

Separable ATPase and Membrane Insertion Domains of the SecA Subunit of Preprotein Translocase*

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The SecA subunit of preprotein translocase drives ATP-dependent translocation of preproteins across the bacterial inner membrane concomitant with cycles of membrane insertion and de-insertion (Economou, A., and Wickner, W. (1994) *Cell* 78, 835–843). We have identified the membrane-inserting region of SecA as a 30-kDa domain in the C-terminal third of the protein beginning at aminoacyl residue 610. Limited proteolysis in the absence of translocation ligands indicates that the SecA monomer is composed of two primary structural domains, the 30-kDa membrane-inserting domain and an N-terminal 65-kDa ATPase domain. This limited protease treatment of SecA results in constitutive ATPase activity, indicating that intramolecular constraints between the two domains may play a role in the regulation of ATP hydrolysis by SecA.

Protein export across the bacterial inner membrane is catalyzed by preprotein translocase, a multisubunit enzyme composed of integral and peripheral membrane domains (1). The essential or “core” subunits of this complex, identified by genetic and biochemical studies, are the membrane-embedded heterotrimer SecYEG and the peripheral membrane SecA protein, which binds to SecYEG on the cytoplasmic surface of the plasma membrane (2, 3). Translocation of preproteins *in vitro* has been achieved using these four subunits reconstituted into proteoliposomes, depends upon the energy of ATP hydrolysis by SecA, and is stimulated by the proton motive force across the membrane (4, 5).

The availability of the purified translocase components make it a particularly attractive system for mechanistic studies of protein translocation. Preproteins bearing an N-terminal leader sequence are held in a translocation-competent conformation by one of a number of different chaperone proteins, most notably the export-specific SecB chaperone (6). SecA, bound to high affinity sites comprised of SecYEG and acidic phospholipids (7, 8), in turn serves as a receptor for the preprotein-SecB complex through its affinity for both SecB and for the leader and mature domains of the precursor (9). Precursor binding induces the ATPase activity of SecA and initiates translocation (10).

Topological studies have revealed that SecA bound with high affinity to SecYEG undergoes repeated cycles of membrane

insertion and de-insertion concomitant with translocation (11). This is in agreement with observations that SecA is accessible to membrane impermeant reagents from the periplasmic side of the membrane (12). ATP binding to the first SecA nucleotide binding domain drives membrane insertion of a 30-kDa region of the SecA polypeptide. Hydrolysis of ATP at this site, as well as additional ATP binding and hydrolysis, results in deinsertion (13). ATP interactions at a proposed second nucleotide binding site may be necessary for the coupling of SecA membrane cycling to preprotein translocation. Significantly, a single round of ATP binding is sufficient for SecA membrane insertion (11, 13). This event is paralleled by the stepwise translocation of discrete lengths (20–30 aminoacyl residues) of preprotein (14, 15), consistent with the hypothesis that ATP-driven translocation is catalyzed by repeated cycles of SecA membrane insertion and de-insertion (11).

Despite recent advances in our understanding of the mechanistic role played by SecA, little is known of the structure-function relationships that promote ATP-driven protein translocation. We now report the identification of the membrane-inserting region of SecA and show that it is a pre-existing domain in the C-terminal third of the polypeptide. Limited proteolysis in solution suggests that the SecA monomer can be divided into two primary structural domains, a 65-kDa N-terminal ATPase domain and the 30-kDa membrane-inserting domain. Cleavage of SecA by limited proteolysis results in unregulated ATPase activity, suggesting that intramolecular constraints between these two regions may play a role in the regulation of ATP hydrolysis during translocation.

EXPERIMENTAL PROCEDURES

Materials—SecA (16), SecB (17, 18), and proOmpA (19) were purified according to previously described methods. Na¹²⁵I (approximately 15 mCi/mg) was purchased from Amersham Corp. Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) was from Pierce. Creatine kinase, creatine phosphate, and Pefabloc® were from Boehringer Mannheim. Arabinose, ATP, dithiothreitol (DTT)¹ and L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin, and soybean trypsin inhibitor were from Sigma. ¹⁴C-labeled molecular weight markers were from Life Technologies, Inc. SecA was iodinated as described previously (11).

Cell Growth and Preparation of Inverted Inner Membrane Vesicles—*Escherichia coli* strain BL21 containing plasmid pHasecEYG (20) was transformed with plasmid pGAP6 bearing the SecD/F operon with regulated expression driven by the Ara regulon.² All media and antibiotics were according to Sambrook *et al.* (30). Cultures were grown at 37 °C with aeration in LB medium containing 100 μ g/ml ampicillin and 50 μ g/ml chloramphenicol. Exponential cultures at $A_{600} = 0.200$ were induced with 1% (w/v) arabinose and growth continued to $A_{600} = 1.0$ – 1.3 . IMVs were prepared by the method of Chang *et al.* (31) as modified by Douville *et al.* (20). IMVs were then treated with 6 M urea (30 min, 0 °C) to inactivate endogenous SecA and remove other peripheral mem-

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¹ The abbreviations used are: DTT, dithiothreitol; IMV, inverted inner membrane vesicle; PAGE, polyacrylamide gel electrophoresis; NBD, nucleotide binding domain.

² F. Duong and W. Wickner, manuscript in preparation.

brane proteins, as described by Cunningham *et al.* (16).

SecA Membrane Insertion Reactions—*In vitro* SecA membrane insertion reactions were performed utilizing a modification of the method of Economou and Wickner (11). Reaction mixtures (1.0 ml) contained either [125 I]SecA (2×10^6 cpm) or unlabeled SecA (100 μ g/ml), 250 μ g/ml urea-washed IMVs, 120 μ g/ml proOmpA, 60 μ g/ml SecB, 2 mM ATP, 1 mM DTT, 5 mM creatine phosphate, and 1 μ g/ml creatine kinase in translocation buffer (50 mM HEPES-KOH, pH 8.0, 5 mM MgCl₂, 50 mM KCl). Insertion reactions were incubated for 15 min at 37 °C, followed by chilling on ice. One-tenth of a reaction containing radioactive SecA was then added to each of the reactions containing unlabeled SecA, and the mixtures were digested with 1 mg/ml trypsin for 15 min. Trypsinolysis was terminated by the addition of Pefabloc® (1 mg/ml). IMVs were then sedimented in a Beckman TLA 120 rotor at $200,000 \times g$ (15 min, 4 °C). The membrane pellets were resuspended in extraction buffer (50 mM Tris-HCl, pH 8.2, 6 M urea) and 1 mg/ml Pefabloc®. After incubation on ice for 30 min, the membranes were again sedimented, and the supernatants were analyzed by SDS-PAGE with silver-staining, autoradiography, and Western blotting with anti-SecA antibodies.

Purification and Sequencing of the SecA Membrane-inserting Domain—For preparative scale purification, 16 complete 1.0-ml SecA membrane insertion reactions were performed as described above. After treatment with 6 M urea, supernatants containing the extracted SecA fragment were pooled and chromatographed on a MonoQ fast protein liquid chromatography anion exchange column (Pharmacia Biotech Inc.) equilibrated with extraction buffer. The column was eluted with a 12 column volume gradient of 0–1.0 M NaCl in extraction buffer. Flow-through and elution fractions were concentrated by trichloroacetic acid precipitation (20) and analyzed by SDS-PAGE with silver-staining and autoradiography. N-terminal peptide sequencing was performed on an Applied Biosystems Model 477A instrument.

Translocation ATPase Assays—Translocation ATPase assays were performed as described (7) with minor modifications. Complete reactions contained 100 μ g/ml urea-washed IMVs, 50 μ g/ml proOmpA, 80 μ g/ml SecB, 20 μ g/ml soybean trypsin inhibitor, 4 mM ATP, and 1 mM DTT in translocation buffer (see above). SecA concentrations used were 10–80 μ g/ml (prior to tryptic cleavage).

Limited Trypsinolysis of SecA—SecA (2 mg/ml) in SecA Buffer (50 mM Tris-Cl pH 8.0, 50 mM KCl, 5 mM MgCl₂, 10% glycerol) was incubated with increasing amounts (final concentration, 0–200 μ g/ml) of trypsin (15 min, 0 °C). Proteolysis was stopped by the addition of soybean trypsin inhibitor (2 mg/ml).

Other Methods—Silver staining of polyacrylamide gels was according to the method of Blum *et al.* (21). Protein concentrations were determined using Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. SDS-PAGE (15% polyacrylamide), electrophoretic transfer of proteins to polyvinylidene difluoride membranes, and Western blot were performed as described previously (22, 23). Anti-SecA antibody was purified from serum by affinity chromatography on Affi-Gel (Bio-Rad) with covalently linked SecA.

RESULTS

Previous work has described an *in vitro* assay for the insertion of SecA into inverted IMVs. Insertion is dependent upon ATP, preprotein, physiological temperature, and the SecYEG integral membrane domain of translocase (11). Membrane insertion of a 30-kDa region of SecA is monitored by inaccessibility to added protease. In the present study, similar reactions were performed on a preparative scale for the purification and identification of the 30-kDa membrane-inserted SecA fragment. The yield of membrane-inserted SecA was maximized through the use of IMVs derived from *E. coli* cells overexpressing the SecYEG and SecD/F components of translocase (13, 20).² SecA insertion reactions containing either [125 I]-labeled SecA as a tracer or 100 μ g/ml unlabeled SecA were incubated separately to allow maximization of both the tracer signal and the chemical yield of membrane-inserted SecA. Aliquots of a complete reaction containing [125 I]SecA were then mixed with reactions containing unlabeled SecA in order to monitor purification of the SecA 30-kDa fragment. The mixed reactions were digested with trypsin, and membranes were sedimented by ultracentrifugation and resuspended in buffer containing 6 M urea to extract the inserted SecA fragment from the membrane. After re-sedimentation, analysis of the urea superna-

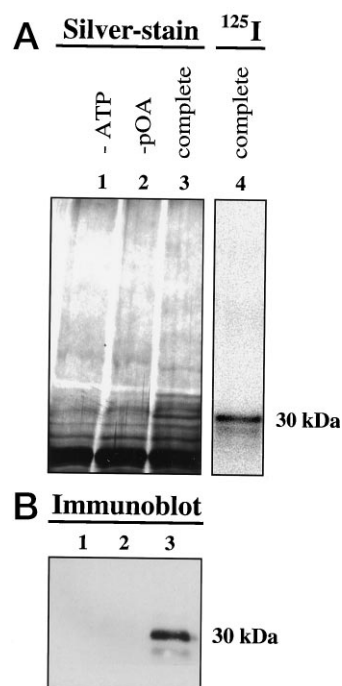


FIG. 1. Visualization of the membrane-protected SecA 30-kDa fragment. SecA membrane insertion reactions were prepared as described under “Experimental Procedures.” After a 37 °C incubation and chilling on ice, 100 μ l of a complete reaction containing [125 I]-labeled SecA was added to control reactions lacking either ATP (lanes 1) or the preprotein proOmpA (lanes 2) and to a complete reaction (lanes 3), all containing 100 μ g/ml unlabeled SecA. After proteolysis, urea extracts were prepared as described and analyzed by SDS-PAGE with silver staining (A), and by Western blotting with affinity-purified anti-SecA antibody (B). Lane 4 shows the autoradiogram of the complete reaction with the [125 I]-labeled SecA 30-kDa fragment indicated.

nts by SDS-PAGE with silver staining reveals a band of 30-kDa present in extracts of the complete reaction (Fig. 1A, lane 3), which corresponds in molecular weight to the radioactive 30-kDa band detected by autoradiography of the same gel (Fig. 1A, lane 4). The silver-stained 30-kDa band is absent in the control reactions lacking either ATP (Fig. 1A, lane 1) or the preprotein proOmpA (Fig. 1A, lane 2), indicating that the generation of this fragment is dependent on the necessary components of translocation and is likely the chemical equivalent of the radioactive 30-kDa fragment (Fig. 1, lane 4) described previously (11). Similar results were seen upon Western blotting the same urea supernatants with anti-SecA antibody (Fig. 1B, lanes 1–3), confirming the identity of the 30-kDa band seen by silver staining and its dependence on complete translocation conditions. The SecA 30-kDa fragment was further purified by MonoQ anion exchange chromatography (Fig. 2A). The prominent radioactive elution peak in the middle of the salt gradient corresponds to the appearance of the 30-kDa fragment detected by autoradiography (Fig. 2B). This band in turn corresponds exactly both in molecular weight and in intensity to the 30-kDa band seen by silver staining of the same gel (Fig. 2, B and C), further confirming that it is the chemical equivalent of the radioactive 30-kDa species. The urea extracts from translocation reactions lacking either ATP or preprotein were also chromatographed on the same Mono Q column and, as expected, no 30-kDa SecA fragment was recovered (data not shown).

Peak fractions containing the 30-kDa SecA fragment were pooled, electrophoresed by SDS-PAGE, and electrotransferred to a polyvinylidene difluoride membrane. After staining with Coomassie Brilliant Blue and autoradiography (not shown), the stained band corresponding to the radioactive 30-kDa band was excised and subjected to N-terminal sequencing. This se-

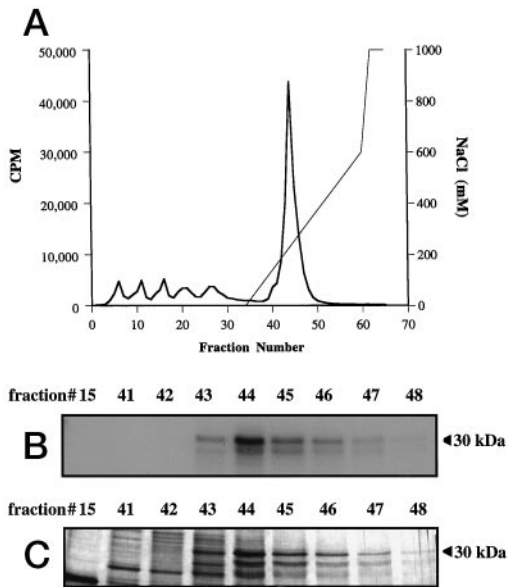


FIG. 2. Purification of the membrane-protected SecA 30-kDa fragment. A, MonoQ chromatography. Complete 1.0-ml SecA membrane insertion reactions were performed as described under "Experimental Procedures" and in the legend to Fig. 1. Urea supernatants containing the SecA 30-kDa fragment were pooled, loaded on a Pharmacia MonoQ column, and eluted with a gradient of 0–1 M NaCl. Eluent fractions were assayed by γ counting. Flow-through and elution peak fractions were then analyzed by SDS-PAGE. B, SDS-PAGE autoradiogram of flow-thru and elution peak fractions. C, the same gel as in B with protein bands visualized by silver staining.

sequence analysis (17 cycles) revealed that the 30-kDa polypeptide is derived from the SecA protein beginning at aminoacyl residue 610 (see Fig. 4B; Ref. 24). Hypothetical extension of this sequence to a molecular mass of 30 kDa would result in termination at approximately amino acid position 870, 31 residues before the C terminus of the full-length SecA protein. In support of this estimation, amino acid analysis of the purified 30-kDa band indicated that no cysteines are present in this SecA fragment (data not shown), although SecA contains cysteinyl residues at positions 98, 885, 887, and 896 (24). Furthermore, an antiserum raised against residues 875–890, capable of immunoprecipitating full-length SecA, was unable to precipitate the 30-kDa fragment.³ These analyses indicate that the C terminus of SecA has been removed by the protease treatment to at least aminoacyl residue 884. Note that a less prominent band of approximately 27–28 kDa, which is seen in some but not all SecA insertion reactions, co-elutes with the 30-kDa fragment (Fig. 2, B and C). N-terminal sequencing of this fragment (eight cycles) revealed a sequence beginning at aminoacyl residue 658 of the SecA protein (data not shown), consistent with the notion that this is a degradation product of the major 30-kDa SecA species.

We have previously reported that limited proteolysis of SecA, in the absence of membranes or other ligands, can also yield a 30-kDa fragment (13). The yield of this fragment was greatly reduced in the presence of normal translocation ligands of SecA, suggesting that formation of this fragment was a consequence of the SecA protein conformation rather than arising from proteolytic specificity. However, it was unclear whether this 30-kDa fragment derived from SecA in solution represented the same 30-kDa domain that inserts into the membrane. To further investigate this possibility, SecA was incubated with increasing concentrations of trypsin in the absence of any translocation components. This treatment generated a

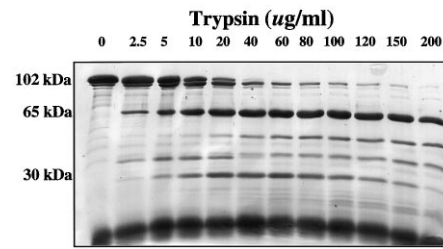


FIG. 3. Limited trypsinolysis of SecA produces two primary fragments. SecA was digested with increasing concentrations of trypsin, as described under "Experimental Procedures." After a 15-min incubation on ice, proteolysis was stopped by the addition of soybean trypsin inhibitor. Samples (25 μ g of each SecA) were analyzed by SDS-PAGE with Coomassie staining. The apparent molecular masses of full-length SecA (102 kDa) and of the two major fragments (65 and 30 kDa) are indicated.

30-kDa fragment of SecA that appeared at a low trypsin concentration (2.5 μ g/ml) and was resistant to proteolysis at up to 100 μ g/ml trypsin but was degraded at higher concentrations (Fig. 3). Strikingly, the N-terminal sequence of this fragment and that of the membrane-inserted 30-kDa fragment are identical (see Fig. 4B). This result, together with the relative resistance of this fragment to further degradation, indicates that the membrane-inserting region of SecA exists as a previously folded domain of the protein. Note that the trypsin concentrations required to generate this fragment in solution (2.5–100 μ g/ml) are relatively low and that higher concentrations, such as the 1 mg/ml trypsin used to define inaccessibility of the membrane-inserted state of the 30-kDa SecA domain, result in complete degradation. Therefore, the protease inaccessibility of the membrane-inserted domain is distinct from formation of the 30-kDa fragment by limited proteolysis, which appears to be a consequence of the folded structure of the SecA protein.

Limited trypsinolysis of SecA also yielded a prominent 65-kDa fragment at the same protease concentrations that produced the 30-kDa fragment. Sequencing indicates that the N terminus of this fragment begins at the fifth aminoacyl residue of the SecA protein (Fig. 4A; Ref. 24). Hypothetical extension of this sequence for 65 kDa would result in its approximate termination at aminoacyl residue 570. This fragment thus contains all of the first nucleotide binding domain of SecA (NBD-1) as well as some or all of the proposed NBD-2 (Fig. 4; Ref. 32).

SecA that had been subjected to limited trypsinolysis was unable to support preprotein translocation or membrane insertion (data not shown). Interestingly, however, this treatment of SecA resulted in unregulated ATP hydrolysis. To investigate this phenomenon, SecA was cleaved in solution with trypsin at a final concentration of 60 μ g/ml (see "Experimental Procedures") to yield the 65- and 30-kDa fragments as the predominant species, with little full-length SecA remaining (Fig. 5A). As a control for the presence of trypsin and trypsin inhibitor in the ATPase reaction mixture, noncleaved SecA was prepared by the addition of trypsin that had first been inactivated with the inhibitor, resulting in no cleavage (Fig. 5A). Noncleaved SecA alone in solution has a very low level of ATP hydrolysis (Ref. 7; Fig. 5B, *open circles*). The addition of membranes and preprotein substrate to form a complete translocation reaction results in a high level ATPase activity referred to as "translocation ATPase" (Ref. 10; Fig. 5B, *closed circles*). In the absence of membranes and preprotein, the rate of ATP hydrolysis by trypsin-cleaved SecA, although substantially lower than the complete reaction containing uncleaved SecA, was approximately 18-fold higher than that of noncleaved SecA under the same conditions (Fig. 5B, *open squares*). However, the addition of membranes and preprotein to cleaved SecA resulted in only a modest (2-fold) stimulation of ATP hydrolysis (Fig. 5, *closed*

³ J. Eichler, personal communication.

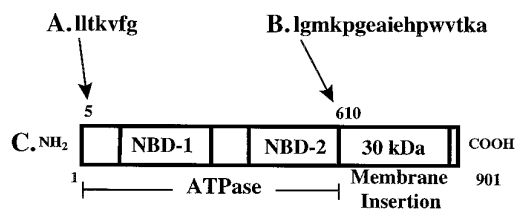


FIG. 4. The SecA monomer contains two primary structural and functional domains. Peptide sequences obtained by N-terminal Edman degradation that identify the SecA 65 kDa tryptic fragment (A) and the SecA 30-kDa fragment seen both by protease inaccessibility during translocation and by limited trypsinolysis (B). C, proposed domain organization of the SecA monomer. Other possible membrane-inserting regions and important functional sites, such as for SecB and preprotein binding, are not shown.

squares), in contrast to the ATPase of uncleaved SecA under complete translocation conditions.

DISCUSSION

Membrane insertion of the 30-kDa domain of SecA was proposed to be directly responsible for ATP-dependent forward movement of preprotein through the membrane (11). We have discovered that the membrane-inserting domain of SecA lies in the C-terminal third of the protein, beginning at aminoacyl residue 610. This and other studies raise a number of interesting questions regarding the structure and function of the SecA ATPase. Of central importance is the mechanism by which the energy released by ATP binding and hydrolysis is converted into the mechanical force that drives membrane insertion. The membrane-inserting domain is proximal to the C-terminal end of the proposed NBD-2, with the two possibly overlapping to a certain extent (Fig. 4; Ref. 32). However, membrane insertion is driven by ATP binding at NBD-1 (13, 33), which is located on the N-terminal side of NBD-2 relative to the membrane-inserting domain (Fig. 4). Although no detailed structural data are yet available concerning the spatial relationships between folded regions of SecA, specific intramolecular interactions between these regions are likely to be relevant to the mechanics of membrane insertion.

What is the environment surrounding the SecA membrane-inserting domain during translocation? The membrane-inserting domain is strikingly devoid of hydrophobic stretches (24). Therefore, this region may be protected from the lipid phase during membrane insertion by the SecYEG subunits of translocase. Alternately, because SecA can bind and partially insert into lipid (7, 34), the membrane-inserting domain may undergo an unusual conformational change to allow for exposure to the lipid phase (27). Both of these possibilities carry significant implications for the route taken by the preprotein through the membrane. Furthermore, our studies do not exclude the possibility that other regions of the SecA polypeptide in addition to the 30-kDa domain may insert into the membrane. Such regions may have escaped detection in our assay system due to very small size, poor stability after proteolysis, or uneven radiolabeling.

We propose from this study that the SecA monomer contains two primary structural domains. Limited trypsinolysis of SecA yields two major fragments (Fig. 3), one of which corresponds to the 30-kDa membrane-inserting domain, the other to a 65-kDa fragment that encompasses all of NBD-1 and some or all of the proposed NBD-2 (Fig. 4; Ref. 32). We observed previously that the 30-kDa domain of SecA becomes more susceptible to proteolysis upon interaction with membranes and preprotein in the absence of translocation (13). This indicates that the formation of these major tryptic fragments is a result of the protein conformation of SecA and not of factors inherent in its amino acid sequence. The relative trypsin resistance of these

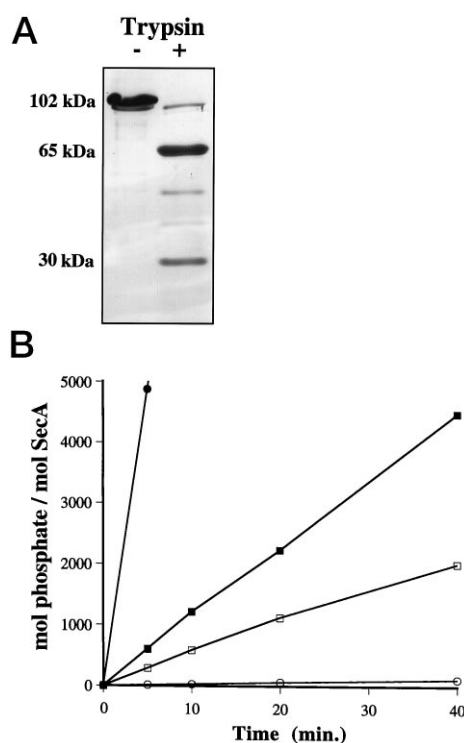


FIG. 5. Tryptic cleavage of SecA results in unregulated ATPase activity. A, SecA was treated with 60 µg/ml trypsin as described in the legend to Fig. 3 to produce the 65- and 30-kDa fragments as the primary cleavage products. B, translocation ATPase. Translocation ATPase assays were performed as described under "Experimental Procedures." *Open circles*, uncleaved SecA minus IMVs and proOmpA. *Closed circles*, uncleaved SecA plus IMVs and proOmpA. For unity of scale, only the first time point is shown for the latter reaction. Note, however, that the reaction is linear to the 20-min time point (not shown). *Open squares*, cleaved SecA minus IMVs and proOmpA. *Closed squares*, cleaved SecA plus IMVs and proOmpA.

two fragments indicates that they are independently folded domains of SecA, and the extreme protease sensitivity of the junction between them suggests that they are separated by a flexible hinge or loop region.

Finally, we have found that limited trypsinolysis of SecA in solution to yield the 65-kDa ATPase and 30-kDa membrane-inserting domains results in unregulated ATPase activity in the absence of normal translocation ligands (Fig. 5). Indeed, previous studies utilizing truncated SecA have demonstrated a role for the extreme C-terminal region in regulating ATPase activity (25, 26). Whereas tryptic cleavage of SecA results in a significant activation of ATP hydrolysis by SecA, the addition of membranes and preprotein to cleaved SecA results in only a modest (approximately 2-fold) additional stimulation and does not approach the level of the complete reaction containing uncleaved SecA (Fig. 5, *open circles*). This weak additional stimulation of ATPase activity by the normal translocation ligands could be due to the small amount of uncleaved SecA remaining after trypsinolysis (Fig. 5A). Therefore, it appears that limited trypsinolysis results in both activation of ATP hydrolysis and insensitivity to further activation by normal translocation ligands. We suggest that this activity may be due to the relief of intramolecular constraints normally present between the two primary domains of the SecA monomer. We further speculate that the relief of these constraints by limited trypsinolysis may be functionally similar to changes in the conformation of SecA imposed by the binding of preprotein, SecYEG, and acidic phospholipids. Thus, the present findings may offer new insights into how these ligands activate the SecA ATPase during translocation.

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