Biophysical Insights into the Oligomerization of Bclxl Apoptotic Repressor

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UNIVERSITY OF MIAMI

BIOPHYSICAL INSIGHTS INTO THE OLIGOMERIZATION OF
BCLXL APOPTOTIC REPRESSOR

By

Vikas Bhat

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BIOPHYSICAL INSIGHTS INTO THE OLIGOMERIZATION OF BCLXL APOPTOTIC REPRESSOR

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The BclXL apoptotic repressor, a member of the B-cell lymphoma 2 family of proteins, plays a central role in determining the fate of cells to live or die during physiological processes such as embryonic development and tissue homeostasis.

Herein, using a wide array of biophysical methods, I investigate the molecular basis of action of BclXL.Briefly, I provide evidence that BclXL bears intrinsic propensity to oligomerize in solution. Importantly, such oligomerization of BclXL is driven by the intermolecular binding of its C-terminal transmembrane (TM) domain to the canonical hydrophobic groove in a domain-swapped trans fashion, whereby the TM domain of one monomer occupies the hydrophobic groove within the other monomer and vice versa. Of particular interest is the observation that acidic pH promotes the assembly of BclXL into a higher-order megadalton aggregate with a plume-like appearance and harboring structural features characteristic of a molten globule.

Moreover, BclXL undergoes irreversible aggregation and assembles into highly-ordered rope-like homogeneous fibrils at elevated temperatures. Remarkably, the formation of such fibrils correlates with the decay of a largely α-helical fold into a predominantly β-sheet architecture of BclXL in a manner akin to the formation of amyloid fibrils. Further interrogation reveals that while BclXL aggregates in solution display diminished affinity toward BH3 ligands, they appear to be optimally primed for insertion into cardiolipin bicelles. This salient observation strongly argues that BclXL
aggregates likely represent an on-pathway intermediate for insertion into mitochondrial outer membrane during the onset of apoptosis.

Collectively, my study sheds light on the propensity of BclXL to aggregate in solution, particularly under acidic conditions and at elevated temperatures—the physical factors that mimic cellular stress—thus bearing important consequences on its mechanism of action in gauging the apoptotic fate of cells in human health and disease.
DEDICATION

To my grandparents, parents, friends and relatives for their continuous support, inspiration, love and blessings.
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Chapter 1: Introduction

1.1 Historical overview of the programmed cell death

Homeostasis is a salient property of healthy multicellular organisms. An excess or dearth of any type of cell can be deleterious. For proper functioning of the organism, cells need to be capable of responding to the external cues that signal damage or stress, at the same time, when necessary, they must have the ability to act upon these signals to execute their own death or commit suicide. This active process of cell death is called as apoptosis. In 1842, Carl Vogt demonstrated the phenomenon of cell death that was later precisely described by Walther Fleming in 1885 (1). However, in the following years the focus shifted primarily to phagocytosis. During mid-twentieth century, Lockshin and Willaims coined the term programmed cell death (PCD) for the first time while they were studying embryogenesis (2). Later in 1972 John Kerr and co-workers coined the term apoptosis (derived from the Greek word meaning “falling of leaves from the tree”) and described morphological characteristics of cell death during development and tissue homeostasis (3). Subsequently, the study of apoptosis became a major field of research and a plethora of studies provided profound understanding of the mechanism of PCD. While these studies have contributed to a broad understanding of apoptosis, we still lack the mechanistic insight of different key regulators involved in this process. Following sections will discuss the process of apoptosis and the different mechanisms involved that have been studied so far.

1.2 Cell death by apoptosis

Apoptosis involves cell death in a highly programmed and coordinated manner characterized by distinct biochemical and morphological changes, that include, condensation of nuclei, DNA fragmentation, chromatin condensation, plasma membrane blebbing, cellular...
shrinkage, mitochondrial disintegration and subsequently generation of apoptotic bodies that contain nuclear fragments and cytoplasmic bodies which are engulfed by macrophages and other neighboring cells (4). Apoptosis is crucial during many physiological processes like embryonic development and tissue homeostasis. Notably, dysfunction of apoptotic machinery can result in many pathological conditions, including cancer, autoimmune diseases and neurodegenerative disorders (5-7). In mammalian cells, apoptosis is mediated by two interconnected pathways: the extrinsic and intrinsic pathways (Figure 1-1). Although each of these pathways is stimulated by distinct stimuli, they both lead to the activation of downstream effectors called as caspases, which are aspartate specific proteases that mediate cell death by demolishing the cellular architecture through cleavage of proteins (8).

1.2.1 Extrinsic death receptor pathway

The extrinsic pathway is activated when extracellular death ligands, such as tumor necrosis factor α (TNFα), Fas ligand also called as Apo-1 or CD95, Apo3 ligand or TNF related apoptosis inducing ligand (TRAIL), bind to specific cell surface receptor, like TNF receptor 1, death receptor (DR) 3 , DR 4 and DR 5 (9, 10). These death ligands are secreted by the neighboring cells in response to different stimuli, like presence of infectious agents or lymphocyte expansion in response to antigens (11, 12). Once released, they mediate communication between cells and can subsequently orchestrate cell death if required. The receptors of death ligands contain a death domain in their intracellular region that can bind to several adaptor proteins forming death inducing signaling complex (DISC). For instance, the Fas receptor DISC consists of Fas-associated death domain protein (FADD), initiator procaspases-8 and -10 and some other regulators and cofactors (13) (Figure 1-1). DISC helps in recruiting procaspase-8 and enables its
autoactivation by autoproteolysis. Caspase-8 further activates downstream caspases such as caspase-3 and caspase-7 through proteolysis. Once activated the downstream caspases start cleaving cell protein and thus leads to cell death. Fas receptor mediated apoptotic signaling is mainly divided into two types. In type I cells, the DISC is formed at a high level thus causing enhanced caspase-8 activation (14). Caspase-8 can then directly activate downstream caspases without a mitochondrial amplification loop as required for type II cells that are dependent on the mitochondrial amplification loop since there is a lower level of DISC formation.

Caspase-8 helps in this process by activatingBid (Bcl2 family protein) through proteolysis to form truncated Bid (tBid), which in turn promotes the release of

**Figure 1-1:** A schematic illustrating the extrinsic and intrinsic pathways involved in apoptosis. Both pathways culminate into activation of caspase-3, 6 and 7 that leads to apoptosis. Fas mediated signaling pathway is chosen as an example of extrinsic pathway. Intrinsic pathway is depicted through the up-regulation of p53 protein due to DNA damage. This triggers activation of Bcl2 family proteins which then through a series of events lead to mitochondrial outer membrane (MOM) permeabilization and release of Cyt c. The released Cyt c activates caspases through APAF1 protein and climactically cell death (15).
apoptogenic factors from the mitochondria, thus also activating the mitochondrial or intrinsic apoptotic pathway. This acts as an amplification loop for the extrinsic pathway in case of type II cells, ultimately resulting in cell death.

1.2.2 Intrinsic mitochondrial dependent pathway

The intrinsic pathway, also known as mitochondrial-dependent pathway is activated in response to a myriad of death stimuli originating within the cell or sometimes externally as explained in previous section. Examples of death stimuli originating within the cell are radiation-induced DNA damage, metabolic stress, reactive oxygen species (ROS) and upregulation of oncogenes (16). Upon activation, the intrinsic pathway leads to permeabilization of mitochondrial outer membrane (MOM) and release of apoptogenic factors such as cytochrome c (cyt c), second mitochondria derived activator of caspases (Smac)/direct inhibitor of apoptosis binding protein with low pI (Diablo), apoptosis inducing factors (AIF) or endonuclease G (EndoG) into the cytosol (17-20) (Figure 1-1).

Of these apoptogenic factors, Cyt c is believed to play a major role in intrinsic pathway by virtue of its ability to activate the apoptotic protease activating factor (Apaf1) and deoxyadenosine triphosphate (dATP) to form the apoptosome (21). The apoptosome triggers the cleavage of pro-caspase-9 to caspase-9, which in turn activates the downstream caspases-3, 6 and 7 that eventually cause cell disruption (22). Importantly, the intrinsic pathway is regulated by members of B-cell lymphoma 2 (Bcl2) protein family, acting as mediators between the two pathways of apoptosis (Figure 1-1). Following section will describe in detail, the Bcl2 family, its structure and its role in regulating apoptosis.
1.3 The Bcl2 family and Apoptosis

Bcl2 family proteins have been implicated in regulating cellular viability by either promoting or suppressing apoptosis in vertebrates (23-25). Bcl2 proteins are characterized by presence of at least one or more of the four Bcl2 homology domains (BH1-BH4) which are important for the homo and hetero dimeric interaction among the members of this family.

1.3.1 Domain organization and functional classification

The Bcl2 proteins can be divided into three major groups based on their domain organization and functional characteristics: activators, effectors and repressors (Figure 1-2). Activators, such as BH3-interacting domain death agonist (Bid), Bcl2-interacting mediator (Bim), Bcl2-antagonist of cell death (Bad), promoter-upregulated modulator of apoptosis (PUMA) and Bcl2 modifying factor (Bmf) proteins are characterized by the presence of single BH3 homology domain and are localized in the cytosol (26). Effectors like Bcl2-associated X (Bax) and Bcl2 antagonist/killer-1 (Bak) proteins are mainly cytosolic in their inactive state. Domain organization of these proteins consists of BH3-BH1-BH2-TM modular architecture, where TM is the carboxy-terminal (CT) hydrophobic transmembrane domain. Both activators and effectors function as pro-apoptotic proteins. Activators act as major sensors for cellular stress and many signaling pathways converge upon these proteins. They in turn activate the effectors to permeabilize the MOM and result in cell death. Repressors are composed of BH4-BH3-BH1-BH2-TM modular organization, with an additional BH4 domain at the N-terminal (NT) end of the protein. Bcl2, B-cell lymphoma (BclW), myeloid lymphoma extra-large (Mcl-1) and B-cell lymphoma extra-large (BclXL) are the major members of
this group. Generally these proteins are integrated with MOM or endoplasmic reticulum (ER) membrane but can also exist in the cytosol (27-29). Repressors act as anti-apoptotic proteins by preventing MOM permeabilization by neutralizing the activity of effectors (23).

### 1.3.2 Mechanism of action and interplay between Bcl2 family members

Different competing theories have been introduced to elucidate the mechanism of Bcl2 protein function. However, the inherent action of these proteins remains ambiguous. A simple biophysical mechanism that is relevant for their action in apoptosis is presented here (Figure1.3). Cellular ratio of activators, effectors and repressors is known to regulate the apoptotic fate of a cell (30, 31). Repressors by virtue of their ability to hetero-associate with effectors through BH3 domains, prevent cell death in normal healthy cells. When apoptotic signals are induced, the activators are stimulated. This in turn, starts a competing process where activators attempt to displace effectors from the repressor-effector hetero-associated complex and, in doing so, redeem the pro-apoptotic action of effectors and suppress the anti-apoptogenic effect of repressors. Consequently, the effectors trigger apoptotic cell death by inserting into the MOM and creating mitochondrial pores. Importantly, this process is analogous to the insertion of bacterial
Figure 1-3: Schematic representation of the mechanism by which different groups of Bcl2 family proteins regulate cell death. In the healthy cells, repressors (R) neutralize effectors (E) by heterodimerization giving rise to equilibrium between the free R and E-R complex. Upon arrival of apoptotic cues, the activators (A) compete with E to bind to R. Thus, the equilibrium shifts towards A-R complex on left. This liberates the E form the E-R complex, which then translocate to MOM. Within the MOM E are believed to form pores through which cytochrome c is released which further activates various caspases that eventually cause cell death by chewing up the cellular protein.

toxins such as diptheria and colicins (32-34). Not only that, the solution conformation of Bcl2 proteins is similar to these toxins, suggesting that Bcl2 proteins may have a similar membrane conformation, or interact with the membrane by a similar mechanism. In addition to disengaging the effectors from the inhibitory action of repressors, activators are also believed to directly bind to effectors and further their participation in the assembly of mitochondrial pores. Homo-association of effectors within the MOM is believed to be the main mechanism of pore formation (35-38). This results in permeabilization of the MOM leading to the release of apoptogenic factors including cytochrome c and Smac/Diablo into the cytosol. Consequently, increasing levels of
apoptogenic factors in the cytosol activates caspases, which triggers a series of cascade reactions as discussed in the previous section, leading to cell death (Figure 1-3). One of the fascinating aspects of Bcl2 family members, especially repressors and effectors is their ability to function both in hydrophilic cytoplasm and within the hydrophobic membranes. This functional duality of Bcl2 family and their ability to form pores in MOM is attributed to its overall structural topology.

1.3.3 Structural studies of Bcl2 family

Structurally, the Bcl2 family is classified into two groups, folded globular and intrinsically unstructured proteins (IUPs). The multi domain repressor and effector proteins belong to the folded globular group. The activators or BH3 only proteins are IUPs and are believed to fold upon binding to globular proteins. Only exception in this case is Bid protein which is known to form a globular structure (25). The first folded globular topology structure was published for BclXL in 1996 (39). To date, the structures of most of the known repressor and effector proteins have been solved and unsurprisingly due to their sequence similarity all of them show a remarkably similar overall topology. The three dimensional structure is characterized by a central, primarily hydrophobic α-helical hairpin "dagger" (α5 and α6) enveloped by six amphipathic α-helices (α1- α4 and α7- α8) of varying lengths forming a "cloak" around it (Figure 1-4a). A long unstructured loop is present between the first two α-helices. Additionally, folded globular proteins have a CT hydrophobic α-helix (α9), commonly known as TM domain and is predicted to be responsible for MOM localization (40-43). By virtue of this "cloak and dagger" topology, Bcl2 members can coexist as soluble factors in the cytoplasm under quiescent state and as membrane channels in MOM upon apoptotic induction. Notably, the
**Figure 1-4**: Schematic representation of the three dimensional structural topology of the folded globular Bcl2 family proteins. (a) "Cloak" and "dagger" topology of Bcl2 family, the central hydrophobic helices α5-α6 (color blue) form the dagger surrounded by the amphiphilic helices (color green) α1-α4 on one side and α7-α8 on the other side. The hydrophobic helix α9 (color yellow) form the TM domain that helps in membrane integration. (b) The hydrophobic groove formed by α2-α5 helices is occupied by the TM domain (α9 helix) in an intra-molecular manner in case of cytoplasmic BclW and Bax (47, 49). (c) BH3 ligand (color red) is bound to the hydrophobic groove of the protein displacing TM domain. The activators compete for the hydrophobic groove and in the process, withdraw the TM domain from the hydrophobic groove. This conformational change is known to activate the pro-apoptotic property of effectors and also neutralize the anti-apoptotic activity of repressors (48).

Hydrophobic dagger is believed to directly participate in the formation of mitochondrial pores. In repressors, α2-α5 helices associate to form a hydrophobic groove that serves as the docking site for the BH3 domain of activators and effectors. Interestingly, effectors also have a hydrophobic groove assembled by α1/α6 helices that acts as the region for activators to bind. This groove is located on the opposite side to that occupied by the hydrophobic groove in repressors (44-46). In case of BclW, Bax and Bak the CT (α9) TM domain is believed to occupy the hydrophobic groove in an intra-molecular manner (Figure 1-4b). Upon apoptotic induction the activators compete for the hydrophobic
groove and in the process, withdraw the TM domain allowing the protein to translocate to the MOM (47-49) (Figure 1-4c). This conformational change is believed to activate the pro-apoptotic property of effectors and also neutralize the anti-apoptotic activity of repressors. However, the precise mechanism that regulates conformational changes, and therefore their activity, remain elusive.

1.4 Conformational changes associated with Bcl2 family proteins

Apoptotic regulation by Bcl2 family proteins is believed to be primarily dependent on solution to membrane translocation. As discussed in previous section, conformational change leads to migration and insertion of some Bcl2 proteins into MOM during apoptosis. Bax and Bak are known to oligomerize and form pores in MOM by the virtue of their ability to undergo conformational change (34, 50-52). Notably, repressor proteins (BclW and BclXL) are also believed to translocate and insert into the MOM upon apoptotic stimulation. BclXL was shown to bind with Bax within the MOM and inhibit its oligomerization and further pore formation (53). Another study showed that BclW was inactivated upon insertion into the MOM (47). Regardless of the contradictory hypothesis about their mechanism of action, many in-vitro studies have shown that the solution-membrane transition and accompanying conformational change of both effectors and repressors is primarily dependent on, acidic pH and presence of lipid vesicles (54), later it was shown that solution pH alone can also change the conformation of these proteins (55-57).

1.4.1 Effect of pH on the conformation of Bcl2 family proteins

Solution pH was shown to modulate the conformation of Bcl2 family members in a manner similar to bacterial toxins by reducing the activation energy for the solution-
membrane conformational change (33, 58-60). The conformational change can be large, altering the quaternary structure, or it can be small changing only the tertiary structure also referred as molten globule conformation. The molten globule is believed to assist in the membrane insertion of proteins by acting as the intermediate between the solution and the membrane conformation (61, 62). In-vitro, many Bcl2 family proteins show enhanced property of homo or hetero association at lower pH (4-5) range. Moreover, the pore forming property of these proteins also showed significant enhancement in this pH range (59, 63-65). Interestingly, many studies have shown that upon apoptotic induction a pH gradient is established across the mitochondria, leading to alkalization of the mitochondrial matrix and acidification of the cytosol. Further, it was shown that the efficiency of caspase activation by cyt c is pH dependent and optimally occurs around pH 6.3-6.8 (55-57). MOM was shown to be decorated with anionic lipids like cardiolipin, creating a negative surface potential thus, increasing the proton concentration near the surface and ultimately lowering the local pH around the membrane surface (66). Many other studies provided further evidences for various stimuli that can cause cytosol acidification, for example, UV-irradiation, etoposide, staurosporine, anti-Fas anti bodies, growth factor deprivation, somatostatin, over-expression of bax, and p53 activation (67-69). All these observations imply that a decrease in pH within the cell due to various reasons act as a switch for activating the apoptotic machinery, since the cell is undergoing a stress condition and if not able to overcome it, should follow a death pathway. Similar kind of stress can be imposed by an increase in temperature, which is shown to alter the conformation and the association property of Bcl2 protein family members leading to their activation which causes cell death (70).
1.4.2  **Effect of temperature on the conformation of Bcl2 family proteins**

Hyperthermia is under investigation for a long time now as a method to induce apoptosis in cancer cells. However, the precise mechanism underlying the process remains unclear. Significant advancement in this field has been made in recent past by studying the effect of temperature on cellular structure and de novo synthesis of DNA and RNA molecules as well as the protein synthesis and changes associated with their structure (71, 72). Mild hyperthermia induces Bax activation and oligomerization in lymphoid cells. Cyt c was shown to be released when Bax and Bak were incubated along with purified mitochondria at elevated temperature in-vitro, suggesting that the proteins undergo a conformational change with an increase in temperature (70, 73). A similar study on lysozyme has shown to enhance its membrane binding ability accompanied by change in conformation (74). Although unrelated to apoptosis, higher temperature is also known to induce the oligomerization by domain swapping in case of bovine pancreatic ribonulcease (RNase A) and cystatins by decreasing the high energy barrier between the monomer and the higher order oligomers (75, 76). Notably, a recent study has shown that mouse BclXL can also form domain swapped dimers at elevated temperature (77).

Interestingly, many proteins upon oligomerization are known to form amyloid-like fibrillar aggregates under different environmental stress conditions, including, higher temperature, low pH or presence of toxic chemicals (78, 79). The deposition of amyloid-like fibrils is believed to play major role in many degenerative diseases like, Parkinson’s disease, Alzheimer’s disease and Huntington’s disease (80). A range of proteins not associated with amyloid diseases are also able to aggregate in-vitro into amyloid fibrils at higher temperature (81-83). The precise role of amyloid fibers formed by these proteins...
within the cell is still not clear but it has been shown that amyloid fibers have the ability to permeabilize the artificial membranes as well as cellular membranes and can be highly cytotoxic \cite{84, 85}. Amyloid fibrils formed by lysozyme are known to induce apoptosis by virtue of their ability to cause membrane damage \cite{74}. Thus, it is a likely possibility that upon induction of apoptosis under stress, Bcl2 family proteins can oligomerize and form amyloid-like fibrils that can form channels in MOM through which apoptogenic factors can be released.

1.5 **Significance of these studies**

Even though Bcl2 family proteins were discovered more than two decades ago, they have not been extensively studied at biophysical level. The molecular basis of protein-protein interactions among various Bcl2 proteins remain poorly understood. Notably, most of the earlier biophysical and structural studies on BclXL and other Bcl2 repressors have been carried out on truncated constructs lacking both the structurally disordered \(\alpha_1-\alpha_2\) loop and the functionally-critical TM domain. Unraveling the molecular mechanism of the full length proteins especially the role of TM domain will shed more light on the structural and functional properties of Bcl2 family proteins. By using a diverse array of biophysical techniques, this thesis aims to further our understanding of the biophysical parameters associated with the full length Bcl2 family proteins by using BclXL apoptotic repressor as the model protein. The goal of the study is to provide mechanistic insights in ligand binding and membrane insertion of the full length protein and how these properties affect the structure of the protein. At the same time, I aspire to understand how different physiological changes in the protein environment will alter its conformation. Such knowledge will not only shed more light on
the underlying molecular mechanisms driving apoptosis but will also deliver more information for the advancement of novel therapies attributed with lower toxicity and higher efficacy for the treatment of many pathological conditions primarily, Alzheimer’s, Parkinson’s and cancer. In an attempt to gain more knowledge and further our understanding about the structural and functional properties of BclXL protein, I set out in this thesis to determine the physicochemical properties associated with this protein.
Chapter 2: Materials and Methods

2.1 Molecular cloning

Human BclXL constructs including BclXL_FL (residues 1–233) and BclXL_dTM (residues 1-200) were cloned into pET30 bacterial expression vectors using Novagen ligation-independent cloning technology. The vector encodes an N-terminal polyhistidine (His)-tag. The His-tag was used to aid in protein purification using Ni-NTA affinity chromatography.

2.2 Protein expression and purification

All BclXL constructs were transformed and subsequently expressed in BL21* (DE3) bacterial strain (Invitrogen). To maximize protein expression, BL21* cells uses the DE3 lysogen to express the recombinant protein and have a truncated RNase E. Cells were cultured in Terrific Broth (TB) media grown at 20ºC to an optical density of greater than unity at 600nm prior to induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacterial cells were further grown overnight at 20ºC to express the protein and were subsequently harvested and resuspended in Lysis Buffer (50 mM Tris, 500mM NaCl, 2M Urea, 2mM β-mercaptoethanol (β-ME), 10% Triton X-100 at pH 8.0). Cells were then disrupted using a Biospec Bead-Beater® and subjected to high speed centrifugation to remove cell debris. Cell lysate thus obtained was then applied to a Ni-NTA affinity chromatography column. Non-specific binding bacterial proteins were removed by washing the column extensively with Wash Buffer (50 mM Tris, 500 mM NaCl, 2M Urea, 20 mM Imidizole, and 2mM β-ME at pH 8.0). Elution Buffer (50 mM Tris, 500 mM NaCl, 2M Urea, 200 mM Imidizole, 2mM β-ME at pH 8.0) was used finally elute the protein from the column. The elutant was dialyzed against an
appropriate physiological buffer. Dialyzed protein was further purified using a HiLoad 26/60 Superdex 200 preparatory grade size exclusion chromatography (SEC) column coupled to a GE Akta FPLC system. Purity of protein was further verified by SDS-PAGE analysis (Figure 2.1). Protein concentrations were determined by fluorescence-based Quant-It assay (Invitrogen) and spectrophotometrically using extinction coefficients of 47,440 M⁻¹cm⁻¹ for BclXL_FL and 41,940 M⁻¹cm⁻¹ for BclXL_dTM constructs. The extinction coefficients were calculated using the online software ProtParam at Expasy Server (86). Final yields were typically between 10-20mg proteins of apparent homogeneity per liter of bacterial culture. Results from both the methods were in excellent agreement.

2.3 SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used technique to separate proteins according to size (87). Being an anionic detergent SDS acts as a protein denaturant. When the protein is subject to 100°C temperature in presence of SDS, it evenly coats the polypeptide backbone with a negative charge proportional to the mass of the protein. An electric field is applied across the gel during PAGE run, SDS-coated proteins are pulled with the same force per unit mass towards the gel apparatus cathode. The proteins separate based on difference in molecular mass of each species since the observed electrophoretic mobility is a linear function of the logarithm of molecular weight. A standardized set of protein markers of known molecular weight then helps to determine the size of separated species.

To check the apparent homogeneity of recombinant protein obtained after Ni-NTA and SEC purification, SDS-PAGE analysis (Figure 2-1) were carried out by loading
Figure 2-1: SDS-PAGE analysis of recombinant BclXL FL (A) and BclXL dTM (B) purified from bacteria using Ni-NTA affinity chromatography. Briefly, total bacterial cell lysate (LYS) was loaded onto a Ni-NTA affinity column. After the passage of flow-through (FT), the column was extensively washed. The fraction eluted from the Ni-NTA affinity chromatography (NAC) column was further subjected to size-exclusion chromatography (SEC). Note that the left lane shows the Promega Broad Range Protein Markers.

sample onto a 12% (w/v) SDS-PAGE gel run at 150V for 60min using a VWR AccuPower power supply and a Bio-Rad Protein Chamber. Protein bands were visualized by staining with 0.1% (w/v) coomassie-blue solution containing 40% (v/v) methanol and 10% (v/v) acetic acid, and then destaining with a destain-solution containing 10% (v/v) acetic acid and 10% (v/v) methanol. Images of the gels were captured using a UVP MultiDoc-It Gel Imaging system.

2.4 Peptide synthesis

HPLC-grade 20-residue peptides corresponding to various BH3 domains within human Bid (H₂N-DIIRNIARHLAQVGDSMDRS-COOH), Bad (H₂N-
AAQRYGRELRRMSDEFVDS-COOH) and Bax (H\textsubscript{2}N-ASTKKLSELSKRGDELDSN-COOH) proteins were commercially obtained from GenScript Corporation. The peptide concentration was measured gravimetrically.

2.5 Bicelles preparation

Phospholipids 1,2-dimyristoyl-sn-glycero-3-Phosphocholine (DMPC), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) and 1,1’2,2’-tetraoleoyl cardiolipin (TOCL), were obtained commercially from Avanti Lipids. Mixed DMPC/DHPC and TOCL/DHPC bicelles were prepared at a final concentration of 30 mM, at DMPC to DHPC and TOCL to DHPC molar ratio of 1:2 and 1:4 respectively, by stirring for 2 h at 37°C in appropriate buffers.

2.6 SEC analysis

Size-exclusion chromatography (SEC) was performed using a HiLoad Superdex200 column coupled to a GE Akta FPLC system equipped with the UNICORN software for automatic operation. SEC is a commonly used technique in which macromolecules, such as proteins are separated based upon their size or hydrodynamic volume. Briefly, protein sample mixture is applied to a column containing a porous gelatinous medium and running at low pressure (~0.2 MPa). The gelatinous medium is made of porous “beads” and act as the stationary phase. Molecules with a diameter smaller than the pores in the beads will enter them and result in larger volume to travel before elution. On the contrary, molecules with a diameter larger than that of the pore won’t be able to enter the gel medium and their flow will not be obstructed leading to their faster elution. This differential elution rate of particles on a stationary phase permits the separation of molecules within a sample based on size only, larger molecules will
elute earlier than smaller molecules. Given that the pore size of the column is not uniform throughout the length of the column and a given protein is a collection of different sized molecules instead of having a unique well-defined size, a normal distribution of the elution volume for a given protein is obtained. The primary advantage of using SEC is that it can be performed under native conditions that do not alter the sample. But a disadvantage associated with this technique is that it primarily determines the molecular weight based on assumption that the molecule entering the stationary phase is completely spherical, the globular molecular shape is not taken into account during the analysis. Thus, its ability to determine the molecular weight of non-spherical molecules is very limited.

After purification to apparent homogeneity using Ni-NTA affinity chromatography, extensive dialysis of recombinant proteins was carried out in appropriate buffer prior to application on Superdex200 column pre-equilibrated in the same buffer at 10°C. The elution of protein was recorded using UV monitor at 280nm and automatically plotted as a function of elution volume in the UNICORN software. The protein identity was further confirmed in elution fractions by SDS-PAGE analysis.

2.7 CD analysis

Circular dichroism (CD) helps to study the secondary and tertiary structural features of different macromolecules. CD analysis was carried on the recombinant proteins in various conditions to analyze their Opticospectroscopic properties. CD is a useful technique for determining the changes associated with the structure of a macromolecule in presence of different ligands or a change in the environment of macromolecule.
CD measurements were conducted on a Jasco J-815 spectrometer thermostatically controlled at specified temperature. Data were acquired using the inbuilt Jasco software. Samples were prepared in appropriate buffers at different pH conditions. For far-UV measurements, experiments were conducted on 5μM of recombinant BclXL_FL and BclXL_dTM protein and data were collected using a quartz cuvette with a 2-mm path length in the 190-250 nm wavelength range. For near-UV measurements, experiments were conducted on 30μM of recombinant BclXL_FL and BclXL_dTM protein and data were collected using a quartz cuvette with a 10-mm path length in the 260-340 nm wavelength range. Data were normalized against reference spectra to remove the contribution of buffers. All data were recorded with a slit bandwidth of 2 nm at a scan rate of 10 nm/min. Each data set represents an average of four scans acquired at 0.1 nm intervals. All data were processed and analyzed using the Microcal ORIGIN software. More detailed and specific procedures can be found in Chapters: 3.3.6, 4.3.5 and 5.3.6.

2.8 ALS measurements

Analytical light scattering (ALS) consists of two types of light scattering techniques—static light scattering (SLS) and dynamic light scattering (DLS). While SLS collects the time-averaged intensity of scattered light, DLS measures the fluctuation of intensity of scattered light with time. Both the techniques are useful to analyze size and molecular mass as well as hydrodynamic properties of proteins at the same time. When light passes through a medium containing small particles (e.g. molecules) whose size is smaller than the wavelength of incident light, there will be scattering in all directions known as Rayleigh scattering. The scattered light can be thoroughly analyzed to obtain fundamental physical properties of the scattering system. SLS measures the angular
dependence in scattering intensity over a range of concentrations of the sample. This helps to determine both molecular weight and radius of gyration \( R_g \) of the scattering system. Multiple detection angles are used to measure the scattering intensity in SLS and the change in intensity with respect to angle permits determination of the radius of gyration \( R_g \). The extent of light scattering is directly proportional to molecular weight and concentration. Using reduced Zimm equation after double extrapolation to zero angle and zero concentration molecular weight of a sample can be easily obtained after measuring the scattering intensity using SLS (88, 89). DLS measures the random motion of particles within the solvent also called as Brownian motion that helps to determine the size of particles by measuring the hydrodynamic radius (\( R_h \)). The particle size is calculated by measuring the translation diffusion coefficient (\( D_t \)) which is inversely proportional to \( R_h \). Time-dependent and concentration dependent fluctuation in scattering intensity is measured by a single detector positioned at 90° with respect to the incident laser beam. Dynamic change in particle motion gives rise to fluctuations. For a system undergoing Brownian motion, scattered light will undergo constructive and destructive interference resulting in fluctuation of measured scattering intensity. The rate of fluctuation intensity will depend on the size of the particles. Since larger particles move slower in a solution relative to smaller particles. Thus, larger particles will cause the intensity to fluctuate slowly than the smaller one corresponding to a smaller \( D_t \) and a larger \( R_h \) and vice-versa. A plot of the logarithm of \( R_h \) versus the logarithm of molecular weight of known protein standards gives information about the degree to which a protein is folded. \( R_h \) values for a corresponding molecular weight which lay off of the standard curve indicate the protein is likely not folded in an entirely compact manner.
ALS experiments were conducted on a Wyatt miniDAWN TREOS triple-angle static light scattering detector and Wyatt QELS dynamic light scattering detector coupled in-line with a Wyatt Optilab rEX differential refractive index detector and interfaced to a Hiload Superdex 200 size-exclusion chromatography column under the control of a GE Akta FPLC system within a chromatography refrigerator at 10°C. The BclXL_FL and BclXL_dTM constructs were prepared in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol at pH 8.0 and loaded onto the column at a flow rate of 1 ml min⁻¹ and the data were automatically acquired using the ASTRA software. The starting concentrations injected onto the column were between 10-50 μM. The angular and concentration dependence of static light scattering (SLS) intensity of each protein species resolved in the flow mode was measured by the Wyatt miniDAWN TREOS detector. The SLS data were analyzed according to the built-in Zimm equation in ASTRA software (89, 90). The time and concentration dependence of dynamic light scattering (DLS) intensity of each protein species resolved in the flow mode was measured by the Wyatt QELS detector positioned at 90° with respect to the incident laser beam. The DLS data were iteratively fit using non-linear least squares regression analysis using the built-in equation in ASTRA software (91-93). More detailed and specific procedures can be found in Chapters: 3.3.5, 4.3.3 and 5.3.4.

2.9 ITC measurements

Isothermal titration calorimetry (ITC) is a paramount technique to study the protein-ligand thermodynamics. ITC helps to understand the comprehensive thermodynamic forces that govern the protein-ligand interactions (94, 95). ITC is a quantitative technique that directly measures heat change associated with a chemical
reaction, such as a bimolecular binding event. Measurement of heat of reaction allows precise determination of enthalpy ($\Delta H$), binding constants ($K_d$) and stoichiometry ($n$) and then can calculate entropy ($\Delta S$) and Gibbs free energy ($\Delta G$). Thus, ITC provides a complete thermodynamic profile of molecular interaction. Protein is titrated with a ligand by a series of injections, each injection yields a heat signal which will approach zero as binding sites on the protein become saturated. The binding isotherm thus obtained is integrated and fitted to a given model. Different thermodynamic parameters associated with the reaction are thus obtained. Moreover, ITC is a useful technique to understand if the reaction between two components is under enthalpic or entropic control. Exothermic reactions are usually controlled by enthalpic component characterized by favorable contacts formed between protein and ligand due to electrostatic interactions and hydrophobic forces. In contrast, endothermic or weakly exothermic reactions are mainly controlled by entropy, where a large positive entropy change of the system due to ordering, disordering or conformational change in the macromolecule upon ligand binding act as the driving force for the reaction.

ITC experiments were performed on Microcal VP-ITC instrument and data were acquired and processed using automated features in Microcal ORIGIN software. All measurements were carried at least three times. Briefly, protein samples and peptide were prepared alone or in presence of bicelles in different buffers containing 5mM $\beta$-mercaptoethanol and de-gassed using the ThermoVac accessory for 10min. The experiments were initiated by injecting $25 \times 10^{\mu l}$ injections from the syringe into the calorimetric cell, at a fixed temperature. The change in thermal power as a function of each injection was automatically recorded using Microcal ORIGIN software and the raw
data were further processed to yield binding isotherms of heat uptake per injection as a function of concentration. The heats of mixing and dilution were subtracted from the heat of binding per injection. A more detailed procedure and specific can be found in Chapters: 3.3.3, 4.3.2 and 5.3.8.

2.10 DSC measurements

Differential scanning calorimetry (DSC) is a powerful analytical technique to determine the stability of macromolecules alone or in presence of different ligands in different buffer conditions. DSC directly measures the heat change in a sample as the temperature is raised or lowered in a controlled fashion. Biomolecules in a solution are always in equilibrium between the native conformation and a collection of unfolded conformations. Different thermodynamic parameters including enthalpy (ΔH) and entropy (ΔS) regulate these conformations of the molecule by determining the effective Gibbs free energy (ΔG) associated with each conformation. DSC measures the enthalpy of unfolding of a molecule due to heat denaturation. As the temperature within the DSC cell increases, the molecule starts to unfold only when the entropic factor (TΔS) overcomes the stabilizing enthalpic forces such as hydrogen bonding, electrostatic interactions and hydrophobic interactions. Thus, giving rise to an endothermic peak, the maxima of which is the transition mid-point also called as Tm which is defined as the temperature where only 50% of the protein is in its native conformation. DSC is also useful to calculate the change in heat capacity (ΔCp) due to thermal unfolding.

DSC experiments were performed on a TA Nano-DSC instrument, and data were acquired and processed using the integrated NanoAnalyze software. All measurements were repeated at least three times. Briefly, protein samples and peptide were prepared
alone or in presence of bicelles in different buffers. All experiments were conducted on 10–50 μM of each protein construct alone, in presence of 10 molar excess of the peptide or 30 molar excess of bicelles in the 40–120 °C temperature range at a heating rate \( dT/dt \) of 1°C min−1 under an excess pressure of 3 atm. The change in thermal power \( dQ/dt \) as a function of temperature was automatically recorded using the NanoAnalyze software. The raw data were further processed to yield the melting isotherms of excess heat capacity (Cp) as a function of temperature (T). A more detailed procedure and specific can be found in Chapters: 3.3.2 and 4.3.4.

2.11 SSF measurements

Steady-state fluorescence (SSF) spectroscopy was employed to analyze the tertiary structural changes associated with the protein alone, in presence of different ligands and in presence of bicelles using different buffer conditions. Intrinsic tryptophan fluorescence was used as the probe to study the changes in the conformation of the protein. An environmental shift surrounding tryptophan directly correlates with change in its fluorescence spectra thus providing incite about protein conformational change. SSF spectra of different dyes such as, 8-anilinonaphthalene-1-sulfonate (ANS) and Thioflavin T (ThT) was also studied in presence of protein using different buffer conditions. These dyes are known to have a characteristic fluorescence spectrum in different wavelength ranges and are useful to understand the tertiary and quaternary structural changes associated with the protein.

SSF spectra were collected on a thermostatically-controlled Jasco FP-6300 spectrofluorometer using a quartz cuvette with a 10-mm pathlength at 25 °C. Briefly, protein samples were prepared in appropriate buffers alone, in presence of different
ligands and in presence of different dyes. All data were recorded using a 2.5-nm bandwidth for both excitation and emission. Data were normalized against reference spectra to remove the contribution of buffer. The reference spectra were obtained in a similar manner and in the same conditions. More detailed procedures can be found in Chapter: 3.3.4, 4.3.6 and 5.3.6.

2.12 Microscopy

Microscopy is employed as a technique to view objects or samples which otherwise cannot be seen by normal eye alone. The object is not visible when its size is too small that it does not fall within the resolution range of the normal eye. Microscopy aids in visualizing these objects and can be carried out in optical, transmission or scanning mode. Optical microscopy uses the visible or fluorescence light that gets transmitted or reflected from the sample. Transmission mode utilizes electromagnetic radiations/electron beam which passes through the sample and gets collected at the detectors to create the image. In the scanning mode fine electromagnetic beam is used to scan over the sample that provides information about the sample topography and composition.

Transmission electron microscopy (TEM) experiments were conducted on a Philips CM-10 electron microscope operating at a voltage of 80 kV, and images were photographed at a magnification of 105,000X. Scanning electron microscopy (SEM) experiments were conducted on a Zeiss Gemini Ultra-55 electron microscope operating at a voltage of 5 kV using the in-lens detector and the images were photographed at a magnification of 50,000X. Fluorescence microscopy (FM) experiments were conducted on a Leica DMI6000 microscope with 10x objective. Images obtained from FM were
analyzed and processed using Leica LAS-AF software. For each technique data were collected on BclXL_FL or BclXL_dTM constructs alone or in presence of 10M excess of the peptide or with 10 mM bicelles. For FM prior to imaging, each sample was stained with 25µM ThT and mounted onto a glass slide. More detailed procedures can be found in Chapter: 3.3.7, 4.3.7 and 5.3.8.

2.13 Molecular modeling

Molecular modeling (MM) was used to build three dimensional structural models of BclXL protein in different conformations using the MODELLER software based on homology modeling (96,97). Briefly, molecular dynamics and simulated annealing protocols are employed by MODELLER software to constitute the modeled structure by adjusting the spatial restraints obtained from amino acid sequence alignment with a corresponding template in Cartesian space. The three dimensional model thus obtained is expected to have similar folds as the template structure excluding specific amino acids which have modified side chains due to the introduction of defined hydrogen bonding, rearrangements of the domain or the modeling of the loops not contributed by the original template structure.

In the current study, the solution structures of truncated BclXL in which the TM domain and the α1–α2 loop are missing (Protein Data Bank (PDB) ID: 1BXL), hereinafter referred to as tBclXL, and the full-length Bax in which the TM domain occupies the canonical hydrophobic groove (PDB ID: 1F16) were used as templates. More specifically, the entire models of BclXL in various conformations were built in homology with tBclXL (PDB ID: 1BXL) except for the TM domain, which was built in homology with the TM domain of Bax. MOLMOL was used to bring various parts and/or
monomers into optimal spatial orientations relative to each other in a rigid-body fashion. For each structural model, a total of 100 atomic models were calculated and the structures with the lowest energy, as judged by the MODELLER Objective Function, were selected for further analysis. RIBBONS was used to render the atomic models (98). All calculations and data processing was done on a Linux workstation equipped with a dual-core processor. Specific modifications made for each model can be found in Chapters: 3.3.8, 4.3.7 and 5.3.2.

2.14 Molecular dynamics

Molecular dynamics (MD) is a computer simulation technique to study the time dependent physical movements of molecular system. MD simulations can provide detailed information about the fluctuations and the conformational changes associated with a macromolecule. MD is based on Newton’s equation of motion, \( F = ma \), where “\( F \)” is the force exerted on the particle, “\( m \)” is the mass of the particle and “\( a \)” is its acceleration. The acceleration of each atom in a system can be determined if the force acting on each atom is known. The trajectories of molecules and atoms that describe their positions, acceleration and velocities can then be determined by integrating the equations of motion. Using this method the state of the system can be predicted by knowing the positions and the velocities of each atom.

MD simulations were performed with GROMACS (99, 100) software utilizing OPLS-AA force field (101, 102). Briefly, the modeled structures of BclXL in different conformations were centered within a cubic box, hydrated using the extended simple point charge (SPC/E) water model (103-105), and the ionic strength of solution was set to 100mM with NaCl. The hydrated structures were energy-minimized with the steepest
descent algorithm prior to equilibration under the NPT ensemble conditions, wherein the number of atoms (N), pressure (P) and temperature (T) within the system were respectively kept constant at ~50000, 1 bar and 300 K. The Particle-Mesh Ewald (PME) method was employed to compute long-range electrostatic interactions with a 10Å cut-off \((10^4)\), and the Linear Constraint Solver (LINCS) algorithm to restrain bond lengths \((10^6)\). All MD simulations were performed under periodic boundary conditions using the leap-frog integrator with a time step of 2 fs. For the final MD production runs, data were collected every 10 ps over a time scale of 100 ns. All simulations were run on a Linux workstation using parallel processors at the High Performance Computing facility within the Center for Computational Science of the University of Miami. Specific modifications made for each model can be found in Chapters: 3.3.9, 4.3.8 and 5.3.3.
Chapter 3: Ligand Binding and Membrane Insertion Compete with Oligomerization of the BclXL Apoptotic Repressor

3.1 Summary

B-cell lymphoma extra-large (BclXL) apoptotic repressor plays a central role in determining the fate of cells to live or die during physiological processes such as embryonic development and tissue homeostasis. Herein, using a myriad of biophysical techniques, we provide evidence that ligand binding and membrane insertion compete with oligomerization of BclXL in solution. Of particular importance is the observation that such oligomerization is driven by the intermolecular binding of its C-terminal transmembrane (TM) domain to the canonical hydrophobic groove in a domain-swapped trans fashion, whereby the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa. Binding of BH3 ligands to the canonical hydrophobic groove displaces the TM domain in a competitive manner, allowing BclXL to dissociate into monomers upon hetero-association. Remarkably, spontaneous insertion of BclXL into DMPC/DHPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dihexanoyl-sn-glycero-3-phosphocholine) bicelles results in a dramatic conformational change such that it can no longer recognize the BH3 ligands in what has come to be known as the “hit-and-run” mechanism. Collectively, our data suggest that oligomerization of a key apoptotic repressor serves as an allosteric switch that fine-tunes its ligand binding and membrane insertion pertinent to the regulation of apoptotic machinery.

3.2 Overview

Apoptosis plays a key role in removing damaged and unwanted cells in a highly programmed and coordinated manner during physiological processes such as embryonic
development and tissue homeostasis. Importantly, deregulation of apoptotic machinery can result in the development of diseases such as cancer and neurodegenerative disorders (5-7). The Bcl2 family of proteins has come to be regarded as a central player in coupling apoptotic stimuli to determining the fate of cells to live or die (23-25, 27, 28, 107-109). The Bcl2 proteins can be divided into three major groups: activators, effectors and repressors. Activators such as Bid and Bad belong to the BH3-only proteins, where BH3 is the Bcl2 homology 3 domain. Effectors such as Bax and Bak contain the BH3-BH1-BH2-TM modular architecture, where TM is the transmembrane domain located C-terminal to Bcl2 homology domains BH3, BH1 and BH2. Repressors such as Bcl2, BclXL and BclW are characterized by the BH4-BH3-BH1-BH2-TM modular organization, with an additional N-terminal Bcl2 homology 4 domain.

According to one school of thought, the apoptotic fate, or the decision of a cell to continue to live or pull the trigger to commit suicide, is determined by the cellular ratio of activator, effector and repressor molecules (30, 31). In quiescent and healthy cells, the effectors are maintained in an inactive state via complexation with repressors. Upon receiving apoptotic cues, in the form of DNA damage and cellular stress, the activators are stimulated and compete with effectors for binding to the repressors and, in so doing, not only do they neutralize the anti-apoptotic action of repressors but also unleash the pro-apoptogenicity of effectors. The effectors subsequently initiate apoptotic cell death by virtue of their ability to insert into the mitochondrial outer membrane (MOM) resulting in the formation of mitochondrial pores in a manner akin to the insertion of bacterial toxins such as colicins and diphtheria (32-34, 62, 110). In addition to freeing up the effectors from the inhibitory effect of repressors, the activators are also believed to
directly bind to effectors and facilitate their participation in the assembly of mitochondrial pores. This provides a route for the release of apoptogenic factors such as cytochrome c and Smac/Diablo from mitochondria into the cytosol. Subsequently, rising levels of apoptogenic factors in the cytosol switch on aspartate-specific proteases termed caspases, which in turn, demolish the cellular architecture by cleavage of proteins culminating in total cellular destruction. Importantly, studies suggest that the release of apoptogenic factors may occur through the so-called voltage-dependent anion channel (VDAC) located within MOM ([111]). Thus, while apoptotic effectors such as Bax and Bak accelerate the opening of VDAC, apoptotic repressors such as BclXL and Bcl2 have been shown to trigger its closing. Although the precise mechanism of how exactly various members of the Bcl2 family execute and regulate apoptosis remains a subject of immense controversy, it is generally agreed that hetero-association between various members of the Bcl2 family is one of the defining events in the decision of a cell to live or die.

Despite their low sequence convergence, all members of Bcl2 family share a remarkably conserved 3D topological fold characterized by a central predominantly hydrophobic α-helical hairpin “dagger” (α5 and α6) surrounded by a “cloak” comprised of six amphipathic α-helices (α1-α4 and α7-α8) of varying lengths ([40]). Additionally, the effectors and repressors also contain a C-terminal hydrophobic α-helix termed α9, or more commonly the TM domain, because it allows these members of the Bcl2 family to localize to MOM upon apoptotic induction ([41, 42, 112]). The “cloak and dagger” structural topology of Bcl2 members is the hallmark of their functional duality in that they are able to co-exist as “soluble factors” under quiescent cellular state and as
“membrane channels” upon apoptotic induction. Notably, the hydrophobic dagger not only provides the bulk of the thermodynamic force in driving the water-membrane transition of various Bcl2 members upon apoptotic induction but also directly participates in the formation of mitochondrial pores that provide a smooth channel for the exit of apoptogenic factors. A prominent feature of repressors is that they contain what has come to be known as the “canonical hydrophobic groove”, formed by the juxtaposition of α2-α5 helices, that serves as the docking site for the BH3 domain (α2 helix) of activators and effectors. In a remarkable twist, the effectors also contain a hydrophobic groove for accommodating the BH3 domain of activators but this “pseudo hydrophobic groove”, formed by the juxtaposition of α1/α6 helices, is geographically distinct in that it is located on the face opposite to that occupied by the canonical hydrophobic groove in repressors (44-46). Surprisingly, in the case of Bax effector, the canonical hydrophobic groove is occupied by its C-terminal TM domain (α9 helix) in an intramolecular manner (113). The binding of activators via their BH3 domains to the pseudo hydrophobic groove within Bax is believed to disengage the TM domain allowing it to translocate to MOM in response to apoptotic signals (44-46). In a manner akin to the autoinhibition of Bax for mitochondrial translocation (113), the canonical hydrophobic groove within the BclW repressor is also not freely available but rather locked down through intramolecular binding of its TM domain (α9 helix) (47, 114). Subsequent binding of the BH3 domain of activators and effectors to the canonical hydrophobic groove within BclW is believed to displace the TM domain so as to allow it to translocate to MOM upon apoptotic induction and, in so doing, neutralize its anti-apoptotic activity (48). In an effort to further understand how the TM domain and MOM modulate the binding of BH3 ligands to
repressors, we set out here to analyze biophysical properties of full-length BclXL construct (BclXL FL) and a truncated BclXL construct (BclXL dTM) in which the TM domain has been deleted alone and their behaviors toward BH3 ligands in solution and in DMPC/DHPC bicelles mimicking MOM (Figure 3-1).

Our study reveals that ligand binding and membrane insertion compete with oligomerization of BclXL in solution. Of particular importance is the observation that such oligomerization is driven by the intermolecular binding of its C-terminal transmembrane (TM) domain to the canonical hydrophobic groove in a domain-swapped trans-fashion, whereby the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa. Binding of BH3 ligands to the canonical hydrophobic groove displaces the TM domain in a competitive manner allowing BclXL to dissociate into monomers upon hetero-association. Remarkably,
spontaneous insertion of BclXL into DMPC/DHPC bicelles results in a dramatic conformational change such that it can no longer recognize the BH3 ligands in what has come to be known as the “hit-and-run” mechanism. Collectively, our data suggest that oligomerization of a key apoptotic repressor serves as an allosteric switch that fine tunes its ligand binding and membrane insertion pertinent to the regulation of apoptotic machinery.

3.3 Experimental Procedures

3.3.1 Protein Preparation

BclXL_FL (residues 1-233) and BclXL_dTM (residues 1-200) constructs of human BclXL were cloned into pET30 bacterial expression vectors with an N-terminal His-tag using Novagen LIC technology (Figure 1a). The proteins were subsequently expressed in Escherichia coli BL21*(DE3) bacterial strain (Invitrogen) and purified on a Ni-NTA affinity column using standard procedures. Briefly, bacterial cells were grown at 20°C in Terrific Broth to an optical density of greater than unity at 600nm prior to induction with 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacterial culture was further grown overnight at 20°C and the cells were subsequently harvested and disrupted using a BeadBeater (Biospec). After separation of cell debris at high-speed centrifugation, the cell lysate was loaded onto a Ni-NTA column and washed extensively with 20mM imidazole to remove non-specific binding of bacterial proteins to the column. The recombinant proteins were subsequently eluted with 200mM imidazole and dialyzed against an appropriate buffer to remove excess imidazole. Further treatment on a Hiload Superdex 200 size-exclusion chromatography (SEC) column coupled in-line with GE Akta FPLC system led to purification of BclXL_FL and BclXL_dTM constructs to an
apparent homogeneity as judged by SDS-PAGE analysis. Final yield was typically between 10-20mg protein of apparent homogeneity per liter of bacterial culture. Protein concentration was determined by the fluorescence-based Quant-It assay (Invitrogen) and spectrophotometrically using extinction coefficients of 47,440 M\(^{-1}\)cm\(^{-1}\) and 41,940 M\(^{-1}\)cm\(^{-1}\) respectively calculated for the BclXL_FL and BclXL_dTM constructs using the online software ProtParam at ExPasy Server (115). Results from both methods were in an excellent agreement. 20-mer peptides spanning various BH3 domains within human Bid, Bad and Bax proteins were commercially obtained from GenScript Corporation. The sequences of these peptides are shown in Figure 1b. The peptide concentrations were measured gravimetrically. Mixed DMPC/DHPC bicelles were prepared in an appropriate buffer at a final concentration of 30mM, at DMPC to DHPC molar ratio of 1:2, by stirring for 2h at 37°C.

3.3.2 Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed on a TA Nano-DSC instrument and data were acquired and processed using the integrated NanoAnalyze software. All measurements were repeated at least three times. Briefly, samples of BclXL_FL and BclXL_dTM constructs alone, in the presence of Bid_BH3 peptide and in the presence of DMPC/DHPC bicelles were prepared in 50mM Sodium phosphate at pH 8.0. All experiments were conducted on 10-50\(\mu\)M of each protein construct alone or at 10-molar excess of Bid_BH3 peptide in the 40-120°C temperature range at a heating rate (dT/dt) of 1°C/min under an excess pressure of 3atm. The change in thermal power (dQ/dt) as a function of temperature was automatically recorded using the NanoAnalyze software. Control experiments on the buffer alone, in the presence of
Bid_BH3_peptide and in the presence of DMPC/DHPC bicelles were also conducted in an identical manner to generate baselines that were subtracted from the raw data to remove contribution due to the buffer and/or due to the peptide or bicelles. The raw data were further processed to yield the melting isotherms of excess heat capacity (C_p) as a function of temperature (T) using the following relationship:

\[
C_p = \frac{(dQ/dt)}{(dT/dt)PV}
\]  

[3-4]

where P is the initial concentration of protein loaded into the calorimetric cell and V is the effective volume of calorimetric cell (0.3ml).

### 3.3.3 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on a Microcal VP-ITC instrument and data were acquired and processed using the integrated Microcal ORIGIN software. All measurements were repeated at least three times. Briefly, samples of BclXL_FL and BclXL_dTM constructs and various BH3 peptides were prepared alone or in presence of DMPC/DHPC bicelles in 50mM Sodium phosphate, 100mM NaCl, 1mM EDTA and 5mM β-mercaptoethanol at pH 8.0. The experiments were initiated by injecting 25 x 10μl aliquots of 0.5-1mM of each BH3 peptide from the syringe into the calorimetric cell containing 1.8ml of 40-50 μM of BclXL_FL or BclXL_dTM construct at 25 °C. The change in thermal power as a function of each injection was automatically recorded using the ORIGIN software and the raw data were further processed to yield binding isotherms of heat release per injection as a function of molar ratio of each BH3 peptide to BclXL_FL or BclXL_dTM construct. The heats of mixing and dilution were subtracted from the heat of binding per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was titrated against each peptide in an
identical manner. To extract binding affinity \((K_d)\) and binding enthalpy \((\Delta H)\), the ITC isotherms were iteratively fit to the following built-in function by non-linear least squares regression analysis using the integrated ORIGIN software:

\[
q(i) = \frac{n\Delta HVP}{2} \left\{ \frac{1}{\left[1 + \frac{L}{nP} + \frac{K_d}{nP}\right]} - \left[\frac{1}{\left[1 + \frac{L}{nP} + \frac{K_d}{nP}\right]^2} - \frac{4L}{nP}\right]^{1/2}\right\} \tag{3-1}
\]

where \(q(i)\) is the heat release (kcal/mol) for the \(i\)th injection, \(n\) is the binding stoichiometry, \(V\) is the effective volume of protein solution in the calorimetric cell (1.46 ml), \(P\) is the total protein concentration in the calorimetric cell and \(L\) is the total concentration of peptide ligand added for the \(i\)th injection. Note that Eq [3-1] is derived from the binding of a ligand to a macromolecule using the law of mass action assuming a one-site model (116). The free energy change \((\Delta G)\) upon ligand binding was calculated from the relationship:

\[
\Delta G = RT \ln K_d \tag{3-2}
\]

where \(R\) is the universal molar gas constant (1.99 cal/K/mol) and \(T\) is the absolute temperature. The entropic contribution \((T\Delta S)\) to the free energy of binding was calculated from the relationship:

\[
T\Delta S = \Delta H - \Delta G \tag{3-3}
\]

where \(\Delta H\) and \(\Delta G\) are as defined above.

3.3.4 Steady-state fluorescence

Steady-state fluorescence (SSF) spectra were collected on a Jasco FP-6300 spectrofluorimeter using a quartz cuvette with a 10-mm pathlength at 25 °C. Briefly, experiments were conducted on 1-5\(\mu\)M of BclXL_FL or BclXL_dTM construct alone, in the presence of Bid_BH3 peptide (10-molar excess) and in the presence of DMPC/DHPC bicelles in 50mM Sodium phosphate, 100mM NaCl, 1mM EDTA and 5mM \(\beta\)-
mercaptoethanol at pH 8.0. The excitation wavelength was 290nm and emission was acquired over the 300-500nm wavelength range. All data were recorded using a 2.5-nm bandwidth for both excitation and emission. Data were normalized against reference spectra to remove the contribution of buffer, Bid_BH3 peptide or DMPC/DHPC bicelles.

3.3.5 Analytical light scattering

Analytical light scattering (ALS) experiments were conducted on a Wyatt miniDAWN TREOS triple-angle static light scattering detector and Wyatt QELS dynamic light scattering detector coupled in-line with a Wyatt Optilab rEX differential refractive index detector and interfaced to a Hiload Superdex 200 size-exclusion chromatography column under the control of a GE Akta FPLC system within a chromatography refrigerator at 10°C. The BclXL FL and BclXL_dTM constructs were prepared in 50mM Sodium phosphate, 100mM NaCl, 1mM EDTA and 5mM β-mercaptoethanol at pH 8.0 and loaded onto the column at a flow rate of 1ml/min and the data were automatically acquired using the ASTRA software. The starting concentrations of both protein constructs injected onto the column were between 10-50μM. The angular- and concentration-dependence of static light scattering (SLS) intensity of each protein construct resolved in the flow mode was measured by the Wyatt miniDAWN TREOS detector. The SLS data were analyzed according to the following built-in Zimm equation in ASTRA software (117, 118):

\[
[Kc/R_0] = \left(\frac{1}{M} + 2A_2c\right)\left[1 + \left(\frac{16\pi^2(R_g)^2}{3\lambda^2}\sin^2(\theta/2)\right)\right]
\]

where \(R_0\) is the excess Raleigh ratio due to protein in the solution as a function of protein concentration \(c\) (mg/ml) and the scattering angle \(\theta\) (42°, 90° and 138°), \(M\) is the observed molar mass of each protein species, \(A_2\) is the second virial coefficient, \(\lambda\) is the
wavelength of laser light in solution (658nm), $R_g$ is the radius of gyration of protein, and $K$ is given by the following relationship:

$$K = \frac{4\pi^2n^2(dn/dc)^2}{N_A\lambda^4}$$  \[3-6\]

where $n$ is the refractive index of the solvent, $dn/dc$ is the refractive index increment of the protein in solution and $N_A$ is the Avogadro's number ($6.02\times10^{23}$mol$^{-1}$). Under dilute protein concentrations ($c \to 0$), Eq [3-5] reduces to:

$$[Kc/R_g] = \frac{1}{M} + \left(\frac{16\pi^2(R_g)^2}{3M\lambda^2}\sin^2(\theta/2)\right)$$  \[3-7\]

Thus, a plot of $[Kc/R_0]$ versus $\sin^2(\theta/2)$ yields a straight line with slope $16\pi^2R_g^2/3M\lambda^2$ and y-intercept $1/M$. Accordingly, $M$ and $R_g$ were respectively obtained in a global analysis from the y-intercept and the slope of linear fits of a range of $[Kc/R_0]-\sin^2(\theta/2)$ plots as a function of protein concentration along the elution profile of each protein species using SLS measurements at three scattering angles. It should however be noted that $R_g$ was only determined for larger species that display angular-dependence of scattered light. Weighted-average molar mass ($M_w$) and number-average molar mass ($M_n$) were calculated from the following relationships:

$$M_w = \frac{\sum(c_iM_i)}{\sum c_i}$$  \[3-8\]

$$M_n = \frac{\sum c_i}{\sum(c_i/M_i)}$$  \[3-9\]

where $c_i$ is the protein concentration and $M_i$ is the observed molar mass at the $i$th slice within an elution profile. Likewise, $R_g$ reported here represents the weighted-average value as defined by the following expression:

$$R_g = \frac{\sum(c_iR_{g,i})}{\sum c_i}$$  \[3-10\]

where $c_i$ is the protein concentration and $R_{g,i}$ is the observed radius of gyration at the $i$th slice within an elution profile. The time- and concentration-dependence of dynamic light
scattering (DLS) intensity fluctuation of each protein construct resolved in the flow mode was measured by the Wyatt QELS detector positioned at 90° with respect to the incident laser beam. The DLS data were iteratively fit using non-linear least squares regression analysis to the following built-in equation in ASTRA software: software (119-121):

\[ G(\tau) = \alpha \text{Exp}(-2\Gamma\tau) + \beta \quad [3-11] \]

where \( G(\tau) \) is the autocorrelation function of dynamic light scattering intensity fluctuation \( I \), \( \tau \) is the delay time of autocorrelation function, \( \Gamma \) is the decay rate constant of autocorrelation function, \( \alpha \) is the initial amplitude of autocorrelation function at zero delay time, and \( \beta \) is the baseline offset (the value of autocorrelation function at infinite delay time). Thus, fitting the above equation to a range of \( G(\tau)-\tau \) plots as a function of protein concentration along the elution profile of each protein species computes the weighted-average value of \( \Gamma \) using DLS measurements at a scattering angle of 90°. Accordingly, the translational diffusion coefficient \( (D_t) \) of each protein species was calculated from the following relationship:

\[ D_t = \left[ (\Gamma\lambda^2)/(16\pi^2n^2\sin^2(\theta/2)) \right] \quad [3-12] \]

where \( \lambda \) is the wavelength of laser light in solution (658nm), \( n \) is the refractive index of the solvent and \( \theta \) is the scattering angle (90°). Additionally, the hydrodynamic radius \( (R_h) \) of each protein construct was determined from the Stokes-Einstein relationship:

\[ R_h = \left[ (k_BT)/(6\pi\eta D_t) \right] \quad [3-13] \]

where \( k_B \) is Boltzman’s constant (1.38x10^{-23}JK^{-1}), \( T \) is the absolute temperature and \( \eta \) is the solvent viscosity. Accordingly, the \( R_h \) reported here represents the weighted-average value as defined by the following expression:

\[ R_h = \sum(c_i R_{h,i})/\sum c_i \quad [3-14] \]
where $c_i$ is the protein concentration and $R_{h,i}$ is the observed hydrodynamic radius at the $i$th slice within an elution profile. It should be noted that, in both the SLS and DLS measurements, protein concentration ($c$) along the elution profile of each protein species was automatically quantified in the ASTRA software from the change in refractive index ($\Delta n$) with respect to the solvent as measured by the Wyatt Optilab rEX detector using the following relationship:

$$c = \frac{\Delta n}{dn/dc} \quad [3-15]$$

where $dn/dc$ is the refractive index increment of the protein in solution.

### 3.3.6 Circular dichroism

Far-UV circular dichroism (CD) measurements were conducted on a Jasco J-815 spectrometer thermostatically controlled at 25°C. Experiments were conducted on 1-5μM of BclXL FL or BclXL dTM construct alone, in the presence of Bid_BH3 peptide (10-molar excess) and in the presence of DMPC/DHPC bicelles in 10mM Sodium phosphate at pH 8.0. Data were collected using a quartz cuvette with a 2-mm pathlength in the 190-250nm wavelength range. Data were normalized against reference spectra to remove the contribution of buffer, Bid_BH3 peptide or DMPC/DHPC bicelles. Data were recorded with a slit bandwidth of 2nm at a scan rate of 10nm/min. Each data set represents an average of four scans acquired at 0.1nm intervals. Data were converted to molar ellipticity, $[\theta]$, as a function of wavelength ($\lambda$) of electromagnetic radiation using the equation:

$$[\theta] = \frac{10^5 \Delta \varepsilon}{cl} \text{deg.cm}^2\text{.dmol}^{-1} \quad [3-16]$$

where $\Delta \varepsilon$ is the observed ellipticity in mdeg, $c$ is the peptide or protein concentration in μM and $l$ is the cuvette pathlength in cm.
3.3.7 Transmission electron microscopy

Transmission electron microscopy (TEM) experiments were conducted on a Philips CM-10 electron microscope operating at a voltage of 80kV and images were photographed at a magnification of 105,000. Data were collected on a 25μM of BclXL_FL construct alone or in the presence of 10-molar excess of Bid_BH3 peptide in 50mM Sodium phosphate at pH 8.0 using negative staining. Briefly, formvar-coated copper grids (150-mesh) were floated on drops of each sample for 2 min. After briefly drying on a filter paper, the grids were immediately placed on drops of 2% phosphotungstic acid at pH 7.3 for 5 min. Excess liquid was wicked away with a filter paper and the grids were dried under vacuum desiccator for 3 days prior to imaging.

3.3.8 Molecular modeling

Molecular modeling (MM) was employed to build structural models of BclXL in various conformations using the MODELLER software based on homology modeling in combination with MOLMOL (122, 123). Briefly, the structures modeled were those of BclXL monomers in which the TM domain is either exposed to solution (BclXL_solTM) or occupies the canonical hydrophobic groove (BclXL_cisTM) as well as the BclXL homodimer in which the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in a domain-swapped trans-fashion (BclXL_transTM). In each case, solution structures of truncated BclXL in which the TM domain and the α1-α2 loop are missing (PDB# 1BXL), hereinafter referred to as tBclXL, and the full-length Bax in which the TM domain occupies the canonical hydrophobic groove (PDB# 1F16) were used as templates. More specifically, the entire models of BclXL in various conformations were built in homology with
tBclXL (PDB# 1BXL) except for the TM domain, which was built in homology with the TM domain of Bax (PDB# 1F16), in a multiple-template alignment manner. Additionally, MOLMOL was used to bring various parts and/or monomers into optimal spatial orientations relative to each other in a rigid-body fashion. For the structural model of BclXL_solTM, the TM domain of Bax (PDB# 1F16) was dislodged away from the canonical hydrophobic groove so as to expose it to solution using MOLMOL prior to homology modeling in combination with tBclXL (PDB# 1BXL) in MODELLER. For the structural model of BclXL_cisTM, the TM domain of Bax (PDB# 1F16) was not physically perturbed from the canonical hydrophobic groove prior to homology modeling in combination with tBclXL (PDB# 1BXL) in MODELLER. In structural models of both BclXL_solTM and BclXL_cisTM, the residues within the α1-α2 loop were modeled without a template through energy minimization and molecular dynamics simulations. For the structural model of BclXL_transTM, pre-built structural models of two individual monomers of BclXL_cisTM were brought together in an optimal orientation in MOLMOL such that the α8-α9 loop within one monomer could be domain-swapped with TM domain of the other monomer without becoming taut. This requirement led to roughly parallel orientation of TM domains such that the sidechain moieties of apolar residues facing outward from the TM domain within one monomer were placed within van der Waals contact distance of sidechain moieties of apolar residues facing outward from the TM domain of the other monomer. Next, the α8-α9 loop preceding the TM domain within each BclXL_cisTM monomer was excised out and the resulting BclXL_cisTM monomers were used as a template to homology model the structure of BclXL_transTM. Notably, the residues within the α8-α9 loop within the BclXL_transTM
structural model were modeled without a template through energy minimization and molecular dynamics simulations. For each structural model, a total of 100 atomic models were calculated and the structure with the lowest energy, as judged by the MODELLER Objective Function, was selected for further analysis. The atomic models were rendered using RIBBONS (124).

3.3.9 Molecular dynamics

Molecular dynamics (MD) simulations were performed with the GROMACS software (99, 125) using the integrated OPLS-AA force field (126, 127). Briefly, the modeled structures of BclXL in various conformations (BclXL_solTM, BclXL_cisTM and BclXL_transTM) were centered within a cubic box and hydrated using the extended simple point charge (SPC/E) water model (128, 129). The hydrated structures were energy-minimized with the steepest descent algorithm prior to equilibration under the NPT ensemble conditions, wherein the number of atoms (N), pressure (P) and temperature (T) within the system were respectively kept constant at ~50000, 1 bar and 300 K. The Particle-Mesh Ewald (PME) method was employed to compute long-range electrostatic interactions with a 10Å cut-off (130) and the Linear Constraint Solver (LINCS) algorithm to restrain bond lengths (131). All MD simulations were performed under periodic boundary conditions (PBC) using the leap-frog integrator with a time step of 2fs. For the final MD production runs, data were collected every 10ps over a time scale of 100ns.
3.4 Results and discussion

3.4.1 TM modulates the binding of BH3 ligands to BclXL

To shed light on the role of TM domain in modulating the binding of BH3 ligands to BclXL, we conducted ITC analysis on BclXL_FL and BclXL_dTM constructs using BH3 peptides derived from Bid and Bad activators and the Bax effector — the three well-characterized physiological ligands of BclXL repressor (25, 108, 109). Figure 3-2 provides representative ITC data for the binding of Bid_BH3 peptide to BclXL_FL and BclXL_dTM constructs, while detailed thermodynamic parameters accompanying the binding of all BH3 peptides are shown in Table 3-1. It is evident from our data that the BH3 peptides bind to the BclXL_dTM construct with affinities that are more than an order of magnitude greater than those observed for their binding to the BclXL_FL construct. That this is so strongly suggests that the TM domain in BclXL is not freely exposed to solution but rather associates with the rest of the protein in a manner that inhibits the binding of BH3 ligands. In light of the knowledge that the TM domain of BclW repressor occupies the canonical hydrophobic groove (47, 48, 114), it can be argued that a similar scenario prevails in the case of BclXL and that the binding of BH3 ligands competes with the dissociation of TM domain from the canonical hydrophobic groove.

In addition to dramatic differences observed in the binding affinities of various BH3 peptides toward BclXL_FL and BclXL_dTM constructs, their intermolecular association is also marked by distinct underlying thermodynamic forces. Thus, while binding of various BH3 ligands to BclXL_FL construct is predominantly driven by favorable enthalpic factors accompanied by entropic penalty, binding to BclXL_dTM construct is
Figure 3-2: ITC analysis for the binding of Bid_BH3 peptide to BclXL_FL (a) and BclXL_dTM (b) constructs. The upper panels show raw ITC data expressed as change in thermal power with respect to time over the period of titration. In the lower panels, change in molar heat is expressed as a function of molar ratio of Bid_BH3 peptide to the corresponding construct. The solid lines in the lower panels show the fit of data to a one-site model, as embodied in Eq [3-1], using the ORIGIN software. The insets show same titrations conducted in the presence of DMPC/DHPC bicelles.

favored by both enthalpic and entropic changes (Table 3-1). These salient observations indicate that the solvation of hydrophobic TM domain following the recruitment of BH3 ligands by the canonical hydrophobic groove most likely mitigates the conformational entropy of BclXL. We believe that such loss in conformational dynamics may aid or prime BclXL for subsequent insertion into MOM so as to allow it to interfere with the formation of mitochondrial pores critical for the release of apoptogenic factors into the cytosol. Our data exquisitely illustrate how thermodynamics may gauge the decision of a cell to live or die. Importantly, previous studies suggest that upon insertion into MOM, repressors undergo substantial conformational change and lose their ability to hold onto BH3 ligands in what has been termed the “hit-and-run” mechanism (114, 132-134). To test the validity of this hypothesis further, we also measured the binding of various BH3 peptides to BclXL_FL and BclXL_dTM constructs pre-equilibrated
Table 3-1
Thermodynamic parameters for the binding of various BH3 peptides to BclXL_FL and BclXL_dTM constructs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_d$ / $\mu$M</th>
<th>$\Delta H$ / kcal.mol$^{-1}$</th>
<th>$\Delta S$ / kcal.mol$^{-1}$</th>
<th>$\Delta G$ / kcal.mol$^{-1}$</th>
<th>$K_d$ / $\mu$M</th>
<th>$\Delta H$ / kcal.mol$^{-1}$</th>
<th>$\Delta S$ / kcal.mol$^{-1}$</th>
<th>$\Delta G$ / kcal.mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bid_BH3</td>
<td>9.97 ± 1.25</td>
<td>-18.74 ± 0.19</td>
<td>-11.91 ± 0.17</td>
<td>-6.82 ± 0.01</td>
<td>0.79 ± 0.08</td>
<td>-6.45 ± 0.07</td>
<td>-1.88 ± 0.03</td>
<td>-8.32 ± 0.04</td>
</tr>
<tr>
<td>Bad_BH3</td>
<td>10.30 ± 1.42</td>
<td>-14.19 ± 0.08</td>
<td>-7.37 ± 0.04</td>
<td>-6.81 ± 0.04</td>
<td>0.89 ± 0.12</td>
<td>-7.84 ± 0.14</td>
<td>+0.46 ± 0.11</td>
<td>-8.26 ± 0.02</td>
</tr>
<tr>
<td>Bax_BH3</td>
<td>35.10 ± 3.72</td>
<td>-19.40 ± 0.30</td>
<td>-13.31 ± 0.25</td>
<td>-6.08 ± 0.06</td>
<td>3.25 ± 0.27</td>
<td>-5.55 ± 0.07</td>
<td>+1.94 ± 0.08</td>
<td>-7.50 ± 0.01</td>
</tr>
</tbody>
</table>

All parameters were obtained from ITC measurements at pH 8.0 and 25°C. All binding stoichiometries were 1:1 and generally agreed to within ±10%. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation.

with DMPC/DHPC bicelles as a mimetic for MOM using ITC (Figure 3-2). Our data reveal that the BH3 peptides do not recognize BclXL within bicelles in the presence or absence of TM domain and thereby further corroborate the hit-and-run model of the binding of repressors to their BH3 ligands preceding their insertion into MOM.

Given that we have relied here on isolated BH3 peptides to mimic intact Bid, Bad and Bax, caution is warranted in that the BH3 domains may depart from their physiological behavior when treated as isolated peptides due to the loss of local conformational constraints that they may be subject to in the context of full-length proteins. Nonetheless, it is well-documented that Bid, Bad and Bax interact with apoptotic repressors primarily through their BH3 domains. Additionally, binding of BH3 peptides to apoptotic repressors with high-affinity and specificity as observed here and elsewhere argues strongly in support of BH3 peptides as bona fide models of intact proteins from which they are derived (135-137). We also note that the BH3 domain of Bid binds to apoptotic repressors only upon cleavage of N-terminal region of the protein (138). In short, the use of isolated BH3 peptides here in lieu of full-length proteins is well
justified and our data presented above are likely to be of physiological relevance.

3.4.2 BclXL associates into higher-order oligomers

Our data presented above suggest strongly that the TM domain competes with the binding of BH3 ligands by virtue of its ability to bind the canonical hydrophobic groove. However, unlike the association of TM domain of BclW repressor and Bax effector via an intramolecular cis-fashion (47, 113, 114), the possibility that BclXL may associate in an intermolecular trans-fashion by virtue of its TM domain so as to form domain-swapped dimers cannot be excluded. To test this notion, we next conducted ALS analysis on BclXL_FL and BclXL_dTM constructs and quantified various physical parameters accompanying the behavior of these protein constructs in solution from the first principles of hydrodynamics without any assumptions (Figure 3-3 and Table 3-2). Remarkably, our data show that while BclXL_FL construct predominantly associates into higher-order oligomers that we herein refer to as multimer (~300kD) and polymer (~3000kD), the BclXL_dTM construct is largely monomeric in solution. This strongly implicates the involvement of TM domain in mediating the formation of domain-swapped dimers of BclXL_FL that further associate into larger oligomeric species. In light of our ITC data presented above, we believe that such oligomerization likely serves as an auto-inhibitory allosteric switch under quiescent cellular state. However, upon the induction of apoptosis, the rising cellular levels of BH3 ligands in the form of activators compete with oligomerization of BclXL so as to dislodge the TM domain from the canonical hydrophobic groove and thereby initiating its translocation into MOM, requisite for its anti-apoptotic behavior. Although a minor fraction of both BclXL_FL

BclXL_dTM constructs is also observed as a dimer, we believe that these homo-
Figure 3-3: ALS analysis for BclXL_dTM and BclXL_FL constructs as indicated. (a) Elution profiles as monitored by the differential refractive index (Δn) plotted as a function of elution volume (V) for BclXL_FL (top panel) and BclXL_dTM (bottom panel) constructs. Note that the elution profile for BclXL_FL construct is shown at both 50μM (black) and 10μM (red) initial protein concentrations loaded onto the Superdex-200 column, while that for BclXL_dTM construct is only shown at 50μM (black). (b) Partial Zimm plots obtained from analytical SLS measurements at a specific protein concentration for BclXL_FL polymer (top panel) and BclXL_dTM monomer (bottom panel). The solid lines through the data points represent linear fits. (c) Autocorrelation function plots obtained from analytical DLS measurements at a specific protein concentration for BclXL_FL polymer (top panel) and BclXL_dTM monomer (bottom panel). The solid lines through the data points represent non-linear least squares fits to Eq [3-11].

dimers are physically-distinct. BclXL_FL dimer is most likely constructed through TM-swapping, such that the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice-versa in an intermolecular trans-fashion, in agreement with our observations that the binding of BH3 ligands to the canonical hydrophobic groove is compromised in the context of full-length BclXL (Table 3-1). This notion is also consistent with previous studies in which the TM domain was shown to promote homodimerization of full-length BclXL within live cells (133, 139). In contrast, the formation of BclXL_dTM dimer is most probably driven through inter-monomer swapping of α6-α8 helices such that the canonical hydrophobic grooves within each monomer remain fully exposed to solution and are available for the binding of BH3
Table 3-2
Comparison of hydrodynamic parameters for BCLXL_dTM and BclXL_FL constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Associativity</th>
<th>M_w / kD</th>
<th>M_n / kD</th>
<th>M_w/M_n</th>
<th>R_g / Å</th>
<th>R_h / Å</th>
<th>R_g/R_h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BclXL_dTM</td>
<td>Monomer</td>
<td>28 ± 2</td>
<td>28 ± 2</td>
<td>1.00 ± 0.00</td>
<td>ND</td>
<td>33 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>54 ± 2</td>
<td>54 ± 2</td>
<td>1.00 ± 0.01</td>
<td>ND</td>
<td>41 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>BclXL_FL</td>
<td>Monomer</td>
<td>31 ± 1</td>
<td>31 ± 1</td>
<td>1.00 ± 0.01</td>
<td>ND</td>
<td>36 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>62 ± 3</td>
<td>61 ± 3</td>
<td>1.01 ± 0.01</td>
<td>ND</td>
<td>47 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Multimer</td>
<td>361 ± 26</td>
<td>321 ± 25</td>
<td>1.14 ± 0.02</td>
<td>107 ± 4</td>
<td>99 ± 2</td>
<td>1.10 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Polymer</td>
<td>3355 ± 188</td>
<td>3134 ± 147</td>
<td>1.17 ± 0.02</td>
<td>198 ± 8</td>
<td>175 ± 6</td>
<td>1.13 ± 0.02</td>
</tr>
</tbody>
</table>

All parameters were obtained from ALS measurements at pH 8 and 10°C. Note that the calculated molar masses of recombinant BclXL_dTM and BclXL_FL constructs from their respective amino acid sequences are 27kD and 31kD, respectively. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation. Note that the R_g parameter could not be determined (ND) for various species due to their lack of angular-dependence of scattered light.

ligands without any restriction as reported earlier in the case of BclXL and BclW constructs in which the TM domain has been truncated (77, 140, 141).

It should also be noted here that when SEC-resolved fractions containing the BclXL_FL monomer and dimer were re-analyzed on SEC column, both the oligomeric species re-appeared in the elution profile. Likewise, re-analysis of SEC-resolved fractions containing the BclXL_FL higher-order oligomers on SEC column also resulted in the appearance of monomeric and dimeric species. Taken collectively, these salient observations suggest strongly that BclXL_FL exists in a reversible monomer-dimer-multimer-polymer equilibrium. The fact that such equilibrium prevails even at lower concentrations of BclXL argues strongly that the ability of BclXL to undergo oligomerization in solution is likely to be physiologically-relevant. In an attempt to gain insights into the conformational heterogeneity of the oligomeric species of BclXL, we also determined the M_w/M_n and R_g/R_h ratios from our hydrodynamic data (Table 3-2).
Figure 3-4: TEM micrographs of negatively-stained BclXL_FL construct alone (a) and in the presence of Bid_BH3 peptide (b).

While the $M_w/M_n$ ratio provides a measure of the macromolecular polydispersity, the $R_g/R_h$ ratio sheds light on the overall macromolecular shape. Our data suggest that while the higher-order oligomers of BclXL display some degree of polydispersity ($M_w/M_n > 1.05$), the monomeric and dimeric forms of BclXL are predominantly monodisperse ($M_w/M_n < 1.05$). Additionally, the higher-order oligomers of BclXL most likely adopt an elongated rod-like shape ($R_g/R_h > 1$) in lieu of a more spherical or disc-like architecture.

That this is so was further confirmed by TEM analysis (Figure 3-4). Thus, while BclXL_FL alone exudes rod-like appearance in solution, addition of Bid_BH3 peptide is concomitant with the disappearance of these rod-like structures, implying that ligand binding most likely results in the dissociation of higher-order oligomers into monomers in agreement with our ITC data.
3.4.3 Ligand binding and membrane insertion modulate thermodynamic stability of BclXL

In light of our data presented above, we next wondered whether the ability of full-length BclXL to associate into higher-order oligomeric species is a manifestation of its enhanced stability and to what extent such thermodynamic advantage may be modulated by ligand binding and membrane insertion. Toward this goal, we conducted DSC analysis on BclXL_FL and BclXL_dTM constructs alone, in the presence of Bid_BH3 peptide and in the presence of DMPC/DHPC bicelles (Figure 3-5). Our analysis suggests that BclXL_FL is significantly more stable than BclXL_dTM. Thus, while the unfolding of BclXL_dTM is accompanied by a melting temperature (T_m) of 72°C, unfolding of BclXL_FL is not observed even when the melting temperature is raised to 120°C (Figures 3-5a and 3-5b). Addition of Bid_BH3 peptide to BclXL_dTM construct raises its T_m value to 78°C, implying that ligand binding enhances the stability of BclXL. In light of our ITC and TEM analysis, binding of BH3 peptide to BclXL_FL might be expected to destabilize this construct. Yet, no thermal melting of liganded BclXL_FL construct is observed in the 40-120°C temperature range in a manner akin to the unliganded BclXL_FL construct. This observation suggests that the liganded BclXL_FL construct is significantly more stable than liganded BclXL_dTM. Surprisingly, the behavior of BclXL_FL and BclXL_dTM constructs within bicelles is mirrored to that observed in solution. Thus, while no thermal melting of BclXL_dTM construct within bicelles is observed in the 40-120°C temperature range, the melting of BclXL_FL construct within bicelles is characterized by three distinct thermal phases, with T_m values of 78°C, 89°C and 96°C. We attribute such multi-phasic thermal transition of BclXL_FL within bicelles to the dissociation of an higher-order oligomer into dimeric and
monomeric species prior to melting. Notably, these observations are not affected when DSC analysis is conducted on BclXL_FL construct at lower protein concentrations (Figure 3-5c), implying that the thermal behavior of BclXL within both solution and bicelles is likely to be of physiological relevance.

Taken collectively, our data suggest that although the BclXL_FL construct exists as an oligomer within bicelles, the physical basis of such oligomerization is likely to be distinct from that observed in solution and thereby supporting the notion that BclXL undergoes conformational change upon membrane insertion in agreement with previous reports (60, 134, 142). Importantly, our data also suggest that although the BclXL_dTM construct is predominantly monomeric in solution, it undergoes oligomerization within bicelles to such an extent that it is stable up to a temperature of 120°C. This observation is in agreement with the view that the TM domain is not critical for the insertion of apoptotic repressors into membranes (134, 142, 143). However, the observation that the
BclXL_dTM oligomers within membranes are more stable than the BclXL_FL oligomers is being reported here for the first time. In light of this novel finding, we hypothesize that the TM domain may not only play a role in regulating ligand binding but that it may also control the degree of BclXL oligomerization within membranes. Importantly, it has been suggested that the TM domain targets BclXL to MOM as opposed to other intracellular membranes (144). Thus, although the lack of TM domain may facilitate oligomerization of BclXL within membranes, the TM domain may play a key role in its correct intermembraneous localization as well as oligomerization relevant to its anti-apoptotic function.

3.4.4 BclXL undergoes tertiary and quaternary structural changes upon ligand binding and membrane insertion

It is believed that apoptotic repressors undergo substantial conformational changes upon insertion into membranes (134, 142). In order to elucidate the role of TM domain in dictating such conformational changes within BclXL upon ligand binding and membrane insertion, we measured SSF spectra of BclXL_FL and BclXL_dTM constructs alone, in the presence of Bid_BH3 peptide and in the presence of DMPC/DHPC bicelles (Figure 3-6). It is important to note that intrinsic protein fluorescence, largely due to tryptophan residues, is influenced by changes in the local environment and thus serves as a sensitive probe of overall conformational changes within proteins. This is further aided by the fact that BclXL_FL and BclXL_dTM constructs respectively contain seven and six tryptophan residues positioned at various strategic positions to monitor conformational changes occurring at both the intramolecular and intermolecular level. Strikingly, our data show that the intrinsic fluorescence of BclXL_FL construct is much higher than that observed for the BclXL_dTM construct (Figures 3-6a and 3-6b). This most likely arises
due to the interfacial burial of solvent-exposed tryptophans on the protein surface upon intermolecular association of BclXL_FL into higher-order oligomers. This is further evidence for the propensity of BclXL_FL construct to undergo oligomerization in solution.

Strikingly, although binding of Bid_BH3 peptide to both BclXL_FL and BclXL_dTM constructs results in the quenching of intrinsic fluorescence, the magnitude of such quenching is much larger for the BclXL_FL construct. These findings suggest that the binding of Bid_BH3 peptide to BclXL_FL construct is coupled to its dissociation into monomers, or in statistical terms, shifts the equilibrium in favor of monomers. In striking contrast to ligand binding, insertion of both BclXL_FL and BclXL_dTM constructs into bicelles results in the enhancement of intrinsic fluorescence in agreement with the overall movement of tryptophan residues from a polar environment to a more hydrophobic milieu. However, the extent of such fluorescence enhancement is much larger for the BclXL_FL construct versus the BclXL_dTM construct. This implies that
although both constructs undergo conformational changes upon insertion into bicelles, the BclXL_FL construct does so more dramatically and possibly resulting in its quaternary structural rearrangement in addition to tertiary structural changes. This salient observation thus further corroborates our DSC data where both BclXL_FL and BclXL_dTM constructs appear to form distinct oligomers within bicelles. Importantly, SEC-resolved fractions containing higher-order oligomers of BclXL_FL construct appear to behave very similar to non-resolved BclXL_FL in solution and within bicelles (Figures 3-6a and 3-6c), implying that the oligomers rapidly re-equilibrate in agreement with our ALS analysis.

3.4.5 BclXL undergoes secondary structural changes upon ligand binding and membrane insertion

To probe secondary structural changes upon ligand binding and membrane insertion, we next measured and compared far-UV CD spectra of BclXL_FL and BclXL_dTM constructs alone, in the presence of Bid_BH3 peptide and in the presence of DMPC/DHPC bicelles (Figure 3-7). Consistent with our SSF analysis above, both BclXL_FL and BclXL_dTM constructs display spectral features in the far-UV region characteristic of an $\alpha$-helical-fold with bands centered around 208nm and 222nm (Figures 3-7a and 3-7b). Upon the addition of Bid_BH3 peptide, there is a noticeable increase in the far-UV spectral intensities of 208-nm and 222-nm bands within both constructs but more so in the case of BclXL_FL, implying that ligand binding is coupled to secondary structural changes. The nature of such increase in $\alpha$-helicity is not clear but it is possible that this increase is in part due to the coil-helix transition of the BH3 peptide upon binding. Interestingly, the nature of secondary structural changes observed upon the addition of bicelles appears somewhat distinct then observed upon ligand binding in both
Figure 3-7: Far-UV CD spectra of BclXL_FL construct at 5μM (a), BclXL_dTM construct at 5μM (b) and SEC-resolved fractions containing higher-order oligomers of BclXL_FL at 1μM (c) alone (black), in the presence of excess Bid_BH3 peptide (red) and in the presence of excess DMPC/DHPC bicelles (green). Thus, while the 208-nm band increases in intensity in the presence of bicelles, the 222-nm band undergoes reduction. Notably, SEC-resolved fractions containing the higher-order oligomers of BclXL_FL construct appear to behave very similar to non-resolved BclXL_FL in solution and within bicelles (Figures 3-7a and 3-7c), implying that the oligomers rapidly re-equilibrate in agreement with our ALS analysis. Taken together, our data suggest that ligand binding and membrane insertion of both BclXL_FL and BclXL_dTM constructs is coupled to secondary structural changes though the precise nature of such structural perturbations remains uncertain.

3.4.6 Structural models provide physical basis of oligomerization of BclXL

In an effort to understand the physical basis of oligomerization of full-length BclXL, we built 3D atomic models of BclXL monomers in which the TM domain is either exposed to solution (BclXL_solTM) or occupies the canonical hydrophobic groove (BclXL_cisTM) as well as the BclXL homodimer in which the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice
versa in a domain-swapped trans-fashion (BclXL_transTM) (Figure 3-8). It is noteworthy that these structural models were derived from the known solution structures of truncated BclXL, in which the TM domain and the α1-α2 loop are missing, and the full-length Bax in which the TM domain occupies the canonical hydrophobic groove (113, 145).

As discussed earlier, the topological fold of BclXL is comprised of a central predominantly hydrophobic α-helical hairpin dagger (α5 and α6) surrounded by a cloak comprised of six amphipathic α-helices (α1-α4 and α7-α8) of varying lengths. Additionally, the C-terminal hydrophobic TM domain (helix α9) may in principle adopt one of the following three conformations: The TM domain may be exposed to solution as depicted in the BclXL_solTM model (Figure 3-8a). However, given its predominantly hydrophobic nature, the TM domain is likely to become unfolded in solution while its association with the canonical hydrophobic groove, formed by the juxtaposition of α2-α5 helices, would be thermodynamically favorable as shown in the BclXL_cisTM model (Figure 3-8b). Such an intramolecular association of TM domain has indeed been previously reported in the case of BclW repressor and Bax effector (47, 113, 114).

Disordered regions such as loops within proteins interconnecting α-helices or β-strands have come to prominence over the past decade or so in their ability to modulate protein structure and function (146-150). Strikingly, the α8-α9 loop preceding the TM domain in BclXL is much longer than that found in Bax effector, while the anomalously long α1-α2 loop (~60 residues) in BclXL is relatively short in both the BclW repressor and the Bax effector. This raises the possibility that the TM domain in BclXL may not only associate with the rest of the protein in an intramolecular cis-manner but rather intermolecular association in a trans-fasion through TM-swapping may be a preferred alternative as
Figure 3-8: Structural models of full-length BclXL in three distinct conformations with respect to the C-terminal TM domain (α9 helix). (a) Monomeric BclXL with the TM domain exposed to solution (BclXL_solTM). (b) Monomeric BclXL with the TM domain bound to the canonical hydrophobic groove (BclXL_cisTM). (c) Homodimeric BclXL with the TM domain bound to the canonical hydrophobic groove but swapped in an intermolecular trans-fashion — the TM domain of one monomer (green) is bound to the other monomer (blue) and vice versa (BclXL_transTM).

suggested by the BclXL_transTM model (Figure 3-8c). This latter notion is not only supported by cell-based studies on full-length BclXL (133, 139), but would also provide
a physical route for the oligomerization of BclXL into higher-order oligomers reported here for the first time. Importantly, our BclXL_transTM model suggests that homodimerization, with the monomers related by a two-fold axis of symmetry, would lead to further thermodynamic stabilization of TM domains. Thus, roughly parallel orientation of TM domains within BclXL_transTM dimer would allow sidechain moieties of apolar residues facing outward from the TM domain within one monomer to engage in van der Waals contacts with sidechain moieties of apolar residues facing outward from the TM domain of the other monomer in a manner akin to hydrophobic interactions stabilizing leucine zippers (151). Prevalence of such additional favorable interactions would clearly favor the docking of TM domain to the canonical hydrophobic groove via a trans-mechanism over intramolecular association.

3.4.7 MD simulations support dimerization of BclXL through domain-swapping

Our structural models of full-length BclXL presented above suggest strongly that the BclXL_transTM homodimeric conformation would be the most preferable in solution and, that the α1-α2 and α8-α9 loops may play an active role in driving such homodimerization. To further test the validity of our structural models and to gain insights into macromolecular dynamics, we next conducted MD simulations over tens of nanoseconds (Figure 3-9) the time regime over which macromolecular motions such as conformational fluctuations and intermolecular movements relevant to their biological function occur. As shown in Figure 3-9a, the MD trajectories reveal that all three conformations of BclXL (BclXL_solTM, BclXL_cisTM and BclXL_transTM) reach structural equilibrium after about 20ns with an overall root mean square deviation (RMSD) between 5-10Å. To understand the rather low stability of these conformations,
we deconvoluted the overall RMSD for the full-length (FL) BclXL spanning residues 1-233 into three constituent regions: (i) the central core (CC) region spanning residues 86-195; (ii) the N-terminal (NT) region, containing the α1 helix (BH4 domain) and the α1-α2 loop, spanning residues 1-85; and (iii) the C-terminal (CT) region, containing the α9 helix (TM domain) and the α8-α9 loop, spanning residues 196-233. To our surprise, we noticed that the overwhelming protein flexibility in all three conformations largely resides in the NT and CT regions, while the CC region displays a very high degree of order with little internal motions. However, the conformational dynamics of the NT and CT regions display discernable differences within the three distinct conformations of BclXL. In the case of BclXL_solTM conformation, both NT and CT regions remain highly mobile, reflecting in part the thermodynamically unfavorable solvation of the hydrophobic TM domain, which also appears to undergo unfolding during the course of MD trajectory. Interestingly, while the NT region remains relatively mobile in both BclXL_cisTM and BclXL_transTM conformations in a manner akin to its mobility observed within BclXL_solTM, the CT region experiences substantial loss of conformational dynamics which can be attributed to the stabilization of the TM domain by the canonical hydrophobic groove either in an intramolecular manner (BclXL_cisTM) or via domain-swapping (BclXL_transTM). Importantly, the CT region appears to be less mobile and more ordered over the course of MD trajectory within BclXL_transTM relative to its mobility within the BclXL_cisTM conformation, arguing in favor of greater stability of homodimeric versus monomeric conformation. An alternative means to assess mobility and stability of macromolecular complexes is through an assessment of the root mean square fluctuation (RMSF) of specific atoms over the course of MD
Figure 3-9: MD analysis on structural models of full-length BclXL in three distinct conformations with respect to the C-terminal TM domain (α9 helix). (a) Root mean square deviation (RMSD) of backbone atoms (N, Cα and C) for residues 1-233 (black), residues 86-195 (red), residues 1-85 (green) and residues 196-233 (blue) within each simulated structure relative to the initial modeled structure of BclXL_solTM, BclXL_cisTM and BclXL_transTM as a function of simulation time. Note that, for each construct, the RMSD of full-length (FL) protein spanning residues 1-233 is also deconvoluted into the central core (CC) region spanning residues 86-195, the N-terminal (NT) region spanning residues 1-85, and the C-terminal (CT) region spanning residues 196-233. (b) Root mean square fluctuation (RMSF) of backbone atoms (N, Cα and C) averaged over the entire course of corresponding MD trajectory of BclXL_solTM, BclXL_cisTM and BclXL_transTM as a function of residue number.

In light of this observation, we believe that the intrinsic flexibility of the α1-α2 loop may be a driving force for the homodimerization of BclXL through favorable entropic contributions and that such intermolecular association may provide a thermodynamic bottle-neck for it to switch to an active conformation. Post-translational phosphorylation of BclXL may induce conformational changes within the α1-α2 loop
that lead to its ordering and thereby remove the bottle-neck promoting its homodimerization and subsequently shifting the equilibrium in favor of monomeric conformation that exudes higher anti-apoptogenicity.

Unlike the enhanced mobility of $\alpha_1-\alpha_2$ loop within BclXL_transTM, the ordering of the $\alpha_8-\alpha_9$ loop appears to provide a mechanism for greater stabilization of TM within BclXL_transTM compared to BclXL_cisTM conformation as evidenced by the RMSF of residues located within the TM domain (Figure 3-9b). Taken together, our MD simulations suggest that the dimeric BclXL_transTM conformation is more stable than either of the monomeric conformations and thereby further support the notion that domain-swapped homodimerization likely plays a key role in the intermolecular association of BclXL into higher-order oligomers.

3.5 Concluding remarks

Despite their discovery more than two decades ago (152-156), members of the Bcl2 family have not been extensively studied using biophysical tools. In particular, previous biophysical and structural studies on BclXL and Bcl2 repressors have heavily relied on truncated constructs devoid of both the structurally-disordered $\alpha_1-\alpha_2$ loop and the functionally-critical TM domain (39, 40, 137). Although the view that structure dictates protein function has been the holy grail of structural biology over the past century, the notion that structurally-disordered regions may also represent hot spots of protein function would have been perceived blasphemous even a decade ago. However, it is now rapidly becoming clear that structurally-disordered regions within proteins hold critical clues to their functional diversity and, in particular, their tight regulation (146-150).
In light of the aforementioned arguments, we undertook here detailed biophysical analysis of the full-length BclXL and investigated the role of the TM domain in dictating structure-function relationships within this important member of Bcl2 family. Our studies reveal for the first time that BclXL displays a high propensity to associate into higher-order oligomers that are likely to be of physiological relevance. In particular, oligomerization of BclXL appears to be driven through domain-swapping such that the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in a trans-fashion. Over the past decade or so, homodimerization of proteins through domain-swapping has emerged as a common mechanism for protein oligomerization (157-162). From a thermodynamic standpoint, such intermolecular association would allow two participating monomers to bury additional surface area culminating in not only enhanced stability but also providing a greater interacting molecular surface for further oligomerization (Figure 3-10a). We believe that such a mechanism also promotes the intermolecular association of BclXL homodimers into higher-order oligomers. Nonetheless, our in vitro and in silico analysis does not exclude the possibility that BclXL oligomerization may also ensue through an alternative interlocking mechanism (Figure 3-10b), whereby the TM domain of one monomer locks onto the canonical hydrophobic groove of another monomer in a head-to-tail fashion in a manner akin to actin polymerization (163). Regardless of the precise mechanism, BclXL oligomerization reported here appears to play a key role in fine-tuning its anti-apoptotic action by virtue of its ability to regulate ligand binding and membrane insertion.

Consistent with this notion, truncation of TM domain completely abolishes oligomerization of BclXL and the resulting truncated construct exudes biophysical
Figure 3-10: Models for BclXL oligomerization and its role in apoptotic regulation. (a) Oligomerization of BclXL via a domain-swapped mechanism. The TM domain of one monomer (green) occupies the canonical hydrophobic groove within another monomer (blue) and vice versa to form a homodimer. The resulting homodimers, due to greater interacting molecular surface area, further self-associate into higher-order oligomers. (b) Oligomerization of BclXL via an inter-locking mechanism. The TM domain of one monomer (green) occupies the canonical hydrophobic groove within another monomer (blue) in a head-to-tail fashion so as to aid the assembly of much larger oligomers.

behavior distinct from the full-length protein including thermal stability, ligand binding and membrane insertion. Importantly, the ability of TM domain to trigger oligomerization of BclXL in solution appears to provide an allosteric switch for its auto-inhibition, activation and subsequent insertion into membranes. Thus, while ligand binding triggers the dissociation of BclXL oligomers into monomers, their subsequent insertion into membrane appear to be coupled to re-oligomerization into a functionally-active conformation.

On the basis of our data presented here, we propose a model to account for the self-association of BclXL into higher-order oligomers in concert with its hetero-
association with repressors and activators and how such cross-talk is finely tuned in quiescent healthy cells versus apoptotic cells (Figure 3-11). In quiescent non-apoptotic cells, BclXL either self-associates into higher-order oligomers and/or hetero-associates with effectors such as Bax and Bak, depending on the relative ratio of their cellular concentrations, to form repressor-effector complexes. In this manner, self-association into higher-order oligomers leads to inactivation of BclXL and hetero-association inactivates effectors. Upon receiving apoptotic stimuli, activators such as Bid and Bad compete with self-association of BclXL into higher-order oligomers and its hetero-association with effectors, leading to the formation of repressor-activator complexes as well as freeing up the effectors, which subsequently insert into MOM. This results in mitochondrial permeabilization leading to the release of apoptogenic factors that in turn induce cells to undergo apoptosis. Additionally, the displacement of the TM domain from the canonical hydrophobic groove within BclXL by BH3-only activators in a competitive manner triggers the translocation of BclXL into MOM via its TM domain (α9 helix) as well as the hairpin dagger (α5/α6 helices). Such solution-membrane transition would result in the disruption of the canonical hydrophobic groove allowing the BH3 ligands to drop off in agreement with the “hit-and-run” mechanism (114, 132-134). Inside MOM, BclXL oligomer may exert its anti-apoptotic action by virtue of its ability to interfere with Bax and other effectors in the creation of mitochondrial pores so as to prevent the cytosolic release of apoptogenic factors and thereby halt the cell to undergo apoptosis. Notably, our model presented above is consistent with previous studies implicating the role of TM domain in mediating membrane insertion of apoptotic repressors (47, 133, 139), but contrasts other studies where regions other than the TM domain have been suggested.
Figure 3-11: A thermodynamic cycle depicting how various linked-equilibria determine the fate of BclXL repressor to self-associate into higher-order [BclXL]ₙ oligomers versus hetero-association with activator (A) and effector (E) molecules in quiescent versus apoptotic cells (see text for more details). (143, 164). More importantly, consistent with our model is the observation that truncation of TM domain in both BclXL and Bcl2 repressors renders them cytosolic and impairs their ability to prevent apoptotic cell death (133, 165). On the other hand, it has also been shown that although BclW repressor associates with membranes in response to apoptotic stimuli, it neither promotes nor inhibits apoptosis (48).

Taken collectively, our study provides new mechanistic insights into the functional regulation of a key member of Bcl2 family and corroborates the notion that the TM domain promotes oligomerization of BclXL as previously reported by Zimmerberg and co-workers (54). Importantly, this salient observation is further supported by studies conducted within live cells (133, 139). However, our study also challenges the findings of other investigators. Notably, Hockenbery and co-workers recently demonstrated oligomerization of a truncated BclXL construct in which the TM domain is deleted (140), while Hill and co-workers reported lack of oligomerization in both the full-length BclXL and a truncated construct devoid of TM domain (60). Although we are unable to account
for the discrepancies observed between our data and those reported by others, we believe that these findings do not necessarily have to be mutually exclusive and the differences are likely to be explained by distinct experimental conditions employed in each study. We believe that the contradictory nature of our findings to those reported earlier will serve as a driving force for further advances in this field.
Chapter 4: Acidic pH Promotes Oligomerization and Membrane Insertion of the BclXL Apoptotic Repressor

4.1 Summary

Solution pH is believed to serve as an intricate regulatory switch in the induction of apoptosis central to embryonic development and cellular homeostasis. Herein, using an array of biophysical techniques, we provide evidence that acidic pH promotes the assembly of BclXL apoptotic repressor into a megadalton oligomer with a plume-like appearance and harboring structural features characteristic of a molten globule. Strikingly, our data reveal that pH tightly modulates not only oligomerization but also ligand binding and membrane insertion of BclXL in a highly subtle manner. Thus, while oligomerization and the accompanying molten globular content of BclXL is least favorable at pH 6, both of these structural features become more pronounced under acidic and alkaline conditions. However, membrane insertion of BclXL appears to be predominantly favored under acidic conditions. In a remarkable contrast, while ligand binding to BclXL optimally occurs at pH 6, it is diminished by an order of magnitude at lower and higher pH. This reciprocal relationship between BclXL oligomerization and ligand binding lends new insights into how pH modulates functional versatility of a key apoptotic regulator and strongly argues that the molten globule may serve as an intermediate primed for membrane insertion in response to apoptotic cues.

4.2 Overview

The Bcl2 family of proteins plays a central role in coupling apoptotic stimuli to the removal of damaged and unwanted cells during physiological processes such as embryonic development and cellular homeostasis (23-25, 27, 28, 107-109). The Bcl2 proteins can be divided into three major groups: activators, effectors and repressors.
Activators such as Bid and Bad belong to the BH3-only proteins, where BH3 is the Bcl2 homology 3 domain. Effectors such as Bax and Bak contain the BH3-BH1-BH2-TM modular architecture, where TM is the transmembrane domain located C-terminal to Bcl2 homology domains BH3, BH1 and BH2. Repressors such as Bcl2, BclXL and BclW are characterized by the BH4-BH3-BH1-BH2-TM modular organization, with an additional N-terminal Bcl2 homology 4 domain. According to one school of thought, the apoptotic fate, or the decision of a cell to continue to live or pull the trigger to commit suicide, is determined by the cellular ratio of activator, effector and repressor molecules (30, 31). In quiescent and healthy cells, the effectors are maintained in an inactive state via complexation with repressors. Upon receiving apoptotic cues, in the form of DNA damage and cellular stress, the activators are stimulated and compete with effectors for binding to the repressors and, in so doing, not only do they neutralize the anti-apoptotic action of repressors but also unleash the pro-apoptogenicity of effectors. The effectors subsequently initiate apoptotic cell death by virtue of their ability to insert into the mitochondrial outer membrane (MOM) resulting in the formation of mitochondrial pores in a manner akin to the insertion of bacterial toxins such as colicins and diphtheria (32-34, 62, 110). This leads to the release of apoptogenic factors such as cytochrome c and Smac/Diablo from mitochondria into the cytosol. Subsequently, rising levels of apoptogenic factors in the cytosol switch on aspartate-specific proteases termed caspases, which in turn, demolish the cellular architecture by cleavage of proteins culminating in total cellular destruction.

Despite their low sequence convergence, all members of Bcl2 family share a remarkably conserved 3D topological fold characterized by a central predominantly
hydrophobic α-helical hairpin “dagger” (α5 and α6) surrounded by a “cloak” comprised of six amphipathic α-helices (α1-α4 and α7-α8) of varying lengths (40). A prominent feature of repressors is that they contain what has come to be known as the “canonical hydrophobic groove”, formed by the juxtaposition of α2-α5 helices, that serves as the docking site for the BH3 domain (α2 helix) of activators and effectors. Additionally, the effectors and repressors also contain a C-terminal hydrophobic α-helix termed α9, or more commonly the TM domain, because it allows these members of the Bcl2 family to localize to MOM upon apoptotic induction (41, 42, 112). The “cloak and dagger” structural topology of Bcl2 members is the hallmark of their functional duality in that they are able to co-exist as “soluble factors” under quiescent cellular state and as “membrane channels” upon apoptotic induction. Notably, the hydrophobic dagger not only provides the bulk of the thermodynamic force in driving the water-membrane transition of various Bcl2 members upon apoptotic induction but also directly participates in the formation of mitochondrial pores that provide a smooth channel for the exit of apoptogenic factors. In particular, the water-membrane transition of effectors and repressors is believed to be driven by acidic pH, and optimally occurs at around pH 4, in a manner akin to pore formation by the bacterial toxins (34, 39, 54, 58, 59, 65, 166, 167). The acidic pH destabilizes the solution conformation of these proteins while at the same time inducing the formation of molten globule, which is believed to serve as an intermediate for subsequent insertion into membranes (61, 62, 168, 169). It should be noted that the molten globule is a partially disordered conformation which contains a native-like secondary structure but without the tightly-packed hydrophobic core comprised of nonpolar residues (170-173). Importantly, several lines of evidence suggest
the formation of a pH gradient across the mitochondria, accompanied by the alkalinization of mitochondrial matrix and acidification of the cytosol, upon the induction of apoptosis (55, 56, 68, 174-176). This observation further corroborates the role of acidic pH in driving apoptotic machinery.

We have previously shown that BclXL displays the propensity to oligomerize in solution and that such oligomerization is driven by the intermolecular binding of its C-terminal TM domain to the canonical hydrophobic groove in a domain-swapped trans-fasion, whereby the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa (177). We postulated that such oligomerization serves as a regulatory switch to turn the anti-apoptotic action of BclXL “off” in quiescent cells but “on” in response to apoptotic cues. In an effort to understand how solution pH modulates oligomerization of BclXL and the effect of such oligomerization on subsequent binding of BH3 ligands in the form of activators and effectors and membrane insertion in the context of apoptosis, we undertook the present study. Herein, we provide evidence that acidic pH promotes the assembly of BclXL apoptotic repressor into a megadalton oligomer with a plume-like appearance and harboring structural features characteristic of a molten globule. Strikingly, our data reveal that pH tightly modulates not only oligomerization but also ligand binding and membrane insertion of BclXL in a highly subtle manner. Thus, while oligomerization and the accompanying molten globular content of BclXL is least favorable at pH 6, both of these structural features become more pronounced under acidic and alkaline conditions. However, membrane insertion of BclXL appears to be predominantly favored under acidic conditions. In a remarkable contrast, while ligand binding to BclXL optimally
occurs at pH 6, it is diminished by an order of magnitude at lower and higher pH. This reciprocal relationship between BclXL oligomerization and ligand binding lends new insights into how pH modulates functional versatility of a key apoptotic regulator and strongly argues that the molten globule may serve as an intermediate primed for membrane insertion in response to apoptotic cues.

4.3 Experimental Procedures

4.3.1 Protein Preparation

Full-length human BclXL (residues 1-233) was cloned into pET30 bacterial expression vector with an N-terminal His-tag using Novagen LIC technology, expressed in Escherichia coli BL21*(DE3) bacterial strain (Invitrogen) and purified on a Ni-NTA affinity column using standard procedures as described previously (177). Protein concentration was determined by the fluorescence-based Quant-It assay (Invitrogen) and spectrophotometrically on the basis of an extinction coefficient of 47,440 M⁻¹cm⁻¹ calculated for the full-length BclXL using the online software ProtParam at ExPasy Server (115). Results from both methods were in an excellent agreement. The 20-mer peptide spanning residues 81-100 corresponding to the BH3 domain within human Bid (H₂N-DIIRNIARHLAQVGDMSDRS-COOH), hereinafter referred to as Bid_BH3 peptide, was commercially obtained from GenScript Corporation. The peptide concentration was measured gravimetrically. Mixed TOCL/DHPC bicelles were prepared at a final concentration of 30mM, at TOCL to DHPC molar ratio of 1:4, by stirring for 2h at 37°C in appropriate buffers. Samples of full-length BclXL, Bid_BH3 peptide and TOCL/DHPC bicelles were prepared under various pH conditions using acetate (pH 4.0), phosphate (pH 6.0), Tris (pH 8.0) and CAPS (pH 10.0) buffers. For ITC and ALS
measurements, all buffers were made up to a final concentration of 50mM containing 100mM NaCl, 1mM EDTA and 5mM β-mercaptoethanol at each pH. For DSC, CD, SSF and SEM experiments, all buffers were made up to a final concentration of 50mM at each pH. All measurements were repeated at least three times.

4.3.2 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on a Microcal VP-ITC instrument and data were acquired and processed using the integrated Microcal ORIGIN software. For peptide binding, experiments were initiated by injecting 25 x 10μl aliquots of 0.5-1mM of Bid_BH3 peptide from the syringe into the calorimetric cell containing 1.8ml of 50μM of BclXL at 25 °C. For membrane insertion, experiments were initiated by injecting 25 x 10μl aliquots of 50μM of full-length BclXL from the syringe into the calorimetric cell containing 1.8ml of 2mM of TOCL/DHPC at 25 °C. In each case, the change in thermal power as a function of each injection was automatically recorded using the ORIGIN software and the raw data were further processed to yield binding isotherms of heat release per injection either as a function of molar ratio of peptide to BclXL or as a function of molar ratio of BclXL to bicelles. The heats of mixing and dilution were subtracted from the heats of peptide binding or membrane insertion per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was either titrated against the Bid_BH3 peptide or BclXL in an identical manner. The apparent equilibrium dissociation constant (K_d) and the enthalpic change (ΔH) associated with peptide binding to BclXL or membrane insertion of BclXL at various pH were determined from the non-linear least-squares fit of data to a one-site
binding model as described previously (116, 177). The binding free energy change ($\Delta G$) was calculated from the following expression:

$$\Delta G = RT \ln K_d$$ \[1\]

where $R$ is the universal molar gas constant (1.99 cal/K/mol) and $T$ is the absolute temperature. The entropic contribution ($T\Delta S$) to the free energy of binding was calculated from the relationship:

$$T\Delta S = \Delta H - \Delta G$$ \[2\]

where $\Delta H$ and $\Delta G$ are as defined above.

4.3.3 Analytical light scattering

Analytical light scattering (ALS) experiments were conducted on a Wyatt miniDAWN TREOS triple-angle static light scattering detector and Wyatt QELS dynamic light scattering detector coupled in-line with a Wyatt Optilab rEX differential refractive index detector and interfaced to a HiLoad Superdex 200 size-exclusion chromatography column under the control of a GE Akta FPLC system within a chromatography refrigerator at 10°C. Briefly, BclXL was loaded onto the column at a starting concentration of 50μM and at a flow rate of 1ml/min. All data were automatically acquired using the ASTRA software. Notably, the angular- and concentration-dependence of static light scattering (SLS) intensity of BclXL resolved in the flow mode was measured by the Wyatt miniDAWN TREOS detector equipped with three scattering angles positioned at 42°, 90° and 138°. The time- and concentration-dependence of dynamic light scattering (DLS) intensity fluctuation of BclXL resolved in the flow mode was measured by the Wyatt QELS detector positioned at 90° with respect to the incident
laser beam. Hydrodynamic parameters $M_w$ (weighted-average molar mass), $M_n$ (number-average molar mass), $R_g$ (weighted-average radius of gyration) and $R_h$ (weighted-average hydrodynamic radius) associated with solution behavior of BclXL were determined by the treatment of SLS data to Zimm model and by non-linear least-squares fit of DLS data to an autocorrelation function as described earlier (117-121, 177). It should be noted that, in both the SLS and DLS measurements, protein concentration ($c$) along the elution profile of BclXL was automatically quantified in the ASTRA software from the change in refractive index ($\Delta n$) with respect to the solvent as measured by the Wyatt Optilab rEX detector using the following relationship:

$$c = \frac{(\Delta n)}{(dn/dc)}$$

[3]

where $dn/dc$ is the refractive index increment of the protein in solution.

4.3.4 **Differential scanning calorimetry**

Differential scanning calorimetry (DSC) experiments were performed on a TA Nano-DSC instrument and data were acquired and processed using the integrated NanoAnalyze software. Briefly, experiments were conducted on 50\,$\mu$M of BclXL in the 40-120$^\circ$C temperature range at a heating rate ($dT/dt$) of 1$^\circ$C/min under an excess pressure of 3atm. The change in thermal power ($dQ/dt$) as a function of temperature was automatically recorded using the NanoAnalyze software. Control experiments on the buffers alone were also conducted in an identical manner to generate baselines that were subtracted from the raw data to remove background contribution due to the buffer. The raw data were further processed to yield the melting isotherms of excess heat capacity ($C_p$) as a function of temperature ($T$) using the following relationship:
\[ C_p = \frac{(dQ/dt)}{(dT/dt)PV} \]  \[4\]

where P is the initial concentration of protein loaded into the calorimetric cell and V is the effective volume of calorimetric cell (0.3ml).

### 4.3.5 Circular dichroism

Circular dichroism (CD) measurements were conducted on a Jasco J-815 spectrometer thermostatically controlled at 25°C. For far-UV measurements, experiments were conducted on 5\(\mu\)M of BclXL and data were collected using a quartz cuvette with a 2-mm pathlength in the 190-250nm wavelength range. For near-UV measurements, experiments were conducted on 30\(\mu\)M of BclXL and data were collected using a quartz cuvette with a 10-mm pathlength in the 260-340nm wavelength range. All data were normalized against reference spectra to remove the contribution of buffers. All data were recorded with a slit bandwidth of 2nm at a scan rate of 10nm/min. Each data set represents an average of four scans acquired at 0.1nm intervals. Data were converted to molar ellipticity, \([\theta]\), as a function of wavelength (\(\lambda\)) of electromagnetic radiation using the equation:

\[ [\theta] = \frac{(10^5\Delta\varepsilon)/cl}{\text{deg.cm}^2.\text{dmol}^{-1}} \]  \[5\]

where \(\Delta\varepsilon\) is the observed ellipticity in mdeg, c is the peptide or protein concentration in \(\mu\)M and l is the cuvette pathlength in cm.

### 4.3.6 Steady-state fluorescence

Steady-state fluorescence (SSF) spectra were collected on a Jasco FP-6300 spectrofluorimeter using a quartz cuvette with a 10-mm pathlength at 25 °C. Briefly, experiments were conducted on 5\(\mu\)M of BclXL alone or in the presence of excess ANS or
acrylamide. For intrinsic protein fluorescence measurements, the excitation wavelength was 290nm and emission was acquired over the 300-500nm wavelength range. For ANS fluorescence, the excitation wavelength was 375nm and emission was acquired over the 400-600nm wavelength range. All data were recorded using a 2.5-nm bandwidth for both excitation and emission. Data were normalized against reference spectra to remove background contribution of appropriate buffers. Fluorescence enhancement (E) of ANS in the presence of BclXL at each pH was calculated from the following equation:

$$E = (\Phi - \Phi_o/\Phi_o) \times 100\%$$  \[6\]

where $\Phi$ is the fluorescence yield of ANS in the presence of BclXL and $\Phi_o$ is the fluorescence yield of ANS alone at corresponding pH. Fluorescence yield ($\Phi$) is defined as the area integrated under the corresponding SSF spectra. Fluorescence quenching (Q) of BclXL in the presence of acrylamide at each pH was calculated from the following equation:

$$Q = (\Phi_o - \Phi/\Phi_o) \times 100\%$$  \[7\]

where $\Phi$ is the fluorescence yield of BclXL in the presence of acrylamide and $\Phi_o$ is the fluorescence yield of BclXL alone at corresponding pH.

### 4.3.7 Scanning electron microscopy

Scanning electron microscopy (SEM) experiments were conducted on a Zeiss Gemini Ultra-55 electron microscope operating at a voltage of 5kV using the in-lens detector and the images were photographed at a magnification of 50,000x. Data were collected either on 25μM of BclXL alone and in the presence of 10-molar excess of Bid_BH3 peptide or on 10mM of TOCL/DHPC bicelles alone and in the presence of
25μM of BclXL at the specified pH. Briefly, 100μl of each sample was deposited onto a carbon-coated copper grid (200-mesh) and incubated for 5-10 min followed by the removal of excess solution. Grids were negatively stained with 1% uranyl acetate. Excess liquid was wicked away with a filter paper and the grids were allowed to air dry prior to imaging.

4.3.8 Molecular modeling

Molecular modeling (MM) was employed to build structural models of BclXL in two distinct oligomeric conformations, BclXL_transTM and BclXL_runawayTM, using the MODELLER software based on homology modeling in combination with MOLMOL (122, 123). In the BclXL_transTM conformation, the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in a domain-swapped trans-fashion. In BclXL_runawayTM conformation, the TM domain of one monomer occupies the canonical hydrophobic groove within the adjacent monomer in a head-to-tail manner and the TM domain of this second monomer in turn occupies the canonical hydrophobic groove within the third monomer in a runaway domain-swapping fashion. In each case, solution structures of truncated BclXL in which the TM domain and the α1-α2 loop are missing (PDB# 1BXL), hereinafter referred to as tBclXL, and the full-length Bax in which the TM domain occupies the canonical hydrophobic groove (PDB# 1F16) were used as templates. Additionally, MOLMOL was used to bring various parts and/or monomers into optimal spatial orientations relative to each other in a rigid-body fashion. First, the structural model of full-length BclXL, in which the TM domain occupies the hydrophobic groove within the same molecule in a cis manner, was built using tBclXL (PDB# 1BXL) and Bax (PDB# 1F16) in a multiple-template alignment
manner and the residues within the α1-α2 loop were modeled without a template through energy minimization and molecular dynamics simulations. Next, pre-built structural models of two individual monomers of full-length BclXL were brought together in an optimal orientation in MOLMOL such that the α8-α9 loop within one monomer could be domain-swapped with TM domain of the other monomer in a trans (BclXL_transTM) or runaway (BclXL_runaway) fashion without becoming taut. This requirement led to either roughly parallel (BclXL_transTM) or series (BclXL_runaway) orientation of TM domains within each monomer. Finally, the α8-α9 loop preceding the TM domain within each BclXL monomer was excised out and the resulting monomers were used as a template to homology model the structures of BclXL_transTM and BclXL_runawayTM, wherein the residues within the α8-α9 loop within each structural model were modeled without a template through energy minimization and molecular dynamics simulations. For each structural model, a total of 100 atomic models were calculated and the structure with the lowest energy, as judged by the MODELLER Objective Function, was selected for further analysis. The atomic models were rendered using RIBBONS (124). All calculations and data processing were performed on a Linux workstation equipped with a dual-core processor.

4.3.9 Molecular dynamics

Molecular dynamics (MD) simulations on BclXL as a function of pH were performed with the GROMACS software (99, 125) using the integrated OPLS-AA force field (126, 127). Briefly, ionizable residues within the modeled structure of BclXL_transTM dimeric conformation were protonated/deprotonated according to their pKa values at pH 4.0, 6.0, 8.0 and 10.0 using the H++ server at
http://biophysics.cs.vt.edu. Next, the pH-adjusted structures were centered within a cubic box, hydrated using the extended simple point charge (SPC/E) water model (128, 129), and the ionic strength of solution was set to 100mM with NaCl. The hydrated structures were energy-minimized with the steepest descent algorithm prior to equilibration under the NPT ensemble conditions, wherein the number of atoms (N), pressure (P) and temperature (T) within the system were respectively kept constant at ~50000, 1 bar and 300 K. The Particle-Mesh Ewald (PME) method was employed to compute long-range electrostatic interactions with a 10Å cut-off (130) and the Linear Constraint Solver (LINCS) algorithm to restrain bond lengths (131). All MD simulations were performed under periodic boundary conditions (PBC) using the leap-frog integrator with a time step of 2fs. For the final MD production runs, data were collected every 100ps over a time scale of 100ns. All simulations were run on a Linux workstation using parallel processors at the High Performance Computing facility within the Center for Computational Science of the University of Miami.

4.4 Results and discussion

4.4.1 pH modulates ligand binding to BclXL

To understand how solution pH may dictate the binding of BH3 ligands to BclXL, we conducted ITC analysis for the binding of a 20-mer BH3 peptide derived from Bid activator to full-length BclXL as a function of pH (Figure 4-1 and Table 4-1). Our data show that the binding of BH3 peptide to BclXL displays a subtle relationship with increasing pH. Thus, while ligand binding optimally occurs at pH 6, it is diminished by nearly an order of magnitude under acidic conditions (pH 4) to more than an order of magnitude under alkaline environment (pH 8 and 10). The effect of pH on the thermody-
Figure 4-1: ITC analysis for the binding of Bid_BH3 peptide to full-length BclXL at pH 4 (a), pH 6 (b), pH 8 (c) and pH 10 (d). The upper panels show raw ITC data expressed as change in thermal power with respect to time over the period of titration. In the lower panels, change in molar heat is expressed as a function of molar ratio of Bid_BH3 peptide to BclXL. The solid lines in the lower panels show non-linear least squares fit of data to a one-site binding model using the ORIGIN software as described earlier (177).

The dynamics of ligand binding is also telling. Although binding under all pH conditions analyzed here is favored by enthalpy accompanied by entropic penalty, it is interesting to note that while increasing pH appears to favor enthalpic contributions to the free energy of binding, these favorable changes are largely opposed by equal but opposite entropic factors in agreement with the enthalpy-entropy compensation phenomenon (178-182). We note that the binding of BH3 peptide to BclXL is not coupled to proton uptake or release since the observed binding enthalpy is independent of the ionization enthalpy of the buffer employed. Importantly, we have previously shown that the TM domain reduces the binding of BH3 ligands to BclXL by an order of magnitude by virtue of its ability to bind to the canonical hydrophobic groove in a competitive manner through domain-swapping and thereby promoting the association of BclXL into higher-order oligomers (177). In light of these observations, our data presented above strongly argue that pH not only modulates ligand binding but that it may also play a key role in the
Table 4-1

pH-dependence of thermodynamic parameters for the binding of Bid_BH3 peptide to full-length BclXL

<table>
<thead>
<tr>
<th>pH</th>
<th>Kd / μM</th>
<th>ΔH / kcal.mol⁻¹</th>
<th>TΔS / kcal.mol⁻¹</th>
<th>ΔG / kcal.mol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8.30 ± 1.10</td>
<td>-9.09 ± 0.20</td>
<td>-2.15 ± 0.03</td>
<td>-6.94 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>1.03 ± 0.10</td>
<td>-13.66 ± 0.32</td>
<td>-5.48 ± 0.15</td>
<td>-8.17 ± 0.09</td>
</tr>
<tr>
<td>8</td>
<td>10.36 ± 1.52</td>
<td>-18.39 ± 0.53</td>
<td>-11.58 ± 0.29</td>
<td>-6.80 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>20.51 ± 3.20</td>
<td>-16.54 ± 0.47</td>
<td>-10.13 ± 0.31</td>
<td>-6.40 ± 0.02</td>
</tr>
</tbody>
</table>

All parameters were obtained from ITC measurements. All binding stoichiometries were 1:1 and generally agreed to within ±10%. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation.

oligomerization of BclXL and that such intramolecular association may in turn modulate ligand binding.

4.4.2 *Acidic pH drives the association of BclXL into a megadalton oligomer*

To test the hypothesis that the oligomerization of BclXL is pH-dependent, we next analyzed the propensity of BclXL to oligomerize as a function of solution pH using ALS and quantified various physical parameters accompanying its solution behavior from the first principles of hydrodynamics without any assumptions (Figure 4-2 and Table 4-2). Remarkably, our data show that BclXL exclusively associates into a megadalton oligomer comprised of more than 1000 monomeric units (~34,000 kD), hereinafter referred to as megamer, under acidic conditions (pH 4). At pH 6 and higher, this megamer dissociates and predominantly exists in an equilibrium between monomer (~31 kD), dimer (~62 kD), and two higher-order oligomers, herein referred to as multimer (~400 kD) and polymer (~3500 kD). However, the ratio of these four species is highly pH-dependent. Thus, while the polymer-multimer-dimer-monomer equilibrium shifts in
Figure 4-2: ALS analysis of full-length BclXL under varying pH as indicated. (a) Elution profiles as monitored by the differential refractive index (Δn) plotted as a function of elution volume (V) at pH 4 (top panel), pH 6 (upper-middle panel), pH 8 (lower-middle panel) and pH 10 (bottom panel). (b) Partial Zimm plots obtained for the oligomeric species as indicated from analytical SLS measurements at pH 4 (top panel), pH 6 (upper-middle panel), pH 8 (lower-middle panel panel) and pH 10 (bottom panel). The red solid lines through the data points represent linear fits. (c) Autocorrelation function plots obtained for various oligomeric species as indicated from analytical DLS measurements at pH 4 (top panel), pH 6 (upper-middle), pH 8 (lower-middle panel panel) and pH 10 (bottom panel). The red solid lines represent non-linear least squares fit of data to an autocorrelation function as described earlier (177).

In favor of the smaller species (monomer and dimer) at pH 6, the larger species (multimer and polymer) are favored at pH 8 and pH 10. These salient observations strongly suggest that while pH 6 destabilizes higher-order oligomers of BclXL, alkaline conditions promote association of BclXL into higher-order oligomers and, under acidic conditions, BclXL exclusively associates into a megadalton oligomer. We note that the truncation of the C-terminal TM domain completely abolished oligomerization of BclXL under all pH conditions, implying that the intermolecular association of BclXL observed here is driven by the TM domain in agreement with our previous study (177). In an attempt to gain insights into the conformational heterogeneity of the oligomeric species of BclXL, we also determined the $M_w/M_n$ and $R_g/R_h$ ratios from our hydrodynamic data (Table 4-2).
### Table 4-2
pH-dependence of hydrodynamic parameters for full-length BclXL

<table>
<thead>
<tr>
<th>pH</th>
<th>Associativity</th>
<th>( M_n / \text{kD} )</th>
<th>( M_n / \text{kD} )</th>
<th>( M_w / M_n )</th>
<th>( R_g / \text{Å} )</th>
<th>( R_h / \text{Å} )</th>
<th>( R_g / R_h )</th>
<th>( P / % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Megamer</td>
<td>33805 ± 2821</td>
<td>33085 ± 2623</td>
<td>1.02 ± 0.01</td>
<td>783 ± 86</td>
<td>324 ± 23</td>
<td>2.40 ± 0.16</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Monomer</td>
<td>32 ± 2</td>
<td>31 ± 2</td>
<td>1.01 ± 0.01</td>
<td>ND</td>
<td>26 ± 1</td>
<td>ND</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>61 ± 3</td>
<td>59 ± 2</td>
<td>1.02 ± 0.01</td>
<td>ND</td>
<td>49 ± 4</td>
<td>ND</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Multimer</td>
<td>595 ± 45</td>
<td>577 ± 39</td>
<td>1.03 ± 0.02</td>
<td>141 ± 5</td>
<td>95 ± 3</td>
<td>1.47 ± 0.02</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Polymer</td>
<td>3521 ± 292</td>
<td>3101 ± 201</td>
<td>1.13 ± 0.03</td>
<td>230 ± 12</td>
<td>187 ± 9</td>
<td>1.22 ± 0.02</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>Monomer</td>
<td>31 ± 1</td>
<td>30 ± 2</td>
<td>1.00 ± 0.00</td>
<td>ND</td>
<td>34 ± 2</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>62 ± 4</td>
<td>61 ± 2</td>
<td>1.01 ± 0.01</td>
<td>ND</td>
<td>48 ± 2</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Multimer</td>
<td>364 ± 32</td>
<td>325 ± 33</td>
<td>1.11 ± 0.01</td>
<td>98 ± 4</td>
<td>90 ± 2</td>
<td>1.08 ± 0.02</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Polymer</td>
<td>3729 ± 246</td>
<td>3226 ± 236</td>
<td>1.15 ± 0.02</td>
<td>218 ± 12</td>
<td>188 ± 11</td>
<td>1.15 ± 0.03</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>Monomer</td>
<td>31 ± 2</td>
<td>32 ± 1</td>
<td>1.00 ± 0.00</td>
<td>ND</td>
<td>32 ± 1</td>
<td>ND</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Dimer</td>
<td>61 ± 3</td>
<td>60 ± 3</td>
<td>1.02 ± 0.01</td>
<td>ND</td>
<td>49 ± 3</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Multimer</td>
<td>461 ± 21</td>
<td>448 ± 23</td>
<td>1.03 ± 0.01</td>
<td>131 ± 5</td>
<td>108 ± 4</td>
<td>1.21 ± 0.01</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Polymer</td>
<td>3189 ± 229</td>
<td>3018 ± 212</td>
<td>1.06 ± 0.02</td>
<td>232 ± 13</td>
<td>202 ± 7</td>
<td>1.14 ± 0.02</td>
<td>36</td>
</tr>
</tbody>
</table>

All parameters were obtained from ALS measurements. The population (P) of each species, as estimated from the integration of corresponding peak in the elution profile (Figure 2a), is provided in the right-most column. Note that the calculated molar mass of recombinant full-length BclXL from amino acid sequences alone is 31kD. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation. Note that the \( R_g \) parameter could not be determined (ND) for various species due to their lack of angular-dependence of scattered light.

While the \( M_w / M_n \) ratio provides a measure of the macromolecular polydispersity, the \( R_g / R_h \) ratio sheds light on the overall macromolecular shape. Our data suggest that while the higher-order oligomers (multimer and polymer) of BclXL display some degree of polydispersity (\( M_w / M_n > 1.05 \)) under all pH conditions (pH 6-10) where they are observed, the monomeric and dimeric forms of BclXL are predominantly monodisperse (\( M_w / M_n < 1.05 \)). Strikingly, BclXL not only exclusively exists as a megadalton oligomer under acidic conditions (pH 4) but it also surprisingly appears to be highly monodisperse.
(M_w/M_n < 1.05). Additionally, the higher-order oligomers (multimer and polymer) of BclXL most likely adopt an elongated rod-like shape (R_g/R_h > 1.05) in lieu of a more spherical or disc-like architecture and such quaternary topology seems to be somewhat more favored at pH 6 than under alkaline conditions (pH 8 and pH 10). Consistent with these observations, the megamer observed at pH 4 also seems to adopt an highly elongated rod-like topology (R_g/R_h > 2) with a radius of gyration of ~100 nm, arguing that it may bear the propensity to assemble into fibrils of up to hundreds of nm in length in a manner akin to amyloid fibrils. Indeed, in a recent development, BclXL was shown to aggregate into amyloid-like fibrils under elevated temperatures (183).

4.4.3 pH destabilizes structure and stability of BclXL

Given that acidic pH promotes the association of BclXL into a megadalton oligomer, we next analyzed the effect of solution pH on the stability of this key apoptotic regulator using DSC (Figure 4-3a). Consistent with our ALS analysis, our data reveal that BclXL is extremely stable under acidic conditions (pH 4) and does not undergo a melting transition even when the temperature is raised to 120°C. As the pH is raised to 6, BclXL exhibits two thermal phases with melting temperature (T_m) of around 55°C and 70°C in agreement with the observation that it exists in an equilibrium between various oligomeric states. We attribute these transitions to the dissociation of BclXL dimer (55°C) into monomers and the subsequent unfolding of these monomers (70°C). Interestingly, the thermal stability of BclXL at pH 8 is indistinguishable from that observed at pH 4, implying that although BclXL does not associate into a 1000-mer observed at pH 4, the much smaller oligomeric species observed at pH 8 are nonetheless highly stable. Finally, melting of BclXL at pH 10 is characterized by two distinct thermal
Figure 4-3: Structure and stability of full-length BclXL monitored at pH 4 (red), pH 6 (green), pH 8 (blue) and pH 10 (magenta) using various techniques. (a) DSC isotherms of BclXL at various pH. (b) Far-UV CD spectra of BclXL at various pH. (c) SSF spectra of BclXL at various pH.

phases accompanied by T_m values of around 60°C and 90°C, which most likely correspond to the dissociation of BclXL dimer into monomers and the subsequent unfolding of these monomers, respectively. Collectively, our DSC data suggest that although BclXL bears the propensity to associate into higher-order oligomers under all pH conditions, the oligomers observed at pH 6 and pH 10 are thermally much less stable than those observed at pH 4 and pH 8.

Next, we wondered whether differential stability of BclXL under various pH conditions also correlates with its structure. Toward this goal, we first measured far-UV CD spectra of full-length BclXL to probe the secondary structure as a function of pH (Figure 4-3b). Notably, BclXL displays spectral features in the far-UV region characteristic of an α-helical fold with bands centered around 208nm and 222nm under all pH conditions. However, there are subtle differences that offer us a key glimpse into how pH affects protein secondary structure. Thus, the intensity of the far-UV spectrum of
BclXL steadily increases with increasing pH, implying that the protein has a higher propensity to adopt \( \alpha \)-helical fold under alkaline than acidic conditions. We believe that such decrease in \( \alpha \)-helical propensity as the pH decreases likely underscores its ability to associate into a megadalton oligomer at pH 4. Spurred on by these promising insights, we also conducted SSF analysis on full-length BclXL to monitor tertiary and quaternary structural changes accompanying BclXL as a function of pH (Figure 4-3c). It is important to note that intrinsic protein fluorescence, largely due to tryptophan residues, is influenced by changes in the local environment and thus serves as a sensitive probe of overall conformational changes within proteins. This is further aided by the fact that there are seven tryptophan residues within BclXL, located at various strategic positions to monitor conformational changes occurring at both the intramolecular and intermolecular level.

In agreement with foregoing argument, our SSF analysis shows that the intrinsic fluorescence of BclXL is highly pH-dependent, implying that protein tertiary and quaternary structures are perturbed by solution pH. Thus, while intrinsic fluorescence of BclXL drops as the pH changes from 4 to 6, it undergoes substantial enhancement at pH 8 only to drop again at pH 10. The enhancement in intrinsic fluorescence is most likely due to the transfer of tryptophan residues to a more hydrophobic environment, while a drop in intrinsic fluorescence could be explained by the greater solvent-exposure of tryptophan residues. Accordingly, one plausible interpretation of these subtle changes in intrinsic fluorescence is that the protein tertiary and quaternary structures experience largest perturbation at pH 6, while experiencing less perturbation at other pH conditions. However, we note that the presence of several tryptophan residues within BclXL may
mask and average out the changes in intrinsic fluorescence observed here instead of providing a site-specific information. This argument is further supported by the fact that the tryptophan emission maximum observed in BclXL lies around 338nm and does not appear to be pH-dependent. It should be noted that the tryptophan emission maximum is highly sensitive to the polarity of the surrounding solvent environment and occurs around 350nm when fully exposed to water and around 330nm when fully buried within the hydrophobic core of a protein. Thus, a value of 338nm observed for the tryptophan emission maximum in BclXL most likely arises from an averaging effect and suggests that while some tryptophan residues may be fully buried within the interior of the protein, others are likely to be solvent-exposed. We note that the tryptophan emission can also be influenced by resonance energy transfer from nearby tyrosine residues. In particular, tyrosine residues within BclXL are likely to exist in the negatively-charged phenolate state due to the ionization of the sidechain hydroxyl moiety at pH 10. Accordingly, changes in the ionization state of neighboring tyrosine residues as the solution pH varies must also influence the intrinsic fluorescence of tryptophan residues in a highly subtle manner. Despite these caveats, the data presented above are consistent with the propensity of BclXL to undergo oligomerization and its affinity toward Bid_BH3 peptide as a function of pH.

**4.4.4 Acidic pH induces the formation of molten globule and promotes membrane insertion of BclXL**

Acidic pH is believed to destabilize the solution conformation of bacterial toxins while at the same time inducing the formation of molten globule, which is believed to serve as an intermediate for subsequent insertion into membranes (61, 62, 168, 169). We wondered whether the propensity of BclXL to associate into a megadalton oligomer with
Figure 4-4: Tertiary structural analysis of full-length BclXL at pH 4 (red), pH 6 (green), pH 8 (blue) and pH 10 (magenta) using various techniques. (a) SSF spectra of ANS in the presence of BclXL at various pH. (b) SSF spectra of BclXL in the presence of excess acrylamide at various pH. (c) Near-UV CD spectra of BclXL alone at various pH. Note that the background fluorescence due to ANS alone (a) and BclXL alone (b) was subtracted from the spectra shown at each pH. In (a) and (b), the upper panels show raw SSF spectra, while corresponding fluorescence enhancement (E) and fluorescence quenching (Q) at each pH are displayed in the lower panels.

A rod-like appearance at pH 4 also manifests in the formation of a molten globule. To test this hypothesis, we determined the effect of binding of ANS to BclXL as a function of pH using SSF (Figure 4-4a). ANS is a hydrophobic fluorescent dye whose fluorescence undergoes substantial enhancement upon binding to exposed apolar surfaces such as those characteristic of molten globule conformations of proteins (184). More importantly, ANS has been widely used as a test for the demonstration of molten globule-like states in proteins. Consistent with this notion, our data show that in the presence of BclXL, ANS experiences close to 15-fold fluorescence enhancement at pH 4 versus a mere 2-fold at pH 6, while under alkaline conditions (pH 8 and 10), its fluorescence undergoes about 5-fold enhancement. That this is so strongly suggests that acidic pH induces the formation of molten globule within BclXL and that megadalton oligomer observed here may be an
on-pathway intermediate primed for insertion into MOM in response to apoptotic cues.
This salient observation is in remarkable agreement with the evidence that upon apoptotic induction, a pH gradient is formed across the mitochondria with alkalinization of mitochondrial matrix and acidification of the cytosol (55, 56, 68, 174-176). We also note that while ANS emission occurs maximally around 515nm in water, it appears to be blue-shifted to around 475nm upon binding to BclXL. This is further evidence for the exposure of hydrophobic surfaces in BclXL under all pH conditions, albeit more so at pH 4.

To further test the notion that pH modulates tertiary and quaternary structure of BclXL, we also monitored the extent of quenching of intrinsic tryptophan fluorescence by acrylamide using SSF (Figure 4-4b). In this assay, the extent of quenching directly correlates with the degree of solvent-exposure of tryptophan residues within a protein. As mentioned earlier, BclXL is decorated with seven tryptophan residues located at various strategic positions to monitor conformational changes occurring at both the intramolecular and intermolecular level. As shown in Figure 4b, our fluorescence quenching analysis with acrylamide reveals that the optimal quenching occurs at pH 6, implying that the tryptophan residues either undergo some level of burial or dehydration under acidic as well as alkaline conditions. These data thus strongly argue that BclXL is characterized by the solvent-exposure of apolar surfaces in a manner akin to a molten globule under acidic conditions, while changes in pH result in perturbation of tertiary and quaternary structure as monitored by the movement of tryptophan residues. This notion is further corroborated by our near-UV CD analysis (Figure 4-4c), which largely monitors the chiral environment of aromatic residues such as tryptophan and tyrosine. Thus, while
BclXL exhibits a strong near-UV CD signal at pH 6, it becomes substantially attenuated under acidic pH. This implies that BclXL loses substantial tertiary structure under acidic conditions in agreement with our view that acidic pH induces the formation of molten globule. Interestingly, the near-UV CD signal is also attenuated under alkaline conditions (pH 8 and 10), arguing that not only acidic but also alkaline pH destabilizes the tertiary structure of BclXL.

To test our hypothesis that the molten globule-like state of BclXL observed under acidic pH may serve as an intermediate for membrane insertion, we next directly analyzed the binding of BclXL to mixed TOCL/DHPC bicelles as a function of pH using ITC (Figure 4-5 and Table 4-3). Our analysis reveals that BclXL binds to TOCL/DHPC bicelles, used here as a model for MOM, only under acidic conditions. Importantly, varying the conditions of ITC experiments such as temperature or ionic strength had no effect on these observations, implying that BclXL indeed bears intrinsic affinity for bicelles only under acidic pH in lieu of lack of any observable change in the heat of binding, a scenario that may prevail for macromolecular interactions under entropic control. Notably, the truncation of the C-terminal TM domain completely abolished the binding of BclXL to bicelles under all pH conditions, implying that the TM domain is a requisite for membrane insertion of BclXL. This salient observation is consistent with previous developments implicating the role of TM domain in mediating membrane insertion of apoptotic repressors (47, 133, 139), but contrasts other studies where regions other than the TM domain have been suggested (143, 164). More importantly, the observation that the truncation of TM domain in both BclXL and Bcl2 repressors renders them cytosolic and impairs their ability to prevent apoptotic cell death may be due to
Figure 4-5: ITC analysis for the binding of full-length BclXL to mixed TOCL/DHPC bicelles at pH 4 (a), pH 6 (b), pH 8 (c) and pH 10 (d). The upper panels show raw ITC data expressed as change in thermal power with respect to time over the period of titration. In the lower panels, change in molar heat is expressed as a function of molar ratio of BclXL to bicelles. In (a), the solid line in the lower panel shows non-linear least squares fit of data to a one-site binding model using the ORIGIN software as described earlier (177).

their inability to insert into MOM upon apoptotic induction (133, 165). We note that while TOCL only comprises about 10% of total phospholipid content of MOM, it is believed to be critical for the mitochondrial targeting of apoptotic regulators and the subsequent release of apoptogenic factors such as cytochrome c (185-189). This is largely due to the highly distinguished structural features of TOCL. Thus, unlike canonical phospholipids, TOCL is a diphospholipid wherein two phosphatidylglycerols connect with a central glycerol backbone to form a dimeric structure. Importantly, the tetraoleoyl fatty acid moieties combined with an acidic head group in TOCL provide a unique chemical and structural configuration for the interaction of MOM with apoptotic regulators and other mitochondrial proteins in a highly specific manner. It is also noteworthy that artificial membranes, such as bicelles and liposomes, devoid of TOCL display little or no affinity toward apoptotic regulators (190-193).
Table 4-3
pH-dependence of thermodynamic parameters for the binding of full-length BclXL to mixed TOCL/DHPC bicelles

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_d$ / $\mu$M</th>
<th>$\Delta H$ / kcal.mol$^{-1}$</th>
<th>$\Delta S$ / kcal.mol$^{-1}$</th>
<th>$\Delta G$ / kcal.mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>$0.17 \pm 0.02$</td>
<td>$-63.00 \pm 4.2$</td>
<td>$-53.73 \pm 4.1$</td>
<td>$-9.26 \pm 0.1$</td>
</tr>
<tr>
<td>6</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>8</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>10</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
</tbody>
</table>

All parameters were obtained from ITC measurements. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation. NB indicates no binding observed.

4.4.5 Ligand binding and membrane insertion are coupled to conformational changes within BclXL

To further shed light on the propensity of BclXL to oligomerize in solution, we conducted SEM analysis as a function of pH on full-length BclXL alone, in the presence of Bid_BH3 peptide and in the presence of mixed TOCL/DHPC bicelles as a mimetic for MOM (Figure 4-6). Consistent with our ALS analysis, our SEM data reveal that BclXL assembles into plume-like soluble aggregates with lengths of up to a few $\mu$m at pH 4 (Figure 4-6a). In contrast, BclXL adopts poorly-defined amorphous structures at pH 6 and pH 8, while much smaller rod-like aggregates are observed at pH 10. Remarkably, the soluble aggregates of BclXL undergo conformational change and appear to dissociate into much smaller oligomers upon the addition of Bid_BH3 peptide under all pH conditions (Figure 4-6b). This change is particularly striking at pH 4, implying that ligand binding and protein oligomerization occur in a competitive manner as reported in our previous study (177). In a manner akin to ligand binding, the interaction of BclXL
with TOCL/DHPC bicelles also appears to dramatically perturb its solution conformation under all pH conditions (Figures 4-6c and 4-6d). However, such solution-membrane transition is most notable at pH 4, where large plume-like aggregates transform into ring-like structures in association with bicelles, whereas interaction of BclXL is much less conspicuous under other pH conditions. The most straightforward interpretation of these data is that the binding of BclXL to bicelles occurs optimally at pH 4 in agreement with our SSF and ITC data presented above.

**4.4.6 Structural models provide physical basis of acid-induced oligomerization of BclXL**

In an effort to understand the physical basis of acid-induced oligomerization of full-length BclXL, we built structural models of BclXL in two distinct conformations, herein referred to as BclXL_transTM and BclXL_runawayTM (Figure 4-7). In
BclXL\textsubscript{trans}TM conformation, the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in a domain-swapped trans-fashion as described earlier (177). In BclXL\textsubscript{runaway}TM conformation, the TM domain of one monomer occupies the canonical hydrophobic groove within the adjacent monomer and the TM domain of this second monomer in turn occupies the canonical hydrophobic groove within the third monomer in a runaway domain-swapping fashion. It is noteworthy that these structural models were derived from the known solution structures of truncated BclXL, in which the TM domain and the \( \alpha1-\alpha2 \) loop are missing, and the full-length Bax in which the TM domain occupies the canonical hydrophobic groove (113, 145). As discussed earlier, the topological fold of BclXL is comprised of a central predominantly hydrophobic \( \alpha \)-helical hairpin dagger (\( \alpha5 \) and \( \alpha6 \)) surrounded by a cloak comprised of six amphipathic \( \alpha \)-helices (\( \alpha1-\alpha4 \) and \( \alpha7-\alpha8 \)) of varying lengths. However, the key to BclXL oligomerization appear to be the TM domain, which we believe undergoes domain swapping either in a trans-fashion (BclXL\textsubscript{trans}TM) or via the runaway mechanism (BclXL\textsubscript{runaway}TM). Over the past decade or so, oligomerization of proteins through domain-swapping has emerged as a common mechanism for the assembly of proteins into higher-order structures (157-162). From a thermodynamic standpoint, such intermolecular association would allow two participating monomers to bury additional surface area culminating in not only enhanced stability but also providing a greater interacting molecular surface for further oligomerization. This could occur either through the formation of TM-swapped dimers (Figure 4-7a), which would serve as building blocks for further oligomerization, or alternatively, the TM domain could promote oligomerization of BclXL in a head-to-tail fashion (Figure 4-7b). More
Figure 4-7: Structural models of full-length BclXL in two distinct oligomeric conformations, herein designated BclXL_transTM (a) and BclXL_runawayTM (b). In BclXL_transTM conformation, the TM domain of one monomer (green) occupies the canonical hydrophobic groove within the other monomer (yellow) and vice versa in a domain-swapped trans-fashion. In BclXL_runawayTM conformation, the TM domain of one monomer (green) occupies the canonical hydrophobic groove within the adjacent monomer (yellow) and the TM domain of this second monomer (yellow) in turn occupies the canonical hydrophobic groove within the third monomer (cyan) in a runaway domain-swapping fashion. In each model, the red spheres denote the C\textalpha atom of Asp/Glu residues and the blue spheres the C\textalpha atom of His residues. Note also that the TM domain (\alpha5 helix), \alpha1-\alpha2 loop and \alpha8-\alpha9 loop are labeled within each monomer.

importantly, our structural models reveal that the surface of BclXL is heavily decorated with ionizable residues such as Asp, Glu and His, which are particularly prevalent in the \alpha1-\alpha2 loop. Accordingly, these ionizable residues must play a key role in the acid-induced association of BclXL into a megadalton oligomer observed here. Thus, under alkaline conditions, the deprotonation of these ionizable residues will likely increase overall negative charge on BclXL and the resulting electrostatic repulsions between neighboring residues may act as a barrier to extensive oligomerization observed at pH 4. On the other hand, under acidic conditions, protonation will result in the neutralization of
negative charge on Asp/Glu residues, while His residues will gain a net positive charge. Such change in electrostatic polarity may not only promote association of BclXL into a megadalton oligomer observed here but would also likely render it thermodynamically more favorable for the protein to “breathe” and “open up” and, in so doing, facilitate the formation of a molten globule required for its insertion into membrane. It is also conceivable that one or more His residues may engage in some sort of ion pairing with Asp/Glu residues at pH 6, where His will be positively charged but Asp/Glu will bear a net negative charge, within BclXL in an intramolecular manner. Such charge-charge interactions could account for the rather low propensity of BclXL to undergo oligomerization at the expense of monomeric conformation at pH 6 (Figure 4-2a). However, as the pH becomes more acidic, the neutralization of negative charge on Asp/Glu residues will disfavor such intramolecular ion pairing with His and may facilitate oligomerization as observed at pH 4. Importantly, such a scenario is plausible in light of our structural models. In particular, a pair of His residues located within the α1-α2 loop (H58/H71) lies within close proximity to D61/D76/E79, all of which would be negatively charged at pH 6. We note that the oligomerization of RNase A through domain-swapping under acidic conditions has also been reported previously (159). While such oligomerization of RNase A proceeds through an unfolded intermediate (194), we do not believe that a similar scenario also prevails in the case of BclXL oligomerization observed here under acidic conditions. This notion is primarily supported by our far-UV CD analysis wherein BclXL retains a native-like secondary structure under both acidic and alkaline conditions (Figure 3b). Although the oligomerization of BclXL may not ensue via an unfolded intermediate, our studies strongly support the role of a molten
globule intermediate in both the oligomerization and membrane insertion of BclXL.

**4.4.7 MD simulations suggest that the atomic fluctuations within BclXL are pH-dependent**

Our structural models of full-length BclXL presented above suggest strongly that the charged residues may play an active role in driving its association into a megadalton oligomer under acidic conditions. To test this hypothesis and to gain insights into macromolecular dynamics of BclXL as a function of pH, we conducted MD simulations on the BclXL_transTM dimeric conformation over tens of nanoseconds (Figure 4-8). As shown in Figure 4-8a, the MD trajectories reveal that while BclXL reaches structural equilibrium under near-neutral conditions (pH 6 and 8) after about 20ns with an overall root mean square deviation (RMSD) of ~8Å, its structural stability is highly compromised under both acidic (pH 4) and alkaline (pH 10) conditions within this time regime. In particular, the poor structural stability of BclXL at pH 4 may account for its ability to associate into higher-order oligomers such as the plume-like aggregates observed here.

An alternative means to assess mobility and stability of macromolecular complexes is through an assessment of the root mean square fluctuation (RMSF) of specific atoms over the course of MD simulation. Figure 4-8b provides such analysis for the backbone atoms of each residue within BclXL. The RMSF analysis reveals that while a majority of residues within BclXL appear to be well-ordered under all pH conditions, the residues within the α1-α2 loop experience rapid fluctuations which are particularly exaggerated at pH 10. Given that the α1-α2 loop is extensively decorated with acidic residues, it would be plausible to suggest that neutralization of negative charge within
Figure 4-8: MD analysis on the structural model of BclXL_transTM dimeric conformation as a function of pH. (a) Root mean square deviation (RMSD) of backbone atoms (N, Cα and C) within each simulated structure relative to the initial modeled structure of BclXL_transTM as a function of simulation time under various pH conditions as indicated. (b) Root mean square fluctuation (RMSF) of backbone atoms (N, Cα and C) averaged over the entire course of corresponding MD trajectory of BclXL_transTM as a function of residue number under various pH conditions as indicated. The shaded vertical rectangular box indicates the position of residues within the α1-α2 loop.

This loop under acidic pH may serve as a signal for the association of BclXL into a megadalton oligomer. It is noteworthy that the deletion of the α1-α2 loop in BclXL augments its anti-apoptogenicity and that the suppressive effect of α1-α2 loop is relieved by its post-translational phosphorylation (195). In light of this observation, we believe that the intrinsic flexibility of the α1-α2 loop may be a driving force for the oligomerization of BclXL through favorable entropic contributions and that such intermolecular association most likely compromises its anti-apoptotic action.

4.5 Concluding remarks

Our earlier studies provided the evidence for the association of full-length BclXL into higher-order oligomers under mildly alkaline conditions (177). In this study, we have demonstrated that the oligomerization of BclXL is highly pH-dependent and that under
acidic conditions, it associates into a megadalton oligomer with a plume-like appearance and harboring molten globule characteristics. Although such acidic conditions are unlikely to be recapitulated globally within the milieu of the living cell, it is highly conceivable that a change in pH of a few units is norm within small localized microenvironments of the cytosol. Indeed, several lines of evidence suggest the formation of a pH gradient across the mitochondria, accompanied by the alkalinization of mitochondrial matrix and acidification of the cytosol, upon the induction of apoptosis (55, 56, 68, 174-176). Additionally, our data also reflect the fact that the acidic conditions employed here may also serve as mimicry for cellular stress. The ability of BclXL to undergo acid-induced oligomerization is thus highly relevant to the situation in vivo. More importantly, previous studies suggest that the molten globule represents a thermodynamically favorable route for the membrane insertion of many other proteins that undergo solution-membrane transition (61, 62, 168, 169). Consistent with this notion, our data argue that BclXL interacts with cardiolipin bicelles optimally under acidic conditions, which favor both its oligomerization and the formation of a molten globule. It is noteworthy that cardiolipin is not only exclusively found within mitochondrial membranes but, upon apoptotic induction, BclXL specifically localizes at the MOM (144), presumably through a physical interaction with cardiolipin. Regardless of the in vivo mechanisms involved in the insertion of BclXL into MOM, the data presented here unequivocally demonstrate that acidic pH promotes the oligomerization of BclXL and that such propensity of BclXL to undergo oligomerization is likely to be relevant to its in vivo function.
Chapter 5: Heat-Induced Fibrillation of BclXL Apoptotic Repressor

5.1 Summary

The BclXL apoptotic repressor bears the propensity to associate into megadalton oligomers in solution, particularly under acidic pH. Herein, using various biophysical methods, we analyze the effect of temperature on the oligomerization of BclXL. Our data show that BclXL undergoes irreversible aggregation and assembles into highly-ordered rope-like homogeneous fibrils with length in the order of mm and a diameter in the μm-range under elevated temperatures. Remarkably, the formation of such fibrils correlates with the decay of a largely α-helical fold into a predominantly β-sheet architecture of BclXL in a manner akin to the formation of amyloid fibrils. Further interrogation reveals that while BclXL fibrils formed under elevated temperatures show no observable affinity toward BH3 ligands, they appear to be optimally primed for insertion into cardiolipin bicelles. This salient observation strongly argues that BclXL fibrils likely represent an on-pathway intermediate for insertion into mitochondrial outer membrane during the onset of apoptosis. Collectively, our study sheds light on the propensity of BclXL to form amyloid-like fibrils with important consequences on its mechanism of action in gauging the apoptotic fate of cells in health and disease.

5.2 Overview

Embryonic development and cellular homeostasis are heavily dependent on the concerted action of Bcl2 family of proteins in what has come to be known as apoptosis (23-25, 27, 28, 107-109). The Bcl2 proteins can be divided into three major groups with respect to their role in the regulation of apoptotic machinery: activators, effectors and repressors. Activators such as Bid and Bad belong to the BH3-only proteins, where BH3 is the Bcl2
homology 3 domain. Effectors such as Bax and Bak contain the BH3-BH1-BH2-TM modular architecture, where TM is the transmembrane domain located C-terminal to Bcl2 homology domains BH3, BH1 and BH2. Repressors such as Bcl2 and BclXL are usually characterized by the BH4-BH3-BH1-BH2-TM modular organization, with an additional N-terminal Bcl2 homology 4 domain.

How do Bcl2 proteins keep apoptosis in check? In a nutshell, the apoptotic fate, or the decision of a cell to live or die, is determined by the cellular ratio of activator, effector and repressor molecules (30, 31). In quiescent and healthy cells, the effectors are maintained in an inactive state via complexation with repressors. Upon receiving apoptotic cues, in the form of DNA damage and cellular stress, the activators are stimulated and compete with effectors for binding to the repressors and, in so doing, not only do they neutralize the anti-apoptotic action of repressors but also unleash the pro-apoptogenicity of effectors. The effectors subsequently initiate apoptotic cell death by virtue of their ability to insert into the mitochondrial outer membrane (MOM) resulting in the formation of mitochondrial pores in a manner akin to the insertion of bacterial toxins such as colicins and diphtheria (32-34, 62, 110). This leads to the release of apoptogenic factors such as cytochrome c and Smac/Diablo from mitochondria into the cytosol. Subsequently, rising levels of apoptogenic factors in the cytosol switch on aspartate-specific proteases termed caspases, which in turn, demolish the cellular architecture by cleavage of proteins culminating in total cellular destruction.

While there is a general consensus that hetero-association between various members of the Bcl2 family represents a defining event in the decision of a cell to live or die, the biophysical basis of such protein-protein interactions remains hitherto poorly
characterized and, in particular, our limited knowledge on the ability of Bcl2 proteins to undergo homo-association into higher-order oligomers and aggregates leaves much to be desired in our quest to further our understanding of apoptosis at molecular level. Toward this goal, our previous studies have shown that BclXL apoptotic repressor bears the propensity to associate into megadalton oligomers in solution, particularly under acidic pH, and that such aggregation is largely mediated by the C-terminal transmembrane (TM) domain (177, 196). Importantly, a truncated construct of BclXL lacking the C-terminal TM domain, was recently shown to form amyloid-like fibrils under elevated temperatures (183). This salient observation invokes a key role of thermal energy in driving the aggregation of BclXL. In an effort to further explore the effect of elevated temperature, we have conducted here detailed biophysical analysis on the propensity of full-length BclXL, harboring the C-terminal TM domain, to undergo oligomerization. It is important to note here that temperature is one of the key physical factors that governs the ability of many proteins to associate into higher-order oligomers. Additionally, elevated temperature should also serve as a mimicry for cellular stress and thus may shed light on how cellular homeostasis may regulate the oligomerization of this key apoptotic regulator.

5.3 Experimental Procedures

5.3.1 Sample Preparation

Full-length human BclXL (residues 1-233) was cloned into pET30 bacterial expression vector with an N-terminal His-tag using Novagen LIC technology, expressed in Escherichia coli BL21*(DE3) bacterial strain (Invitrogen) and purified on a Ni-NTA affinity column using standard procedures as described previously (177, 196). Protein
concentration was determined by the fluorescence-based Quant-It assay (Invitrogen) and spectrophotometrically on the basis of an extinction coefficient of 47,440 M$^{-1}$cm$^{-1}$ calculated for the full-length BclXL using the online software ProtParam at ExPasy Server (115). Results from both methods were in an excellent agreement. The 20-mer peptide spanning residues 81-100 corresponding to the BH3 domain within human Bid (H$_2$N-DIIRNIARHLAQVGDSMDRS-COOH), herein referred to as Bid$_{BH3}$, was commercially obtained from GenScript Corporation. The peptide concentration was measured gravimetrically. Mixed TOCL/DHPC bicelles were prepared at a final concentration of 30mM, at TOCL to DHPC molar ratio of 1:4, by stirring for 2h at 37°C. For biophysical experiments described below, all protein, peptide and bicelle samples were prepared in 50mM Sodium phosphate buffer containing 100mM NaCl (except for CD measurements) at pH 8.0. Except for transient measurements, samples of BclXL were pre-incubated overnight at various temperatures ranging from 20°C to 80°C prior to each experiment. All measurements were repeated at least three times.

5.3.2 Molecular dynamics

Molecular dynamics (MD) simulations were performed with the GROMACS software (99, 125) using the integrated OPLS-AA force field (126, 127).Briefly, the BclXL$_{transTM}$ structural model was centered within a cubic box, hydrated using the extended simple point charge (SPC/E) water model (128, 129), and the ionic strength of solution was set to 100mM with NaCl. The hydrated structure was energy-minimized with the steepest descent algorithm prior to equilibration under the NPT ensemble conditions, wherein the number of atoms (N), pressure (P) and temperature (T) within the system were kept constant. The Particle-Mesh Ewald (PME) method was employed to
compute long-range electrostatic interactions with a 10Å cut-off \((130)\) and the Linear Constraint Solver (LINCS) algorithm to restrain bond lengths \((131)\). All MD simulations were performed under periodic boundary conditions (PBC) at 20°C, 40°C, 60°C and 80°C using the leap-frog integrator with a time step of 2fs. For the final MD production runs, data were collected every 100ps over a time scale of 100ns. All simulations were run on a Linux workstation using parallel processors at the High Performance Computing facility within the Center for Computational Science of the University of Miami.

5.3.3 Molecular modeling

Molecular modeling (MM) was employed to build a domain-swapped structural model of BclXL homodimer, herein referred to as BclXL_transTM, using the MODELLER software \((122, 123)\). Briefly, in the BclXL_transTM structural model, the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in a domain-swapped trans-fashion as described earlier \((177, 196)\). The structural model was rendered using RIBBONS \((124)\).

5.3.4 Analytical light scattering

Analytical light scattering (ALS) experiments were conducted on a Wyatt miniDAWN TREOS triple-angle static light scattering detector and Wyatt QELS dynamic light scattering detector coupled in-line with a Wyatt Optilab rEX differential refractive index detector and interfaced to a Hiload Superdex 200 size-exclusion chromatography column under the control of a GE Akta FPLC system within a chromatography refrigerator at 10°C. Briefly, pre-heated samples of 10μM BclXL at various temperatures ranging from 20°C to 80°C were loaded onto the column at a flow rate of 1ml/min and the data were automatically acquired using the ASTRA software. The angular- and concentration-
dependence of static light scattering (SLS) intensity of BclXL resolved in the flow mode was measured by the Wyatt miniDAWN TREOS detector equipped with three scattering angles positioned at 42°, 90° and 138°. The time- and concentration-dependence of dynamic light scattering (DLS) intensity fluctuation of BclXL resolved in the flow mode was measured by the Wyatt QELS detector positioned at 90° with respect to the incident laser beam. Hydrodynamic parameters $M_w$ (weighted-average molar mass), $M_n$ (number-average molar mass), $R_g$ (weighted-average radius of gyration) and $R_h$ (weighted-average hydrodynamic radius) associated with solution behavior of BclXL were determined by the treatment of SLS data to Zimm model and by non-linear least-squares fit of DLS data to an autocorrelation function as described earlier (177, 196). It should be noted that, in both the SLS and DLS measurements, protein concentration ($c$) along the elution profile of BclXL was automatically quantified in the ASTRA software from the change in refractive index ($\Delta n$) with respect to the solvent as measured by the Wyatt Optilab rEX detector using the following relationship:

$$c = (\Delta n)/(dn/dc)$$

[1]

where $dn/dc$ is the refractive index increment of the protein in solution.

5.3.5 **Circular dichroism**

Circular dichroism (CD) measurements were conducted on a thermostatically-controlled Jasco J-815 spectrometer at 25°C. For far-UV steady-state measurements, experiments were conducted on pre-heated samples of 10μM BclXL alone, pre-equilibrated with 100μM Bid_BH3 peptide, or pre-equilibrated with 2mM TOCL/DHPC bicelles at various temperatures ranging from 20°C to 80°C and data were collected using a quartz cuvette with a 2-mm pathlength in the 195-255nm wavelength range. For near-
UV steady-state measurements, experiments were conducted on pre-heated samples of 50μM BclXL alone at various temperatures ranging from 20°C to 80°C and data were collected using a quartz cuvette with a 10-mm pathlength in the 255-315nm wavelength range. In each case, a slit bandwidth of 2nm was used and data were recorded at a scan rate of 10nm/min. All spectral data were normalized against reference spectra to remove the background contribution of buffer. Each spectral data set represents an average of four scans acquired at 0.1nm intervals. All data were converted to mean ellipticity, [θ], as a function of wavelength (λ) of electromagnetic radiation using the equation:

\[
[\theta] = \left[\frac{(10^5 \Delta \theta)/c}{l}\right] \text{deg.cm}^2\text{.dmol}^{-1}
\]  

where \(\Delta \theta\) is the observed ellipticity in mdeg, \(c\) is the protein concentration in μM and \(l\) is the cuvette pathlength in cm. For far-UV transient measurements, freshly purified samples of 10μM BclXL alone, pre-equilibrated with 100μM Bid_BH3 peptide, or pre-equilibrated with 1mM TOCL/DHPC bicelles at 20°C were placed in a quartz cuvette with a 2-mm pathlength and the change in spectral intensity at 222nm, \([\theta]_{222}\), was monitored over three consecutive temperature steps (herein denoted Steps I-III) as a function of time for 90min: in Step I, the temperature was ramped up from 20°C to 80°C at a ramp rate of 2°C/min over the time period 0-30min; in Step II, the temperature was held constant at 80°C over the time period 31-60min; in Step III, the temperature was ramped down from 80°C to 20°C at a ramp rate of 2°C/min over the time period 61-90min

5.3.6 **Steady state fluorescence**

Steady-state fluorescence (SSF) spectra were collected on a thermostatically-controlled Jasco FP-6300 spectrofluorimeter using a quartz cuvette with a 10-mm
pathlength at 25°C. Briefly, experiments were conducted on pre-heated samples of 10μM BclXL pre-equilibrated with 100μM ANS, pre-equilibrated with 10μM ThT, or pre-equilibrated with 10μM ThT and 100μM Myr at various temperatures ranging from 20°C to 80°C. For ANS fluorescence, the excitation wavelength was 375nm and emission was acquired over the 400-700nm wavelength range. For ThT fluorescence, the excitation wavelength was 420nm and emission was acquired over the 430-650nm wavelength range. All data were recorded using a 2.5-nm bandwidth for both excitation and emission. Data were normalized against reference spectra to remove background contribution of protein and buffer. Fluorescence enhancement (E) of ANS or ThT in the presence of BclXL at each incubation temperature was calculated from the following equation:

\[ E = \left[ \frac{\Phi - \Phi_o}{\Phi_o} \right] \times 100\% \]  

where \( \Phi \) is the fluorescence yield of ANS or ThT in the presence of BclXL and \( \Phi_o \) is the fluorescence yield of ANS or ThT alone at corresponding incubation temperature. Fluorescence yield (\( \Phi \)) is defined as the area integrated under the corresponding emission spectra.

### 5.3.7 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on a Microcal VP-ITC instrument at 25 °C. Briefly, experiments were conducted to probe the binding of Bid_BH3 peptide and mixed TOCL/DHPC bicelles to pre-heated samples of 50μM BclXL at various temperatures ranging from 20°C to 80°C. For peptide binding, experiments were initiated by injecting 25 x 10μl aliquots of 1mM of Bid_BH3 peptide from the syringe into the calorimetric cell containing 1.8ml of pre-heated samples of 50μM BclXL. For membrane insertion, experiments were initiated by injecting 25 x 10μl
aliquots of pre-heated samples of 50μM BclXL from the syringe into the calorimetric cell containing 1.8ml of 2mM of TOCL/DHPC. In each case, the change in thermal power as a function of each injection was automatically recorded using the ORIGIN software and the raw data were further processed to yield binding isotherms of heat release per injection either as a function of molar ratio of peptide to BclXL or as a function of molar ratio of BclXL to bicelles. The heats of mixing and dilution were subtracted from the heats of peptide binding or membrane insertion per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was either titrated against the Bid_BH3 peptide or BclXL in an identical manner. The apparent equilibrium dissociation constant (K_d) and the enthalpic change (∆H) associated with peptide binding to BclXL or membrane insertion of BclXL were determined from the non-linear least-squares fit of data to a one-site binding model as described previously (116, 177). The binding free energy change (∆G) was calculated from the following expression:

\[ \Delta G = RT\ln K_d \]  \hspace{1cm} [4]

where R is the universal molar gas constant (1.99 cal/K/mol) and T is the absolute temperature. The entropic contribution (T∆S) to the free energy of binding was calculated from the relationship:

\[ T\Delta S = \Delta H - \Delta G \]  \hspace{1cm} [5]

where ∆H and ∆G are as defined above.

5.3.8  **Fluorescence microscopy**

Fluorescence microscopy (FM) experiments were conducted on a Leica DMI6000 microscope with 10x objective. All images were analyzed and processed using Leica
LAS-AF software. Data were collected on pre-heated samples of 25\(\mu\)M BclXL alone, pre-equilibrated with 250\(\mu\)M Bid_BH3 peptide, or pre-equilibrated with 2mM TOCL/DHPC bicelles at various incubation temperatures ranging from 20\(^\circ\)C to 80\(^\circ\)C. Prior to imaging, each sample was stained with 25\(\mu\)M ThT and mounted onto a glass slide.

5.4 Results and discussion

5.4.1 BclXL harbors intrinsic propensity to aggregate

On the basis of previous x-ray crystallographic and molecular modeling analysis (40, 145, 177, 196), the 3D structural topology of BclXL is characterized by a central predominantly hydrophobic \(\alpha\)-helical hairpin “dagger” (\(\alpha5\) and \(\alpha6\)) surrounded by a “cloak” comprised of six amphipathic \(\alpha\)-helices (\(\alpha1\)-\(\alpha4\) and \(\alpha7\)-\(\alpha8\)) of varying lengths. The so-called “canonical hydrophobic groove”, that serves as the docking site for the BH3 domain of activators and effectors, is formed by the juxtaposition of \(\alpha2\)-\(\alpha5\) helices. Additionally, BclXL is decorated with a C-terminal hydrophobic \(\alpha\)-helix termed \(\alpha9\), or more commonly the TM domain, which is believed to facilitate localization of BclXL to MOM upon apoptotic induction (41, 42, 112).

Importantly, we have previously shown that BclXL displays the propensity to oligomerize in solution and that such oligomerization is driven by the intermolecular binding of its C-terminal TM domain to the canonical hydrophobic groove in a domain-swapped trans-fashion (177, 196), whereby the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in what we refer to as the BclXL\textsubscript{transTM} conformation (Figure 5-1a). We further postulated that such homodimerization could in turn drive the association of BclXL into higher-order mega-
Figure 5-1: In silico analysis of BclXL. (a) A structural model of a dimeric conformation of BclXL (BclXL_transTM), where the TM domain of one monomer (cyan) occupies the canonical hydrophobic groove within the other monomer (blue) and vice versa in a domain-swapped trans-fashion swapping as described earlier (177, 196). (b) Plots showing the propensity of BclXL to aggregate into amyloid-like fibrils as predicted by AMYLPRED (red) and AGGRESCAN (green). Note that the BH4-BH3-BH1-BH2-TM modular architecture of BclXL is overlaid for direct correlation of aggregation propensity to specific domains within BclXL.

dalton aggregates. In light of the knowledge that a wide range of proteins share the ability to aggregate into amyloid-like fibrils under environmental stresses such as acidic pH and
elevated temperatures (78, 79, 197, 198), we also analyzed the intrinsic propensity of BclXL to aggregate into fibrils using aggregation predictors such as AMYLPRED (199) and AGGRESCAN (200). As shown in Figure 5-1b, our in silico analysis reveals that BclXL indeed harbors intrinsic propensity to aggregate and that the residues that drive such aggregation primarily reside within the BH1 (α4-α5) and TM (α9) domains. While the involvement of BH1 domain in promoting the aggregation is somewhat surprising, the role of TM domain is in full agreement with our previous studies demonstrating that its deletion abolishes the association of BclXL into larger aggregates (177, 196).

5.4.2 Thermal motions appear to destabilize the structural architecture of BclXL

Our previous studies have shown that the BclXL apoptotic repressor bears the propensity to associate into megadalton aggregates in solution, particularly under acidic pH (177, 196). To understand the extent to which elevated temperature may also contribute to such aggregation, we conducted MD simulations on the BclXL_transTM dimeric conformation over tens of nanoseconds at various temperatures (Figure 5-2a). As shown in Figure 2a, the MD trajectories reveal that while BclXL reaches structural equilibrium after about 20ns under all temperatures, its stability is compromised under elevated temperatures. Thus, while the root mean square deviation (RMSD) of BclXL at structural equilibrium fluctuates around 8Å at low temperatures (20°C and 40°C), it rises to around 12Å under elevated temperatures (60°C and 80°C). This strongly argues that the poor structural stability of BclXL due to enhanced thermal motions under elevated temperatures may account for its ability to associate into higher-order aggregates.

An alternative means to assess mobility and stability of macromolecular complexes is through an assessment of the root mean square fluctuation (RMSF) of
Figure 5-2: MD analysis of BclXL_transTM conformation at various temperatures as indicated. (a) Root mean square deviation (RMSD) of backbone atoms (N, C$\alpha$ and C) within each simulated structure relative to the initial modeled structure of BclXL_transTM as a function of simulation time. (b) Root mean square fluctuation (RMSF) of backbone atoms (N, C$\alpha$ and C) averaged over the entire course of corresponding MD trajectory of the modeled structure of BclXL_transTM as a function of residue number. Note that the shaded vertical rectangular boxes indicate the residue boundaries of the α1-α2 loop as well as the BH1 and TM domains.

Specific atoms over the course of MD simulation. Figure 5-2b provides such analysis for the backbone atoms of each residue within BclXL. The RMSF analysis shows that while a majority of residues within BclXL appear to be well-ordered under all temperatures, the exaggerated at 20°C but become more widespread at 80°C. Accordingly, the change in motional properties of residues within the α1-α2 loop under elevated temperatures could trigger the association of BclXL into larger aggregates. It is noteworthy that the deletion of the α1-α2 loop in BclXL augments its anti-apoptogenicity and that the suppressive effect of α1-α2 loop is relieved by its post-translational phosphorylation (195). In light of this observation, we believe that the intrinsic flexibility of the α1-α2 loop may be a
driving force for the aggregation of BclXL through favorable entropic contributions and that such intermolecular association most likely compromises its anti-apoptotic action. Interestingly, our in silico analysis presented above reveals that the major determinants of the propensity of BclXL to aggregate most likely reside within the BH1 and TM domains in lieu of the α1-α2 loop (Figure 5-1b). However, residues within the BH1 and TM domains show no observable change in their backbone dynamics in response to changes in temperature. This likely suggests that the molecular origin of factors promoting the aggregation of BclXL is highly complex and may not necessarily be governed by changes in thermal motions. Nevertheless, our MD simulations provide molecular insights into the effect of temperature on the motional properties of BclXL.

5.4.3 Elevated temperature shifts the equilibrium of BclXL into megadalton aggregates

To directly test the extent to which temperature may promote the association of BclXL into larger aggregates, we conducted ALS analysis on pre-heated samples of BclXL at various temperatures ranging from 20°C to 80°C and quantified physical parameters accompanying its solution behavior from the first principles of hydrodynamics without any assumptions (Figure 5-3 and Table 5-1). Our data indicate that BclXL exists in various associative conformations at 20°C, ranging from monomer (31kD) and dimer (62kD) to higher-order oligomers, herein referred to as multimer (~400kD) and polymer (~4000kD). At 40°C, the dimer and multimer conformers appear to shift in the direction of the polymeric conformation. Remarkably, under elevated temperatures (60°C and 80°C), BclXL appears to largely exist in a large aggregate that we refer to herein as megamer. This strongly suggests that elevated temperature
facilitates association of BclXL into megadalton aggregates. Notably, the truncation of C-terminal TM domain completely abolished oligomerization of BclXL under low temperatures (20°C and 40°C), while only small aggregates were observed under elevated temperatures (60°C and 80°C). These observations are in general agreement with previous studies showing that a C-terminally truncated construct of BclXL forms amyloid-like fibrils under elevated temperatures (183). However, our data presented above implicate a key role of TM domain in driving the intermolecular association of BclXL into large aggregates in agreement with our previous studies (177, 196). In an attempt to gain insights into the conformational heterogeneity of the oligomeric species of BclXL, we also determined the $M_n/M_w$ and $R_g/R_h$ ratios from our hydrodynamic data.
Table 5-1
Hydrodynamic parameters for BclXL pre-incubated at the indicated temperatures

<table>
<thead>
<tr>
<th>Associativity</th>
<th>M_w / kD</th>
<th>M_n / kD</th>
<th>M_w/M_n</th>
<th>R_g / Å</th>
<th>R_h / Å</th>
<th>R_g/R_h</th>
<th>P / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer 20°C</td>
<td>34 ± 3</td>
<td>33 ± 3</td>
<td>1.02 ± 0.01</td>
<td>ND</td>
<td>25 ± 2</td>
<td>ND</td>
<td>14</td>
</tr>
<tr>
<td>Dimer 20°C</td>
<td>59 ± 4</td>
<td>57 ± 4</td>
<td>1.03 ± 0.01</td>
<td>ND</td>
<td>45 ± 3</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Multimer 20°C</td>
<td>374 ± 23</td>
<td>353 ± 15</td>
<td>1.06 ± 0.02</td>
<td>101 ± 9</td>
<td>87 ± 3</td>
<td>1.25 ± 0.02</td>
<td>39</td>
</tr>
<tr>
<td>Polymer 20°C</td>
<td>3787 ± 242</td>
<td>3227 ± 185</td>
<td>1.16 ± 0.04</td>
<td>248 ± 31</td>
<td>184 ± 9</td>
<td>1.33 ± 0.15</td>
<td>44</td>
</tr>
<tr>
<td>Monomer 40°C</td>
<td>30 ± 2</td>
<td>29 ± 2</td>
<td>1.03 ± 0.01</td>
<td>ND</td>
<td>28 ± 4</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>Polymer 40°C</td>
<td>8729 ± 797</td>
<td>7660 ± 948</td>
<td>1.15 ± 0.05</td>
<td>407 ± 20</td>
<td>223 ± 15</td>
<td>1.82 ± 0.16</td>
<td>80</td>
</tr>
<tr>
<td>Megamer 60°C</td>
<td>24895 ± 1463</td>
<td>24145 ± 1039</td>
<td>1.04 ± 0.02</td>
<td>598 ± 28</td>
<td>274 ± 24</td>
<td>2.17 ± 0.10</td>
<td>100</td>
</tr>
<tr>
<td>Megamer 80°C</td>
<td>61010 ± 7624</td>
<td>60730 ± 7741</td>
<td>1.01 ± 0.01</td>
<td>709 ± 86</td>
<td>341 ± 48</td>
<td>2.08 ± 0.17</td>
<td>100</td>
</tr>
</tbody>
</table>

All parameters were obtained from ALS measurements. The population (P) of each species, as estimated from the integration of corresponding peak in the elution profile (Figure 5-3), is provided in the right-most column. Note that the calculated molar mass of recombinant full-length BclXL from amino acid sequence alone is 31kD. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation. Note that the R_g parameter could not be determined (ND) for various species due to their lack of angular-dependence of scattered light.

(Table 5-1). It should be noted that while the M_w/M_n ratio provides a measure of the macromolecular polydispersity, the R_g/R_h ratio sheds light on the overall macromolecular shape. Our data suggest that while the higher-order oligomers (multimer and polymer) of BclXL display some degree of polydispersity (M_w/M_n > 1.05) under all temperatures, the monomeric and dimeric forms of BclXL are predominantly monodisperse (M_w/M_n < 1.05). Strikingly, BclXL not only exclusively exists as a megadalton oligomer under elevated temperatures (60°C and 80°C) but it also surprisingly appears to be highly monodisperse (M_w/M_n < 1.05). Additionally, the higher-order oligomers (multimer and polymer) of BclXL most likely adopt an elongated rod-like shape (R_g/R_h > 1.05) in lieu
of a more spherical or compact structure. Consistent with these observations, the megameric species observed under elevated temperatures (60°C and 80°C) also seem to adopt an highly elongated rod-like architecture ($R_e/R_h > 2$) with a radius of gyration of ~100 nm, arguing that it may bear the propensity to assemble into fibrils of up to hundreds of nm in length in a manner akin to amyloid fibrils. It should be noted that the actual size of BclXL aggregates observed under elevated temperatures is likely to be much larger due to the fact that the various hydrodynamic parameters reported here exceed the upper limit of detection of ALS. Additionally, hydrodynamic properties of such BclXL aggregates are most likely underestimated here due to the filtration of protein samples prior to ALS analysis, implying that larger aggregates most likely never reach the ALS detectors. We also note that our ALS analysis of BclXL in the presence of TOCL/DHPC bicelles—as a model for MOM—was complicated by the fact that the scattering of light by bicelles swamped the protein signal, thereby rendering it very difficult to analyze the effect of bicelles on BclXL aggregates.

5.4.4 BclXL undergoes structural transition at elevated temperature

It is well-documented that many proteins that aggregate into amyloid-like fibrils adopt cross β-sheet structure combined with the loss of globular fold (201-205). Thus, we wondered whether the ability of BclXL to associate into large aggregates under elevated temperatures is also coupled to such structural changes. To address this question, we carried out CD analysis on BclXL pre-heated overnight at various temperatures ranging from 20°C to 80°C (Figure 5-4). Our far-UVCD analysis shows that BclXL displays spectral features characteristic of an α-helical fold with bands centered around 208nm and 222nm at lower temperatures (Figure 5-4a, top panel). Remarkably, under elevated
temperatures, the α-helical spectral features of BclXL disappear at the expense of appearance of a new band around 216nm, which is characteristic of β-sheet architecture. These salient observations suggest that BclXL undergoes structural transition from a predominantly α-helical fold to a largely β-sheet conformation.

To monitor how elevated temperature affects tertiary structure of BclXL, we next conducted near-UV CD analysis in a similar manner (Figure 5-4b, top panel). Unsurprisingly, BclXL displays spectral features in the near-UV region characteristic of a well-folded globular protein with bands emanating from the chiral environment surrounding aromatic residues such as phenylalanine (258nm), tyrosine (282nm) and tryptophan (293nm) at lower temperatures. Consistent with our far-UV CD analysis presented above, these bands either largely disappear or become substantially attenuated.
at elevated temperatures. This is evidence that BclXL aggregates lose their native tertiary structure and adopt a more fibrillar conformation that presumably lacks a well-defined tertiary structure under elevated temperatures. Importantly, the lack of a single isosbestic point in both the far-UV and near-UV spectra recorded for BclXL at various temperatures strongly argues that the conversion of an α-helical fold into β-sheet architecture occurs via at least one intermediate step (Figures 5-4a and 5-4b, top panels). This view is further corroborated by the observation that the dependence of spectral intensities at 222nm (monitoring secondary structural changes) and 282nm (monitoring tertiary structural changes) displays multiphasic behavior with increasing temperature in lieu of a linear relationship (Figures 5-4a and 5-4b, bottom panels). Taken together, our far-UV and near-UV CD data strongly suggest that BclXL aggregates observed under elevated temperatures most likely adopt a cross β-sheet structure characteristic of amyloid-like fibrils.

5.4.5 BclXL undergoes distinct structural transition upon interaction with BH3 ligand and MOM mimetic

To understand how BH3 ligands and MOM mimetics modulate the extent to which the BclXL interconverts from an α-helical fold to a β-sheet conformation, we conducted far-UV CD analysis on BclXL pre-equilibrated overnight either with a 20-mer BH3 peptide derived from Bid activator (Bid_BH3) or mixed TOCL/DHPC bicelles—used here as a model for MOM—at various temperatures ranging from 20°C to 80°C (Figure 5-5). Remarkably, our analysis reveals that while BclXL adopts a predominantly α-helical fold with minima centered around 210nm and to a lesser extent at 222nm in the presence of Bid_BH3 peptide and TOCL/DHPC bicelles at 20°C (Figures 5-5a and 5-5b), it undergoes structural transition in which the minima around 210nm and 222nm are more
Figure 5-5: Steady-state CD analysis of BclXL pre-equilibrated with Bid_BH3 peptide (a) or TOCL/DHPC bicelles (b) overnight at various temperatures (T). (a) Far-UV spectra of BclXL pre-equilibrated with Bid_BH3 peptide (top panel) and the dependence of mean ellipticity at 222nm, $[\theta]_{222}$, on temperature (bottom panel). (b) Far-UV spectra of BclXL pre-equilibrated with TOCL/DHPC bicelles (top panel) and the dependence of mean ellipticity at 222nm, $[\theta]_{222}$, on temperature (bottom panel). In the top panels in (a) and (b), the spectra shown were recorded at 20°C (black), 30°C (red), 40°C (green), 50°C (blue), 60°C (cyan), 70°C (magenta) and 80°C (brown). In the bottom panels in (a) and (b), the data points are connected with a solid line for clarity. The error bars were calculated from three independent measurements to one standard deviation. (c) Comparison of mean ellipticity at 222nm ($[\theta]_{222}$), and 210nm ($[\theta]_{210}$, for BclXL alone (Free), BclXL pre-equilibrated with Bid_BH3 peptide (+Peptide), or BclXL pre-equilibrated with TOCL/DHPC bicelles (+Bicelles) overnight at 80°C.

or less preserved but experience a loss in spectral intensity at elevated temperatures. This suggests strongly that the peptide and the bicelles induce a structural transition within BclXL that is distinct from that observed for BclXL alone at elevated temperatures. We interpret such structural transition from a partial loss of $\alpha$-helical fold to a coiled-coil conformation in sharp contrast to the $\beta$-sheet architecture observed for BclXL alone at elevated temperatures. Such differences in the structural transition are further highlighted by the differential changes observed in the ellipticity at 222nm as a function of temperature for BclXL alone (Figure 5-4a, bottom panel) versus those observed in the presence of Bid_BH3 peptide (Figure 5-5a, bottom panel) and TOCL/DHPC bicelles (Figure 5-5b, bottom panel). Notably, comparison of mean ellipticity at 210nm and
222nm for BclXL alone, BclXL pre-equilibrated with Bid_BH3 peptide, and BclXL pre-equilibrated with TOCL/DHPC bicelles overnight at 80°C further corroborates the notion that BclXL undergoes distinct structural changes at elevated temperatures depending on whether it is heated alone in solution or in the presence of its ligands (Figure 5-5c).

5.4.6 Aggregation of BclXL under elevated temperature represents a kinetic trap

Our steady-state CD data presented above suggest that the α-β structural transition of BclXL alone, in the presence of Bid_BH3 peptide or TOCL/DHPC bicelles at elevated temperatures overnight is an irreversible process in that the protein aggregates retain their integrity and β-sheet structure when cooled down to a temperature of 25°C (Figures 5-4 and 5-5). In an attempt to directly gauge the kinetics and reversibility of temperature-induced aggregates of BclXL on a shorter time scale, we next transiently monitored the far-UV CD spectral intensity at 222nm, [θ]222, of BclXL alone, pre-equilibrated with Bid_BH3 peptide, or pre-equilibrated with mixed TOCL/DHPC bicelles at 20°C as a function of temperature in the 20-80°C range over a time period of 90min (Figure 5-6). Consistent with our far-UV CD data presented above, [θ]222 of BclXL alone increases with increasing temperature from 20°C to 80°C (Figure 5-6a), implying that BclXL undergoes α-β transition under elevated temperatures.

Importantly, [θ]222 of BclXL alone exquisitely plateaus out as the temperature reaches a constant value of 80°C and the resulting plateau is unaffected upon the reversal of the temperature from 80°C to 20°C. This salient observation further corroborates the notion that the temperature-induced formation of BclXL aggregates is an irreversible process that results in a kinetic trap. Interestingly, when BclXL is pre-equilibrated with Bid_BH3 peptide, [θ]222 shows no change as a function of time (Figure 5-6b), implying
Figure 5-6: Transient CD analysis of BclXL alone (a), pre-equilibrated with Bid_BH3 peptide (b), or pre-equilibrated with TOCL/DHPC bicelles at 20°C (c). Briefly, changes in mean ellipticity at 222nm, [θ]_{222}, were monitored for each sample over three consecutive temperature steps (herein denoted Steps I-III) as a function of time for 90min: in Step I, the temperature was ramped up from 20°C to 80°C at a ramp rate of 2°C/min over the time period 0-30min; in Step II, the temperature was held constant at 80°C over the time period 31-60min; in Step III, the temperature was ramped down from 80°C to 20°C at a ramp rate of 2°C/min over the time period 61-90min. In each panel, the red solid line shows the change in temperature (T) as a function of time (t) and the three temperature steps are demarcated by vertical dashed lines.
that the binding of BH3 ligands to BclXL slows down the aggregation of BclXL. Finally, pre-equilibration of BclXL with TOCL/DHPC bicelles does not appear to dramatically affect the aggregation of BclXL as monitored by changes in $\theta_{222}$ (Figure 5-6c).

5.4.7 BclXL harbors structural features characteristic of amyloid fibrils under elevated temperature

In light of the knowledge that many proteins that aggregate into amyloid-like fibrils adopt cross $\beta$-sheet structure with exposed hydrophobic surfaces (201-205), we next analyzed the ability of BclXL to aggregate under various temperatures ranging from 20°C to 80°C using fluorescent hydrophobic dyes in combination with SSF (Figure 5-7). It is well-documented that the fluorescence of hydrophobic dyes such as ANS and ThT undergoes enhancement upon binding to the canonical cross $\beta$-sheet topology and the exposed hydrophobic surfaces characteristic of amyloid-like fibrils (184, 206-209). Consistent with this notion, our analysis reveals that while ANS fluorescence undergoes nearly two-fold enhancement when BclXL is pre-heated to 80°C relative to incubation at 20°C (Figure 5-7a), ThT experiences close to an order of magnitude fluorescence enhancement (Figure 5-7b). We note that while the emission of ANS and ThT occurs maximally around 500-515nm in water, it appears to be blue-shifted to around 475nm upon binding to BclXL. This is further evidence for the exposure of hydrophobic surfaces in BclXL, which apparently becomes more exaggerated under elevated temperatures. Importantly, polyphenols such as Myr have been shown to destabilize amyloid fibrils (210). We wondered whether Myr may also have a similar effect on the fibrillar aggregates observed for BclXL under elevated temperatures. Indeed, when BclXL is pre-equilibrated with ThT in the presence of Myr prior to heating at various temperatures, the fluorescence enhancement of ThT is substantially reduced under elevated temperatures (Figure 5-7c).
Figure 5-7: SSF analysis of BclXL pre-heated overnight at various temperatures. (a) Fluorescence spectra of ANS in the presence of BclXL (top panel) and the dependence of ANS fluorescence enhancement (E) on temperature (bottom panel). (b) Fluorescence spectra of ThT in the presence of BclXL (top panel) and the dependence of ThT fluorescence enhancement (E) on temperature (bottom panel). (c) Fluorescence spectra of ThT in the presence of BclXL pre-equilibrated with Myr (top panel) and the dependence of ThT fluorescence enhancement (E) on temperature (bottom panel). (d) Fluorescence spectra of ThT in the presence of lysozyme (top panel) and the dependence of ThT fluorescence enhancement (E) on temperature (bottom panel). In the top panels, the fluorescence spectra shown were recorded at 20°C (black), 30°C (red), 40°C (green), 50°C (blue), 60°C (cyan), 70°C (magenta) and 80°C (brown). Note that the dashed lines indicate the background fluorescence spectra of ANS (a), ThT (b and d) and ThT pre-equilibrated with Myr (c) in buffer alone. In the bottom panels, the data points are connected with a solid line for clarity. The error bars were calculated from three independent measurements to one standard deviation.

It is also important to note that the dependence of fluorescence enhancement of ANS and ThT displays multiphasic behavior with increasing temperature in lieu of a linear trend (Figures 5-7a-5-7c), bottom panels). As noted above, this implies that the decay of an α-helical fold into β-sheet architecture occurs via at least one intermediate step upon the heating of BclXL.

To further corroborate the notion that the enhancement of hydrophobic dyes such as ThT upon binding to BclXL correlates with the formation of amyloid fibrils, we also used lysozyme as a positive control. Notably, it is widely-documented that lysozyme forms amyloid fibrils at elevated temperatures (211-213). Consistent with this knowledge, our analysis shows that ThT experiences close to two-fold fluorescence enhancement when lysozyme is pre-heated to 80°C relative to incubation at 20°C in a
manner akin to that observed for BclXL (Figure 5-7d). However, the fact that the ThT fluorescence enhancement observed for lysozyme is much less than that noted for BclXL under similar conditions suggests that BclXL fibrils are likely to be much larger in size than those of lysozyme. It is noteworthy that SSF analysis of BclXL in the presence of TOCL/DHPC bicelles was complicated by the fact that the hydrophobic dyes ANS and ThT strongly bound to the bicelles and, in so doing, swamped the fluorescence changes due to their binding to BclXL alone under various conditions. Accordingly, such binding overlap prevented us from conducting any reliable measurements on BclXL in the presence of bicelles. Nonetheless, our data presented above strongly support the credence that elevated temperatures promote the aggregation of BclXL into amyloid-like fibrils.

5.4.8 Aggregation compromises the binding of BclXL to BH3 ligands

During apoptosis, BclXL exerts its suppressive effect by virtue of its ability to recruit the BH3 domains of apoptotic effectors such as Bax and Bak and, in so doing, neutralizes their pro-apoptotic function (30, 31). However, our data presented above suggest that BclXL undergoes structural transition from a largely α-helical fold into a cross β-sheet structure characteristic of amyloid-like fibrils under elevated temperatures. Accordingly, we would predict that the formation of such fibrillar aggregates is likely to be directly coupled to the loss of ligand binding to BclXL, since the above-mentioned structural transition would compromise the integrity of the canonical hydrophobic groove within BclXL required for ligand binding. To test this hypothesis, we conducted ITC analysis for the binding of Bid_BH3 peptide to BclXL pre-incubated at various temperature ranging from 20°C to 80°C (Figure 5-8 and Table 5-2). Our data show that the binding of Bid_BH3 peptide to BclXL becomes progressively attenuated by more
Figure 5-8: ITC analysis for the binding of Bid_BH3 peptide to BclXL pre-heated overnight at 20°C (a), 40°C (b), 60°C (c) and 80°C (d). The upper panels show raw ITC data expressed as change in thermal power with respect to time over the period of titration. In the lower panels, change in molar heat is expressed as a function of molar ratio of Bid_BH3 peptide to BclXL. The solid lines in the lower panels show non-linear least squares fit of data to a one-site binding model using the ORIGIN software as described earlier (177, 196).

than an order of magnitude as the incubation temperature is raised from 20°C to 60°C and becomes completely abolished when BclXL is pre-heated to 80°C. It should be noted here that the stoichiometries for the binding of Bid_BH3 peptide to BclXL were fixed to unity during the fit of the ITC data at all temperatures to allow for the loss of an incompetent fraction of protein unable to bind ligand. However, when the stoichiometries were allowed to float, there was little or negligible change in the values of the binding constants or the underlying thermodynamic parameters as reported in Table 5-2. Interestingly, the loss of ligand binding with increasing incubation temperature correlates with both the loss of favorable enthalpic change and unfavorable entropy, implying that BclXL undergoes more “ordered” structure at elevated temperatures in agreement with its propensity to aggregate into amyloid-like fibrils. Collectively, our data suggest that BclXL loses the ability to recognize BH3 ligands upon aggregation and such behavior
Table 5-2
Thermodynamic parameters for the binding of Bid_BH3 peptide to BclXL pre-incubated at the indicated temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$K_d$ / μM</th>
<th>$\Delta H$ / kcal.mol$^{-1}$</th>
<th>$T\Delta S$ / kcal.mol$^{-1}$</th>
<th>$\Delta G$ / kcal.mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>8.90 ± 1.71</td>
<td>-19.11 ± 0.63</td>
<td>-12.15 ± 0.52</td>
<td>-6.90 ± 0.11</td>
</tr>
<tr>
<td>25°C</td>
<td>10.90 ± 2.41</td>
<td>-18.75 ± 0.19</td>
<td>-11.96 ± 0.06</td>
<td>-6.78 ± 0.13</td>
</tr>
<tr>
<td>37°C</td>
<td>38.13 ± 7.95</td>
<td>-16.03 ± 0.20</td>
<td>-9.99 ± 0.32</td>
<td>-6.04 ± 0.12</td>
</tr>
<tr>
<td>40°C</td>
<td>48.39 ± 9.51</td>
<td>-14.26 ± 0.61</td>
<td>-8.37 ± 0.49</td>
<td>-5.89 ± 0.12</td>
</tr>
<tr>
<td>60°C</td>
<td>147.51 ± 30.28</td>
<td>-5.54 ± 0.11</td>
<td>-0.31 ± 0.02</td>
<td>-5.23 ± 0.13</td>
</tr>
<tr>
<td>80°C</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
</tbody>
</table>

All parameters were obtained from ITC measurements. The stoichiometries for the binding of Bid_BH3 peptide to BclXL were fixed to unity at all temperatures to allow for the loss of an incompetent fraction of protein unable to bind ligand. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation. NB indicates no binding observed.

should also be expected to result in the loss of its anti-apoptotic function.

5.4.9 Aggregation promotes the insertion of BclXL into lipid bicelles

In light of the knowledge that the amyloid-like fibrils bear the potential to permeabilize cellular membranes and lipid bilayers (84, 85, 214-216), we next wondered whether the fibrillar aggregates observed here under elevated temperatures may also represent a facilitated route for the entry of BclXL into MOM. To test this hypothesis, we analyzed the binding of BclXL pre-incubated at various temperatures ranging from 20°C to 80°C to mixed TOCL/DHPC bicelles using ITC (Figure 5-9 and Table 5-3). Remarkably, our analysis reveals that BclXL binds to TOCL/DHPC bicelles, used here as a model for MOM, only when pre-heated to temperatures of 40°C and above. Importantly, titration of BclXL aggregates into the calorimetric cell containing the buffer alone resulted in little or negligible change in thermal power (Figures 5-9a-5-9d), implying that the observed heat change is not due to dissociation of BclXL aggregates but
Figure 5-9: ITC analysis for the binding of mixed TOCL/DHPC bicelles to BclXL pre-heated overnight at 20°C (a), 40°C (b), 60°C (c) and 80°C (d). The upper panels show raw ITC data expressed as change in thermal power with respect to time over the period of titration. In the lower panels, change in molar heat is expressed as a function of molar ratio of BclXL to bicelles (●). Titration of BclXL pre-heated overnight at 20°C (a), 40°C (b), 60°C (c) and 80°C (d) into buffer alone is also shown as a control (○). The solid lines in the lower panels show non-linear least squares fit of data to a one-site binding model using the ORIGIN software as described earlier (177, 196).

rather results from a direct and specific interaction between BclXL and bicelles. We interpret such BclXL-lipid interaction in terms of the insertion of BclXL into bicelles in light of the knowledge that BclXL not only contains a C-terminal TM domain that spontaneously inserts into synthetic membranes but it also localizes to MOM during the onset of apoptosis (60, 65, 112, 144). It should be noted that the stoichiometries for the binding of BclXL to TOCL/DHPC bicelles were typically around 0.001 at all temperatures. This implies that the binding of one molecule of BclXL requires about 1000 molecules of lipids and, in so doing, this gives rise to rather low protein-lipid stochiometries observed here. Notwithstanding these limitations, our data suggest that BclXL fibrils represent an on-pathway intermediate state primed for insertion into MOM with important consequences on cellular physiology. Could BclXL harbor functional duality in its ability to act as anti-apoptotic under one state (globular) and pro-apoptotic
Table 5-3
Thermodynamic parameters for the binding of mixed TOCL/DHPC bicelles to BclXL pre-incubated at the indicated temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( K_d ) (μM)</th>
<th>( \Delta H ) (kcal.mol(^{-1}))</th>
<th>( T\Delta S ) (kcal.mol(^{-1}))</th>
<th>( \Delta G ) (kcal.mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>25°C</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>37°C</td>
<td>4.1 ± 0.84</td>
<td>-44.0 ± 1.41</td>
<td>-36.63 ± 1.29</td>
<td>-7.36 ± 0.12</td>
</tr>
<tr>
<td>40°C</td>
<td>2.8 ± 0.42</td>
<td>-59.5 ± 2.12</td>
<td>-51.9 ± 2.03</td>
<td>-7.58 ± 0.09</td>
</tr>
<tr>
<td>60°C</td>
<td>2.5 ± 0.49</td>
<td>-79.0 ± 2.82</td>
<td>-71.3 ± 2.70</td>
<td>-7.66 ± 0.12</td>
</tr>
<tr>
<td>80°C</td>
<td>2.7 ± 0.35</td>
<td>-115.0 ± 3.32</td>
<td>-107.4 ± 3.10</td>
<td>-7.62 ± 0.07</td>
</tr>
</tbody>
</table>

All parameters were obtained from ITC measurements. The stoichiometries for the binding of BclXL to TOCL/DHPC bicelles were typically around 0.001 (one molecule of BclXL bound per 1000 molecules of bicelles) at all temperatures. Errors were calculated from at least three independent measurements. All errors are given to one standard deviation. NB indicates no binding observed.

(fibrillar) under another? In this regard, it is interesting to note that caspase-induced N-terminal cleavage of BclXL within the cellular milieu renders it pro-apoptotic (54, 217, 218). Thus, it is conceivable that the propensity of BclXL to aggregate into fibrils may represent an alternative mechanism to trigger its pro-apoptotic action.

Importantly, we also note that while the binding of BclXL pre-heated to temperatures of 37°C and above to TOCL/DHPC bicelles occurs with similar affinities, the underlying thermodynamics governing this membrane-protein interaction bear substantial differences. Thus, for example, while the favorable enthalpy change accompanying this membrane-protein interaction more than doubles in magnitude from an incubation temperature of 37°C to 80°C, exactly the opposite trend is observed in the case of unfavorable entropic contribution such that it compensates any net gain in the free energy and hence the binding affinity. This trend is due to the phenomenon of enthalpy-
entropy compensation that widely governs the thermodynamic behavior of macromolecular interactions.

5.4.10 Aggregation results in the formation of highly-ordered rope-like homogeneous fibrils of BclXL

To understand the morphological and structural features of fibrillar aggregates of BclXL and how they may be modulated by ligand binding and membrane insertion, we conducted FM analysis on samples of BclXL pre-heated at various temperatures ranging from 20°C to 80°C and pre-stained with ThT (Figure 5-10). Our data show that while no observable fibrils are detected at lower temperatures (20°C and 40°C), BclXL forms highly-ordered rope-like homogeneous fibrils at higher temperatures (60°C and 80°C) with length in the order of mm and a diameter in the μm-range (Figure 5-10a). It should be noted that while electron microscopy analysis on BcXL revealed the formation of small non-fibrillar aggregates at lower temperatures (20°C and 40°C) in agreement with our ALS analysis (Figure 5-3), the rather large fibrils observed at higher temperatures (60°C and 80°C) were better suited for FM analysis.

Importantly, amyloid-like fibrils hitherto reported for other proteins typically tend to be less than μm in length and nm in diameter (78, 79, 197, 198, 219-222). The fact that BclXL fibrils observed here under elevated temperatures are three orders of magnitude larger in size than anything ever reported before is highly surprising and of particular significance. Interestingly, while the rope-like morphological features of BclXL fibrils under elevated temperatures were by and large unaffected in the presence of Bid_BH3 peptide (Figure 5-10b), the addition of mixed TOCL/DHPC bicelles apparently abolished their formation (Figure 5-10c). Of particular note here is the observation that while the lack of ability of Bid_BH3 peptide to halt the formation of BclXL fibrils is
consistent with our far-UV CD data (Figure 5-5a), the apparent loss of fibrillar architecture upon interaction of BclXL with TOCL/DHPC bicelles is highly surprising (Figure 5-5b). In order to reconcile the discrepancy between our FM and CD data, we reason that while the insertion of BclXL fibrils into TOCL/DHPC bicelles appears to be coupled with its $\alpha$-$\beta$ structural transition as observed in our far-UV CD (Figure 5-5b), the resulting $\beta$-sheet structure within mixed bicelles is unlikely to bear the hallmarks of a fibrillar architecture in agreement with our FM analysis (Figure 5-10c).

Taken together, these data demonstrate that while BH3 ligands may not affect its ability to form fibrillar aggregates, the insertion of BclXL into MOM likely is highly preferred over its ability to undergo fibrillation. This salient observation further corroborates the notion that the BclXL fibrils may serve as an on-pathway intermediate
for membrane insertion.

5.5 Concluding remarks

Although the central role of Bcl2 proteins in orchestrating apoptosis has been known for more than two decades (152-156), the underlying mechanisms remain far from understood. Previous studies have shown that truncated constructs of BclXL apoptotic repressor display the propensity to homodimerize in solution (54, 63, 140). These observations are further supported by studies conducted within live mammalian cells (133, 139). More recently, a truncated construct of BclXL lacking the C-terminal TM domain, was shown to form amyloid-like fibrils under elevated temperatures (183). However, biophysical work from our laboratory on purified recombinant full-length BclXL to apparent homogeneity is beginning to provide new insights into the role of C-terminal TM domain in driving the aggregation of this key apoptotic repressor into higher-order oligomers (177, 196). In a continuing theme, we have examined here the effect of temperature on the propensity of full-length BclXL to undergo such oligomerization.

The conventional wisdom in molecular biophysics is that heating proteins results in their irreversible and amorphous aggregation due to the loss of intramolecular forces such as hydrogen bonding, ion pairing and van der Waals contacts required for the integrity of native fold. In this study, we have demonstrated that the BclXL apoptotic repressor undergoes transformation to another “ordered” secondary structure characteristic of amyloid-like fibrils instead of amorphous aggregation when subjected to elevated temperatures. Amyloid fibrils typically display a characteristic cross-β sheet structure, which is essentially comprised of an array of β-sheets running perpendicularly
along the fibril axis (219-222). It is important to note that a wide range of proteins are known to aggregate under environmental stresses such as acidic pH and elevated temperatures into amyloid fibrils (78, 79, 197, 198). In particular, the deposition of amyloid-like fibrils is believed to play a central role in the pathogenesis of many diseases such as α-synuclein in Parkinson's disease, tau protein in Alzheimer's disease, prion in bovine spongiform encephalopathy, and huntingtin in Huntington's disease (80, 223-225).

Strikingly, while amyloid fibrils implicated in such diseases typically tend to be μm in length, the ability of BclXL to form elongated fibrils up to mm in length is highly surprising. Interestingly, the structure-inducing osmolyte TMAO has been previously shown to induce the formation of rope-like tropoelastin fibrils comparable in size to those observed here for BclXL (226). Whether TMAO may exert a similar effect on BclXL under ambient temperature remains to be seen.

It is telling that a truncated construct of BclXL devoid of the C-terminal TM domain has also been shown to form amyloid-like fibrils, albeit much smaller than those observed here, under elevated temperatures (183). This suggests that while TM domain likely facilitates the aggregation of BclXL into amyloid-like fibrils, other regions also harbor intrinsic aggregation propensity in agreement with our in silico analysis presented here. While our study does not demonstrate the physiological relevance of the ability of BclXL to aggregate into fibrils, the fact that such fibrils appear to be primed for insertion into cardiolipin bicelles provides an interesting scenario. Thus, under cellular stress mimicking elevated temperatures, the BclXL fibrillar aggregates may insert into MOM resulting in the formation of mitochondrial pores, thereby leading to the release of apoptogenic factors from mitochondria into the cytosol and triggering the induction of
apoptosis in a manner akin to Bax and Bak effectors (30, 31). Alternatively, the formation of rope-like fibrils under cellular stress may enable BclXL to physically damage the intracellular membranes and/or interfere with the ability of actin cytoskeleton to orchestrate cellular signaling involved in a diverse array for processes central to the maintenance of a healthy environment.

Could BclXL have a functional duality in that it may antagonize apoptotic machinery in quiescent healthy cells but drive apoptosis under cellular stress? This notion gains further momentum in light of the fact that amyloid-like fibrils share the ability to permeabilize cellular membranes and lipid bilayers, implying that this may represent the primary toxic mechanism of amyloid pathogenesis (84, 85, 214-216). More importantly, lysozyme fibrils have been shown to induce apoptotic cell death by virtue of their ability to induce membrane damage (74). Finally, caspase-induced cleavage of α1-α2 loop of BclXL within mammalian cells has been shown to convert BclXL from being a pro-survival to pro-apoptotic factor (227). Thus, the daring possibility that BclXL fibrils may also promote apoptosis warrants further inquiry in vivo. While this work is beyond the scope of our current study, it is set to take center stage in our future efforts directed at unraveling the mysteries of this key apoptotic player.
Chapter 6: Conclusion

Apoptosis plays a vital role in normal development of multi-cellular organisms. Bcl2 family proteins are known to be the central players that coordinate this process. Generally, it is considered that various members of Bcl2 family associate in homo or hetero fashion to coordinate the timing of cell death. However, even after being discovered more than two decades ago, the mechanistic knowledge of Bcl2 family protein interaction and function at biophysical level is still limiting. In particular, many previous biophysical and structural studies on different proteins of this family have relied mostly on truncated constructs (39, 40, 137). In light of these limiting observations, we aimed in this thesis to further our understanding about the structural and functional properties of Bcl2 family proteins using full-length BclXL as the model protein.

Using an array of biophysical techniques, we have demonstrated that BclXL protein can associate into physiologically relevant higher-order oligomers, by virtue of its TM domain. Previous studies conducted on cultured mammalian cells also showed the formation of oligomers by BclXL within the cell (133, 139), however, the precise mechanism of formation of these oligomers was not clear at that time. Furthermore, we were able to show that this oligomerization is regulated by changes in physiological conditions like pH and temperature. We have also demonstrated the effect of differential oligomerization on ligand binding and membrane insertion. Our studies on the full-length BclXL protein sheds light on the role of the structurally-disordered α1-α2 loop and the functionally-critical TM domain thus corroborating the growing consensus about the distinct role of structurally-disordered regions within proteins (146-150), that have traditionally been considered to be nonfunctional. We were able to establish through our
molecular models for the first time the role of TM domain in oligomerization of BclXL through formation of domain swapped dimers. Our molecular models show that the TM domain of one monomer occupies the canonical hydrophobic groove within the other monomer and vice versa in a trans-fashion. This is important in light of previous studies that suggest homo-dimerization through domain swapping is a common mechanism for protein oligomerization (157-162). We were successfully able to extend this theory in case of BclXL protein oligomerization. However, the possibility of alternative interlocking mechanism similar to actin polymerization is not excluded from our study. Regardless of the mechanism of association, the oligomerization of BclXL reported in our studies so far may seems to play an important role in regulating the anti-apoptotic function of BclXL by virtue of its ability to regulate ligand binding and membrane insertion. In light of our current study on BclXL protein, we propose a dual role of BclXL in mammalian cells. In healthy cells, BclXL can either homo-oligomerize or bind to effector proteins such as Bak and Bax by hetero-association, which leads to inactivation of BclXL as well as the effector proteins. Upon apoptotic induction, activator proteins such as Bid and Bad can compete with the homo and hetero association of BclXL protein, causing a release of effector proteins from the inhibitory effect of BclXL protein, following which they can insert themselves into the MOM and mediate apoptosis. Also, binding of activators to BclXL causes displacement of its TM domain from the canonical hydrophobic groove triggering its translocation into MOM via TM domain, consistent with previous studies showing the role of TM domain in membrane translocation (133, 165). Collectively, our biophysical studies sheds light on the functional regulation of BclXL protein and at the same time substantiate the notion of domain swapping as one of
the mechanisms for the formation of higher order oligomers. Given that most of the structural studies so far on BclXL protein has been carried out on truncated constructs lacking the TM domain and intervening loop between BH1 and BH2 domain, the use of full length BclXL protein in our experiments makes this study more physiologically relevant and paves the way for further research on BclXL in mammalian cells.

Following our detailed structural analysis and characterization of full length BclXL protein, we were motivated to study if and how pH can modulate the associativity and consequently, the structural and functional properties of BclXL protein. Studies have shown that water-membrane transition of effectors and repressors are driven by acidic pH which destabilizes the solution conformation of the protein (34, 39, 54, 58, 59, 65, 166, 167). Consistent with the previous studies, our data suggest that acidic pH causes conformational change in BclXL protein and induces the formation of molten globule, which we observed can act as the intermediate for membrane insertion. Our data show that oligomerization and ligand binding properties of BclXL is highly pH dependent and by virtue of its TM domain BclXL is able to form megadalton oligomers with plume-like appearance at acidic conditions. pH also appears to affect ligand binding and membrane insertion properties of the protein by virtue of its ability to form molten globule intermediate at low pH conditions. It can be argued that acidic conditions as low as pH 4 are unlikely to be present in or around a living cell, however, small changes in pH have been reported in the cytoplasm particularly in cells undergoing apoptosis (55, 56, 68, 174-176). Moreover, the acidic conditions used in this study may even be considered to mimic cellular stress. It has been shown by many previous studies that UV-irradiation, etoposide, staurosporine, or growth factor deprivation can cause cytosol acidification as
the cell undergoes stress due to these factors (67-69). Cellular stress can thus lead to activation of apoptotic machinery and one way to do that can be promotion of BclXL oligomerization which can act as the switch to turn off its anti-apoptotic action and further promote apoptosis by creating pores in MOM by virtue of its ability to form molten globule. Thus, acid induced oligomerization of BclXL may be highly relevant to the in vivo condition. More importantly, the formation of molten globule and its interaction with cardiolipin bicelles at acidic condition, as shown by our ITC experiments in Chapter 4, further provides evidence that low pH conditions can promote MOM insertion of the protein through interaction with cardiolipin present in MOM. A similar study using mammalian cells grown under various cellular stress conditions could shed more light on the physiological role of BclXL oligomerization and its membrane association. Our future research efforts will mainly focus to address the in-vitro observations in different mammalian cell systems.

The homo-association of BclXL into megadalton oligomers at acidic pH condition motivated us to further explore the role of other stress conditions that can affect protein structure and function. To understand this, we studied the effect of temperature on the associativity and functional properties of BclXL protein. Temperature is a key environmental factor that can regulate the association property of proteins. At the same time extreme temperature conditions used in our experiments can mimic cellular stress and shed light on how cellular homeostasis may regulate the association of BclXL protein. Our results are contrary to the conventional knowledge of irreversible and amorphous aggregation of protein at elevated temperature. The data show that elevated temperature causes a change in the secondary structure of BclXL protein from α-helix to
an ordered β sheet, characteristic of amyloid fibrils known to have a cross-β sheet structure formed by a series of β-sheets running perpendicular along the axis of fibril (220). Surprisingly the amyloid-like fibrils formed by full length BclXL in our studies were several times larger than other proteins that show a propensity to form amyloid fibers. We postulate that the C-terminal TM domain drives the aggregation and formation of large elongated fibrils of BclXL protein as shown by our molecular models and in-silico studies in chapter 5. The notion was further confirmed by cleaving the TM domain which abolished the formation of large fibrils under elevated temperature conditions. However, smaller fibrils in μm range were formed by the truncated construct which was also reported previously (183). Our study further showed that amyloid like fibers have an enhanced propensity to permeabilize cardiolipin bicelles, which can be important with respect to formation of MOM pores under stress conditions by BclXL, thus promoting apoptosis. Although, we were not able to demonstrate the physiological relevance of BclXL fibrillar aggregates in this study, future studies in-vivo can throw more light on the formation and function of amyloid fibers by this key apoptotic protein. Particularly, knowing that amyloid-like fibrils formed by different proteins are deposited in various diseases for instance, α-synuclein in Parkinson's disease, tau protein in Alzheimer's disease, prion in bovine spongiform encephalopathy and huntingtin in Huntington's disease (80, 223, 224) The finding that BclXL has the propensity to form amyloid like fibrils which can interact with cardiolipin bicelles gains more importance as it further suggests that BclXL has functional duality in that it can prevent apoptosis in healthy cells but under cellular stress can drive cell death by MOM permeabilization. The formation of amyloid fibers in-vitro by BclXL warrants a thorough in-vivo study to
understand the stress factors that can induce their formation and further what role these fibers plays in regards to cell death.

In conclusion, this study demonstrates how the TM domain exquisitely regulates the associativity of BclXL protein. Through molecular modeling and extensive biophysical techniques, we demonstrated that BclXL protein has the propensity to associate into large oligomers and further showed how this associativity is regulated by different external factors, contributing significantly to our understanding of the structural and functional properties of a key member of Bcl2 family protein. By showing that BclXL undergoes tertiary and secondary structural changes under stress conditions to form molten globule and amyloid like fibrils respectively and that these structural changes can enhance the binding of BclXL protein with cardiolipin membranes we were able to propose the dual function of BclXL protein. However, the study needs further work to establish the role of molten globule and amyloid like fibrils formed within the cell. Since BclXL plays an important role in different types of cancer, it would be interesting to determine the precise role and conditions that leads to structural change in BclXL protein. This may open up a new avenue of drug targeting and disease regulation in case of cancer. As often happens in science, the results of this study so far beg more questions than it answers. Despite that, these studies further enhance our understanding of the molecular mechanism and structural properties of BclXL apoptotic regulator protein. Furthermore, novel therapeutic avenues for targeting cells that have perturbed apoptotic mechanism may open up one day as a result of these studies.
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