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Effect of divalent cations on the ATPase activity of *Escherichia coli* SecA

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Abstract It was found that Ca^{2+} stimulates the intrinsic SecA ATPase activity in the absence as well as in the presence of liposome. On the other hand, Mg^{2+}, the general cofactor for ATPase, did not affect the intrinsic SecA ATPase but reduced the portion of ATPase activity enhanced by Ca^{2+}. The enhancement of SecA ATPase activity correlated well with the increase in 8-anilino-1-naphthalene-sulfonic acid binding of SecA, suggesting that increased exposure of hydrophobic residues stimulates the enzyme activity. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: SecA; Intrinsic ATPase activity; Calcium ion; Magnesium ion; 8-Anilino-1-naphthalene-sulfonic acid binding

1. Introduction

SecA protein, an ATPase, undergoes cyclic motions, inserting into the membrane in the presence of preprotein and ATP and deinserting in the course of the hydrolysis of ATP [1,2]. The hydrolysis of ATP generates the driving force for the translocation of preprotein from the cytoplasm to the periplasm of *Escherichia coli* [3]. In the absence of translocation ligands such as preprotein, SecYEG or liposome, SecA protein has a low basal ATPase activity due to the inhibitory effect of C-terminus which is in close proximity with the active center in the N-terminal half of the native protein [4,5]. The ATPase activity is increased in the presence of liposome because the C-terminus of the SecA protein is separated from the active center when it binds to the liposome and is thus unable to down-regulate the intrinsic ATPase [6,7]. The SecA ATPase activity brings about the preprotein translocation only in the presence of other translocation components [3].

Many substances such as substrate and product of the ATPase as well as other agents are known to induce structural change of SecA. Both ATP and ADP can produce the conformational change of SecA into a form which is resistant to the proteolytic digestion [8]. On the other hand, SecA in the presence of preprotein and phospholipids is more susceptible to the proteolytic digestion than the intact SecA protein [8]. Divalent ions such as Mg^{2+} and Zn^{2+} are also known to bind to SecA protein [9,10].

Earlier, it was observed that the addition of non-lamellar-prone lipids to the liposome increases the SecA ATPase activity [11]. It was confirmed that the non-lamellar-prone lipids induce phase separation of negatively charged phospholipids such as PG. Since Ca^{2+} alone also increased the SecA ATPase activity, it was concluded that the clustering of PG as the result of the phase separation is responsible for the enhanced ATPase activity. Now, it will be shown that Ca^{2+} has an intrinsic ability to increase the SecA ATPase activity with or without the presence of liposome. Also, it will be shown that Mg^{2+} inhibits the effect of Ca^{2+} on SecA ATPase activity.

2. Materials and methods

2.1. Materials

Total lipid extract from *E. coli* membrane was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and was used without further purification. 8-Anilino-1-naphthalene-sulfonic acid (ANS) was purchased from Sigma Chemical Company. All other chemicals were of the highest grade commercially available. In all experiments, solutions were treated with Chelax-100 resins to prevent contamination of metal ions.

2.2. SecA and pRBP preparation

Wild type SecA protein and mutant SecA proteins (SecA D209N and SecA R509K) were purified from SecA-overproducing strains (*E. coli* RR1/pMAN400, *E. coli* BL21.19 D209N, and *E. coli* BL21.14 R509K) according to the procedure described previously [12]. SecA D209N and SecA R509K, kindly provided by Prof. Oliver, have single point mutations at nucleotide binding site (NBS) I and NBS II of SecA, respectively [13]. The precursor form of ribose binding protein (pRBP) was purified from *E. coli* s IQ87/pTS128 by ion exchange chromatography as described elsewhere [14]. The obtained proteins were electrophoretically homogeneous and the protein concentration was determined by the Bradford method using bovine serum albumin as a standard [15].

2.3. IMV preparation

The inverted membrane vesicles (IMVs) were prepared from *E. coli* CP626, which does not produce RBP, as described elsewhere [16], and were suspended in 50 mM Tris–HCl, pH 7.5. Protein concentration of IMV was determined by the Lowry method using bovine serum albumin as a standard [17].

2.4. ATPase activity assay

The ATPase assay was performed in 50 μl of reaction buffer. The sample solutions containing 2.5 μg of SecA protein were incubated at 30°C for 20 min prior to the analysis of the released inorganic phosphate (P_i) as described [18]. Reaction mixtures were preincubated for 15 min at 30°C. Reactions were initiated with the addition of ATP. The reactions were stopped with the addition of 800 μl of a color reagent (0.034% malachite green and 10.5 g/l ammonium molybdate in 1 N HCl and 0.1% Triton X-100) and 100 μl of 34% citric acid

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Abbreviations: NBS, nucleotide binding site; pRBP, precursor form of ribose binding protein; IMV, inverted membrane vesicle; P_i, inorganic phosphate; ANS, 8-anilino-1-naphthalene-sulfonic acid; T_m, the midpoint temperature of the thermal denaturation curve

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solution. After standing for 30 min at room temperature, the absorbance was measured at 660 nm. One unit of ATPase activity was defined as the hydrolysis of 1 nmol of ATP/min/nmol of SecA protein.

2.5. Liposome preparation

Vesicles of *E. coli* total lipid extract were freshly prepared. *E. coli* total lipid extract was evaporated under a stream of Ar gas. The dried lipid was hydrated in 50 mM Tris–HCl, pH 7.5, followed by a vigorous vortexing and a brief sonication in a bath-type sonicator for 30 s. To obtain homogeneous large unilamellar vesicles, the dispersion was frozen and thawed five times and passed 25 times through two 100-nm pore size polycarbonate membranes in an Extruder Lipo-Fast (Avestin Inc., Ottawa, Canada). The concentration of liposome was 1 mg/ml throughout these investigations.

2.6. Thermal unfolding

The thermal unfolding experiments of 0.5 mM SecA proteins were performed in 50 mM Tris–HCl, pH 7.5 and the temperature was increased from 20°C to 60°C at a constant rate of 0.33°C/min using a MONO-TECH MRC 1011D refrigerated bath circulator. For the temperature scanning of fluorescence, the Trp fluorescence intensities, excited at 295 nm and measured at 340 nm, were collected at every 2°C increment.

2.7. ANS binding assay

The conformation changes of SecA by ATP binding were monitored by ANS binding. The ANS fluorescence spectra (excitation at 374 nm) were measured on a Shimadzu RF-5301PC spectrofluorometer equipped with a constant-temperature cell holder. The spectra of all samples were obtained after the incubation of samples at 30°C for 10 min. SecA protein concentration in the samples was 0.05 mg/ml in 50 mM Tris–HCl, pH 7.5, and the concentration of ANS was 200 μM.

3. Results

3.1. Effect of divalent cations on the ATPase activity of SecA

Previously, it was observed that the ATPase activity of SecA present together with liposomes composed of acidic phospholipid PG plus either PE or PC is increased when Ca^{2+} is added [11]. Here, we show that Ca^{2+} has a stimulating effect on the SecA ATPase activity even in the absence of liposome. Fig. 1A gives the Ca^{2+}-induced increase in the SecA ATPase activity obtained with and without liposomes made of the total lipid extract from *E. coli*. The previous

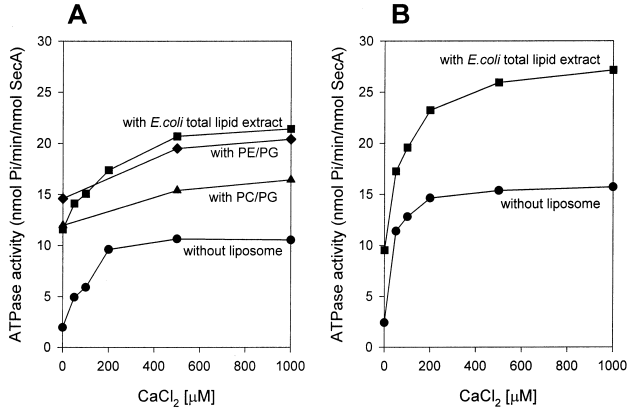

Fig. 1. The effect of Ca^{2+} on the ATPase activity of SecA in the presence and absence of liposomes. The data for PE/PG (60:40) and PC/PG (60:40) are taken from the previous report [11]. The units for the ATPase activity used in the earlier report were converted into the present ones by recalculating the original raw data for comparison. The ATPase activity of SecA with liposome or without liposome was measured with increasing concentration of $CaCl_2$ in the presence of 2 mM ATP and 2 mM $MgCl_2$ (A) or in the presence of only 2 mM ATP (B) at 30°C.

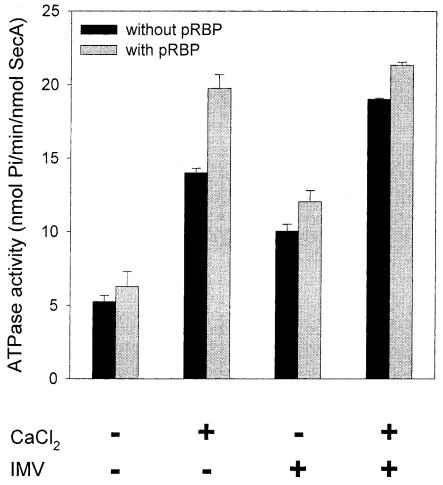

Fig. 2. The effect of Ca^{2+} on the ATPase activity of SecA in the presence of translocation ligands. The effect of Ca^{2+} (200 μM) on the SecA was measured in the presence of pRBP or IMV. pRBP (1 mg/ml) was diluted 20 times into the reaction mixture including SecA (0.05 mg/ml) and/or IMV (0.1 mg/ml) for the refolding after the unfolding with 1 M GdnHCl at 30°C for 1 h. 0.05 mM GdnHCl was included in each reaction mixture. The reaction was initiated by the addition of ATP after the incubation of the reaction mixture at 30°C for 15 min. The averages of three independent measurements with standard deviation are shown.

results are also shown in the figure for comparison. In the present experiments, 2 mM $MgCl_2$ is also added to the solution to provide the same condition as in the previous experiments [11]. The liposomes alone stimulate the ATPase activity but the magnitude of the increase is similar when Ca^{2+} concentration is increased in all four cases in Fig. 1A. The enhancing effect of Ca^{2+} on the activity with PC/PG is smaller than that of PE/PG or total lipid extract. The closeness of the curves for the systems containing the *E. coli* total lipid extract and PE/PG reflect their similar lipid compositions. In order to eliminate the complicating effect of Mg^{2+}, the experiments were repeated without this cation and the results are given in Fig. 1B. Here again, it can be seen that Ca^{2+} has a stimulating effect on SecA ATPase activity regardless whether liposomes are present or not. In the previous report [11], the Ca^{2+}-induced increase in the SecA ATPase activity was attributed to the clustering of the negatively charged lipids as the result of the phase separation in the presence of the cation. This conclusion may not be valid because the Ca^{2+} concentration-dependent increases of the enzyme activity of systems with and without liposomes are about the same suggesting that Ca^{2+} exerts a direct effect.

SecA ATPase activity is also affected by pRBP. Fig. 2

Table 1
K_M and V_{max} of SecA ATPase activity in the presence of various concentrations of calcium

$CaCl_2$ (μM)	K_M [a,b]	V_{max} [a,b]
0	17	3.7
10	51	7.1
50	247	14.9
200	813	24.8

[a] K_M and V_{max} are calculated from the fitting to the Lineweaver–Burk plot.
[b] The units for K_M and V_{max} are μM and nmol P_i/min/nmol SecA, respectively.

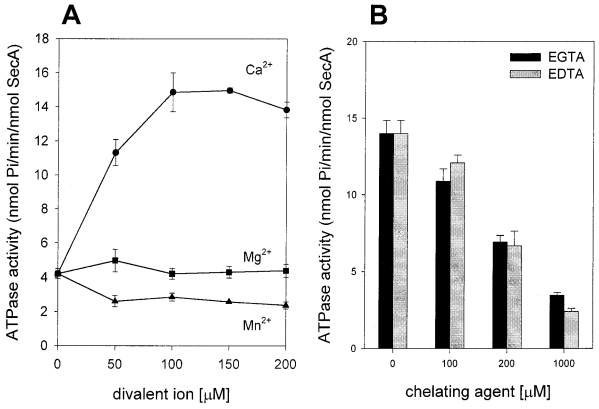

Fig. 3. The effect of divalent ions on the intrinsic ATPase activity of SecA without liposome. The ATPase activity of SecA without liposome was measured with increasing concentration of $CaCl_2$ (closed circle), $MgCl_2$ (closed square) or $MnCl_2$ (closed triangle) (A). Effect of chelating agent, EDTA (gray bar) or EGTA (black bar), on the enhanced ATPase activity in the presence of 200 μM $CaCl_2$ was examined (B). The averages of three independent measurements with standard deviation are shown.

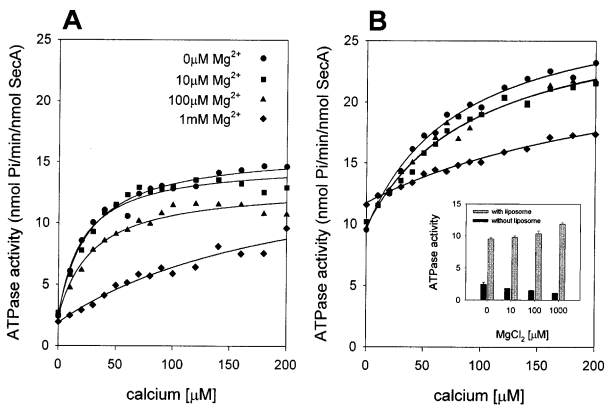

Fig. 5. The effect of Ca^{2+} on the ATPase activity of SecA in the presence of various concentrations of Mg^{2+} without liposome (A) and with liposome (B). The data are fitted to three-parameter hyperbolic equation ($y = a+(bx)/(c+x)$). The inset shows the ATPase activity of SecA without Ca^{2+} in the presence (gray bar) or in the absence (black bar) of liposome. In the inset, the averages of five measurements with standard deviation are shown.

shows the effect of Ca^{2+} on the ATPase activity in the presence of preprotein or IMV. In the presence of 200 μM Ca^{2+}, the ATPase activity without the translocation ligand is smaller than that in the presence of preprotein or IMV. It seems that the structural changes of SecA by the binding of the translocation ligands such as preprotein and IMV affect the enhanced ATPase activity by Ca^{2+}. Especially, the ATPase activity by Ca^{2+} was greatly increased by the presence of pRBP (Fig. 2).

Fig. 3A compares the effect of Mg^{2+} and Mn^{2+} on the SecA ATPase activity in the absence of liposomes with that of Ca^{2+}. It is of interest that Mg^{2+}, which is a general cofactor for ATPases, has no effect on the ATPase activity and Mn^{2+} even inhibits the ATPase activity slightly. The enhancing effect of Ca^{2+} was abolished in the presence of EDTA or EGTA (Fig. 3B).

Fig. 4 shows the dependence of ATPase activity on the substrate concentration at various Ca^{2+} concentrations. The values of K_M and V_{max} were estimated form this curve and

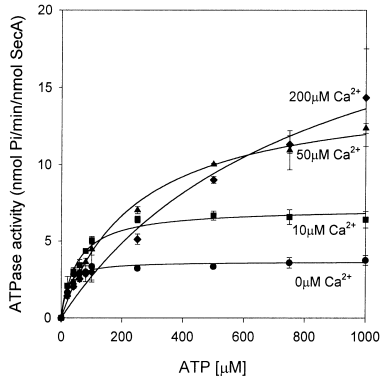

Fig. 4. The ATP concentration-dependent ATPase activity of SecA in the presence of various concentrations of Ca^{2+}. The ATPase activities of SecA were measured in the presence of various concentrations of ATP at several Ca^{2+} concentrations (0, 50, 100, or 200 μM). The averages of three measurements with standard deviation are shown. The data are fitted to the Lineweaver–Burk plot for the calculation of K_M and V_{max}.

given in Table 1 which shows increased K_M and V_{max} as the concentration of Ca^{2+} was increased. K_M and V_{max} without Ca^{2+} were 17 μM and 3.7 units, respectively. With 200 μM of Ca^{2+}, K_M and V_{max} were 813 μM and 24.8 units, respectively. This may mean that the Ca^{2+} brings about changes in the active site of SecA ATPase to enhance the ATPase activity.

Fig. 5 shows that the portion of ATPase activity of SecA protein enhanced by Ca^{2+} was inhibited by Mg^{2+} both in the absence and presence of liposome. It was already shown that Mg^{2+} by itself had no effect on the intrinsic ATPase activity of SecA (Fig. 3). The inset in Fig. 5B shows the Mg^{2+}-dependent ATPase activity in the absence of Ca^{2+}. When no liposome is present the ATPase activity decreases with increasing Mg^{2+} concentration, but an opposite effect is apparent when liposomes are present. The significance of this is not clear at the moment.

3.2. *Effects of ATP and divalent ions on ANS binding of SecA*

In order to correlate the Ca^{2+}-induced ATPase activity increase with possible structural change of the SecA, the effect of Ca^{2+} on the fluorescence and CD spectra of SecA was checked but no discernible change was observed (data not shown). This lack of overall structural change was corroborated by the thermal unfolding experiments as given in Table 2. Addition of either Ca^{2+} or Mg^{2+} had no effect on the the midpoint temperature of the thermal denaturation curve (T_m) of SecA but there was a 2°C increase in the T_m value in the presence of 1 mM ATP suggesting tightening of the SecA structure by ATP. Both Ca^{2+} and Mg^{2+} did not affect the ATP-induced T_m increase.

A significant influence of Ca^{2+} on the binding of ANS,

Table 2
T_m for the thermal unfolding of SecA in the presence or in the absence of ATP and divalent ions

Divalent ions	Without 1 mM ATP (°C)	With 1 mM ATP (°C)
None	40.4	42.5
Mg^{2+}	40.7	42.9
Ca^{2+}	39.9	42.6

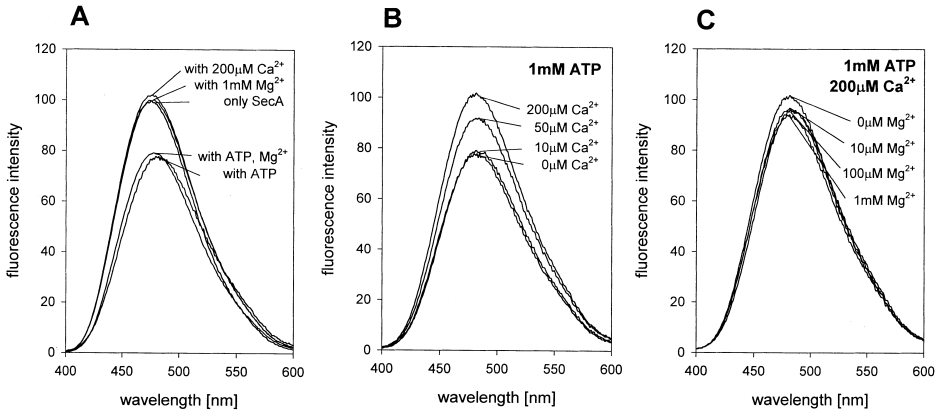

Fig. 6. The ANS binding of SecA. The fluorescence spectra due to the ANS binding are shown as the difference between the spectra in the presence of SecA and the spectra in the absence of SecA. The spectra were measured after the incubation of samples at 30°C for 15 min. The spectra were obtained in the presence of 200 μM Ca^{2+}, 1 mM Mg^{2+} or 1 mM ATP (A). The ANS binding of SecA in the presence of 1 mM ATP increased as the concentration of Ca^{2+} increased (B). Mg^{2+} inhibited the effects of Ca^{2+} (C).

however, was observed indirectly through fluorescence experiments. Fig. 6A shows that SecA binds ANS appreciably but the extent of binding decreases in the presence of ATP. Apparently, ATP makes the SecA structure more compact lowering the exposure of hydrophobic residues. This result is in agreement with the thermal unfolding experiments (Table 2).

It was observed that Ca^{2+} itself does not affect the ANS binding by SecA (Fig. 6A) but compensates the reducing effect of ATP on the ANS binding (Fig. 6B). As the concentration of Ca^{2+} is increased to 200 μM, the reducing effect of 1 mM ATP on the ANS binding is completely nullified.

Mg^{2+} alone, again, does not affect the ANS binding of SecA but, unlike Ca^{2+}, up to 1 mM of Mg^{2+} does not counteract the effect of ATP on the ANS binding as shown in Fig. 6A. It is of interest, however, that Mg^{2+} partially overcomes the Ca^{2+} effect (Fig. 6B) as shown in Fig. 6C. The parallel correlation between the extent of ANS binding and the ATPase activity is apparent when Fig. 6B is compared with Fig. 4 and Fig. 6C with Fig. 5A.

3.3. *Effect of Ca^{2+} on the mutant SecA proteins*

We have tested the effect of Ca^{2+} on the individual SecA ATP binding sites of NBS I and NBS II using mutant SecA of D209N and R509K. SecA D209N was obtained by replacing the Glu-209 in the NBS I region of the wild type SecA with a Gln while R509K has a Lys instead of Arg at the residue position of 509 in the NBS II region. SecA D209N binds only ADP with low affinity whereas SecA R509K binds ADP with high affinity, but neither of them displays ATPase activity [13]. In contrast to the wild type SecA, the presence of Ca^{2+} had no effect on the ATPase activities of both SecA D209N and SecA R509K (Fig. 7A). Also, Ca^{2+} did not affect the ANS binding profiles of mutant SecA proteins (Fig. 7B). It seems that only one intact NBS sequence is not sufficient to enhance the ANS binding of SecA by Ca^{2+} in the presence of ATP.

4. Discussion

SecA itself has a basal intrinsic ATPase activity when there is no other translocation ligand present and this activity is stimulated when it is bound to the liposome [3]. SecA has two ATP binding sites, NBS I and NBS II [13]. NBS I of SecA has an important role for the translocation of preprotein [2] as well as the intrinsic ATPase activity [19]. The SecA ATPase activity is down-regulated by two independent areas of the sequence [19]. The C-terminus of the SecA protein inhibits the intrinsic ATPase activity, and its deletion or unfolding stimulates activity [6,20]. The binding of SecA to the liposome appears to allow the partially unfolded C-domain of SecA to penetrate the lipid bilayer [21]. This, in turn, separates the NBS I from the inhibiting C-terminus thus allowing the ATPase activity to be stimulated. NBS II of SecA also down-regulates the intrinsic ATPase activity of NBS I of SecA [19].

The main conclusion that can be drawn from the present investigation is that Ca^{2+} alone also stimulates the SecA ATPase activity and this appears to be related to the exposure of the hydrophobic residues to the surface of this protein. That the Ca^{2+}-induced activity stimulation is basically different from the liposome-induced enhancement can be seen from the fact that the extent of activity increment for a given Ca^{2+} concentration is about the same regardless whether liposome is present or not.

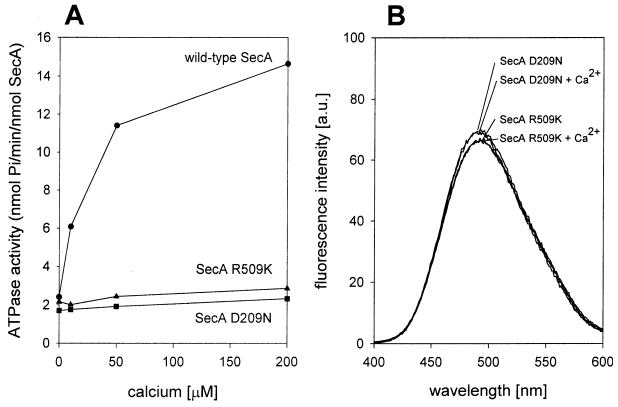

Fig. 7. The effects of Ca^{2+} on the mutants, SecA D209N and SecA R509K. Ca^{2+} had no effect on the ATPase activities of SecA D209N and SecA R509K (A). Also, Ca^{2+} did not change the ANS binding of SecA D209N and SecA R509K (B).

It is interesting that the ANS binding of SecA correlates well with the ATPase activity of SecA (Fig. 6B). It appears that ATP has a compacting effect on SecA. This coincides with the proteolytic digestion pattern of SecA protein in the presence of ATP and preprotein ligand [8]. In the presence of only ATP, SecA is more resistant to the protease and has a low ATPase activity. On the other hand, SecA with preprotein or liposome is more susceptible to the protease and has a high ATPase activity. Both ANS binding (Fig. 6) and the proteolytic digestion pattern shows that SecA has smaller ATPase activity as the conformation of SecA becomes more compact. The increase in the SecA T_m value in the presence of ATP supports the compacting effect of ATP. This may have a self-regulation effect on the ATPase activity bringing it down to the basal activity. The Ca^{2+} appears to counteract at least partially the compacting effect of ATP enhancing the ATPase activity. The counteraction may be a subtle process and only the exposure of the hydrophobic residues, but not the major conformational change manifested by change in CD and fluorescence spectra and T_m, can be detected.

Although the mechanism of Ca^{2+}-induced enhancement of basal ATPase activity appears to be different from that for liposome induced increase, it is not quite clear whether the effect of Ca^{2+} is localized or generalized. It is, however, likely that Ca^{2+} acts in the active center because it inhibits the ATP effect and also there seems to be competition between Ca^{2+} and Mg^{2+}, the latter being known to interact with the active center [9]. The change of K_M and V_{max} values due to Ca^{2+} may mean the conformational change of active sites. Whether the increased ANS binding caused by Ca^{2+} occurs at the localized event at the active center or more widespread is not certain.

Mg^{2+} did not quench the enhanced ANS binding of SecA by Ca^{2+} (Fig. 6C) although it inhibited the enhanced ATPase activity by Ca^{2+} (Fig. 5A). If Ca^{2+} competes with Mg^{2+} for the same binding site, Mg^{2+} must overcome the Ca^{2+} effect on the ANS binding although the Ca^{2+} effect on the ATPase activity is decreased. These results suggest that Mg^{2+} inhibits the Ca^{2+} effect by binding at the other sites.

Whether the enhancing effect of Ca^{2+} on SecA ATPase activity in vitro has any hearing on the Ca^{2+} in vivo situation is uncertain because the cytoplasmic concentration of Ca^{2+} of *E. coli* is very low (200–300 nM), which raises 2–7 times according to the cell growth stage or the outer Ca^{2+} concentration [22]. However, SecA can penetrate into the membrane during the active state [23,24] and it is possible that the peripheral Ca^{2+} can interact with SecA. And, it is possible that Mg^{2+} contributes to the regulation of the intrinsic ATPase activity by the stabilization of the conformation induced by ATP binding.

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