Energetic Requirements for Bacterial Protein Export

John Andrew Corbett

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ENERGETIC REQUIREMENTS FOR BACTERIAL PROTEIN EXPORT

by

John Andrew Corbett

A dissertation submitted in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1990
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John A. Corbett
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ABBREVIATIONS USED

ACMA  9-amino-6-chloro-2-methoxy-acridine
ATP  adenosine-5'-triphosphate
ATPase  ATP phosphohydrolase
BCA  bicinchoninic acid
BLA  β-lactamase
CTFs  cytoplasmic translocation factors
DCCD  N,N'-dicyclohexylcarbodiimide
DES  diethylstilbestrol
DNA  deoxyribonucleic acid
ER  endoplasmic reticulum
FFT  firefly tails
Hepes  N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
IPTG  isopropyl-β-D-thiogalactoside
LSBP  leucine-specific binding protein
mRNA  messenger ribonucleic acid
NADH  reduced nicotinamide adenine dinucleotide
Oxonol VI  bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol
SRP  signal recognition particle
TCA  Trichloroacetic acid
TDAB  tetradecyltrimethylammonium bromide
tris  tris-(hydroxymethyl)aminomethane
TS  temperature sensitive
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<td>Vi</td>
<td>sodium orthovanadate</td>
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<tr>
<td>2-D</td>
<td>two-dimensional</td>
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<tr>
<td>Δp</td>
<td>total proton motive force</td>
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<td>ΔΨ</td>
<td>transmembrane electrical potential</td>
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<td>ΔpH</td>
<td>transmembrane pH gradient</td>
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ABSTRACT

Energetic Requirements for Bacterial Protein Export

by

John Andrew Corbett, Doctor of Philosophy

Utah State University, 1990

Major Professor: Dr. Bruce R. Copeland
Department: Chemistry and Biochemistry

Bacterial protein export involves the translocation of precursor proteins across the inner cytoplasmic membrane. Over 100 proteins are exported from Escherichia coli. This study showed that energy in the form of ATP and membrane gradient energy is essential for the export of leucine specific binding protein and β-lactamase precursors. Ionophores or combinations of ionophores (SF6847, valinomycin/nigericin and valinomycin/monensin) which dissipate protonmotive force inhibit protein export. Valinomycin alone also inhibits export, but not as well as reagents which dissipate protonmotive force. Nigericin or monensin alone slightly stimulate protein export. These results suggest that the transmembrane electrical potential (ΔΨ) is the component of membrane gradient energy necessary for precursor protein export.

ATP is necessary for the export of precursors. In the absence of ATP, in vitro export of leucine specific binding protein and β-lactamase precursors is not observed. An upper limit of 10μM was determined for the effective Km for ATP
during in vitro protein export. It was also shown that ATP is consumed during the export process. The SecA protein was shown to contain an ATPase activity that is stimulated by the presence of inverted membranes and purified LSBP precursors.

Vanadate and diethylstilbestrol, inhibitors of ATPases, inhibit in vitro protein export. Vanadate also inhibits SecA ATPase activity which depends on membranes and precursors. Vanadate is a specific inhibitor of P-type ion translocating ATPases. This study showed primary sequence homology between part of the SecA protein and the phosphorylation sequence of P-type ATPases. Sequence homology, vanadate inhibition of SecA ATPase activity, and vanadate inhibition of in vitro protein export suggest that SecA may function by a mechanism similar to the E₁,E₂ mechanism found in P-type ATPases.

Phosphorylation of two proteins with apparent sizes of 62 and 37 kDa is observed to occur in an export-associated fashion. This phosphorylation is dependent on membranes and precursors, is sensitive to hydroxylamine, and is sensitive to inhibitors of protein export, including valinomycin/nigericin and vanadate. Furthermore, phosphorylation of the 62 kDa protein is dependent on the presence of SecA. The phosphate linkage appears to be an acyl phosphate based on hydroxylamine sensitivity and reduction of the acyl phosphate linkage by NaCNBH₃. Both proteins appear to be peripherally associated with the cytoplasmic face of the inner membrane, which is also consistent with a possible role in the bacterial protein export process.
CHAPTER I
INTRODUCTION

Literature Review

Introduction—Bacterial protein export is initiated in the cellular cytoplasm with the synthesis of proteins destined for secretion to extra-cytoplasmic locations. The bacterial cytoplasm is bound by a hydrophobic inner membrane. *Escherichia coli*, a gram negative bacteria, also contains an outer membrane, which serves as a second barrier to the movement of macromolecules. Located between these membrane barriers is the periplasmic space. Bacterial protein export involves the translocation of exported proteins across the inner membrane to destinations which include the periplasmic space, outer membrane, or in some cases the exterior of the cell (1-4).

Proteins destined for export are synthesized in a precursor form containing an amino terminal extension of 20-40 amino acid residues. The peptide extension has been termed a leader or signal sequence because it serves as a molecular beacon distinguishing exported from nonexported proteins. Following translocation of the secreted protein across the bacterial inner membrane, a specific leader (signal) peptidase removes the leader peptide. Cleavage of the leader sequence renders the process irreversible.

Proteins undergoing export traverse the membrane in a unidirectional fashion which is both rapid and precise. Over 100 *E. coli* proteins are exported. The biochemical functions of exported proteins are diverse and include the binding and transport of amino acids, regulation of osmolarity, and participation in
metabolism. In this study the export of two proteins, leucine specific binding protein (LSBP) and β-lactamase (BLA), was investigated. LSBP is responsible for leucine binding and transport, while BLA confers penicillin and ampicillin resistance to bacterial strains carrying the bla gene on a plasmid.

Comparison of Bacterial Protein Export and Eukaryotic Protein Secretion—
Bacteria provide a relatively simple system in which to identify and determine the molecular events necessary for translocation of proteins across biological membranes. *E. coli* is an excellent choice because of the ease of genetic manipulation and its biochemical simplicity, combined with the vast amount of biochemical and genetic information already obtained (5).

Determination of export events in *E. coli* also has a broad application to eukaryotic protein secretion (translocation of proteins across eukaryotic membrane systems). Many of the events appear to be similar, including

1) Most exported and secreted proteins are synthesized in a precursor form containing an amino terminal leader or signal sequence of 20 to 40 amino acids in length (6).

2) Leader sequences are functionally conserved in that eukaryotic signal sequences function in bacteria (7) while bacterial leader sequences function in eukaryotes (8).

3) Additional sequence information necessary for protein export and secretion is contained in the mature domains of exported and secreted proteins (9).
4) Specific leader or signal peptidase cleave the leader sequence during or following translocation of precursor proteins (10).

5) ATP is required for protein translocation across all membrane systems studied to date (reviewed, 11).

There are also some differences between bacterial protein export and eukaryotic secretion. Protonmotive force appears to be required for optimal export of bacterial proteins, while it is not required for protein secretion across the endoplasmic reticulum (ER) (12). Also, a signal recognition particle (SRP), which functions exclusively during precursor protein synthesis, has been isolated in eukaryotic secretion systems (4). Neither this protein nor a protein which functions in the same manner has been observed in bacterial systems. These differences are minor in comparison to the similarities between eukaryotic secretion and bacterial protein export. Thus, information obtained in the bacterial system should be directly applicable to eukaryotic secretion.

**Early Models of Export**—Pioneering work by George Palade laid the foundation for protein export studies (13). Palade traced the eukaryotic cellular routing of noncytoplasmic proteins using autoradiography and cell fractionation. He found that ribosomes bound to the endoplasmic reticulum (rough ER) are the site where secreted protein synthesis occurs, and proteins destined to be secreted are never found in the cytoplasm in their completed form but are immediately segregated into the lumen of the rough ER (13). Palade’s work was of vast importance; however, it stopped short
of addressing the location of information determining the specificity and selectivity of protein export.

Work by Palade and others suggested that eukaryotic protein secretion was tightly coupled to protein synthesis. These findings, as well as the discovery of signal sequences, led to the introduction of the signal hypothesis by Blobel and Dobberstein (14). Redman and Sabatini (15) had shown that truncation of exported proteins by the action of puromycin had no effect on their localization into the lumen of the ER, while Milstein et al. (16) had demonstrated the existence of a peptide extension on the amino terminal end of immunoglobulin G. The existence and importance of amino terminal leader sequences was not widely accepted until Blobel and Dobberstein (17) developed the first in vitro system for the study of protein secretion. Using the eukaryotic in vitro system comprised of a cell-free translation system and microsomal vesicles, it was shown that precursor protein synthesis in the presence of microsomal vesicles resulted in removal of the amino terminal leader sequence (processing) from presecretory proteins (17). However, precursor protein processing was not observed if microsomal vesicles were added following the completion of synthesis. Furthermore, the processed form of the presecretory protein was protected within the lumen of the ER when soluble protease was added, showing that translocation of the presecretory protein correlates with processing of the leader peptide. These results led to the formation of the signal hypothesis.

The signal hypothesis stated that proteins destined for export are initially synthesized in a larger precursor form containing an N-Terminal 20-40 amino acid
extension (leader sequence). The leader peptide was proposed to function in the attachment of the translation complex to the membrane, forming a pore through which the precursor proteins are exported. The precursor protein chain was believed to pass through this pore as peptide chain elongation proceeded, possibly using the energy of translation to drive protein export. A specific leader peptidase was proposed to remove the leader sequence, most likely before translation of the precursor protein was complete (14, 17).

In support of the signal hypothesis, Walter and Blobel isolated a signal recognition partial (SRP) that was found to interact with the leader peptide as it emerged from the ribosome translation complex (18). Binding of SRP to the emerging precursor protein was found to halt any further translation. Meyer and Dobberstein isolated a second membrane associated component, the docking protein, that relieved the translation block (19). This work represented the first experimental evidence supporting the full signal hypothesis.

The signal hypothesis explained accumulating eukaryotic evidence that suggested a cotranslational protein secretion process, but concurrent studies on the export of M13 coat protein suggested that bacterial protein export could occur in a posttranslational fashion (20). M13 coat protein contains a 23 amino acid leader sequence in its precursor form (procoat), while in its mature form it exists transiently in the inner membrane of E. coli before incorporation into phage heads (21). Date and Wickner (20) showed that in vitro export of procoat could occur after synthesis was completed. It was shown that procoat irreversibly binds to added proteoliposomes and
is inserted into the lipid vesicles following synthesis. This was demonstrated by resistance to proteolytic digestion from added soluble protease (22).

Results showing that M13 procoat could be translocated in a posttranslational fashion led to the development of the membrane trigger hypothesis proposed by Wickner (23). This model stated that the leader peptide allows the growing peptide chain to fold in a manner compatible with the aqueous environment of protein synthesis. Following synthesis the precursor protein was proposed to interact with the membrane. This interaction was suggested to involve the leader peptide as well as some portion of the mature protein, and features of the membrane that could be either lipid, protein, or properties of the lipid phase. Binding of the precursor protein to the appropriate membrane recognition site was suggested to cause a spontaneous conformational change in the precursor protein allowing for its insertion into the membrane.

These early models have served as a direction in which to attack the problem of protein export. They have led to a proliferation of data which suggest that both models in their simplest form correctly describe different examples of protein export and secretion. They also illustrate the difficulty and complexity in devising one unified model describing protein export.

Energy Requirements for Bacterial Protein Export—Energy in the form of ATP and protonmotive force is required for the export of bacterial proteins. The requirement for ATP appears to be global for all protein secretion and export systems which have been studied (12), while the participation of membrane gradient energy appears to be
specific for individual systems (12). Membrane energy was initially shown to be involved in the bacterial protein export process by the action of protonophores and ionophores which dissipate protonmotive force and cause accumulation of precursor forms of all exported proteins in vivo (1, 24, 25). The later development of in vitro protein export systems has made it possible to identify the importance of ATP during bacterial protein export (26). These systems have also made it possible to better characterize the role of membrane energy during protein export.

Most in vitro export systems consist of some form of precursor protein which is added to inverted membrane vesicles (posttranslational translocation), or the in vitro synthesis of precursor protein in the presence of inverted membrane vesicles (cotranslational translocation). For posttranslational translocation, precursor proteins can be prepared by in vitro synthesis in an E. coli cell lysate that is void of membranes. Following precursor synthesis, the small molecule pool required for protein synthesis is usually removed by desalting gel exclusion chromatography. Addition of desalted precursor protein to inverted membrane vesicles makes it possible to study protein translocation in the absence of precursor protein synthesis. Recent advances have led to a more purified posttranslational translocation system which utilizes purified precursor proteins (27). Cotranslational translocation systems are comprised of an E. coli cell lysate capable of plasmid or mRNA directed synthesis in the presence of inverted membrane vesicles. This system allows for the examination of protein export while synthesis is occurring. However, some of the export observed in this type of system may be posttranslational.
Using a posttranslational translocation system, Chen and Tai (26) showed that ATP is absolutely required for protein translocation and suggested that membrane energy may participate but was not essential for in vitro bacterial protein export. These conclusions were based on the lack of protein export inhibition by protonophores when membranes devoid of an active F<sub>1</sub> component of the F<sub>1</sub>F<sub>0</sub> ATPase were used. Controversy ensued over the role of membrane energy, as this result contradicted many studies which showed that uncoupling of membrane energy inhibits protein export (24, 25). It is now clear that both membrane energy and ATP are required for optimal in vitro bacterial protein export. Geller et al. (28), and Yamane et al. (29) showed that optimal export of OmpA and OmpF-Lpp requires both membrane energy and ATP. In the absence of membrane energy a reduced level of translocation is observed. Addition of NADH (to facilitate the formation of membrane energy) results in optimal translocation of these proteins. Also, Yamane et al. (29) showed that in vitro protein export could be completely inhibited by the presence of reagents which dissipate membrane energy in membranes prepared from mutants devoid of the F<sub>1</sub>F<sub>0</sub> ATPase.

Protonmotive force is composed of a transmembrane electrical potential and a transmembrane pH gradient. The effects of the individual components of protonmotive force on bacterial protein export have been examined under both in vivo and in vitro conditions. Bakker and Randall (30) have shown that both components of protonmotive force are required for protein export under in vivo conditions, while Mizushima and associates (11) have obtained similar results under in vitro conditions. These results
suggest that both the transmembrane electrical potential and the transmembrane pH gradient function as the membrane energy source required for bacterial protein export.

The requirements for membrane energy appear to differ depending on the exported protein (31). Export of the outer membrane protein OmpA is only marginally dependent on membrane energy (26, 32), while LSBP and BLA are highly dependent on the presence of membrane energy (33). In a recent review, Mizushima and Tokuda (11) claim that the presence of a proline residue near the leader sequence processing site may reduce the requirement for membrane energy in protein export. The strong helix-breaking ability of proline was suggested as the reason for the reduced membrane energy dependence in the export of proteins containing proline at this location.

ATP is of paramount importance in protein export since all known export systems require ATP (12). ATP is believed to be consumed during the protein export process, since non-hydrolyzable analogues of ATP have been shown to inhibit bacterial protein export (34). The F₁F₀ ATPase is not the site at which ATP is consumed during protein export as E. coli strains containing a deletion of the F₁F₀ ATPase genes can export proteins in a normal fashion (26, 28, 29). The SecA protein has been implicated as the site of ATP consumption during protein export (see page 12 and reference 35). A stoichiometry of 5000 molecules of ATP consumed by SecA per precursor protein translocated has been determined (36). SecA also has been shown to have a Km for ATP of 0.23mM (36). These results suggest that much of the characterization of ATP in the export process has been achieved, but a direct coupling between SecA catalyzed
ATP hydrolysis and protein export has not been shown. Also, the mechanism by which SecA functions, and identification of other export components which may contain ATPase activity involved in protein export, have not been determined.

Identification of Components of the Bacterial Protein Export Process—Genetic methods have proven to be very productive in identifying genes which code for components involved in the bacterial protein export. Two different methods have been employed. The first method utilizes lacZ fusion technology to obtain mutants which contain pleiotropic protein export defects (37). The genes isolated by this method have been termed the sec genes (for secretion). This fusion technology involves the in-frame coupling of a functional lacZ gene behind the portion of a gene coding for the amino terminal end of exported proteins. The rationale behind lacZ gene fusion is that the lacZ gene product is not exportable, apparently due to protein folding (38). When lacZ is fused to genes coding for exported proteins such as LamB (outer membrane maltoporin) or MalE (periplasmic maltose binding protein) the fusion product is embedded in the membrane. This results in jamming of the export machinery. Mutations in secretion components can be selected if they reverse jamming of the export machinery. Many reviews have been written to describe the process, the most recent being by Bieker et al. (38). Genes coding for export components identified by this method include secA (39), secB (40), secD (41), secE (42), and secY (43).

A second method has also been used to isolate genes coding for protein export components. It involves the identification of genes which suppress mutations in the signal (leader) sequence (44). Mutations in signal sequences result in an inhibition or
reduced level of export of these proteins. Mutations in other genes which suppress the original signal sequence mutation (export defect) should code for components involved in the export process. Genes identified in this fashion have been termed prl genes (for protein localization). Three genes identified by this method have been shown to suppress mutations in sec genes (38). These include prlA, prlD, and prlG which are suppressor alleles of secY, secA, and secE, respectively. Identification of secY, secA, and secE genes by both genetic methods is strong evidence for involvement of their gene products in the bacterial protein export process.

Biochemical studies have also been used to identify protein export components. The development of in vitro systems has greatly facilitated the process. Protein export components identified by biochemical characterization include cytoplasmic translocating factors (CTFs) (45), and the chaperone proteins trigger factor (27) and GroEl (46).

Function of Identified Components of the Bacterial Protein Export Machinery

One shortcoming of genetic methods is the difficulty of determining biochemical functions of identified protein export components. Only recently has research been directed at determining the biological function of many protein export components. The current state of our knowledge on the function of protein export components is discussed below.

Leader Peptidase—Two distinct leader peptidases have been identified in E. coli. Leader peptidase I was the first bacterial protein export component to be characterized biochemically (47). It is responsible for the correct processing of most exported proteins including LSBP and BLA (which have been used in this research as model...
proteins). Signal peptidase II or lipoprotein-specific signal peptidase is responsible for the processing of glyceride-modified lipoprotein precursors (48). In this review the properties of Signal peptidase II will not be discussed.

Leader peptidase I is a single subunit, 36 kDa protein encoded by the lepB gene (49). The active site of the protein is located on the exterior of the E. coli inner membrane, where it is anchored by a short amino terminal section of the protein (50). Most of the protein is exposed to the periplasim.

SecA—Genetic studies indicate that the secA gene product is essential for protein export. Temperature sensitive mutations in secA resulted in a partial export defect at 30°C (permissive temperature), while export is severely defective at 37-42°C (nonpermissive temperature) (37). Cell death also ensues when the temperature sensitive mutants are grown at the nonpermissive temperature (37). Nine independent secA(Ts) mutations have been analyzed, and five of these mutations reside in the first 170 amino acid residues of the 901 amino acid residue SecA protein (51). Located within this sequence region is a predicted ATP binding site (37). Furthermore, three different signal sequence suppressor mutations have been mapped to the secA gene, with one of these suppressor mutations located 5 amino acid residues away from the predicted ATP binding site (52).

Sodium azide-resistant mutations are some of the oldest known mutations in E. coli (53). It has been found that azide resistance and phenethyl alcohol resistance (an inhibitor of bacterial protein export) are allelic (54). Oliver and coworkers have analyzed four different azide-resistance mutations and found them to be allelic to
secA (52). Addition of sodium azide to wild-type *E. coli* cell cultures results in a nearly complete block in protein export after one minute. It now appears that azide inhibition of protein export is due to inhibition of SecA.

The secA temperature sensitive mutants have been used to gauge the requirement of an exported protein for the protein export machinery. In all cases examined to date a requirement for the SecA protein has been accompanied by a requirement for the SecY protein (52). Several patterns exist for secA-dependent export. SecA is required for the export of most *E. coli* outer membrane proteins, periplasmic proteins, and integral membrane proteins containing a cleavable or uncleavable leader sequence and a substantial exported segment (52). Only two types of exported proteins have been shown to be secA-independent. The first type include small integral membrane proteins (usually less than 75 amino acid residues) such as M13 procoat (55). The second type consists of unusual proteins, such as haemolysins, which appear to require special machinery for their export (56).

Biochemical studies have shown that two major forms of SecA exist in approximately equal abundance. One form of the protein is peripherally associated with the cytoplasmic face of the *E. coli* inner membrane, while the second form appears to be soluble (57). The function of the soluble form of SecA is unknown, while the membrane-associated form appears to be directly involved in the bacterial protein export process. Studies with secA-lacZ fusions indicate that the first 250 amino terminal residues of the SecA protein sequence contain several membrane binding elements (52). It is not clear if SecA has a membrane receptor for binding, but it has
been established that SecA can bind and insert into liposomes which contain acidic phospholipid (58). Several reports indicate that SecA may bind to the integral membrane protein SecY (see below). The secY24(Ts) mutation results in accumulation of precursors when membranes prepared from this strain are incubated at the nonpermissive temperature (59). Addition of purified SecA protein to inactive secY24 membranes restores their ability to support the export of precursor proteins (60). Furthermore SecA ATPase activity has a requirement for a functional SecY protein (35, to be discussed). These data are suggestive of an interaction between SecA and SecY, however direct proof of a physical interaction between the two proteins has not been established.

SecA has also been shown to bind to precursors of exported proteins. Chemical cross-linking of SecA to the chimeric protein OmpF-Lpp was shown to be dependent on the leader peptide and mature regions of OmpF-Lpp (61). Cross-linking was not observed either when the leader peptide was removed or with other nonsecretory proteins. Cross-linking of SecA to precursor proteins was enhanced when the positive charge at the amino-terminal region of the leader peptide was increased (61). The level of chemical cross-linking between SecA and precursor protein also paralleled the level of translocation (61). These results taken in total suggest that SecA interacts with exported proteins and that the interaction is dependent on the charge at the amino terminal region of the leader peptide as well as elements in the mature region of exported proteins.
SecA contains a low level of endogenous ATPase activity which is stimulated by the addition of purified precursors to exported proteins and membrane vesicles (35). The stimulation also requires the presence of functional SecY protein in the membranes. SecA ATPase activity is inhibited in the presence of SecY antibodies and in the presence of membranes prepared from secY24 at the nonpermissive temperature (35). SecA ATPase activity has been termed "translocation ATPase," by Wickner and coworkers, because of its dependence on precursors to exported proteins and inverted membrane vesicles (35). However, it is not apparent that SecA ATPase activity coincides with the actual translocation of precursor protein across inverted membrane vesicles. Some evidence suggests that SecA ATPase activity is responsible for translocation because photocrosslinking of SecA with 8-azido-ATP inhibits SecA dependent *in vitro* translocation and ATPase activity with nearly identical kinetics (35). Furthermore, it has recently been possible to reconstitute SecA ATPase activity in liposomes containing acidic phospholipids and in the presence of purified precursor protein (58). These results suggest that SecA ATPase activity is at least coupled to protein export, although they do not supply direct proof of precursor translocation coupled to ATPase activity.

Sequence analysis has shown that SecA contains three predicted ATP binding sites (52). This prediction is consistent with photocrosslinking studies which reveal that SecA crosslinks to 8-azido-ATP at two sites, while ATPase activity still remains, suggesting three ATP binding sites (35). Further characterization of ATP binding has shown that SecA interacts with ATP within the first 200 amino acid residues of the
protein (62). SecA contains a sequence in this region which shares some homology to ATP binding consensus sequences (62). Mizushima and coworkers claim that interactions of SecA with precursor proteins also occurs in the amino terminal region of SecA, suggesting that the amino terminus of SecA is responsible for its function during protein export (62).

SecY—The secY gene codes for a 49 kDa integral membrane protein which appears to have 10 membrane spanning domains (63). Mutational studies have shown that cells containing the secY24(TS) mutation accumulate precursor proteins when grown at the nonpermissive temperature (64). The effect of the mutation can be relieved by the introduction of a plasmid bearing the wild type secY gene (64). In vitro studies have shown that membranes prepared from the secY24 mutant grown at the nonpermissive temperature are unable to support protein export (59). Furthermore, membranes prepared from this strain grown at the permissive temperature support export, but when the same membranes are incubated at the nonpermissive temperature protein export is inhibited (59). These results indicate that precursor protein accumulation and loss of in vitro protein export ability of membranes prepared from secY24 at the nonpermissive temperature are due to the effect of the secY24 mutation.

The secY24 mutation is a substitution of aspartic acid for glycine at a position believed to reside on the cytoplasmic face of the protein (65). The site of the mutation has led to speculation about a possible defective interaction between SecY and another component of the export machinery (59, 66). Suppression of the secY24 mutation was shown to occur when purified SecA protein was added, suggesting that the mutation
results in inefficient binding of SecA to the integral membrane protein SecY (59). Detailed studies of this possible interaction, until recently, have proven difficult due to the inability to purify active SecY protein (67).

**SecE**—The secE gene was identified by lacZ fusion methods (42). The gene encodes a 127 amino acid integral membrane protein predicted to span the membrane three times (38). Sequence analysis of SecE indicates that some homology to SecY exists, however the significance of this homology is unknown (68). Genetic analysis reveals that SecE interacts with SecY directly, and that it may function before SecY during the protein export process (69).

**SecB**—The secB gene codes for a 16 kDa protein that does not appear to be essential for cell growth (70) or export of all *E. coli* proteins (71). The SecB protein appears to function in the folding and translocation of a specific class of exported proteins (72). SecB is capable of suppressing the secY24 mutation, but only 30% as well as SecA (73). The site of this suppressor activity is not at the membrane since incubation of SecB with secY24 membranes (treated at the nonpermissive temperature) does not restore translocation activity. The suppressor activity may be due to antifolding activity (to be discussed), or to a yet unidentified activity of the SecB protein.

**SecD** and **SecF**—The secD gene was isolated as a cold-sensitive lethal mutation that caused accumulation of precursor proteins (41). The secF gene was discovered during DNA sequence analysis of the secD gene (38). Based on sequence analysis and
phoA fusion studies it is believed that both genes code for cytoplasmic membrane proteins (38). At present the biochemical functions of these proteins are unknown.

**Trigger Factor and GroEL**—Trigger factor is a 63 kDa protein isolated by affinity chromatography using OmpA precursor proteins conjugated to a Sepharose matrix (27). Further characterization has shown that trigger factor can be removed from the 50S ribosome subunit by treatment with LiCl. The 50S subunit is the site of exit for nascent polypeptides (74). Trigger factor appears to interact with the mature regions of precursor proteins stabilizing an open or unfolded conformation (75).

GroEL is an *E. coli* heat shock protein which also appears to have antifolding activity (75). It was initially implicated in the protein export process by photocrosslinking to precursors of BLA (46). It has since been shown to stabilize open or unfolded structures of a number of precursor proteins (75).

**CTFs**—Cytoplasmic factors have been shown to stimulate translocation *in vitro* (45). These factors, termed CTFs, have been isolated by fractionation of the cytoplasmic contents of the cell. Purification of CTFs have indicated that one component is SecB, while two others are believed to be 60 kDa and 120 kDa proteins (76). The functions of these two proteins are unknown, however it has been speculated that CTFs operate in a manner similar to SecB (76).

**Precursor Protein Conformation**—Randall and Hardy have shown that precursors of exported proteins fold into structures characteristic of the mature protein (tightly folded). When folded in a mature-like structure, reduced levels of export are observed (77). Further analysis has revealed that the leader sequence may play a
fundamental role in governing the folding kinetics of the newly synthesized precursor protein (78). In the absence of a leader peptide, maltose binding protein folds rapidly into a mature-like structure, while the folding is considerably slowed in the presence of the leader peptide (79).

Trigger factor, SecB, and GroEL also appear to reduce the rate of precursor protein folding. When purified OmpA precursor protein is diluted from urea into an aqueous solution containing trigger factor or GroEL it will bind to either of these proteins (75). This interaction maintains a loose "export competent" conformation which increases the level of in vitro translocation of the precursor protein (75). Proteins which interact with precursors to stabilize "export competent" conformations have been termed chaperone proteins (80). Evidence also indicates that individual chaperone proteins function on only specific classes of exported proteins. For example trigger factor is unable to stabilize precursors of the outer membrane pore protein PhoE, while it functions as a chaperone for the outer membrane protein OmpA (81). SecB also appears to maintain an export competent conformation for many export precursor proteins (75). However it does not appear to stabilize precursors of LamB (72), ribose binding protein (72), or LSBP (Ben Owens personal communication).

Leader Sequences—It has been stated by Randall and Hardy that the leader (signal) sequence provides "unity in function in spite of a diversity of sequence" (82). Leader sequences are essential for protein export and appear to be the mechanism by which exported proteins are selectively sorted at the membrane, however, there is a large divergence in primary sequence of leader peptides (6). In spite of the lack of
sequence homology there are strong similarities in residue types. Leader sequences contain between 15 and 30 amino acid residues located at the amino terminal end of precursor proteins. Within the leader sequence three conserved regions exist; the basic amino terminus, the hydrophobic core, and the processing site.

The basic amino terminus contains 1-3 positively charged amino acids residues (6). Using site directed mutagenesis to remove or replace the basic amino terminus with neutral or acid amino acid residues, the level of precursor protein synthesis as well as processing and export is reduced (2). The basic charge on the amino terminus appears to be essential for recognition and binding to SecA (61). Increasing the basic charge found in this region results in a higher level of in vitro translocation of precursors (83). These results indicate that the charge on the amino terminus appears to affect the rate of synthesis and export of precursor proteins.

The hydrophobic core is usually 7-16 amino acid residues long, and rich in apolar residues (6). Protein structure prediction analysis of this region suggest alpha helix or beta sheet formation (6), which has been confirmed by spectroscopic techniques for leader peptides (84). Mutagenesis in this region can disrupt signal peptide function and cause the accumulation of precursors to exported proteins in vivo. A summary of the mutagenesis results suggest that insertion of charged amino acid residues or deletions of amino acid residues in the hydrophobic core disrupt the required hydrophobicity causing an inhibition of export. Insertion of helix destabilizing amino acid residues such as proline and glycine also disrupt secondary structures necessary for leader sequence function, resulting in a reduced level of export (6).
The processing site consists of 6-8 amino acid residues following the hydrophobic core. All naturally occurring cleavage sites conform to the -3,-1 rule, where the -1 and -3 amino acid residues must be small and uncharged (serine, alanine, glycine, cysteine) (85). Amino acids which correspond to -3 and -1 are located with in the leader sequence. Residue 1 corresponds to the first amino acid residue of the mature protein. In many cases mutations at -1 or -3 either slow down processing or eliminate processing, but do not tend to affect export of the protein (85).

The conserved regions of similarity play an important role in the nature and function of leader sequences, however their function is not fully understood. A great deal of work has been done on the structure of signal sequences. Biophysical approaches have shown that tertiary structure of the leader sequence can change depending on its environment (84). The importance of insertion into the lipid protein bilayer has been shown (86). It also appears that leader sequences play an integral role in folding of the precursor protein prior to export (79). Present data taken together suggest signal sequences function in at least three different stages of the export process: folding of precursor proteins, entry into the export pathway, and during translocation across the membrane.

**Current Model of Bacterial Protein Export**–It is possible to separate the current understanding of bacterial protein export into three different events: precursor protein synthesis, binding to a "translocator", and precursor protein translocation. The events are believed to be identical whether export occurs coupled to synthesis (cotranslational)
or uncoupled from synthesis (posttranslational). The only difference between the two types of export appears to be chaperone interaction with precursor proteins.

Precursor protein synthesis is the initial step of protein export. Following protein synthesis the precursor is believed to leave the translation complex and interact with the appropriate chaperone protein. Chaperone proteins include SecB, trigger factor, GroEL, and possibly CTFs. Chaperone proteins appear to stabilize an "export competent" conformation of the precursor protein. Under cotranslational translocation conditions the precursor may not interact with a chaperone protein, but rather proceed directly to the second stage of export.

In the second stage of protein export, the precursor or chaperone/precursor complex interacts with the translocator. The translocator appears to be a complex of the integral membrane proteins SecY, SecE, the peripheral membrane protein SecA, and other unidentified components. The precursor protein binds to SecA (both the leader peptide and mature portions of the precursor protein appear to be involved [61]) and stimulates SecA ATPase activity. The ATPase activity may be necessary for activation of other export components or for the translocation of the precursor.

The final stage of the export process is the translocation of the precursor protein across the membrane. SecY, SecE, and other membrane components may constitute a membrane pore through which the precursor protein is exported. Conversely, translocation may also occur directly across the lipid protein membrane bilayer. Leader peptidase removes the leader peptide from the precursor protein rendering the process irreversible.
Membrane energy and ATP are essential for the export of precursor proteins. ATP is utilized at SecA, and the energy obtained from ATP hydrolysis may drive the translocation event. SecA ATPase activity may also be used to activate or regulate other components. Activation by SecA may result in consumption of ATP by a second component which drives translocation. In any event, ATP consumption is necessary for export to occur. Membrane energy may be used for the assembly of the translocator, to direct precursor proteins to the translocator, or to move the precursor protein across the membrane.

As should be evident from the current model, many unanswered questions remain. These include the actual roles of membrane energy and ATP during bacterial protein export, and the mechanism by which SecA consumes ATP. The nature of interactions between precursor proteins and translocator complex, and the mechanism of translocator complex formation needs to be elucidated. Furthermore, additional components of the bacterial protein export process which have proven to be elusive need to be identified. Addressing these unanswered questions will aid in the determination of how proteins are exported.

References


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CHAPTER II

ATP AND MEMBRANE ENERGY ARE ESSENTIAL FOR IN VITRO TRANSLOCATION OF LEUCINE SPECIFIC BINDING PROTEIN AND $\beta$-LACTAMASE PRECURSORS

Introduction

All forms of protein translocation across biological membranes require energy in the form of high energy phosphate (ATP), membrane gradient energy, or both (1, 2). Early studies showed that ionophore addition to intact *E. coli* cells results in the accumulation of nearly all proteins destined for export, including M13 procoat (3), outer membrane protein A (OmpA) (4), alkaline phosphatase (5), BLA (6), LSBP (7), and leucine isoleucine valine binding protein (7). In a more rigorous study, Bakker and Randall (8) concluded that protein export requires membrane gradient energy in the form of total protonmotive force (the gradient of proton electrochemical potential across the membrane).

The development of *in vitro* protein export systems has made it possible to explore the energy requirements of bacterial protein export in greater detail. Cotranslational and posttranslational translocation systems are the two most commonly used *in vitro* protein export systems. The cotranslational translocation system carries out plasmid or mRNA directed precursor protein synthesis using an *E. coli* cell lysate containing inverted inner membrane vesicles. With the cotranslational system it is
possible to study protein export while precursor protein synthesis is occurring. The posttranslational protein translocation system combines inverted cytoplasmic membrane vesicles, and some exogenous source of precursor protein. In most cases the precursor protein is made separately by plasmid or mRNA directed precursor protein synthesis with a membrane-free E. coli cell lysate (9). Addition of precursor protein to inverted membrane vesicles allows for the study of protein export independent of protein synthesis.

Using a posttranslational translocation system with in vitro synthesized precursor protein in which ATP had been removed by gel exclusion chromatography, Chen and Tai showed that export of OmpA and alkaline phosphatase requires ATP (10). The requirement for ATP was also observed for cotranslational translocation (11). The requirement for ATP during protein export has since been established for many exported E. coli proteins (12). Furthermore, the addition of non-hydrolyzable analogues of ATP was shown to inhibit the export of OmpA (13). These results suggest that ATP hydrolysis is required for in vitro protein export.

In vitro studies of the membrane energy requirement for protein export have been controversial. Chen and Tai (10) found that OmpA can be translocated into inverted unc- membrane vesicles (devoid of the F1 ATPase) in the presence of ATP and a protonophore, leading the authors to conclude that membrane energy may participate in but is not required for bacterial protein export. Conversely, Geller et al. (14), and Yamane et al. (15) showed that OmpA requires membrane energy in addition to ATP for optimal in vitro translocation. It is currently believed that both membrane energy
and ATP are required for optimal translocation of bacterial proteins, but the requirement for membrane energy may differ depending on the precursor protein species (2).

In this chapter the energy requirements for the export of LSBP and BLA precursor proteins have been investigated. It was confirmed that both LSBP and BLA precursor proteins require ATP for in vitro export, and an effective Km for ATP during export was determined. Furthermore, membrane gradient energy was shown to be necessary for optimal translocation of both proteins, with the transmembrane electrical potential appearing to be the most important component of membrane gradient energy.

**Materials and Methods**

**Materials**—Valinomycin, nigericin, monensin, gramicidin, sodium ATP, NADH, Nonidet P-40, Brij-35, firefly lantern tails, tris base, and proteinase K were purchased from Sigma. Reagents for Bradford and BCA protein concentration determination were purchased from Pierce. SF6847 was a gift from Dr. Jack Lancaster. All other chemicals were of reagent grade. *E. coli* strain 1100 ΔuncBC (bgIR, thi-1, rel-1, HfrH, ΔuncB-uncC) (16) was provided by Dr. Robert D. Simoni. *E. coli* strain AE1 (7) was provided by Dr. Dale Oxender.

**Cell Extracts**—S-30 extract was prepared by a modified version of the method of Wilcox et al. (17). *E. coli* strain AE1 (18) was used for all assays which required S-30 extract. Cells were grown to an $A_{600}$ of 3-4 in PYEG medium (28.9g K$_2$HPO$_4$, 5.6g KH$_2$PO$_4$, 9g yeast extract, 1.8g casamino acids, 18mg tryptophan, 12mg thiamine, 0.9% glucose and 1mmol FeCl$_3$ per liter). Cultures were chilled on ice for 20 min and then
harvested by centrifugation (6000 x g, 15 min, 4°C). Cells were washed once in TMK buffer (50mM tris acetate, 60mM potassium acetate, 15mM magnesium acetate, pH 7.8) and suspended in an equal weight of the same buffer containing 2mM dithiothreitol. In some cases 125mM sucrose was also included in this buffer. The cells were lysed by two passes through a chilled French pressure cell (Aminco) at 7500 psi. The cell lysate was centrifuged twice at 30,000 x g for either 30 min (Sorvall SS-34 rotor) or 12 min (Beckman TLA 100.3 rotor) at 4°C. The supernatant was incubated for 80 min at 37°C with an additional 1mM dithiothreitol, 3mM magnesium acetate, 10 M of each of the 20 amino acids, 0.8mM ATP, and 100mM tris acetate, pH 7.8 (final concentration). The S-30 extract was dialyzed for 6 hours against TMK buffer containing 1mM dithiothreitol and stored at -70°C.

S-100 extract was obtained by centrifugation of S-30 extract (100,000 x g, 90 min, 4°C). The pellet containing ribosome and membrane was discarded, and the supernatant was stored at -70°C.

Inverted inner membrane vesicles were prepared by a modified version of the method of Muller and Blobel (19). Cells were grown to an $A_{600}$ of 3-4 in PYEG for E. coli strain AE1, or to mid log phase in LBT (20) for E. coli strain 1100 $\Delta$uncBC. Cells were harvested, washed, and resuspended in 50mM triethanolamine, 250mM sucrose, 2mM dithiothreitol, pH 7.5 as stated for S-30 preparation. Cells were lysed in a chilled French pressure cell at 4000 psi, and the cellular debris was removed by centrifugation (8000 x g, 20 min, 4°C). Inverted membranes were isolated by centrifugation in a Beckman TLA 100.3 rotor (60,000 rpm, 70 min, 4°C). This
centrifugation step results in two membrane pellets, a soft pellet located above a tightly packed hard pellet. Translocation activity was found to be associated with the soft pellet. Membranes obtained from the soft pellet were diluted to approximately 20mg/ml in buffer and stored at -70°C.

Ribosomes were prepared by the detergent lysis method of Godson and Sinsheimer (9) with the following modifications. Brij-35 was used in place of Brij-58. The cell lysate was centrifuged in a 50 Ti rotor (125,000 x g, 4 min, 4°C). Ribosomes were isolated from the resulting supernatant by a second centrifugation (125,000 x g, 60 min, 4°C) and suspended at 220 A_{260} units/ml in 50mM triethanolamine acetate, 15mM magnesium acetate, 60mM potassium acetate, pH 7.8. Ribosomes were stored at -70°C.

DNA from pOX7 (18) was isolated by alkaline lysis and purified by cesium chloride gradient centrifugation (21).

Cotranslational Translocation—Cotranslational translocation reactions were composed substantially as described by Zubay (22) except that 2.5% glycerol was used in place of 16mg/ml polyethylene glycol. Reactions of 25 ml total volume contained 6mg/ml S-30 extract, 800ng pOX7 plasmid DNA and all amino acids except methionine. Reactions were preincubated for 8 min at 37°C before the start of synthesis. Synthesis and translocation was initiated by the addition of 20 Ci of L-[\text{35S}]methionine (1100Ci/mMol). Incubation continued for 20 min at 37°C. Where indicated ionophores were vacuum dried from ethanol solutions onto polypropylene centrifuged tubes before the addition of other reaction components.
Posttranslational Translocation Posttranslational translocation reactions contained 5-10 l of labeled LSBP and BLA precursors, 1mg/ml inverted membrane vesicles, 3mM ATP, 3mM NADH, 60mM potassium acetate, 12mM magnesium acetate, and 50mM potassium N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes) pH 7.6, in a final volume of 25 l. Reactions were incubated at 37 °C for 20 min. Reactions done in the presence of an ATP regeneration system also contained 15mM phosphoenolpyruvate and 15 g/ml pyruvate kinase. Where indicated, ionophores were added as described under cotranslational translocation.

In vitro LSBP and BLA precursors were prepared by plasmid directed synthesis using an S-100 cell extract and ribosomes. Synthesis was carried out in the same fashion as cotranslational reactions, except that reactions contained 5mg/ml S-100 extract and 0.22 A_{260} units of ribosomes in place of S-30. In a few cases cold methionine at 2.5 M was used in place of [35S]methionine. Synthesis was stopped by the addition of 250 g/ml chloramphenicol and 250 g/ml streptomycin sulfate from 50mg/ml stock solutions in dimethyl sulfoxide followed by chilling on ice. Synthesis reactions were then passed through desalting gel columns at 4 °C by the method of Chen and Tai (10) to remove the small molecule pool necessary for synthesis. Columns (10 times the reaction volume) were prepared from Bio-Gel P-6DG, equilibrated in 50mM sodium Hepes, 2.5% glycerol, 1mM dithiothreitol, pH 7.6. Immediately after recovery of the precursor from the column, chloramphenicol and streptomycin sulfate were added to 250 g/ml, and the precursor was stored at -70 °C.
Protease Protection—Where indicated, completed cotranslational or posttranslational translocation reactions were digested for 20 min at 37°C with 200 g/ml proteinase K and 125 mM added NaCl. Digests were chilled on ice, and trichloroacetic acid was added to a final concentration of 10% to halt proteolysis and precipitate protein. Precipitates were incubated on ice for 10 min and then isolated by centrifugation (12,000 x g, 5 min, 4°C). The precipitates were washed two times with ice cold acetone (5 times the original volume) and centrifuged as before. Residual acetone was removed under vacuum for 30 min.

Determination of ATP Concentration—ATP concentrations were measured by luminescence using the firefly tail method (23). The firefly tail method is a coupled enzyme system where a linear relationship exists between light generated by luciferin-leuciferase and the ATP concentration. Activated firefly tail (FFT) extract was prepared by homogenation of 50 mg of firefly lanterns in 5 ml of 20 mM sodium glycylglycine, 20 mM magnesium sulfate, 50 mM sodium arsenate, pH 7.4, on ice and centrifuged (18,000 x g, 10 min, 4°C). The supernatant was activated by the addition of 80 mg of Ca$_3$(PO$_4$)$_2$, incubated for 10 min at room temperature, and centrifuged (400 x g, 2 min, 4°C). The supernatant was treated with Ca$_3$(PO$_4$)$_2$ a second time, centrifuged (18,000 x g, 10 min, 4°C), and the supernatant (activated FFT extract) was stored at -20°C. Samples were prepared for ATP determination by precipitation with 3 M perchloric acid and 0.005% phenolphthalein, which was then neutralized to the phenolphthalein endpoint with 7.5 M KOH and 50 mM KH$_2$PO$_4$ and incubated on ice for 10 min. The supernatant was collected by centrifugation (12,000 x g, 10 min, 4°C).
ATP was measured by the addition of 0.9ml of 5mM sodium arsenate pH 8.0, 4mM magnesium sulfate, 20mM glycylglycine pH 8.0, to 50 l of sample. Activated FFT extract was added (50 l), and the samples were incubated for 20 seconds at room temperature, followed by counting for 30 seconds on the $^3$H plus $^{14}$C channel of a Beckman LS 100C scintillation counter. ATP concentrations were determined by comparison to an ATP standard curve. All ATP concentration determinations were repeated three separate times. Results of individual determinations are shown, with the error being the standard deviation of 3 separate measurements.

SDS-Polyacrylamide Gel Electrophoresis—Samples were prepared for electrophoresis by the addition of an equal volume of sample mix containing 250mM tris-HCl buffer, 4% SDS, 20% (W/V) β-mercaptoethanol, pH 6.6. Samples were boiled for 4 min, and then 1/10th volume of 0.5% bromphenol blue dye in 80% glycerol (W/V) was added. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (24) in gels (1.5mm x 14cm x 30cm) containing 10% total acrylamide. Following electrophoresis, gels were fixed in 10% trichloroacetic acid, 10% (V/V) glacial acetic acid, 30% (V/V) methanol, then washed in water, dried, and autoradiographed.

Protein Concentration—Protein concentration was determined by the Bradford (25) or bicinechoninic acid (BCA) method (26).

Densitometry—Densitometry was done on autoradiographs using an ISCO gel scanner model 1312. Area of the peaks was determined by integration using Inject computer software program (Interactive Microware, 1988).
Results

ATP Is Necessary for the Posttranslational Translocation And Processing of LSBP and BLA Precursors—Using an *in vitro* posttranslational translocation system, the requirement for ATP during the translocation of LSBP and BLA precursor proteins was investigated. Figure II.1 shows that processing and translocation of $^{35}\text{S}$methionine-labeled LSBP and BLA precursors requires ATP. In the presence of ATP, processing (lane 6) and translocation (lane 3) of LSBP and BLA precursors are observed. However, in the absence of ATP, neither processing (lane 5) nor translocation (lane 2) of LSBP or BLA precursors are observed. Processing refers to the removal of the leader sequence resulting in the mature protein which is smaller than the precursor and therefore will migrate faster on SDS gels.

To determine if precursor protein processing corresponds with translocation into the inverted membrane vesicles, soluble protease was added following translocation. Addition of protease results in digestion of precursor proteins which have not been translocated (Figure II.1, lanes 1-3), while the mature translocated protein is protected from the added protease within the inverted membrane vesicles (lane 3). There is some incomplete protease digestion of LSBP precursors as evidenced by the band which runs just below the mature form of LSBP (Figure II.1). This protein fragment is present in
FIG. II.1. ATP dependent posttranslational translocation and processing of LSBP and BLA precursors. Posttranslational translocation reactions containing [35S]methionine-labeled LSBP and BLA precursors were carried out in the presence of 3mM ATP (lanes 1, 3, 4, 6) and inverted membranes (lanes 2, 3, 5, 6) as indicated. Translocation reactions were incubated for 20 min at 37°C followed by digestion with 200µg/ml proteinase K (lanes 1-3) for 20 min at 37°C. Trichloroacetic acid (TCA) precipitates from these reactions were subjected to SDS polyacrylamide gel electrophoresis and autoradiography. Precursor and mature proteins are indicated by p and m respectively. BLA is indicated by β-Lactam.
the absence of membranes (lane 1) indicating that it is a protease resistant from of LSBP precursor and not a processed form of LSBP. Incomplete digestion of LSBP is probably due to folding of the precursor protein into a conformation that is partially resistant to protease. However, untranslocated precursor proteins are accessible to protease, even though they are not completely digested. The level of protected or translocated protein corresponds very well with the level of processing. In most further studies processing of the leader peptide will be used as a measure of protein translocation.

The maximum level of processing shown in Figure II.1 is approximately 25-30% of total precursor protein. Reasons for incomplete translocation of precursor proteins using in vitro protein export systems are not understood, but the system does function in a fashion analogous to in vivo protein export. Precursor proteins are translocated across the membrane, translocation is dependent on energy, and the precursors are processed to their mature form.

Effects of ATP Concentration on the Level of Precursor Protein Translocation

The dependence of in vitro protein export on ATP concentration was examined using the posttranslational translocation system. Figure II.2 shows that ATP concentrations below 0.1mM do not support processing of LSBP and BLA precursor proteins (lanes 2-4). A low level of processing of both precursors is observed in the presence of 0.1mM ATP (lane 5). Near maximal processing of LSBP and BLA precursor proteins is evident at ATP concentrations of 1mM and 3mM (lanes 6 and 7). A band which migrates to approximately the same location as the mature form of LSBP is present in
FIG. II.2. Effects of different ATP concentrations on posttranslational processing of LSBP and BLA precursors. Posttranslational translocation reactions containing [35S]methionine-labeled LSBP and BLA precursors were carried out in the absence of ATP (lane 1 and 2) or the presence of the following ATP concentrations: lane 3, 1µM; lane 4, 10µM; lane 5, 100µM; lane 6, 1mM; lane 7, 3mM. Membrane vesicles were absent from lane 1, but present in lanes 2-7. Reaction conditions were as stated in "Materials and Methods." Samples were subjected to SDS gel electrophoresis and autoradiography. Precursor and mature proteins are indicated by p and m respectively.
FIG. II.3. Effects of different ATP concentrations on posttranslational processing of LSBP and BLA precursors in the presence of an ATP regeneration system. Posttranslational translocation reactions with [35S]methionine-labeled LSBP and BLA precursors were carried out as stated in "Materials and Methods." Translocation reactions in lanes 2-7 contained 15mM phosphoenolpyruvate and 15µg/ml pyruvate kinase, and 1mg/ml inverted membrane vesicles. ATP was absent from reactions in lanes 1 and 2, and present at the following concentrations in the remaining lanes: lane 3, 1µM; lane 4, 10µM; lane 5, 100µM; lane 6, 1mM; lane 7, 3mM. Samples were subjected to SDS gel electrophoresis and autoradiography. Precursor and mature forms are indicated p and m respectively.
lanes 1-4 (or at ATP concentrations below 0.1mM), but this band does not appear to be due to processing of LSBP precursors in this experiment as it is present in the absence of added membranes (lane 1). Furthermore, it is clear that processing of BLA precursors does not appear to occur at ATP concentrations below 0.1mM (lanes 1-4). These results could mean that the effective Km for ATP during protein export is between 0.1mM and 1mM. Alternatively, it is possible that ATP is being consumed to the point that concentrations of ATP needed to support translocation are exhausted.

To further investigate these possibilities, an ATP concentration curve was done in the presence of an ATP regeneration system consisting of phosphoenolpyruvate and pyruvate kinase (Figure II.3). A nearly maximal level of LSBP and BLA precursor protein processing is observed at all ATP concentrations from 1 M to 3mM (lanes 3-7). In the absence of added ATP and an ATP regeneration system, processing of LSBP and BLA is not observed (lane 1). Addition of an ATP regeneration system in the absence of added ATP results in processing of both precursor proteins to approximately the same level as observed in the presence of added ATP (lane 2). These results are consistent with a low effective Km for ATP and the rapid hydrolysis of ATP in the protein export process. However, they also suggest that some amount of nucleotide contamination is present, since protein export is observed in the reaction which contained only an ATP regeneration system (lane 2). The ATP concentration was directly measured to be 10 M in a translocation reaction containing the ATP regeneration system and no added ATP. This confirms that translocation reactions are
contaminated with some nucleotide. It also establishes an upper limit of 10 M for the effective Km.

Role of ATP in Cotranslational Protein Translocation—To examine ATP requirements for cotranslational translocation, synthesis/translocation assays were run at different concentrations of added ATP. Under cotranslational translocation conditions the concentration of nucleotides other than ATP is 0.56mM, and regeneration of ATP is possible due to the presence of phosphoenolpyruvate and pyruvate kinase. Figure II.4A indicates that synthesis increases linearly for both LSBP and BLA precursors from 1 M to 270 M ATP (lanes 1-4). This was confirmed by densitometry done on both the precursor and mature forms of LSBP (Figure II.4C). Using the densitometry data the effective Km for ATP during precursor protein synthesis, or the ATP concentration which supports half maximal synthesis, was estimated to be 300 M (Figure II.4C). At 1.1mM ATP the level of synthesis appears to have reached saturation, while at 2.2mM ATP the level of synthesis is slightly lower than at 1.1mM ATP (Figure II.4A lanes 6 and 7, and Figure II.4C). BLA precursor synthesis is slightly greater than LSBP at all ATP concentrations assayed, however the ATP concentrational dependence of synthesis appears to parallel LSBP synthesis.

Processing of LSBP and BLA precursors is observed to be nearly optimal in the presence of 100 M, 1.1mM and 2.2mM ATP (Figure II.4A, lanes 5-7). Below 100 M ATP little can be determined about processing due to the low intensities of the precursor and mature bands of both LSBP and BLA precursor proteins (lane 1-4). To investigate processing of LSBP and BLA precursors at ATP concentrations below
FIG. II.4A. Effects of different ATP concentrations on protein synthesis and cotranslational processing of LSBP and BLA precursors. Cotranslational translocation reactions were run as stated in "Materials and Methods" in the presence of 20µCi [35S]methionine and the following ATP concentrations: lane 1, 1µM; lane 2, 10µM; lane 3, 100µM; lane 4, 270µM; lane 5, 550µM; lane 6, 1.1mM; lane 7, 2.2mM. Samples were subjected to SDS gel electrophoresis and autoradiography. Precursor and mature forms are indicated p and m respectively.
FIG. II.4B. Different exposures were taken of the SDS gel shown in FIG. II.4A. in an attempt to observe processing at low ATP concentrations (lanes 1-4). Autoradiographs of 6 hours (lanes 5-7), 15 hours (lanes 3 and 4), and 210 hours (lanes 1 and 2) exposures were taken. Precursor and mature bands are indicated by p and m respectively.
FIG. II.4C. This figure shows a plot of the level of LSBP precursor protein synthesis at different ATP concentrations. Densitometry was done as stated in "Materials and Methods" on both the mature (m) and precursor (p) bands of LSBP from the autoradiograph in FIG. II.4A. The sum of the densities of both the precursor and mature bands of LSBP was plotted against the ATP concentration.
FIG. II.4D. This figure shows a plot of the % of LSBP processing at different ATP concentrations. Densitometry was done as stated in "Materials and Methods" on both the mature and precursor bands of LSBP. The % processing is the density of the mature protein divided by the sum of the densities of the mature and precursor forms of LSBP.
100 M, different radiographic exposures were used to obtain approximately equal intensities of the precursor protein bands. The results (Figure II.4B), indicate that the percentage of precursor protein processing for both LSBP and BLA is nearly identical from 10 M to 2.2mM added ATP (lanes 2-7). AT 1 M ATP a much lower level of processing is observed for both LSBP and BLA precursors. To further confirm the processing results densitometry was done on the precursor and mature bands of LSBP (Figure II.4D). The densitometry results show that a much lower level of processing is observed in the presence of 1 M added ATP, than is observed in the presence of added ATP at concentrations ranging from 10 M to 1.1mM added ATP.

To confirm the concentration of ATP present in these reactions a firefly luminescence assay was done. The assay worked well for direct determination of ATP concentrations in reactions containing 0.57mM or greater added ATP. In reactions containing less ATP, the presence of other nucleotides necessary for synthesis made it difficult to accurately measure ATP. However, it is possible to use the densitometry data (Figure II.4C) for protein synthesis as a probe for ATP concentration. This standard curve allows the concentration of ATP to be estimated in reactions containing 1 M and 10 M added ATP. These concentration were determined to be 8 M ATP in the reaction which contained 10 M added ATP, and 0.1 M ATP in the reaction which contained 1 M added ATP. These results for synthesis and processing of LSBP and BLA precursor proteins establish 10 M ATP as the upper limit for the effective Km during cotranslational translocation. It should be noted that ATP concentrations
determined at the low end of the curve are approximations used to confirm the level
of added ATP, and contain at least 10% error.

Effects of Ionophores on In Vitro Protein Translocation in a Cotranslational
Translocation System—To determine if membrane energy is required for the
translocation of LSBP and BLA precursors, cotranslational translocation reactions were
done in the presence of various ionophores (Figure II.5). The protonophore SF6847
and the ionophore combinations valinomycin/nigericin and valinomycin/monensin all
cause nearly complete inhibition of LSBP and BLA precursor protein processing (lanes
5, 3, and 8 respectively). These ionophores or combinations of ionophores are believed
to dissipate total protonmotive force (27). The effects of valinomycin, nigericin, and
monensin were also examined individually. Valinomycin functions as an electrogenic
K⁺-specific transporter which under these conditions should dissipate the transmembrane
electrical potential (ΔΨ). Valinomycin inhibits processing of both LSBP and BLA
precursor proteins, although the level of inhibition (lane 2) is slightly less than the
inhibition observed in the presence of protonmotive force dissipators. Nigericin and
monensin catalyze the electroneutral exchange of H⁺ for K⁺ or Na⁺, respectively (27).
Under most conditions these ionophores will dissipate the transmembrane pH gradient.
In contrast to valinomycin these ionophores do not appear to inhibit processing of either
LSBP or BLA (lane 4 and 7). In fact, a low level of stimulation of LSBP and BLA
precursor processing activity appears to occur in the presence of nigericin and
FIG. II.5. Effects of ionophores and ionophore combinations on cotranslational processing of LSBP and BLA precursors. Cotranslational translocation reactions were carried out as stated in "Materials and Methods" in the presence of 20µCi of [35S]methionine. The following ionophores were added to cotranslational translocation reactions: no ionophore (lane 1); 2µM valinomycin (lane 2), 2µM valinomycin and 1µM nigericin (lane 3), 1µM nigericin (lane 4), 200nM SF6847 (lane 5), 1µM gramicidin D (lane 6), 1µM monensin (lane 7), and 2µM valinomycin and 1µM monensin (lane 8). Samples were subjected to SDS gel electrophoresis and autoradiography. The precursor and mature forms are indicated by p and m respectively.
Table II.I.

Effects of ionophores or combinations of ionophores on ATP concentrations during cotranslational translocation reactions

Cotranslational translocation reactions containing 2.2mM ATP were run in parallel with reactions done in FIG. II.4, except cold methionine was used. ATP concentrations were determined by the firefly tail assay as stated in "Materials and Methods." Concentrations of ionophores: valinomycin 2µM, nigericin 1µM, SF6847 200nM. Error represents the standard deviation of 3 replicate measurements.

<table>
<thead>
<tr>
<th>Ionophore</th>
<th>Measured ATP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Valinomycin and</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Nigericin</td>
<td></td>
</tr>
<tr>
<td>Nigericin</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>SF6847</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>
FIG. II.6. Effects of ionophores on posttranslational translocation of LSBP and BLA precursors. Posttranslational translocation reactions containing [35S]methionine-labeled LSBP and BLA precursors were carried out as stated in "Materials and Methods." All reactions contained 3mM ATP and 3mM NADH, and lanes 2-8 contained inverted membrane vesicles. The following ionophores were added to these reactions; no ionophore (lane 1 and 2); 2µM valinomycin (lane 3), 2µM valinomycin and 1µM nigericin (lane 4), 1µM nigericin (lane 5), 200nM SF6847 (lane 6), 1µM monensin (lane 7), 2µM valinomycin and 1µM nigericin (lane 8). Samples were subjected to SDS gel electrophoresis and autoradiography. The precursor and mature forms are indicated by p and m for both LSBP and BLA.
monensin. The effect of the channel former gramicidin D is also shown. Gramicidin also appears to have an inhibitory effect on processing of LSBP and BLA (lane 6).

**Effects of Ionophores on ATP Levels During Cotranslational Translocation**—It has been suggested that inhibition of protein export by uncouplers of protonmotive force might be a consequence of ATP depletion resulting from the uncoupling action of the ionophores (10). To examine this possibility the concentration of ATP was measured in cotranslational translocation reactions containing ionophores and combinations of ionophores (Table II.1). In all cases the measured ATP concentration in the presence of ionophores or ionophore combinations is nearly identical to the level of ATP measured in the absence of these reagents. Furthermore, the concentrations are all well above the ATP concentration which supports optimum synthesis and processing of both precursors (Figure II.4).

**Effects of Ionophores on In Vitro Posttranslational Precursor Protein Processing**
The effects of ionophores and combinations of ionophores on posttranslational translocation were also examined (Figure II.6). The protonophore SF6847 and the ionophore combinations valinomycin/nigericin, and valinomycin/monensin inhibit processing of LSBP and BLA precursor proteins (lanes 6, 4, and 8, respectively). Valinomycin also inhibits processing of both proteins (lane 3), but the level of inhibition is less than the inhibition observed in the presence of reagents which dissipate total protonmotive force. Nigericin and monensin appear to slightly stimulate processing of LSBP and BLA precursor proteins (lanes 5 and 7). These results are
consistent with those obtained under cotranslational translocation conditions (Figure II.5).

The Individual Effects of ATP and Membrane Gradient Energy on Posttranslational Translocation—Using the posttranslational translocation system it is possible to examine the individual effects of ATP and membrane gradient energy on the export of LSBP and BLA precursor proteins (Figure II.7). The posttranslational translocation system is ideally suited for this study because energy necessary for precursor protein synthesis (including ATP) is removed by gel exclusion chromatography, and membranes prepared from the *E. coli* strain 1100 ΔuncBC (lacking the F₁ ATPase) prevent formation of a membrane energy gradient by ATP. In the presence of ATP and NADH optimal processing of both LSBP and BLA is observed (lane 3). ATP alone supports nearly optimal processing of both LSBP and BLA precursors (lane 2). NADH alone supports a low level of LSBP and BLA precursor protein processing (lane 4). In the absence of added energy a much lower level of processing is observed for BLA or LSBP precursors (lane 1). These results suggest that optimal processing of LSBP and BLA requires both ATP and NADH, while ATP alone can support nearly optimal export.
FIG. II.7. Individual effects of ATP and membrane gradient energy on the posttranslational processing of LSBP and BLA precursors. Posttranslational translocation reactions containing desalted [35S]methionine-labeled precursors of LSBP and BLA were carried out in the absence of added ATP or NADH (lane 1), or with 3mM ATP (lane 2), 3mM ATP and 3mM NADH (lane 3), or 3mM NADH (lane 4). Translocation reactions were run for 20 min at 37°C, followed by SDS gel electrophoresis and autoradiography. Precursor and mature forms of LSBP and BLA are indicated by p and m.
Discussion

Results presented in this chapter are consistent with many lines of evidence suggesting that both ATP and membrane energy are required for optimal translocation of many *E. coli* exported proteins (10-15). *In vitro* posttranslational translocation studies show that ATP alone can support translocation of LSBP and BLA precursor proteins; however in the absence of ATP, processing is not observed for either protein (Figure II.1). This result is consistent with published results showing ATP dependent *in vitro* translocation of many *E. coli* export proteins (12).

Chen and Tai have shown that non-hydrolyzable analogues of ATP inhibit *in vitro* bacterial protein translocation (13), suggesting that ATP hydrolysis occurs during the export process. Consistent with this interpretation is the observation of precursor protein processing at low concentrations of ATP (1 M added) in the presence of an ATP regeneration system (Figure II.3). In the absence of an ATP regeneration system processing of LSBP and BLA precursor proteins was not observed at ATP concentrations below 0.1mM (Figure II.2). Although these experiments were done using membranes containing the F$_1$ ATPase, consumption of ATP by the F$_1$ ATPase does not appear to be responsible for ATP hydrolysis since nearly identical results were obtained using membranes prepared from an *E. coli* strain containing a deletion of the F$_1$ ATPase genes (data not shown). This result indicates that protein export requires consumption of ATP at a site other than the F$_1$ ATPase.
In this chapter the effective Km for ATP was determined for the *in vitro* export of proteins under cotranslational and posttranslational translocation conditions. Under posttranslational translocation conditions in the presence of an ATP regeneration system, processing of both LSBP and BLA precursor proteins was at least half maximal at the lowest ATP concentration examined. The concentration was directly measured to be 10 M. This result suggests that the effective Km for ATP during posttranslational translocation can not be higher than 10 M.

Determination of the effective Km for ATP under cotranslational translocation conditions is complicated by an ATP requirement for precursor protein synthesis. Nevertheless, it is possible to bracket the ATP concentration which supports half maximal processing activity. This was accomplished by doing cotranslational translocation reactions at various concentrations of added ATP. The percentage of LSBP and BLA precursor protein processing is virtually identical at ATP concentrations ranging from 10 M to 2.2mM (Figure II.4B). At 1 M ATP, less than half maximal processing is observed for both LSBP and BLA precursors. This result suggests that the effective Km for ATP under cotranslational translocation conditions is between 1 M and 10 M. Protein synthesis, which has an effective Km for ATP determined to be approximately 0.3mM, proved to be a good indicator of the ATP concentration (Figure II.4C). Using protein synthesis as a probe for ATP concentration, the ATP level at the upper limit of the effective Km value for processing was confirmed to be about 10 M. This value is roughly the same as the effective Km for ATP in translocation determined under posttranslational translocation conditions, indicating that ATP may be utilized in
the same manner for both types of \textit{in vitro} protein export. Furthermore, this same effective Km was determined for the export of two different proteins, LSBP and BLA.

The SecA protein, an essential component of the protein export machinery (28), has recently been shown to contain an ATPase activity (29). Kinetic characterization of SecA ATPase activity indicates a Km for ATP of 0.23mM (30). This value differs significantly from the effective Km for ATP during the \textit{in vitro} export of LSBP and BLA precursor proteins determined in this study. The Km determined for SecA ATPase is the concentration of ATP which supports half maximal ATPase activity. Conversely, the Km determined for translocation is the concentration of ATP which supports half maximal precursor protein processing. If SecA is the only protein which utilizes ATP during the protein export process the Km for ATP in SecA ATPase activity would be expected to fall in the same range as the Km for ATP determined for \textit{in vitro} protein export. The much higher value for the SecA ATPase may be due to the method used to determine the constant. SecA ATPase activity was measured in the presence of urea-treated membrane vesicles, and purified precursor protein without an ATP regeneration system (30). It is possible that membrane proteins which regulate SecA ATPase activity are removed or inactivated by urea treatment. The effective Km determined in this chapter is based on the level of precursor protein processed at different ATP concentrations in the presence of an ATP regeneration system.

Membrane energy appears to be required for optimal translocation of both LSBP and BLA precursor proteins, however its exact role in the export process is unknown. Ionophores or combinations of ionophores which dissipate total protonmotive force
(SF6847, valinomycin/nigericin, and valinomycin/monensin) inhibit protein translocation almost completely (Figures II.5 and II.6). This effect was observed in both the cotranslational and posttranslational translocation systems. Inhibition of precursor protein export is not a result of ATP consumption due to the uncoupling action of the ionophores, since the presence of ionophores has no effect on the level of LSBP and BLA precursor protein synthesis (Figure II.5). Also, the measured ATP levels in the presence of the ionophores are not substantially different from the measured levels in the absence of the ionophores (Table II.I). These results indicate that membrane gradient energy is necessary for protein export.

Protonmotive force ($\Delta p$) is composed of an electrical component (transmembrane electrical potential, $\Delta \Psi$) and the chemical pH gradient across the membrane ($\Delta p$H). It can be described by the following relationship

$$\Delta p = \Delta \Psi - (2.303RT/F)\Delta p$H.$$

It is experimentally difficult to separate the roles of $\Delta p$, $\Delta \Psi$, and $\Delta p$H under conditions used to study in vitro protein export. Ionophores which dissipate $\Delta p$, or one of its two components, $\Delta \Psi$ or $\Delta p$H, have been employed in the past. Several problems exist with this approach. It is extremely difficult to determine what effect the dissipation of one gradient has on the two related gradients. An example of such a problem is the observed stimulation of LSBP and BLA precursor protein processing in the presence of nigericin and monensin. Nigericin and monensin should dissipate the transmembrane pH gradient, but their effects on $\Delta \Psi$ and $\Delta p$ are unknown. A second problem is that the effects of some ionophores (valinomycin, nigericin, and monensin as examples)
depend on the magnitude and direction of ion gradients (K⁺, and Na⁺) other than the H⁺ gradient. In many cases these gradients are unknown.

Noting these concerns, the data presented in Figures II.6 and II.7 indicate that the individual component of total protonmotive force necessary for in vitro protein export may be ΔΨ. This conclusion is based on the following reasoning. Valinomycin functions as an electrogenic K⁺ transporter, usually dissipating the transmembrane electrical potential. Valinomycin also inhibits processing of both LSBP and BLA precursors under cotranslational and posttranslational translocation conditions (Figure II.6 and II.7). The level of inhibition of precursor protein processing is less than in the presence of ionophores which uncouple total protonmotive force. Incomplete inhibition of protein export by valinomycin could be the result of a transmembrane K⁺ gradient that is partially equilibrated with ΔΨ (27). In contrast, nigericin and monensin stimulate processing of LSBP and BLA precursors. These ionophores function as electroneutral exchangers (27) thereby dissipating ΔpH. Since dissipation of ΔΨ inhibits processing of LSBP and BLA precursors, and dissipation of ΔpH slightly stimulates processing of both proteins, it is suggested that the component of total protonmotive force necessary for the export of LSBP and BLA is the transmembrane electrical potential gradient. The stimulation of precursor protein processing by the dissipation of ΔpH is presumed to result from the interconversion of ΔpH into ΔΨ. Once again, caution must be used with this interpretation for reasons mentioned previously.
This interpretation does appear to conflict with observations made by Yamane et al. (15) which show that nigericin strongly inhibits translocation of the chimeric protein OmpF-Lpp. Valinomycin also inhibits translocation of this protein to approximately the same level. These results led the authors to conclude that total protonmotive force is necessary for in vitro bacterial protein export. However, Yamane et al. (15) indicate that ionophores are added to posttranslational translocation reactions from ethanolic solutions. Ethanol at concentrations greater than 0.5% inhibits processing and translocation of LSBP and BLA precursors (personal communication Bruce Copeland). It is possible that inhibition of OmpF-Lpp translocation by nigericin may be due to the presence of ethanol rather than dissipation of the transmembrane pH gradient. Alternatively export of OmpF-Lpp may require different membrane energy gradients than needed for the export of LSBP and BLA.

Development of posttranslational translocation systems has made it possible to examine the role of ATP and membrane energy separately during in vitro protein export. ATP alone is capable of supporting translocation of LSBP and BLA precursors. Membranes prepared from E. coli strain 1100 ΔuncBC (the F₁ ATPase genes are deleted in this strain) were used to prevent membrane energy formation by ATP at the F₁F₀ ATPase. NADH alone does support a low level of LSBP and BLA precursor protein processing, but it is much less than the level observed in the presence of ATP. Processing of LSBP and BLA in the presence of NADH alone may be the result of ATP contamination in the posttranslational translocation system. The combination of ATP and NADH results in the highest level of processing. These results indicate that
ATP alone is capable of supporting processing and translocation of LSBP and BLA precursors, but optimal translocation requires the presence of both ATP and NADH. These results are also consistent with observations made by Geller et. al (14), and Yamane et. al (15) which show that OmpA and the chimeric protein OmpF-Lpp require both membrane energy and ATP for optimal in vitro protein translocation.

References


CHAPTER III

INHIBITION OF IN VITRO BACTERIAL PROTEIN EXPORT BY VANADATE AND DIETHYLSILBESTROL

Introduction

Chapter II demonstrated that ATP is required for the in vitro export of LSBP and BLA precursors. In fact an ATP requirement appears to exist for translocation of almost all exported proteins across biological membranes (1). Recently Lill et al. (2) showed that SecA is capable of ATP hydrolysis, suggesting that SecA may be one site which utilizes ATP during bacterial protein export.

SecA is a peripheral membrane protein essential for protein export in E. coli (3). It is found in two equally abundant forms, membrane associated and soluble (4). The membrane associated form of SecA is believed to interact with SecY, acidic phospholipids, and possibly other membrane associated export components forming a protein export complex (5, 6). The role of soluble SecA is unknown. SecA was initially shown to have an endogenous ATPase activity which was stimulated by the addition of purified OmpA precursors and inverted membrane vesicles containing functional SecY protein (2). The dependence of SecA ATPase activity on membrane and precursor protein makes it very likely that SecA is one site which utilizes ATP during protein export. A stoichiometry of 5000 molecules of ATP hydrolyzed per translocated molecule of proOmpA has been determined (7). The Km for ATP at the SecA ATPase was determined to be 0.23mM (7). This value is much higher than the
effective ATP Km (1-10 M), determined for the translocation of LSBP and BLA precursor proteins (Chapter II). Characterization of SecA with 8-azido-ATP showed that it contains 3 ATP binding sites, at least one of which is responsible for ATPase activity (2). This conclusion is consistent with the primary sequence data predicting three ATP binding sites for SecA (8). Oliver et al. (8) have suggested that SecA ATPase and the *E. coli* F$_1$ ATPase may function by analogous mechanisms. Both proteins apparently possess three ATP-binding sites (2, 9), both have some connection to sodium azide inhibition (8, 10), and both are enzymes which couple the movement of molecules through membrane pores (protons through the F$_0$ for the F$_1$ ATPase, and proteins through possibly SecY and other integral membrane export components for SecA [2, 9]). This interpretation is also consistent with the observation that DCCD, an inhibitor of F-type ATPases, inhibits precursor protein translocation in membranes prepared from an *E. coli* strain containing a deletion of the F$_1$ ATPase genes ([11], for a review of 3 major ion transport ATPases see reference [12]). On the other hand, DCCD is not a specific inhibitor of F-type ATPases, as it has been shown to inhibit all major classes of ion transport ATPases (13). SecA has never been shown to be capable of ATP synthesis (an enzymatic activity expected for only F-type ATPases), and sodium azide inhibition of protein export has been mapped to the secA gene locus, but azide has not yet been shown to inhibit SecA ATPase activity (2). These inconsistencies suggest that the mechanistic action of the SecA ATPase is still very much in question.
In this chapter the ATPase inhibitors vanadate and diethylstilbestrol (DES) were shown to inhibit precursor protein translocation. Vanadate was also shown to inhibit SecA ATPase activity that depends on LSBP precursor protein and inverted membranes. Vanadate is an inhibitor of many phosphohydrolases (14). Amongst the ion transport ATPases, vanadate is a specific inhibitor of the P-type class (13). It is believed to inhibit because of structural similarity to the transition state structure of phosphate (14). The results presented in this chapter suggest that SecA may function by a mechanism which is more similar to P-type ion transport ATPases than to the F-type ATPases.

**Materials and Methods**

**Materials**—In addition to materials as described in Chapter II, 9-amino-6-chloro-2-methoxy acridine (ACMA) was from Molecular Probes Inc, sodium orthovanadate was from Aldrich, diethylstilbestrol was from Sigma, and bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol (Oxonol VI) was a gift from Dr. Jack Lancaster. *E. coli* strain BL21(ΔDE3)/PT7-secA (3) was provided by Dr. Don Oliver.

**Cell Extracts**—S-30, S-100, ribosomes, pOX7 plasmid DNA, and inverted membranes were prepared as described in Chapter II. Urea-treated ΔuncBC inverted membrane vesicles were prepared by treatment with 6M urea, 30mM tris HCl, 1mM disodium EDTA, pH 8.0 for 30 min on ice. Membranes were isolated by centrifugation (Beckman TLA 100.3 rotor, 75,000 rpm, 1.5 hours, 4°C). The membrane pellet was resuspended in a minimal volume of 50mM triethanolamine acetate, 2mM ditiothreitol, pH 7.5 and treated a second time with 4M urea as stated above. Membranes were
isolated by centrifugation (same conditions as above) and resuspended in 50mM triethanolamine acetate, 250mM sucrose, and 2mM dithiothreitol, pH 7.5 to a final concentration of approximately 5mg/ml. Urea-treated membranes were stored at -70°C. Urea-treatment of inverted membranes results in quantitative removal of SecA as determined by two dimensional SDS gel electrophoresis.

SecA protein was purified from the E. coli SecA overproducing strain BL21(λDE3)/PT7-secA (3) by the method of Kawasaki et al. (15). Cells were grown in TYPN medium (20g tryptone, 10g yeast extract, 5g Na₂HPO₄, pH 7.4 with concentrated HNO₃) containing 25 g/ml ampicillin to an A₆₀₀ of 1.0, at which point over-production of SecA was induced by addition of 2mM isopropyl-β-D-thiogalactoside (IPTG). After 3 hours, cells were harvested by centrifugation (6000 x g, 15 min, 4°C) and resuspended in an equal weight of TMK buffer (Chapter II, S-30 extract) containing 1mM dithiothreitol. Cells were lysed in a chilled French pressure cell (Aminco) by 3 passes at 8000 psi and the cell lysate was centrifuged (2.5 hours at 150,000 x g, 4°C). The supernatant was dialyzed against TMK buffer containing 1mM dithiothreitol and subjected to ammonium sulfate fractionation. The 40-55% saturated fraction was dialyzed against the same buffer and chromatographed on Sephacryl 300, or in some cases Sephacryl 250. Fractions containing approximately 90-95% pure SecA (as determined by gel electrophoresis) were pooled and stored at -70°C.

LSBP precursor was purified from E. coli strain JM109 carrying the overproducer plasmid p18-livK. This plasmid was constructed by insertion of the livK
gene into pUC18 behind the lac promoter (personal communication Ben Owens). LSBP precursors were accumulated by overproduction of the livK gene product after induction with IPTG. Overproduction causes compartmentalization of LSBP precursor into inclusion bodies. Inclusion bodies were isolated by centrifugation from lysed cells and were then solubilized in 8M urea. LSBP precursor was further purified by ammonium sulfate fractionation and stored at -70°C in 8M urea, 50mM tris HCl, 2mM EDTA, pH 8.0. LSBP precursor protein purified in this fashion was determined to be approximately 90% pure by gel electrophoresis. When used in translocation or ATPase assays precursor was diluted at least 10-fold from the urea solution into an appropriate buffer and then added to reactions. The final concentration of urea in translocation or ATPase assays was never more than 0.75M. A complete description of LSBP precursor protein purification and overproducing strain construction will be described elsewhere (Ben Owens and Bruce Copeland, manuscript in preparation).

**Cotranslational Translocation**—Cotranslational translocation reactions were run as described in Chapter II. Ionophores and DES were added prior to reactions from ethanol stock solutions, with the ethanol removed under vacuum. Vanadate was added by dilution from a stock solution buffered with 50mM potassium Hepes, pH 7.6.

**Posttranslational Translocation**—Posttranslational translocation reactions were as described in Chapter II. Inhibitors were added as stated above.

**Determination of ATP Concentration**—The concentration of ATP was determined as described in Chapter II.
Oxonol VI and ACMA Fluorescence—Fluorescence of oxonol VI and ACMA was measured on a Gilford Fluoro IV fluorometer. Cuvettes contained 45mM tris acetate, 27mM ammonium acetate, 45mM sodium acetate, 2.5% glycerol, 1mM dithiothreitol, and 20 l of S-30 extract in a total volume of 2.5ml. Fluorescence of 1 M oxonol VI was calibrated to 100% with excitation at 580nm and emission at 640nm. Fluorescence was calibrated to 100% for 1M ACMA with excitation at 420nm and emission at 480nm. DES was added to fluorescence reactions in Me₂SO. The final concentration of Me₂SO was never greater than 0.1%.

ATPase Assays—ATPase activity was determined by a modified version of the method of Lill et al. (16) ATPase activity was measured in reactions containing 50mM potassium Hepes pH 7.0, 30mM KCl, 30mM NH₄Cl, 5mM magnesium acetate, 1mM dithiothreitol, 120 g/ml urea-treated membranes, 40 g/ml purified LSBP precursor, 20 g/ml purified SecA, and 4mM ATP. Components were mixed on ice and incubated at 37°C for 20 min. LSBP precursor was diluted from urea into buffer as stated above. Inorganic phosphate release was analyzed by the colorimetric method of Lanzetta et al. (17). Reactions were stopped by the addition of 0.8ml of color reagent (0.034% malachite green, 10.5g/l ammonium molybdate in 1M HCl, and 0.1% Triton X-100, filtered through a Whatman number 5 filter). After a 1 min incubation at room temperature, 0.1ml of 34% citric acid was added. Color was allowed to develop for 30-40 min at room temperature before the volume was increased to 3ml with distilled H₂O. The absorbance at 660nm was compared to a phosphate standard curve. ATPase
activity is defined as pmoles of inorganic phosphate released per 1 of reaction per 20 min.

Determination of ATPase activity in the presence of the ATPase inhibitors vanadate and DES was slightly modified. All reaction components and inhibitors except LSBP purified precursor were preincubated on ice for 20 min. Precursor protein was then added and reactions were carried out as stated above. No background ATPase activity was observed during the 20 min preincubation on ice, and preincubation had no effect on SecA ATPase activity in the absence of inhibitors. DES, valinomycin, and nigericin were added as stated above. ATPase activity experiments were repeated 3 times. Values reported are the average of three replicates of individual experiments. Error was estimated as the standard deviation of three replicates.

**Densitometry**—Densitometry was done as described in Chapter II.

**SDS-Polyacrylamide Gel Electrophoresis**—SDS gel electrophoresis was done as described in Chapter II.

**Protein Concentration Determination**—Protein concentration was determined as described in Chapter II.
Results

Effects of Vanadate and DES on In Vitro Posttranslational Translocation—To further characterize the role of ATP in bacterial protein export, the effects of vanadate and DES on in vitro posttranslational protein export were examined (Figure III.1A). Vanadate inhibits posttranslational processing of $^{35}$S-labeled precursors to LSBP and BLA at a concentration of 100 M (lanes 3 and 8). Vanadate at a concentration of 1 mM further inhibits processing (lanes 4 and 9). DES inhibits processing and translocation of LSBP and BLA precursors at a concentration of 100 M (lanes 6 and 11), while 10 M DES does not appear to effect processing of either precursor protein (lanes 5 and 10). In these experiments membranes, ATP, NADH, and inhibitors were preincubated together for 20 min on ice prior to the addition of precursor protein. Preincubation does not appear to effect precursor protein translocation or processing in the absence of the inhibitors (lanes 2, and 7), but does increase the level of inhibition observed in the presence of the inhibitors (data not shown).

The level of vanadate and DES inhibition of LSBP and BLA precursor protein processing was determined using densitometry (Figure III.1B). Quantitation reveals that vanadate at 1 mM inhibits 50% of the processing observed for both proteins. Vanadate at 100 M inhibits nearly 40% of the processing activity. DES at 100 M inhibits 60% of the processing of BLA and nearly 20% of LSBP processing. DES at 10 M inhibits 20% of BLA precursor processing, but does not appear to inhibit processing of LSBP precursors.
FIG. III.1. Effects of vanadate and DES on the posttranslational translocation and processing of LSBP and BLA precursors. A. Posttranslational translocation reactions were carried out as stated in "Materials and Methods." All reactions contained 3mM ATP and 3mM NADH. Inverted membrane vesicles were present in lanes 2-11. Inhibitors were added at the following concentrations: 100 M vanadate (lanes 3 and 8), 1mM vanadate (lanes 4 and 9), 10 M DES (lanes 5 and 10), and 100 M DES (lanes 6 and 11). All lanes were incubated with ATP, NADH and the indicated amounts of inhibitor for 20 min on ice prior the addition of [35S]methionine-labeled LSBP and BLA precursor proteins. Translocation was allowed to proceed for 20 min at 37°C, at which time proteinase K (200 g/ml) digestion was carried out for 20 min at 37°C (lanes 2-6). TCA precipitates were subjected to SDS gel electrophoresis and autoradiography. Precursor and mature forms are indicated by p and m respectively. B. The % inhibition of processing is indicated in the presence of inhibitors. Densitometry was done as stated in "Materials and Methods" on lanes 7-11. The % inhibition is defined as the % processing in the presence of inhibitor divided by the % processing in the absence of inhibitor minus 1 times 100.
FIG. III.2. **Effects of vanadate and DES on cotranslational LSBP and BLA precursor protein processing.** Cotranslational translocation reactions were carried out in the presence of [35S] methionine as stated in "Materials and Methods." Lane 1, standard cotranslational translocation reaction containing ATP; lane 2, standard reaction containing ATP and 1mM NADH; lane 3, conditions as in lane 2 plus 200nM SF6847; lane 4, conditions in lane 2 plus 2µM valinomycin; lane 5, conditions as in lane 2 plus 2µM valinomycin and 1µM nigericin; lane 6, conditions as in lane 2 plus 1mM vanadate; lane 7, conditions as in lane 2 plus 100µM DES. Samples were subject to SDS gel electrophoresis and autoradiography. Precursor and mature forms are indicated by p and m respectively.
Effects of Vanadate and DES on Cotranslational Translocation—Nearly identical inhibition of LSBP and BLA precursor protein processing was observed using the cotranslational translocation system (Figure III.2). Inhibition of processing is observed for both LSBP and BLA precursors in the presence of 1mM vanadate or 100 M DES (lanes 6 and 7). For reference, the inhibition of precursor protein processing by ionophores or combinations of ionophores is also shown (lanes 3-5). Vanadate and DES (lanes 6 and 7) inhibit processing of both LSBP and BLA to approximately the same level as SF6847 (lane 5) or valinomycin/nigericin (lane 4), and to a slightly greater level than valinomycin alone (lane 3). In these experiments the respiratory substrate NADH was added to aid in production of membrane energy (lanes 2-7). In the absence of inhibitors, the level of processing with ATP and NADH (lane 2) is nearly identical to the level of processing with ATP alone (lane 1).

Effects of Vanadate and DES on Membrane Gradient Energy and ATP Levels
Since bacterial protein export is inhibited by agents which dissipate protonmotive force (Chapter II), the effects of vanadate and DES on membrane energy were examined. The transmembrane electrical potential (ΔΨ) was monitored by fluorescence quenching of oxonol VI (Figure III.3). The addition of ATP to inverted membrane vesicles results in development of ΔΨ as shown by fluorescence quenching. Vanadate (1mM) or DES (100 M) slightly reduced the level of ΔΨ generated in the presence of ATP. However, addition of the respiratory substrate NADH in the presence of vanadate or DES and ATP results in development of a larger ΔΨ than in the presence of ATP.
FIG. III.3. Effects of vanadate and DES on \( \Delta \Psi \) as monitored by quenching of oxonol VI fluorescence. Fluorescence measurements were made as described in "Materials and Methods" using 1\( \mu \)M oxonol VI and S-30 extract as a source of membranes. Curves show time courses of oxonol fluorescence in the presence of membranes with sequential addition of substrates, inhibitors, and ionophores, as indicated. Additions were made at the following final concentrations: 2mM ATP, 1mM NADH, 1mM vanadate, 100\( \mu \)M DES, 2\( \mu \)M valinomycin and 1\( \mu \)M nigericin. Oxonol VI, DES, valinomycin, and nigericin were added from concentrated ethanol stocks to give final ethanol concentrations not exceeding 0.25% (V/V).
Table III.I.

Effects of vanadate and DES on the concentration of ATP in cotranslational translocation reactions

Cotranslational translocation reactions were run in parallel to reactions shown in Figure 2 except cold methionine was used in place of \([^{35}S]\)methionine. After a 20 min incubation at 37°C, the concentration of ATP was determined as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ATP Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>1mM Vanadate</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>100µM DES</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>
alone. This result in conjunction with the observation that precursor protein export is virtually identical in the presence of ATP alone or ATP and NADH (Figure III.2), show that inhibition of processing by vanadate and DES is not due to dissipation of membrane energy. These results were also confirmed with the fluorescent probe ACMA. ACMA has previously been regarded as a probe for total protonmotive force, although most acridine probes should in principle detect $\Delta p$H (18). The results with ACMA (data not shown) completely parallel the results shown in Figure III.3, confirming that inhibition of precursor protein processing by vanadate and DES is not due to a reduction in membrane energy.

To verify that inhibition of in vitro protein export by vanadate and DES is not due to a reduction in the level of ATP during translocation reactions, ATP concentrations were directly measured in reactions run in parallel to Figure III.2 (Table III.1). Inclusion of vanadate (1mM) or DES (100 M) in cotranslational translocation reactions has little effect on the concentration of ATP as compared to the control reaction which did not contain either inhibitor. The concentration of ATP directly measured in the control reaction was 1.9mM ATP, while the ATP concentrations in the presence of vanadate or DES were determined to be 1.3 and 1.4mM respectively. All of these concentrations are far above the minimum concentration of ATP necessary to support optimal processing (or synthesis) of LSBP and BLA precursors (Chapter II). Therefore, inhibition of translocation by vanadate and DES is apparently not due to decreases in ATP levels.
Membrane and Precursor Dependent SecA ATPase Activity—To determine what effects vanadate and DES have on SecA ATPase activity, a SecA ATPase assay was developed. The system is comprised of purified SecA, inverted membrane vesicles prepared from the *E. coli* strain 1100 ΔuncBC (lacking genes for the F₁ ATPase) which were urea-treated to remove SecA, and purified LSBP precursor. Using a similar system Wickner and coworkers have shown that SecA ATPase activity depends on the presence of purified OmpA precursors and urea-treated inverted membrane vesicles (2). The dependence of SecA ATPase activity on purified LSBP precursor protein and urea-treated membranes was therefore examined. SecA ATPase activity was assayed using an approach very similar to one developed by Lill *et al.* (16), which measures ATPase activity by the colorimetric determination of released inorganic phosphate. Membranes prepared from the *E. coli* strain 1100 ΔuncBC were used for SecA ATPase assays to reduce the level of ATPase activity associated with membranes. Table III.II shows that SecA alone contains an ATPase activity which is stimulated by the addition of urea-treated membranes, and further stimulated by the addition of purified LSBP precursor protein. LSBP purified precursor protein appears to have a very low level of ATPase activity, while inverted membrane vesicles contain a slightly higher level of ATPase activity. The addition of purified LSBP precursor protein to SecA alone, or to the inverted membrane vesicles in the absence of SecA does not appear to result in any increase in ATPase activity. These results are qualitatively consistent with previous results obtained for proOmpA precursor (2). Since SecA ATPase activity is stimulated
ATPase activity was determined in the presence or absence of 120µg/ml ΔuncBC urea-treated inverted membranes, 20µg/ml purified SecA protein, 40µg/ml purified LSBP precursor protein and 4mM ATP. Reaction components were added on ice and incubated for 20 min at 37°C followed by determination of phosphate as described in "Materials and Methods." ATPase activity is pmoles of inorganic phosphate released per µl of reaction per 20 min. Purified LSBP precursor (LSBP) is indicated.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>SecA</th>
<th>(-)pLSBP</th>
<th>(+)pLSBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>none</td>
<td>95 ± 0</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>520 ± 25</td>
<td>525 ± 30</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>365 ± 10</td>
<td>335 ± 25</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>750 ± 15</td>
<td>930 ± 40</td>
</tr>
</tbody>
</table>
by precursors and membranes this activity will be referred to as translocator-SecA ATPase activity.

Effect of Vanadate and DES on SecA ATPase Activity—The effects of vanadate and DES on SecA ATPase activity are shown in Table III.III. In the presence of SecA, purified LSBP precursor protein, and inverted membrane vesicles, 660 pmoles of phosphate per 1 of reaction per 20 min of SecA ATPase activity was observed. Addition of 100 M vanadate inhibits this ATPase activity to 450 pmoles of phosphate per 1 of reaction per 20 min, while 1mM vanadate reduces the level to 390 pmoles of phosphate per 1 of reaction per 20 min. DES at a concentration of 100 M, which inhibits SecA-dependent posttranslational precursor protein translocation, has little effect on ATPase activity. DES at a concentration of 1mM has a slightly inhibitory effect on SecA ATPase activity, but this concentration of DES is much higher than the concentration needed to inhibit protein export (Figure III.1). The time course of ATPase activity in the presence or absence of inhibitors was linear for 40 min (data not shown).

Effect of Vanadate on Translocator-SecA ATPase Activity—Vanadate inhibits SecA ATPase activity to approximately 400 pmoles of phosphate per 1 of reaction per 20 min or roughly the ATPase activity endogenous to urea-treated inverted membranes. Vanadate inhibition of SecA ATPase activity could be due to inhibition of ATPase activity endogenous to the membranes, or ATPase activity associated with SecA in the absence of membrane. To determine if vanadate is inhibiting endogenous membrane ATPase activity, SecA ATPase activity in the absence of membranes, or precursor and
Table III.III.

Effects of vanadate and DES on SecA ATPase activity

SecA ATPase activity was determined in the presence of 120µg/ml urea treated ΔuncBC membranes, 20µg/ml purified SecA protein, 40µg/ml purified LSBP precursor protein, 4mM ATP, and the indicated amount of the inhibitors. Reactions were preincubated with or without inhibitors for 20 minutes on ice prior to the addition of purified LSBP precursor. ATPase activity is pmoles of inorganic phosphate released per µl of reaction per 20 min. ATPase activity was determined as stated in "Materials and Methods."

<table>
<thead>
<tr>
<th>SecA</th>
<th>Inhibitor (mM)</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>none</td>
<td>380 ± 60</td>
</tr>
<tr>
<td>+</td>
<td>none</td>
<td>660 ± 20</td>
</tr>
<tr>
<td>+</td>
<td>0.1 vanadate</td>
<td>450 ± 35</td>
</tr>
<tr>
<td>+</td>
<td>1.0 vanadate</td>
<td>390 ± 40</td>
</tr>
<tr>
<td>+</td>
<td>0.1 DES</td>
<td>640 ± 10</td>
</tr>
<tr>
<td>+</td>
<td>1.0 DES</td>
<td>570 ± 10</td>
</tr>
</tbody>
</table>
FIG. III.4. Effects of vanadate on ATPase activity at various membrane concentrations. Curves above the dashed line show ATPase activity in the presence of 20µg/ml SecA, 40µg/ml purified LSBP precursors, the indicated amount of urea-treated membrane, and 4mM ATP (●,○). Curves below the dashed line show ATPase activity measured in the presence of 40µg/ml purified LSBP precursors, the indicated amounts of urea-treated inverted membranes, and 4mM ATP (Δ,▲). The inset shows ATPase activity in the presence of 20µg/ml SecA, 40µg/ml LSBP precursors, and 4mM ATP. The dashed line represents the ATPase activity associated with SecA and LSBP precursors. All types of ATPase activity were measured with (●,▲) or without (●,○) 1mM vanadate (Vi, as indicated in the inset). All components except LSBP precursors were preincubated on ice for 20 min with or without vanadate. Following preincubation LSBP precursors were added and the reactions were incubated for 20 min at 37°C. The release of inorganic phosphate was measured as stated in "Materials and Methods." ATPase activity is pmoles of phosphate per µl of reaction per 20min.
membrane dependent SecA ATPase activity (translocator-SecA ATPase activity), each activity was assayed with and without vanadate (Figure III.5). Translocator-SecA ATPase activity is sensitive to vanadate at all membrane concentrations assayed (upper half of Figure III.5). Vanadate appears to inhibit this activity to approximately the same level as SecA ATPase activity in the absence of membrane. Endogenous membrane ATPase activity is insensitive to vanadate at all membrane concentrations assayed (lower half of Figure III.5). ATPase activity associated with SecA in the absence of membranes is also insensitive to vanadate (inset). These results show that vanadate inhibits translocator-SecA ATPase activity (ATPase activity which is stimulated by precursors and membranes), but has no effect on endogenous membrane or SecA ATPase activity in the absence of membranes.

**Determination of Vanadate Concentration Required for Half Maximal Inhibition of SecA**—The effect of various vanadate concentrations on translocator-SecA ATPase activity was used to determine the vanadate concentration which inhibits translocator-SecA ATPase activity half maximally (Figure III.6). Translocator-SecA ATPase activity, or ATPase activity which corresponds to precursor protein export (membrane and precursor dependent), was subtracted from ATPase activity associated with SecA in the absence of membranes and ATPase activity endogenous to membranes alone. Half maximal inhibition was determined to occur at approximately 20 M vanadate.

**Effect of the Ionophore Combination Valinomycin/Nigericin on SecA ATPase Activity**—In combination, valinomycin and nigericin inhibit protein export to near completion (Figure III.2, and Chapter II). This inhibition is presumably due to
FIG. III.5  Effect of vanadate concentration on translocator-SecA ATPase activity. Translocator-SecA ATPase activity was assayed in the presence of different concentrations of vanadate. Reactions components, 120µg/ml urea-treated membranes, 25µg/ml SecA, 4mM ATP and the indicated concentrations of vanadate were preincubated on ice for 20 min prior to the addition of 40µg/ml LSBP precursor. Phosphate release was measured as stated in "Materials and Methods", after a 20 min incubation at 37°C. ATPase activity, pmoles of phosphate per µl of reaction per 20 min, of inverted membranes alone, and SecA alone was subtracted from the ATPase activity associated with membranes, SecA, purified LSBP precursor. This activity is plotted against the log of the vanadate concentration (µM).
Table III.IV.

Effect of the ionophore combination valinomycin and nigericin on SecA ATPase activity

SecA ATPase activity was determined in the presence of 40µg/ml purified LSBP precursor protein, 4mM ATP, and where indicated 120µg/ml urea treated ΔuncBC membranes, 20µg/ml purified SecA protein, and 2µM valinomycin and 1µM nigericin. ATPase activity was determined as stated in "Materials and Methods." ATPase activity is pmoles of inorganic phosphate released per µl of reaction per 20 min.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>SecA</th>
<th>Val/Nig</th>
<th>ATPase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>455 ± 50</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>877 ± 50</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>847 ± 50</td>
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<tr>
<td>+</td>
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<td>+</td>
<td>315 ± 15</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>300 ± 10</td>
</tr>
</tbody>
</table>
dissipation of membrane energy. The effect of this ionophore combination on SecA ATPase activity was examined (Table III.IV). The ionophore combination valinomycin/nigericin has little effect on translocator-SecA ATPase activity or endogenous membrane ATPase activity.

**Discussion**

Results presented in this chapter show that vanadate and DES inhibit posttranslational and cotranslational translocation of LSBP and BLA precursor proteins. Using both cotranslational and posttranslational *in vitro* translocation systems, protein export is inhibited by 50% in the presence of 1mM vanadate, while 100 M vanadate inhibits posttranslational translocation of LSBP and BLA precursor proteins by approximately 40%. DES also substantially inhibits protein export at a concentration of 100 M. Inhibition of protein export by vanadate and DES is not due to inhibitor effects on ATP concentration or dissipation of membrane gradient energy. The measured concentrations of ATP are not substantially different whether vanadate or DES are present or absent, and sufficient membrane energy for translocation LSBP and BLA precursors is observed in the presence of vanadate or DES, ATP and NADH. Vanadate and DES are known ATPase inhibitors (13). These results, coupled with reports that DCCD (an ATPase inhibitor) inhibits protein export in membranes devoid of the F₁ component of the F₁F₀ ATPase (11), suggest that some ATPase other than the F₁ ATPase is involved in the bacterial protein export process.
The SecA protein is a peripheral membrane protein which is essential for bacterial protein export (4). Treatment of inverted membrane vesicles with urea results in near quantitative removal of SecA, and renders the vesicles unable to export precursor proteins. Addition of purified SecA protein to the membrane vesicles restores their ability to export precursors of LSBP (data not shown). Wickner and coworkers have shown that SecA contains an ATPase activity which is stimulated by the addition of membranes, and further stimulated by addition of purified OmpA precursors (2). Results presented in this chapter using purified LSBP precursor qualitatively confirm this result. Vanadate substantially inhibits precursor and membrane stimulated SecA ATPase activity, while DES has no apparent inhibitory effect at concentrations which inhibit precursor protein translocation. Membranes, and SecA in the absence of membranes have ATPase activities which are insensitive to vanadate. Since SecA ATPase activity depends on purified precursor protein and membranes it has been termed translocator-SecA ATPase activity.

Wickner and coworkers have designated SecA ATPase activity as "Translocation ATPase" (2) because of its dependence on precursors and membranes. There is a clear correlation between requirements for SecA ATPase activity and in vitro protein export. Since SecA protein is essential for export, and since ample evidence demonstrates ATP hydrolysis is necessary for export (Chapter II, and 1), there can be little doubt that SecA ATPase activity is important for protein export. However, SecA ATPase activity in this assay system (urea-treated membranes, purified SecA, and purified precursor) may not be tightly coupled to precursor translocation even though it requires many of
the same conditions. This activity is not substantially inhibited by either uncouplers of protonmotive force or DES, even though both of these inhibit in vitro protein export. Moreover, the ATPase activity is linear for at least 40 minutes under these assay conditions (data not shown, and 16), while translocation and processing of LSBP precursors normally ends after 10 minutes (data not shown and personal communication Ben Owens). Under these conditions it may be more appropriate to view SecA ATPase activity as a meaningful activity associated with export, but not an activity which is completely sufficient for translocation in this assay system. This makes the dual sensitivity of precursor translocation and translocator-SecA ATPase activity to vanadate all the more interesting. No other inhibitor or uncoupler has been shown to inhibit both precursor translocation and SecA ATPase activity, making it likely that vanadate is a direct inhibitor of SecA function, thereby inhibiting protein export.

Vanadate is an inhibitor of numerous enzymes, including many phosphohydrolases (14). It is believed to inhibit by functioning as a transition state analogue of phosphate. Of the 3 major types of ion transport ATPases, vanadate is a specific inhibitor of the P-type ATPases (13). Inhibition of P-type ATPases is believed to result from vanadate binding at the active site of the enzyme more tightly than the enzyme substrate (19).

Characteristic of P-type ATPases is an acyl phosphate (phosphorylated) intermediate. The phosphorylated intermediate appears to cause a conformational change necessary for enzyme function (19). Phosphorylation of P-type ATPases occurs in a specific sequence domain conserved in all known P-type ATPase. The SecA
protein contains a sequence which displays some homology to the phosphorylation sequence of P-type ATPases (Figure III.7). The SecA sequence contains relatively conservative substitutions of neutral amino acids leucine for glycine, and alanine for leucine at two positions in the sequence. The major difference in the two sequences is at the location where phosphorylation occurs in P-type ATPases. In P-type ATPases this is at an aspartyl residue. The SecA sequence contains a glycine residue at this position, preceded by glutamate. This is a large enough difference to make SecA distinct from P-type ATPases. However, the similarities in the sequences are sufficient that the SecA sequence could easily participate in the same type of chemistry as the P-type phosphorylation sequence. The greater length of the glutamate side chain may offset the extra distance along the peptide backbone resulting from the glycine separating glutamate and lysine. Furthermore, glycine has no side chain to interfere sterically.

Experimental evidence also suggests that this sequence region is important for SecA function. The prlD4 mutation is a substitution of threonine by asparagine at position 111 in the SecA protein sequence (20). This mutation has been shown to suppress maltose binding protein signal sequence mutations (20). Also, Matsuyama et. al (21) have identified this sequence region by crosslinking to 8-azido-ATP. While neither of these observations supply direct proof that this sequence is involved in a P-type ATPase mechanism, they do indicate the importance of this sequence region in SecA.
<table>
<thead>
<tr>
<th>Residue Number</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>367</td>
<td>Asp-Lys-Thr-Gly-Thr-Leu-Thr</td>
<td>Na,K, α</td>
</tr>
<tr>
<td>379</td>
<td>Asp-Lys-Thr-Gly-Thr-Leu-Thr</td>
<td>H,K-</td>
</tr>
<tr>
<td>345</td>
<td>Asp-Lys-Thr-Gly-Thr-Leu-Thr</td>
<td>Ca-</td>
</tr>
<tr>
<td>372</td>
<td>Asp-Lys-Thr-Gly-Thr-Leu-Thr</td>
<td>H-, yeast</td>
</tr>
<tr>
<td>301</td>
<td>Asp-Lys-Thr-Gly-Thr-Ile-Thr</td>
<td>Kdp, β</td>
</tr>
<tr>
<td>273</td>
<td>Asp-Lys-Thr-Gly-Thr-Leu-Thr</td>
<td>K-, S.f.</td>
</tr>
<tr>
<td>102</td>
<td>Glu-Gly-Lys-Thr-Leu-Thr-Ala-Thr</td>
<td>SecA</td>
</tr>
</tbody>
</table>

FIG. III.6. Primary sequence comparisons between the phosphorylation domains of P-Type ATPases and the SecA protein. References to sequences: Na,K-ATPase, α subunit (26); Ca-ATPase (27); H,K-ATPase (28), H-ATPase yeast (29), Kdp-ATPase, β-subunit E. coli (30); K-ATPase, S. faecalis (31); SecA, E. coli (32). The P-type ATPase phosphorylation site is denoted by *. Predicted phosphorylation site in SecA is denoted by ***.
If SecA functions by a P-type ATPase mechanism, a phosphorylated intermediate of the SecA protein should exist. Phosphorylation of SecA has been investigated, but never observed (personal communication Don Oliver, and data not shown). It is possible that SecA functions by a mechanism similar to P-type ATPases, but transfers phosphorylation to a second protein necessary for SecA function. SecA dependent phosphorylation of a 62 kDa protein has been observed (Chapter IV). This protein may interact with SecA through a pseudo-P-type mechanism, thereby modulating SecA ATPase. Alternatively, the inability to detect phosphorylation of SecA could be due to instability of glutamylphosphate intermediates of SecA. Comparisons of synthetic peptides reveals that the phosphoaspartyl linkage is more stable than the phosphoglutamyl linkage (22). Glutamic acid residues have also proven to be a major source of problems for peptide synthesis (23). Inability to synthesize peptides containing glutamate is believed to be due to the formation of six-membered ring structures (23). The ability to cyclize may contribute to the instability of glutamylphosphates.

Eukaryotic P-type ATPases are inhibited half maximally by submicromolar to micromolar concentrations of vanadate (14). *E. coli* contains one known P-type ATPase, the K⁺ translocating Kdp ATPase, which is inhibited half maximally by 30 M vanadate (24). Results presented in this study show that translocator-SecA ATPase activity is inhibited half maximally by approximately 20 M vanadate (Figure III.6). This vanadate concentration is considerably higher than needed to inhibit most P-type ATPases of eukaryotic origins (14). However, the relatively high concentration of
vanadate required to inhibit both the Kdp ATPase and SecA ATPase may be a common feature associated with bacterial enzymes which function by a P-type ATPase mechanism.

Evidence presented in this chapter suggests that SecA functions by a mechanism similar to P-type ATPases. The evidence includes sequence similarity between SecA and the phosphorylation domain of P-type ATPases, and vanadate inhibition of protein export and translocator-SecA ATPase activity. However, the evidence is not perfect. All known P-type ATPases are approximately 100 kDa integral membrane proteins (13). While SecA is similar in size it is only peripherally associated with the membrane (25), however SecA ATPase activity does show "surface dilution kinetics" characteristic of membrane bound enzymes (16). Phosphorylation of P-type ATPases occurs at an aspartic acid residue (19), whereas the proposed phosphorylation site in SecA is a glutamic acid residue. The vanadate concentration required to inhibit SecA half maximally is much higher than required to inhibit most P-type ATPases. These differences suggest that SecA may function by a mechanism which is similar, but not strictly identical to the proposed E₁,E₂ mechanism of P-type ATPase (19).

It is clear that further research on the function of SecA is necessary to determine its mechanism of action. Clues to the mechanism of SecA and SecA interactions with the protein export machinery might be found by characterizing the inhibitory effects of DES on protein export. DES has no effect on translocator-SecA ATPase activity, but inhibits precursor protein export. These results suggest that DES inhibits a component of the export machinery which functions after or independent of SecA. This result is
of interest, as it might lead to the characterization of a protein export component which has not been previously identified.

References


CHAPTER IV
PHOSPHORYLATION ASSOCIATED WITH IN VITRO PROTEIN EXPORT

Introduction

Chapter III showed that SecA contains an ATPase activity that is dependent on purified precursor protein and membranes and is sensitive to vanadate. Vanadate was also shown to inhibit translocation and processing of LSBP and BLA precursors. Sequence analysis indicates that SecA contains some homology to the consensus sequence found in the phosphorylation domain of P-type ATPase. Furthermore, the concentration of vanadate required to inhibit SecA ATPase activity half maximally is in the same range as the concentration required to inhibit the E. coli P-type ATPase, Kdp-ATPase (1).

P-type ATPases undergo phosphorylation which is essential for enzymatic activity (2). This phosphorylation occurs at a conserved aspartic acid residue forming an acyl phosphate linkage. Since vanadate inhibits protein export perhaps by inhibition of the SecA ATPase, and since SecA contains some sequence homology to the phosphorylation domain of P-type ATPases (Chapter III), it is reasonable that phosphorylation might play a role in the bacterial protein export process. Phosphorylation of proteins during bacterial protein export has been speculated for many years (3, 4).
In this chapter phosphorylation of 62 and 37 kDa proteins was observed in a membrane and precursor protein dependent fashion. Phosphorylation of the 62 kDa protein was also dependent on the SecA protein. The phosphorylation appears to be an acyl phosphate linkage in both proteins. A rapid method for mapping proteins with acyl phosphate phosphorylation by 2-D gel electrophoresis was developed. Using this technique the 62 and 37 kDa proteins were characterized.

Materials and Methods

Materials—\( ^{32}\text{P}\)ATP and \( ^{3}\text{H}\)NaCNBH₃ were purchased from Amersham; L-\([^{35}\text{S}]\)-methionine was obtained from Dupont/NEN. All other chemicals were of reagent grade obtained from commercial sources, or as described in Chapter II. Purified SecA protein and \textit{E. coli} strain BA13 were provided by Dr. Don Oliver. Trigger factor antiserum was a kind gift from Dr. William Wickner.

Cell Extracts—Ribosomes, pOX7 plasmid DNA, S-30, S-100, and inverted membrane vesicles were prepared substantially as described in Chapter II. S-30, S-100, and membranes from \textit{E. coli} strain BA13 (temperature sensitive for SecA production, [5]) were prepared with a slight modification in growth conditions. BA13 cells were grown at 30 C and shifted to 42 C for 2.5 hours before harvest. The shift results in the production of an inactive form of SecA. In vitro synthesized LSBP and BLA precursors were prepared as stated in Chapter II, except that S-100 prepared from \textit{E. coli} strain BA13 was used where indicated.
Phosphorylation—Inverted *E. coli* membranes (1.5mg/ml) were incubated immediately before use for 1 min at 37°C in the presence of 3mM NADH, 12mM magnesium acetate, 55mM potassium acetate, 24mM potassium N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes) pH 7.6, and 5 M ATP (AE1 system) or 200 M ATP (BA13 system). This incubation allows for translocation of any residual nascent precursors. Precursor protein (2mg/ml) was then added and incubated at 37°C for 30 sec (AE1 membrane/precursor system) or 2 min (BA13 membrane/precursor system), followed by chilling on ice for 3 min. Radiolabeled phosphorylation was initiated by addition of 10-15 Ci of 5000Ci/mmol $\gamma^{32}$P]ATP per each 25 l reaction. After incubation for 10 sec at room temperature, the reactions were quenched by addition of 30 l of 15mM unlabeled ATP, 15mM KH$_2$PO$_4$, and 16% trichloracetic acid, and chilled on ice for 5 min. Protein was isolated by centrifugation at 45,000 x g for 5 min at 4°C. Pellets were washed two times with 150 l of 15mM ATP, 30mM KH$_2$PO$_4$, and 16% trichloroacetic acid, two times with 250 l of ice cold diethylether, and one time with 150 l of 50mM KH$_2$PO$_4$, pH 2.0 (same centrifugation conditions).

Posttranslational Translocation—Posttranslational translocation reactions were run as described in Chapter II with minor modifications. LSBP and BLA precursor protein, and inverted membranes were prepared from the *E. coli* strain BA13 grown at the nonpermissive temperature. Purified SecA protein was added to a final reaction concentration of 15 g/ml.

Protease Protection—Protease protection was done as described in Chapter II.
**Electrophoresis**—Acid/TDAB gel electrophoresis was carried out using the gel system of Amory et al. (6). Pellets from phosphorylation reactions were solubilized in 50 l of sample buffer (100mM KH₂PO₄, 250mM sucrose, 5% β-mercaptoethanol, 35mM TDAB, 20 g/ml malachite green, pH 3.0) at 4°C. Electrophoresis was carried out at 5V/cm for 10 hours at 4°C. Gels were washed in 2% glycerol for 5 min and autoradiographed at -70°C either wet or after vacuum drying. Sodium dodecylsulfate (SDS) gel electrophoresis was carried out according to the method of Laemmli (7) as described in Chapter II.

Two-dimensional gel electrophoresis was done as described by Copeland et al. (8) except that 0.3% SDS was included during sample solubilization. Visualization was accomplished by autoradiography (³⁵S and ³²P) or fluorography (³H).

**Reductive Tritiation**—Phosphorylated protein bands were excised from wet acid/TDAB gels and placed in 100 l of dimethyl sulfoxide. Reduction was accomplished by incubation with 10 Ci of [³H]NaCNBH₃ for 1 hour at room temperature. Gel slices were rinsed one time with 150 l of dH₂O, two times with 150 l of isopropyl alcohol, and two times with 150 l of dH₂O (10 min each time). For standard SDS gel electrophoresis, tritiated acid/TDAB gel slices were soaked in SDS sample mix for 30 min prior to loading. For two-dimensional electrophoresis, acid/TDAB gel slices were loaded on the first dimension isoelectric focusing gel along with a 2-D protein sample equivalent to the protein composition of a posttranslational translocation reaction (or phosphorylation reaction).
Protein Concentration—Protein concentration was determined by the Bradford method (9).

Results

Membrane and precursor dependent phosphorylation—The search for phosphorylation involved in bacterial protein export was started by examining phosphorylation of proteins trapped by acid precipitation at low temperature. In order to visualize any phosphorylation that might be labile under alkaline conditions, a low temperature acid/tetradecyltrimethylammonium bromide (TDAB) gel electrophoresis system was employed. This system has been previously used to characterize P-type ion translocating ATPases (6). In this gel system, the cationic detergent TDAB is used to separate proteins according to molecular weight. Figure IV.1 shows an autoradiogram of an acid/TDAB gel in which phosphorylation reactions were carried out with an in vitro posttranslational translocation system containing [γ32P]ATP. The translocation system consisted of inverted E. coli membrane vesicles, NADH as a respiratory substrate, and desalted in vitro synthesized precursors of LSBP and BLA. This figure shows that proteins indicated by A and B are phosphorylated in the presence of LSBP and BLA precursor protein and membranes (lane 1). The sizes of these proteins were determined to be 69 and 49 kDa by comparison to protein molecular weight standards on the acid/TDAB gel system. Essentially no phosphorylation of either protein is observed when membranes (lane 2), or precursor protein is absent (lane 3). Moreover, no phosphorylation of either protein is observed if the precursor/synthesis mixture is
FIG. IV.1. Visualization of in vitro export-associated phosphorylation of proteins by acid/TDAB gel electrophoresis: Membrane and precursor protein dependence. Proteins were phosphorylated in the presence of [γ³²P]ATP, separated by acid/TDAB gel electrophoresis, and autoradiographed as described in "Materials and Methods." Phosphorylation reactions were carried out with inverted E.coli membrane vesicles and in vitro synthesized precursor fraction (lane 1), precursor fraction only (lane 2), membranes only (lane 3), or membranes and synthesis mixture without precursor (lane 4). Synthesis mixture is identical to precursor fraction except that synthesis of precursors was blocked by inclusion of chloramphenicol and streptomycin. The locations of two protein bands that are phosphorylated in the presence of precursor protein and membranes are indicated by A and B respectively. The approximate sizes of band A and band B proteins are 69 and 49 kDa, as determined by comparison to molecular weight standards on stained acid/TDAB gels.
replaced with an otherwise equivalent synthesis mixture where precursor synthesis has been blocked (lane 4). Other phosphorylated bands are observed in Figure IV.1, but phosphorylation of these proteins does not appear to correlate with protein export.

Phosphorylation of both proteins is highly sensitive to the assay conditions. Times and temperatures used for phosphorylation reactions were selected from measurements of ATP turnover rates and export temperature/time courses (data not shown). These conditions were selected to make export slow enough that most but not all $\gamma^{32}$P]ATP is consumed, and to increase the likelihood that export events are initiated but not extensively completed during the phosphorylation reaction.

**Effects of Protein Export Inhibitors and Hydroxylamine on Phosphorylation**—The response of phosphorylation to hydroxylamine as well as inhibitors of protein export was examined (Figure VI.2). Lane 2 shows that phosphorylation of band A and B protein species disappears upon treatment with hydroxylamine (NH$_2$OH). Loss of phosphorylation upon treatment with hydroxylamine is a standard criterion used to demonstrate existence of acyl phosphates (2, 10, 11). Notably in lane 2, this treatment does not lead to the disappearance of other phosphorylated bands in the sample. Lane 3 shows that phosphorylation of both band A and B proteins is almost completely inhibited in the presence of the ionophore combination valinomycin/nigericin. This ionophore combination, like other uncouplers of protonmotive force, inhibit processing and translocation of both LSBP and BLA precursors (Chapter 2). Lanes 4 and 5 show that phosphorylation of band A and B is significantly reduced in the presence of 100 M vanadate, while nearly complete inhibition is observed with 1mM vanadate. At these
FIG. IV.2. Response of phosphorylation to hydroxylamine and inhibitors of protein export. Phosphorylation was carried out as described in Figure IV.1 using precursor protein and membranes (lane 1), or washed with 50 mM hydroxylamine sulfate, 100 mM potassium Hepes, pH 6.0 in place of the final 50 mM KH₂PO₄ wash (lane 2), phosphorylated in the presence of 2 µM valinomycin and 1 µM nigericin (lane 3), phosphorylated in the presence of 100 µM sodium orthovanadate (lane 4), phosphorylated in the presence of 1 mM sodium orthovanadate (lane 5). Locations of the phosphorylated protein bands are indicated A and B.
concentrations, vanadate inhibits \textit{in vitro} protein export and SecA ATPase activity (Chapter III).

**SecA-dependent Phosphorylation**—To further establish that the phosphorylation is export-specific, a SecA-deficient \textit{in vitro} export system was developed. The system contained LSBP and BLA precursor protein and membranes prepared from BA13, a temperature sensitive \textit{E. coli} secA mutant strain that does not produce active SecA protein when grown at its restrictive temperature (5). Phosphorylation of both band A and B protein is undetectable in this SecA-deficient system (Figure VI.3a, lane 1), but addition of purified SecA protein results in phosphorylation of band A protein (Figure VI.3a, lane 2). Phosphorylation conditions used with the BA13 system were modified slightly (see "Materials and Methods") to optimize phosphorylation. Phosphorylation of the band B protein has never been observed using the BA13 extracts in phosphorylation reactions.

To confirm SecA dependence, processing and translocation of $^{35}$S-labeled precursors of LSBP and BLA were examined in the BA13 system (Figure VI.3b). Processing and translocation of BLA precursors are dependent on addition of purified SecA protein in the SecA-deficient system (lanes 2,4). In the absence of purified SecA neither processing nor translocation of BLA precursors is observed (lanes 1,3). Also, in the absence of SecA a protein band is observed which migrates to the location of the mature form of the LSBP protein (lane 1). However this band is not protected from added protease indicating that it is not a translocated form of LSBP (lane 2). Addition of SecA stimulates processing and translocation of LSBP. It should be noted that there
FIG. IV.3. **SecA dependence of in vitro phosphorylation and protein translocation.** (a) Phosphorylation in a SecA-defective precursor/membrane system in the absence (lane 1) or presence (lane 2) of 20 µg/ml purified SecA protein. The phosphorylated protein is designated by A. (b) Precursor processing (lanes 1 and 3) and protease protection (lanes 2 and 4) in the SecA-defective system without (lanes 1 and 2) or with (lanes 3 and 4) 20 µg/ml purified SecA protein. SecA-deficient inverted membrane vesicles were prepared from E. coli strain BA13, and 35S-labeled precursors of LSBP and BLA (β-Lactam) were synthesized using SecA-deficient synthesis extracts from BA13. Phosphorylation, acid/TDAB gel electrophoresis, precursor protein translocation, and SDS gel electrophoresis were carried out as described in "Materials and Methods." Precursor and mature forms of proteins are indicated by p and m. (Note: proteinase K digestion of LSBP precursor gives a protein fragment that is only slightly smaller than the undigested mature form.)
FIG. IV.4. **Reductive tritiation of the band A protein.** Acid/TDAB gel band slices of the band A phosphoprotein region (69 kDa) were reductively tritiated and subjected to SDS gel electrophoresis as described in "Materials and Methods." The SDS gel shows levels of reductive tritiation from acid/TDAB gel slices where good phosphorylation of the protein had been observed (lane 1), where negligible phosphorylation had been observed (lane 2); or where the acid/TDAB gel slice was from a sample which had not been phosphorylated (lane 3). The location of the band A reductively tritiated protein (69 kDa) is indicated.
is incomplete protease digestion of LSBP precursors as evidenced by the band which runs just below the mature form of LSBP.

**Reductive Tritiation of the Phosphorylated Proteins**—Since phosphorylation of the protein bands A and B was sensitive to the strong nucleophile hydroxylamine (Figure IV.2 lane 3), it is likely that the phosphoproteins contain acyl phosphate linkages. This interpretation is further supported by the thermal and alkali lability of the phosphorylation. Phosphorylation of band A and B proteins was not detectable on conventional sodium dodecylsulfate (SDS) gels (7) at 4°C or on acid/TDAB gels at room temperature (data not shown). Existence of an acyl phosphate can be confirmed by reductive tritiation with $[^3\text{H}]\text{NaCNBH}_3$, which produces a triitated homoserine from aspartyl phosphate or aminohydroxyvaleric acid from glutamyl phosphate (10, 12). This method also has the advantage that it replaces the unstable $^{32}\text{P}$ acyl phosphate linkage with a stable tritium label. Figure IV.4 shows the results of reductive tritiation on acid/TDAB gel band slices of the band A (69 kDa) protein region. Autoradiography of acid/TDAB gels was used to determine the extent of phosphorylation of the band A protein prior to reductive tritiation. Lane 1 demonstrates tritium incorporation in a sample where there had been good phosphorylation prior to tritiation. Lane 2 shows faintly detectable tritiation of a sample in which there was faint phosphorylation prior to tritiation. Lane 3 shows the absence of any detectable tritiation in a sample that had not been subjected to phosphorylation. Nearly identical results were obtained for the reductive tritiation of the band B (49 kDa) protein (data not shown). These results
FIG. IV.5. 2-D gel mapping of the reductively tritiated phosphorylated protein species. The phosphorylated protein bands A and B were excised from acid/TDAB gels, reductively tritiated, and comigrated with 2-D translocation reaction samples as described in "Materials and Methods." Proteins were visualized by silver staining. The reductively tritiated band A and band B protein species were visualized by fluorography. The locations of the proteins on the silver stained pattern are indicated by circles. Note that the size of the reductively tritiated band A protein is 62 kDa, and the band B protein is 37 kDa on the 2-D SDS gel.
provide strong evidence for acyl phosphate linkages in the band A and band B phosphoproteins.

Mapping of the Phosphorylated Proteins by 2-D gel Electrophoresis—To identify and further characterize the both band A and B phosphorylated species, acid/TDAB gel samples of reductively tritiated band A and B gel regions were mixed with total protein from a standard posttranslational translocation reaction and subjected to O’Farrell (13) two-dimensional (2-D) gel electrophoresis. The 2-D gel protein pattern is shown in Figure IV.5. The locations of the reductively tritiated proteins on the 2-D protein pattern are indicated by circles. The apparent sizes of the reductively tritiated proteins on the 2-D SDS gel are 62 and 37 kDa. The slight difference in sizes of the two proteins as determined by acid/TDAB gel electrophoresis presumably reflects differences in migration in the two gel systems. Since SDS gel electrophoresis is the commonly used method for molecular weight determination, the band A and B proteins (69 kDa and 49 kDa by acid/TDAB electrophoresis) will be referred to by their size determined on 2-D gels (62 kDa and 37 kDa respectively).

Examination of the 2-D protein map of *E. coli* (14) indicates that these proteins (coordinates: [73,100] for the 62 kDa protein and, [88,94] for the 37 kDa protein) have not been previously identified. Furthermore, the location of the 62 kDa protein does not correspond to the 2-D location of trigger factor (62 kDa) as determined by Western blot analysis (data not shown). The acidic location of the 37 kDa protein indicates that it is probably not the 37 kDa SecY protein which runs at a very basic location on 2-D gels (15).
Cellular Localization of the 62 kDa and 37 kDa Phosphorylated Proteins—2-D gel electrophoresis was used to determine the cellular location of both the 37 and 62 kDa proteins. Components of the posttranslational translocation system were run on 2-D gels and the presence or absence of both proteins was determined visually. Results indicate that both the 62 kDa and 37 kDa phosphoprotein are primarily associated with inverted membrane vesicles, while neither protein was found in the in vitro synthesized precursor protein fraction (data not shown). To determine if these proteins are integral membrane components or peripherally associated, membranes were treated with increasing amounts of NaCl (Table IV.I). Addition of 1.5M NaCl to the inverted membranes results in nearly complete removal or stripping of both proteins, 1.0M NaCl removes approximately 75% of both proteins, and at 0.5M NaCl approximately 50% of both proteins are stripped from the membranes.
Table IV.I.

Effect of NaCl on removal of the 37 kDa and 62 kDa proteins from inverted membrane vesicles

Inverted membrane vesicles were prepared as stated in "Materials and Methods." The membranes were treated with the indicated amounts of NaCl and incubated on ice for 30 min. The membranes were pelleted by centrifugation (70,000 rpm, 60 min, 4°C in a Beckman TLA 100.3 rotor) followed by 2-D gel electrophoresis of both the supernatant and pellet. The amounts of both the 62 and 37 kDa proteins on the 2D-gels were determined visually. Complete removal of the indicated proteins (protein found exclusively in the supernatant fraction) corresponds to (++++).

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<thead>
<tr>
<th>NaCl (M)</th>
<th>37 kDa</th>
<th>62 kDa</th>
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<tr>
<td>none</td>
<td>none</td>
<td>none</td>
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<tr>
<td>0.5</td>
<td>++</td>
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<tr>
<td>1.0</td>
<td>+++</td>
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<td>1.5</td>
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Discussion

Data presented in this chapter indicate that a 62 kDa protein is phosphorylated in an export associated fashion. Phosphorylation of the 62 kDa protein is believed to be involved in the protein export process based on three lines of evidence: 1) phosphorylation is dependent on the presence of both inverted membrane vesicles and precursor protein (Figure IV.1); 2) phosphorylation is inhibited by the dissipation of protonmotive force (presence of valinomycin and nigericin) and vanadate which is consistent with their inhibitory effects on protein export (see Chapters 2 and 3 respectively); 3) phosphorylation is dependent on the presence of a functional translocation system as shown by the absence of phosphorylation in the SecA defective BA13 system. Also consistent with a possible role in protein export is the localization of the 62 kDa protein on the cytoplasmic side of the inner membrane.

A 37 kDa protein also appears to be phosphorylated in an export-associated fashion. Phosphorylation of this protein is dependent on the presence of precursor protein and inverted membrane vesicles, and is sensitive to compounds which inhibit protein export in vitro (Figure IV.3). Localization of this protein on the cytoplasmic side of inverted membrane vesicles is also consistent with a role in bacterial protein export. However, phosphorylation does not appear to show the same SecA dependence as the 62 kDa protein. In the SecA defective BA13 system, phosphorylation of this protein is not observed in either the presence or absence of SecA. 2-D gel electrophoresis reveals that the 37 kDa protein is present in the BA13 membranes (data not shown), indicating that phosphorylation conditions used in the BA13 system either
do not elicit phosphorylation of this protein, or that phosphorylation occurs but is not stable enough to be observed under these conditions. Due to the lack of SecA dependence of the 37 kDa protein, further characterization is necessary to demonstrate an involvement in protein export.

Information about the possible role of phosphorylation during protein export can be inferred from the nature of the phosphate linkage and its stability in these proteins. The phosphate linkages of both the 37 and 62 kDa proteins differ from the more commonly known examples of phosphorylation in biological systems, which involve phosphoesters of serine, tyrosine, or threonine. Phosphoester linkages are stable at neutral pH and elevated temperatures, insensitive to hydroxylamine, and are not reactive with NaCNBH₃ (12, 16). In contrast, the phosphate linkages in the 62 and 37 kDa proteins are unstable at high temperatures and alkaline pH, sensitive to hydroxylamine, and are reducible by NaCNBH₃. These are characteristics of acyl phosphates, which contain mixed phosphoanhydrides.

Acyl phosphates vary considerably in their stability, and the stability appears to correlate with function. The *E. coli* NR₁ protein, which is phosphorylated during regulation of nitrogen assimilation (17), contains a phosphoaspartyl residue that is detectable on Laemmli electrophoresis gels at room temperature (6). On the other hand, mechanistic phosphoaspartyl linkages in P-type ion transport ATPases are too labile to detect on Laemmli gels even at lower temperatures (6). It might be expected that acyl phosphate linkages involved in regulation would be more stable at physiological temperatures and pH than mechanistic linkages. The phosphorylation
detected here has a stability similar to phosphorylations in P-type ATPases (1, 6), suggesting that export-related phosphorylation is mechanistic.

Two-dimensional gel electrophoresis was used to further characterize the 62 and 37 kDa proteins on protein patterns where over 600 E. coli protein have been mapped previously (14). To locate the two proteins on the 2-D gel patterns a reductive tritiation method was developed because the instability of the acyl phosphate linkage under standard SDS gel electrophoresis conditions would make direct identification impossible. The method consists of reducing the $^{32}\text{P}$-protein linkage with $[^{3}\text{H}]\text{NaCNBH}_3$ in gel slices removed from acid/TDAB gels. NaCNBH$_3$ selectively reduces acyl phosphate linkages, replacing the labile $^{32}\text{P}$ with a covalently stable $^{3}\text{H}$ (12). The amount of tritium incorporated into the protein corresponds very well with the original extent of phosphorylation observed in the acid/TDAB gel (Figure IV.4). Straightforward identification of the proteins was obtained when the tritiated gel bands were subjected to 2-D gel electrophoresis with a representative mixture of unlabeled E. coli protein (Figure IV.5). This method is suggested as a general procedure for rapid identification and characterization of acyl phosphate containing proteins.

Characterization of both proteins by 2-D gel electrophoresis reveals that these proteins have not been previously identified on 2-D gels or characterized as protein export components. The 62 kDa protein does not appear to be trigger factor (based on western blot analysis), or GroEL (previously mapped by 2-D electrophoresis, [14]).
The 37 kDa protein does not appear to be SecY which runs at a very basic location on 2-D gels (15).

ATP turnover is required for protein export (Chapter II, and [18]), and is also required for phosphorylation observed here. Recent evidence suggests that SecA is at least one site at which ATP is utilized during protein export (Chapter III, and 19). SecA contains an ATPase activity, dependent on inverted membrane vesicles and purified precursor protein, which is sensitive to vanadate (Chapter III). Vanadate also inhibits posttranslational precursor protein translocation (Chapter III). Furthermore, phosphorylation of the 62 kDa protein is dependent on SecA. These results suggest that SecA ATPase activity and phosphorylation of the 62 kDa protein are related.

As discussed in Chapter III, there is some sequence homology between SecA and the phosphorylation domain of P-type ATPases. However, the conserved aspartic acid phosphorylation site is not observed in the SecA protein. Instead, a glutamic acid residue is found one residue away from phosphorylation site in SecA. It is possible that glutamic acid is phosphorylated since it contains a carboxyl group side chain necessary for the formation of an acyl phosphate. Under the phosphorylation conditions used in this study, phosphorylation of SecA was never observed. This result is consistent with failures by other workers to detect phosphorylation of SecA (personal communication D. Oliver). Inability to observe SecA in a phosphorylated form is not surprising. Phosphorylation of a glutamic acid residue has never been observed in P-type ATPases. The predicted phosphorylation domain found in SecA could, however, be essential for the observed phosphorylation of the 62 kDa protein. It is possible that
SecA is phosphorylated at the glutamic acid residue and the acyl phosphate is transferred to the 62 kDa protein in a concerted fashion. In this respect, the phosphorylated form of the 62 kDa protein would be expected to regulate or function in concert with SecA. Inhibition of SecA ATPase activity and phosphorylation of the 62 kDa by vanadate, and SecA dependent phosphorylation of the 62 kDa protein are all in agreement with this interpretation.

Inconsistent with this interpretation is the observation that ionophores (for example valinomycin/nigericin) which inhibit phosphorylation of the 62 kDa protein have no effect on SecA ATPase activity (Chapter III). This would seem to indicate that the two events may be unrelated. However, it is possible that phosphorylation of the 62 kDa protein is the link between membrane energy and SecA ATPase activity during protein export. Urea treatment of inverted membrane vesicles (necessary for SecA ATPase activity determination) results in near quantitative removal of the 62 kDa protein (data not shown). Removal of the 62 kDa protein from SecA ATPase reactions may result in an unregulated or membrane energy independent SecA ATPase activity. In this light, the high Km (0.23mM) for ATP in SecA ATPase activity (20) may be the Km value for SecA ATPase activity independent of membrane energy. Whereas, the effective Km value determined for protein export (1-10 M, Chapter II) may be the Km for ATPase activity which is dependent on membrane energy.

Alternatively the 62 kDa protein may function as an ATPase involved in the export process. The nature of the phosphate linkage and its stabilities are consistent with a mechanistic function for the protein. Characterization of the functions associated
with the 62 kDa and 37 kDa phosphorylated proteins will have to await purification and reconstitution in appropriate in vitro protein export systems. This approach should yield further information about the functions of these proteins and may provide insight about the regulation of ATPase activity during protein export.

References


CHAPTER V

SUMMARY OF CONCLUSIONS

1. ATP and membrane gradient energy are necessary for optimal in vitro export of LSBP and BLA precursors.

2. The upper limit for the effective Km for ATP in processing of LSBP and BLA precursors under in vitro protein export conditions is 10µM.

3. The transmembrane electrical potential ($\Delta \Psi$) appears to be the membrane energy component necessary for export of LSBP and BLA precursors.

4. The ATPase inhibitors vanadate and DES inhibit in vitro protein export of LSBP and BLA precursors.

5. SecA ATPase activity that is dependent on membranes and precursor is sensitive to vanadate, with half maximal inhibition of SecA ATPase activity observed at approximately 20µM vanadate.

6. Part of the SecA protein sequence has homology to the phosphorylation sequence in P-type ATPases.

7. Two proteins with apparent sizes of 62 and 37 kDa are phosphorylated in a precursor and membrane dependent fashion.

8. Phosphorylation of both the 62 and 37 kDa proteins is sensitive to uncouplers of membrane gradient energy and vanadate, consistent with effects of these reagents on in vitro bacterial protein export.

9. Phosphorylation of the 62 kDa protein is SecA-dependent.
10. Reductive tritiation of both the 62 and 37 kDa proteins and hydroxylamine sensitivity indicate acyl phosphate linkages.

11. Both the 62 and 37 kDa proteins correspond to *E. coli* proteins previously unidentified on 2-D gels.

12. The 62 and 37 kDa proteins are peripherally associated with the cytoplasmic face of *E. coli* membranes.
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Publications:

Corbett, J A., Copeland B.R. (1990) Phosphorylation of a 69kDa Protein Associated With Bacterial Protein Export. EMBO J. submitted

Corbett, J.A., Copeland, B.R. (1990) Inhibition of Bacterial Protein Export by Vanadate and DES. In Preparation


Presentations:
