier (2, 13). For preparing membranes and extracts devoid of F_1F_0-H^+-ATPase, CK1801 [F^- *ΔlacU169 araD139 thiA rpsL relA Δ(uncB-uncC)*], an MC4100 derivative with a deletion of the whole *unc* operon (obtained from C. Kumamoto, Stanford University), was subcultured and maintained in minimal medium with 0.4% glucose and 100 μg of streptomycin per ml. The $ATPase^-$ phenotype was confirmed by the inability to grow with succinate as the carbon source.

Preparation of various fractions. Inverted membrane vesicles, mRNA, and S30 extracts were prepared as described previously (2, 13). When membranes were prepared from strain CK1801, a small portion of each culture was checked before harvesting for the $ATPase^-$ phenotype by plating on medium containing glucose or succinate. Membranes prepared from this strain could utilize D-lactate but not ATP to generate membrane potential (4). All membranes were washed and suspended in 50 mM potassium phosphate or in 10 mM Tris hydrochloride–50 mM KCl, all at pH 7.6.

Protein synthesis and posttranslational translocation. In vitro protein synthesis was done at 40°C for 15 min, and the translation mixture was centrifuged through a Sephadex column to remove small molecules as described earlier (3), except that the Sephadex column was equilibrated with 50 mM potassium phosphate buffer (pH 7.6) without $MgSO_4$.

Translocation mixtures (0.1 ml) contained, unless otherwise stated, 50 mM potassium phosphate (pH 7.6), 1 mM spermidine phosphate, 8 mM putrescine phosphate, 0.1 mg of chloramphenicol per ml, 0.15 A_{280} units of membrane vesicles, 80 μl of the Sephadex-centrifuged precursor preparation, and 3 mM ATP. Translocation was allowed to occur at 40°C for 15 min. Samples were then exposed to pronase (0.3 mg/ml) for 15 min at 0°C, membrane vesicles were isolated, and the translocated products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (3).

Quantitation of fluorograms. For quantitation of protein translocation, fluorograms were scanned with an LKB Zeinek Soft-Laser Scanning Densitometer as described earlier (2, 3).

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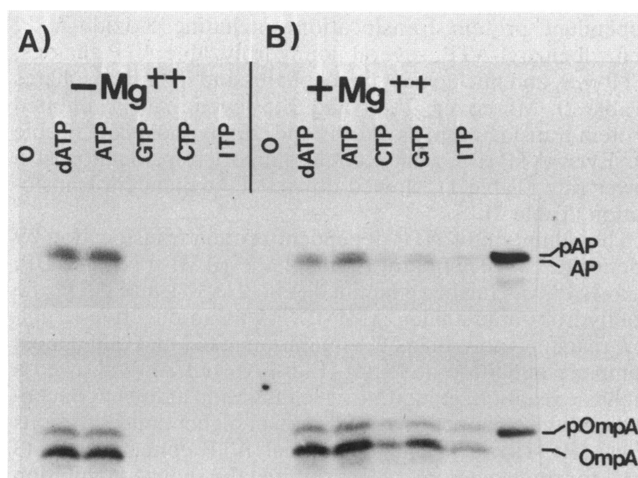

FIG. 1. Ability of nucleotides to support protein translocation. Translocation was carried out without or with 5 mM Mg^{2+} in the absence or presence of 5 mM nucleoside triphosphates. The translocated products were electrophoresed and fluorographed as described in the text. The upper and lower parts of the gel were exposed to Kodak XAR-5 film for 9 and 5 h, respectively. pAP, Precursor of alkaline phospatase (AP); pOmpA, precursor of OmpA protein (OmpA).

Quantitation of ATP hydrolysis. The reaction mixtures were identical to the translocation mixtures, except that 1 mM or 3 mM [α-^{32}P]ATP (1 μCi/0.1 ml) was used and the precursor preparations did not contain pre-alkaline phosphatase (synthesized from mRNA without alkaline phosphatase). At various times, the reaction was stopped by adding phosphoric acid and cooling on ice, and the samples, together with cold standards of ATP, ADP, and AMP, were spotted on Brinkman PEI-F cellulose thin-layer chromatography plates and developed with 0.65 M KH_2PO_4 (pH 3.5). The positions of ATP, ADP, and AMP were located under short-wave UV light (R_fs, 0.323, 0.579, and 0.726, respectively) and confirmed by exposure to Kodak X-ray film. Quantitation of the radioactive nucleotides was carried out by cutting out the corresponding spots and counting them in a scintillation counter.

Chemicals. Translational-grade [^{35}S]methionine was obtained from New England Nuclear Corp. 3′-*O*-(4-Benzoyl)benzoyl ATP was prepared by L. Ge essentially by the method of Williams and Coleman (17). Formycin A-5′-triphosphate was a gift of N. Sarkar, ATP-α-S (S isomer) was from Boehringer Mannheim Biochemicals, and all other ATP analogs were from Sigma Chemical Co. All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Protein translocation energized by ATP or dATP. We showed previously that the posttranslational translocation of the precursors of alkaline phosphatase and OmpA protein into *E. coli* membrane vesicles was stringently dependent on an exogenous energy source in the form of ATP or dATP (3). In this system, translocation occurs when materials (precursor and mature proteins) resist pronase digestion and sediment with membrane vesicles; the efficiency of translocation is about 10 to 25% for alkaline phosphatase and 25 to 50% for OmpA protein.

Many nucleoside and deoxynucleoside diphosphates and triphosphates could partially replace ATP for translocation (Fig. 1B; see also reference 3), presumably due to their conversion by a highly active nucleoside diphosphokinase in the presence of Mg^{2+} (6). However, in the absence of added Mg^{2+}, while ATP or dATP still promoted protein translocation (Fig. 1A), other nucleotides, including ADP, were no longer active (Fig. 1A and Table 1). These results provide additional evidence that among the nucleotides, ATP or dATP is the active substrate as a source of energy to drive proteins across the membranes. Other experiments showed that phosphoenolpyruvate could not replace ATP for protein translocation (data not shown). Since ATP-dependent protein translocation occurred with or without added Mg^{2+} (see also reference 4), all further studies were carried out in the absence of added Mg^{2+}.

ATP hydrolysis. Experiments were carried out to determine whether there is a correlation between ATP hydrolysis and the extent of protein translocation. However, in the current system, most ATP hydrolysis seemed to be unrelated to translocation, since ATP hydrolysis continued when translocation had stopped; in addition, few differences in ATP hydrolysis could be seen with or without translocation of the OmpA precursor, much of the ATP hydrolysis occurring with S30 extracts alone, with some enhancement by the addition of membranes (data not shown). The hydrolysis of ATP was probably not due to residual H^+-ATPase activity, as it was also observed with extracts and membranes prepared from a mutant (CK1801) with a deletion of all structural genes for H^+-ATPase (data not shown).

Functional ATP analogs. It was found earlier that ATP-γ-S cannot replace ATP for protein translocation (3), but 5 mM ATP-γ-S (S isomer) was active (Table 1), although somewhat less so than ATP. Among the other ATP analogs tested, formycin A-5′-triphosphate, an analog with a reversal of the C-8 and the N-9 of the purine ring, was almost as active as ATP for protein translocation; N^1-oxide-ATP was partially active (Table 1), with slower kinetics (data not shown), but adenosine 5′-tetraphosphate or AMP plus PP_i was not (Table 1). These results, combined with those reported above, indicate specificity for both the adenosine and phosphate moieties of ATP for supporting translocation.

Inhibitory ATP analogs. Other nucleotides and ATP analogs were inactive in the absence of Mg^{2+} (Table 1 and Fig.

TABLE 1. Capability of analogs to replace ATP in protein translocation

Compound	% Relative translocation activity of[a]:		
	APase	pOmpA	OmpA
ATP	100	100	100
Formycin A-5′-triphosphate	81	88	95
N^1-Oxide-ATP	15	32	55
ATP-α-S	18	25	26
ATP-γ-S	0	0	0
N^1,N^6-Etheno-ATP	0	0	0
Adenosine 5′-tetraphosphate	0	0	0
ADP or AMP	0	0	0
Adenosine or adenine	0	0	0
PP_i or tripolyphosphate	0	0	0
AMP + PP_i	0	0	0

[a] Translocation was carried out with each compound at 5 mM without added Mg^{2+} as described in the text. Translocation of alkaline phosphatase (APase), OmpA protein (OmpA), and its precursor (pOmpA), each with ATP as its energy source, was taken as 100%.

TABLE 2. Inhibitory ATP analogs in ATP-dependent protein translocation

Addition (mM)	% Inhibition of translocation activity of[a]:		
	APase	pOmpA	OmpA
AMP-PNP (5)	100	100	100
AMP-PCP (5)	84	71	75
AMP-CPP (5)	100	100	100
ATP-γ-S (3)	100	100	100
ATP-α-S (3)	38	51	47
Nucleoside triphosphates (3)	65–84	46–61	47–66
8-Azido-ATP (3)	82	49	45
3'-*O*-(4-Benzoyl)benzoyl ATP (3)	81	77	50
GMP-PCP (3)	87	90	91
ADP (3)	91	80	76
AMP (3)	100	100	100
GMP (5)	60	61	61
GDP (3)	73	76	74
2',3'- or 3',5'-Cyclic AMP (3)	100	100	100
Tripolyphosphate (3)	96	98	97
PP_i (1)	70	80	77
PP_i (2)	95	91	96

[a] Translocation with 1 mM ATP together with the additions at the concentrations indicated was assayed without added Mg^{2+}. The nucleoside triphosphates tested were GTP, CTP, TTP, and ITP. The reduction of ATP-dependent translocation with the analogs was analyzed. APase, Alkaline phosphatase; pOmpA, precursor of OmpA protein; OmpA, OmpA protein.

1). To determine whether they might compete with ATP and inhibit ATP-dependent translocation, we carried out translocation assays with ATP in the presence of the analogs. Since translocation was optimal at 3 to 5 mM ATP and decreased at higher ATP concentrations (4), competition experiments were done with 3 or 5 mM analogs and with suboptimal or near-optimal (1 or 3 mM) ATP. For more efficient competition, most studies were carried out with 1 mM ATP, but the partial competition was also confirmed at 3 mM ATP.

In the absence of added Mg^{2+}, all other nucleoside triphosphates not only could not replace ATP or dATP (Fig. 1) but also inhibited their ability to drive protein translocation (Table 2). Other ATP analogs were found to inhibit ATP-dependent protein translocation, including 8-azido-ATP, benzylbenzoyl-ATP, several nonhydrolyzable ATP analogs, ATP-γ-S, and nucleoside diphosphates and monophosphates (Table 2). Moreover, 2',3'- and 3',5'-cyclic AMP inhibited protein translocation, as did PP_i and tripolyphosphate (Table 2). Even ATP-α-S, which could support translocation at a lower rate (Table 1), slowed down ATP-dependent translocation (Table 2).

The inhibition of ATP-dependent protein translocation by adenosine 5'-[β,γ-imido]triphosphate (AMP-PNP), ADP, and AMP was further examined (Fig. 2). At 1 mM ATP, the nonhydrolyzable analog AMP-PNP was an effective inhibitor, reaching more than 50% inhibition at 0.5 mM and almost complete inhibition at 1 mM (Fig. 2A), but ADP showed a biphasic reaction, exhibiting little inhibition at lower concentrations and a cooperative inhibition at higher concentrations (Fig. 2B). However, at an optimal ATP concentration (3 mM) for translocation, AMP exhibited the reduced inhibition expected for a competitive antagonist (Fig. 2C), but ADP was a stronger inhibitor and no longer showed a biphasic reaction (Fig. 2B).

Nonfunctional compounds. Several ATP analogs and adenine-related compounds neither supported protein translocation nor inhibited ATP-dependent translocation, even at a molar ratio of 5:1 (with 1 mM ATP). These included adenine, adenosine, adenosine 5'-tetraphosphate, and N^1,N^6-etheno-ATP (Tables 1 and 2).

Inactivation of membranes by photoaffinity labeling with ATP analogs. The inhibition data suggested that protein translocation involved at least an ATP-binding site with specificity for both the adenosine and phosphate moieties of ATP and that ATP hydrolysis may play a role in the process. To determine whether the membrane contains an ATP-binding site and whether irreversible binding of a nucleotide renders a membrane inactive for subsequent ATP-dependent protein translocation, we treated membranes with photoactivable 8-azido-ATP, which partially inhibited protein translocation (Table 2). Membranes irradiated with the analog lost their activity for subsequent protein translocation even when supplemented with ATP (Table 3). Photoaffinity labeling with 8-azido-AMP at higher concentrations also rendered membranes inactive. The photoinactivation by the

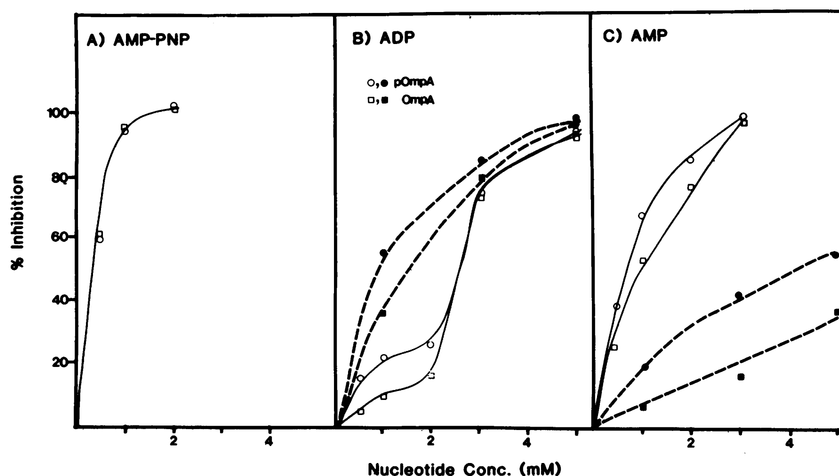

FIG. 2. Effects of nucleotides on ATP-dependent translocation. Translocation was carried out with 1 mM (solid lines) or 3 mM (dashed lines) ATP and with other analogs at the indicated concentrations (Conc.). To simplify the data, only the effects on OmpA and its precursor (pOmpA) are presented. Similar results were obtained for alkaline phosphatase, except that the inhibitory action was more pronounced.

TABLE 3. Inactivation of membranes by photoaffinity labeling with azido-nucleotides

Treatment (mM)	% Translocation of[a]:		
	APase	pOmpA	OmpA
Buffer	100	100	100
Azido-ATP (1)	17	47	21
Azido-ATP (3)	5	11	2
Azido-AMP (3)	12	13	32
Azido-AMP (5)	0	4	2
Azido-ATP (1) and:			
ATP (1)	58	67	55
ATP (3)	65	85	66
AMP (3)	42	77	54
ATP (3)	116	126	117

[a] All membranes (10 A_{280} units per 0.1 ml of buffer containing 10 mM Tris hydrochloride (pH 7.6) and 50 mM KCl) were placed in a 96-well microtiter plate with the indicated additions and irradiated with long-wave UV (at 366 nm) in a Chromato-vue cabinet (model C-70; Ultra-violet Products, Inc.) at a distance of 10 cm for 10 min at 0°C. After irradiation, samples were diluted to 1 ml with the buffer, and each was centrifuged through a 2-ml 0.25 M sucrose cushion in a Beckman 50 Ti rotor at 45,000 rpm for 90 min. The membranes were resuspended in the buffer and assayed for protein translocation with 3 mM ATP in the absence of Mg^{2+} as described in the text. APase, Alkaline phosphatase; pOmpA, precursor of OmpA protein; OmpA, OmpA protein.

analogs was partially prevented by the presence of ATP or AMP during irradiation (Table 3), demonstrating that the inactivation of the membranes was site specific. These results showed that there is at least an ATP-binding site in the membrane involved in protein translocation.

DISCUSSION

Previous studies established that in the absence of a functional H^+-ATPase, ATP hydrolysis is essential for protein translocation (3, 4). The data presented here showed that in the absence of Mg^{2+}, protein translocation occurred only with ATP or dATP and not with other natural nucleotides (Fig. 1), further substantiating an earlier, similar conclusion based on the use of *E. coli* glycerol kinase to specifically deplete ATP (3). These results suggest that the requirement of ATP for protein translocation involved the specific recognition of both the adenosine and phosphate moieties of ATP.

The structural features required for supporting protein translocation were studied by the use of ATP analogs. Formycin A-5′-triphosphate was as effective as ATP and N^1-oxide-ATP, and ATP-α-S also showed activity, but nonhydrolyzable ATP analogs (AMP-PNP, adenosine 5′-[β,γ-methylene]triphosphate [AMP-PCP], adenosine 5′-[α,β-methylene]triphosphate [AMP-CPP]), ATP-γ-S, and ADP could not support protein translocation. These results are consistent with the notion that the hydrolysis of the terminal phosphate moiety of ATP plays a role in the protein translocation process. It was of interest that the nonhydrolyzable ATP analogs as well as a number of other substances that failed to promote protein translocation (nucleoside triphosphates, 8-azido-ATP, AMP, cyclic AMP, PP_i, and tripolyphosphate) inhibited the ATP-dependent process. This result suggested that these substances, although nonfunctional, could nevertheless bind to a site essential for translocation. Since adenine, adenosine, and adenosine 5′-tetraphosphate had no effect on protein translocation, either alone or in the presence of ATP, it was possible to conclude that the process required a nucleotide-binding site and that binding required a phosphate moiety and was further enhanced by the presence of an adenosine moiety.

The effective inhibition of ATP-dependent protein translocation by the nonhydrolyzable analog AMP-PNP (Fig. 2A) suggested that the analog binds more strongly than ATP. In contrast, the inhibition by AMP was consistent with but not proof of its being a competitive inhibitor (Fig. 2C). The inhibition by ADP revealed a biphasic reaction (Fig. 2B), suggesting that there are two modes of interaction and that there are two ATP-binding sites: a regulatory site which binds the nucleotide and a catalytic site involving hydrolysis. However, the rapid hydrolysis of ATP to ADP complicates interpretation, and the biphasic inhibition by ADP and its unusual response to ATP concentration (Fig. 2B) suggested a greater complexity of the interaction than can be explained simply by assuming two different binding sites. That at least an ATP-binding site is involved in protein translocation is supported by photoaffinity labeling with an ATP analog. The photo-cross-linking of 8-azido-ATP or 8-azido-AMP rendered the membrane inactive for subsequent translocation in the presence of ATP, even after the unreacted analog was removed. Photoinactivation was reduced by the presence of ATP or AMP, indicating a specific effect on an ATP-binding site in the membrane (Table 3).

The exact functions of ATP and its hydrolysis in protein translocation are not known: the high level of nonspecific ATP hydrolysis in the present crude translocation system hindered our attempt to correlate quantitatively ATP hydrolysis with translocation. This question must await further purification of the system. We had earlier suggested (4) that the function of ATP includes at least two components: one is essential for protein translocation, and the other facilitates the process and can be replaced by proton motive force. The second component may function by maintaining the overall membrane topology and proper protein conformation involved in protein translocation across membranes. The ATP-specific component may involve ATP hydrolysis and could possibly function in a process similar to vesicle acidification during organelle membrane fusion (5). Another possible role of ATP hydrolysis is in the unfolding of precursors at or near the site of their interaction with the membrane to a conformation or to a domain suitable for translocation. A third possibility, which may not necessarily exclude the others, is the phosphorylation and dephosphorylation of a membrane protein(s) to regulate the initial interaction of precursors with a putative membrane receptor or the subsequent transit of precursors through the membrane. In support of this notion (but not proof for the process), we found that a few membrane proteins underwent rapid phosphorylation and dephosphorylation by [γ-^{32}P]ATP but not by [γ-^{35}S]ATP, and several inhibitors of protein translocation affected the level of phosphorylation (unpublished observations). Whether these membrane proteins are involved in protein translocation remains to be determined.

A requirement for ATP for posttranslational translocation has been reported for chloroplasts (7). We suggested earlier (3) that ATP may also be required for protein transport into mitochondria and endoplasmic reticulum, thus eliminating an apparent difference in the source of energy for protein translocation in eucaryotic and procaryotic cells. Indeed, recent studies have shown that the tight coupling of translation and translocation (15) is not observed in mammalian cell-free systems (9) and that translocation across canine endoplasmic reticulum membranes and yeast membranes can proceed in the absence of peptide chain elongation and also requires ATP (8, 10, 12, 16). It is thus possible that

posttranslational translocation in procaryotic as well as in eucaryotic systems is directly driven by ATP and involves the same mechanism. Whether cotranslational translocation also requires ATP (2, 3) remains to be determined.

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