ADP-dependent Conformational Changes Distinguish *Mycobacterium tuberculosis* SecA2 from SecA1*

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**Background:** *Mycobacterium tuberculosis* possesses a second SecA ATPase that is required for bacterial virulence.

**Results:** ADP binding by SecA2 causes a conformational change observable by biochemical methods.

**Conclusion:** ADP binding closes the clamp in SecA2. This is not observed in SecA1 or *E. coli* SecA.

**Significance:** Nucleotide-dependent clamp closure suggests a mechanism by which mycobacterial SecA2 is distinguished from SecA1 by the translocation machinery.

In bacteria, most secreted proteins are exported through the SecYEG translocon by the SecA ATPase motor via the general secretion or “Sec” pathway. The identification of an additional SecA protein, particularly in Gram-positive pathogens, has raised important questions about the role of SecA2 in both protein export and establishment of virulence. We previously showed in *Mycobacterium tuberculosis*, the causative agent of tuberculosis, the accessory SecA2 protein possesses ATPase activity that is required for bacterial survival in host macrophages, highlighting its importance in virulence. Here, we show that SecA2 binds ADP with much higher affinity than SecA1 and releases the nucleotide more slowly. Nucleotide binding also regulates movement of the precursor-binding domain in SecA2, unlike in SecA1 or conventional SecA proteins. This conformational change involving closure of the clamp in SecA2 may provide a mechanism for the cell to direct protein export through the conventional SecA1 pathway under normal growth conditions while preventing ordinary precursor proteins from interacting with the specialized SecA2 ATPase.

According to the World Health Organization, approximately one-third of the world’s population is infected with *Mycobacterium tuberculosis* (TB). TB is the leading cause of death by a bacterial pathogen; in 2011 alone, it claimed the lives of 1.4 million people. Additionally, the emergence of multidrug-resistant strains has caused worldwide concern. In 2011, of the 310,000 cases of multidrug-resistant TB, 9% had extensively drug-resistant TB. Despite substantial efforts to forge effective countermeasures, the development of antibacterial drugs and vaccines has been hampered because the survival strategies employed by *M. tuberculosis* and its mechanisms of pathogenesis are not well understood.

Protein export pathways are promising therapeutic targets that have yet to be fully exploited in this bacterium (1). The *M. tuberculosis* SecA2 protein is one such target. In fact, a ΔsecA2 strain of *M. tuberculosis* promotes apoptosis of infected macrophages and shows enhanced priming of CD8+ T cells in mice (2). The combination of lysine auxotrophy and secA2 deletion to produce a ΔsecA2 ΔlysA strain of *M. tuberculosis* was shown to be a strong vaccine candidate and was safer and more effective than the traditional Bacille Calmette-Guérin vaccine for tuberculosis (3). However, because the role of SecA2 in the mycobacteria and its implications for virulence are not well understood, the development of this vaccine is hindered by stringent pharmacological guidelines. Here, we report extensive biochemical characterization of SecA2 that sets the stage for elucidation of SecA2’s role in mycobacterial protein secretion.

In bacteria, the majority of secreted proteins synthesized by the ribosome are exported across the membrane via the general “Sec” pathway. Proteins destined for export are synthesized with N-terminal signal sequences (4). In *Escherichia coli* and other Gram-negative bacteria, some of these “precursor proteins” are maintained in the unfolded form by the chaperone SecB (5). SecB delivers the precursor protein to a SecA ATPase, which interacts with the translocon SecYEG to drive the export of precursors across the channel by the energy of ATP hydrolysis (6).

Several bacterial species, including *M. tuberculosis*, have been shown to possess an additional SecA protein called SecA2 (7–9). The mycobacterial *secA1* and *secA2* genes are genetically distinct and cannot complement each other or deletion of the essential *E. coli* *secA* gene (8, 10). SecA2 from *M. tuberculosis* only shares about 50% sequence similarity to SecA1 and has a molecular mass of 87 kDa, which is about 20 kDa smaller than SecA1. In addition to SecA2, some *Streptococcus* and *Bacillus* sp. also contain a SecY2 protein, which is thought to function as a translocon in concert with SecA2, as well as a few accessory secretion proteins (9, 11). Such SecA2/Y2 systems in streptococci are dedicated to the secretion of a single unique precursor protein (9). In these systems, the genetic loci containing the accessory *sec* genes are highly conserved (11).
Here, we show that *M. tuberculosis* SecA2 has a significantly higher affinity for ADP, and it releases the nucleotide extremely slowly, compared with SecA1. In addition, we find that SecA2 undergoes a large conformational change upon ADP binding that is easily observable with standard biochemical techniques. Our data suggest that this structural rearrangement is due to closure of the clamp in SecA2, which may be unique to the accessory SecA class of proteins, as nucleotide binding does not induce this structural change in *M. tuberculosis* SecA1 or *E. coli* SecA. This may serve as a mechanism for distinction between the two SecA proteins by the mycobacterial translocation machinery.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Plasmids for the expression of SecA1 and SecA2 with C-terminal hexahistidine tags were constructed by PCR amplification of *secA1* and *secA2* genes from *M. tuberculosis* H37Rv genomic DNA (a generous gift from Miriam Braunstein). The amplified products were digested with restriction enzymes (New England Biolabs) and inserted between the NdeI and HindIII sites of pET30b (Novagen). This construct adds a hexahistidine tag at the C terminus of the protein with the sequence LKLAAALEHHHHHH. In case of SecA2, the start site used is a GTG start site located at nucleotide position 91 from the annotated start site (NCBI accession number NP_216337) because this site represents the true start site (15). An ATG start codon that codes for methionine was added before the GTG codon to ensure translation in *E. coli*. The plasmids containing the *secA1* or *secA2* genes were used as templates for site-directed mutagenesis to generate all other variants used in this study. However, the double cysteine mutants SecA1 D318C/E810C and D318C/K463C and SecA2 S332C/D713C and S332C/D580C were generated after substituting the cysteines in the wild-type protein to serines (C111S in SecA1 and C381S and C667S in SecA2). The respective plasmids were transformed into *E. coli* Rosetta2(DE3) pLysS competent cells (Novagen) for expression. The plasmid for *E. coli* SecA was a gift from Linda Randall (21) and was transformed into *E. coli* BL21(DE3) cells for expression.

**Protein Purification**—For SecA1 expression, the cells were grown at 30 °C in LB medium to mid-log phase, induced with 0.5 mM isopropyl β-D-thiogalactopyranoside, and grown for 4 h before harvesting. For SecA2 expression, cells were grown similarly, except at mid-log phase the temperature was lowered to 16 °C, induced with isopropyl β-D-thiogalactopyranoside, grown at 16 °C overnight, and harvested. All purification steps were carried out at 4 °C. Cell pellets were suspended in Wash 1 buffer (10 mM imidazole, pH 8.0, 20 mM Na2HPO4, 300 mM NaCl) containing 1× protease inhibitor mixture (Sigma) and frozen at −80 °C. Cells were thawed on ice followed by the addition of 1× protease inhibitor mixture, 450 μg/ml each of DNase and RNase and 10 mM MgCl2 (final concentrations). The cells were lysed with a French pressure cell (SLM-Aminco) at 20,000 pounds/inch2. The lysates were centrifuged at 26,000 × g for 15 min followed by ultracentrifugation at 162,000 × g for 3 h. The clarified lysate was applied to a column with Talon Superflow metal affinity resin (Clontech). Unbound protein was eluted with Wash 1 followed by Wash 2 buffer (25
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mm imidazole, pH 8.0, 20 mm Na2HPO4, 300 mm NaCl). The tagged proteins were eluted with 250 mm imidazole, pH 8.0, 20 mm Na2HPO4, 300 mm NaCl, and fractions were analyzed by SDS-PAGE. Fractions containing pure protein were pooled and concentrated using an Amicon centrifugal filter device with a molecular mass cutoff of 30,000 Da (Millipore) and dialyzed against 10 mm HEPES, pH 7.6, 25 mm KCl, and 0.5 mg/ml activated charcoal to remove bound nucleotide. This was followed by dialysis against buffer without charcoal. When >95% nucleotide removal was required in the case of SecA2, the protein was applied to a Blue-Sepharose column. Once pure protein was obtained, aliquots were made, frozen at –80 °C, and were never refrozen once thawed. E. coli SecA, without a hexahistidine tag, was purified as described previously (22).

Affinity Chromatography—Blue-Sepharose (GE Healthcare) chromatography was used to separate the nucleotide-bound form of SecA2 from the nucleotide-free form. After purification using the Talon column, dialysis against buffer with charcoal, SecA2-(His)6 was loaded onto a 10-ml Blue-Sepharose column, washed with 25 mm Tris, pH 7.6, followed by a linear gradient from 25 mm Tris, pH 7.6, with no salt to 2 mM NaCl over 6 column volumes. Nucleotide-free SecA2 was then eluted with 3 column volumes of 2 mM NaCl. Fractions of 1.5 ml were collected throughout the run and also during the wash steps. Samples were taken and subjected to denaturing SDS-PAGE as well as nondenaturing gel electrophoresis (agarose gel electrophoresis). For denaturing SDS-PAGE, a 10% BisTris gel (Invitrogen) was used. Electrophoresis was carried out using 50 mM MOPS, pH 7.4, 5% glycerol, 0.05% SDS, and 0.05% Triton X-100 (TTX) as the running buffer. Fractions containing nucleotide-free SecA2 were pooled, dialyzed against 10 mM HEPES, pH 7.6, 25 mM KCl, and stored as aliquots at –80 °C.

Size Exclusion Chromatography—Purified SecA proteins were characterized by size exclusion chromatography using a BioLogic Duo-Flow system (Bio-Rad). Protein was applied at ~20 mg/ml to a Superdex 200 10/300 GL column (GE Healthcare) and eluted at 0.25 ml/min in either low salt buffer (10 mm HEPES, pH 7.6, 25 mm KCl, 1 mm MgCl2, 0.1 mm tris(carboxymethyl)phosphine (TCEP-HCl) (Pierce)) or high salt buffer (10 mm HEPES, pH 7.6, 300 mm KCl, 1 mm MgCl2, 0.1 mm TCEP-HCl). Ultrapure ATP or ADP (Sigma) was added to the buffer at a final concentration of 1 mm for chromatography with nucleotide. The UV absorbance was recorded on a BioPhotometer at 280 nm. The molecular weight of SecA2 was estimated based on high molecular weight calibration standards ferritin, catalase, aldolase, and creatine phosphokinase (GE Healthcare).

Ion Exchange Chromatography—Nucleotide-free or nucleotide-bound SecA2 was loaded onto a 1-ml Hi-Trap Q column (GE Healthcare) in 25 mm Tris, pH 7.6, using a BioLogic Duo-Flow system (Bio-Rad). The column was washed with 5 ml of 25 mm Tris, pH 7.6. A linear gradient from no salt to 1.5 mm NaCl in 25 mm Tris, pH 7.6, was run over 25 ml followed by 5 ml of 1.5 mm NaCl. Fractions of 1.5 ml were collected throughout the run. Where required, nucleotide-free SecA2 was incubated with a 10-fold molar excess of ADP on ice for 30 min before being loaded onto the Hi-Trap Q column.

Agarose Gel Electrophoresis—Protein-agarose gel electrophoresis was carried out using 1% SeaKem HGT-agarose (Lonza) in 40 mm Tris, 20 mm acetic acid, pH 8.0, using a horizontal gel apparatus (Bio-Rad). Samples were prepared at room temperature in low salt buffer with the final protein concentration at 6 μM monomer. Wherever necessary, nucleotides or nucleotide analogs were added to a final concentration of 0.5 mm. The samples were incubated for ~30 min at room temperature before the addition of native gel sample buffer. The gel was run at 100 V, followed by staining with Coomassie Blue to visualize the bands.

Nucleotide Binding Kinetics—Kinetics of nucleotide binding and release were studied by fluorescence resonance energy transfer (FRET) between the tryptophans of SecA and N'-methylanthraniloyl (MANT) nucleotides (Molecular Probes) in an Aminco Bowman Series 2 luminescence spectrometer. The excitation and emission monochromators were set to 295 and 450 nm, respectively, with 4 nm bandpass. The sample chamber was maintained at 20 °C. Varying concentrations of SecA from 0.05 to 0.5 μM were assessed, keeping the MANT nucleotide concentration in excess at 1.2 μM. Because there was no change in the kinetic constants for this SecA concentration range tested, further kinetic experiments were performed using 0.5 μM SecA to improve the fluorescence signal. The SecA protein was incubated in buffer (10 mm HEPES, pH 7.6, 25 mm KCl, 1 mm MgCl2) in a 1-cm cuvette with stirring. After the addition of MANT nucleotide, the increase in MANT fluorescence was monitored with time. Once saturation was reached, 1 mm unlabeled nucleotide was used to compete the MANT nucleotide from the SecA proteins. The curves were fit to a first order rate equation to give the apparent rate constants for the association reaction (k on) and reverse reaction (k off). The rate constant k off was calculated using Equation 1,

\[ k_{\text{off}} = k_{\text{on}}[\text{MANT nucleotide}] + k_{\text{off}} \]  

(Eq. 1)

The dissociation constant (K d) was calculated by dividing the k off by the k on (23, 24). To verify pseudo-first order kinetics, the k obs was determined at varying MANT-ADP concentrations with the SecA proteins held at 0.5 μM. From the plots of k obs versus MANT-ADP, the kinetics parameters k on and k off were obtained from the slope and y intercept, respectively, when fitted by linear regression.

Thrombin Proteolysis—SecA2 (0.8 mg/ml) was digested with 0.08 units of biotinylated thrombin (Novagen) in the presence or absence of 2 mm ADP in 20 mm sodium phosphate buffer, pH 7.6, supplemented with 1 mm MgCl2 at room temperature. Aliquots in duplicate were taken at various times and added to SDS sample buffer with β-mercaptoethanol. The digested products were separated by SDS-PAGE. One set of samples on the gel were stained with Coomassie to visualize the bands, whereas the second set was transferred to a PVDF membrane and stained with Coomassie Blue. The membrane was destained with methanol to remove excessive background stain. Four proteolytic fragments were cut from the membrane, and the sequence of the N terminus was determined using four cycles of automated Edman degradation (Tufts University Core facility).
RESULTS

SecA2 Shows a Unique Structural Change upon Binding to ADP—Conventional SecA proteins have been shown to undergo conformational changes upon nucleotide binding and hydrolysis. These changes are closely coupled with precursor binding and release, as well as association of SecA with the translocon (16–18). The presence of two SecAs in mycobacteria made us question how these homologs are regulated in the cell. Because both proteins are ATPases with significant sequence homology, could unique conformational changes upon binding nucleotides dictate specific interactions with other translocation components?

To investigate potential structural changes, the SecA proteins were incubated in buffer or in the presence of hydrolyzable nucleotides (ADP or ATP) or fluorescently labeled hydrolyzable analogs such as N’-methylanthraniloyl (MANT)-ADP or MANT-ATP before being subjected to native gel electrophoresis. Because the effect of nucleotides on the conformation of E. coli SecA has been studied extensively, E. coli SecA was used as a control. No shift in the mobility of the protein bands for either E. coli SecA or SecA1 in response to binding of any nucleotide was observed (Fig. 2A). The SecA2 protein, however, was observed to exist as two species in equilibrium, seen as two bands on the agarose gel. In the absence of nucleotides, the slow migrating species predominates. The addition of nucleotides shifts the equilibrium to the faster migrating form. In addition to hydrolyzable nucleotides, this mobility shift required the presence of magnesium ions and was not observed when magnesium was substituted with other divalent cations or when the chelator EDTA was added to the reaction (data not shown). WT SecA2 without a hexahistidine tag also undergoes the mobility shift when incubated with nucleotide (data not shown). A Walker A box variant of SecA2 (K115R), which we have previously shown does not bind ATP (15), did not show a mobility shift on the agarose gel, regardless of the presence of nucleotides (Fig. 2A).

Our data indicate that the mobility shift of SecA2 is solely Mg$^{2+}$ nucleotide-dependent. Additionally, the observation of two discrete bands on the agarose gel suggests the exchange between the species is slow relative to the rate of electrophoresis.

We reasoned that SecA2, being an ATPase, would hydrolyze ATP to ADP. If ADP release were rate-limiting, as is the case with E. coli SecA (25, 26) then the change in SecA2 mobility observed on the agarose gel might be induced by ADP rather than ATP. To distinguish the ADP-bound from the ATP-bound form, SecA2 was incubated with the slowly hydrolyzable ATP analog AMP-PNP and subjected to agarose gel electrophoresis. In the presence of AMP-PNP, only a small fraction of
SecA2 shows a mobility shift compared with SecA2 bound to hydrolyzable nucleotides. This observation suggests the mobility shift is not due to binding of ATP, but it must occur further along the enzymatic cycle of SecA2. To verify that SecA2 did in fact bind AMP-PNP, we used fluorescently labeled MANT AMP-PNP. The MANT AMP-PNP binding kinetics were followed by FRET between the intrinsic tryptophan residues of SecA2 and the MANT nucleotide analog. From the fluorescence traces, it is evident that SecA2 bound MANT AMP-PNP and released it when excess unlabeled ATP was added (Fig. 2B). Nevertheless, a mobility shift as seen with hydrolyzable nucleotides was not observed when MANT AMP-PNP was incubated with SecA2 and subjected to agarose gel electrophoresis, consistent with results for unlabeled AMP-PNP (Fig. 2A).

These data indicate that SecA2 can exist in two structural forms that are in equilibrium with each other. Binding of ADP, but not ATP, causes structural rearrangements in SecA2 and shifts the equilibrium such that one conformation is favored over the other. Furthermore, this change is unique to SecA2, as SecA1 and *E. coli* SecA do not show it, despite their ability to bind nucleotides with high affinity. Even though *E. coli* SecA has been shown to undergo conformational changes upon nucleotide binding (20, 27, 28), these changes do not cause a gel mobility shift in our experiments.

**ADP-bound SecA2 Can Be Separated from Nucleotide-free SecA2 Using Blue-Sepharose Chromatography**—Because SecA2 in the presence of nucleotide shows a mobility shift on an agarose gel, a distinctly nonequilibrium condition, we reasoned the affinity of SecA2 for ADP must be quite high. This observation likewise indicated that a fraction of SecA2 might remain ADP-bound even after purification on the metal affinity column. To separate the ADP-bound fraction from the nucleotide-free protein, we used a Cibacron Blue F3G-A-Sepharose column (Blue-Sepharose), which associates with the nucleotide fold found in ATP-binding proteins (29). The column fractions were run on a 10% BisTris gel, and SecA2 was observed to elute in two peaks. A portion of SecA2 eluted immediately in the flow-through, corresponding to SecA2 that did not bind to the blue column. A larger portion of SecA2 bound to the column tightly and eluted in 2 M NaCl (Fig. 2C). An aliquot from each of the two peaks was run on a native agarose gel. Based on the mobility of the SecA2 band, it is evident that the first peak of SecA2 did not bind to the Blue-Sepharose column because it was ADP-bound and showed the distinct faster mobility on the agarose gel, while the fraction of SecA2 that bound tightly to the column was ADP-free and showed slower mobility (Fig. 2D).

**SecA2 Binds ADP with High Affinity and Releases It Significantly Slowly Compared with SecA1**—To characterize the interaction between SecA2 and ADP, the kinetics of nucleotide binding were assessed using FRET between the intrinsic tryptophans of SecA2 (donor) and MANT nucleotides (acceptor). Because MANT nucleotides were able to induce a structural rearrangement in SecA2 in a manner similar to unlabeled nucleotides (Fig. 2A), the binding of MANT nucleotides to SecA1 and SecA2 likely mimics the association of the unlabeled nucleotides with these proteins. Additionally, MANT-ATP has been shown to support translocation of precursors by the *E. coli* SecA ATPase in an *in vitro* translocation assay, highlighting that substitution of ATP with MANT nucleotides allows for physiologically relevant activity (30).

The tryptophans of *M. tuberculosis* SecA proteins were excited, and upon binding of MANT nucleotides, the increase in MANT fluorescence due to energy transfer was monitored with time (Fig. 3). The data were fit to a single exponential function to obtain the apparent rate constant for association ($k_{\text{obs}}$). After binding was saturated, excess unlabeled nucleotide was added to compete the MANT nucleotide from the protein. The decrease in MANT fluorescence was followed with time. These data were fit with the equation for a first order decay to determine the dissociation rate constant ($k_{\text{off}}$). These constants were further used to calculate the association rate constant ($k_{\text{on}}$) and the dissociation constant ($K_d$) as described under “Experimental Procedures.” To verify pseudo-first order kinetics, $k_{\text{obs}}$ was determined at varying MANT-ADP concentrations, whereas the concentrations of SecA1 or SecA2 were held constant. The plot of $k_{\text{obs}}$ versus [MANT-ADP] was linear for both SecA1 and SecA2 (Fig. 3C), indicating that binding of MANT-ADP to the SecA proteins follows pseudo-first order kinetics.

From analysis of the rate constants obtained from single exponential fits (Table 1), SecA2 showed very slow release of bound ADP ($k_{\text{off}}$ $1 \times 10^{-4}$ s$^{-1}$) compared with SecA1 ($k_{\text{off}}$ $1 \times 10^{-3}$ s$^{-1}$). Also, the affinity of SecA2 for MANT-ADP ($K_d \sim 0.05$ mM) was about 120-fold tighter than that of SecA1 ($K_d \sim 6$ mM). The kinetic constants were similar to those obtained from linear regression when $k_{\text{obs}}$ was plotted as a function of MANT-ADP concentration (Table 1) consistent with pseudo-first order kinetics. From these data, we suggest the slow release of ADP from SecA2 may regulate its function in vivo.

**Structural Change in SecA2 Is Not Because of Changes in Oligomeric State or Surface Charge**—Change in the oligomeric state could reasonably cause a shift in its mobility on an agarose gel, and SecA proteins are well documented to undergo such association reactions (31, 32). To investigate the possibility that the observed structural rearrangements in SecA2 were due to a change in the oligomeric state caused by the presence of nucleotides, SecA2 was subjected to size exclusion chromatography. This was done in the presence of buffer supplemented with 25 or 300 mM KCl because the presence of high salt has been shown to dissociate the *E. coli* SecA dimer (31, 33). The elution profiles for SecA2 in the presence of high salt or low salt buffers were consistent with the proteins being predominantly monomeric in high salt and dimeric in low salt based on calibration standards run over the column (Fig. 4A). When the low salt buffer was supplemented with ADP or ATP, there was no shift in the elution peak of dimeric SecA2. Similarly, no change in the elution of the SecA2 monomer peak was observed when the high salt buffer was supplemented with nucleotides. Monomeric SecA2, in high salt buffer, is able to bind nucleotides with similar affinities to SecA2 in low salt buffer (data not shown). These data show that nucleotides do not change the oligomeric state of SecA2.

Another determinant of migration on a native agarose gel is surface charge of a protein. Because ADP-SecA2 migrates further on the agarose gel compared with apo-SecA2, we speculated the conformational change induced by ADP could lead to...
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To test this possibility, ion exchange chromatography of SecA2 at pH 7.6 using a Hi-Trap Q anion exchange column was performed. We looked for altered binding to the Q-column in the presence of ADP, which would be indicative of a significant change in surface charge. The elution profiles show no shift in the ADP-bound SecA2 peak (Fig. 4B) even though when fractions from each of the peaks were run on an agarose gel the samples corresponding to the ADP-bound SecA2 showed the distinct mobility shift, confirming that ADP remained bound through the column. Even though binding of proteins to an ion exchange column is dependent on the average surface charge and a shift in elution peak on such a column would only occur in the case of a drastic overall surface charge change, the gel mobility shift we observe in response to nucleotides in SecA2 seems like it might require such a dramatic charge or conformational change. However, our data indicate that the ADP-induced conformational change in SecA2 is likely due to a structural rearrangement resulting in a change in the overall shape of the protein, rather than a change in oligomeric state or surface charge.

ADP Binding Confers Protection to Regions of the Pre-protein Binding Domain in SecA2—To investigate the ADP-dependent conformational change, SecA2 was subjected to limited proteo-

![Figure 3: SecA2 binds ADP with high affinity.](image)

**TABLE 1**

| Protein | Kinetic constants determined from direct fits to rate equations | Kinetic constants determined from plots of $k_{obs}$ versus concentration of MANT-ADP |
|---------|---------------------------------------------------------------|------------------------------------------------------------------|
| SecA1   | $k_{obs} \times 10^{-3}$ | $k_{off} \times 10^{-3}$ | $k_{on}$ | $K_d$ | $k_{obs} \times 10^{-3}$ | $k_{off} \times 10^{-3}$ | $k_{on}$ | $K_d$ |
| SecA2   | 1.3 | 1 | 223 | 2 × 10^3 | 0.05 | 1.6 × 10^3 | 0.25 |
Lysis by thrombin in the presence or absence of ADP. The proteolytic fragments were analyzed by SDS-PAGE (Fig. 5A). When ADP-bound, SecA2 showed significant protection to cleavage by thrombin in the presence of ADP. The arrowheads show fragments that were subjected to N-terminal sequencing. Fragments shown with blue arrowheads had the N-terminal sequence IAQL. A predominant thrombin cleavage site with the N-terminal IAQL sequence is shown in blue ball representation on a model of M. tuberculosis SecA2 (orange) generated using the program 3D-JIGSAW (34). The SecA2 model has been overlaid with the structure of T. maritima SecA in the open form in gray on the left (PDB code 3JUX (18)), and on the right, the closed form (PDB code 3DIN (20)). Based on sequence alignment, the corresponding thrombin cleavage site on T. maritima SecA is shown in purple. The UCSF Chimera program (43) was used to generate the figures.

Three (blue arrowheads in Fig. 5A) out of the four fragments had the sequence IAQL at the N terminus, indicating that the predominant cleavage site on SecA2 was the bond between Arg-335 and Ile-336, although the fourth fragment (black arrowhead) had the sequence TTRA, the first threonine being the 4th residue from the N terminus of the protein. The mass of the fragment starting at TTRA is consistent with the C-terminal cut site at Arg-335. The highest molecular weight band starting with IAQL (Fig. 5A, top blue arrowhead) is consistent with the entire C terminus after the cleavage. There is another

FIGURE 4. ADP-induced rearrangement is not due to a charge or oligomeric state change. A, Superdex 200 size exclusion column profiles of SecA2 in the presence of low or high salt buffer either with or without added ADP. The elution volumes of the peaks of the calibration standards are marked along the top x axis along with their corresponding molecular weight. B, Hi-Trap Q-column elution profiles for SecA2 in the presence or absence of ADP. SecA2 was loaded onto the column under three conditions as follows: 1) mixture of ADP- and apo-SecA2; 2) apo-SecA2; 3) ADP-free SecA2 preincubated with ADP for 30 min before being loaded onto the Q column. The column was eluted with a linear salt gradient. An aliquot from each SecA2 peak was run on an agarose gel (inset). A.U., arbitrary units.

FIGURE 5. Limited proteolysis of SecA2. A, cleavage of SecA2 by thrombin with time in the absence and presence of ADP. Cleavage products were separated on a 10% SDS gel. SecA2 shows increased protection to thrombin in the presence of ADP. The arrowheads show fragments that were subjected to N-terminal sequencing. Fragments shown with blue arrowheads had the N-terminal sequence IAQL. B, predominant thrombin cleavage site with the N-terminal IAQL sequence is shown in blue ball representation on a model of M. tuberculosis SecA2 (orange) generated using the program 3D-JIGSAW (34). The SecA2 model has been overlaid with the structure of T. maritima SecA in the open form in gray on the left (PDB code 3JUX (18)), and on the right, the closed form (PDB code 3DIN (20)). Based on sequence alignment, the corresponding thrombin cleavage site on T. maritima SecA is shown in purple. The UCSF Chimera program (43) was used to generate the figures.
predicted thrombin cleavage site in SecA2 located between Arg-502 and Gly-503, which would explain the lowest molecular weight fragment. The ~40-kDa fragment must be caused by a noncanonical cut site. Regardless, the Arg-335 site is protected in SecA2 bound to ADP but exposed in apo-SecA2.

When mapped onto a structural model of SecA2 generated using 3D-JIGSAW (34) the site (blue ball representation) generating IAQL fragments is located in the probable PPXD of SecA2 (Fig. 5B). The model of SecA2 is overlaid with the structure of *T. maritima* SecA in the open clamp form (18) as well as in the closed clamp conformation of *T. maritima* SecA when bound to the translocon channel (20). From a sequence alignment of *T. maritima* SecA with *M. tuberculosis* SecA2, the corresponding thrombin cleavage site on *T. maritima* SecA was identified and mapped onto the structures (Fig. 5B, purple ball representation). With comparison of the two overlays, it is evident that this region undergoes a dramatic conformational change. We postulate that in the ADP-bound conformation of SecA2, this site could be protected if it comes in close proximity to the nucleotide-binding domain (NBD2) in a manner similar to *T. maritima* SecA (Fig. 5B). Based on our data, we hypothesize that for SecA2, binding of ADP might induce the movement of the PPXD such that SecA2 exists in the “closed clamp” state when ADP-bound.

**ADP Binding Closes the Clamp of SecA2 but Not of SecA1**—To test whether the mobility shift shown by SecA2 on an agarose gel in response to ADP binding could be due to movement of the PPXD to the closed clamp state, the native cysteine residues in SecA1 and SecA2 were mutated to serines, and two sets of double cysteine mutants were introduced in both SecA1 and SecA2 to restrain the PPXD by disulfide bond formation in a manner done by the Collinson and co-workers (35). Because of high sequence homology between *E. coli* SecA and *M. tuberculosis* SecA1, we were easily able to make the corresponding mutants in SecA1 representing the open clamp (D318C/E810C) and closed clamp (D318C/K463C). Position Asp-318 is in the PPXD; Glu-810 is located in the helical scaffold domain, and Lys-463 is in NBD2 (Fig. 6A). In SecA2, however, due to
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![Schematic representation of M. tuberculosis SecA proteins in the open or closed clamp forms by disulfide bond formation between pairs of cysteines introduced by site-directed mutagenesis. A, 1% native agarose gel shows SecA1 only shifts mobility to a faster migrating form upon forced closure of the clamp by air oxidation of the cysteine residues. SecA2 shows a shift in mobility in the presence of ADP or if its clamp is forced closed by oxidation of the introduced cysteines by air. The faster band that corresponds to the oxidized form in the closed clamp mutant of SecA2 shifts back to the slow migrating species in the presence of 20 μM TCEP.

FIGURE 7. Binding of ADP induces movement of the PPXD to close the clamp of SecA2 but not in SecA1. A, schematic representation of M. tuberculosis SecA proteins in the open or closed clamp forms by disulfide bond formation between pairs of cysteines introduced by site-directed mutagenesis. B, 1% native agarose gel shows SecA1 only shifts mobility to a faster migrating form upon forced closure of the clamp by air oxidation of the cysteine residues. SecA2 shows a shift in mobility in the presence of ADP or if its clamp is forced closed by oxidation of the introduced cysteines by air. The faster band that corresponds to the oxidized form in the closed clamp mutant of SecA2 shifts back to the slow migrating species in the presence of 20 μM TCEP.

Absence of sequence homology in these regions, as well as the lack of a crystal structure of any SecA2 protein, generation of cysteine pairs that would mimic the respective conformations proved challenging.

We used S332C/D713C to probe the open clamp conformation of SecA2 but were required to generate several combinations of cysteine mutants for the closed clamp, which were then tested for disulfide bond formation. Finally, we generated S332C/D580C (Fig. 6A). Position Ser-332 is modeled to be located in the PPXD of SecA2, and Asp-713 is modeled in the helical wing domain, and Asp-580 is in NBD2. The PPXD in each mutant protein was restrained by disulfide bonds between the cysteines by air oxidation or released using reducing agent. All variant proteins were able to bind nucleotide in a manner similar to wild type.

Strikingly, when the open (D318C/E810C) and closed (D318C/K463C) clamp mutants of SecA1 were subjected to agarose gel electrophoresis in the oxidized state, a mobility shift was seen when the clamp was forced closed. This was similar to our observations with ADP-bound WT SecA2 (Fig. 7B). Consistent with our earlier results, ADP alone did not induce this mobility shift in WT SecA1.

By comparison, in SecA2 the oxidized open clamp mutant did not show a mobility shift on the agarose gel. However, a fraction of the closed clamp mutant SecA2 showed faster migration. This is consistent with the observation that ADP-bound WT SecA2 shifted almost entirely to the fast migrating form. When the oxidized SecA2 closed clamp mutant was reduced with TCEP, the faster migrating band is lost to the slower migrating band (Fig. 7B). The SecA2 mutants in both the open (data not shown) and closed forms are able to shift completely to the faster migrating form on binding ADP, indicating that we have not affected the ability of the protein to undergo the conformational change. Our results show that the faster migrating form is only present when the closed clamp mutant was air-oxidized but was absent when reduced. We expected the closed clamp mutant of SecA2 to shift entirely to the fast migrating form when oxidized; however, only a fraction shifted. This suggests that the open conformation of SecA2, in which the PPXD remains close to the helical wing domain, is favored until the protein is ADP-bound. Alternatively, in SecA2 the sites chosen for the closed clamp cysteines might not be a perfect representation of the true closed clamp. Regardless, these results are consistent with the hypothesis that the faster migrating species observed on the gel corresponds to SecA2 in its closed clamp form. Furthermore, in SecA2, but not in SecA1, movement of the PPXD is regulated by nucleotide binding.

DISCUSSION

The identification of an additional SecA protein in several pathogenic bacteria has raised questions regarding the role of the accessory SecA in precursor translocation. In these systems, because SecA1 and SecA2 are functionally distinct (8, 9), how are pre-proteins targeted to their respective SecA proteins given the redundancy in signal sequences? We set out to investigate some basic differences between the two SecA proteins of M. tuberculosis with the aim that our study might provide insight into the differences in regulation of the two SecA homologs. Our data show that SecA2 binds ADP with high affinity and that binding of ADP induces a large conformational change in SecA2. Interestingly, nucleotide binding does not induce this change in SecA1.

Conventional SecA proteins have been shown to undergo rapid ATP hydrolysis at the membrane ("translocation ATPase" activity) (36) in a reaction where ADP release is rate-limiting (25). Association of SecA with the SecYEG translocon (26, 30) promotes ADP release, and this step is closely coupled to translocation of precursor proteins (37, 38). From kinetic data using MANT-ADP, it is evident that SecA2 releases ADP much more slowly than SecA1; consequently, the affinity of SecA2 for ADP is much higher than SecA1. E. coli SecA has been reported to bind MANT-ADP with a $k_{\text{on}}$ of $7.8 \times 10^4$ M$^{-1}$ s$^{-1}$ and $k_{\text{off}}$ of $6.8 \times 10^{-4}$ s$^{-1}$ resulting in a $K_d$ of 9 nM (30). Despite higher affinity of E. coli SecA for ADP compared with SecA2
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to accommodate the pre-protein and target it to the membrane for translocation. Our results provide the first evidence of biochemical differences between the two SecA homologs, which suggests a mechanism for the cell to distinguish between the proteins during translocation.

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(Kₐ ≈ 50 nM), indicating that it would be ADP-bound in our agarose gel experiments. E. coli SecA does not show a nucleotide-dependent shift like SecA2.

The ADP-dependent shift we observe for SecA2 on an agarose gel is a unique feature of mycobacterial SecA2. Crystal structures of M. tuberculosis SecA1 in the apo-form and when bound to ADP/βS do not show any significant differences, except for a few residues that are directly involved in nucleotide binding, suggesting that there were no significant structural changes in SecA1 upon ADP binding, including in the PPXD (39). This is consistent with our observation that SecA1 does not change mobility on the agarose gel with binding of the nucleotide.

The most dramatic conformational change seen in conventional SecA proteins involves the ~80° movement of the PPXD with respect to the helical wing domain and NDB2 that leads to opening or closure of the clamp (Fig. 1) (19, 20, 35). The clamp is suggested to hold the pre-protein in a manner that facilitates interaction of the pre-protein with the tip of a helix known as the "two-helix finger" that directs it into the channel during translocation (20, 40). How movement of the clamp is regulated in conventional SecA proteins is not known.

Because there is no structure of any SecA2 protein to date, analysis of the conformational change in SecA2 by mutagenesis was challenging. Based on sequence homology between SecA1 and SecA2, the greatest variation between the two proteins lies in the C-terminal domain, although the nucleotide binding domains are highly conserved. This led us to believe that the overall domain architecture of SecA2 would be similar to SecA1. Consequently, the possibility of a large unique conformational change in SecA2 completely different from that seen in conventional SecA proteins seems unlikely, although it cannot be entirely ruled out. Based on biochemical and biophysical studies on conventional SecA proteins and analysis of crystal structures, it seems likely that the mobility shift we observe in SecA2 on an agarose gel involves movement of the PPXD. Consistent with structural data, our biochemical data suggest that the clamp in M. tuberculosis SecA1 remains open in both the ADP-bound and apo-forms of the protein, as seen with E. coli and T. maritima SecA proteins. In contrast, in SecA2 binding to ADP appears to cause closure of the clamp.

Based on the high affinity of SecA2 for ADP, and the slow k_{off}, SecA2 in the mycobacterial cytosol would be primarily ADP-bound even in the presence of the 1 mM cellular concentration of ATP (15). Because the clamp of SecA2 would be closed in this form, it could serve as a mechanism to prevent binding of SecA1-dependent pre-proteins to SecA2. In a study done by Braunstein and co-workers (41) to determine the subcellular localization of the SecA proteins in Mycobacterium smegmatis, SecA1 was found equally distributed between the membrane and the cytosol, although SecA2 was predominantly cytosolic.

Under normal growth conditions, SecA1 functions like conventional SecA proteins in translocating the majority of pre-proteins across the Sec translocon. Because SecA2 is not required for general protein export, but is implicated in the export of virulence factors (12, 42), we hypothesize that under certain conditions, when SecA2 is required, binding to an unknown factor or pre-protein signal sequence could promote ADP release, thereby opening the clamp of SecA2 to enable it to accommodate the pre-protein and target it to the membrane for translocation.
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