In vitro protein translocation into inverted membrane vesicles prepared from *Vibrio alginolyticus* is stimulated by the electrochemical potential of Na^+ in the presence of *Escherichia coli* SecA

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A protein translocation system was reconstituted from inverted membrane vesicles prepared from Na^+ pump-possessing *Vibrio alginolyticus* and purified *Escherichia coli* SecA. The translocation required ATP and was stimulated by the functioning of the Na^+ pump, suggesting that the electrochemical potential of Na^+, but not that of H^+, is important for protein translocation in *Vibrio*.

SecA protein; Protein secretion; Na^+ pump; *Escherichia coli; Vibrio alginolyticus*

1. INTRODUCTION

Protein translocation across the membrane of *E. coli* requires both ATP and the electrochemical potential of H^+ [1]. SecA, a peripheral protein [2], possesses ATPase activity [3], which is most likely involved in protein translocation. Little is known of the role of the electrochemical potential of H^+ in protein translocation in *E. coli*.

Vibrio alginolyticus, a marine bacterium, possesses a unique respiratory Na^+ pump that extrudes Na^+ as the direct result of respiration, leading to the generation of the electrochemical potential of Na^+ [4]. The active transport of amino acids [5], flagella motility [6,7] and growth [8] are supported by the electrochemical potential of Na^+. To examine the effect of the Na^+ pump on protein translocation in *V. alginolyticus*, the construction of an in vitro assay system was attempted. In this paper, we show that protein translocation into inverted membrane vesicles prepared from *V. alginolyticus* took place when the system was supplemented with SecA purified from *E. coli* and that the activity was stimulated by the functioning of the Na^+ pump present in the membrane vesicles.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

V. alginolyticus 138-2 (wild type) and a Na^+ pump-defective mutant, Nap1 [9], were used. These strains were grown on a complex medium as described in [8].

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2.2. *Preparation of SecA and inverted membrane vesicles*

SecA was purified from a cell lysate of *E. coli* RR1/pMAN400, a SecA-overproducing strain [10], by ammonium sulfate fractionation and subsequent hydroxylapatite column chromatography. Inverted membrane vesicles were prepared from *V. alginolyticus* 138-2 and Nap1 as described in [11].

2.3. *Transcription, translation and translocation reaction*

Uncleavable OmpF-Lpp, a model secretory protein for the in vitro *E. coli* assay system [12], was used as the substrate for translocation experiments. In vitro transcription and translation in the presence of Tran^{35}S-label (ICN Radiochemicals; 1000 Ci/ml, 1 Ci = 37 GBq) were performed as described in [12]. [^{35}S]Met-labeled uncleavable OmpF-Lpp was partially purified by gel filtration and then dissolved in 50 mM potassium phosphate (pH 7.5) as described in [1]. The translocation reaction was carried out at 37°C in 0.2 M potassium phosphate (pH 7.5), 6 mM $MgSO_4$, 6 mM Na_2SO_4 containing 0.4 mg/ml of inverted membrane vesicles. ATP and NADH were added to final concentrations of 2 and 5 mM, respectively. SecA was added at 60 μg/ml, unless otherwise specified. The assay was started by the addition of [^{35}S]Met-labeled uncleavable OmpF-Lpp (about 4×10^6 cpm/ml). The translocated protein, which was resistant to treatment with 0.8 mg/ml of proteinase K on ice for 30 min, was detected on SDS-polyacrylamide gels by means of fluorography as described in [13]. The amount of radioactivity was determined densitometrically with a Shimadzu CS-930 dual wavelength chromatoscanner.

3. RESULTS AND DISCUSSION

3.1. *Inverted membrane vesicles prepared from* V. alginolyticus *translocate protein in the presence of* E. coli *SecA*

The translocation of uncleavable OmpF-Lpp was examined with inverted membrane vesicles prepared from the Na^+ pump-deficient mutant, Nap1 (Fig. 1). No proteinase K-resistant band appeared unless SecA and ATP were added to the reaction mixtures (lanes 2, 4 and 6). In contrast, the omission of NADH had little

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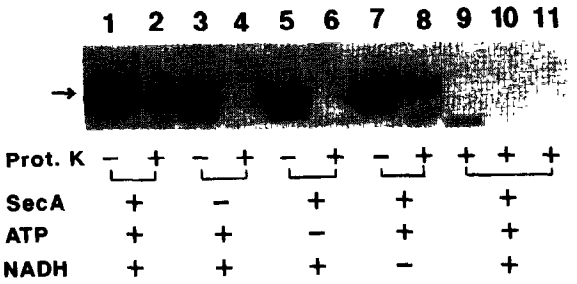

Fig. 1. SecA-dependent translocation of uncleavable OmpF-Lpp into inverted membrane vesicles prepared from Napl, a Na^+ pump-deficient mutant of *V. alginolyticus*. Translocation of [^{35}S]Met-labeled uncleavable OmpF-Lpp was examined at 37°C for 10 min with inverted membrane vesicles prepared from Napl. SecA (60 μg/ml), ATP (2 mM) and NADH (5 mM) were added to the reaction mixtures as indicated. After the translocation reaction, samples were treated with or without proteinase K (0.8 mg/ml), as indicated. Where specified, proteinase K-treatment was performed in the presence of 1% Triton X-100 (lane 9). The translocation reaction was also examined in the absence of membrane vesicles with (lane 11) or without (lane 10) liposome (0.8 mg/ml) prepared from soybean phospholipids. The translocation was analysed on an SDS-polyacrylamide gel by means of fluorography.

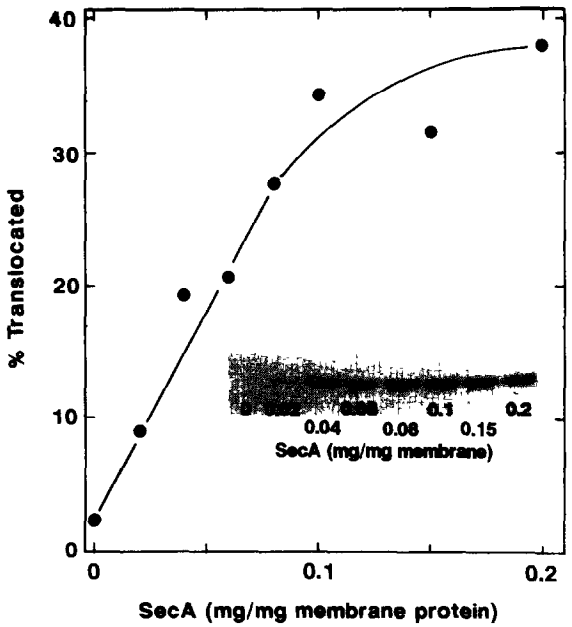

Fig. 2. Effect of the SecA concentration on the translocation activity. The translocation of uncleavable OmpF-Lpp into membrane vesicles prepared from Napl was examined in the presence of various concentrations of SecA at 37°C for 10 min. The amounts of translocated protein determined from the fluorograph (inset) are expressed as percentages of the input uncleavable OmpF-Lpp.

effect on the appearance of the band (lane 8). When proteinase K treatment was performed in the presence of 1% Triton X-100 (lane 9), or when the translocation was assayed in the absence of membrane vesicles (lane 10), the band did not appear at all. Moreover, when membrane vesicles were replaced with liposomes, no proteinase K-resistant band was detected (lane 11). These results indicate that the proteinase K-resistant band represents the true translocated protein, which had become inaccessible to external proteinase K. CTP, GTP or UTP added at 2 mM in place of ATP did not support the translocation (data not shown).

The amount of translocated substrate linearly increased with an increase in the amount of SecA until the latter reached about 10% of that of membrane vesicles (Fig. 2). The absolute requirement of SecA may be attributed to the fact that the precursor protein used in this assay was a chimeric protein of OmpF and a major lipoprotein, both of which are outer membrane proteins of *E. coli*.

3.2. *The Na^+ pump stimulates the SecA-dependent protein translocation into* Vibrio *membrane vesicles*

The Na^+ pump is coupled to only one of the two NADH:quinone oxidoreductase segments of NADH oxidase present in the wild type strain [14]. The segment present in Napl, which lacks the Na^+-motive segment, generates neither the electrochemical potential of Na^+ nor that of H^+. In contrast, both the wild type and Napl generate the electrochemical potential of H^+ at the site of terminal oxidase. Therefore, the direct result of NADH oxidation in wild type membrane vesicles is the generation of both the electrochemical potential of Na^+ and that of H^+, whereas only the latter is generated in Napl membrane vesicles (unpublished data).

The effect of the Na^+ pump on the protein translocation was examined by using membrane vesicles prepared from the wild type and Napl. NADH had little stimulatory effect on the protein translocation into Napl membrane vesicles (Fig. 3A). In marked contrast, when wild type membrane vesicles were used, NADH caused more than 2-fold enhancement of the initial rate of the protein translocation (Fig. 3B). Essen-

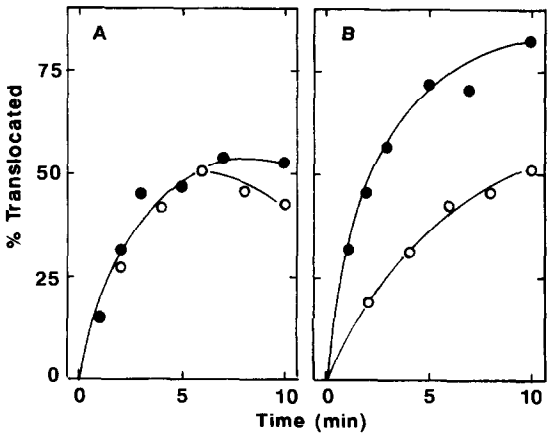

Fig. 3. Effect of the Na^+ pump on the protein translocation in *V. alginolyticus*. The translocation of uncleavable OmpF-Lpp was examined using membrane vesicles prepared from Napl (A) or the wild type (B) in the presence of 60 μg/ml of SecA and 2 mM ATP with (●) or without (○) 5 mM NADH. The amounts of translocated substrate are expressed as percentages of the input uncleavable OmpF-Lpp.

tially the same results were obtained with membrane vesicles treated with *N,N'*-dicyclohexylcarbodiimide (DCCD) (data not shown), indicating that generation of the electrochemical potential of H^+ by F_0F_1-ATPase, which is sensitive to DCCD, had little effect on the protein translocation. Moreover, the collapse of the proton motive force caused by carbonylcyanide *m*-chlorophenylhydrazone, a proton conductor, had no inhibitory effect on the protein translocation in the presence of NADH (data not shown). These results suggest that the electrochemical potential of Na^+, but not that of H^+, plays an important role in protein translocation in *V. alginolyticus*.

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