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Maximal Efficiency of Coupling between ATP Hydrolysis and Translocation of Polypeptides Mediated by SecB Requires Two Protomers of SecA

Running title: Maximal Efficiency Requires Two Protomers of SecA

Chunfeng Mao, [1] Simon J. S. Hardy, [1,2] and Linda L. Randall [1,*]

[1]Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA

[2]Department of Biology, University of York, York YO10 5DD, UK

[*]Correspondence: 117 Schweitzer Hall, University of Missouri, Columbia, MO 65211, USA; craneje@missouri.edu; Tel: 573-884-4160; Fax: 573-882-5635

ABSTRACT

SecA is the ATPase that provides energy for translocation of precursor polypeptides through the SecYEG translocon in *Escherichia coli* during protein export. We have previously shown that when SecA receives the precursor from SecB the ternary complex is fully active only when two protomers of SecA are bound. Here we have used variants of SecA and of SecB that populate complexes containing two protomers of SecA to different degrees to examine both the hydrolysis of ATP and the translocation of polypeptides. We conclude that the low activity of the complexes with only one protomer is the result of a low efficiency of coupling between ATP hydrolysis and translocation.

INTRODUCTION

The general secretory or Sec system in *E. coli* translocates precursors of proteins across the cytoplasmic membrane into the periplasmic space (for review see (21)). Some proteins, such as alkaline phosphatase and the binding proteins for amino acids and sugars, function as soluble proteins in the periplasm; others, such as OmpA, are inserted into the outer membrane. The path across the cytoplasmic membrane barrier is provided by a translocation channel comprising a heterotrimeric complex, SecY, SecE, and SecG (SecYEG). The energy for the movement is supplied by protonmotive force and the hydrolysis of ATP by SecA, which is a peripheral component of the membrane-associated translocon. The Sec system can export polypeptides only if they are devoid of stable tertiary structure. SecB, a small cytosolic chaperone, acts to capture precursors before they acquire stable structure and introduces them into the secretory pathway by delivering them to SecA. SecA can also bind precursors directly as evidenced by the viability of strains of *E. coli* that lack SecB. However, efficiency of export is drastically reduced for many proteins (13).

Crystal structures of both SecB and SecA have been solved. SecB is a tetramer (monomer, 17 kDa) organized as a dimer of dimers (6, 33). There are six dimeric forms of SecA (2, 11, 20, 27, 30, 34) that differ greatly in the contacts that stabilize the dimeric interface. However, the structures of the protomers are all closely related and display only two different conformations, an open state (18) and a closed state (11). In free solution SecA undergoes an equilibrium between monomer and dimer characterized by an equilibrium constant of 0.1 μM to 1 μM depending on the ionic strength and temperature of the solution (31). When SecA interacts with

SecB, two protomers must be bound to SecB for the complex to be active as assessed *in vitro* (24).

Here we have asked why two protomers of SecA are required. We have examined the translocation into inverted cytoplasmic membrane vesicles of two natural ligands, one a soluble periplasmic protein, galactose-binding protein, and the other an outer membrane protein, OmpA. We conclude that the need for two protomers of SecA in the complex is to achieve maximal coupling efficiency between ATP hydrolysis and translocation.

MATERIALS AND METHODS

Materials

[γ-^{32}P] ATP was purchased from either PerkinElmer (Boston, MA) or GE Healthcare (Pittsburgh, PA), [^{35}S]- methionine from PerkinElmer, pre-coated polyethyleneimine cellulose thin layer chromatography plates from Merck KGaA (Darmstadt, Germany), trypsin from Millipore Corporation (Freehold, NJ), bovine pancreatic trypsin inhibitor (aprotinin), and deoxyribonucleaseI (DNaseI) from Sigma-Aldrich (St.Louis, MO), NAP^{TM}10 and HiTrap^{TM} Blue HP columns from GE Healthcare Bio-sciences AB (Uppsala, Sweden), N-[(2-pyridyldithio)ethyl]-4-azidosalicylamide (AET) from Toronto Research Chemicals Inc. (Canada), Precision Plus Protein^{TM} Standards (all blue) from Bio-Rad (Hercules, CA) and *Staphylococcus aureus* micrococcal nuclease from Worthington (Lakewood, NJ).

Protein Purification

SecA, SecAC4, SecAdN7, SecAdN10, SecB, and SecBL75Q were purified from strains of *E.coli* harboring plasmids that express the proteins as previously described (24, 25) except that cells expressing wild-type SecA and those expressing SecAdN10 were suspended at 0.5 gram wet weight of cell pellet per mL of buffer and disrupted using a French Press at 8000 psi. The P11 column was omitted from the SecAdN10 purification. For cross-linking experiments, SecAC4S350C and SecAC4I641C were purified from strains harboring plasmids derived from plasmid pT7*secAC4* that carries a gene for SecA in which the four native cysteine residues were substituted by serine (23). For each SecA species a single cysteine was introduced at the site of interest by site-directed mutagenesis (Quickchange, Stratagene). Proteins were purified as described (24) with minor changes. Micrococcal nuclease (314 units/mL final) was included along with DNaseI (5000 units/mL final) to degrade nucleic acids and decrease the viscosity of the lysate. SecAC4S350C was purified by chromatography on a HiTrap QAE column followed by a HiTrap Blue™ HP instead of P11. For purification of SecAC4I641C only a HiTrap Blue™ HP column was used. Precursor galactose-binding protein was purified as described (29). The precursor of OmpA labeled with [^{35}S] –methionine was produced from strains harboring plasmid pET503, which encodes proOmpA with the substitution C290S, and was purified as described (7). The culture was grown in the M9 minimal medium supplemented with glycerol (0.4% w/v) as carbon source, thiamine (4 μg/mL) and ampicillin (0.1 mg/mL). When the culture reached an optical density of 0.6 at 560 nm, IPTG was added to 0.1 mM to induce proOmpA. Eighty minutes after induction, 1 mCi [^{35}S] – methionine (90 μL) mixed with 900 μL of 30 μM nonradioactive methionine was added to the culture. For use in the cross-linking experiments a species of proOmpA with both native cysteines changed to serine, C290S and C302S, was

constructed by site-directed mutagenesis (Quickchange, Stratagene) and purified as described (7). The precursors were stored in buffers containing denaturant: 1 N guanidine hydrochloride (GuHCl), 10 mM HEPES-KOH, pH 7.6, 0.3 M KOAc, 5 mM $Mg(OAc)_2$, 1 mM EGTA for precursor galactose-binding protein and 4 M urea, 10 mM HEPES-KOH, pH 7.6, 0.1 M KOAc for proOmpA. All proteins were stored at -80ºC.

Cytoplasmic Membrane Vesicles

Inverted cytoplasmic membrane vesicles were prepared as described (32) from *E.coli* strain HB3616, harboring plasmids that express SecE and SecG (pMAN809 with *tac-SecG* insertion (16)) and SecY (pMAN510 (16)) under the control of the *tac* promoter, except that to induce the proteins IPTG was added to 1 mM. In order to remove endogenous SecA the isolated vesicles were exposed to 5 M urea in 50 mM TrisCl, pH 8, 2 mM dithiothreitol (DTT) on ice for 30 min, centrifuged (65000 rpm, 30 min, 4°C, type 65 rotor, Beckman Coulter, Fullerton, CA), and suspended in 10 mM HEPES-KOH, pH 7.6, 0.3 M KOAc, 5 mM $Mg(OAc)_2$, 2 mM DTT. The vesicles were tested for translocation of precursors and for ATPase activity in the absence of added SecA. There was no protection of either precursor ligand and no detectable hydrolysis of ATP within the first 2 minutes. All calculations of efficiency of coupling were done within this time frame.

Translocation and ATPase Assays

Both assays were done in the same reaction mixture, which was made up in glass tubes (12 x 75 mm) so that temperature equilibration would occur rapidly. All mixtures contained 13 mM HEPES-KOH, pH 7.6, 250 mM KOAc, 5 mM $Mg(OAc)_2$, 2 mM DTT, 3.3 mM [γ-^{32}P] ATP

(specific activity: 1.8 Ci/mole), and 2 μM SecA dimer. When specified, SecB was added to 2 μM tetramer and urea-treated inverted membrane vesicles to a final concentration of 0.6 mM lipid. The reactions were initiated by dilution of precursor (precursor galactose-binding protein or [^{35}S]-methionine proOmpA) to 2 μM from denaturant and the glass tubes were immediately transferred to a water bath at 30°C. For reaction mixtures containing precursor galactose-binding protein the final concentration of GuHCl was 12 mM and for those containing proOmpA the final urea concentration was 36 mM. For the experiments in which we determine the ratio of ATP hydrolysis to translocation we do not provide NADH or an ATP regenerating system because we are measuring hydrolysis of ATP using [γ-^{32}P] ATP.

For assessment of both translocation and ATPase activity from the same reaction mixture, samples of 10 μL (from a total mixture of 100 μL) were taken into tubes held on ice at times as indicated, from which samples of 2 μL were immediately removed to tubes with 2 μL 0.1 M EDTA on ice for the ATPase assay. To assess translocation, trypsin (5 mg/mL in 1 mM HCl) was added to a final concentration of 0.5 mg/mL to the tubes containing 8 μL reaction mixture and the samples were incubated for 15 min on ice at which time proteolysis was stopped by addition of bovine pancreatic trypsin inhibitor (44 mg/mL in H_2O) to a final concentration of 5 mg/mL. For determination of the total amount of precursor added to the assay, 10 μL samples were taken at time points 1 min and 6 min and no trypsin was added. Non-reducing SDS gel sample buffer containing N-ethylmaleimide (8 mM) was added and the samples were boiled immediately for 3 min and analyzed by SDS polyacrylamide gel electrophoresis on the same day to avoid sample degradation.

Analyses by SDS Gel Electrophoresis, Immunoblotting and Thin Layer Chromatography

Polyacrylamide (10%, w/w) gels for experiments with [^{35}S] -methionine labeled proOmpA were dried and exposed to an imaging plate (Fuji Film, Stamford, CT) overnight, scanned using a Phosphor Imager (Fuji Film, Stamford, CT), and analyzed with ImageGauge 4.0 (Fuji Film, Stamford, CT). Immunoblots of 10% w/w gels were used to analyze all precursor galactose-binding protein experiments and the experiments presented in Fig. 3. Blots were processed by incubation with a rabbit antiserum raised to appropriate purified protein, then with goat antibodies raised to rabbit IgGs and conjugated with horseradish peroxidase (Bio-Rad), followed by staining with a 4-chloro-1-naphthol/hydrogen peroxide solution. All samples for precursor galactose-binding protein were electrophoresed on the same gel as the purified precursor applied in quantities of 5 ng, 10 ng, and 20 ng to generate a standard curve. The amount of protein at each time point was determined using only those intensities that were within the linear range of the standard. A Kodak EDAS 290 digital camera was used to capture images of the immunoblots and TotalLab software (version 2.01; Nonlinear Dynamics Ltd.) was used to quantify the band intensities. The concentration of the precursor added to the assay in combination with the percentage of protein protected from trypsin digestion was used to determine the concentration of precursor protected for both ligands.

Thin layer chromatography was used to analyze the hydrolysis of ATP. One microliter of the samples taken into EDTA for the ATPase assay as described above was applied to a pre-coated thin layer chromatography plate and dried. After application of all samples the plate was developed in 125 mM KH_2PO_4 (14). After drying, the plates were exposed and scanned using the

Phosphor Imager. The ATPase activity was estimated from the proportion of total radioactivity that migrated as inorganic phosphate (R_f ~ 0.59).

Calculation of the Efficiency of Coupling ATP Hydrolysis to Translocation

The coupling of the hydrolysis of ATP to the translocation of precursor polypeptides was calculated using time points taken within the first 2 minutes. Early time points were used for two reasons. Firstly, ADP has higher affinity for SecA than does ATP (9). Therefore, the accumulation of ADP at later times would be expected to suppress activity. We did not include an ATP regenerating system since we use the appearance of ^{32}P-phosphate as the assay for hydrolysis. Secondly, as the process of translocation approaches a plateau the translocation ATPase activity will be replaced by membrane ATPase activity since SecA without precursor bound will still bind SecYEG. This would result in false values for the coupling.

Cross-linking with a Photoactivatable Reagent

Each of the SecA variants, SecAC4S350C and SecAC4I641C, has a single cysteine as specified. Each variant was labeled with the sulfhydryl specific photoactivable reagent, N-[(2-pyridyldithio)ethyl]-4-azidosalicylamide (AET) (1). The protein to be labeled was exchanged into 100 mM $Na_2B_4O_7$, 100 mM KOAc, pH 8.3 using a NAP^{TM}10 column. All subsequent steps were done in the dark. AET (stored at 45 mM in DMSO at -80ºC) was added at a 10-fold molar excess over cysteine in the SecA and the mixture was incubated for 2 hours at room temperature followed by 1 hour on ice. Free AET was removed by exchange of the protein into 10 mM HEPES-HOAc, pH 6.7, 300 mM KOAc, 5 mM $Mg(OAc)_2$ using a NAP^{TM}10 column. The AET modification was confirmed by MALDI mass spectrometry. Mixtures (~ 30 μL) of the AET-

labeled SecA (12 μM dimer) and precursor (either precursor galactose-binding protein or proOmpA at 12 μM) in 10 mM HEPES-HOAc, pH 6.7, 300 mM KOAc, 5 mM $Mg(OAc)_2$ were prepared and placed in the shallow spots of a porcelain spot plate held on ice and irradiated with a mercury lamp for 1 min. Samples were analyzed by SDS polyacrylamide (10%, w/w) gel electrophoresis using both reducing and non-reducing sample buffers. Gels (both reduced and non-reduced samples) were run in duplicate and subjected to immunoblotting using antisera to purified SecA as well as to the relevant precursor.

Characterization of Protein Preparations and Determination of Lipid Concentration

Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficients of 47,600 $M^{-1}cm^{-1}$ for SecB tetramer, 157,800 $M^{-1}cm^{-1}$ for SecA dimer, 52,955 $M^{-1}cm^{-1}$ for proOmpA and 37,410 $M^{-1}cm^{-1}$ for precursor galactose-binding protein. Since modification by AET changes the absorbance of the SecA variants, those concentrations were determined by electrophoresis of at least three samples of the protein in increasing quantity on the same SDS polyacrylamide (14%, w/w) gel as three quantities of pure SecA of known concentration to generate a standard curve. The amount of protein in the standards and the variants was determined as described for quantification of protein on immunoblots. Concentration of lipid in the vesicle preparation was determined as described on the Avanti Polar Lipids website (www.avantilipids.com) using an average molar mass for *E.coli* lipids of 741 Da.

All protein preparations used in this study were subjected to rigorous characterization. They were analyzed by mass spectrometry. The only degradation detected was in SecAdN10 where approximately 10% of the protein was cleaved to generate two fragments of 67,000 Da and

33,000 Da. The ratio of absorbance at 280 nm to 260 nm for all preparations was 2.0 except for SecAC4, which was 1.8, thus there is no significant contamination by nucleic acid or nucleotides. Column chromatography used with a static light scatter detector and analytical centrifugation showed that the protein preparations contained no aggregation and formed the expected complexes with stoichiometry of either A1:B4 or A2:B4 complex consistent with our previously published results (24). The purity of all preparations was between 80% and 90%.

RESULTS

Translocation of Precursors and ATP Hydrolysis Mediated by SecA Variants with Different Dimer Equilibria

Complexes between wild–type SecA and SecB as well as between variants were assayed for both translocation of precursors and the associated translocation ATPase activity to determine the effect of varying the number of protomers of SecA in the complex. The species of SecA studied were wild-type SecA and three variants of SecA that have been characterized previously (5, 24), SecAdN10 (amino acids 2 through 11 deleted), SecAdN7 (amino acids 2 through 8 deleted) and SecAC4, which lacks zinc. In addition to the complex between wild-type SecA and SecB we studied a complex containing a SecB variant, SecBL75Q, which carries a substitution that interferes with binding the zinc site on SecA. In earlier work (24) we used size-exclusion chromatography coupled with static light scatter to demonstrate that the complex between SecB and SecA has a mass of 272 kDa, indicating that the stoichiometry of the wild-type complex is two protomers of SecA (mass, 204 kDa for dimer) bound to a tetramer of SecB (mass, 69 kDa), referred to hereafter as A2:B4. It is possible to populate complexes containing only one protomer of SecA because although SecA and SecB both display two-fold symmetry the A2:B4 complex is

stabilized by contacts that are distributed asymmetrically. Only one protomer of SecA can bind if the area of contact that is between residues in the β-sheets that form the flat sides of SecB and the C-terminal zinc domain of SecA is eliminated. Here we have examined complexes that have that contact disrupted in two different ways: wild-type SecA bound to a mutant of SecB that has a residue located on the flat β-sheet changed, SecBL75Q, and wild-type SecB bound to a SecA variant, SecAC4, that lacks zinc because the cysteines that coordinate the zinc are replaced by serines. Both complexes were shown to have a stoichiometry of A1:B4 (24), i.e. a molar mass of 171 kDa.

Complexes with a stoichiometry of A1:B4 can also be populated by using species of SecA that have the monomer – dimer equilibrium altered by truncation at the N-terminus. Deletion of amino acids 2 through 8, SecAdN7, shifts the equilibrium toward monomer (K_d ~ 24 μM) (5), whereas deletion of amino acids 2 through 11 results in a species, SecAdN10, that exists only as a monomer (K_d > 230 μM) (5). SecAdN10 was shown to form a complex with SecB of mass 169 kDa corresponding to a stoichiometry of A1:B4 (24), whereas the mass observed for a mixture of SecAdN7 and SecB was 235 kDa indicating an equilibrating population that contains both A1:B4 and A2:B4. It is not possible to estimate the amount of any one species in such a mixture because it would contain not only complexes with stoichiometry A1:B4 and A2:B4 but also free SecA which would itself be in equilibrium between monomer and dimer.

The various combinations of SecA and SecB displaying different equilibrating mixtures of complexes with A1:B4 and A2:B4 stoichiometries were tested for the translocation of precursor into inverted membrane vesicles and for the coupled hydrolysis of ATP. Translocation of the

precursor of the outer membrane protein OmpA, proOmpA, mediated by SecAdN10:SecB complexes (A1:B4) is 22% of that mediated by complexes containing wild-type SecA (A2:B4), 0.19 μM versus 0.86 μM, respectively at the 6 min time point; whereas ATP hydrolysis is 52% of the activity of the wild-type complex (Figs. 1A and 1B). Similar results are seen for translocation of precursor galactose-binding protein (Figs. 2A and 2B); translocation of precursor by the SecAdN10:SecB complex is approximately 24% of that mediated by a wild-type complex, whereas ATP hydrolysis is 37% of wild-type activity. These data indicate that the efficiency of coupling of hydrolysis of ATP to movement of the precursor through the translocon is much poorer for the SecA:SecB complex which has a stoichiometry of A1:B4 than it is for complexes having two protomers bound to SecB as in the wild-type complexes. The translocation activity of complexes containing SecB and SecAdN7 (Figs. 1A and 2A) lies between the activity of wild-type complexes and that of complexes containing SecAdN10. As described above, the equilibrating population of complexes between SecAdN7 and SecB contains both A1:B4 and A2:B4; therefore, we conclude that shifting the equilibrium to complexes containing two protomers of SecA results in more robust translocation. In addition the efficiency of coupling of ATP hydrolysis to translocation is improved. The data in Figures 1 and 2 were used to calculate the efficiency of coupling of hydrolysis of ATP to translocation of a precursor polypeptide (Table 1, See Materials and Methods for calculations). The efficiency of coupling for proOmpA was the highest for the A2:B4 wild-type complex which hydrolyzed approximately 2500 moles of ATP per mole of precursor protected. Complexes with SecAdN7, which contain a mixture of A1:B4 and A2:B4, displayed a coupling of 3600 moles ATP hydrolyzed per mole precursor protected and the lowest efficiency was observed with A1:B4 complexes: SecAdN10 and SecB, zincless SecAC4 and SecB, and SecA and SecBL75Q. The coupling efficiency for precursor

galactose binding protein showed the same trend, most efficient for wild-type complexes, intermediate for complexes formed with SecAdN7 and lowest for those complexes which populate only A1:B4 complexes (Table 1).

Since the stoichiometry between SecA and SecB has been varied using both full length SecA as well as a truncated species and wild-type SecB as well as a variant of SecB, it is safe to conclude that the observed decrease in efficiency of the coupling of ATP hydrolysis to translocation results from a decrease in occupancy of complexes having a stoichiometry of A2:B4 and not from either the truncations of SecA or the mutation of SecB.

Stoichiometry of Complexes between SecA and Precursors

Although SecA mediates a higher level of translocation when it receives precursors via a SecB:precursor complex as opposed to binding precursor directly, it does function *in vitro* in the absence of SecB (Fig. 3). SecAdN10, which is monomeric shows no detectable processing in the absence of SecB; whereas SecAdN7, which does populate dimer but to a lesser extent than does wild-type (24), shows a low level of translocation which is greatly enhanced by addition of SecB. SecA can mediate translocation in the absence of SecB *in vivo* as evidenced by the viability of SecB null strains (13). Therefore, it is of interest to determine the stoichiometry of a complex between SecA and precursors in the absence of SecB. We have not been able to demonstrate a complex between SecA and precursors using size exclusion chromatography; therefore, we can not use the approaches we have applied previously to reveal stoichiometry by determination of molar mass using static light scatter (24). As an alternative we have used cross-linking. We chose a photoactivatable cross-linking reagent, N-[(2-pyridyldithio)ethyl]-4-

azidosalicylamide (AET) to determine whether one protomer of SecA or two interact with one precursor polypeptide. The reagent was attached via a disulfide exchange reaction to two SecA variants, each having a single cysteine, one at position 350 and the other at position 641. These sites were identified as contact sites between SecA and its precursor ligands by site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy (3). Irradiation of solutions that contained complexes between the derivatized SecA variants and either proOmpA or precursor galactose-binding protein generated covalent linkages between the proteins. Analyses of the irradiated samples by SDS polyacrylamide gel electrophoresis followed by immunoblotting using antisera to SecA and to each of the precursors revealed that the complex formed between SecA and either proOmpA or precursor galactose-binding protein had a molar mass of approximately 250 kDa (Fig. 4). This was true whether the reagent was linked to SecA at amino acid position 350 or at position 641. Addition of reducing agent to the SDS gel sample buffer caused disappearance of the high molecular weight cross-linked species (data not shown) as expected since the AET moiety is linked to SecA via a disulfide bond. The ability of the AET-labeled SecAC4I641 to cross-link to SecB was tested as a control for specificity. EPR studies (3) show that SecAC4I641 is not a site of contact within the SecA-SecB complex. As expected no cross-linked species was observed (data not shown). Bands migrating with an apparent mass of approximately 150 kDa that appeared upon irradiation even in the absence of a precursor ligand are likely to be cross-linked dimers of the two SecA species, SecAC4S350AET and SecAC4I641AET. The aberrant migration of the dimers can be explained because the pairs of SecA polypeptides are tethered at two internal positions thereby preventing the chain from becoming fully extended in SDS.

We conclude that each precursor species binds two protomers of SecA to give the observed mass, approximately 250 kDa (204 kDa for SecA dimer and 37 kDa for proOmpA or 36 kDa for precursor galactose-binding protein). Since chemical cross-linking results in an irreversible reaction we have no information relating to the affinity of SecA for the precursors or whether the protomers of SecA bind to the precursor sequentially or as a dimer.

DISCUSSION

We have previously shown that formation of a complex between SecA and SecB that is capable of mediating a high level of translocation of precursors requires two protomers of SecA bound to a tetramer of SecB. Within the complex there are three areas of contact. One site involves the C-terminal zinc containing region of SecA which interacts with negatively charged region on the flat β-sheets that form the sides of the SecB dimer of dimers (4). A second interaction is between the extreme C-terminal flexible tail of SecB and the amino-terminal 11 amino acids of SecA. A third area of contact that provides energy of stabilization is less defined but involves residues lying on the β-sheets of SecB as well as along the interface of the dimer of dimers (22). Even though each of the binding partners displays two-fold symmetry, the contacts between them are distributed asymmetrically. When the contact between the zinc domain on SecA and the side of SecB is disrupted or when 10 amino acyl residues are deleted from the amino terminus of SecA, only one protomer of SecA is bound yielding a complex of stoichiometry A1:B4 that displays a very low activity both *in vivo* (12) and *in vitro* (10, 24, 25, 32).

Here we have asked why two protomers of SecA must be present for full activity in the reaction cycle of SecA and SecB during translocation. We have made use of variants of both SecA and

SecB and the complexes they form, all of which are well characterized. Assays of the extent of translocation of precursors and the associated translocation ATPase activity have allowed us to establish a correlation between an increase in the efficiency of coupling of ATP hydrolysis to translocation of polypeptides and an increase in the population of complexes containing two protomers of SecA bound to SecB. We have established this correlation by varying the population of complexes that have the stoichiometry of A1:B4 and A2:B4 by using species of SecA truncated at the extreme amino terminus with wild-type and a truncated SecB, wild-type SecA with a mutant of SecB, and wild-type SecB with full-length SecA containing no zinc. In every case the higher efficiency of coupling occurs when the A2:B4 complex is more populated. The construct with amino acids 2 through 8 deleted (SecAdN7) was previously studied by Mori et al (17) under the name of SecA N-8. The two preparations differ in that our purified protein retains the N terminal methionine whereas theirs does not. It was shown that SecA N-8 was defective in protein translocation, the translocation ATPase activity and the topological inversion of SecG. The investigators concluded that the amino-terminal region of SecA is involved in functional interaction with SecG. In our work described here we have eliminated the possibility that the effects we observe are specific for deletions at the amino terminus. It seems possible that the effects observed by Mori et al (17) also result from the oligomeric state of SecA and not directly from the lack of seven amino acyl residues. Perhaps the inversion of SecG involves two protomers of SecA.

By what mechanism might two protomers of SecA increase the coupling of ATP hydrolysis to movement? Lill *et al.* (15) showed that in a system using inverted membrane vesicles, such as that we use here, SecA demonstrates a high rate of non-productive hydrolysis, hydrolyzing more

than 1000 moles of ATP per mole of precursor translocated, a level similar to that observed here for wild-type SecA (Table 1) as well as that observed by others (28). It was subsequently shown by Schiebel *et al*. (26) that the poor coupling is the result of a backward slippage of the precursor undergoing translocation. It might be that the two protomers of SecA are required to work together to prevent the backward movement. One protomer might insert a segment of the bound precursor polypeptide into the translocon channel and release it upon hydrolysis of ATP; the second protomer would remain bound to the next more distal segment of the precursor polypeptide and, if also bound to SecYEG, would prevent backward movement of the polypeptide chain. This idea is consistent with observations by others (8, 19). Osborne and Rapoport (19), based on cross-linking of SecA and proOmpA to a covalent dimeric form of SecYEG, proposed that one copy of SecYEG serves as the translocation channel and the other provides a static binding site for SecA. These authors proposed that only a single protomer of SecA is involved, but a slight modification adapts this model to the idea we have put forth here, two protomers of SecA act together to translocate precursors, one staying bound while the other releases the polypeptide into the channel and dissociates from SecYEG. Duong (8) observed association of both monomeric and dimeric SecA to the translocon using the same covalent dimeric SecYEG, but upon addition of ATP only SecA monomers remained associated.

SecA can mediate translocation of precursors through the Sec secretory pathway in the absence of SecB both *in vivo* (13) and *in vitro.* It is currently debated whether when acting in the absence of SecB, SecA functions as a monomer or dimer. Although the *in vitro* translocation activity is very low without SecB, the trend is the same as seen with SecB complexes: SecAdN7 shows very low but detectable activity whereas SecAdN10 shows none. This correlation suggests that

although monomers do function the dimers are more efficient. We have shown here that even when SecA interacts directly with precursors two protomers can associate with each precursor polypeptide. Since it is unlikely that SecA has a different mechanism of coupling hydrolysis to movement of the polypeptide through the translocon in the presence and absence of SecB, the presence of two protomers might act together as proposed here to prevent backward slippage.

We conclude that maximal efficiency of translocation is achieved when two protomers of SecA in complex with SecB act together. A single protomer of SecA can perform the necessary conversion of chemical energy (hydrolysis of ATP) to mechanical work (movement of the precursor through the translocon), but tight coupling of this conversion requires two protomers. An exciting direction for future research is elucidation of the movement within SecA at the resolution of aminoacyl sidechains that transduces the chemical energy of hydrolysis of ATP to insertion of precursors into and through the translocon.

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FIGURE LEGENDS

Figure 1. Translocation of proOmpA mediated by A1:B4 and A2:B4 complexes. A. Protection of proOmpA. B. Associated ATPase activity of the translocation. *In vitro* assays contained proOmpA, SecA species and SecB species at 2 μM each expressed as dimeric SecA and tetrameric SecB: wild-type SecA (circles), SecAdN7 (down triangles), and SecAdN10 (squares) with wild-type SecB; SecAC4 with wild-type SecB (up triangles); wild-type SecA with SecBL75Q (diamonds). The data here and in Figures 2 and 3 were fitted to a hyperbola using SigmaPlot 2001 software. The error bars shown are standard deviations. All assays were done at least three times. When error bars are not obvious, they fall within the symbols. For all fits, 0.45 min was used as the intercept on the x-axis.

Figure 2. Translocation of precursor galactose-binding protein mediated by A1:B4 and A2:B4 complexes. A. Protection of precursor galactose-binding protein (pGBP). B. Associated ATPase activity of the translocation. Concentrations of proteins and symbols in the figures are the same as in Figure 1. The samples were analyzed by immunoblotting.

Figure 3. Translocation of proOmpA in the presence and absence of SecB. *In vitro* translocation of 2 μM proOmpA by SecA wild-type (circles) or SecAdN7 (triangles) at 2 μM SecA expressed as dimer was carried out in the presence (filled symbols) or absence (open symbols) of 2 μM SecB expressed as tetramer. The efficiency of translocation without SecB is extremely low; therefore, to maximize activity for these experiments 1.7 mM NADH, 7.5 mM phosphocreatine, and 37 mg/mL creatine phosphokinase were included in the *in vitro* system to regenerate ATP. The samples were analyzed by immunoblotting.

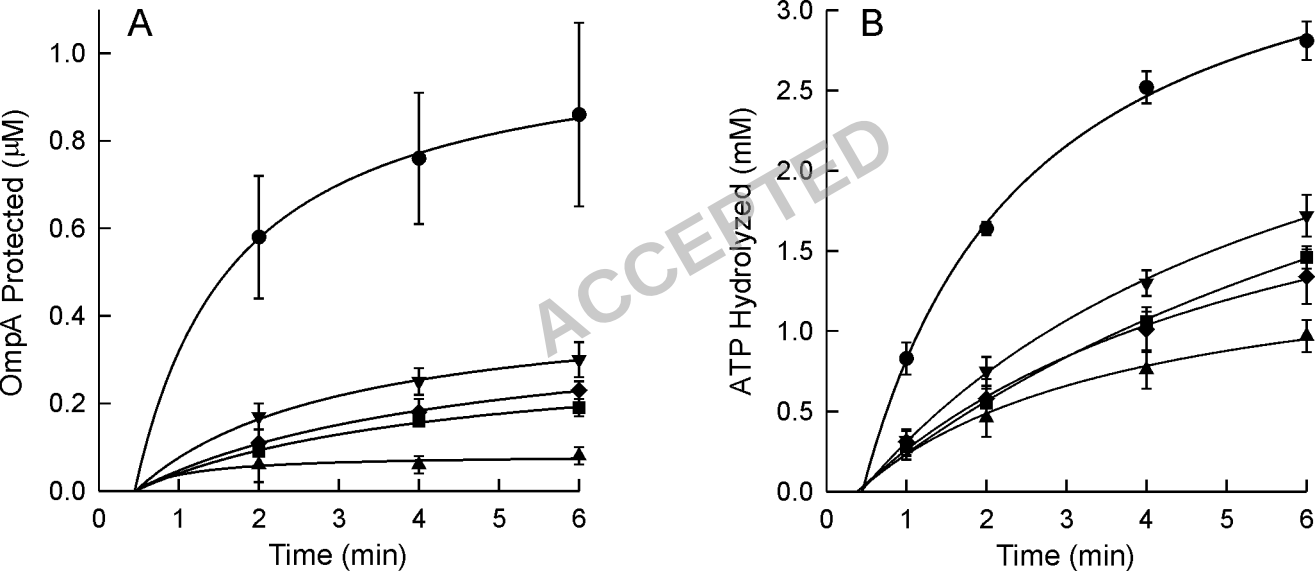
Figure 4. Cross-linking of AET-SecA to precursors. A. Cross-linking to proOmpA. B. Cross-linking to precursor galactose-binding protein (pGBP). Immunoblots using antisera to SecA are shown in the upper panels and those using antisera to the precursors in the lower panels. Cross-linked complexes are indicated by dots and irradiation by hν. Molecular mass markers are shown to the right.

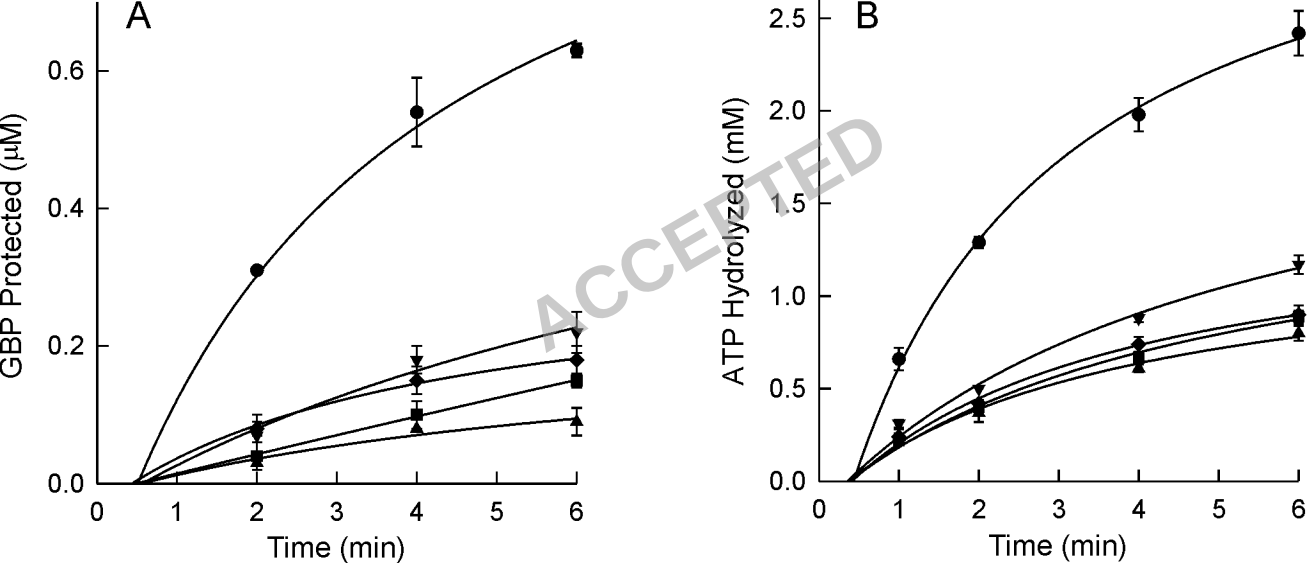
Table 1. Coupling of ATP hydrolysis to translocation[a]

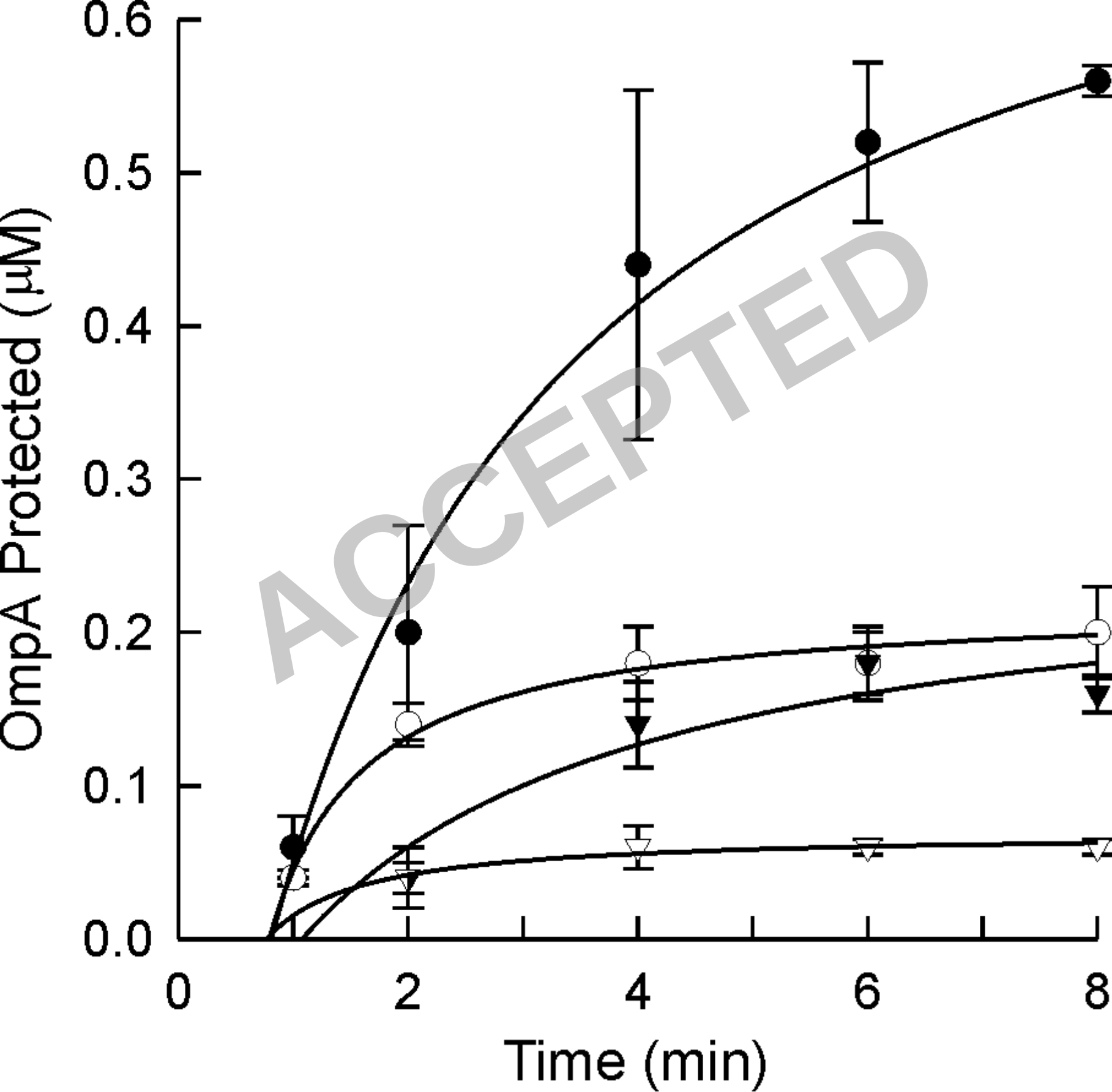
Precursor	SecA and SecB complexes tested				
	SecA WT SecB WT	SecAdN7 SecB WT	SecAdN10 SecB WT	SecAC4 SecB WT	SecA WT SecBL75Q
proOmpA	2500	3600	5800	5800	5600
pGBP	4200	6800	11500	11500	N.D.[b]

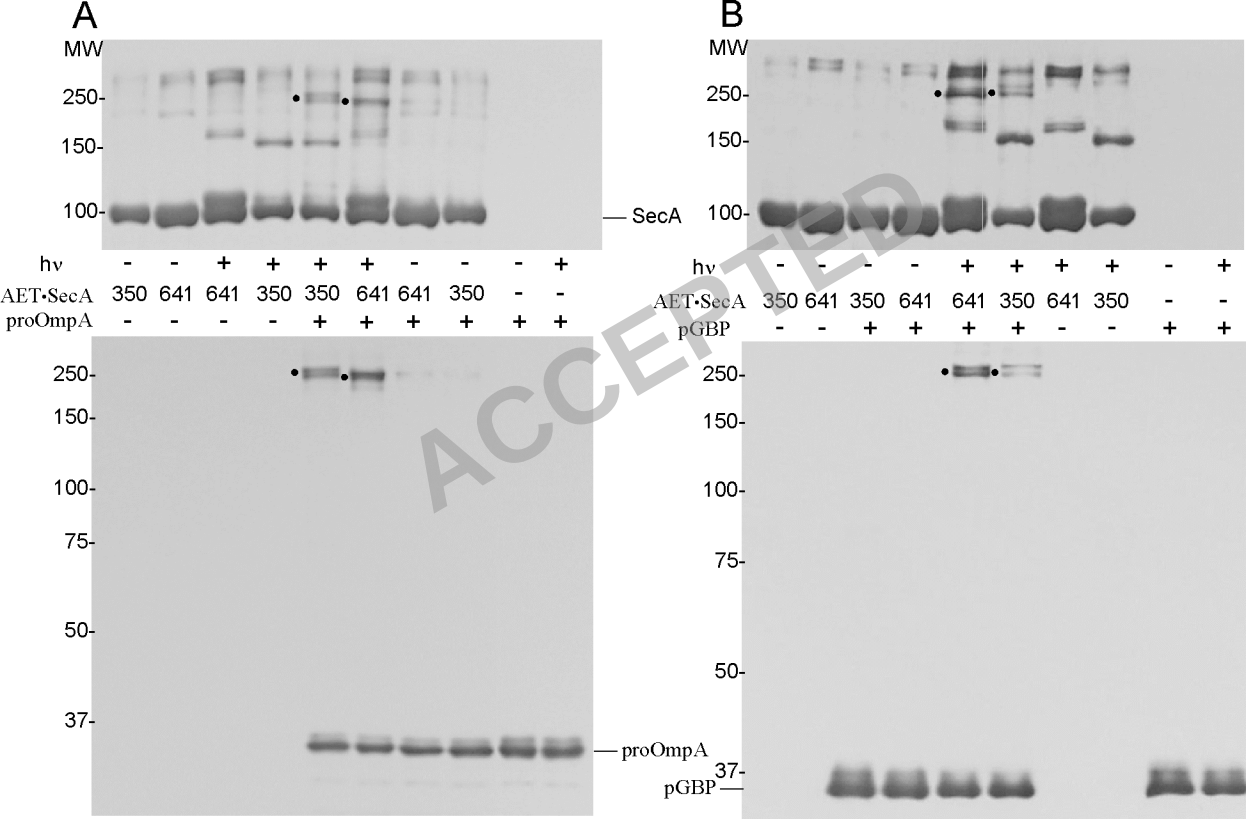
[a] Mole ATP hydrolyzed per mole precursor protected

[b] Not determined, reliable results could not be generated because of the low level of precursor protected


A
OmpA Protected (μM)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Time (min)
B
ATP Hydrolyzed (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
Time (min)


A
GBP Protected (μM)
0.0
0.2
0.4
0.6
0
1
2
3
4
5
6
Time (min)
B
ATP Hydrolyzed (mM)
0.0
0.5
1.0
1.5
2.0
2.5
0
1
2
3
4
5
6
Time (min)




A
MW
250-
150-
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— SecA
hν - - + + + + - - - +
AET•SecA 350 641 641 350 350 641 641 350 - -
proOmpA - - - - + + + + + +
250-
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MW
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AET•SecA 350 641 350 641 641 350 641 350 - -
pGBP - - + + + + - - + +
250-
150-
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50-
37-
pGBP —