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SECA OF *ESCHERICHIA COLI* TRAVERSES LIPID BILAYER OF PHOSPHOLIPID VESICLES

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Summary : SecA protein of *Escherichia coli*, when added externally to the vesicles composed of phosphatidylethanolamine, dioleoylphosphatidylglycerol and cardiolipin, was found to be fragmented by trypsin encapsulated within the vesicles. In the presence of ATP or its non-hydrolyzing analogue, ATP-γS, the number of fragments and extent of hydrolysis occurred much less than in the absence of these compounds. When ADP was added, however, the hydrolysis products were similar to those when no nucleotide was present. Quenching of SecA fluorescence by vesicle-entrapped iodide corroborated the digestion results. These experiments demonstrated that the SecA protein traverses the lipid bilayer and its membrane topology depends on the kind of nucleotide present.

SecA, the central component of the protein translocation machinery of the plasma membrane of *Escherichia coli*, is present in both free and membrane bound forms (1). It is a homodimer of a subunit with a molecular weight of 102 kDa (2,3). *In vivo*, about half of the protein is present in the cytoplasm while the rest is attached to the membrane (1). A part of the membrane-attached SecA is "integral", that is, it cannot be dislodged from the membrane with a high concentration of salt solution (1,5). SecA is an ATPase which is stimulated by the precursor proteins, SecY/E and negatively charged phospholipids. There are a number of experimental observations that suggest a direct involvement of SecA in the translocation of precursor proteins (3,4,5). For this reason, the determination of the membrane topology of the "integral" SecA protein is of vital importance. Also, since most of the ATPases involved in membrane transport or locomotion have ATP/ADP cycles which are closely linked to their cyclic change in conformation, it is possible that the ATP/ADP cycle of the SecA may be coupled to changes in conformation as well as membrane topology.

Recently, Oliver and coworkers, using fluorescence quenchers located at different levels of the acyl chains of the lipid, observed that SecA penetrates deep into the bilayer of model membrane vesicles (6). However, their methods could not distinguish between penetration half way into the bilayer and complete crossing of the bilayer by SecA. This problem was addressed in this study by externally adding the SecA to the phospholipid vesicles entrapped with trypsin. It was found that the SecA indeed traverses the vesicle bilayer and exposes a part of the polypeptide chain to the internal trypsin. Also the digestion pattern of SecA by entrapped trypsin was dependent on the presence of either an ATP analog

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or ADP. These results were confirmed from the quenching of Trp fluorescence of externally added SecA by vesicle-entrapped iodide.

Materials and Methods

SecA protein was purified from a SecA-overproducing strain (RR1/pMAN400) (7) using the method described by Akita *et al* (8) and its purity was found to be more than 98% as assayed by a densitometer. Large unilamellar vesicles of mixed composition were prepared with PE (from bovine brain), DOPG and cardiolipin (from bovine heart) (60:30:10, by weight) by reverse phase evaporation method (9). The phospholipid concentration was determined using the method of Vaskovsky *et al* (10). Trypsin was encapsulated within the vesicles by the method used by Dumont and Richards (11) and by Rietveld *et al* (12). The trypsin digestion was carried out at 30 °C in a 30µl buffer (1 mM DTT, 2 mM $MgCl_2$, 20 mM HEPES, pH 7.5) containing 3 µM of SecA protein, 0.6 mM of phospholipid and 10 µM of trypsin inhibitor. The amount of encapsulated trypsin in each experiment was 0.6 µg. At specified times, the trypsin digestion was terminated by adding SDS sample buffer containing 3 mM phenylmethanesulfonyl fluoride and then placing the reaction vessels in an ice bath. The samples were boiled for 5 min prior to the electrophoresis on a denaturing 12% (w/v) polyacrylamide gel. The staining was done with Coomassie Brilliant Blue.

The fluorescence quenching of SecA tryptophan residues by iodide entrapped within the vesicles were determined with a solution containing 0.7 µM of SecA, 0.4 mM of lipid and 1 mM of nucleotides. The iodide was entrapped within the vesicles using the same method as the trypsin encapsulation and the unentrapped iodide was removed by passing through a Sephadex G-50 column and then pelleting by ultracentrifugation. The liposome used for the control experiments contained only 50 mM of NaCl to maintain the osmotic equilibrium. All fluorescence data were acquired on a Jasco FP770 spectrofluorometer with 295 nm excitation wavelength at 30 °C.

Results and Discussion

Figure 1A shows the SDS-PAGE of the digestion products after SecA was incubated for different time periods with vesicles entrapped with trypsin. Fragments of approximately 70 kDa, 50 kDa, 45 kDa, 35 kDa and 32 kDa were formed at the expense of SecA and the digestion stopped after about 30 min of incubation reaching a plateau. It is clear that a part of SecA traversed the lipid bilayer and was exposed to the trypsin inside. Figure 1A also shows that about 40% of the SecA originally added remained intact and, in view of the numerous trypsin-susceptible sites in the sequence, the fragments formed were apparently protected from further digestion into much smaller units. This may mean that the fragments, once produced, exist within the bilayer and/or on the surface of the bilayer. The 0.6 µg of trypsin completely digested 3µM of SecA within 10 min in the absence of the vesicles. The fragments shown in figure 1A are not the result of the digestion of SecA present entirely outside of the vesicles by the trypsin leaked out. This conclusion was drawn from a control experiment with externally added bovine serum albumin (BSA) which does not penetrate the bilayer. There was no digestion of BSA under this condition (data not shown).

When ATP was also present the number and amount of SecA fragments were reduced significantly (Figure 1B). Only 70 kDa and 45 kDa fragments plus some minor ones were detectable. Since this is a complicated system where ATP is also being hydrolyzed, we repeated the experiment in the presence of ATP-γS, a non-hydrolyzing ATP analogue. There was less digestion of SecA by entrapped trypsin although the 70 kDa and 45 kDa units were still the main fragments produced (Figure 1C). Furthermore, the amount of the fragments did not change after the first sampling at 2 min of incubation. It is clear that the SecA with the bound ATP-γS also traverses the bilayer. This result

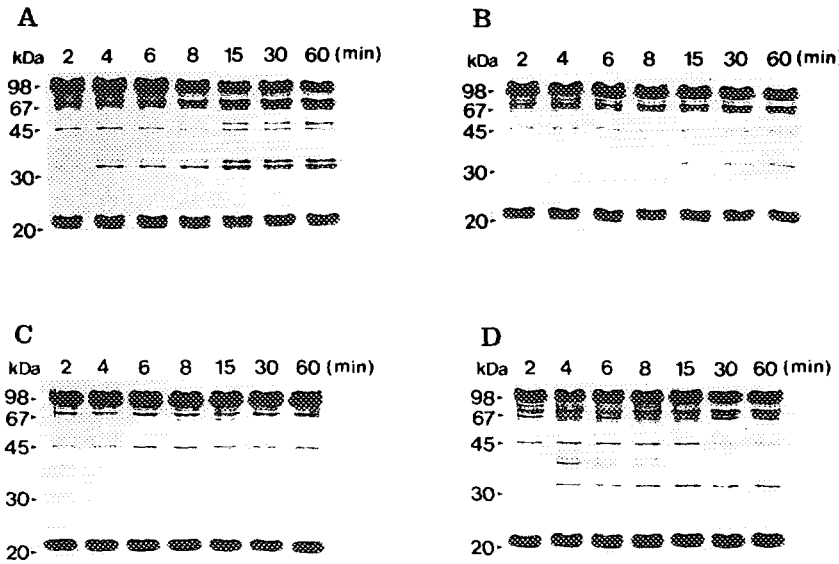

Figure 1. SDS-PAGE of the digestion products of externally added SecA protein by the vesicle-entrapped trysin. Proteolytic digestion of SecA was performed for the indicated time periods in the presence of trypsin-entrapped vesicle. Each reaction mixture (30 μl) contained 3 μM of SecA, 10 μM of trypsin inhibitor, 0.6 mM of phospholipid and 0.6 μg of entrapped trypsin. The digestion was carried out at 30 °C. Samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. The position of trypsin inhibitor is indicated with (▷).
(A) SecA alone. (B) SecA plus 1 mM ATP. (C) SecA plus 1 mM ATP-γS. (D) SecA plus 1 mM ADP.

suggests that the membrane topology of SecA in the presence of ATP or ATP-γS is different from the case without either of them.

The digestion experiment was also repeated in the presence of ADP and the result is shown in Figure 1D. The SDS-PAGE pattern is similar to the case when only SecA was present but there are two additional minor bands between 45 kDa and 35 kDa. Here again, the amount of the fragments did not increase after the first sampling.

These results indicate that the externally added SecA penetrates and traverses the lipid bilayer under the conditions studied here. But the topology seems to be different for each case. More extensive exposure of the SecA was shown for the case of SecA alone or in the presence of ADP than when the ATP analogue was present. It was shown that the SecA binds less to liposomes in the presence of ATP (13). This may be related to an earlier observation that ATP binding gives more compact and stable structure to the SecA (14).

Figure 2 shows the intrinsic fluorescence spectra of SecA tryptophan residues in the presence of vesicles entrapped with iodide. The light-scattering effect of the vesicle was corrected with blank experiments in the presence of vesicles alone. The fluorescence from the nucleotide was negligible. The fluorescence intensity of SecA tryptophan in the presence of iodide-entrapped vesicles was much smaller than that seen with the same amount of iodide-free vesicles. The addition of 1 mM ATP or ATP-γS to the SecA solution containing iodine-entrapped vesicles increased fluorescence intensity considerably. However, the addition of the same amount of ADP had no appreciable effect on this spectrum. These

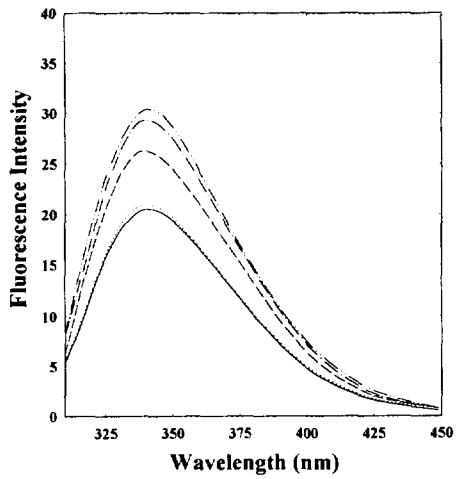

Figure 2. Fluorescence spectra of SecA which were bound to phospholipid vesicles entrapped with iodides. The curve — represents the difference between the spectrum of SecA/vesicle suspension and the spectrum of vesicle suspension. The curves ·····, - - - and -·-·- represent the difference spectra obtained by subtracting the spectra of ADP/vesicles suspension, ATP-γS/vesicle suspension and ATP/vesicle suspension from corresponding suspension with SecA. The curve -·-· represents the difference between the spectrum of SecA/iodide free vesicle suspension and the spectrum of iodide free vesicle suspension.

results support the tryptic digestion experiments which showed a more extensive exposure of SecA to the interior aqueous phase in the presence of ADP or when no nucleotide is present as compared to the cases when either ATP or ATP-γS is present. The control experiments of adding these nucleotides to the system containing SecA and iodide-free vesicles showed no change in the fluorescence intensity.

The results obtained in this study suggest a possible cyclic change in the membrane topology of SecA which may be linked to the ATP/ADP cycle of this ATPase. This cyclic change in topology, in turn, may bring about the translocation of the precursor proteins, SecA acting as a "shuttle".

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