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MINI-REVIEW

In Vitro Translocation of Bacterial Secretory Proteins and Energy Requirements

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Abstract

The recent establishment of *in vitro* assay systems has made biochemical studies on the process of membrane translocation of secretory proteins possible. This review summarizes what we have learned, using these *in vitro* systems, concerning the biochemical process of protein translocation, with special reference to energy requirements. Both ATP and the protonmotive force participate in the translocation reaction. The requirement of ATP is obligatory, whereas that of the protonmotive force differs, in terms of its level, with the secretory protein species. The possible roles of ATP and the protonmotive force in protein translocation are discussed with special reference to the function of SecA, an essential component of the secretory machinery. The effect of positive charges, which precede or follow the hydrophobic domain of signal peptides, on translocation is also discussed.

Key Words: Protein secretion; *in vitro* secretion; ATP; protonmotive force; membrane potential; SecA; signal peptide; positive charge.

Introduction

The translocation of secretory proteins across the cytoplasmic membrane in prokaryotes shares common features with that across the endoplasmic reticulum membrane in eukaryotes. Bacterial cells, especially *Escherichia coli* cells, are advantageous for studying such translocation machinery in that genetic and gene engineering methods can easily be applied to these organisms. A number of secretion mutants have been isolated and well characterized genetically (Beckwith and Ferro-Novick, 1986). From the biochemical

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point of view, however, research on prokaryotes was once behind that on eukaryotes, largely due to a lack of an *in vitro* system suitable for quantitative and biochemical studies.

This review summarizes the recent progress as to *in vitro* protein translocation systems and our current knowledge of the molecular mechanism underlying the translocation of secretory proteins across the bacterial cytoplasmic membrane, with special reference to energy requirements. Readers are also referred to a recent review on the energetics of protein translocation across membranes (Tai, 1989). The effect of positive charges on secretory proteins on translocation will also be discussed in this review.

Improvement of an *In Vitro* Translocation System

A system for *in vitro* protein translocation in prokaryotes requires inverted (inside-out) cytoplasmic membrane vesicles, which are equivalent to microsome vesicles prepared from eukaryotic cells. Methods for preparing *E. coli* inverted membrane vesicles were first developed by Hertzberg and Hinkle (1974) and Futai (1974). Such vesicles have been used successfully in *in vitro* biochemical studies on the translocation process. With these *in vitro* systems, however, the efficiency of protein translocation into membrane vesicles are usually not high enough.

A method for preparing membrane vesicles exhibiting efficient and quantitative protein translocation was developed recently (Yamada *et al.*, 1989a). The procedure involves the disruption of spheroplasts in a French pressure cell and subsequent fractionation by sucrose gradient centrifugation. Inverted membrane vesicles prepared from spheroplasts exhibit higher translocation efficiency than ones directly prepared from intact cells, despite the fact that the cytoplasmic membrane of spheroplasts was reported to be less efficiently inverted than that of intact cells with the French pressure cell treatment (Yamato *et al.*, 1978). On the other hand, the diameter of the former vesicles (0.3–0.5 μm) (Yamada *et al.*, 1989a) was considerably larger than that of the latter ones. This may somehow be related to the high efficiency of translocation.

Another important factor affecting the efficiency of *in vitro* translocation is the species of secretory protein to be used as a substrate. So far, only limited species of secretory protein precursors have been successfully used as substrates for *in vitro* translocation. These include OmpA, MalE, LamB, and OmpF-Lpp. OmpF-Lpp is a model secretory protein, in which the signal peptide and the cleavage site are derived from proOmpF and the following mature region from the major lipoprotein (Yamane *et al.*, 1987). On the other hand, the translocation of OmpF, OmpC, PhoA, and Bla, major secretory

proteins of *E. coli*, were not detected at all or only weakly detected *in vitro*. It is unclear whether this is due to a lack of factor(s) required to keep these proteins in a translocation-competent conformation or one constitutes an essential portion of the translocation apparatus for them.

In an *in vitro* translocation reaction, a relatively large fraction of a mature protein, which has been processed for the signal peptide, is often found not to be translocated into membrane vesicles. This is assumed to be due to premature processing before the translocation of precursor proteins. As one way to overcome this problem, the use of a secretory protein possessing an uncleavable signal peptide was proposed (Yamane *et al.*, 1988). The signal peptide cleavage site of an OmpF-Lpp chimeric protein was changed from Ala-Ala to Phe-Pro to construct "uncleavable OmpF-Lpp." The mutant protein was translocated into membrane vesicles much faster and more quantitatively than the wild-type OmpF-Lpp (Yamada *et al.*, 1989a). It should be mentioned, however, that uncleavability does not always mean increased translocation efficiency (H. Lu, H. Yamada, and S. Mizushima, unpublished results).

With the combination of the fractionated membrane vesicle preparation and an appropriate secretory protein, it became possible to carry out precise *in vitro* kinetic studies on the protein translocation process. An example is shown in Fig. 1.

Translocation and Soluble Factors

Several cytosolic soluble proteins have been reported to be involved in the *in vitro* protein translocation reaction: the trigger factor for OmpA (Crooke *et al.*, 1988; Lill *et al.*, 1988), SecB for MalE and LamB (Weis *et al.*, 1988; Watanabe and Blobel, 1989), and GroEL for Bla (Bochkareva *et al.*, 1988). These cytosolic factors were suggested to maintain precursor proteins in a translocation-competent state.

The requirement of cytosolic proteins for the translocation of uncleavable OmpF-Lpp, a small and hydrophilic protein, was studied quantitatively (Matsuyama and Mizushima, 1989). As much as 80% translocation was observed within 6 min, when uncleavable OmpF-Lpp purified by means of immunoaffinity chromatography was used. Even the precursor protein, which was highly purified through the combination of immunoaffinity chromatography and SDS-polyacrylamide gel electrophoresis, could still be efficiently translocated into the membrane vesicles, suggesting that cytosolic proteins are not an essential component that is directly involved in the translocation process. Since OmpF-Lpp is a small and hydrophilic protein, it may be able to retain such a conformation unaided.

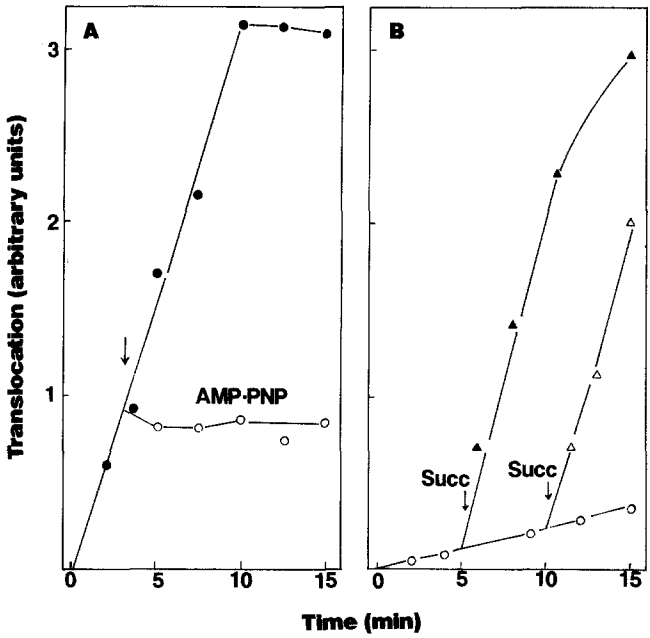

Fig. 1. Kinetic studies showing that protein translocation into *E. coli* membrane vesicles requires both ATP and $\Delta\bar{\mu}H^+$. Translocation of [^{35}S]Met-labeled cleavable OmpF-Lpp was examined at 37°C in 25 μl of 50 mM potassium phosphate, pH 7.5, containing 2 mM $MgSO_4$, 1 mM ATP, and 5 μg protein of inverted membrane vesicles prepared from *E. coli* K003 (*ΔuncB-C*). (A) The reaction mixture also contained 5 mM NADH (●) to generate $\Delta\bar{\mu}H^+$. At 3 min, AMP-PNP was added at the final concentration of 10 mM in order to inhibit ATP hydrolysis (○). The results indicate that the hydrolysis of ATP is essential for protein translocation even in the presence of $\Delta\bar{\mu}H^+$. (B) The translocation was examined in the absence of respiratory substrates (○). As indicated by the arrows, succinate (5 mM) was added at 5 (▲) or 10 (△) min in order to generate $\Delta\bar{\mu}H^+$. The results indicate that the rate of translocation was remarkably stimulated by the generation of $\Delta\bar{\mu}H^+$.

Translocation Requires Both ATP and the Protonmotive Force

It became generally accepted that energy is required for the translocation of a protein across a biological membrane. With an *in vivo* prokaryotic secretory system, the requirement of the protonmotive force ($\Delta\bar{\mu}H^+$) was observed (Randall, 1986). With an *in vitro* system, on the other hand, it was demonstrated that translocation was dependent on both ATP and $\Delta\bar{\mu}H^+$ (Geller *et al.*, 1986; Yamane *et al.*, 1987). ATP, most likely its hydrolysis, is absolutely required. ATP could be replaced by a tenfold higher concentration of CTP or UTP, whereas GTP was completely inactive (Matsuyama and Mizushima, 1989). The molecular mechanism of the ATP requirements will be discussed in a following section in relation to the SecA function.

The requirement of $\Delta\bar{\mu}H^+$, on the other hand, has been the subject of controversy. Some *in vitro* studies demonstrated the involvement of $\Delta\bar{\mu}H^+$ in translocation (Geller *et al.*, 1986; Yamane *et al.*, 1987), whereas another did not (Chen and Tai, 1985). Recently, it became clear that the requirements of the $\Delta\bar{\mu}H^+$ differed, depending on the precursor protein species (Yamada *et al.*, 1989a). The removal of the F_1 complex from F_1F_0-ATPase by urea makes the membrane vesicles permeable to protons (stripped membrane vesicles) (Patel *et al.*, 1975). Experiments with stripped membrane vesicles revealed that the translocation of OmpF-Lpp possessing a cleavable signal peptide absolutely required $\Delta\bar{\mu}H^+$, whereas that of uncleavable OmpF-Lpp or OmpA took place significantly, although at a slower rate, even in the complete absence of $\Delta\bar{\mu}H^+$ (Yamada *et al.*, 1989a). A large variety of mutant precursor proteins, both cleavable and uncleavable OmpF-Lpps, were then constructed and examined as to the $\Delta\bar{\mu}H^+$ requirement. It was suggested that the presence of the proline residue, a strong helix breaker, near the cleavage site may make $\Delta\bar{\mu}H^+$ less essential (H. Lu, H. Yamada, and S. Mizushima, unpublished results). All the uncleavable signal peptide-containing OmpF-Lpp, so far constructed and tested, and proOmpA possess the proline residue at this region.

Different Requirements for ATP and $\Delta\bar{\mu}H^+$ in the Process of Translocation

Although $\Delta\bar{\mu}H^+$ is not obligatory for the *in vitro* translocation of OmpA, the rate of translocation is significantly reduced with the transient accumulation of a possible translocation intermediate in its absence (Tani *et al.*, 1989). The intermediate can be detected on a polyacrylamide gel as a proteinase K-resistant band that accounts for 72% of the mature domain from the amino-terminus. The appearance of this band was completely inhibited in the absence of ATP or in the presence of AMP-PNP [adenosine 5′-(β,γ-imino) triphosphate]. Upon the addition of NADH, which energizes the membrane, the intermediate was converted to the translocated form of OmpA, even in the presence of AMP-PNP. These results interestingly indicate that ATP is absolutely required for the early stage, i.e., up to the stage of the intermediate accumulation, whereas $\Delta\bar{\mu}H^+$ is more critically required for the late stage of the translocation. It is also noticeable that the conversion of the intermediate, which no longer possesses the signal peptide, to the completely translocated OmpA most likely does not require ATP, namely, the process is not coupled with ATP hydrolysis. A similar conclusion was reached by Geller and Green (1989) recently.

Both the Membrane Potential and ΔpH, Which Constitute $\Delta\bar{\mu}H^+$, Participate in Translocation

$\Delta\bar{\mu}H^+$ is composed of an electrical component (membrane potential) and a chemical component (ΔpH). The effects of the individual components on translocation were examined using an *in vitro* system (K. Shiozuka, K. Tani, S. Mizushima, and H. Tokuda, unpublished results). When the membrane potential was specifically collapsed, the rate of translocation of cleavable OmpF-Lpp decreased to about 20% of the normal rate. The collapse of ΔpH caused an about 50% decrease in the rate of translocation. When both the membrane potential and ΔpH were collapsed, more than 90% inhibition was attained. These results indicate that not only the membrane potential but also ΔpH participates in the process of protein translocation. It is highly likely, therefore, that $\Delta\bar{\mu}H^+$ is not required as a driving force for the electrophoretic movement of proteins. On the *in vivo* examination of the energetics of protein translocation, a similar conclusion was reached (Bakker and Randall, 1984).

SecA Function and $\Delta\bar{\mu}H^+$ Requirement

Genetic studies have clearly shown that the *secA* gene is involved in protein secretion in *E. coli* (Oliver and Beckwith, 1982; Michaelis and Beckwith, 1982). A high-expressive plasmid for the *secA* gene was constructed, and the SecA protein was overproduced and then purified (Cabelli *et al.*, 1988; Kawasaki *et al.*, 1989; Cunningham *et al.*, 1989). The purified SecA stimulated the *in vitro* translocation of secretory proteins, when inverted membrane vesicles, from which SecA was depleted by means of treatment with urea (Kawasaki *et al.*, 1989; Cunningham *et al.*, 1989) or an anti-SecA antibody (Cabelli *et al.*, 1988), were used. Interestingly, a large amount of SecA induced the $\Delta\bar{\mu}H^+$-independent translocation of the wild-type OmpF-Lpp, whose translocation into inverted membrane vesicles highly depends on $\Delta\bar{\mu}H^+$ (Yamada *et al.*, 1989b). The $\Delta\bar{\mu}H^+$ requirement was also partly suppressed with a high concentration of ATP or in the presence of an ATP-generating system composed of creatine phosphate and creatine kinase, suggesting that the affinity of the SecA-containing translocation machinery for ATP, which is low in the absence of $\Delta\bar{\mu}H^+$, increases upon the removal of ADP (K. Shiozuka, K. Tani, S. Mizushima, and H. Tokuda, unpublished results). Detailed kinetic studies indeed revealed that K_m of the translocation machinery for ATP is higher by two orders of magnitude in the absence of $\Delta\bar{\mu}H^+$ than in its presence. The presence of an ATP-generating system also resulted in the increase in the affinity of the machinery for ATP in the absence

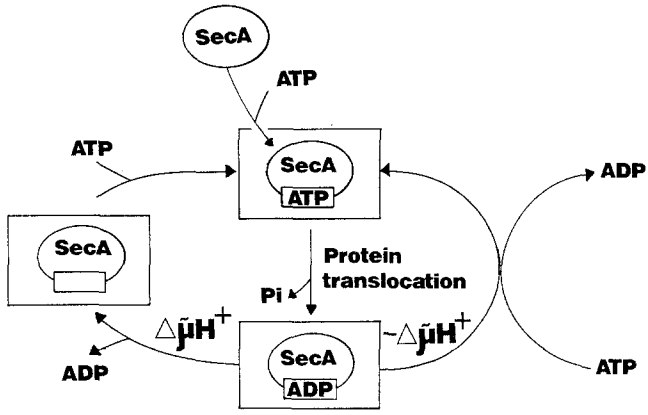

Fig. 2. A possible role of $\Delta\tilde{\mu}H^+$ in the ATP-dependent protein translocation. The protein translocation machinery, containing SecA, hydrolyzes ATP in coupling with protein translocation. ADP thus formed is rapidly released from the machinery in the presence of $\Delta\tilde{\mu}H^+$, and the high-affinity binding of ATP takes place to recycle the protein translocation reaction. In the absence of $\Delta\tilde{\mu}H^+$, on the other hand, the ADP release is suppressed, and a higher concentration of ATP is required to replace ADP. An ATP-generating system also removes ADP from the machinery to allow the high-affinity binding of ATP, although this is not shown in the figure.

of $\Delta\tilde{\mu}H^+$. Furthermore, the site of action of ATP for the translocation reaction is SecA, as discussed in the next section.

Taking all the facts together, we speculate that one of the roles of $\Delta\tilde{\mu}H^+$ in protein translocation is to facilitate the release of ADP, the product of ATP hydrolysis, from SecA in the translocation machinery to reactivate the SecA for the next cycle of the translocation reaction. In the presence of a large amount of SecA, such recycling may be unnecessary. This postulated view is schematically illustrated in Fig. 2. Tai (1989) proposed that the physiological significance of the $\Delta\tilde{\mu}H^+$ may lie in its ability to reduce the ATP concentration needed for the optimal activity.

SecA Function and ATP Hydrolysis

The relationship between the SecA function and the ATP requirement was directly demonstrated by Lill *et al.* (1989). They measured ATP hydrolysis, which is most likely coupled to protein translocation. The ATP hydrolysis required membrane vesicles, SecA, and a precursor protein (proOmpA in their case). Since the isolated SecA itself exhibits ATPase activity, although it is weak, it is reasonable to assume that SecA plays a central role, as the protein translocation ATPase, in coupling the hydrolysis of ATP to the translocation of secretory proteins across the membrane.

Since the SecA requirement for the early stage of the translocation has been suggested, the above vi ' is consistent with the fact that ATP is essentially required for the early s ge of the translocation (Tani *et al.*, 1989).

The binding of ATP to SecA was directly observed by means of flow dialysis (H. Tokuda, S. Kawasaki, and S. Mizushima, unpublished results). The SecA–ATP interaction was also demonstrated by means of photoreactive labeling with [^{32}P]ATP (S. Matsuyama, K. Kimura, and S. Mizushima, in press). To determine the site of ATP binding, the *secA* gene was engineered so as to code for SecA fragments of different sizes, either from the N-terminus or the C-terminus. Almost all of the N-terminal fragments were photoreactively labeled with ATP when the fragments were complimented with a C-terminal fragment, which was large enough to cover the deleted C-terminal region (S. Matsuyama, K. Kimura, and S. Mizushima, in press). A peptide mapping experiment revealed that the site of ATP binding is the same in both native SecA and SecAs "reconstituted" from the deletion fragments. The N-terminal region possesses a sequence which is partly homologous to the consensus sequence for ATP binding (Gay and Walker, 1983). Although this biochemical complimentation experiment revealed only one ATP-binding region, the possible interaction of ATP with other regions on the SecA molecule cannot be excluded. It is also unclear whether or not the binding site represents the site for ATP hydrolysis for translocation. SecA has several possible ATP-binding sequences. Recently, the dimeric structure was proposed for SecA (M. Akita, S. Matsuyama, and S. Mizushima, unpublished results). This would account for why the biochemical complimentation, as to photoreactive labeling with ATP, took place between N-terminal and C-terminal fragments when both fragments shared a common region.

SecA is suggested to interact with SecY, an integral membrane protein, which is essential for protein secretion in *E. coli* (Fandl *et al.*, 1988). The interaction of SecA with secretory proteins, with the recognition of positive charges at the amino-terminus of the signal peptide, was also demonstrated in *E. coli* (M. Akita, S. Sasaki, S. Matsuyama, and S. Mizushima, in press). Thus it became clearer that SecA plays a central role in the translocation of secretory proteins across the membrane by interacting with almost all of the proteins and other factors involved in the process.

Role of Positive Charges in Protein Translocation

The signal peptides of secretory proteins of *E. coli* generally contain one or two basic amino acid residues at their amino terminus (von Heijne, 1985), which are most likely localized on the cytoplasmic side of the membrane during the translocation reaction (Inukai and Inouye, 1983). *In vivo* studies

have suggested that the positively charged amino acid residues in the amino-terminal region of signal peptides play an important role in protein secretion (Vlasuk *et al.*, 1983; Iino *et al.*, 1987). Recent *in vitro* kinetic studies clearly demonstrated the positive charge requirement and, furthermore, that the rate of translocation is roughly proportional to the number of positively charged groups, irrespective of the amino acid species that donates the charge (Sasaki *et al.*, 1990).

The total charge of the first 10 residues of the amino terminus of the mature domain, on the other hand, is almost zero or slightly acidic (von Heijne, 1986a). Contrary to the positive effect of positive charges in the amino-terminal region of the signal peptide, the introduction of positive charges in this region resulted in strong inhibition of the translocation across the membrane both *in vivo* (Yamane and Mizushima, 1988; Li *et al.*, 1988) and *in vitro* (Yamane and Mizushima, 1988).

It should be noted in this connection that the stop-transfer hydrophobic domains of membrane proteins are often followed by positively charged residues (Sabatini *et al.*, 1982; Yamane and Mizushima, 1988). Studies on the topology of some membrane proteins suggest that some of the hydrophobic regions with amino termini directed toward the cytoplasm have a signal peptide-like function, whereas those with carboxyl termini directed toward the cytoplasm function as a stop-transfer sequence (for example, see Akiyama and Ito, 1987). It should also be noted here that, in the case of membrane proteins carrying plural hydrophobic domains, hydrophilic segments facing the cytoplasm are generally positively charged (von Heijne, 1986b).

Taking all the evidence together, we assumed that the positive charges, which are somehow stabilized on the cytosolic surface of membranes, have a positive effect, together with the following hydrophobic domain, as the signal peptide and have a negative effect, together with the preceding hydrophobic domain, on the translocation of the following polypeptide chain. Such possible dual effects of the positive charges on hydrophobic domains may account for the orientation of hydrophobic domains in membranes and the uneven distribution of positively charged amino acid residues across membranes (Yamane and Mizushima, 1988).

Concluding Remarks

The recent establishment of *in vitro* assay systems made the biochemical analysis of the mechanism of protein translocation possible. Using such a system, SecA was found to be essential for the translocation of various model proteins and native proteins. Furthermore, precise kinetic analysis of the process of protein translocation became possible with the improvement

of *in vitro* assay systems, as described in the text. It was shown that ATP is essential but that $\Delta\bar{\mu}H^+$ is not obligatory for protein translocation in *E. coli*. The essential requirements of ATP and SecA are obviously correlated with each other, and support the recent observation that SecA exhibits ATP-hydrolyzing activity (Lill *et al.*, 1989). Although $\Delta\bar{\mu}H^+$ was found to increase the efficiency of protein translocation, it is still not clear how $\Delta\bar{\mu}H^+$ has such an effect. Moreover, membrane components necessary for protein translocation have not been identified biochemically, though genetic data indicate that SecY (Shiba *et al.*, 1984) and probably SecE (Riggs *et al.*, 1988) are essential. Since $\Delta\bar{\mu}H^+$ is a transmembrane energy, components that directly respond to it seem to exist in membranes. In order to clarify the overall mechanism of protein transocation, it is therefore necessary to construct an assay system with which the functions of membrane factors can be examined. In this connection, we recently succeeded in reconstituting a protein translocation system (H. Tokuda and S. Mizushima, unpublished results). The absolute requirements of SecA and ATP were confirmed using reconstituted proteoliposomes. Anti-SecY IgG inhibited the protein translocation by the proteoliposomes. This successful reconstitution will open the way to thorough analysis of the membrane components required for the translocation of secretory proteins.

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