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Production, purification and crystallization of membrane integrated multimeric Bax

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PRODUCTION, PURIFICATION, AND CRYSTALLIZATION OF MEMBRANE
INTEGRATED MULTIMERIC BAX

by

Adelbert Villosio

Bachelor of Science
University of Nevada, Las Vegas
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A thesis submitted in partial fulfillment
of the requirements for the

Master of Science Degree in Biological Sciences
School of Life Sciences
College of Sciences

Graduate College
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December 2010

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THE GRADUATE COLLEGE

We recommend the thesis prepared under our supervision by

Adelbert Viloso

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ABSTRACT

Production, Purification, and Crystallization of Membrane Integrated Multimeric Bax

by

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Dr. Eduardo Robleto, Examination Committee Chair
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Apoptosis, or programmed cell death, is a vital process intimately involved in the embryonic development and homeostatic maintenance of all multicellular organisms. The committing step to apoptosis is regulated by a key protein, Bax, and its ability to integrate and form a pore structure at the outer mitochondrial membrane.

Unfortunately, the molecular details of apoptosis remain largely unclear due to the lack of structural data of integral membrane (IM) Bax. Experimental limitations of membrane protein production have slowed the pursuit of an IM-Bax structure simply because standard protocols for producing recombinant IM-Bax are inefficient in producing adequate quantities of IM-Bax protein. This complication makes it difficult to study Bax in its activated oligomeric state and essentially impossible to develop a protein crystal for x-ray structure analysis.

In this thesis, we have developed solutions to these substantial problems by utilizing the membrane affinity properties of the protein Mistic to promote the membrane integration and efficient protein production of Bax in an *E. coli* expression system. In addition, we have discovered detergent conditions optimized for high protein yield and stability of an activated trimeric Bax. The activated trimer complex was further

characterized by size-exclusion chromatography and multi-angle light scattering techniques to detail the molecular contributions of protein in the detergent-protein complex. Most importantly, the effectiveness of our protocols has produced the first x-ray diffracting crystal of an activated Bax multimer, an invaluable tool for the solution structure of activated Bax complex. This hallmark in apoptosis research will further complete the understanding of apoptosis mechanism and ultimately lead to the development of a new realm of effective pharmaceuticals.

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I am also grateful for the unfailing support of Dr. Eduardo Robleto. You have always been my mentor through my entire university career. Your advice was the initial push to pursuing the graduate school path and your guidance was invaluable through it.

I thank Dr. Wing, Dr. Andres, and Dr. Abel-Santos for every word of constructive critique. I cannot express how helpful your participation in my committee was and I account my success to listening to your commentary.

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LIST OF ABBREVIATIONS

Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 Homology
CMC	Critical Micelle Concentration
CSC	Critical Solubilization Concentration
DDM	n-Dodecyl-B-D-Maltopyranoside
DM	n-Decyl-B-D-Maltopyranoside
IM	Integral Membrane
LAPAO	N-[3-(dimethylamino)propyl]dodecanamide N-oxide
LDAO	n-Dodecyl-n,n-Dimethylamine-n-Oxide
MALS	Multi-Angle Light Scattering
MHC	Major Histocompatibility Complex
MOMP	Mitochondrial Outer Membrane Permeabilization
SEC	Size Exclusion Chromatography
UPP2	Uridine Phosphorylase 2
UDM	n-Undecyl-B-D-Maltopyranoside

CHAPTER 1

INTRODUCTION

1.1 Purpose of Study

Apoptosis is a strictly regulated and complex program to regulate the homeostasis of a developing organism and eliminate diseased cells that may potential harm the body. For understanding diseases of embryonic development, neurodegeneration, autoimmune deficiencies, and cancer; obtaining the molecular structures of proteins involved in apoptosis is an immense priority (Danial and Korsmeyer, 2004; Carrington et al., 2010). In mitochondrial outer membrane permeabilization (MOMP) mediated apoptosis, the formation of a pore on the mitochondrial membrane is the committing passage to cell death and is strictly regulated by the B-cell lymphoma 2 (BCL-2) family of proteins. These pores allow for cytochrome c to diffuse from the inside of the mitochondria into cytoplasmic space in the cell. These events activate apoptosome signaling cascades which eventually lead to cell death.

Oligomerization of Bcl-2-associated X (Bax) of the Bcl-2 family is responsible for the apoptosis inducing pore and its activation is the final checkpoint before the cell commits to cell death. The structural details of the Bax oligomer are essential to understanding the mechanism of apoptosis. Clarification of this information would in turn, allow the science community the ability to troubleshoot apoptosis malfunctions.

Moreover, the realm of membrane integrated proteins is largely untouched (Wallin and Von Heijne, 1998; Krogh et al., 2001). 30 to 45% of human proteins are membrane-embedded or membrane-associated, yet membrane protein structures account for less than 1% of known protein structures listed in the Protein Data Bank (Berman et

al., 2002). This statistic is astonishing in that at least 50% of pharmaceuticals interact with membrane proteins (Klabunde and Hessler, 2002).

In pursuit of what is referred to as the “holy grail of apoptosis,” the purpose of this thesis is to further understand the biochemical mechanisms of Bax oligomerization and therefore the foundational details of mitochondrial apoptosis (Youle and Strasser, 2008). We hypothesize that we have developed an integral membrane conformation of multimeric Bax. Through SEC-MALS, we have quantified the molecular weight of protein contribution to the Bax/detergent complex and conclude that the protein oligomer is trimeric.

In addition, we hypothesize that oligomeric Bax is able to form diffracting protein crystals. The x-ray solution of oligomeric Bax is extremely valuable for molecular clarity of apoptosis as well as providing blueprints for rational drug designers in cancer pharmaceuticals.

This thesis develops techniques for relatively high yields of IM Bax and prompts the finding of an inevitable x-ray solution. The protocol used to obtain these data displays the effectiveness of the fusion partner Mystic and should push science to study IM proteins that were previously impossible to study *in vitro* due to protein expression limitations. With a deeper mechanistic understanding of Bax oligomerization and the eventual high resolution structure of IM Bax, we hope to contribute to the improvement of human health by developing the protein blueprints for rational drug designers. The wealth of knowledge from a Bax structure will initiate a new stage of production of highly specific and efficient pharmaceuticals to solve problems of aberrant apoptosis.

Although the development of a Bax protein crystal is a significantly ambitious task, the probability for success is greatly enhanced by our recent experiences in obtaining the first crystal structure of the human protein uridine phosphorylase 2 (UPP2). Publication details can be seen in the appendix section of this thesis.

1.2 Significance to Development and Disease

Within an entire lifetime, the innumerable events of cell proliferation, the growing biochemical inefficiencies occurring with age and the unavoidable exposure of DNA damaging elements, the occurrence of biochemical mistakes seem inevitable in cellular replication. Surprisingly, despite the trillions of mutable cells the propagation of cancer can originate from, cancer on average occurs less than once in a lifetime (Lowe et al., 2004). Multicellular organisms in response to these highly detrimental events have evolved networks of aberrant tumor control, one of the most important being apoptosis. Cancer has taken the spotlight for focus to discern the mechanisms of apoptosis. Currently, popular methods of treating cancer include surgery, chemotherapy, and radiation therapy and although effective in eliminating cancer cells, the loose specificity of these treatments has long been a major drawback as they can result in collateral damage to healthy cells.

It is evident that in terms of molecular biology, there is much to be learned in the treatment of cancer. For example, it was originally believed that a chemotherapeutic barrage of damage on the cellular components of a tumor defined the lethal factor in eliminating cancerous cells. It is now known that chemotherapeutic drugs do not directly kill but instead trigger the same innate tumor-suppressor networks that halt the events of cancer proliferation, or in other words, chemotherapy activates tumor self destruction

(Lowe et al., 2004). Interestingly, the decision for cells to proliferate or execute apoptosis is controlled by connected pathways. Chemotherapy can potentially disable proliferation checkpoints which undesirably uncouple apoptosis pathways, creating unresponsive tumors (Lowe et al., 2004).

It is also shown that anti-apoptotic proteins are overexpressed in both solid tumors and hemotologic malignancies (Korsmeyer, 1999; Cory et al., 2003). Accumulated genetic errors resulting in gene amplification in Bcl-2 genes is observed in almost all follicular lymphomas and approximately 20% of diffuse B-cell lymphomas (Yunis et al., 1987; Yang and Korsmeyer, 1996). Growing studies on apoptosis and the Bcl-2 family have also detailed other survival mechanisms of tumor proliferation. Errors in upstream factors such as AKT possibly promote tumor survival by decreasing transcription of pro-apoptotic proteins (Grad et al., 2000). Transcription factors such as STAT, Rel/NF-[kappa]B, and Ets have been shown to modulate the expression levels of Bcl-XL and in turn over sequester pro-apoptotic signals (Wendel et al., 2004). The gained ability for cancer to sequester pro-apoptotic signals with anti-apoptotic proteins is speculated to be a survival strategy for tumors, allowing for immunity to death signals and chemotherapeutics acting on pro-apoptosis pathways.

The repeating pattern of high expression of pro-survival proteins in cancers has inevitably led to the development of ABT-737, a BH3-only mimetic capable of high affinity binding to anti-apoptotic proteins. The binding of ABT-737 liberates sequestered pro-apoptotic proteins to induce apoptotic signals. Although effective independently, ABT-737 also acts as a sensitizer to increase the effects of other chemotherapeutic compounds such as proteosome inhibitors. The strategy of orchestrating attacks at

multiple pathways is promising, enabling the elimination of cancerous cells while preventing them from resisting treatment (Oltersdorf et al., 2005).

With many varying characteristics of cancer tissue types, focus on treating a commonality between all cancers is vital. A faulty apoptosis program is one common theme of proliferating cancer, therefore the details of the mechanism of apoptosis are urgent for advancements in oncology.

The Bcl-2 family possesses considerable responsibility in maintaining the immune system as well. Lymphocytes must maintain criteria that act to further increase reactivity to foreign substances while remaining tolerant to self; this is the central principle of a functioning immune system (Carrington et al., 2010). The proapoptosis signals are activated in developing lymphocytes failing to meet standards that conserve proper immune system integrity. For example, if newly created B or T cells fail to undergo productive rearrangement of their T-cell receptor genes, they cannot receive signals through the antigen receptors and as a result are signaled for apoptosis. Qualified immature thymocytes with productive TCR gene rearrangements must express receptors that bind host MHC class I or class II molecules. Receptors that cannot bind MHC class I or class II will fail to respond to foreign antigens presented by host MHC molecules and are therefore signaled for destruction. B cells undergo a similar checkpoint that they must pass in order to proceed in maturity. Lastly, B and T cells that recognize self-antigens are negatively selected and eliminated. Remarkably, after the battery of checkpoints the B and T cells undergo, only 10% meet the strict immunological criteria (Marsden and Strasser, 2003).

Apoptosis also has a role in the halting of an immune response. Once a pathogen is successfully eliminated, the Bcl-2 family is also responsible for the termination of active effector plasma cells and (Sprent and Tough, 2001). A lingering immune response can have deleterious damage. For example, diseases such as the lymphocytic choriomeningitis virus elicit a extremely self-damaging immune system reaction (Gallimore et al., 1998).

In contrast, alteration of apoptosis for the inhibition of a proper immune response can be beneficial for patients of organ transplantation due to the elimination of immune-mediated rejection (Carrington et al., 2010). Treatment of ABT-737 on immature B and T cell populations in mouse immune systems selectively reduced leukocyte populations, inhibited humoral immunity, and slowed cytotoxic T lymphocyte response. This special modulation of the Bcl-2 family allowed for the protection of transplanted islet allografts, leading to glucose processing and the essential reversal of diabetes (Carrington et al., 2010).

Lastly, apoptosis is crucial in all steps in embryo development. The apoptotic process is seen as early as the blastocyst phase, where it shaping the inner cell mass and trophoctoderm by controlling cell numbers (Hardy, 1997). Later in development, apoptosis is responsible for the elimination of abnormal or misplaced cells and also sculpts key biological structures (Jacobson, 1997). For example, the role of apoptosis is seen in the development of the fingers in the hand. Cells that develop in between each digit are eliminated through apoptosis (Mori, 1995).

1.3 Known Structural Characteristics of the Bcl-2 family and Bax

To date, our best solution structure of Bax has been the inactivated, monomeric conformation obtained through NMR spectroscopy (Suzuki et al., 2000). Four sequence conserved alpha helical domains, labeled "Bcl-2 homology" (BH1-4) domains, define the Bcl-2 family (Petros et al., 2004). The Bcl-2 family is further categorized by function: the pro-apoptotic multidomain, anti-apoptotic, and BH3-only proteins. The anti-apoptosis proteins (Bcl-2, Bcl-XL, A1/BF1, Bcl-W) show sequence conservation in three to four BH domains and function as to bind pro-apoptotic proteins, inactivating them (Youle and Strasser, 2008). Second, pro-apoptosis proteins Bak and Bax are divided into multiple BH domains containing three conserved domains (BH1-3) and function by homo-oligomerizing at the mitochondrial membrane to form a cytochrome c releasing pore (Youle and Strasser, 2008). Lastly, the BH3-only proteins only show homology in the BH3 domain and are theorized to either release anti/pro complexes to allow pro-apoptotic proteins to commit to pore formation or activate pro-apoptotic proteins directly (Youle and Strasser, 2008).

A Bax monomer is composed of domains BH1-BH3 and is assembled by 9 alpha helices. $\alpha 4$ - $\alpha 5$ compose the BH1 domain, $\alpha 7$ and $\alpha 8$ the BH2, and $\alpha 2$ the BH3. The conformational change of the monomeric Bax marks its activation, allowing for translocation, and oligomerization with other Bax molecules. Bax is structurally similar to anti-apoptotic protein Bcl-XL. Both proteins form a hydrophobic groove in their BH1-3 domains, however, unlike Bcl-XL, Bax occupies this hydrophobic groove with its own $\alpha 9$ or C-terminal tail (CT) (Muchmore et al., 1996; Suzuki et al., 2000).

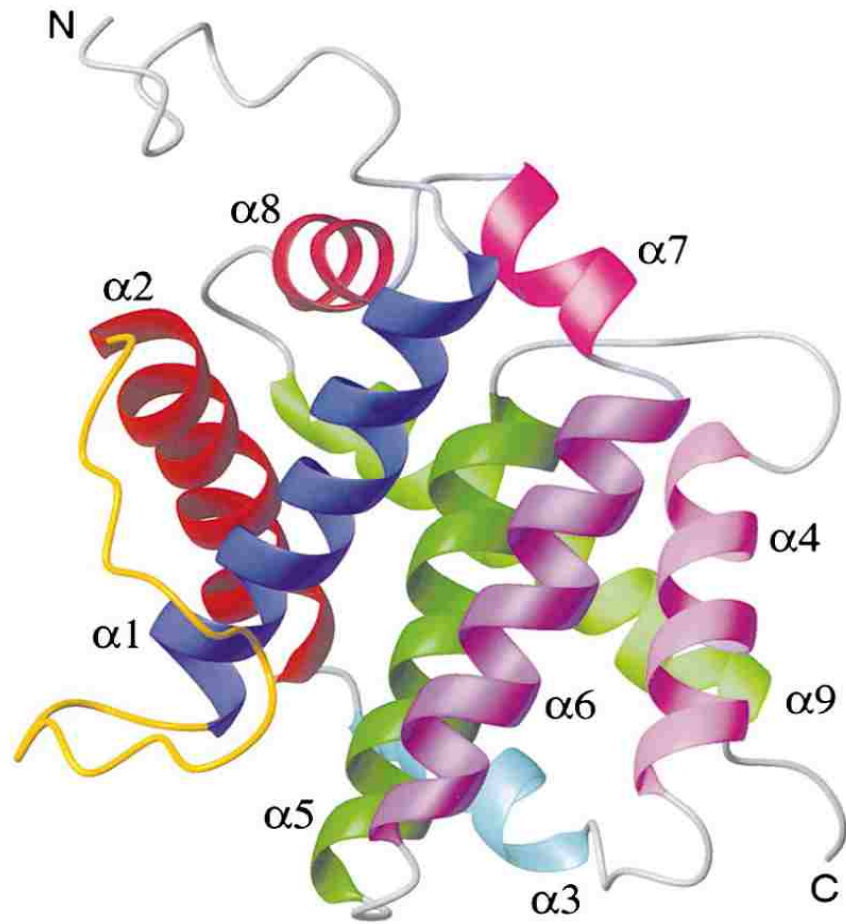


Figure 1: Structure of monomeric Bax solved by NMR spectroscopy (Suzuki et al., 2000).

Hydrophobic $\alpha 5$ composes the core of Bax which is surrounded by the remaining eight amphipathic alpha helices. $\alpha 2$ contains the BH3 domain and together with $\alpha 4$ and $\alpha 5$, face the hydrophobic core of the protein. $\alpha 4$ and $\alpha 5$ are also known as the homo-oligomerization domain, responsible for the oligomerization to other Bax molecules (George et al., 2007).

Helices $\alpha 6$ and $\alpha 9$ are independently important for membrane translocation. Knock-out studies removing individual $\alpha 6$ and $\alpha 9$ helices do not abolish translocation, however removal of both helices halts ability of Bax to translocate to the mitochondrial membrane (George et al., 2010). A Bax molecule contains two cysteine residues, Cysteine-62 at the center of $\alpha 2$ and Cysteine-126 at the end of $\alpha 5$. Both cysteine residues are potentially available for disulfide bridges between other Bax molecules or heterodimerization with other members of the Bcl-2 family (D'Alessio et al., 2005).

Activation of monomeric Bax induces a conformational shift in the molecule. This disengages the $\alpha 9$ from the hydrophobic groove, exposing the BH3 domain to trigger translocation to the mitochondrial membrane which allow oligomerization of Bax molecules or pro-survival proteins (Dewson and Kluck, 2009).

The remaining helices hold the important function of maintaining the monomeric, inactive integrity of Bax. Surprisingly, disruption of the helices causes conformational change and spontaneous activation of Bax (George et al., 2010).

1.4 Bcl-2 Regulation and Bax Activation

Once surpassing anti-apoptotic signals, pro-apoptotic proteins localize to the mitochondrial membrane forming pore structures. The pore structures allow cytochrome

c to diffuse out of mitochondrial space into the cytosol. There, it will activate apoptosome complexes and a subsequent caspase cascade, ultimately leading to cell death (Reed, 2004)

Cellular stresses, such as DNA damage, hypoxia, and oncogene activation are the initial signals of apoptosis activation. These signals activate tumor suppressor p53, which is a direct transcription factor of Bax, as well as BH3-only proteins Puma, Noxa, and Bid (Nakano and Vousden, 2001; Hemann and Lowe, 2006).

The complex interactions of pro-apoptotic multidomain, anti-apoptotic, and BH3-only proteins dictate cell survival or death in MOMP apoptosis (Bleicken et al., 2009). Pro-apoptotic multidomain proteins consist of 2 members, Bcl-2 homologous antagonist/killer (Bak) and Bcl-2-associated X (Bax). Though redundant in their functions, inactivated Bak is a constituent of the mitochondrial membrane while, inactivated Bax is cytosolic and neither protein interacts with each other (Wei et al., 2001).

The exact mechanism of Bax activation awaits clarification though there are three supported theories. The direct activation theory suggests that BH3-only proteins, such as truncated-Bid (t-Bid), Bim, Puma, and RACK1, act as direct pro-apoptotic signals. Contact with BH3-only proteins cause a conformational change in Bax to allow for a series of structural transitions causing homo-oligomerization and subsequent translocation to the mitochondrial membrane (Letai et al., 2002; Certo et al., 2006; Walensky et al., 2006; Wu et al., 2010).

Since a heterodimer of a BH3-only protein interacting with Bax has never been

observed, a “hit-and-run” mechanism has been proposed. This mechanism suggests the Bax/BH3-only interactions are transient. For example, the “hit” may occur when a Bim protein attaches to the back of a Bax monomer, which displaces the C-terminal tail of Bax (Gavathiotis et al., 2008). Subsequently, the BH3 region of a separate Bax molecule may disrupt the groove allowing for the formation of a homo-oligomer while simultaneously displacing tBid, allowing it to “run” away (Wei et al., 2000; Gavathiotis et al., 2008).

A second theory called the displacement model suggests that the presence of anti-apoptotic proteins continuously sequester pro-apoptotic Bax and Bak from activation. Only when a BH3-only protein competes and binds anti-apoptotic proteins does the anti/pro-complex separate, allowing Bax to continue with the default pore formation (Willis et al., 2005; Willis et al., 2007; Fletcher et al., 2008).

A third theory called the "embedded together" model combines both direct and indirect activation theories and suggests that lipids and proteins in the mitochondrial outer membrane are the key to coordinating MOMP (Leber et al., 2007). Evidence in this thesis supports the importance of the membrane in MOMP.

In any case, the end point is the formation of a Bax-only pore that allows cytochrome c from intermitochondrial space to diffuse out. Cytosolic cytochrome c binds Apaf-1 which leads to the assembly of the apoptosome and activation of caspase 9. Caspase 9 then activates caspase-3, caspase 6, and caspase 7 for rapid cleavage of multiple targets leading to the packaging and phagocytosis of the apoptotic cell (Luthi and Martin, 2007; Ravichandran and Lorenz, 2007).

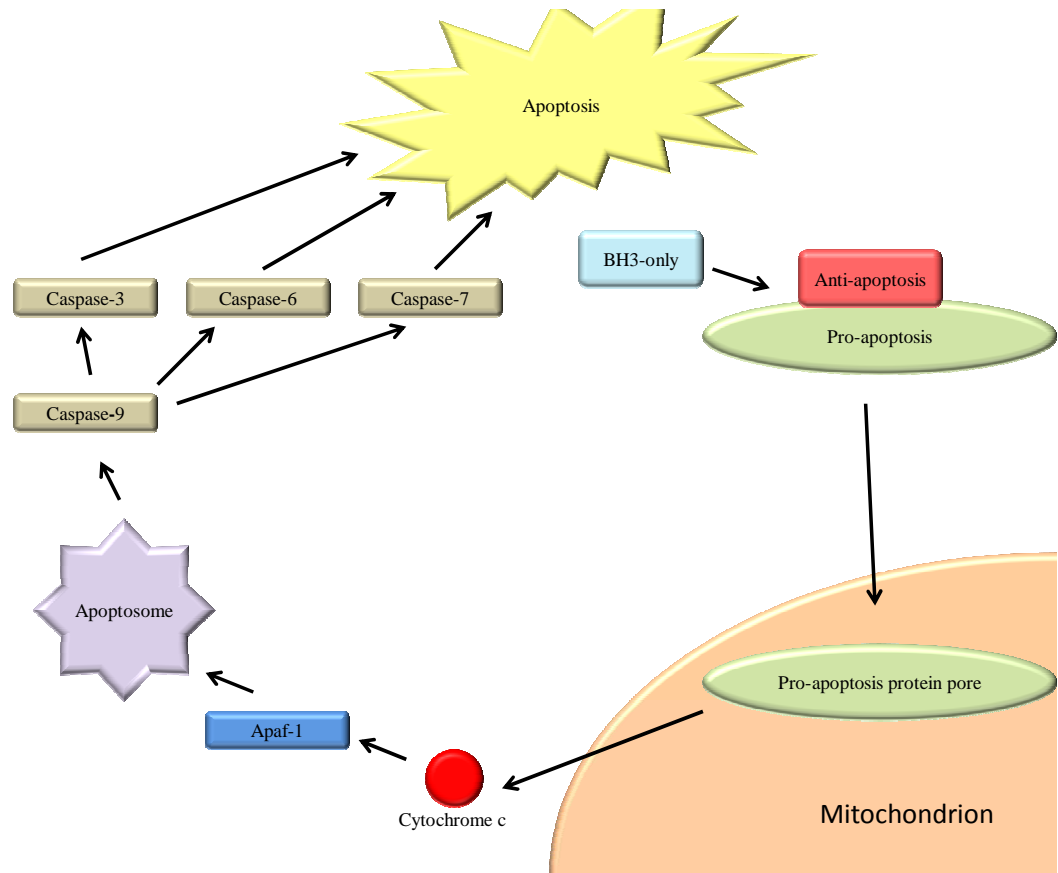


Figure 2: Bcl-2 regulation of mitochondrial outer membrane permeabilization.

1.5 Present Problems in IM Protein Production and a Mystic solution

The halting bottleneck in the elucidation of recombinant IM proteins is simply producing adequate amounts for experimentation. Inducible expression systems in *E.coli* of soluble proteins are extremely reliable in producing high yields. Adversely, a reliable system for expressing IM proteins is lacking, making IM protein production extremely difficult (Tate, 2001). There are two theories for the IM protein production limitation. One, IM protein translocation may require targeting signals that may not be recognized by the bacterial host, making membrane integration impossible. Second, newly translated membrane proteins require chaperones in order to be directed from the ribosome to the cellular membrane. As membrane protein expression increases, the host cells translocation machinery becomes increasingly overwhelmed with the movement of recombinant IM proteins. This results in the neglect of vital host IM proteins. The essential hijacking of translocon machinery leads to cell toxicity (Roosild et al., 2005).

To solve complications of both IM protein signal recognition and translocation overloading, we utilized the fusion partner protein Mystic as an independent translocation system. Discovered in *Bacillus subtilis*, Mystic is a 13 kDa protein and has an innate affinity to the lipid bilayer. Linking Mystic to the N-terminus of human Bax protein allows for the translated protein to be independently moved from the ribosome to the lipid bilayer which allows Bax to be in close proximity to the membrane for integration.

The fact that no known accessory proteins or chaperones are required for proper Bax folding supports the probability that Bax will integrate correctly into the membrane during Mystic translocation.

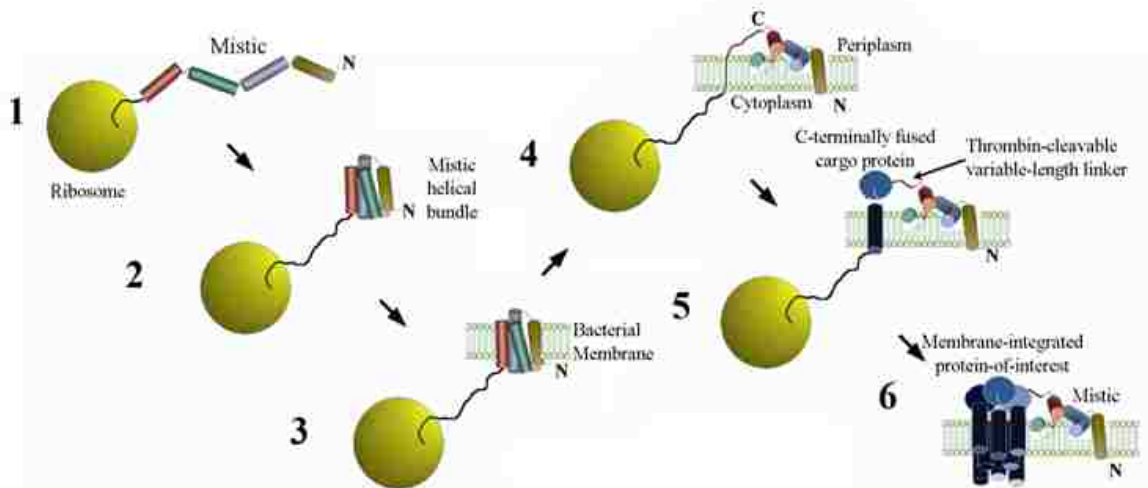


Figure 3: A step-by-step progression of Mistic-fused translation. Mistic is expressed at the N-terminus of a target protein. The innate affinity of Mistic to the bacterial membrane trails the target protein allowing for membrane integration of the protein-of-interest.

1.6 Detergents

When embedded in their native membranes, IM proteins remain properly folded and active. Once extracted from the membrane, the lack of the amphipathic environment causes protein misfolding, loss-of-function, and aggregation (Prive, 2007). Studies on IM proteins have facilitated the use of non-ionic detergents to act as membrane surrogates to maintain protein stability. Detergents are water-soluble, surface-active agents that due to their amphiphilic character will self-aggregate in solution to form micelles (Sigma-Aldrich, 2004).

The problem of determining a compatible detergent is not simple. The numbers of detergents available for *in vitro* studies are enormous, with each detergent having its own complex properties. Generally, detergents have two segments which are common to all detergents. The first segment, called the hydrophilic head group, have a strong influence on interactions with proteins. The second segment, called the hydrophilic alkyl chain, affects the detergent critical micelle concentration (CMC) and aggregation number. Composition of either segment causes diverse interactions with IM proteins.

The CMC is the minimal concentration of detergent in order for micelles to form. The aggregation number is a property unique to each detergent and signifies the number of detergent molecules in a micelle. For general purposes, concentrations of buffers containing detergent should be well above the CMC in order to be an effective solubilizer (Prive, 2007). During the extraction of membrane proteins, the critical solubilization concentration (CSC), which is the minimal detergent concentration required to disrupt membranes into a micellar dispersion, must also be surpassed.

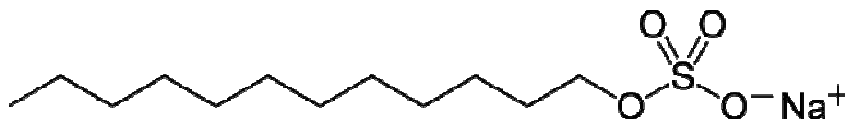
The use of a “gentle” detergent is essential for protein crystallization. Strong detergents such as sodium dodecyl sulfate (SDS) contain reactive head groups and are a protein denaturant. Therefore the use of SDS is undesirable in maintaining protein stability and is not used when attempting to create a protein crystal. On the other hand, weaker detergents such as octyl-glucoside have non-reactive head groups but due to the long alkyl chain, high concentrations are required in order to keep membrane proteins in solution which may be detrimental to protein crystallization (Prive, 2007).

Unfortunately, the optimal detergent for membrane protein extraction or even maintaining protein stability is not necessarily optimal for protein crystallization. The protein, detergent, and aqueous buffer comprise the three components in a membrane protein crystal lattice and are referred to as the protein-detergent complex (PDC). Each component of the PDC has independent physical properties but together must form stable complexes. For example, protein-protein contacts form the primary scaffolding of the lattice however, in order to form crystals, IM proteins must interact within embedded flexible detergent micelles. Small micelle detergents like LDAO form small belts around the transmembrane region of proteins allowing for greater protein-protein interactions however, these detergents are often destabilizing. In contrast, large micelle detergents are much gentler to proteins but the large size of the detergent prevents protein-protein interactions (Prive, 2007).

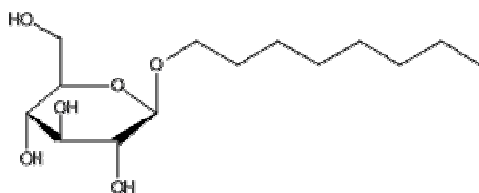
Protein aggregation is usually an irreversible process that is a result of using an incompatible detergent. Aggregation seems to occur with even minor denaturing effects. Small misfolds may lead to non-specific interactions of exposed surfaces meant to be buried in the protein complex. Also, if the detergent does not fully mask the

transmembrane region of IM proteins, the hydrophobic regions of the IM proteins may associate to cover their surfaces from the aqueous buffer. In brief, the flexible and dynamic nature of the detergent belt surrounding the protein makes the formation of a strong crystal lattice very difficult (Prive, 2007).

The ability to shift from cytosolic to membrane-bound conformation makes Bax an extremely interesting IM protein. Studies speculate that a hydrophobic active site is shielded when Bax is in its cytosolic, inactive form. Once activated by a BH3-only protein, Bax shifts to an open conformation. This open conformation exposes its hydrophobic surface to interactions with other activated Bax molecules forming oligomers.



Sodium dodecyl sulfate



Octyl glucoside

Figure 4: Structural Comparison of Strong and Weak Detergents (ChemBook, 2008).

Luckily, strong evidence has also shown that Bax in non-ionic detergent micelles shifts conformation into an activated form. This will allow for *in vitro* oligomerization without the need for BH3-only proteins (Antonsson et al., 2000; Le Maire et al., 2000).

1.7 Size-Exclusion Chromatography and Multi-angle Light Scattering Analysis

Aggregates, heterogeneous protein sample, and any minute contaminants are inevitable in a protein preparation yet are highly detrimental to protein crystallography. A series of purification methods must be taken at every step to protein crystallization trials.

Size-exclusion chromatography (SEC), or gel filtration, is a method widely used in protein purification. In brief, a heterogeneous protein sample is run through a gel matrix packed column. The matrix is composed of porous spherical particles that allow buffer and sample to diffuse through while it is traveling the column. Larger molecules, having difficulty diffusing into the matrix, will elute faster while smaller molecules will spend more time inside the matrix before finally eluting off. The timing at which molecules elute off the column is used to predict the molecular weight of cytosolic proteins.

Unfortunately, the elution timing in SEC to determine molecular mass of a protein becomes unreliable when studying IM proteins. In comparison to cytosolic proteins, detergent-protein complexes will have altered adsorption behavior to the SEC column due to the presence of detergent. More importantly, the presence of detergents increase the molecular mass of IM proteins, making proteins elute much faster, falsely indicating a larger protein.

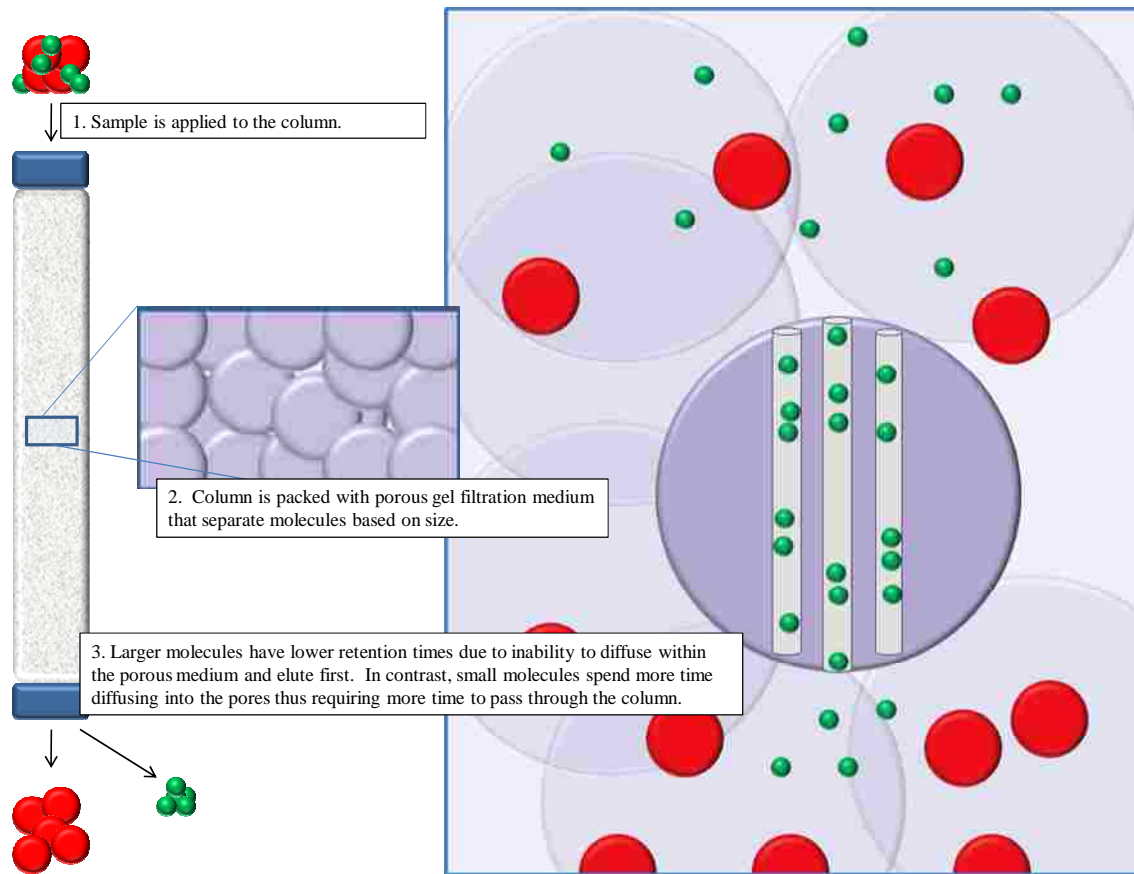


Figure 5: Outline of Size-exclusion chromatography (GE Healthcare, 2002). Timing of protein sample elution is predictable and based on molecular size. Larger molecules have lower retention times due to inability to be “trapped” within the porous medium. In contrast, small molecules spend more time diffusing into the pores thus requiring more time to pass through the column.

To obtain an accurate measurement of molecular masses of both the detergent and protein components, multi-angle light scattering in conjunction with SEC must be used in order to obtain light scattering, UV absorption, and differential refractive index measurements. For simplicity, we use the commercially available Astra software package (Wyatt technologies) to discern the molecular mass contribution of protein and detergent in these complexes. In short, by combining the change in refractive index as a function of protein concentration (dn/dc_{protein}), the change in refractive index as a function of detergent concentration ($dn/dc_{\text{detergent}}$), and measuring the refractive index and UV absorption, Astra can determine detergent concentration (δ) of a given solution. δ is then used to find the refractive index increment (dn/dc_{complex}), the change in refractive index of the solution as a function of protein concentration, using the light scattering and refractive index measurements to determine the molecular mass of the protein/detergent micelle. Again, δ is then used to determine the contributions of protein and detergent to the molecular mass of the complex. Ultimately, these readings are used to determine protein contribution to the protein-detergent complex (Wyatt-Technology, 2010).

1.8 Protein Crystallography

Unfortunately, the biggest drawback of NMR in the study of oligomeric molecules is their large sizes. Larger complexes, like a trimeric Bax (75 kDa) will have slower tumbling rates and shorter NMR signal relaxation times causing poor resolution of the protein. Also, increased molecular weight introduces complexity due to the increased interactions amongst the NMR-active nuclei (Tamm and Liang, 2006). Due to these paralyzing limitations, we turn to protein crystallography in attempt to solve the structure of oligomeric Bax.

Protein crystallography is a technique over 170 years old historically used to purify proteins from a heterogeneous mixture and to measure purity of a given protein preparation (McPherson, 1999). Presently, protein crystallography with the use of x-ray diffraction has grown to be one of the most valuable tools in generating high resolution three dimensional structures. Sadly, the production of a protein crystal is a stifling hurdle in structural biochemistry simply because we lack the insight to know what crystallizes a protein. The dynamic nature of proteins makes it impossible to predict what parameters stabilize each unique protein in order for it to crystallize. In addition, lessons learned from one crystallization success do not translate to even similar proteins due to the fact that each amino acid sequence possesses its own three dimensional structure and therefore surface characteristics. Scientists have even gone to great distances in attempts to eliminate variables such as gravity, leading to crystallography laboratories in space.

For a crystal to form, the properties of the solution and, in the case of IM proteins, the detergent must allow interactions between protein molecules to form stable geometric arrangements. Unlike inorganic molecules, the robustness and flexibility of proteins hinder its ability to organize in geometric lattices. Moreover, many proteins are composed of multiple subunits or form oligomeric complexes, each partition having its own physical properties and sometimes connected by flexible regions. In order to “stiffen” protein complexes, researchers are known to truncate flexible proteins regions. The practice of doing so is controversial, however, since it can be argued that all regions affect the protein complex and therefore no regions should be omitted. This is especially true in Bax studies where truncation of the flexible $\alpha 9$ destroys the oligomerization capabilities of Bax (Ivashyna, 2009).

Due to the tremendous variables scientists must account for, many techniques to obtain a protein crystal are extremely time consuming and are determined empirically. During typical crystallography trials, a crystallographer endures a repeated series of testing conditions and observing results. The formation of any potential “hits” may take weeks to months and even after, rounds of optimization trials may be just as time consuming. It is very common for protein crystallographers to process tens of thousands conditions to obtain a protein crystal.

Also, the appearance of crystals do not equate to success. There is an unfortunate possibility that the crystallographer has used conditions that favor the crystallization of salts in the solution and may only realize it when no diffraction pattern is obtained. Another false positive may come in the form of a protein crystal that cannot diffract x-rays. There are techniques to defend crystallographers from these devastating results such as protein crystal dyes and crystal shatters tests, though the appearance of a diffraction pattern when placed in an x-ray beam is the absolute confirming test that the sample is in fact protein.

Physical factors

Temperature
Methodology
Time

Pressure
Gravity, convection and sedimentation
Vibration and sound
Magnetic fields
Electric fields
Dielectric properties
Viscosity
Equilibration rate
Nucleants
Volume
Particulate/amorphous material
Surface of crystallization device
Sample handling

Chemical factors

Precipitant type
Precipitant concentration
pH and buffer

Ionic strength
Reducing/oxidizing environment
Sample concentration
Metal ions
Detergents
Small molecule impurities
Polyions
Crosslinkers
Heavy metals
Reagent source
Reagent purity
Reagent formulation

Biochemical factors

Sample purity
Macromolecular impurities
Aggregation
Posttranslational modifications

Sample source
Sample storage
Proteolysis
Chemical modifications
Sequence modifications
Sample symmetry
Sample pI
Sample history
Ligands, co-factors, inhibitors
Microbial contamination
Purification methods

Table 1: Major variables affecting protein crystallization (Leunissen, 2001).

CHAPTER 2

METHODOLOGY

2.1 Mystic-fused Bcl-2 family Constructs

Previous to this thesis, no study of the effectiveness of Mystic towards Bcl-2 protein expression had been conducted. Since many of the Bcl-2 family members are speculated to integrate into the mitochondrial membrane, a library of Mystic-fused Bcl-2 family proteins was created to test for Mystic compatibility with Bcl-2 family members for high yield protein expression. The library consisted of an array of full-length Bcl-2 family proteins cloned, using the recombination “LR reaction” of the Gateway Pro Kit (Invitrogen, Carlsbad, CA) to the 3’ of a Gateway adapted pMis vector.

To form the construct library, 1 μL of entry pMis vectors were incubated with 1 μL of one of the target Bcl-2 destination vectors and 2 μL of TE buffer, pH 8.0. Then, 1 μL of the provided “LR Clonase II Enzyme Mix” was added to the mix and allowed to incubate at 25°C. Subsequently, 1 μL of the provided Proteinase K solution was added and allowed to incubate for 10 minutes at 37°C. Transformation was executed and described in section 2.3 “Plasmid Transformation” using Novablue Competent Cells.

To amplify that the recombination reaction, four individual colonies were cultured in 10 mL of Terrific Broth (Novagen, Gibbstown, NJ) at 37°C in a 14 mL Polystyrene Round-Bottom Tube (Becton Dickinson Labware, Franklin Lakes, NJ) overnight. A GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Saint Louis, MO) was utilized to purify the plasmid from each of the four cultures.

Cells were pelleted by in the 14 mL Polystyrene Round-Bottom Tube at 3000 rpm using a Allegra X-15R Centrifuge (Beckman Coulter, Brea, CA). Remaining media was

decanted and the pellet was resuspended using 200 μ L of the provided “Resuspension Solution”, vortexed vigourously and transferred into a 2.5 mL centrifuge tube. Cells were lysed using the 200 μ L of the provided “Lysis Solution” and the contents were inverted 5 times. Cell debris was precipitated by adding 350 μ L of the “Neutralization/Binding Solution” and the contents were inverted 5 times and centrifuged using the Centrifuge 5424 (Eppendorf, Hauppauge, NY) at 12,000 rpm for 10 minutes. A provided “Miniprep Binding Column” was prepared by adding 500 μ L of the “Column Preparation Solution” and centrifuging at 12,000 rpm two times in 30 second intervals before decanting the run-through. The lysate was loaded into the prepared binding column and centrifuged at 12,000 rpm for 30 seconds. The column was transferred to a new 2.5 mL centrifuge tube and 50 μ L of water was added and centrifuged at 12,000 rpm for 1 minute. Plasmid sequences were verified by sending them to Nevada Genomics Center for analysis.

The pMis vector is an IPTG inducible system, possessing a T7 promoter and an ampicillin resistance gene. Expression of the Mystic-fused Bcl-2 family protein produced an amino acid chain consisting of an octahistidine tag, followed by Mystic, the linker, a thrombin cleavage site, the Bcl-2 family member target protein, and finally a “Strep-Tag II” (GE Healthcare, Piscataway, NJ). The linker is a variable length amino acid chain meant to optimize the positioning of the target protein in relation to the membrane. They are notated small (7 residues), medium (14 residues), or large (21 residues) and can have an orientation reversing transmembrane helix. Constructs

displayed differing expression behavior however, due to the success in protein expression and significance that Bax has in the MOMP pathway; we focused our research attempts on the characterization of Bax.

A



B

MK[HHHHHHHH]¹GG[LVPRGS]²HG[MKVTSEEKEQLSTAIDRMNEGLDVFIQFY
 NESEIDEPLIQLEDDTADLMKQARDLYGQEKLNEKLNNTIIKQILSISLSGEGEKE]³[
TSDYKDDDDK[LVPRGS]²[TSLGGSKKAGGT]⁷]⁴[MDGSGEQPRGGGPTSSEQIMK
 TGALLLQGFIQDRAGRMGGEAPELALDPVPQDASTKKLSECLKRIGDELDSNME
 LQRMIAAVDTDSPREVFVRVAADMFSDFGNFNWGRVVALFYFASKLVLKALCTK
 VP ELIRTIMGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGVLT
 ASLTIWKKMG]⁵N[PAFLYKVV]⁷DDPNSSS[WSHPQFEK]⁶

Figure 6: A. Model of MysticBcl-2 construct. B. Sequence of Bax with long-linker. [1]

Histidine-tag [2] Thrombin Cleavage Site [3] Mystic [4] Large Linker (underlined) [5]

Bax [6] Strep-Tag II [7] Residual recombination artifact

Once the protein expression yield for the initial MysticBax with a long linker construct reached a plateau (~ 2.5 mgs/ per 8 liters), we opted to send our MysticBax sequence to DNA 2.0 (Menlo Park, CA) to be synthesized into bacterial expression optimized vectors in attempts to increase our protein yield. In short, DNA 2.0 assembles

short single-stranded oligonucleotides to form the MysticBax sequence. This synthesized sequence is subsequently cloned into a pJexpress411 vector. Major changes in the synthesized plasmids in comparison to the Gateway-adapted pMis are the removal of one thrombin site within the Bax gene, and the replacement of the Ampicillin resistance gene to a Kanamycin resistance.

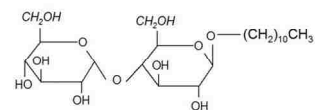
MK[HHHHHHHH]¹GGSGGSGSHG[MKVTSEEKEQLSTAIDRMNEGLDVFIQFYNE
SEIDEPLIQLEDDTADLMKQARDLYGQEKLNEKLNNTIHKQILSISLSGEGEKE]²[TS
DYKDDDDK[LVPRGS]⁶[TSLGGSKKAGGT]⁷]³[MDGSGEQPRGGGPTSSEQIMKTG
ALLQGFIQDRAGRMGGEAPELALDPVPQDASTKKLSECLKRIGDELDSNMELQ
RMIAAVDTSPREVFFRVAADMFSNGNFWGRVVALFYFASKLVLKALCTKVP
ELIRTIMGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGVLTAS
LTIWKKMG]⁴N[PAFLYKVV]⁷DDPNSSS[WSHPQFEK]⁵

Figure 7: Sequence of Synthesized MysticBax sequence. [1] Histidine-tag [2] Mystic [3] Large Linker (underlined)[4] Bax [5] Strep-Tag II [6] Thrombin cleavage[7] Residual recombination artifact

2.2 Detergent Screen

5 detergents were selected based on prior successes of other membrane protein studies and the detergent LAPAO was selected due to its possession of a zwitterionic head group and structural similarity to LDAO.

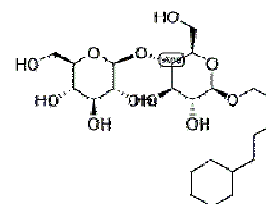
The screening process to determine a compatible detergent was implemented at the 2nd Nickel Bead wash described below in section 2.5 “Protein Extraction and Purification” until the SEC run. The effectiveness of the detergent was determined by the presence of a single UV absorption peak during the SEC run.



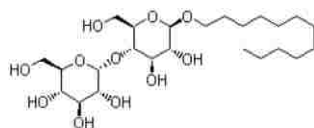
n-Undecyl-B-D-Maltopyranoside (UDM)



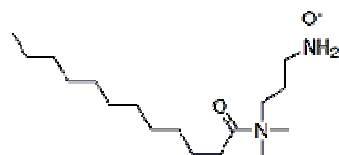
n-Dodecyl-n,n-Dimethylamine-n-Oxide (LDAO)



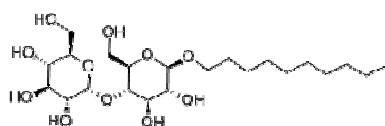
Cymal-5



n-Dodecyl-B-D-Maltopyranoside (DDM)



N-[3-(dimethylamino)propyl]dodecanamide N-oxide (LAPAO)



n-Decyl-B-D-Maltopyranoside (DM)

Figure 8: Detergent Structures (ChemBook, 2008)

2.3 Plasmid Transformation

One μL of the MisticBcl-2 vector was incubated with two aliquots of 20 μL competent cells BL21 (Novagen, San Diego, CA) ,or NovaBlue competent cells when verifying the sequence, each in a 2.5 mL centrifuge tube on ice for 30 minutes. The incubated competent cells were heat-shocked at 42°C water bath for 30 seconds then placed on ice for 1 minute. A 90 μL aliquot of the provided SOC media was added to each centrifuge tube and then incubated at 37°C on an ThermoMixer R (Eppendorf, Hauppauge, NY) for 1 hour. While the 37°C incubation is taking place, two Kanamycin LB agar plates (Teknova, Hollister, CA) are pre-warmed in the 37°C plate incubator. The entire contents of each culture are then transferred to 2 agar plates and spread until absorbed into the agar. The plates were incubated upside-down at 37°C overnight.

2.4 Culture and Protein Induction

Transformed cells containing the inducible vector were scraped off using a single edge industrial razor blade (VWR, West Chester, PA) and transferred into a 50 mL Polypropylene Conical Tube (Becton Dickinson Labware, Franklin Lakes, NJ). The 50 mL tube was then filled with 33 mL of Terrific Broth, vigorously vortexed, and incubated in a 37°C water bath for 10 minutes.

A 4 mL aliquot of the culture was transferred into 8, 2800 mL Erlenmeyer flasks containing 1 liter of terrific broth with 50 $\mu\text{g}/\text{mL}$ Kanamycin. The flasks were incubated at 37°C and shaken at 260 rpm.

Cultures were induced with 0.1 mM isopropyl-B-D-Thiogalactopyranoside (EMD Chemicals, Gibbstown, NJ) when the optical density at 595nm reached approximately

0.60 to 1.00 measured with a spectrophotometer (Fisher Scientific, Hampton, NH).

Once isopropyl-B-D-thiogalactopyranoside was added, the 8 liter cultures were incubated at 18°C for 4 hours.

2.5 Protein Extraction and Purification

Cultures were transferred from the 2800 mL Erlenmeyer flasks into 1 liter spin bottles designed for the RC-3B Refrigerated centrifuge with H6000 rotor and buckets (Block Scientific, Bohemia, NY). The cultures were centrifuged for 10 minutes at 3000 rpm. Media was decanted and the remaining pellets were scraped off and evenly distributed into 4, 50 mL polypropylene conical tubes. Sonication buffer composed of 300 mM Potassium chloride, 50 mM Trizma Base pH 8, 20 mM Imidazole, and 10% glycerol was added to the polypropylene conical tubes until a total volume of approximately 45 mL was reached and then the contents were vigorously vortexed and placed on ice. The addition of 0.5 mM phenylmethylsulfonyl fluoride and 2 mM benzamide protease inhibitors were required to protect our target protein from any detrimental protease activity.

From this point on, the cultures were constantly on ice. Cultures were lysed using a probe Sonicator 3000 (Misonix Inc, Farmingdale, NY) at maximum power for a burst of 5 seconds and subsequent cooldown for 5 seconds for total of 1 minute of sonication (2 minutes total time).

The cell lysates were evenly transferred into 26.3 mL polycarbonate centrifuge tubes (Beckman Instruments Inc. Palo Alto, CA) and placed in a Type Ti-70 Rotor constructed for the Optima L-80 XP Ultracentrifuge (Beckman Coulter, Brea, CA). The lysates were centrifuged at 45,000 rpm for approximately an hour.

The supernatant was decanted. Care was taken not to decant the loose membrane near the pellet. The remaining pellet and membrane were transferred to 50 mL polycarbonate conical tubes. LDAO sonication buffer containing 300 mM Potassium chloride, 50 mM Trizma Base (Sigma-Aldrich, Saint Louis, MO) pH 8, 20 mM Imidazole, 10% glycerol and 20 mM N-N-dimethyldodecylamine-N-oxide (Affymetrix, Santa Clara, CA) was added to each tube until a total volume of 45 mL was reached. Pellets were solubilized by sonication in 5 second bursts with subsequent 5 second cooldown for a total of 1 minute of total sonication (2 minutes total time). The solubilized pellets were transferred into the same 26.3 mL polycarbonate centrifuge tubes, and placed in the Type Ti-70 Rotor. Solubilized pellets were centrifuged for 45,000 rpm for 30 minutes.

The supernatant containing the detergent solubilized membrane was equally decanted into 4, 50 mL conical tubes and 1 mL of His-Select Nickel Affinity Gel (Sigma-Aldrich, Saint Louis, MO) was added to each of the 4 tubes and placed on a plate shaker at very low speeds for 30 minutes at 4°C.

All tube contents were passed through 1 polypropylene chromatography column, allowing the excess buffer to pass and the nickel beads containing bound protein to remain. Nickel beads were washed with 10 mL of LDAO Sonication Buffer [300 mM Potassium chloride, 50 mM Trizma Base pH 8, 20 mM Imidazole, 10% glycerol and 20 mM N-N-dimethyldodecylamine-N-oxide] three times. On the 2nd wash, 10 mL of Sonication Buffer containing one of 6 detergents in the screen (300 mM Potassium chloride, 50 mM Trizma Base pH 8, 20 mM Imidazole, 10% glycerol and 20 mM of detergent from screen (Affymetrix, Santa Clara, CA) was transitioned when 2 mL of

LDAO wash buffer remained in the column. This was followed by 3 more 10 mL detergent screen buffer washes.

Elution of the protein was conducted using 9 mL of elution buffer [300 mM Potassium chloride, 50 mM Trizma Base pH 8, 20 mM Imidazole, 10% glycerol and 20 mM of detergent in screening, 880 mM Imidazole]. The entire elution buffer was added to the column and then collected. 1 mM tris(2-carboxyethyl)phosphine hydrochloride was added to the elution to prevent oxidative damage (Sigma-Aldrich, Saint Louis, MO).

2.6 Size-Exclusion Chromatography and Multi-Angle Light Scattering Analysis

The gel column, Superdex 200 prep grade (GE Healthcare, Piscataway, NJ) was equilibrated with reaction buffer (300 mM Potassium chloride, Trizma Base pH 8, 1 mM tris(2-carboxyethyl)phosphine hydrochloride, and 3 mM of detergent in screening).

The 9 mL eluate from the extraction purification protocol was concentrated down to 2 mL using an Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA). Protein preparation was loaded into the reaction buffer equilibrated Superdex 200 prep grade column and eluted at a rate of 1 mL/minute and fractions were collected in 2 mL intervals with a simultaneous MALS analysis using the minDAWN™ TREOS scanning detector (Wyatt Technology, Santa Barbara, CA).

Two fractions, or 4 mL, at the sharpest absorbance peak were collected and 20 μ L was used to determine protein concentration using Protein Assay Dye (Bio-Rad, Hercules, CA) and 595 nm absorbance was measured with a spectrophotometer (Fisher Scientific, Hampton, NH) to calculate protein concentration by the Bradford method (Bradford, 1976).

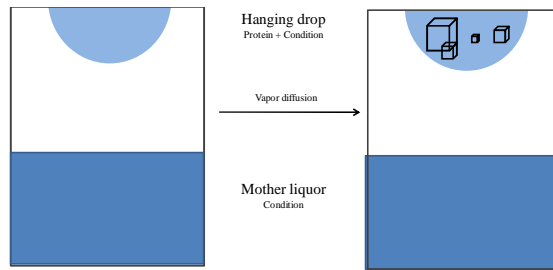
$$\text{Protein in milligrams} = \frac{\text{absorbance at } 595\text{nm} \times 30}{\text{volume of protein in microliters} \times \text{milliliters taken from fractions}}$$

2.8 Protein Dialysis

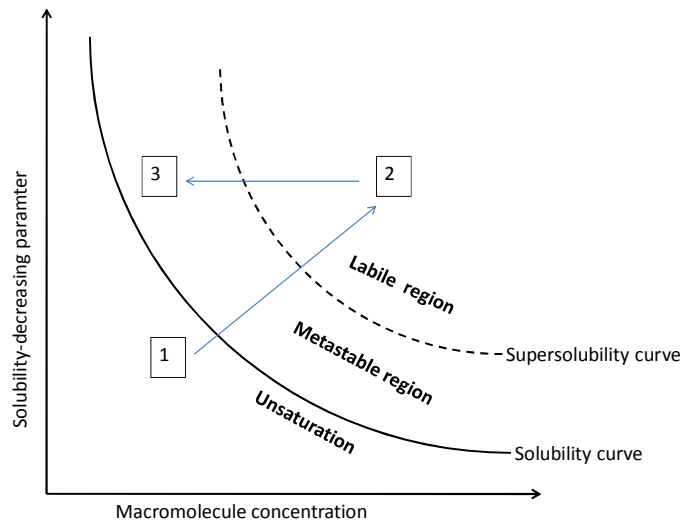
Protein fractions were collected and concentrated in an Amicon Ultra-15 Centrifugal Filter Device to 5 to 10 mg/mL. The protein sample was then transferred into a pre-rinsed dialysis membrane with a molecular weight cut off of 50,000 (Spectrum Labs, Greensboro, NC) to allow excess detergent micelles to diffuse out. The membrane containing protein was dialyzed in 500 mL of dialysis buffer [10 mM Trizma Base pH 8, 1 mM tris(2-carboxyethyl)phosphine hydrochloride, 3 mM N,N-Dimethylauramidopropylamine N-oxide] at 4°C overnight.

2.9 Protein Crystal Screening and Optimization

Initial crystal condition screening was performed using the JCSG+ Suite (Qiagen, Valencia, CA) using the hanging drop method of vapor diffusion. The hanging drop diffusion method involves a silicone cover slide containing 2 μ L of the protein sample mixed with 2 μ L of a testing condition suspended over the “mother liquor” or 1 mL of the testing condition. Through vapor equilibration of the unsaturated droplet and the mother liquor reservoir, the protein solution transitions to super saturation with the ultimate goal of producing protein crystals.



A



B

Figure 9: A. Hanging drop method for protein crystal production. B. Solubility versus protein. 1. Initial protein concentration in the hanging drop is in an unsaturated, soluble state. 2. Through vapor-diffusion, solubility of the protein sample decreases allowing the hanging drop to transition into the labile region where stable nuclei spontaneously form and grow. 3. After equilibration, the hanging drop may go into the metastable region where stable nuclei continue to grow but do not initiate (Adapted from Leunissen, 2001).

2.10 SDS-Page Analysis

SDS-PAGE gels were custom made. The 5 mL resolving gel was composed of 10% acrylamide (EMD Chemicals, Gibbstown, NJ), 1.3 mL of 1.5M Trizma Base pH 8.8, 10% Sodium dodecyl sulfate (Sigma-Aldrich, Saint Louis, MO), 10% Ammonium persulfate (Sigma-Aldrich, Saint Louis, MO) . Polymerization was induced by 1 μ L of 2, 3-dimethyl-2-butene (Sigma-Aldrich, Saint Louis, MO).

The 2 mL stacking gel was composed of 5% acrylamide (EMD Chemicals, Gibbstown, NJ), 1.5 mL of 1.5M Trizma Base pH 6.8, 10% Sodium dodecyl sulfate (Sigma-Aldrich, Saint Louis, MO), 10% Ammonium persulfate (Sigma-Aldrich, Saint Louis, MO). Polymerization was induced by 1 μ L of 2, 3-dimethyl-2-butene (Sigma-Aldrich, Saint Louis, MO). Protein was visualized using Coomassie blue staining.

2.11 X-ray Diffraction Analysis

Successful protein crystals were sent to Stanford Synchrotron Radiation Lightsource for x-ray analysis.

CHAPTER 3

ANALYSIS AND CRYSTALLIZATION OF A BAX MULTIMER

3.1 MisticBcl-2 Family Members Expression Behavior and Protein Expression Requires a Long Linker

An array of Bcl-2 family constructs was expressed to test their ability to cooperate with a Mistic leader. Criteria for success were graded by the intensity and homogeneity of the gel band, qualitatively characterized by SDS-PAGE.

The preliminary expression results show that Bax with a long linker was a successful candidate for further study. The significance of Bax in MOMP as well as its uncharacterized oligomeric state prompted us to focus our crystallization studies solely on Bax.

Optimization of the linker length is vital in the expression of Bax signifying that conformation in the cell membrane is essential. The target protein being trailed by Mistic requires correct positioning in relation to the membrane in order to properly fold. In theory, if the construct lacks a linker or the linker is too short the Bax protein may be in close proximity to the membrane but may never embed. On the other hand, a linker excessive in length is prone to proteolytic degradation where Bax may be cleaved before ever reaching the membrane.

We facilitated the use of linker sequences between Mistic protein and the target Bax protein to obtain this membrane bound complex. Linker constructs (small, medium, and large) were constructed and expressed with LDAO as the stabilizing detergent which is further detailed in section 3.2 “LDAO is Optimal for Membrane Extraction”. The relative yields were qualitatively measured using SDS-PAGE

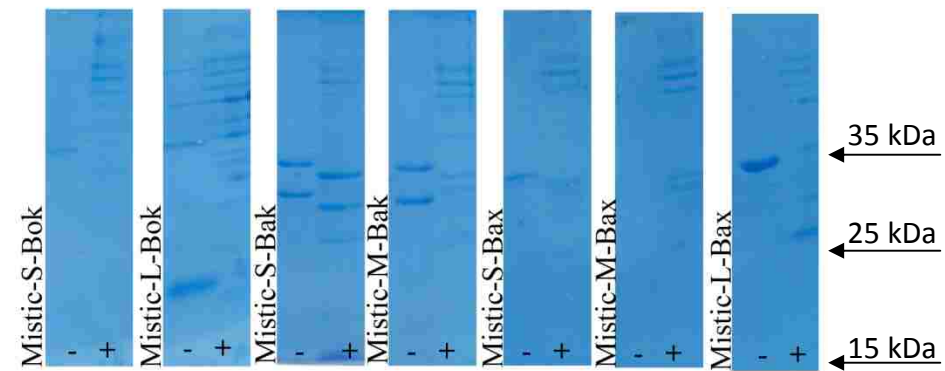
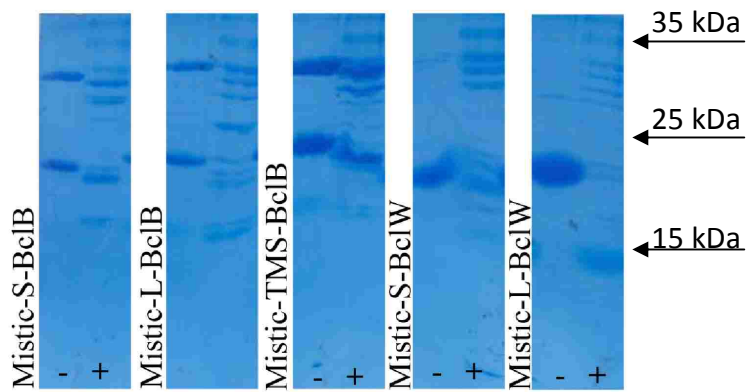
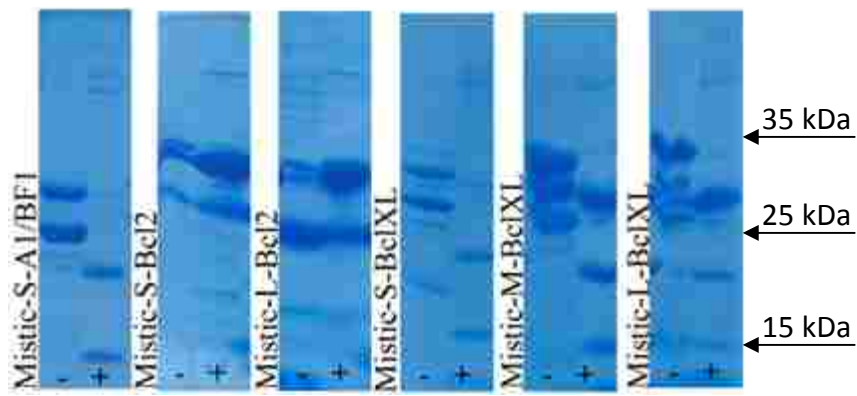


Figure 10: MisticBcl-2 family expression library with differing linker lengths. L=large linker, M=Medium linker, S= small linker TM= transmembrane helix. - = no thrombin + = thrombin cut

Standard bacterial protein expression protocols were ineffective at producing workable quantities of Bax (<1 mg per 8 liter preparation). Optimization of the protein preparation revealed significantly higher yields when membrane extraction and protein elution occur 4 hours after IPTG induction versus the standard overnight time period. It is speculated that increasing aggregation of Bax at the bacterial membrane is toxic, leading to lower protein yields at time points exceeding 4 hours.

BaxMistic with a long linker (MisticBaxL) is the most efficient construct for expression. Higher average yields of MisticBaxL (2.5 mgs to 4 mgs) was obtained with by sending the sequence for synthesis (DNA 2.0) that are optimized for bacterial expression. The decision to stop utilizing thrombin to remove Mistic was made due to the incomplete or partial cutting due an unforeseen inhibition of thrombin when utilizing the detergent LAPAO. Also, the C-terminus Strep-Tag II was not removed. Constructs with a stop codon immediately after the Bax sequence interestingly destabilized Bax significantly.

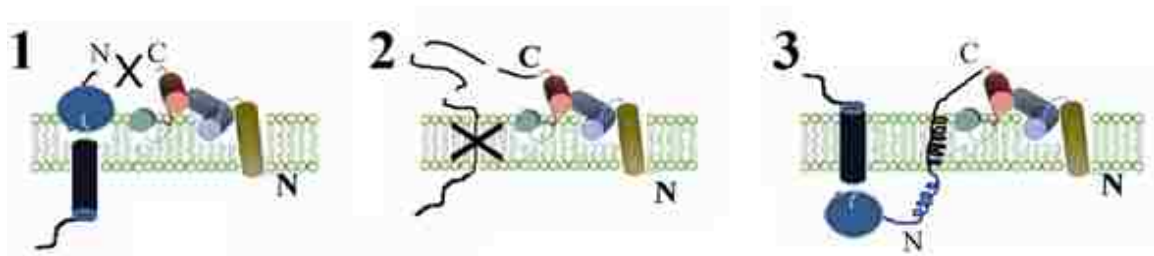


Figure 11: The linker length may affect protein expression. 1. A linker too short may cause the target protein to preclude the cellular membrane. 2. A linker too long is prone to proteolytic degradation. 3. The use of a transmembrane helix may be necessary for correct orientation of the trailing protein to embed into the membrane.

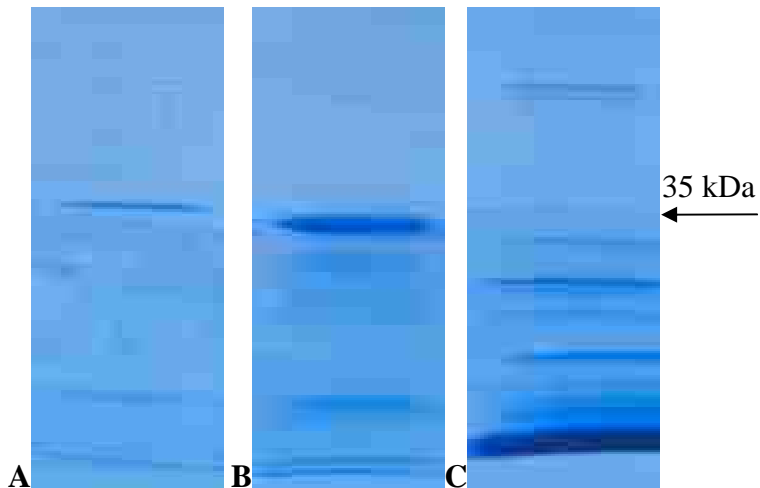
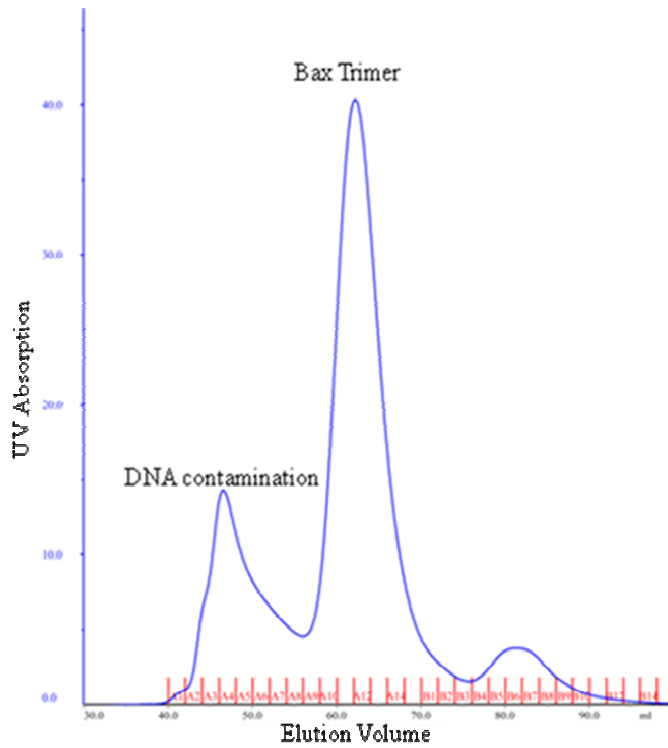


Figure 12: SDS PAGE of MysticBax Constructs. A. Original MysticBaxL B. Synthesized MysticBaxL C. Synthesized MysticBaxL without Strep-Tag II

3.2 LDAO is Optimal for Membrane Extraction

Six detergents were tested to optimize expression yield and Bax complex stability. Protein samples were prepared as described in the methods section and the size exclusion Superdex 200PG column was equilibrated with the reaction buffer containing the same detergent. Protein yield and stability was best attained when LDAO was initially used for membrane extraction and then the detergent was switched to LAPAO starting at the 2nd Ni+ column wash. In an interesting note, what appears to be a higher-order form of Bax actually is an aggregation of nucleic acids. This DNA peak can be maximized when the entire protein preparation is executed at 4°C with or without the attachment of Mystic. The cause of the nucleic acid contamination is unknown.

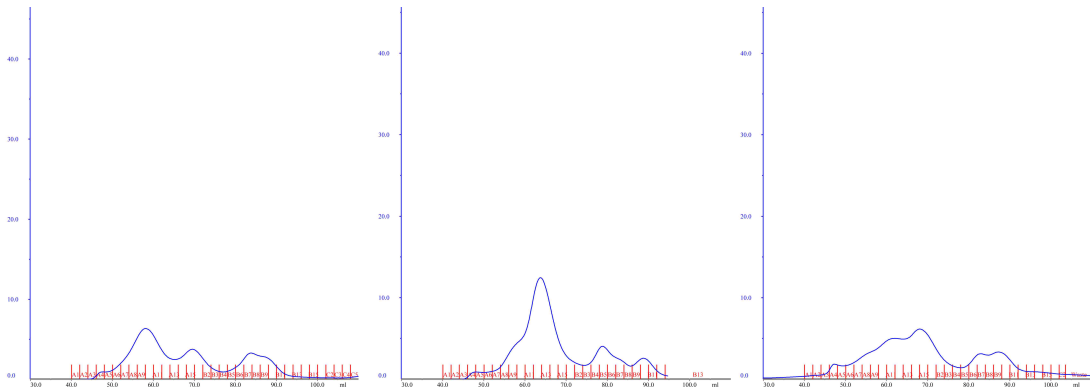
A



B



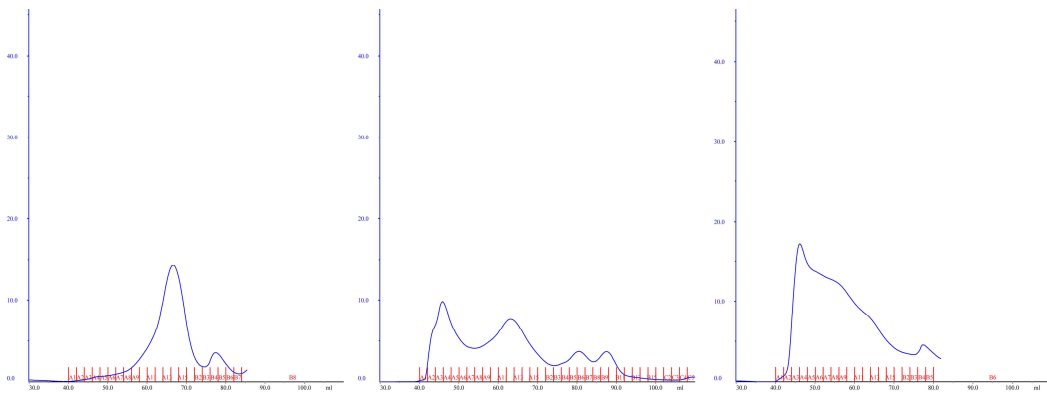
Figure 13: A. LDAO extraction produces homogenous Bax. A sharp oligomeric Bax peak is obtained in SEC analysis when membrane extraction is conducted with LDAO as the detergent and LAPAO as the solubilizing detergent. B. DNA contamination. The peak eluting before oligomeric Bax is a large aggregation of nucleic acids as demonstrated by DNA gel electrophoresis.



ME: LDAO SD: Cymal-5

ME: LDAO SD: DDM

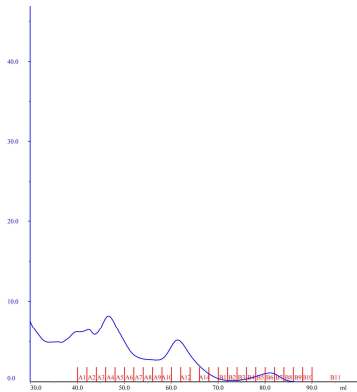
ME: LDAO SD: DM



ME: LDAO SD: LDAO

ME: LDAO SD: UDM

ME: LDAO SD: No Detergent



ME: LAPAO SD: LAPAO

Figure 14: Detergents affect stability of protein sample. ME= Detergent used during membrane extraction SD= Detergent used to solubilize protein. X-axis = elution volume, Y-axis =UV absorbance (mAU).

3.3 Bax not affected by Octahistidine-tag or Mystic

The decision to stop using thrombin to cleave the octahistidine tag and Mystic with linker was made due to the difficulty in cleaving the thrombin site. Even at high concentration of thrombin (100 units), cleavage was incomplete. SEC analysis shows that even with octahistidine tag and Mystic with linker still attached, Bax remained in oligomeric conformation.

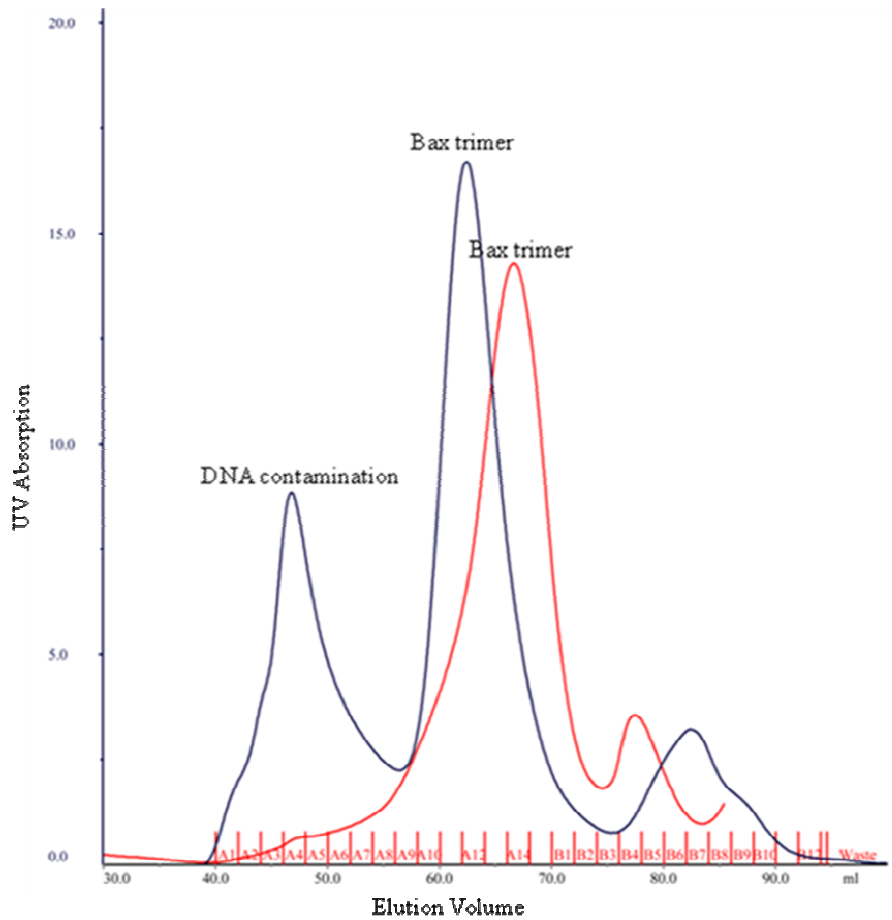


Figure 15: Bax oligomerization not affected by octahistidine tag or Mystic with linker.

Black = MysticBax. Red = Bax

3.4 Bax Oligomers Form Trimers

A SEC-MALS measurement was taken under a Bax preparation with DDM as the solubilizing detergent. As shown in figure 14, the over-estimation of SEC to the molecular weight of protein/detergent complex run is apparent with the molecular mass reading at ~170 kDa. In contrast, the multi-angle light scattering measurement of protein concentration shows that the protein/detergent complex is composed of almost equal parts protein and detergent at 75 kDa for a total of 150 kDa. With Bax monomer being a ~23 kDa protein, the molecular weight of this complex allows for three Bax monomers.

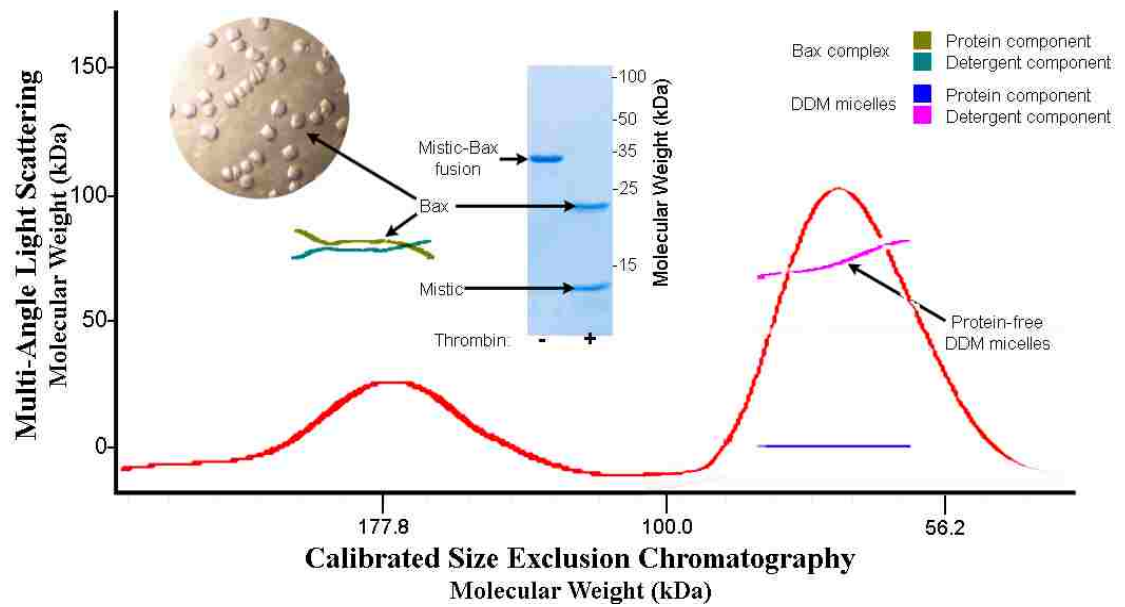


Figure 16: SEC-MALS measurement showing trimeric Bax.

3.5 LAPAO is the Optimal Detergent for Crystal Trials and Produces X-ray diffracting Crystals

Crystal trials commenced using LDAO as membrane solubilizers were unsuccessful. None of the 96 crystal trials in the JCSG+ suite produced any potential hits despite the relatively high yield and stable protein conformation. This prompted us to remain using LDAO for membrane extraction while screening for another detergent capable of maintaining Bax in a trimeric conformation while promoting crystal growth.

The first potential candidate for Bax solubilization was UDM. Bax solubilized in UDM met the criteria for trimer stabilization in accordance to SEC and under hanging drop well conditions of 0.2M Magnesium chloride, 0.1M HEPES pH 7.5, and 30% v/v PEG 400 produced large crystals (approximately 20 x 15 x 10 μ M).

Unfortunately, physical handling of the crystal proved extremely difficult and can be described as touching an “ordered gel”. Pursuit of a UDM-Bax crystal was finally halted when the protein crystal did not diffract X-rays.

We were prompted to change protein detergents due to repeated formations of the “ordered gel” morphology. The change to LAPAO as a protein solubilizer produced tiny “needles” in multiple +JCSG Suite conditions. Subsequently, these conditions were pursued and optimized.

One condition under 0.20M Potassium formate and 20% PEG3350 at 25°C produced sharp rod crystals (5 x 15 x 5 μ M). Use of the same protein preparation under an optimizing trial produced reproducible crystals. The high percentage PEG3350 served as a cryoprotectant and the crystals were shipped to Stanford Synchrotron Radiation Lightsource for x-ray diffraction analysis.

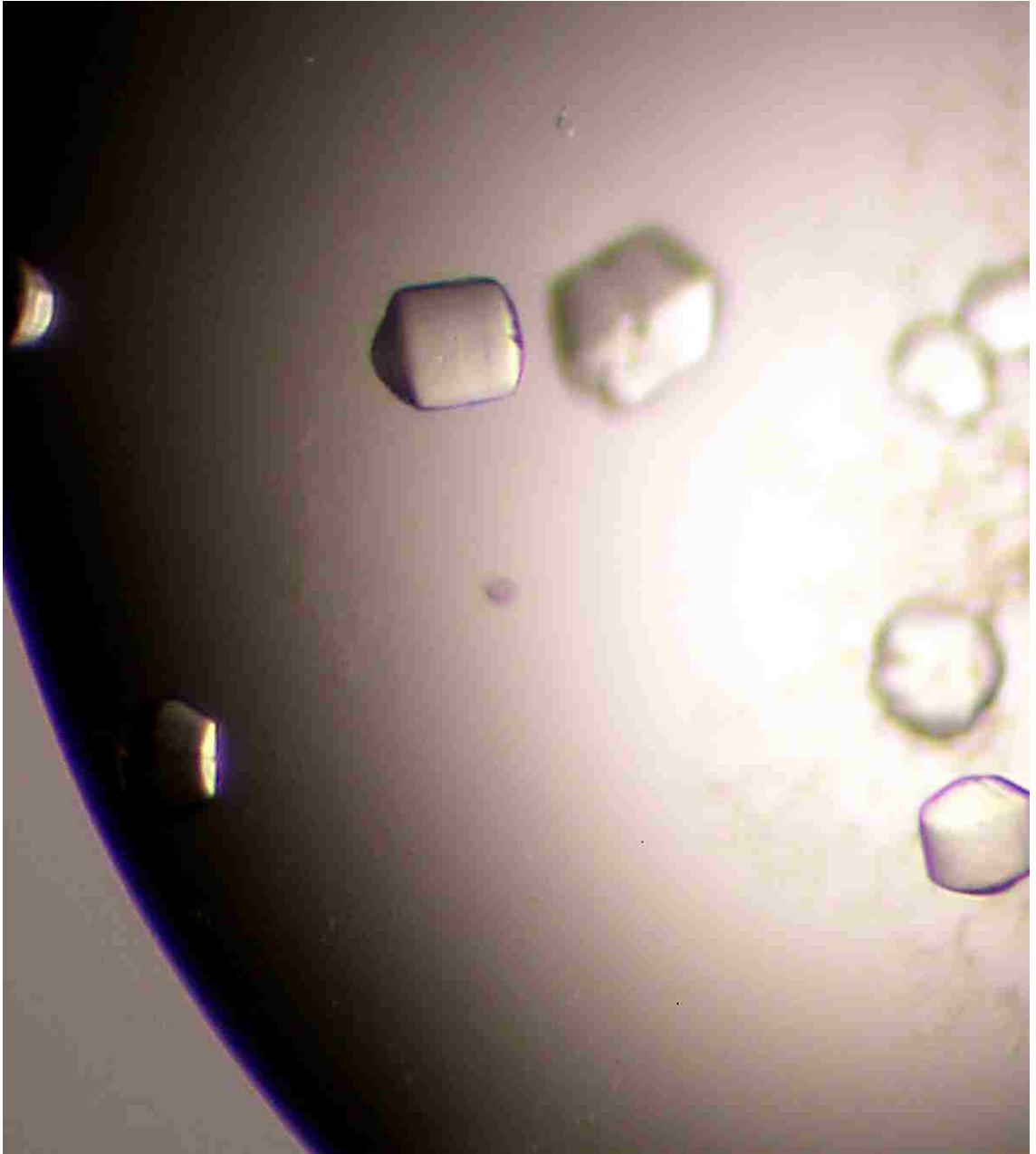


Figure 17: Bax crystals produced with UDM. Condition: 0.1M HEPES pH 7.5, 30% v/v PEG 400, 0.2M Magnesium chloride at 25°C.

Unfortunately, conditions were unique to this protein preparation because crystals independent of this preparation could not be obtained. After numerous attempts, we were forced to rescreen using the JCSG+ suite. Luckily, more screening hits appeared when trials were incubated at 4°C. We have recently obtained needle-like crystals in 20% Glycerol, 30% PEG 1000, and 0.30M Yttrium (II) chloride and are currently optimizing crystallization conditions.

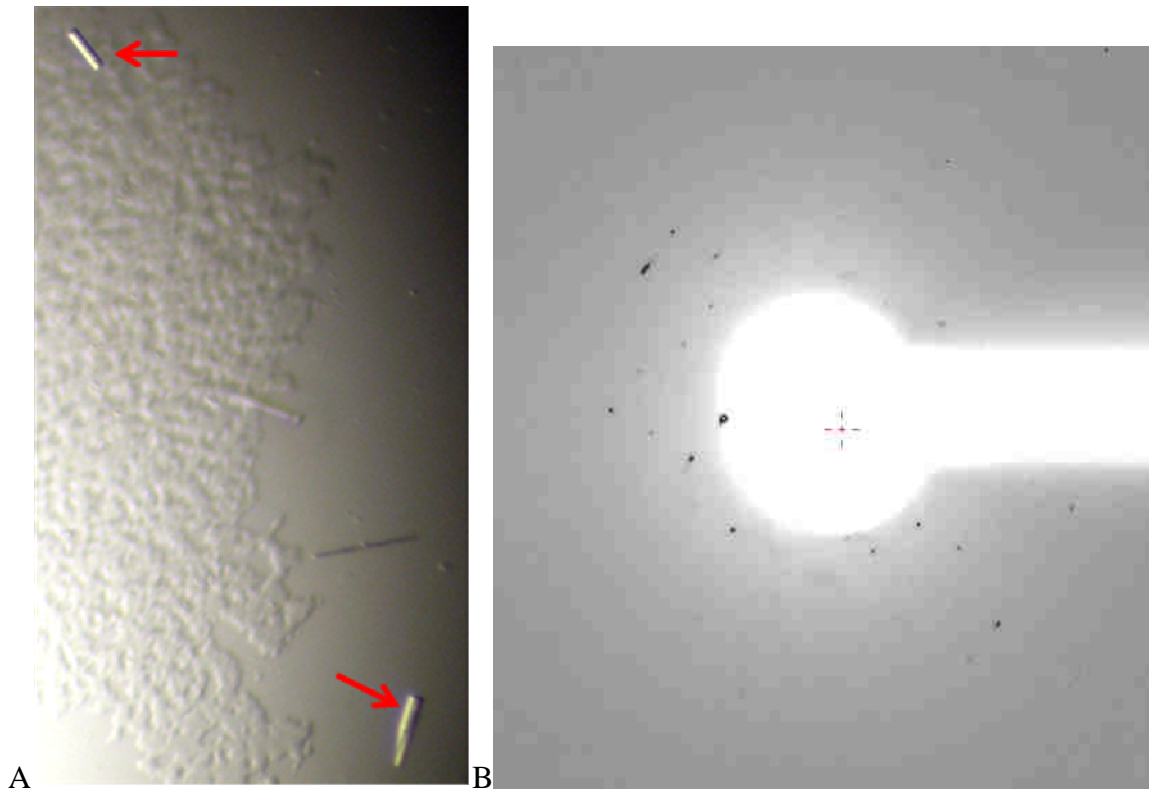


Figure 18: A. MysticBax rod crystals with LAPAO. Condition: 0.20M Potassium formate and 20% PEG3350. B. X-ray diffraction pattern.

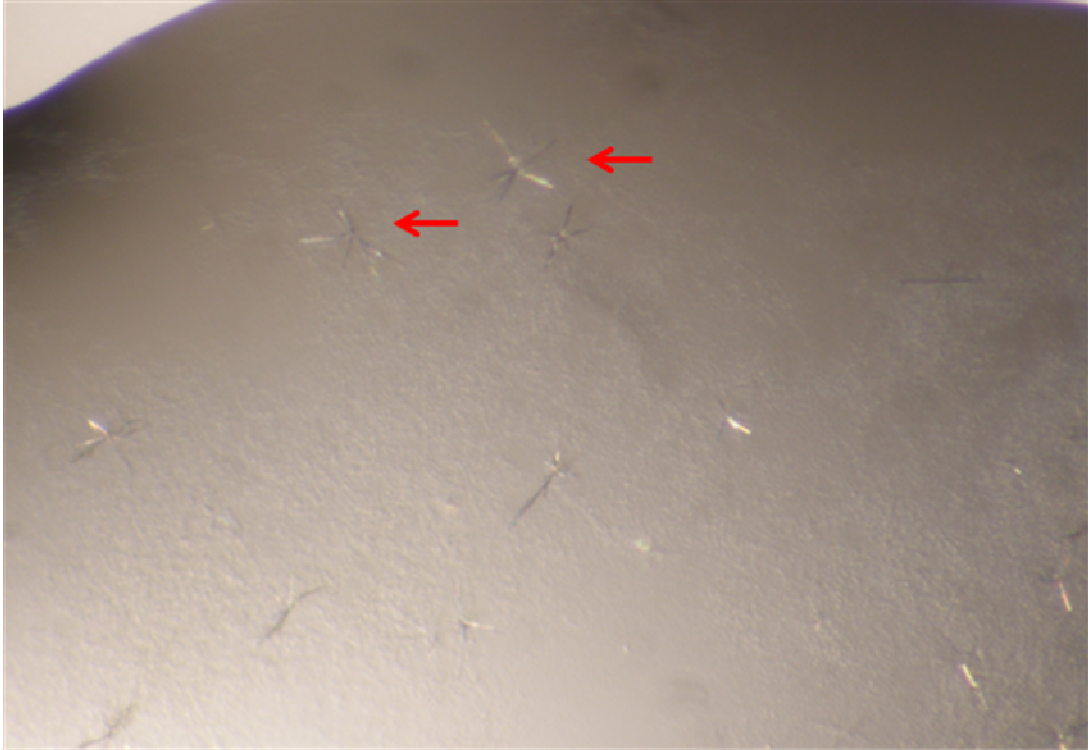


Figure 19: MysticBax needle crystals. Condition: 20% Glycerol, 30% PEG 1000, and 0.30M Yttrium (II) chloride.

CHAPTER 4

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

A recent study using a fluorescent Bax mutant lacking $\alpha 9$ for fluorescence correlation spectroscopy and fluorescent-intensity distribution analysis has shown that soluble monomeric Bax remains monomeric even after integrating into the detergent micelles (Ivashyna et al., 2009). In our research with full-length Bax, molecular weight contribution of detergent is approximately equal to protein contribution according to MALS. This is in direct contradiction to those previous findings of monomeric Bax embedded within a large detergent micelle. Our studies cannot support the formation of activated monomeric Bax embedded in a large detergent micelles and concretely reinforces the importance of $\alpha 9$ and in turn, full-length Bax when studying homo-oligomerization of Bax proteins.

The details of the multistep process of Bax activation leading to pore formation are highly disputed, but we are slowly chipping away at the details. Until recently, how Bax activation, suppression, and oligomerization occurred with only one known protein assembly surface caused substantial confusion of the regulation mechanism of Bax. Some clarity was obtained after the discovery of a novel interaction site opposite of the BH3 hydrophobic domain, capable of binding a stabilized α -helix of Bcl-2 (SAHB) domains of the BH3-only protein Bim. This study showed that conformational change induced from an interaction site opposite of the BH3 hydrophobic domain was capable of dislodging the $\alpha 9$ helix and potentially allowing the oligomerization of other Bax proteins (Gavathiotis et al., 2008). The study also suggests there must be at least one more

unidentified surface in addition to the BH3-hydrophobic domain and novel SAHB site on Bax that mediate homo-oligomerization. What is glaringly missing from the current models of high order Bax oligomerization is the presence of a cell membrane in the conformational change of Bax oligomers. Our formation of a “never before seen” Bax trimer is evidence that membrane induced conformational change exists.

In the presence of a membrane, detergents produced stable trimers, even when using some of the same detergents, such as LDAO and DDM, used in “membraneless” dimer studies. The importance of a membrane is further reinforced with studies able to form functional Bax pore structures in a liposome (Bleicken et al., 2009). It is therefore essential that further research of the pore forming Bax oligomers require a cell membrane for higher-order activation.

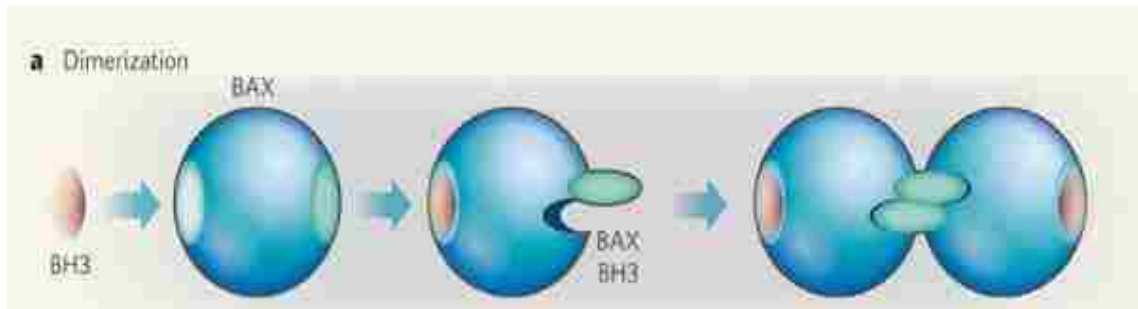


Figure 20: Mechanism of Bax dimerization. A BH3-only molecule binds to a novel site on the opposite side to the BH3 hydrophobic domain of Bax. This dislodges $\alpha 9$, exposing the BH3 hydrophobic surface for homo-oligomerization (Adapted from Gavathiotis et al., 2008)

Presuming the end product of Bax oligomer is a ring structure that allows the diffusion of cytochrome c, we theorize the dimer Bax model must be a transient structure

seen only *in vivo* during cytosol to mitochondrial membrane translocation. We propose that once Bax reaches the membrane, another membrane-induced conformational change occurs allowing for additional Bax monomers to attach from 2 sides of each Bax molecule. The initial state leads to a pre-pore trimer but binding of BH3 molecules to the complex allows for the addition of Bax subunits to the oligomeric complex. This continues until a pore large enough for cytochrome c to diffuse through is formed leading to the cell death. The proposed mechanism is outlined in Figure 21.

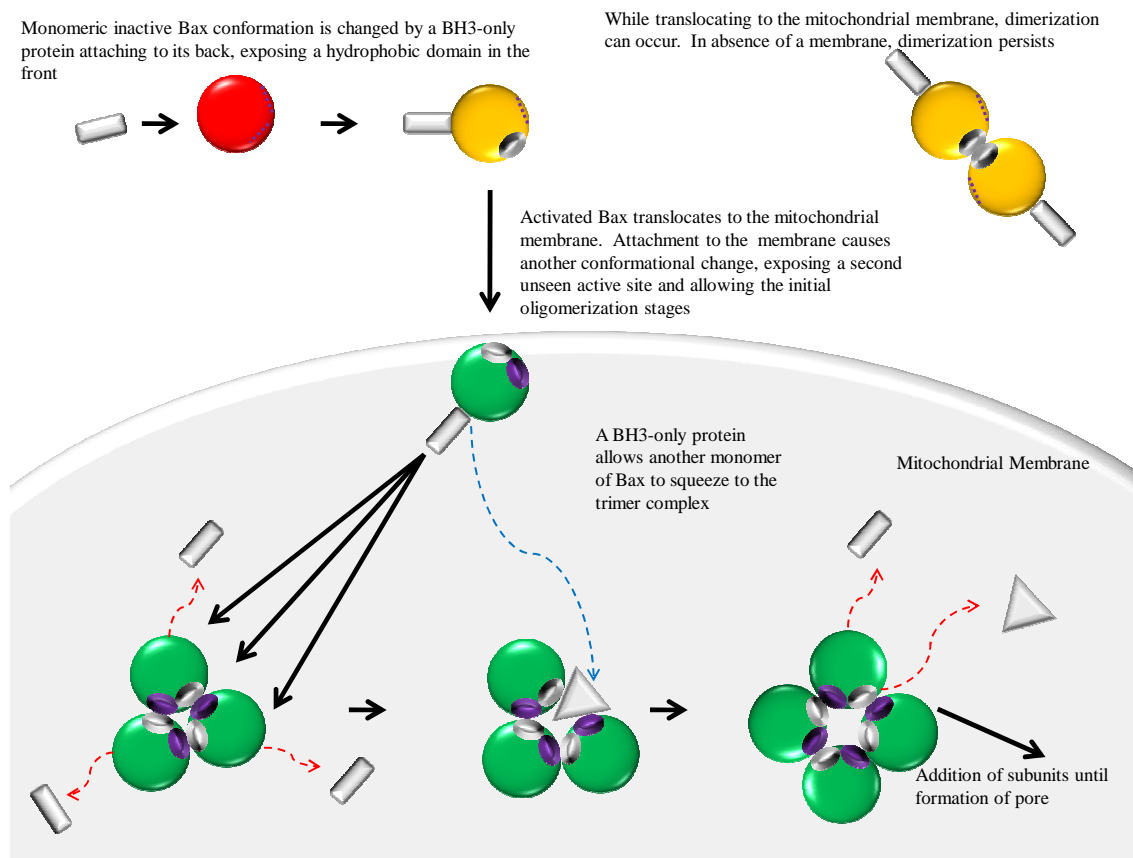


Figure 21: Proposed Bax oligomerization mechanism

In pursuit of x-ray diffracting protein crystals of Bax, we have overcome many pitfalls that plague the field of protein crystallography. Membrane protein production is an early hurdle for many IM protein studies yet, the effectiveness of Mystic allowed for the production of the first x-ray diffracting protein crystal of an oligomeric Bax. We have tested over 8,700 total crystallization condition trials. Approximately 40% were screening trials with the remaining trials optimizing “hits” of the conducted screen. Although 95% of the trials conducted were in room temperature conditions, recently, cold room trials appear to be the most promising as the small crystals are highly reproducible. With typical protein crystallography projects ranging in the tens of thousands of trials to solve a structure, we remain more than optimistic that we will obtain multiple protein crystals of Bax for a complete high resolution 3D protein structures.

APPENDIX

My experiences with success in developing protein purification protocols and executing effective protein crystallography trials has expanded to other proteins other than Bax described in this thesis. I have assisted in the production of two protein structures of the human protein uridine phosphorylase 2 (UPP2) in active conformation with bound 5-benzylacyclouridine and also the inactive conformation of UPP2 with bound 5-benzylacyclouridine. Both structures have been published in the RCSB Protein Data Bank (PDB IDs 3P0E & 3P0F) as well as a pending publication in *The Journal of Biological Chemistry* entitled “A Novel Structural Mechanism for Redox Regulation of Uridine Phosphorylase 2 Activity”.

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