Allosteric Regulation of SecA

MAGNESIUM-MEDIATED CONTROL OF CONFORMATION AND ACTIVITY

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In bacteria, the SecA protein associates with a ubiquitous protein channel SecYEG where it drives the post-translational secretion of pre-proteins across the plasma membrane. The high-resolution structures of both proteins have been determined in their resting states; however, the mechanism that couples ATP hydrolysis to active transport of substrate proteins through the membrane is not well understood. An analysis of the steady-state ATPase activity of the enzyme reveals that there is an allosteric binding site for magnesium distinct from that associated with hydrolysis of ATP. We have demonstrated that this regulation involves a large conformational change to the SecA dimer, which exerts a strong influence on the turnover and affinity for ATP, as well as the affinity for ADP. The strong inhibitory influence of magnesium on the ATPase activity can be countered by cardiolipin and conditions that promote protein translocation.

Chemo-mechanical protein machines have evolved to couple energy derived from chemical reactions to mechanical motion. They have been exploited to drive a number of processes that are central to cell biology, such as protein translocation.

Membrane and secretory proteins are targeted to specific membranes before they pass through or into the bilayer. A ubiquitous protein channel is found in the plasma membrane of archaea and bacteria, and also in the endoplasmic reticulum of eukaryotes. Co-translational and post-translational targeting systems converge on the Sec complex whereupon the unfolded protein substrate is driven through the membrane (1). The structure of the protein channel has been determined in its resting state to high resolution (2, 3), and its active and membrane-bound state is most likely a dimer (3–7).

In bacteria, post-translational translocation of secretory proteins requires that they are maintained in an unfolded conformation, before they are passed on to a soluble ATPase SecA (8). Upon receiving the substrate, SecA associates with the protein-conducting channel, the SecYEG complex, where it harnesses the energy from ATP binding and hydrolysis to actively push it across the membrane (9–11). A model has been proposed for a multistep reaction for the transport of proteins across the membrane (12), regulated by distinct ATP binding and hydrolysis events, translocating ~25 amino acids (2.5 kDa) in each step (13). However, another study estimates that 5 ATP molecules are required to send each amino acid across the membrane (14).

Unliganded SecA exists in solution in a monomer-dimer equilibrium (15, 16), and is predominantly dimeric (16). However, the precise oligomeric state of SecA that binds to the protein channel and drives the translocation reaction has formed the subject of conflicting accounts. Several studies reported that a dimer with two active subunits was required for active translocation (17) and in support, a later study demonstrated that covalently cross-linked dimers were able to sustain ATP hydrolysis and protein translocation (18, 19). Other investigators showed that acidic phospholipids, detergent, signal peptide, and SecYEG apparently bring about a dissociation of the dimer (20). Following on from this, a mutant of SecA (∆11/N95) was constructed with truncations to the N and C termini (∆2–11 and ∆832–901), which was shown not to form inter-subunit cross-links, and to sediment through sucrose cushions as if it were a monomer (21). An analysis of complexes formed between SecYEG and SecA by analytical ultracentrifugation showed that in the absence of nucleotide one SecA bound to the channel, whereas two bound in the presence of the ATP analogue AMPPPNP2 (7).

Several different structures of SecA have now been determined, five dimers (22–26) and one monomer (27). Four of the dimers revealed the SecA protomers packed head-to-tail in the crystal lattice, all in different antiparallel conformations (22–24, 26), another has a parallel arrangement of the subunits (25). These differences obviously add further confusion to our picture of the nature of the oligomeric state and the conformation of the active form of the protein. The monomer structure, and subsequently the latest dimer were found to exhibit a large domain movement, exposing a cleft that has been proposed to form a polypeptide binding site (26, 27). Interestingly, the conformation of the nucleotide binding fold does not seem to be affected by the presence of bound nucleotide (22–24, 26, 27).

The ATPase activity of SecA has to be regulated in some fashion, to prevent futile hydrolysis of ATP during moments of rest. The basal ATPase activity of SecA is partially increased by the presence of acidic phospholipids and then fully stimulated by addition of vesicles containing SecYEG and precursor protein (28, 29). The mechanism for this regulation is not clear.

* The on-line version of this article (available at http://www.jbc.org) contains supplemental data and equations.

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2 The abbreviations used are: AMPPPNP, adenosine 5′-[(β,γ-methylene)-triphosphate; C12E9, polyoxyethylene(9)dodecyl ether; ATP-S, adenosine 5′-O-(thiotriphosphate).

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There have been several observations with respect to magnesium and its effects on the regulation of SecA, which have not been adequately explained. Removal of the cation from a binding site, distinct from the nucleotide binding folds, can result in a 10-fold stimulation of ADP release (30). The activity of SecA and the elevated activity found in a complex of SecA and SecYEG were both stimulated by the removal of Mg$^{2+}$, which also served to stabilize the latter complex in the presence of ATP (or ATPγS) (5). Mg$^{2+}$ has also been shown to modulate fluorescence anisotropy measurements, as well as the fluorescence observed from intrinsic and extrinsic probes of the enzyme (27, 31, 32).

Despite the fact that the structures of both SecA and SecYEG have been determined, we are still a long way from understanding their concerted mechanism of action. To reach this point, we need to understand the nature and timing of the conformational changes within SecA, and how they are affected by the ATPase cycle. In the light of the conflicting and fragmented accounts of the reaction cycle, we decided to conduct a thorough analysis of the kinetics of the protein translocation process. As a first approach to this characterization we have performed a steady-state analysis of SecA in isolation, together with a study of its oligomeric state by equilibrium and velocity sedimentation using analytical centrifugation. An analysis of the wild-type and proposed monomeric mutant SecA-Δ11/N95 (21) in this way generated some new findings on the oligomeric state of SecA, its ATPase activity, and the influence of magnesium and lipids.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Biochemicals—Escherichia coli** polar lipid and *E. coli* cardiolipin were purchased from Avanti, and were prepared at 10 mg/ml in 50 mM triethanolamine, pH 7.5, 50 mM KCl. Polyoxyethylene(9)dodecyl ether (C12E9) was purchased from Sigma and its effects on the regulation of SecA, which have not been determined, we are still a long way from understanding the nature and timing of the conformational changes within SecA, and how they are affected by the ATPase cycle. In the light of the conflicting and fragmented accounts of the reaction cycle, we decided to conduct a thorough analysis of the kinetics of the protein translocation process. As a first approach to this characterization we have performed a steady-state analysis of SecA in isolation, together with a study of its oligomeric state by equilibrium and velocity sedimentation using analytical centrifugation. An analysis of the wild-type and proposed monomeric mutant SecA-Δ11/N95 (21) in this way generated some new findings on the oligomeric state of SecA, its ATPase activity, and the influence of magnesium and lipids.

**Kinetic Analysis of SecA**

**Pyruvate Kinase/Lactate Dehydrogenase Assay**—Standard assay components were 1000 units/ml lactate dehydrogenase, 700 units/ml pyruvate kinase, 2 mM phosphoenolpyruvate, and 0.2 mM NADH in TKM buffer (50 mM triethanolamine, pH 7.5, 50 mM KCl, 2 mM MgCl$_2$). 0.3 μM SecA or SecA-Δ11/N95 (monomer concentration) was used in each reaction, and the assays were initiated by addition of ATP.

**EnzChek Assay**—The standard assay components were 0.2 mM 2-amino-6-mercapto-7-methylpurine riboside and 1 unit of purine nucleoside phosphorylase in TK buffer (50 mM triethanolamine, pH 7.5, 50 mM KCl). 0.3 μM SecA or SecA-Δ11/N95 was used (monomer concentration) in each reaction, and ADP, Mg$^{2+}$, and *E. coli* lipids were added to the concentrations indicated in the text, prior to the initiation of the reaction by addition of ATP.

**Determination of Residual Magnesium Concentration**—The amount of Mg$^{2+}$ contained within the standard assay conditions (TK buffer) was calculated using inductively coupled plasma-atomic emission spectroscopy, using a Jobin Yvon Horiba Ultima 2 sequential spectrometer fitted with a Bergher Mira Mist Nebulizer and automated with a Jobin Yvon AS421 autoSampler.

Curve fitting and analysis for all kinetic data, including that of the global fits is shown under supplemental materials.

**Analytical Ultracentrifugation**—SecA stock solutions of wild-type and SecA-Δ11/N95 were dialyzed extensively against either TK or TKM buffers. These were subjected to sedimentation velocity and sedimentation equilibrium centrifugation experiments using either an XL-A or XL-1 centrifuge (Beckman Instruments) equipped with standard Epon centerpieces and an An-60Ti rotor. SecA or SecA-Δ11/N95 (0.4–13 μM) were used and scans were recorded at either 230 or 280 nm. Sedimentation velocity experiments were carried out at 25 °C and 130,000 × g. The scans were fitted to a continuous c(s) model, using Ultrascan (34). The data were then extrapolated to zero concentration to yield $g_{s0,w}^0$. Equilibrium scans were taken at 16 and 20 h, at speeds of 3,000, 5,800, and 8,000 × g at 25 °C. The data were analyzed using a variety of models, the data shown are that of a 1-component ideal species model, which yielded the most appropriate fit using Ultrascan (34). The data were then subjected to a Monte Carlo analysis, to reveal the statistical values for all parameters.

**In Vitro Translocation Assays**—SecYEG was reconstituted into *E. coli* polar lipid proteoliposomes as described previously (35). Translocation of proOmpA into SecYEG proteoliposomes was assayed essentially as described (36), except that protease-protected proOmpA was detected by Western blot using an antibody raised against proOmpA.
RESULTS

SecA Activity Is Affected Dramatically by Magnesium Acting at a Location Distinct from the Hydrolytic Site—Steady-state ATPase assays were used in a range of conditions to characterize the kinetics of ATP turnover by both wild-type SecA and the proposed monomeric mutant SecA-Δ111/N95 (21). In 2 mM Mg\(^{2+}\), ATP binding is tight and substrate turnover is slow (Fig. 1A, Table 1). However, when no Mg\(^{2+}\) was included in the buffer (thus in the presence of only residual magnesium), the behavior of SecA is dramatically altered, the affinity for ATP being weakened by ~150-fold, and the turnover increased by a factor of 30 (Fig. 1B, Table 1); both wild-type and the SecA-Δ111/N95 behave essentially in an identical manner.

The residual magnesium concentration in the assay buffer was calculated using inductively coupled plasma-atomic emission spectroscopy to be 1 μM, ~3 times greater than the SecA concentration in the experiment (0.3 μM). Unsurprisingly, addition of EDTA to the reaction inhibited the activity completely (data not shown). Characteristic of all ATPases, SecA uses Mg\(^{2+}\) as a cofactor coordinating the γ-phosphate of ATP required for hydrolysis.

Accordingly, the apparent inhibitory effect of Mg\(^{2+}\) was examined in more detail and it was shown to be relatively unaffected by a 10-fold increase in the concentration of ATP (Fig. 2, Table 2). The affinity of Mg\(^{2+}\) for ATP is relatively high (\(K_d\) [Mg\(^{2+}\)-ATP] = 20 μM) (37), therefore the fact that the inhibition is not strongly coupled to the concentration of ATP indicates that the inhibition occurs via Mg\(^{2+}\), and not by Mg\(^{2+}\)-ATP. In other words, it is acting at a location distinct from the nucleotide binding site and the effect is allosteric. Moreover, it can be deduced that Mg\(^{2+}\)-ATP is not the substrate for SecA, as the Mg\(^{2+}\) required for catalysis must be always bound at the NBD, as hydrolysis can occur when ATP is in great excess over Mg\(^{2+}\) (1 mM ATP to 1 μM Mg\(^{2+}\), Fig. 1B). The actual binding affinity for Mg\(^{2+}\) at this second allosteric site must be much higher than the affinity Mg\(^{2+}\) has for ATP, given the fact that it remains inhibitory when ATP is more than 500 times in excess (Fig. 1B).

The data were fitted according to a global fit, which incorporates a matrix of Mg\(^{2+}\) and ATP concentrations and the respective steady state rate kinetics (Fig. 2B and supplemental materials). This procedure made an adjustment for the chelating effect of ATP on Mg\(^{2+}\) and also incorporated the data for both weak and tight binding of ATP with residual and 2 mM Mg\(^{2+}\), respectively. The fit matched the data well, and a simple model has been proposed to explain the allosteric influence of Mg\(^{2+}\) on the reaction cycle of SecA (Fig. 3).

The Competitive Inhibition of SecA by ADP Is Affected by Magnesium—The effects of product inhibition were studied both in the presence and absence of Mg\(^{2+}\) (Fig. 4 and Table 3). ADP exhibited a classic competitive inhibition effect with residual Mg\(^{2+}\); there was a 10-fold increase in \(K_{(app)}\) at a 10-fold higher ATP concentration (Table 3). The affinity of SecA for ADP, given by \(K_d\) [ADP], was calculated at different concentrations of ATP and Mg\(^{2+}\), taking into account the increased affinity for ATP at the higher Mg\(^{2+}\) concentration (see supplemental materials and Table 3). As with affinity of SecA for ATP, in the presence of 2 mM Mg\(^{2+}\) the affinity for ADP was greatly increased, by ~200-fold (Tables 1 and 3).

The Inhibitory Effect of Magnesium Can Be Alleviated by Cardiolipin—Preliminary experiments indicated that cardiolipin binds to SecYEG and stabilizes the dimeric conformation,

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**TABLE 1**

Calculated values of the \(K_m\) [ATP] and \(V_{max}\) for wild-type SecA and SecA-Δ111/N95 with and without added 2 mM Mg\(^{2+}\).

|                  | \(K_m\) [ATP] + Mg\(^{2+}\) | \(V_{max}\) + Mg\(^{2+}\) | \(K_m\) [ATP] − Mg\(^{2+}\) | \(V_{max}\) − Mg\(^{2+}\) |
|------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| SecA             | 0.32 ± 0.03                 | 0.56 ± 0.01                 | 50.8 ± 3.2                  | 22.36 ± 0.28                |
| SecA - Δ111/N95  | 0.40 ± 0.03                 | 0.96 ± 0.02                 | 47.7 ± 3.7                  | 30.16 ± 0.47                |
Kinetic Analysis of SecA

FIGURE 2. Inhibition of ATP turnover by Mg

A

mM Mg

B

mM Mg

FIGURE 3. The SecA binding cycle for ATP substrate and the non-catalytic regulatory Mg

TABLE 2

Calculated values for the apparent $K_{\text{app}}(\text{Mg}^{2+})$ of the ATPase reaction in the presence of 1 mM and 100 mM ATP

The data were collected according to Fig. 2A, and fitted to Equation 1 as described under supplemental materials. S.E. from the fitting procedure is shown.

| $K_{\text{app}}(\text{Mg}^{2+}, 1 \text{ mM ATP})$ | $K_{\text{app}}(\text{Mg}^{2+}, 100 \text{ mM ATP})$ |
|---------------------------------|---------------------------------|
| SecA                            | 

which is proposed to be the active form. Cardiolipin is also known to be a chelator of divalent cations (38). In addition, acidic phospholipids have been shown to be important for protein translocation (39). Therefore, we reasoned that the influence of cardiolipin on protein translocation might be an indirect effect mediated by Mg$^{2+}$, and decided to determine the effect of cardiolipin on SecA activity. The experiment was conducted with and without added 2 mM Mg$^{2+}$; the detergent C$_{12}$E$_{9}$ was used to solubilize the lipid, and was shown to change neither the affinity of SecA for ATP nor the turnover rate (Table

TABLE 3

Calculated values for the $K_{\text{app}}(\text{ADP})$ determined with different concentrations of Mg$^{2+}$ and ATP

The data were collected according to Fig. 4, and fitted to Equation 4 as described under supplemental materials. Actual values for $K_{\text{app}}(\text{ADP})$, taking into account the altered $K_{d}(\text{ATP})$ at high concentrations of Mg$^{2+}$, were calculated by fitting the data in Fig. 4 to Equation 4 as described under supplemental materials. S.E. from the fitting procedure is shown.

| $K_{\text{app}}(\text{ADP})$ | $K_{d}(\text{ATP})$ |
|--------------------------|--------------------------|
| $-\text{Mg}^{2+}, 1 \text{ mM ATP}$ | $0.54 \pm 0.06$ | $26.25 \pm 3.09$ |
| $-\text{Mg}^{2+}, 100 \text{ mM ATP}$ | $0.051 \pm 0.007$ | $17.08 \pm 2.21$ |
| $+\text{Mg}^{2+}, 100 \text{ mM ATP}$ | $0.023 \pm 0.001$ | $0.1 \pm 0.004$ |
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4). In low concentrations of Mg$^{2+}$, cardiolipin was found to inhibit the ATPase activity (Fig. 5A), presumably due to chelation of the Mg$^{2+}$ required for the hydrolysis of ATP. At higher concentrations of Mg$^{2+}$ (100 μM) there was a striking increase in activity peaking at ~2.4 μM cardiolipin, followed by a gradual reduction (Fig. 5A), indicating that the effect cannot be simply explained by the chelation of Mg$^{2+}$. The effects of increasing cardiolipin in a yet higher concentration of Mg$^{2+}$ resulted in a less pronounced rise and fall of ATPase activity. The inhibitory phase of cardiolipin in the presence of added Mg$^{2+}$ may have been the result of the aggregation of SecA-lipid complexes, because it cannot be the result of Mg$^{2+}$ chelation at this high a concentration.

Next, we determined the $K_m$ value for ATP binding with high (2 mM) Mg$^{2+}$ and high (36 μM) cardiolipin (asterisk in Fig. 5A and B, and Table 4); both the $K_m$ and $V_{max}$ were characteristic of conditions having only a low concentration of Mg$^{2+}$. Therefore, we can infer from this data that cardiolipin counteracts the inhibition caused by Mg$^{2+}$. Total E. coli polar lipids had a less dramatic effect (Fig. 5C). An ~10-fold higher concentration was required to reach an equivalent enhancement. Therefore, we assumed that this was a result of the cardiolipin (9.8%, w/w) contained in this fraction.

Magnesium Alters the Conformation of the SecA Dimer, but Not the Oligomeric State—To determine whether the observed change in activity was affected by a change in the oligomeric state of SecA, sedimentation equilibrium and sedimentation velocity experiments were carried out by analytical ultracentrifugation. Sedimentation equilibrium revealed unambiguously that SecA is a dimer within a 0.4–13 μM concentration range (Fig. 6 and Table 5), and also in the presence of 2 mM EDTA (data not shown). Rather surprisingly, SecA-Δ11/N95, previously reported to be a monomer (21), was found to be a dimer as well. In both cases, the monomers were in such rarity that they could not be detected with sufficient accuracy to determine a dissociation constant, an indication that this value must therefore be in the low nanomolar range.

Subsequently, sedimentation velocity also revealed that there is essentially only a dimeric species present (Fig. 7 and Table 6). However, it could be shown for SecA and SecA-Δ11/N95 that the sedimentation properties, and hence shape, were sensitive to Mg$^{2+}$. An increase of 0.54 (SecA) and 0.11 (SecA-Δ11/N95) $s_{20,w}$ units (the sedimentation coefficient $S$, expressed in terms of a water solvent at 20 °C and extrapolated to zero concentra-

### Table 4

| Concentration | $K_m$ (μM) | $V_{max}$ (mol ATP hydrolyzed mol SecA$^{-1}$ min$^{-1}$) |
|---------------|------------|------------------------------------------------------|
| +Mg$^{2+}$    | 0.32 ± 0.03| 0.56 ± 0.01                                           |
| -Mg$^{2+}$    | 50.8 ± 3.2 | 22.36 ± 0.28                                          |
| +Mg$^{2+}$; 36 μM cardiolipin | 10.4 ± 1.0 | 11.33 ± 0.26                                          |
| -Mg$^{2+}$; 0.64% C$_{12}$E$_9$ (control) | 61.3 ± 6.4 | 18.07 ± 0.40                                          |

FIGURE 5. Cardiolipin relieves Mg$^{2+}$ inhibition through the formation of a ternary SecA-Mg$^{2+}$-cardiolipin complex. A, the ATPase activity of purified wild-type SecA was measured in the presence of 100 μM ATP in TK buffer with residual Mg$^{2+}$ (open circles, solid line), 100 μM Mg$^{2+}$ (filled triangles, dashed line), or 2 mM Mg$^{2+}$ (filled circles, dotted line), with increasing concentrations of cardiolipin. B, the ATPase activity of purified wild-type SecA was measured with 36 μM C$_{12}$E$_9$-solubilized cardiolipin in TKM buffer (asterisk in A), over a range of ATP concentrations to determine the Michaelis-Menten parameters. Calculated values of $K_m$ and $V_{max}$ are shown in Table 4. C, the ATPase activity of wild-type SecA was measured in the presence 100 μM ATP and 100 μM Mg$^{2+}$ with increasing concentrations of cardiolipin and total E. coli polar lipids, which contain 9.8% cardiolipin. Error bars represent S.D. from four to six replicates.
tion) was observed in the presence of 2 mM Mg$^{2+}$, compared with residual Mg$^{2+}$, indicative of a more extended shape.

The Influence of Magnesium Is Countered by Conditions That Promote Protein Translocation—The protein translocation reaction driven by wild-type SecA and SecA-$\Delta$11/N95 through SecYEG were compared and the mutant was found to have a significant proportion of the wild type activity. The amount of proOmpA translocated by wild type and by $\Delta$11/N95 is approximately the same after 5 and 15 min, respectively (Fig. 8A). Mg$^{2+}$ was titrated into the protein translocation assay and its effects monitored (Fig. 8B). There is a difference in the dependence of Mg$^{2+}$ in translocation compared with the ATPase activity of isolated SecA, indicating that conditions that promote translocation also alleviate the inhibitory effect of Mg$^{2+}$.

**TABLE 5**
Molecular weight determined by equilibrium velocity centrifugation for wild-type SecA and SecA-$\Delta$11/N95 with and without added 2 mM Mg$^{2+}$

The data were collected according to Fig. 6, and fitted to a 1-component ideal species model using ultrascans (34). The molecular weight of a monomer of each species, based on the amino acid sequence, is shown in parentheses.

| Molecular mass (kDa) | Oligomeric state |
|----------------------|------------------|
| SecA (102 kDa)       | +Mg$^{2+}$       |
|                     | $s_{20w}^{0}$    |
|                     | $s_{20w}^{0}$    |
|                     | 200.1 ± 7.1      |
|                     | 201.6 ± 6.4      |
|                     | Dimer            |
|                     |                |
| SecA-$\Delta$11/N95 (94 kDa) | +Mg$^{2+}$       |
|                     | $s_{20w}^{0}$    |
|                     | $s_{20w}^{0}$    |
|                     | 184.6 ± 7.1      |
|                     | 181.5 ± 8.3      |
|                     | Dimer            |
|                     |                |

**DISCUSSION**

Our understanding of the molecular mechanism of Sec-dependent translocation and the specific role of SecA is in its infancy. There are now six structures available of this protein, five dimers and one monomer. There is, for instance, no consensus with respect to the active oligomeric form of the protein, the processive nature of the reaction and the stoichiometry of ATP/amino acid translocated (see Introduction). These uncertainties are understandable in view of the fact that there is little data to describe the interactions that occur between SecYEG, SecA, and substrate. There is also a paucity of information on the kinetics and the hydrolytic cycle of SecA and the timing and nature of the reactions that bring about the conformational changes that must ultimately drive the vectorial passage of proteins through the membrane.

The experiments described in this paper address this fundamental aspect of the system, the reaction cycle of SecA. We begin at the simplest level of the system and elucidate the basic ATPase mechanism. We have characterized SecA by steady-state ATPase assays and related the observed kinetics to structural changes by analytical ultracentrifugation.

The divalent metal cation Mg$^{2+}$ was found to have a powerful effect on the binding affinity and turnover of ATP: tight and slow in the presence of high concentrations, loose and fast with trace amounts. The simplest way of explaining this...
Kinetic Analysis of SecA

A

| Time (min) | 0 | 1 | 2 | 5 | 10 | 15 | 0 | 1 | 2 | 5 | 10 | 15 |
|------------|---|---|---|---|----|----|---|---|---|---|----|----|
| SecA       |   |   |   |   |    |    |   |   |   |   |    |    |
| wild-type  | + | + | + | + | +  | +  | + | + | + | + |    |    |
| Δ11/N95    |   |   |   |   |    |    |   |   |   |   |    |    |

B

| [Mg²⁺] μM | 0 | 1 | 2 | 5 | 10 | 25 | 50 | 100 | 200 | 400 | 400 | 4000 |
|-----------|---|---|---|---|----|----|----|-----|-----|-----|-----|------|
| ATP       | + | + | + | + | +  | +  | +  |    |    |    |    |     |

FIGURE 8. Translocation of proOmpA through SecYEG by wild-type SecA and SecA-Δ11/N95. A, translocation of proOmpA into SecYEG-containing proteoliposomes using 0.2 μM wild-type SecA or SecA-Δ11/N95. Successfully translocated (protease-protected) proOmpA was detected by Western blot. B, translocation assays performed with a range of magnesium acetate concentrations, including 1 mM ATP. The sample applied to the far right-hand lane of the blot in both panels had not been protease treated and was loaded as a measure of 10% of the total pro-OmpA used in each reaction.

The observed change in affinity for ATP caused by Mg²⁺ indicates that a conformational change occurs upon binding. This prediction was tested by analytical ultracentrifugation to determine the extent of the rearrangement. In potentially oligomeric proteins, such rearrangements could take the form of changes in the state of assembly, but sedimentation equilibrium data showed that the molecular mass of the protein (200 kDa) was unchanged by Mg²⁺ and was consistent with a dimeric quaternary structure. However, the results of sedimentation velocity reveal that structural rearrangements of the dimer are quite large, with the Mg²⁺-bound form of the protein having a significantly larger (~6%) Sedberg constant. Interpreted empirically this is equivalent to the difference in sedimentation behavior between bovine carboxypeptidase A and bovine superoxide dismutase (45). These proteins have the same molecular mass and partial specific volumes but the latter is 40% longer in its longest axis (from 50 to 72 Å) (45). Hence, the change in sedimentation coefficient observed in SecA could represent a considerable opening of the structure.

The mutant SecA-Δ11/N95 is also a dimeric species but does not undergo such a large conformational change, probably due in part to its smaller size, and possibly also to the participation of the C terminus in this rearrangement, which has been truncated in the mutant form. This could explain why the mutant behaves in an identical manner in terms of ATPase activity, but has a reduced activity in terms of translocation. One slightly surprising aspect of this analysis was the indication that the predicted monomeric mutant SecA-Δ11/N95 (42) is a dimer, in contrast to previous findings. However, this is not to say that a monomeric form of SecA plays no part in the transport process.

The different structures of SecA all reveal essentially the same nucleotide binding fold. Insofar as we can tell most crystals were grown in the presence of inhibitory Mg²⁺ concentrations. Therefore the activated state of SecA with a dramatically reduced affinity for ATP that we have identified kinetically, may not be represented in the structural data base.

The concentration of free Mg²⁺ in bacterial cells is ~1 mM (46), sufficient to effectively inhibit the reaction and prevent futile ATP consumption by SecA. The curious observation that cardiolipin is able to counteract the Mg²⁺-induced inhibition might reflect a natural function or regulatory process, important for protein translocation. Conditions that promote protein translocation are insensitive to high concentrations of Mg²⁺.

SecA is water-soluble, but due to the nature of its activity has an intimate association with the lipid bilayer. It has been reported that acidic lipids and those that do not form lamellae increase SecA ATPase activity (28, 29). Moreover, reports show that acidic phospholipids are required for protein translocation (39). There have also been accounts of lipid binding sites on SecA (33, 47). The ability of lipids such as cardiolipin to chelate divalent cations (38) might indicate that the activation in ATPase activity is not directly affected by the lipid, but by its potential to extract Mg²⁺ from the allosteric binding site. This effect cannot be by a simple bulk chelation of Mg²⁺, as we observe the activation with only 2.4 μM cardiolipin in the presence of 100 μM Mg²⁺. Instead, the effect is likely to occur upon the formation of a SecA-Mg²⁺-cardiolipin ternary complex, in which the Mg²⁺ may be shifted from the allosteric site by local interaction with the cardiolipin. It is tempting to propose that this kind of regulation might be important in the activation of the enzyme as it delivers substrate protein in the vicinity of the membrane and the Sec complex. Preliminary experiments show that cardiolipin stabilizes the dimeric and active form of the SecYEG complex in E. coli, raising the possibility that this
activation could be promoted by a specific cardiolipin encountered only during the initiation of protein translocation (Fig. 9).

The results presented here describe a comprehensive analysis of the steady-state kinetics of the isolated motor domain of the major bacterial protein translocation apparatus. A conformational change in the dimer regulated by Mg$^{2+}$ brings about a large shift in the properties of the nucleotide binding affinity and turnover of the enzyme, and it is likely that these events are coupled to the protein translocation reaction. More detailed mechanistic understanding requires an analysis of the transient kinetics of ATP binding and hydrolysis that can then be extended to include the protein substrate and SecYEG.

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