SecB Modulates the Nucleotide-Bound State of SecA and Stimulates ATPase Activity†

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ABSTRACT: In *Escherichia coli*, the formation of SecA−SecB complexes has a direct effect on SecA ATPase activity. The mechanism of this interaction was evaluated and defined using controlled trypsinolysis, equilibrium dialysis at low temperature, and kinetic analyses of the SecA ATPase reaction. The proteolysis data indicate that SecB and the nonhydrolyzable ATP analogue AMP-P-C-P induce similar conformational changes in SecA which result in a more open or extended structure that is suggestive of the ATP-bound form. The effect is synergistic and concentration-dependent, and requires the occupation of both the high- and low-affinity nucleotide binding sites for maximum effect. The equilibrium dialysis experiments and kinetic data support the observation that the SecB-enhanced SecA ATPase activity is the result of an increased rate of ATP hydrolysis rather than an increase in the affinity of ATP for SecA and that the high-affinity nucleotide binding site is conformationally regulated by SecB. It appears that SecB may function as an intermolecular regulator of ATP hydrolysis by promoting the ATP-bound state of SecA. The inhibition of SecA ATPase activity by sodium azide in the presence of IMVs and a functional signal peptide further indicates that SecB promotes the ATP-bound form of SecA.

Many proteins which are destined for extracytoplasmic locations in *Escherichia coli* are exported by the Sec pathway, a multicomponent preprotein translocase. The SecYEG complex is the centerpiece of the translocation channel through the inner membrane, and SecA is the molecular motor with its associated ATPase activity responsible for driving the movement of proteins through the channel (*1*−*6*). Some proteins are targeted to the translocase via an interaction with the signal recognition particle (SRP), yet other proteins are delivered through an interaction with the chaperone SecB (*7*).

Several studies have established that SecB has at least two functions (*6*, *8*); it serves to keep the preprotein in an unfolded, translocation-competent state (*9*), and it facilitates targeting of the preprotein to membrane-associated SecA (*10*). Soluble SecA−SecB complexes have been observed (*10*, *11*) that have a much lower affinity ($K_d = 1.6\ \mu M$) than those formed at the membrane, K_d approximately 30 nM (*12*, *13*). This substantial difference in affinity, coupled with the increased concentration of SecA at the membrane, may be required to direct the cytosolic chaperone and preprotein to membrane-embedded SecA and to limit the binding of SecB to cytosolic SecA. Furthermore, it has been suggested that the SecA−SecB interaction is required to dissociate the mature domain of the preprotein from SecB and that binding of the signal peptide to SecA is necessary to ensure efficient transfer of the preprotein to the translocase (*14*).

Using in vivo analysis, previous studies have noted that preprotein translocation becomes more efficient in the presence of SecB (*15*−*17*). This result is consistent with a funneling effect by which soluble preprotein−SecB complexes are concentrated at translocation sites through interactions with SecA. However, recent experiments indicate that this interaction has additional consequences with respect to the activity of SecA. It has been shown that SecB directly promotes the ATPase activity of SecA and its presence enhances signal peptide-stimulated SecA/lipid ATPase activity (*18*). This, in turn, could contribute to the efficiency of preprotein translocation in the presence of SecB in vivo. Thus, the ATPase activity of SecA is exquisitely sensitive to interaction with its ligands, including anionic lipids (*19*), SecYEG (*19*), preprotein (*20*), signal peptide (*21*, *22*), and SecB (*18*).

SecA has both high-affinity (NBD1) and low-affinity (NBD2) nucleotide binding domains (*23*). Binding of ATP to SecA produces a substantial conformational change to a more elongated form (*24*) and promotes insertion of SecA well into the lipid bilayer (*25*). Subsequent hydrolysis of ATP is accompanied by release of the preprotein, deinsertion of SecA from the membrane, and the return to a more globular, compact SecA form (*1*−*5*). Successive cycles may propel the complete translocation of the entire polypeptide (*26*).

In this study, we examine the basis for the SecB stimulation of SecA ATPase activity previously reported (*18*). Data obtained from the proteolysis, equilibrium dialysis, and kinetic studies indicate that SecB interacts with SecA, inducing a conformational alteration in the enzyme to a more extended structure which favors ATP binding and subsequent hydrolysis. During this process, the high-affinity nucleotide

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binding site is conformationally regulated by SecB. We also demonstrate that the ATPase inhibition observed in the presence of NaN_3[1] and SecB is SecYEG-dependent and that the presence of SecB renders SecA more susceptible to inhibition.

EXPERIMENTAL PROCEDURES

Materials. TPCK-trypsin (EC 3.4.21.4), reactive blue 4 agarose, ADP, PMSF, DTT, AMP-P-C-P, and [^{3}H]ADP (25.3 Ci/mmol) were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP (3000 Ci/mmol) was from Perkin Elmer (Boston, MA). Acetone-precipitated and ether-extracted *E. coli* phospholipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). The *E. coli* wild-type alkaline phosphatase signal peptide, MKQSTIALALLPLLFTPVTKAC-NH_2, and the nonfunctional 1K2L peptide, MKQQQAALAAAA-LAASSSASAC-NH_2, were synthesized and purified as described previously (*27, 22*).

Protein Purification. SecA was overexpressed and purified from S300 fractions of *E. coli* strain BL21.14pCS1 by affinity chromatography on reactive blue 4 agarose essentially as reported earlier (*23*). $His_{(6)}$-tagged SecB was isolated from *E. coli* strain TG1(pREP4, pQE9secB) under nondenaturing conditions as described (Qiagen, Valencia, CA) (*18*). Both proteins were dialyzed against either 25 mM Tris-HCl (pH 7.5), 25 mM KCl, 0.5 mM EDTA, 5 mM β-mercaptoethanol, and 0.5 mM PMSF or 50 mM Hepes−KOH (pH 7.0), 30 mM KCl, 30 mM NH_4Cl, 0.5 mM magnesium acetate, and 1 mM DTT. Protein concentrations were determined by the Bradford assay at 595 nm (*28*) using BSA as the standard. Working stock solutions of both SecA and SecB (1−5 mg/mL) were stored at 4 °C while ammonium sulfate precipitates were kept at −70 °C for long-term storage.

Inner Membrane Vesicle (IMV) Isolation. Inverted inner membrane vesicles (IMVs) were prepared essentially as described (*29, 30*) from either wild-type *E. coli* strain MC4100 or the SecYEG-overexpressing strain CK1801-(pSE420secYEG) (*31*). SecA-depleted IMVs were obtained by treating the membrane preparations with freshly prepared 6 M urea in 50 mM Tris-HCl (pH 8.0) for 30−60 min at 0 °C (*32*). Subsequently, membranes were collected by ultracentrifugation (40000*g* for 90 min), washed once with 20 mM HEPES−KOH (pH 7.5), 250 mM sucrose, 1 mM DTT, and recentrifuged, and the pellet was resuspended in the same buffer. Aliquots of about 5 mg/mL were stored at −70 °C. The amount of membrane protein was determined according to a modified Lowry procedure (*33*) using BSA as the standard.

SecA ATPase Assay. SecA ATPase activity in the presence of IMVs was determined essentially as described previously for SecA/lipid ATPase activity (*21, 22*) with minor modification. Briefly, SecA, synthetic signal peptide, and SecB were added to the reaction buffer (50 mM Hepes−KOH, pH 7.5, containing 30 mM KCl, 30 mM NH_4Cl, 0.5 mM magnesium acetate, and 1 mM DTT) and preincubated on ice for 15−30 min to establish complex formation. After the addition of IMVs (100−200 μg of membrane protein/mL) or *E. coli* phospholipids (small unilamellar vesicles, 320 μg/mL), the reactions were initiated with 4 mM ATP and further incubated at 37 °C for 40 min before determining the ATPase activity, using the malachite green colorimetric method (*34*). All values are corrected for endogenous activity, and BSA was omitted from all assays containing IMVs and SecB. NaN_3 was added as indicated in the legend for Figure 6.

Kinetic Analyses. Multiple-turnover SecA ATPase activity was determined as follows. Reaction mixtures (50 μL) were essentially the same as those described above for the SecA ATPase assay except that ATP (10 μM to 2 mM) was present in substantial excess over SecA (0.4 μM), and SecB (10 μM, monomer) was added when needed. After a short preincubation on ice, the reactions were initiated with the desired concentration of ATP. Incubation was continued for 30−40 min at 37 °C, and the released Pi was colorimetrically determined as described above.

To evaluate the involvement of the high-affinity binding site, reactions at low ATP concentrations were performed using [γ-^{32}P]ATP essentially as described (*35*) with minor modification. Reaction mixtures (25 μL), containing [γ-^{32}P]-ATP and unlabeled ATP at the desired concentrations (20 nM to 2 μM), SecA (4 nM), phospholipids (3 μg/mL), and SecB (1 μM) when needed were incubated at 37 °C for 10 min in the ATPase assay buffer. ATP hydrolysis was stopped by mixing 15 μL of the reaction mixture with 300 μL of a 5% suspension of activated charcoal in 20 mM phosphoric acid at 0 °C. After incubation for 10 min on ice, the charcoal and the remaining substrate were pelleted by a 10 min centrifugation at 4 °C. Aliquots (50 μL) of the supernatant, containing the released Pi, were analyzed by liquid scintillation counting. The amount of Pi released was background-corrected for ATP hydrolysis in the absence of the enzyme. The data were analyzed using a nonlinear regression analysis with Prism 2.0 (GraphPad Software, Inc., San Diego, CA) using the Michaelis−Menten equation, and k_{cat} values were calculated using $k_{cat} = V_{max}/[\text{SecA}]$.

SecA Trypsinolysis. Proteolytic digestions (up to 2 h) of SecA were carried out with trypsin at 0 °C essentially as described (*36, 37*) with minor modification. All reactions contained SecA (10−20 μg in 25−50 μL, 2 μM dimer), 50 mM Tris-HCl, pH 7.8, 50 mM KCl, 0.5 mM $MgCl_2$, and 1 mM DTT. Nucleotides (ADP, ATP, AMP-P-C-P) and SecB were added as indicated in the figure legends. Following a 30 min preincubation at 0 °C, trypsin was added (25−600 ng/25 μL) and the incubation continued. Aliquots of each reaction were taken at successive time intervals and the reactions terminated by the addition of an equal volume of 2× SDS−PAGE loading mixture (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue). Samples were boiled for 3 min and stored at −70 °C until subjected to SDS−PAGE (*38*) on 10% or 12.5% polyacrylamide mini-gels. Independent experiments were repeated at least 3 times with high reproducibility. Gel densitometric scans were obtained using an LKB Ultrascan XL laser densitometer or by NIH IMAGE 1.62f.

[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; AMP-P-C-P, adenylylmethylenediphosphonate; AMP-P-N-P, 5′-adenylylimidodiphosphate; IMVs, inverted inner membrane vesicles; NaN_3, sodium azide; Pi, inorganic phosphate.

Table 1: SecB-Induced Increase in SecA ATPase Activity (Relative %)

peptide	IMVs[a]	IMVs[b]
none	9	13
wild type[c]	19	34
1K2L	5	17

[a] IMVs prepared from wild-type *E. coli* strain MC4100. [b] IMVs prepared from *E. coli* strain pSE420secYEG which overexpresses SecYEG. [c] Wild-type signal peptide of *E. coli* alkaline phosphatase. All reactions were conducted essentially as described under Experimental Procedures. The final concentration of the wild-type and 1K2L synthetic signal peptides was 20 μM. Data are given for the percent increase in SecA ATPase activity in the presence of SecB relative to that in its absence.

Equilibrium Dialysis. The effects of SecB and the nonhydrolyzable ATP analogue, AMP-P-C-P, on the binding and release of ADP from SecA were evaluated using equilibrium dialysis at 4 °C. The dialysis buffer was comprised of 50 mM potassium phosphate, pH 7.5, supplemented with 0.5 mM $MgCl_2$ and 2 mM DTT. SecA (1 μM, monomer), buffer, and [^{3}H]ADP (0.125 μM) were placed in one chamber of the dialysis unit while the opposite chamber contained buffer only. A dialysis membrane with a 12 000−14 000 molecular weight cutoff was used. After an overnight incubation at 4 °C, AMP-P-C-P at 0.13 or 340 μM was added to the chamber containing SecA and [^{3}H]ADP. An additional overnight equilibration was performed before SecB was added to a final concentration of 30 μM. At each step of the dialysis, triplicate aliquots of each chamber were analyzed by liquid scintillation counting to determine the amount of bound and free [^{3}H]-ADP. At the end of each experiment, the integrity of SecA and SecB was monitored by SDS−PAGE. No evidence of proteolytic digestion was observed even after extended incubation at 4 °C.

RESULTS

Recently, it was found that SecB, even in the absence of preprotein, directly enhances SecA ATPase activity either in an aqueous environment or in the presence of liposomes. Furthermore, SecB stimulates signal peptide-induced SecA/lipid ATPase activity in the absence of the mature portion of the preprotein (*18*). A comparable effect of SecB on SecA associated with its biological membrane was confirmed using urea-treated IMVs from an *E. coli* strain expressing wild-type levels of the translocation components as well as membranes from an *E. coli* strain that overexpresses SecYEG. As shown in Table 1, SecB enhanced the ATPase activity of SecA incubated with wild-type IMVs in the presence of signal peptide and, under identical conditions, a consistently higher activity was observed with membranes isolated from a SecYEG-overexpressing strain. The data are consistent with studies which indicate that SecB has a higher affinity for IMV-bound SecA in the presence of the signal peptide region of proOmpA (*14*). It also suggests that the enhanced preprotein-stimulated SecA ATPase activity observed in the presence of SecB (*13*) may arise in part due to a direct affect of SecB on SecA, since in this study the mature region of the preprotein was not present and the signal peptide region apparently does not bind to SecB (*39*). SecB also increased the level of SecA ATPase activity in the presence of a weak signal peptide, such as 1K2L, however,

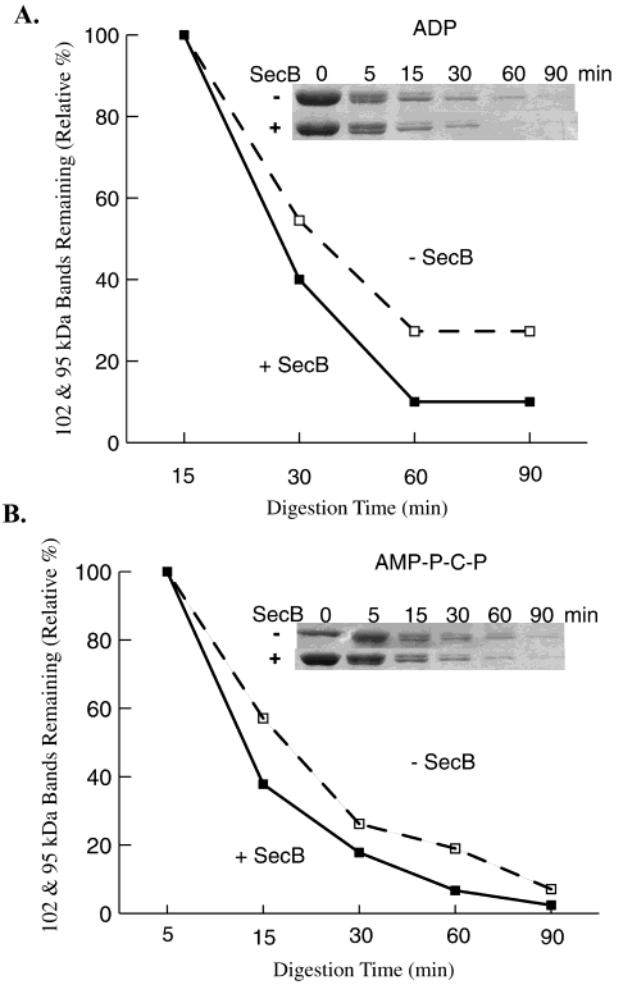


FIGURE 1: SecB effect on SecA trypsinolysis at 37 °C in the presence of AMP-P-C-P or ADP. Proteolysis was conducted as described under Experimental Procedures. All reactions were preincubated at 0 °C for 30−60 min prior to trypsinolysis at 37 °C for the indicated times. The concentrations used are as follows: SecA (2 μM dimer), SecB (47 μM tetramer), ADP (5 mM) (panel A), and AMP-P-C-P (5 mM) (panel B). The trypsin to SecA ratio used was either 1:200 (panel A) or 1:400 (panel B). The amounts of 102 plus 95 kDa bands at 15 min (panel A) and 5 min (panel B) were equated to 100%.

to a lesser extent (Table 1). The IMV data suggest that, in vivo, both a functional signal peptide and SecB can effectively contribute to the enhancement of membrane-associated SecA ATPase activity even in the absence of mature protein.

To probe the mechanistic basis for the SecB-induced enhancement in SecA ATPase activity, the proteolysis sensitivity of SecA in the presence and absence of SecB was examined. As shown in Figure 1, intact SecA (102 kDa) and a 95 kDa fragment were more readily hydrolyzed by trypsin at 37 °C in the presence of SecB and either 5 mM ADP or AMP-P-C-P relative to that with nucleotide alone. This indicates that SecB is promoting a more open, trypsin-accessible form of SecA which is structurally related to the membrane-inserted and ATP-bound form of the protein (*24*).

Limited trypsinolysis of SecA at 0 °C generates fragments of 67, 46, 34, 30, and 16 kDa (*36*, *37*). The formation of the 46 kDa fragment, which is derived from the amino-terminal 67 kDa domain, is dependent on the presence of nonhydrolyzable ATP analogues, AMP-P-N-P or AMP-P-C-P, but is

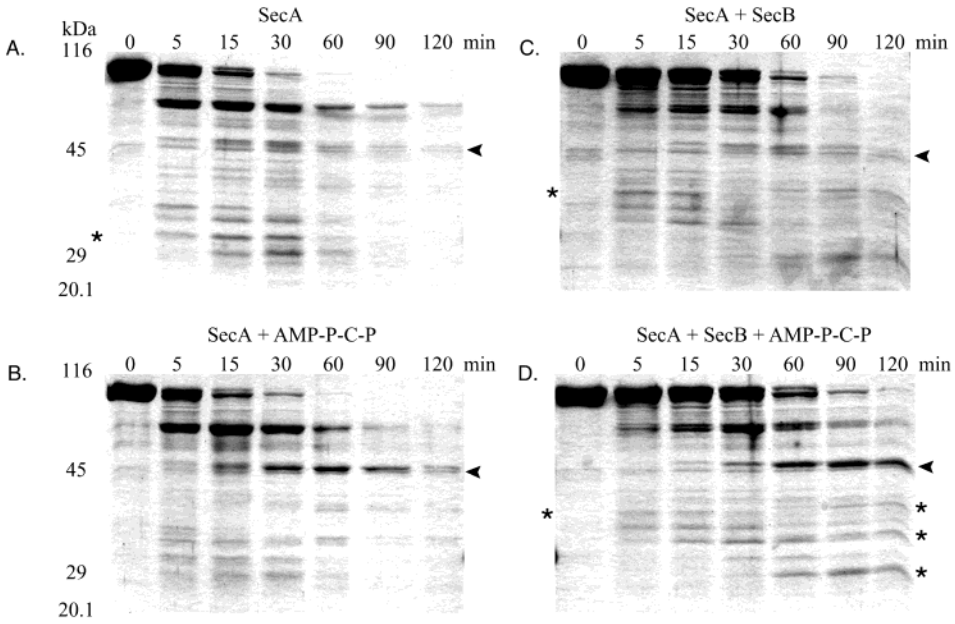


FIGURE 2: Effects of SecB and AMP-P-C-P on the trypsinolysis of SecA at 0 °C. The controlled trypsinolysis of SecA dimer (2 μM) in the presence of AMP-P-C-P (1 mM) and/or SecB tetramer (47 μM) was conducted as described under Experimental Procedures. The trypsin to SecA ratio used was 1:33.3 (600 ng of enzyme and 20 μg of SecA). The time of digestion (min) is given at the top of each panel. SDS−PAGE was done on 12.5% polyacrylamide mini-gels. The solid arrowhead designates the 46 kDa fragment. Asterisks indicate differences in proteolytic fragments occurring in the 29−40 kDa region.

Table 2: Relative Change of the 46 kDa Trypsinolysis Fragment at 0 °C in the Presence of Different Ligands[a]

digestion time (min)	ligands		
	SecB	AMP-P-C-P	SecB+AMP-P-C-P
60	34	150	224
90	41	227	617
120	18	134	589

[a] The intensity of the 46 kDa band was normalized to that of zero time (intact SecA), and the relative increase (percent) at each time point in the presence of ligands (SecB and/or AMP-P-C-P) versus their absence is given.

not produced appreciably in the presence of ATPγS, ADP, or ATP (*37*). This fragment is used as an indicator of the presence of the ATP-bound form of SecA. A comparison of the effects of AMP-P-C-P and SecB on controlled SecA trypsinolysis at 0 °C is shown in Figure 2. A prominent 46 kDa fragment (designated by a solid arrowhead) was generated in the presence of either AMP-P-C-P (Figure 2B) or, to a lesser degree, SecB (Figure 2C). However, in the presence of both components (Figure 2D), this fragment was more stable and resistant to proteolysis (Table 2). Under the same conditions, when SecB (p*I* 4.0) was replaced with BSA which has a similar p*I* value, 4.8 (*17, 40*), no effect on the production of the 46 kDa fragment was evident (data not shown). The results suggest that the presence of SecB promotes a more open SecA species (which gives rise to the stable 46 kDa fragment) suggestive of the ATP-bound form (*24, 41*). Other differences in the protein banding profiles, occurring in the 29−40 kDa region and indicated by asterisks in Figure 2, are also readily apparent and support a SecA−SecB interaction as well as different SecA conformations in the presence and absence of the chaperone. Although SecB associates primarily with the C-terminal 22 amino acyl residues of SecA (*13, 42*), we cannot emphatically rule out the possibility that binding involves the region in which trypsin-sensitive residues, such as Arg 420, are involved (*37*) and that some stabilization of SecA occurs in the presence of SecB. Nevertheless, the above data are consistent with the hypothesis that SecB shifts the equilibrium toward the ATP-bound form of SecA, poising SecA for transfer of the preprotein and a subsequent round of ATP hydrolysis.

The dose-dependent responses of SecB and AMP-P-C-P on SecA trypsinolysis at 0 °C and at 90 min of treatment are shown in Figure 3. The intensity of the 46 kDa fragment is dependent on the concentrations of both AMP-P-C-P (Figure 3A) and SecB (Figure 3B), with maximal responses observed at 5 mM and 80 μg, respectively. The synergistic effect of both SecB and AMP-P-C-P is striking (Figure 3C,D). About a 4-fold increase in the intensity of the 46 kDa fragment was observed when 1 mM AMP-P-C-P and 80 μg of SecB are both present in the reaction mixture (Figure 3D). No significant 46 kDa fragment was observed with either 5 mM ATP (which can be hydrolyzed to ADP) or 5 mM ADP (data not shown).

Since the above data suggest that SecB binding favors the nonhydrolyzed ATP-bound form of SecA, we considered that SecB would also promote the release of ADP. The effects of SecB and AMP-P-C-P on the binding and release of ADP from SecA were evaluated by equilibrium dialysis at 4 °C, and the results are shown in Figure 4. In the presence of 340 μM AMP-P-C-P, a low level of ADP release from SecA was apparent. However, upon addition of SecB (30 μM), a greater than 2-fold stimulation in nucleotide release was observed. In comparison, no significant SecB-induced release of ADP was observed when 0.13 μM AMP-P-C-P was used. When the same amount of ADP was used instead of AMP-P-C-P, no SecB-induced ADP release was detected (data not shown). It should be emphasized that ATP and ADP bind to SecA with essentially the same affinity while that for

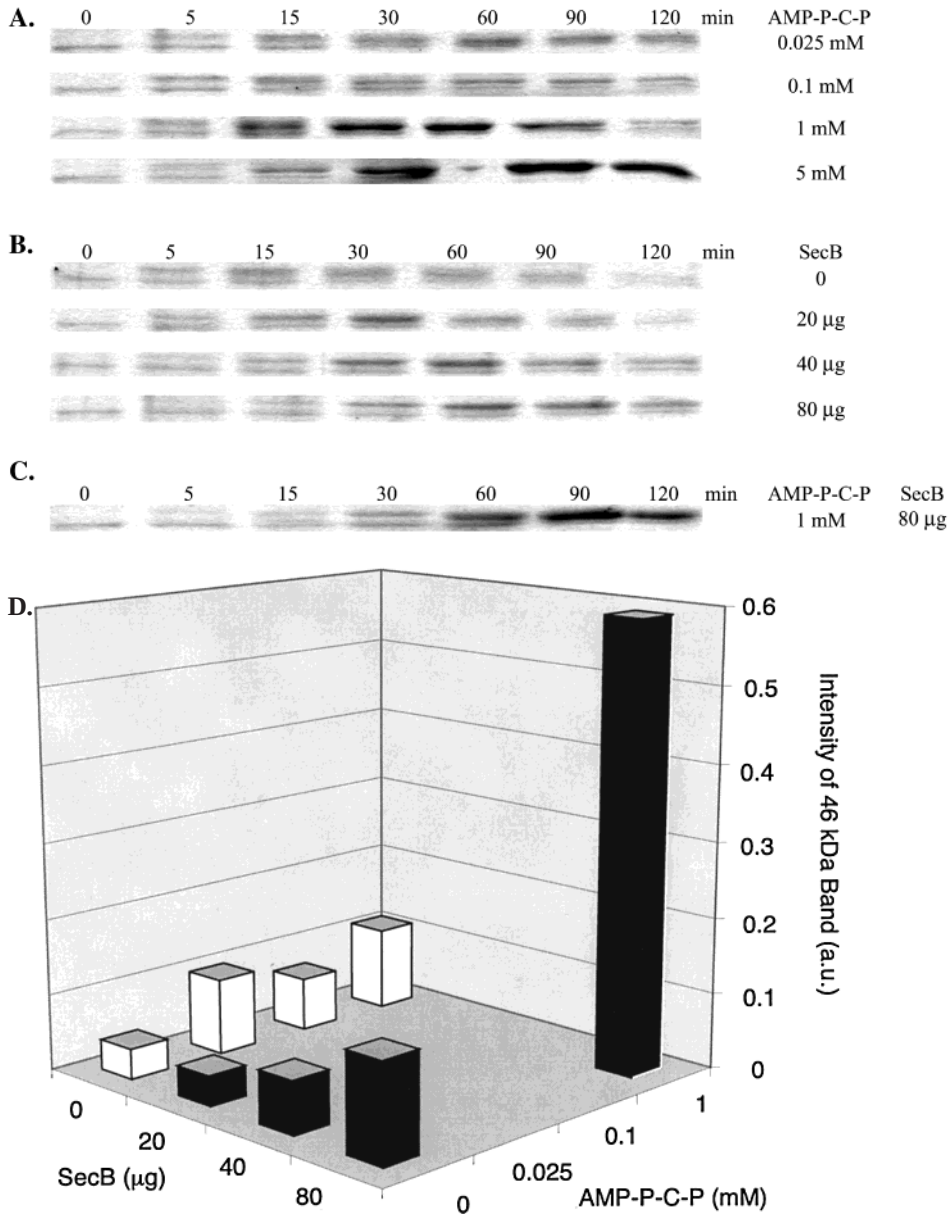


FIGURE 3: Effects of AMP-P-C-P and SecB concentrations on the 46 kDa SecA fragment from trypsinolysis at 0 °C. Trypsin digestion was conducted as described under Experimental Procedures. The time of digestion (min) is given at the top of panels A, B, and C. The enzyme to SecA ratio used was the same as given in Figure 1. SDS−PAGE was done on either 10% or 12.5% polyacrylamide mini-gels. Data of the 90 min values from densitometric scanning of the gel profiles shown in panels A, B, and C are presented in panel D. In panel A, no sample was loaded on the gel at 60 min in the presence of AMP-P-C-P (5 mM).

AMP-P-N-P is approximately 100-fold lower (*43*), which is similar to that estimated for AMP-P-C-P (data not shown). This would explain why, at equilibrium, not all of the bound ADP is released from NBD-I in the presence of 340 μM AMP-P-C-P (Figure 4). Other factors that have been implicated in ADP release from secretory components are $\Delta\mu_{H^+}$, a high concentration of ATP or an ATP-generating system (*44*), preproteins (*45*), synthetic signal peptides (unpublished results), and anionic phospholipids (*46*). An enhanced ADP release would allow increased ATP binding and turnover.

To examine the latter possibility, kinetic studies on the effect of SecB on the multiple-turnover ATPase activity of SecA in phospholipid vesicles were conducted. As shown in Figure 5, the V_{max}, but not the K_M, was increased in the presence of SecB. Using an ATP concentration range of 10 μM−2 mM, the V_{max} was found to be 30% higher in the presence of SecB (Figure 5A). It appears that the SecB effect is exhibited when a majority of the low-affinity binding sites are occupied; for example, 75% at 1 mM ATP, 85% at 2 mM ATP (*24*). Under these conditions, the high-affinity binding sites were fully saturated. To investigate the potential involvement of the high-affinity binding sites in the SecB effect (Figure 5B), an ATP concentration of 20 nM−2 μM was used, necessitating the use of [γ-^{32}P]ATP and 4 nM SecA to make accurate determinations. In the presence of SecB and phospholipid vesicles, the V_{max} increase was more substantial, over 2-fold, indicating a more pronounced affect of the chaperone on the high-affinity binding site. In this experiment, essentially none of the low-affinity binding sites should have been occupied since the highest concentration of substrate used was only 2 μM.

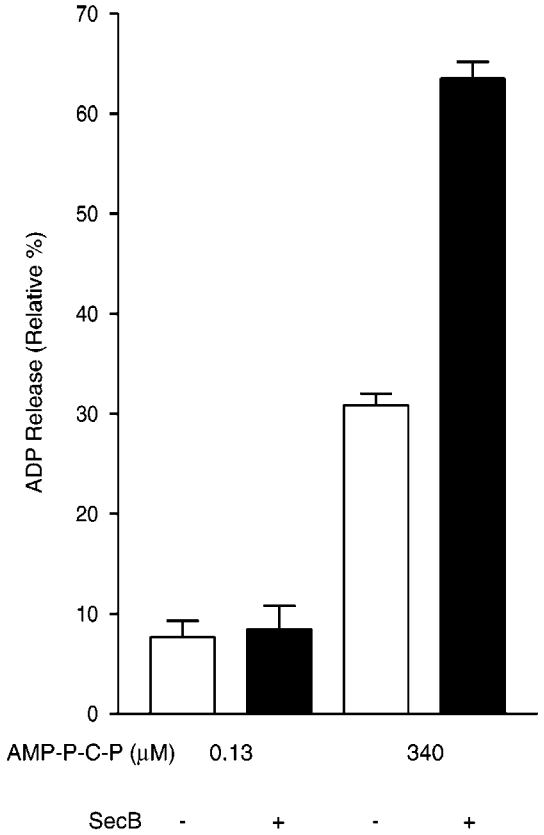
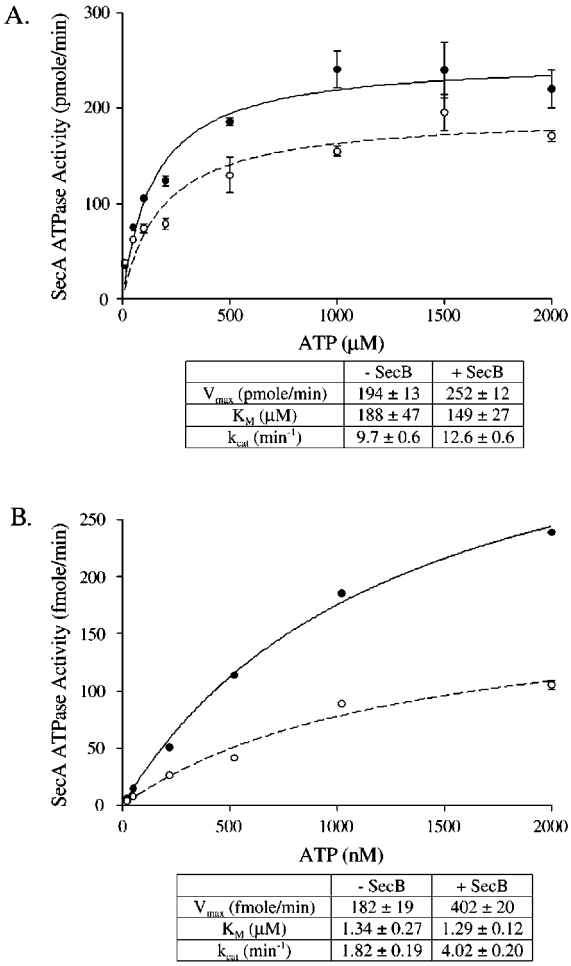


FIGURE 4: SecB-induced release of ADP from SecA in the presence of AMP-P-C-P. The equilibrium dialysis conditions used are described under Experimental Procedures. SecA monomer (1 μM) was equilibrated with either 0.13 or 0.25 μM [^{3}H]ADP at 4 °C overnight. After addition of either 0.13 or 340 μM AMP-P-C-P and further equilibration, SecB (final concentration 30 μM monomer) was added. Aliquots from each chamber, separated by a 12 000−14 000 MWCO dialysis membrane, were analyzed for radioactivity as described. The amounts of free [^{3}H]ADP prior to the addition of AMP-P-C-P and SecB were set as 100%.



	- SecB	+ SecB
V_{max} (pmole/min)	194 ± 13	252 ± 12
K_M (μM)	188 ± 47	149 ± 27
k_{cat} (min^{-1})	9.7 ± 0.6	12.6 ± 0.6

	- SecB	+ SecB
V_{max} (fmole/min)	182 ± 19	402 ± 20
K_M (μM)	1.34 ± 0.27	1.29 ± 0.12
k_{cat} (min^{-1})	1.82 ± 0.19	4.02 ± 0.20

FIGURE 5: Kinetics of the SecA ATPase reaction in the presence and absence of SecB. The enzyme assays were conducted as described under Experimental Procedures. All reactions contained 50 mM Hepes−KOH (pH 7.5), 30 mM KCl, 30 mM NH_4Cl, 0.5 mM magnesium acetate, 1 mM DTT. SecA monomer (0.4 μM), *E. coli* phospholipids (320 μg/mL), and SecB monomer (10 μM) when desired were used with ATP (10−2000 μM) in the reaction (50 μL) (panel A). The kinetics of the high-affinity binding site (panel B) were evaluated using [γ-^{32}P]ATP as described under Experimental Procedures. Briefly, ATP (20−2000 nM), SecA (4 nM), phospholipids (3 μg/mL), and SecB (1 μM) when needed were included in the reaction (25 μL). Each data point represents an average of triplicate assays ± SE. Open circles, SecA alone; filled circles, SecA plus SecB. The V_{max} values are reported in pmol of Pi/min (panel A) or fmol of Pi/min (panel B).

It has been well documented that NaN_3 inhibits SecA function and is an effective inhibitor of preprotein translocation both in vivo and in vitro (*47*, *48*). Using IMVs prepared from a SecYEG-overexpressing *E. coli* strain, the SecA ATPase activity in the presence of synthetic wild-type signal peptide was inhibited by NaN_3 in a concentration-dependent manner (Figure 6A). At a NaN_3 concentration of 20 mM, the SecA activity was inhibited 26% in the presence of SecB, but, interestingly, only a 9% reduction in activity was observed when SecB was absent. Furthermore, the SecB stimulatory effect on SecA was also reduced by the presence of NaN_3; i.e., 20 mM NaN_3 induced a 39% decrease in the SecB enhancement in SecA ATPase activity relative to that in the absence of azide. In contrast, when liposomes from *E. coli* phospholipid were used instead of IMVs, the SecB effect on SecA remained almost constant, regardless of the presence of NaN_3 (Figure 6B). In the liposome system, NaN_3 also showed essentially no inhibition of the SecA activity, which is consistent with a previous report using large unilamellar vesicles composed of *E. coli* phospholipids and preprotein in the translocation ATPase assay (*49*). It is clear that the NaN_3 effect on the ability of SecB to stimulate SecA activity is specific for SecA that is associated with SecYEG, and it is not derived from another component associated with IMVs since the endogenous ATPase activity of the membrane was comparatively very low.

DISCUSSION

SecA, the pivotal component of *E. coli* translocase, is the molecular motor that powers the membrane translocation of preproteins. Previous studies have shown that its ATPase activity is crucial for the protein translocation process and is modulated by multiple biological factors (*19*, *21*, *22*). As shown in Table 1 and Figure 6, there is also a consistent increase in SecA activity induced by SecB under different conditions. Since, in our study, there was a lack of preprotein mature region to interact with SecB, and because SecB does not specifically recognize signal peptide (*39*), the observed effect may reflect a role of the protein that is different from the chaperone and membrane-targeting functions previously

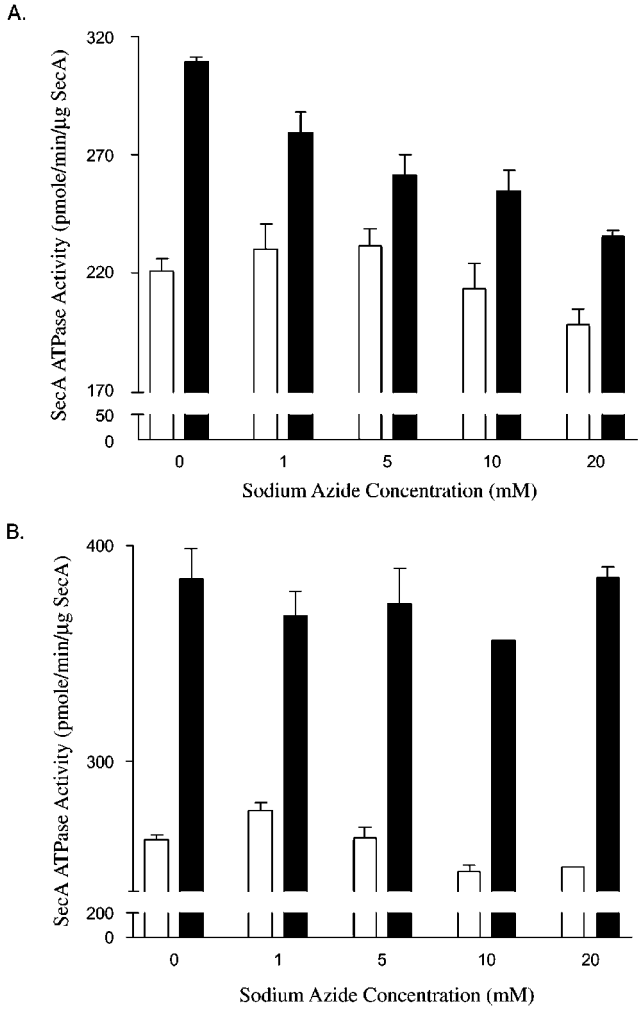


FIGURE 6: Sodium azide inhibition of SecA ATPase activity. All assays (total volume 25 μL) were conducted in 50 mM Hepes−KOH (pH 7.5), containing 30 mM KCl, 30 mM NH_4Cl, 0.5 mM magnesium acetate, 1 mM DTT, SecA monomer (0.4 μM), wild-type signal peptide (20 μM), SecB monomer (7 μM), and IMVs (200 μg of membrane protein/mL) (A) or 320 μg/mL *E. coli* phospholipids (B) as described under Experimental Procedures. NaN_3 was added, at the concentrations indicated, before the addition of ATP. Each data point represents an average of triplicate assays ± SE. White bar, absence of SecB; black bar, presence of SecB.

described. Clarifying the molecular basis for the SecB affect on SecA ATPase activity is needed to better understand the involvement of the chaperone in the protein transport process.

Previous studies have identified at least two distinct conformational states of SecA: a closed, compact conformation which is favored at low temperature or upon ADP binding; and a more open, elongated one which is favored at higher temperature or upon interacting with signal peptide, preprotein, anionic phospholipids, or the nonhydrolyzable ATP analogues AMP-P-N-P or AMP-P-C-P (*19, 21, 22, 24, 41, 50*). The extended conformation is considered to represent a transition form of SecA that resembles the membrane-inserted form of the protein. Since the compact ADP-bound form of SecA is the predominant species at low temperature, SecB may be needed to alter the conformation of the protein to a more extended structure which is more readily inserted into the membrane. It has previously been shown that SecB, as well as SecG, SecD, and SecF, markedly enhances protein translocation, specifically, at lower temperatures (*51*−*53*).

Sodium azide markedly inhibits Sec-dependent preprotein membrane translocation in vivo and in vitro. It has been shown that SecA translocation ATPase and in vitro translocation are azide-inhibited via blocking the SecA membrane deinsertion step, leaving SecA in the extended membrane-inserted state at SecYEG (*49*). Our data are consistent with the possibility that NaN_3 stops the deinsertion of SecA by preventing it from adopting an ADP-bound form. This is in agreement with our hypothesis that SecB poises SecA in favor of an extended form that resembles the ATP-bound conformation which would be particularly susceptible to NaN_3 inhibition. This correlates well with in vivo results previously reported (*15*) that NaN_3 sensitivity is increased for SecB-dependent PhoA mutant proteins compared to wild-type PhoA which does not require SecB for efficient protein transport.

Both the equilibrium dialysis and kinetic data are consistent with a SecB−SecA interaction resulting in an increase in the number of available ATP binding sites. Since ADP and ATP have similar affinities for SecA (*43*) and ADP release is the rate-limiting step in hydrolysis (*37*), the exchange of ADP with ATP may be too slow to support the biological function of SecA. SecB may be required to promote conversion of SecA to a form which is more accessible to ATP binding. A similar relationship has been observed for the modulation of the ATPase activity of the bacterial heat shock protein hsp70 analogue DnaK in the presence of both the DnaJ and GrpE accessory proteins (*54*). DnaJ binds to DnaK and accelerates the rate of ATP hydrolysis by increasing the V_{max} without affecting the nucleotide affinity while the subsequent binding of GrpE increases the release of bound ADP.

Since the primary SecB recognition elements within SecA reside at the C-terminal extreme (22 amino acyl residues) of the protein (*13, 42*), it is unlikely that SecB directly affects the ATP hydrolysis reaction per se which would require it to contact or interact with the NBD1 site. It is more likely that SecB stimulates SecA ATPase activity by binding to a region other than the ATP binding domain and inducing a structural change in the dimer which exposes previously hidden or buried high-affinity nucleotide binding sites. Recent studies have shown that the C-terminal domain (34 kDa) of SecA contains an intramolecular regulator of ATP hydrolysis (IRA1) which interacts with the N-domain and couples ATP binding and hydrolysis to SecA membrane insertion/deinsertion and preprotein translocation (*37*). The N-terminal domain has been reported to include a newly defined IRA2 region which overlaps with NBD2 and interacts directly with NBD1 and regulates ATPase activity by controlling ADP release and ATP hydrolysis at the high-affinity binding site (*55*). The collective data indicated that SecB may also impact ATP hydrolysis through these proposed regulatory regions. Schmidt et al. (*46*) reported that the high-affinity binding site was conformationally regulated by temperature and other factors and suggested that other Sec proteins may act in a similar manner.

In summary, in addition to its chaperone activity, membrane targeting role, and involvement in modulating SecA conformation, SecB also enhances the formation of the ATP binding form. Therefore, it appears that SecB primes SecA for membrane insertion and thus increases the efficiency of translocation activity at a critical point in the secretion

process, namely, the delivery of preprotein to membrane translocation sites.

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REFERENCES

1. Wickner, W., and Leonard, M. R. (1996) *J. Biol. Chem. 271*, 29514−29516.
2. Driessen, A. J. M., Fekkes, P., and van der Wolk, J. P. W. (1998) *Curr. Opin. Microbiol. 1*, 216−222.
3. Schmidt, M. G., and Kiser, K. B. (1999) *Microbes Infect. 1*, 993−1004.
4. Economou, A. (1999) *Trends Microbiol. 7*, 315−320.
5. Fekkes, P., and Driessen, A. J. M. (1999) *Microbiol. Mol. Biol. Rev. 63*, 161−173.
6. Kim, J., and Kendall, D. A. (2000) *Cell Stress Chaperones 5*, 267−275.
7. Valent, Q. A., Scotti, P. A., High, S., de Gier, J.-W. L., von Heijne, G., Lentzen, G., Winermeyer, W., Oudega, B., and Luirink, J. (1998) *EMBO J. 17*, 2504−2512.
8. Driessen, A. J. M. (2001) *Trends Microbiol. 9*, 193−196.
9. Diamond, D. L., and Randall, L. L. (1997) *J. Biol. Chem. 272*, 28994−28998.
10. Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J. P., and Wickner, W. (1990) *Cell 63*, 269−279.
11. Hoffschulte, H. K., Drees, B., and Müller, M. (1994) *J. Biol. Chem. 269*, 12833−12839.
12. den Blaauwen, T., Terpetschnig, E., Lakowicz, J. R., and Driessen, A. J. M. (1997) *FEBS Lett. 416*, 35−38.
13. Fekkes, P., van der Does, C., and Driessen, A. J. M. (1997) *EMBO J. 16*, 6105−6113.
14. Fekkes, P., de Wit, J. G., van der Wolk, J. P. W., Kimsey, H. H., Kumamoto, C. A., and Driessen, A. J. M. (1998) *Mol. Microbiol. 29*, 1179−1190.
15. Kim, J., and Kendall, D. A. (1998) *J. Bacteriol. 180*, 1396−1401.
16. Kumamoto, C. A., and Beckwith, J. (1983) *J. Bacteriol. 154*, 253−260.
17. Weiss, J. B., Ray, P. H., and Bassford, P. J., Jr. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8978−8982.
18. Kim, J., Miller, A., Wang, L., Müller, J. P., and Kendall, D. A. (2001) *Biochemistry 40*, 3674−3680.
19. Lill, R., Dowhan, W., and Wickner, W. (1990) *Cell 63*, 271−279.
20. Cunningham, K., and Wickner, W. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8630−8634.
21. Miller, A., Wang, L., and Kendall, D. A. (1998) *J. Biol. Chem. 273*, 11409−11412.
22. Wang, L., Miller, A., and Kendall, D. A. (2000) *J. Biol. Chem. 275*, 10154−10159.
23. Mitchell, C., and Oliver, D. (1993) *Mol. Microbiol. 10*, 483−497.
24. den Blaauwen, T., Fekkes, P., de Wit, J. G., Kuiper, W., and Driessen, A. J. M. (1996) *Biochemistry 35*, 11994−12004.
25. Ulbrandt, N. D., London, E., and Oliver, D. (1992) *J. Biol. Chem. 267*, 15184−15192.
26. Economou, A., and Wickner, W. (1994) *Cell 78*, 835−843.
27. Izard, J. W., Doughty, M. B., and Kendall, D. A. (1995) *Biochemistry 34*, 9904−9912.
28. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
29. Müller, M., and Blobel, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7421−7425.
30. de Vrije, T., Tommassen, J., and de Kruijff, B. (1987) *Biochim. Biophys. Acta 900*, 63−72.
31. Dapic, V., and Oliver, D. (2000) *J. Biol. Chem. 275*, 25000−25007.
32. Cunningham, K., Lill, R., Crooke, E., Rice, M., Moore, K., Wickner, W., and Oliver, D. (1989) *EMBO J. 8*, 955−959.
33. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.
34. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem. 100*, 95−97.
35. Miller, J. D., Berstein, H. D., and Walter, P. (1994) *Nature 367*, 657−659.
36. Price, A., Economou, A., Duong, F., and Wickner, W. (1996) *J. Biol. Chem. 271*, 31580−31584.
37. Karamanou, S., Vrontou, E., Sianidis, G., Baud, C., Roos, T., Kuhn, A., Politou, A. S., and Economou, A. (1999) *Mol. Microbiol. 34*, 1133−1145.
38. Laemmli, U. K. (1970) *Nature 227*, 680−685.
39. Randall, L. L., Topping, T. B., and Hardy, S. J. S. (1990) *Science 248*, 860−863.
40. Akita, M., Sasaki, S., Matsuyama, S., and Mizushima, S. (1990) *J. Biol. Chem. 265*, 8164−8169.
41. van der Does, C., Manting, E. H., Kaufmann, A., Lutz, M., and Driessen, A. J. M. (1998) *Biochemistry 37*, 201−210.
42. Breukink, E., Nouwen, N., van Raalte, A., Mizushima, S., Tommassen, J., and de Kruijff, B. (1995) *J. Biol. Chem. 270*, 7902−7907.
43. Matsuyama, S., Kimura, E., and Mizushima, S. (1990) *J. Biol. Chem. 265*, 8760−8765.
44. Shiozuka, K., Tani, K., Mizushima, S., and Tokuda, H. (1990) *J. Biol. Chem. 265*, 18843−18847.
45. Shinkai, A., Mei, L. H., Tokuda, H., and Mizushima, S. (1991) *J. Biol. Chem. 266*, 5827−5833.
46. Schmidt, M., Ding, H., Ramamurthy, V., Mukerji, I., and Oliver, D. (2000) *J. Biol. Chem. 275*, 15440−15448.
47. Oliver, D., Cabelli, R. J., Dolan, K. M., and Jarosik, G. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8227−8231.
48. Rusch, S. L., and Kendall, D. A. (1994) *J. Biol. Chem. 269*, 1243−1248.
49. Eichler, J., Rinard, K., and Wickner, W. (1998) *J. Biol. Chem. 273*, 21675−21681.
50. Ding, H., Mukerji, I., and Oliver, D. (2001) *Biochemistry 40*, 1835−1843.
51. Nishiyama, K. I., Hanada, M., and Tokuda, H. (1994) *EMBO J. 13*, 3272−3277.
52. Pogliano, J. A., and Beckwith, J. (1994) *EMBO J. 13*, 554−561.
53. Shimizu, H., Nishiyama, K., and Tokuda, H. (1997) *Mol. Microbiol. 26*, 1013−1021.
54. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2874−2878.
55. Sianidis, G., Karamanou, S., Vrontou, E., Boulias, K., Repanas, K., Kyrpides, N., Politou, A. S., and Economou, A. (2001) *EMBO J. 20*, 961−970.

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