Structural plasticity of BCL-2 family proteins and its functional implication in apoptosis

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**STRUCTURAL PLASTICITY OF
BCL-2 FAMILY PROTEINS AND ITS FUNCTIONAL
IMPLICATION IN APOPTOSIS**

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SCHOOL OF BIOLOGICAL SCIENCES

2018

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BCL-2 FAMILY PROTEINS AND ITS FUNCTIONAL
IMPLICATION IN APOPTOSIS**

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Abbreviations

3DDS	Three-dimensional Domain Swapping
A.A	Amino Acid
ATP	Adenosine Triphosphate
Bcl-2	B Cell Lymphoma 2
BH	Bcl-2 Homolog
BLR	Bcl-2 Like Region
BMH	Bismaleimidohexane
BN	Blue Native
CD	Circular Dichroism
cFLIP	Cellular FLICE-like Inhibitory Protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	Critical Micelle Concentration
CNS	Crystallography & NMR System
CSP	Chemical Shift Perturbation
CV	Column Volume
ddH ₂ O	Double-distilled H ₂ O
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DMPC	Dimyristoylphosphatidylcholine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
DPC	Dodecylphosphocholine
DR	Death Receptor
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid

FACS	Fluorescence-activated Cell Sorting
FADD	Fas-associated Death Domain
FITC	Fluorescein Isothiocyanate
FL	Full Length
FPLC	Fast Protein Liquid Chromatography
FT	Flow-through
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GST	Glutathione S-Transferase
HADDOCK	High Ambiguity Driven protein-protein Docking
HRP	Horseradish Peroxidase
HSQC	Heteronuclear Single Quantum Coherence
IAP	Inhibitors of Apoptosis Protein
ICAD	Inhibitor of Caspase-Activated Deoxyribonuclease
IPTG	Isopropyl β -D-1-Thiogalactopyranoside
Kda	Kilodalton
KO	Knockout
LB	Lysogeny Broth
LDAO	Lauryldimethylamine Oxide
LMPC	Lysomyristoylphosphatidylcholine
MEF	Mouse Embryonic Fibroblast
MOMP	Mitochondrial Outer Membrane Peameabilization
Mpa	Megapascal
MPT	Mitochondrial Permeability Transition
MRE	Molar Residue Ellipticity
MS	Microsecond
Ni ²⁺ -NTA	Ni ²⁺ -Nitrilotriacetic Acid
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Spectroscopy
NP	N-terminal Peptide
OD	Optical Density

OG	Octyl β -D-Glucopyranoside
OM	n-Octyl β -D-Maltoside
OMM	Outer Mitochondrial Membrane
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PI	Propidium Iodide
PMSF	Phenylmethylsulfonyl Fluoride
PPM	Parts-Per Million
PS	Phosphatidylserine
REM	Restrained Energy Minimization
RMSD	Root-Mean-Square Deviation
RPM	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
STS	Staurosporine
TAE	Tris Acetate-EDTA
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween20
TCEP	Tris(2-Carboxyethyl)Phosphine
TFE	Trifluoroethanol
TM	Transmembrane
TNF	Tumor Necrosis Factor
TOCSY	Total Correlation Spectroscopy
UV	Ultraviolet
VDAC	Voltage-Dependent Anion Channel
WT	Wildtype

Abstract

Dysregulated apoptosis has been implicated in the development of several diseases including cancer and neuronal degenerative diseases. Structural studies of Bcl-2 family members have provided mechanistic insights into their underlying molecular mechanism regulating mitochondrial apoptosis through homo- and hetero dimerization between pro- and anti- apoptotic Bcl-2 proteins. Bcl-2 family proteins undergo structural transition between solution and membrane states. Currently, the structural plasticity and three-dimensional structural portraits of Bcl-2 family of proteins in membrane environment remain poorly characterized. The first part of this thesis is devoted to address these largely unanswered questions. Bcl-xL, which is an anti-apoptotic Bcl-2 family member, was chosen and its structural behaviors were examined by employing multiple biochemical and biophysical approaches including pH, heat, chemical crosslinking, detergents, size-exclusion column chromatography and native gel. The results showed that Bcl-xL's structural transition is induced in response to such external stimuli. As the structural transition of Bcl-xL was notably induced by n-Octyl β -D-Maltoside (OM), the focus of this study was on the structural characterization of Bcl-xL in OM as a membrane-mimicking environment. Three-dimensional structural analysis revealed that Bcl-xL undergoes structural transition by forming a three-dimensional domain-swapped (3DDS) dimer in the presence of n-octyl- β -D-Maltoside detergent. Subsequently, 3DDS-resistant mutants have been generated and their ability to inhibit structural transition confirmed. This further prompted us to investigate whether this structural transition of Bcl-xL plays a regulatory role in maintaining its apoptotic ability in response to external apoptotic stimuli perturbing cellular homeostasis. Our collaborators Carl white and his colleagues revealed that Bcl-xL knockout mouse embryonic fibroblast (MEF) cells expressing 3DDS-resistant mutants exhibited lower mitochondrial calcium uptake than wild type MEF cells in response to changes in external $[Ca^{2+}]$. This implies that structural transition of Bcl-xL might have functional implication in facilitating calcium homeostasis in mitochondria.

The second part of this study is devoted to the molecular interaction between Bcl-xL and Voltage-Dependent Anion Channel (VDAC). While VDAC has been reported to interact with Bcl-xL to regulate mitochondrial calcium homeostasis, detailed molecular

basis of the interaction between the two molecules currently remains elusive. To this end, we have characterized synthetic peptides derived from the N-terminal region of VDAC and determined the NMR structure of VDAC2-SG20 peptide in the presence of Bcl-xL. Intriguingly, our NMR structural analysis showed that the VDAC2 N-terminal peptide adopts an alpha helical conformation in complex with Bcl-xL. It also showed that the peptide binds to the canonical hydrophobic binding pocket for BH3 peptides of the Bcl-2 family proteins. Functional implications of these molecular interactions in the mechanism of VDAC function in Bcl-2-related apoptotic regulation are discussed.

Introduction

1.1 Apoptosis

Apoptosis, also known as a program cell death, is a mechanism involved in several biological events in multicellular organisms (Elmore, 2007; Hengartner, 2000; Lockshin and Williams, 1965). Kerr, Wyllie, and Currie first coined the term ‘apoptosis’ after observing a common pattern of programmed cell death found in various tissues and cells (Kerr et al., 1972). The authors noticed the unique features of apoptotic cell death that were distinct from necrotic cell death. Apoptosis differs from necrosis in its mechanism and morphology of cell death (Fink and Cookson, 2005). Differences of morphology between apoptosis and necrosis are listed in **Table 1**.

Table 1 Difference between apoptosis and necrosis.

	Apoptosis	Necrosis
Cause	Physiological stimuli	Pathologic (infection, toxin, trauma, etc)
Trigger	Both intrinsic and extrinsic	Extrinsic only
Number of cells affected	Single cell	Many cells at once
Cell volume	Cell shrinkage	Cell swelling
Inflammatory response	No	Yes
Membrane integrity	Maintain membrane integrity with blebbing observed	Loss of membrane integrity
DNA degradation	Chromatin condensation and DNA fragmentation (non-random)	Random DNA degradation
Organelle	Preserved organelles	Disrupted organelles
Cell death	Formation of apoptotic bodies ingested by neighboring cells	Cell lysis and release of cellular contents

Apoptosis functions as a defense mechanism to protect the organism by eliminating abnormal or damaged cells that are considered harmful to the organism (Adachi-Yamada and O'Connor, 2004; Norbury and Hickson, 2001). Apoptosis also plays a role in sculpting the organs during embryonic development (Penaloza et al., 2006). In addition, by regulating the population of cells through apoptosis, tissues such as gastrointestinal tissues or thymic tissues can maintain cell homeostasis (Paganelli et al.,

2008; Renehan et al., 2001). Since apoptosis is involved in numerous biological events as mentioned above, strict regulation of apoptosis is essential for proper development and function of organisms. There are at least two main pathways in mammals which are involved in regulation of apoptosis: the extrinsic pathway (receptor-mediated pathway) and the intrinsic pathway (mitochondrial pathway). It is highly important to understand the mechanisms of apoptosis, as they are attributed to the pathogenesis of many diseases including various types of cancers and autoimmune disease (Barr and Tomei, 1994; Paganelli et al., 2008; Wong, 2011).

1.2 Apoptotic pathways

1.2.1 Extrinsic pathway

The extrinsic pathway of apoptosis is associated with the activation of death signaling pathway triggered by the binding of the extracellular death ligand to the transmembrane death receptors expressed on the surface of cells. Death receptors (DR) are protein members of the tumor necrosis factor (TNF) receptor superfamily (Locksley et al., 2001). To date, several death ligand/receptors have been well characterized, which include FasL/FasR and TNF- α /TNFR (Beg and Baltimore, 1996; Wajant, 2002). Upon binding of FasL or TNF- α to their corresponding receptors, adaptor proteins such as Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD) bind to cytoplasmic domain of the death receptors. Binding of TRADD results in recruitment of FADD (Hsu et al., 1996). FADD contains death effector domain (DED) which allows association with procaspase-8 via dimerization of DED. Upon binding of procaspase-8 to DED of FADD, death inducing signaling complex (DISC) is assembled at the cytoplasmic tail of TNFR/FasR, which results in the activation of procaspase-8 via autocatalytic reaction through induced proximity (Kischkel et al., 1995). Caspase-8 activation can be inhibited by cFLIP protein via its binding to the DED of FADD and caspase-8 (Irmeler et al., 1997). Activation of caspase-8 triggers downstream activation of executioner caspases including caspase-3, caspase-6, and caspase-7. This process is also called as 'execution phase' of apoptosis, where both extrinsic and intrinsic pathways merge and actual cell destruction begins. Executioner caspases induce apoptosis by several mechanisms (Slee et al., 2001; Thornberry and Lazebnik, 1998).

One of the mechanisms is to inactivate proteins such as ICAD which protect cells against cell death (Enari et al., 1998). In living cells, ICAD is associated with caspase-activated deoxyribonuclease (CAD) to inhibit its DNase activity. During apoptosis, ICAD is cleaved by caspase-3, which results in releasing CAD to an active form. Active CAD is responsible for DNA fragmentation found in apoptotic cells. Caspases also induce apoptosis by destroying cellular structures. During apoptosis, caspase-6 cleaves lamin A proteins, which results in destruction of nuclear lamina, contributing to chromatin condensation (Ruchaud et al., 2002). Lastly, caspases induce apoptosis by cleaving anti-apoptotic Bcl-2 family proteins to inactivate their anti-apoptotic activity, as well as to produce a fragment which triggers cell death (Cheng et al., 1997).

1.2.2 Intrinsic pathway

The intrinsic pathway of apoptosis, also known as mitochondrial pathway, is triggered by both extracellular and internal stimuli such as growth factor withdrawal, radiation, toxins, DNA damage, viral infections, etc. Initiation of intrinsic pathway of apoptosis results in opening of the mitochondrial permeability transition (MPT) pore at the inner mitochondrial membrane, which increases the permeability of mitochondria membranes to allow passage of molecules of less than 1500 Dalton (Lemasters et al., 2009). Pro-apoptotic molecules including Smac/DIABLO and cytochrome c are released from mitochondria to cytosol through MPT pore (Saelens et al., 2004). Cytochrome c associates with Apaf-1 and formation of ‘apoptosome’ is facilitated (Chinnaiyan, 1999). Apoptosome activates procaspase-8, which subsequently activates caspase-3 (Riedl and Salvesen, 2007). Smac and DIABLO promote mitochondrial apoptosis by antagonizing inhibitors of apoptosis protein (IAP) which inhibits activation of caspase-3. Both intrinsic and extrinsic pathways converge at the level of caspase-3 activation, and downstream execution pathway leads to apoptotic cell death (Hengartner, 2000). The Bcl-2 family of proteins regulate cytochrome c release from mitochondria and thus control mitochondrial pathway of apoptosis (Cory and Adams, 2002). To date, there are several mechanisms that have been suggested to explain how members of Bcl-2 family regulate mitochondrial permeability and cytochrome c release (Antonsson and Martinou, 2000; Gross et al., 1999; Reed, 1997; Shimizu et al., 1999). One proposed mechanism is that Bcl-2 proteins undergo structural changes to form channels, insert into the outer

mitochondrial membrane (OMM). This model has been proposed based on the structural similarity of anti-apoptotic Bcl-xL with the transmembrane domain of diphtheria toxin, which forms a pH-dependent membrane pore (Bennett et al., 1994; London, 1992; Muchmore et al., 1996). Another suggested mechanism is that pro-apoptotic Bax and Bak proteins form large oligomeric pores in the OMM, which results in permeabilization of the OMM during apoptosis. According to this model, Bax and Bak remains inactive and localized in the cytosol and in the OMM, respectively, in the absence of apoptotic stimuli (Cheng et al., 2003; Wolter et al., 1997). In response to cell death signals, Bax and Bak form BH3-in-groove homodimers, and assembled into higher oligomers to form large pores in the OMM with a diameter of 30~60Å (Czabotar et al., 2013; Dewson et al., 2008; Martinez-Caballero et al., 2009). This model is supported by several studies on the structural mechanism of Bak/Bax homodimerization and oligomerization (Aluvila et al., 2014; Czabotar et al., 2013; Mandal et al., 2016; Salvador-Gallego et al., 2016). On the other hand, it has been also suggested that BH3-in-groove homodimers of Bax and Bak may induce MOMP through membrane disintegration rather than pore formation (Czabotar et al., 2013; Fuhrmans and Marrink, 2012). In addition, it has been reported that the members of Bcl-2 family can insert into synthetic lipid membranes to form ion channels (Minn et al., 1997; Schendel et al., 1997). However, it is still unclear whether the size of pore formed by Bcl-2 family proteins would be big enough for passage of proteins. Another possible mechanism of Bcl-2 family proteins to regulate mitochondrial apoptosis is by interacting with other pore-forming proteins at the OMM. One such example is the voltage-dependent anion channel (VDAC), as it has been reported that Bcl-2 family proteins interact with VDAC to regulate its channel activity and mitochondrial apoptosis (Arbel et al., 2012; Huang et al., 2013; Shimizu et al., 1999).

Figure. 1.1 Extrinsic and intrinsic pathways of apoptosis in mammalian cells.

Both receptor-mediated pathway and mitochondrial pathway are presented in the cartoon. (Figure is adapted from Hengartner, 2000 (Hengartner, 2000))

It has also been suggested that the release of cytochrome c can occur via mitochondrial outer membrane permeabilization (MOMP) regulated by pro- and anti-apoptotic Bcl-2 family proteins (Chipuk and Green, 2008; Korsmeyer et al., 2000; Moldoveanu et al., 2013). In this model, Bid, a 'BH3 domain only' pro-apoptotic member of the Bcl-2 family, is activated by caspase-8 via its cleavage. The truncated Bid (tBid) subsequently translocates to mitochondria and inserts into the OMM, where it interacts with pro-apoptotic Bcl-2 family proteins, Bak and Bax to induce structural change and oligomerization of Bak/Bax.

The release of cytochrome c passes through the pore formed by the multimerization of Bak and Bax at OMM, and is released to the cytosol, proceeding to subsequent apoptotic

pathways. This model integrates both receptor-mediated apoptotic pathways which leads to activation of caspase-8, and mitochondrial apoptotic pathway starting from truncation of Bid, resulting in MOMP and cytochrome release (Korsmeyer et al., 2000). Hence, it is suggested that extrinsic and intrinsic pathways are not entirely separate pathways, but are engaged in ‘cross-talk’ at the level of Bid truncation.

1.3 Bcl-2 family proteins

As mentioned in the previous chapter, proteins of the Bcl-2 family are involved in regulation of MOMP and release of apoptogenic molecules such as cytochrome c during intrinsic apoptotic pathway. In human, there are more than seventeen Bcl-2 family proteins, which have similar three-dimensional structure or predicted secondary structure with Bcl-2 (Dewson and Kluck, 2010). The protein family of Bcl-2 can be further divided into three subsets of families, based on their homology and function in regulation of apoptosis (Youle and Strasser, 2008): (i) Anti-apoptotic Bcl-2 family including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, which inhibit apoptosis and contain up to four conserved sequence homology motifs also known as Bcl-2 homology (BH) domains; (ii) Pro-apoptotic multidomain Bcl-2 family including Bax and Bak, which promote apoptotic cell death and display all four BH domains; (iii) Pro-apoptotic BH3 only proteins including Bim, Bid, Bad, Puma, and Noxa, which possess pro-apoptotic properties and show sequence homology only at one BH domain. (**Figure 1.2**)

Figure. 1.2 Classification of the Bcl-2 family proteins.

Three subsets of the Bcl-2 family – anti-apoptotic, pro-apoptotic multidomain, pro-apoptotic BH3 only, are presented as a cartoon with the homology domains and the protein members of each family.

1.3.1 Regulation of mitochondrial apoptotic pathway by Bcl-2 family proteins

Studies on pro-apoptotic multidomain Bcl-2 family proteins, Bak and Bax, revealed that mouse embryonic fibroblast (MEF) cells lacking both Bak and Bax proteins exhibit resistance to tBid-induced cytochrome c release during mitochondrial apoptosis, as well as to several apoptotic stimuli including staurosporine, UV radiation, etoposide, etc (Wei et al., 2001). Also, it has been shown that Bax^{-/-}Bak^{-/-} mice displayed impaired apoptosis during development, which resulted in several developmental defects (Lindsten et al., 2000). These reports suggest that activation of either Bak or Bax protein is essential in MOMP during mitochondrial apoptosis. The BH3-only proteins of pro-apoptotic Bcl-2 family promote apoptosis by either activating pro-apoptotic multidomain proteins (Bak, Bax), or inactivating anti-apoptotic multidomain proteins (Bcl-2, Bcl-xL, Mcl-1) (**Figure 1.3**) (Chen et al., 2015). The ‘activator’ BH3-only molecules (Bim, Puma, and Bid) bind to Bak/Bax to directly activate them by inducing conformational change of Bak/Bax proteins, resulting in oligomerization of Bak/Bax and MOMP (Chipuk and Green, 2008; Wei et al., 2000). Anti-apoptotic Bcl-2 family members inhibit the activation of pro-apoptotic Bak and Bax by sequestering the activator BH3-only molecules (Emily et al., 2001). Another group of BH3 only proteins,

the ‘inactivator’ BH3-only molecules (Noxa and Bad), bind to anti-apoptotic Bcl-2 family sequestering the activators, resulting in the release of the activators and Bak/Bax activation (Chen et al., 2015). In this ‘hierarchical model of regulation of apoptosis by the Bcl-2 family’, the activator BH3-only proteins act downstream of inactivator BH3-only proteins to activate Bax/Bak (Chen et al., 2015; Kim et al., 2006).

Figure. 1.3 Mechanism of Bak/Bax activation and MOMP during intrinsic apoptosis.

A cartoon depicting the hierarchical model of regulation of apoptosis by the Bcl-2 family is presented.

As mentioned above, both pro- and anti- apoptotic Bcl-2 family proteins play a vital role in modulation of mitochondrial changes during intrinsic apoptosis pathway. Intrinsic pathway of apoptosis is controlled by harmony of pro- and anti- apoptotic members of the Bcl-2 family antagonizing each other. Thus, understanding the biochemical characteristic of both pro- and anti- apoptotic Bcl-2 family proteins is essential to comprehend the complicated apoptosis machinery.

1.3.2 Structural characteristic of Bcl-2 family proteins

Protein members of both pro- and anti- apoptotic Bcl-2 family, despite the difference of their roles in regulating apoptosis, share a striking similarity in three-dimensional structure. All multidomain Bcl-2 family proteins are globular proteins in which two hydrophobic alpha helices are surrounded by six to seven amphipathic alpha helices. (**Figure 1.4**) Also, on the surface of the multidomain Bcl-2 proteins, there is a hydrophobic pocket consisting of the BH1, BH2, and BH3 domains. This hydrophobic groove serves as a binding site for BH3 domains of another protein members of Bcl-2

family, and it has been reported that the anti-apoptotic Bcl-2 family proteins have high selectivity in binding to BH3-only ligands.

The solution structure of Bcl-xL was solved by NMR spectroscopy and X-ray crystallography in 1996 (Muchmore et al., 1996) (**Figure 1.4.A**). Bcl-xL was the first member of Bcl-2 family of which the three-dimensional structure was determined. Bcl-xL has two large hydrophobic helices, $\alpha 5$ and $\alpha 6$, in the center of protein structure, which are flanked by $\alpha 3$ and $\alpha 4$ on one side and $\alpha 1$, $\alpha 2$, $\alpha 8$ on the other side. Unlike other Bcl-2 family proteins, Bcl-xL possesses a large loop region connecting helices $\alpha 1$ and $\alpha 2$, indicated with red arrow in **Figure 1.4.A**. This loop is thought to be unstructured and flexible, due to the lack of electron density and medium- and long- range NOEs in this loop region. In previous studies, it was shown that deletion of this loop did not affect the ability of Bcl-xL to protect the cells against apoptosis (Muchmore et al., 1996). Hence, it is suggested that the loop of Bcl-xL is dispensable for the protein's anti-apoptotic property, and the loop-deleted mutant Bcl-xL are commonly used for structural and biochemical studies of Bcl-xL.

Figure. 1.4 Three-dimensional structures of anti-apoptotic Bcl-2 family proteins.

Ribbon and surface representation of Bcl-xL (PDB ID: 1LXL) (A), Bcl-2 (PDB ID: 1G5M) (B), Mcl-1 (PDB ID: 2MHS) (C), and Bcl-w (PDB ID: 1MK3) (D). All alpha helices are numbered as indicated, and BH domains are color-coded (BH1: yellow, BH2: red, BH3: green, BH4: blue).

The solution structures of Bcl-2 isoforms were determined by NMR spectroscopy (Petros et al., 2001) (**Figure 1.4.B**). Like Bcl-xL, Bcl-2 has two hydrophobic helices surrounded by six amphipathic helices. As the wild type Bcl-2 showed poor solubility, the structure of Bcl-2 was determined by replacing a part of the unstructured loop of Bcl-2 with a shorten loop region of Bcl-xL. Since the wild type protein had an isoelectric point (pI) very close to the pH of NMR buffer, the loop replacement of wild type could enhance the solubility of protein. The resulting chimeric protein had a lower pI compared to the wild type, improving its solubility.

The three-dimensional solution structure of Mcl-1 has also been determined by NMR spectroscopy (Day et al., 2005) (**Figure 1.4.C**). For structural study, N- and C-terminal regions of Mcl-1 were truncated to enhance the solubility of protein. Mcl-1 has a central hydrophobic helix ($\alpha 5$) flanked by amphipathic helices, and an additional hydrophobic pocket on the surface of the protein. Despite its low sequence homology with other Bcl-2 family members, the overall fold of Mcl-1 was similar to other Bcl-2 proteins.

The structure of Bcl-w in solution was solved by using NMR spectroscopy (Denisov et al., 2003b) (**Figure 1.4.D**). Bcl-w shares a structural similarity with other members of the Bcl-2 family, as it has two hydrophobic alpha helices in the center of the protein, surrounded by amphipathic helices. However, Bcl-w has unusual structural feature compared to other Bcl-2 family proteins. Bcl-w has an additional alpha helix ($\alpha 9$) at the C-terminal, which occupies the hydrophobic pocket on the protein surface. This unique structure is similar to the structure of pro-apoptotic Bax protein (Suzuki et al., 2000) (**Figure 1.5.A**). As the hydrophobic pocket of Bcl-w protein is inaccessible, BH3-only pro-apoptotic proteins have lower affinity to the hydrophobic pocket of Bcl-w compared to other Bcl-2 family proteins. Hence, it has been suggested that this C-terminal alpha helix of Bcl-w protein might play a role in modulating protein-ligand interaction (Hinds et al., 2003).

The solution structure of Bax, a member of pro-apoptotic multidomain Bcl-2 family, was solved by NMR spectroscopy (Suzuki et al., 2000) (**Figure 1.5.A**). Bax has nine alpha helices, and the overall assembly of alpha helices from $\alpha 1$ to $\alpha 8$ highly resembles that of Bcl-xL. In addition, like Bcl-w, C-terminal $\alpha 9$ helix of Bax sits in the hydrophobic cleft on the surface of the protein. This binding occurs in a similar way

how BH3-only peptides bind to anti-apoptotic Bcl-xL, even though the direction of $\alpha 9$ helix is opposite to that of BH3-only peptide.

The three-dimensional solution structure of pro-apoptotic Bak was determined by both NMR spectroscopy and X-ray crystallography (Moldoveanu et al., 2006; Wang et al., 2009) (**Figure 1.5.B**). Bak protein has globular structure made up of eight alpha helices, which is similar to the overall folds that have been observed in other members of Bcl-2 family. However, compared to other Bcl-2 family proteins, Bak has a hydrophobic cleft on its surface that is sterically occluded by side-chain stacking of residues Arg88 and Tyr89, hindering the binding of BH3-only peptides to the pocket.

Figure. 1.5 Three-dimensional structures of pro-apoptotic Bcl-2 family proteins.

Ribbon and surface representation of Bax (PDB ID: 1F16) (A), and Bak (PDB ID: 2IMS) (B). All alpha helices are numbered as indicated, and the BH domains are color-coded (BH1: yellow, BH2: red, BH3: green).

Most of the multidomain Bcl-2 family proteins contain C-terminal transmembrane (TM) domain, which is assumed to be responsible for membrane anchoring of Bcl-2 family proteins. However, except Bax and Bcl-w, most of the structures of Bcl-2 family proteins solved so far, lack the C-terminal transmembrane domains. In normal cells, Bcl-2 are mainly localized in membranes, including nuclear outer membrane, endoplasmic reticulum (ER) membrane and mitochondrial outer membrane (Akao et al., 1994). Bcl-w, like its relative Bcl-2, is found to be present in nuclear envelope and mitochondrial membrane (O'Reilly et al., 2001). Localization of Mcl-1 is also similar to Bcl-2, as Mcl-1 shows prominent localization at mitochondria (Yang et al., 1995). Bcl-xL, on the other hand, are present both in the cytosol and membranes. Upon induction of apoptosis, cytosolic Bcl-xL proteins are translocated to mitochondrial outer membrane, where they interact with pro-apoptotic Bcl-2 family proteins to regulate MOMP (Hsu et al., 1997). Pro-apoptotic Bcl-2 family, Bax and Bid, show prominent localization at cytosol in the absence of apoptotic signal. Upon protein activation during mitochondrial apoptosis, such pro-apoptotic proteins move to the mitochondrial membrane (Wolter et al., 1997). In a recent study, Barbara Schellenberg and her coworkers suggested that Bax is targeted to mitochondria in both normal cells and apoptotic cells, but in normal cells, it is continuously translocated back to the cytosol (Schellenberg et al., 2013).

As mentioned earlier, multidomain Bcl-2 family proteins possess a hydrophobic pocket consisting of BH1, BH2, and BH3 domains on the surface of the protein, which provides a binding site for BH3 domain of another protein member of Bcl-2 family (**Figure 1.6.A and B**). Anti-apoptotic Bcl-2 family proteins protect cells against apoptosis by interacting with pro-apoptotic Bcl-2 proteins, and the molecular basis of this interaction lies on the BH3 domain of one Bcl-2 family protein binding to a hydrophobic pocket on the surface of another Bcl-2 family member. Hence, it has been suggested that the hydrophobic cleft mediates heterodimer formation among Bcl-2 family proteins. To date, several structures of anti-apoptotic Bcl-2 family proteins in complex with BH3-only peptides or BH3 mimetic ligands have been solved by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography (Lee et al., 2016; Sattler et al., 1997) (**Figure 1.6.C and D**). Also, it has been reported that pro-apoptotic BH3-only proteins such as Bim, Bad, and Bmf are intrinsically disordered, and binding

to hydrophobic pocket enhances the folding of BH3-only protein to adopt alpha helical conformation, which in turn improves its binding affinity to the pocket (Hinds et al., 2007).

Figure. 1.6 Hydrophobic cleft of Bcl-2 family proteins binds to BH3 peptide or its own C-terminal alpha helix.

Surface representation of Bcl-xL (PDB ID: 1MAZ) (A), Bcl-2 (PDB ID: 1G5M) (B), Bcl-xL in complex with Bid BH3 (PDB ID: 4QVE) (C), Bcl-xL and Bak BH3 (PDB ID: 5FMK) (D), Bax (1F16) (E), and Bcl-w (1MK3) (F). Hydrophobic amino residues are colored in blue, and either BH3 peptide or C-terminal alpha helix which binds to the hydrophobic groove is represented as a red colored ribbon.

On the other hand, the three-dimensional structure of Bcl-w shows its C-terminal alpha helix sitting on its own hydrophobic cleft, hindering access of other BH3 domain to the cleft. (**Figure 1.6.F**) Also, instead of BH3 only proteins, the hydrophobic cleft of Bax protein is occupied with its own C-terminal TM domain. In the absence of apoptotic signal, Bax sequesters its C-terminal TM domain in the hydrophobic pocket, adopting 'TM:groove' conformation (Suzuki et al., 2000). (**Figure 1.6.E**) Upon initiation of apoptosis, Bax is translocated to the mitochondrial region and TM domain of Bax is inserted into mitochondrial membrane. Hence, it is suggested that the hydrophobic pocket not only regulates apoptosis by modulating interactions among Bcl-2 family proteins, but also regulates apoptosis by modulating subcellular localization of pro-apoptotic Bax protein (Nechushtan et al., 1999).

1.3.3 Oligomerization of Bcl-2 family proteins

As mentioned earlier, heterodimer formation of Bcl-2 family protein via BH3 domain binding to the hydrophobic cleft plays a vital role in controlling MOMP and mitochondrial apoptosis. To date, structural and functional aspect of such heterodimers have been well characterized in several studies. In addition, it has been also reported that some of the Bcl-2 family protein members including Bax, Bak, Bcl-w, and Bcl-xL forms homodimers in certain environments. In these proteins, dimerization could be either induced by detergent, changes in pH or temperature, or it could occur without any inducement (Denisov et al., 2007; Hsu and Youle, 1997; O'Neill et al., 2006; Xie et al., 1998).

Homodimerization and oligomerization of Bax and Bak has been extensively studied, together with its structure and function in regulation of mitochondrial apoptosis (Aluvila et al., 2014; Czabotar et al., 2013; Kuwana et al., 2016; Mandal et al., 2016; Moldoveanu et al., 2006; Salvador-Gallego et al., 2016). The crystal structure of Bak homodimer with inhibitory Zinc binding site has been reported. In this report, it has been argued that Zn^{2+} binds to the inhibitory binding site on Bak protein and mediates homodimerization of Bak, and Zn^{2+} -trapped Bak proteins are not capable of causing cytochrome c release from mitochondria (Moldoveanu et al., 2006). (**Figure 1.7.A**) For Bax, on the other hand, two types of protein dimerization have been reported by Peter M. Colman and his coworkers (Czabotar et al., 2013; Dewson et al., 2012). First, they have shown that two

Bax molecules swap $\alpha 6$ - $\alpha 8$ helices to form domain swapped dimer in the presence of n-Octyl- β -D-Maltopyranoside (OM) detergent. They named this domain swapped dimer as ‘core/latch’ dimer, and the crystal structure of this dimer has been determined. (**Figure 1.7.B**) However, it has been suggested that the core/latch dimer is off pathway, as its structure is not consistent with the previous reports on interfaces in Bax oligomers formed during apoptosis (Bleicken et al., 2010; Dewson et al., 2012; Zhang et al., 2010). Another form of Bax homodimer is ‘BH3:groove’ dimer, where one protein inserts its BH3 domain into the hydrophobic groove of another protein, forming a symmetric dimer (Dewson et al., 2008; Dewson et al., 2012). It has been also proposed that BH3:groove dimers oligomerize via an interface between $\alpha 6$ helices and permeabilize mitochondrial outer membrane (Dewson et al., 2009).

Figure. 1.7 Crystal structure of Bak and Bax dimer.

For figure legend, see next page.

A) Cartoon representation of Bak homodimer. (PDB ID: 2IMS) Inhibitory zinc binding site is indicated with a circle. All alpha helices are labeled with their numbers, and the residues interacting with Zn^{2+} (D160 and H164) are shown as sticks in the Zn^{2+} binding site. B) Cartoon representation of the Bax domain swapped dimer. (PDB ID: 4BD7) All alpha helices are labeled with their numbers, and two monomeric folds of Bax are separated by a dashed line.

The crystal structure of Bcl-w dimer has also been reported by Lee and her coworkers (Lee et al., 2011) (**Figure 1.8.B**). The hinge regions connecting $\alpha 2$ - $\alpha 3$ and $\alpha 4$ - $\alpha 5$ of Bcl-w undergo conformational change, which results in the angle of helix $\alpha 2$ and $\alpha 3$ changing from $\sim 90^\circ$ to $\sim 240^\circ$. Through this structural transition, alpha helices $\alpha 3$ and $\alpha 4$ hinge away from one Bcl-w to another Bcl-w protein to form domain-swapped dimer (**Figure 1.8.A**). Unlike Bcl-xL which requires certain stimulus for protein dimerization, Bcl-w forms domain-swapped dimers without any inducement, and this dimerization is more likely to be dependent on the amino acid sequence of Bcl-w. Lee and her coworkers showed in the report that the hinge region connecting $\alpha 4$ and $\alpha 5$ (indicated with an arrow in **Figure 1.8.B**) is essential for domain swapping of Bcl-w protein. They generated chimeric Bcl-w by replacing the amino acid residues at this linker region with corresponding residues of Bcl-2 or Bcl-xL, and it was shown that the chimeric protein was mainly observed as monomeric form. They also showed the possible influence of structural plasticity at the $\alpha 4$ - $\alpha 5$ linker region on protein function. Mouse embryonic fibroblast expressing Bcl-w chimera showed enhanced anti-apoptotic capacity compared to wild-type when exposed to apoptotic stimuli such as ABT-737 or etoposide. Hence, it has been suggested that structural flexibility of Bcl-w at the linker region $\alpha 4$ - $\alpha 5$ has certain influence on the functional aspect of Bcl-w protein, even though the exact mechanism is still unclear.

Figure. 1.8 Crystal structure of Bcl-w domain-swapped dimer.

A) Cartoon representation of monomer Bcl-w (PDB ID: 1MK3) on the left and one half of Bcl-w domain-swapped dimer on the right (PDB ID: 2Y6W). Alpha helices $\alpha 3$ and $\alpha 4$ are colored magenta. B) Cartoon representation of the Bcl-w domain-swapped dimer. (PDB ID: 2Y6W)

Like Bax and Bcl-w, Bcl-xL also has been shown to form three-dimensional domain-swapped dimer, and its crystal structure has been determined by O'Neill and his coworkers (O'Neill et al., 2006) (**Figure 1.9.C**). However, formation of Bcl-xL domain-swapped dimer requires physico-chemical changes in environment as an inducement, such as heat or pH change, whereas Bcl-w could dimerize through domain-swapping in the absence of any stimulus (Denisov et al., 2007; Xie et al., 1998). Domain swapping of Bcl-xL occurs by reciprocal exchange of C-terminal alpha helices ($\alpha 6$ - $\alpha 8$) between monomer subunits. During dimerization, hinge region connecting $\alpha 5$ - $\alpha 6$ (indicated with an arrow in **Figure 1.9.A**) undergoes dramatic conformational change, where the $\alpha 5$ - $\alpha 6$

hairpin is converted to an extended alpha helix, as shown in **Figure 1.9.B**. Together with structural characteristic of Bcl-xL dimers, O'Neill and his coworkers also proposed a possible function of this protein dimer. Previously, it was reported that Bcl-xL could form ion channel in synthetic lipid membranes (Minn et al., 1997). O'Neill and his coworkers examined the pore-forming capacity of Bcl-xL monomer and domain-swapped dimer using liposome leakage assay. Bcl-xL dimers showed enhanced pore-forming activity compared to monomers. Hence, they suggested that Bcl-xL domain-swapped dimers might function as intermediates in the formation of ion channel.

Figure. 1.9 Crystal structure of Bcl-xL domain-swapped dimer.

A) Cartoon representation of Bcl-xL monomeric proteins (PDB ID: 1MAZ). B) Cartoon representation of one half of Bcl-xL domain-swapped dimer. Alpha helices $\alpha 6$ - $\alpha 8$ involved in domain-swapping are colored in magenta. (PDB ID: 2B48) C) Cartoon representation of Bcl-xL domain-swapped dimer. (PDB ID: 2B48) Bcl-xL dimer consists of two monomeric folds of Bcl-xL, and each monomeric fold is colored blue and magenta.

In summary, there have been numerous reports on the oligomerization of Bcl-2 family proteins including Bax, Bak, Bcl-w, and Bcl-xL. Also, it has been suggested that the dimeric and oligomeric Bcl-2 family proteins might have distinctive roles in the regulation of apoptosis as well as cellular ion homeostasis via pore formation (Dewson et al., 2009; Dewson et al., 2008; Lee et al., 2011; O'Neill et al., 2006). However, structural mechanisms underlying protein oligomerization are different among the family members.

In addition, it has been shown that certain Bcl-2 family proteins require physico-chemical environmental changes as inducement for oligomerization (Czabotar et al., 2013; Denisov et al., 2007; Hsu and Youle, 1997; Xie et al., 1998). However, these reports are mainly focused on one member of Bcl-2 family protein with one particular inducement respectively, and there has been no report on screening several Bcl-2 family proteins regarding their oligomerization with different sets of inducement yet. Therefore, it is difficult to discern if Bcl-2 family proteins have a common pattern in oligomerization.

1.4 Voltage-Dependent Anion Channel (VDAC)

1.4.1 Interaction of Bcl-2 family proteins with VDAC

Bcl-2 family of proteins regulate mitochondrial apoptosis not only by controlling MOMP, but also by regulating mitochondrial calcium homeostasis (Baffy et al., 1993; Murphy et al., 1996; Pinton et al., 2008; Zhu et al., 1999). Mitochondrial calcium overload results in the swelling of mitochondria, leading to the rupture of MOM and release of mitochondrial apoptotic factors into the cytosol (Rizzuto et al., 2008). Hence, maintenance of mitochondrial calcium homeostasis is essential to protect cell against apoptosis.

In the mitochondrial outer membrane, there are many proteins involved in modulation of mitochondrial calcium homeostasis. Voltage dependent anion channel (VDAC), also known as mitochondrial porin (Shoshan-Barmatz et al., 2010b), is one of such proteins. VDAC is located at the mitochondrial outer membrane, facilitating passage of ions and small metabolites between mitochondria and cytosol. The published structure of VDAC consists of a beta barrel made of 19 β strands and an alpha helix at the N-terminus

(Bayrhuber et al., 2008; Hiller et al., 2008; Schredelseker et al., 2014), and several scientific reports have been proposing that N-terminal alpha helix might have role in regulating the channel activity of the protein (Geula et al., 2012; Mannella, 1998; Popp et al., 1996).

Interestingly, many scientists have been also reporting the interaction between Bcl-xL and N-terminal helix of VDAC, and its importance in modulating mitochondrial calcium uptake, as well as regulating mitochondrial apoptosis (Arbel et al., 2012; Huang et al., 2013). In these reports, interaction of Bcl-xL with VDAC N-terminal helix was demonstrated by pull-down assay, western blot, Acridine Orange(AcOr)/ethidium bromide (EtBr) cell staining, and mitochondrial $[Ca^{2+}]$ measurement. So far, many cell-based assays have been done to investigate the functional implication of the interaction between the two proteins, but the structural information on the VDAC N-terminal helix binding and interacting with Bcl-xL is not available. In order to understand the mechanism how Bcl-xL works with VDAC N-terminal region to modulate mitochondrial calcium homeostasis, it is crucial to gain insight into the structural perspective of the interaction.

1.4.2 Biochemical and structural characteristic of VDAC

VDAC was first found by Schein, Stanley J., Marco Colombini, and Alan Finkelstein in 1976 (Schein et al., 1976), and its biochemical and structural characteristic has been extensively studied to this day. VDAC, also known as mitochondrial porin, facilitates passage of ions and small metabolites across the OMM, allowing molecule exchanges between cytosol and mitochondria. As the name suggests, VDAC has voltage-dependent ion channel activity. At low membrane potential below 10 mV, VDAC adopts open-state conformation, showing high conductance and anion selectivity. When the membrane potential is increased above 30 mV, conformation of VDAC is changed to closed-state, showing low conductance and cation selectivity. Simple salts can pass through VDAC at both open and closed states, but passage of mitochondrial metabolites, especially organic anions, is significantly different between the two states (Colombini et al., 1996).

In mammals, there are three VDAC isoforms (VDAC1, VDAC2, and VDAC3), and pairwise sequence identity of human VDAC1/VDAC2, VDAC1/VDAC3, and

VDAC2/VDAC3 are 75%, 67%, and 74%, respectively (**Figure 1.10**). Compared to VDAC1 or VDAC3, VDAC2 has additional 11 amino acid residues at the N-terminus. Despite the high degree of sequence homology, expression level and the distribution of each isoform varies in different cell types. Among all three VDAC isoforms, VDAC1 has been most extensively characterized structurally and functionally.

hVDAC1	-----MAVPPTYADLGKSARDVFTKGYGFGGLIKLDLTKSENGLEFTSSGSANT	49
hVDAC2	MATHGQTCARPMCIPPSYADLGKAARDIFNKGFGGLVKLDVTKSCSGVEFSTSGSSNT	60
hVDAC3	-----MCNTPTYCDLGKAAKDVFNKGYGFGMVKIDLTKSCSGVEFSTSGHAYT	49
	*. *:*.*****:*.*:*.*****:*.*:*****. *:***:*. *	
hVDAC1	ETTKVTGSLETKYRWTEYGLTFTEKWNTDNTLGTEITVEDQLARGLKLTFDSSFSPTNGK	109
hVDAC2	DTGKVTGTLETKYKWCEYGLTFTEKWNTDNTLGTEIAIEDQICQGLKLTFTDTTSPNTGK	120
hVDAC3	DTGKASGNLETKYKVCNYGLTFTQKWNTDNTLGTEISWENKLAEGCLKLTLDITFVPNTGK	109
	:* *:*.*****: *****:*****:*****: *::..*****:*. * *****	
hVDAC1	KNAKIKTGYKREHINLGCMDDFDIAGPSIRGALVLGYEGWLGYQMNFFETAKSRVTQSNF	169
hVDAC2	KSGKIKSSYKRECINLGCDDVDFDFAGPAIHGSAVFGYEGWLGYQMTFDSAQSKLTRNNF	180
hVDAC3	KSGKIKASYKRDCFSVGSNVDFDFSGPTIYGWAVLAFEGWLGYQMSFDTAKSKLSQNNF	169
	*. *:*.*****: ..*:..*:..*:..*:..* * *:..*****:*. *:***:..**	
hVDAC1	AVGYKTDEFQLHTNVNDGTEFGGSIYQKVNKKLETAVNLAWTAGNSNTRFGIAAKYQIDP	229
hVDAC2	AVGYRTGDFQLHTNVNDGTEFGGSIYQKVCEDLDTSVNLAWTSGTNCTRFGIAAKYQLDP	240
hVDAC3	ALGYKAADFQLHTNVNDGTEFGGSIYQKVNKKIETSLINLAWTAGNSNTRFGIAAKYMLDC	229
	*:***: *****:*****:*****: ..*:..*:..*:..*:..* ***** *	
hVDAC1	DACFSAKVNNSSLIGLGYTQTLKPGIKLTLSALLDGKNVNAGGHKLGLEFQA	283
hVDAC2	TASISAKVNNSSLIGVGYTQTLRPGVKLTLSALVDGKSNAGGHKVGLEALEEA	294
hVDAC3	RTSLSAKVNNASLIGLGYTQTLRPGVKLTLSALIDGKNFSAGGHKVGLEFELEA	283
	:. :*****:*****:*****:*.*****:***..*****:*. *:***:	

Figure. 1.10 Amino acid sequence alignment of human VDAC isoforms.

Amino acid sequence of the three human VDAC isoforms are aligned using Clustal Omega (Chenna et al., 2003) from EMBL website. (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)

Three-dimensional structures of human VDAC1 (hVDAC1) (**Figure 1.11.A and B**), mouse VDAC1 (mVDAC1) (**Figure 1.11.C**), and zebra fish VDAC2 (zVDAC2) (**Figure 1.11.D**) have been determined by X-ray crystallography and/or NMR spectroscopy (Bayrhuber et al., 2008; Hiller et al., 2008; Schredelseker et al., 2014; Ujwal et al., 2008). VDAC1 and VDAC2 have similar three-dimensional fold, as both proteins have one beta barrel with a diameter of 20 to 30 Å, made of 19 beta strands, and one amphipathic alpha helix from N-terminal region, located inside the pore. The structure of VDAC3 has not been determined yet.

The N-terminal alpha helix of VDAC has been shown to play an important role in regulation of channel gating. Truncation mutation of mouse VDAC1 at N-terminal region leads to loss of voltage-dependence of the channel protein, exhibiting high conductance regardless of voltage (Abu-Hamad et al., 2009). To date, several mechanisms have been suggested to explain how the N-terminal alpha helix modulate channel activity of VDAC protein. (**Figure 1.12**) Firstly, it has been proposed that at high membrane potential, the N-terminal alpha helix located within the lumen moves from the beta-barrel wall towards the center of the pore, blocking the passage of molecules through the pore (Ujwal et al., 2008) (**Figure 1.12.A**). Also, unfolding of the N-terminal alpha helix has been suggested as a mechanism of channel gating, allowing it to interact with the opposite side of the barrel wall across the channel lumen (Bayrhuber et al., 2008; Hiller et al., 2008) (**Figure 1.12.B**). Lastly, it has been suggested that translocation of N-terminal alpha helix into and out of the pore might regulate channel activity by blocking the channel or causing conformational change of beta barrel (Zachariae et al., 2012) (**Figure 1.12.C and D**).

In addition, there have been several reports proposing the role of VDAC N-terminal region in regulation of mitochondrial apoptosis. T-REx293 cells that were silenced for hVDAC1 and reconstituted with mouse VDAC with a N-terminal truncation ($\Delta(26)$ mVDAC1) could not undergo apoptosis in response to apoptosis-inducing agents including staurosporine (STS), curcumin, As_2O_3 , and cisplatin (Abu-Hamad et al., 2009; Shoshan-Barmatz et al., 2010a). Also, cytochrome c release was inhibited in the cells expressing $\Delta(26)$ mVDAC1, indicating that the N-terminal region of VDAC1 is necessary for cytochrome c release and mitochondrial apoptosis (Abu-Hamad et al., 2009).

Figure. 1.11 NMR and crystal structure of VDAC isoforms.

A) Cartoon representation of NMR structure of hVDAC1 in LDAO micelles (PDB ID: 2K4T), side and top view. B) 3D structure of hVDAC1 in LDAO or Cymal-5 micelles determined conjointly by x-ray crystallography and NMR spectroscopy, (PDB ID: 2JK4) side and top view. B) Cartoon representation of mVDAC1 crystal structure in DMPC/CHAPSO bicelles (PDB ID: 3EMN). D) Cartoon representation of zVDAC2 in *n*-undecyl- β -D-thiomaltopyranoside solved by X-ray crystallography (PDB ID: 4BUM). All structural figures are colored from blue to red (N- to C- terminus), and their N- and C- terminus are labelled with 'N' and 'C', respectively.

Figure. 1.12 Suggested mechanisms of VDAC channel gating by the N-terminal alpha helix.

A) Gating model proposed by Ujwal et al. B) Gating model proposed by Bayrhuber et al. and Hiller et al. C) and D) Gating model proposed by Zachariae et al. Left side of the panel represents open state of VDAC, and right side of the panel indicates closed state of VDAC.

1.4.3 VDAC interacts with Bcl-2 family proteins to regulate mitochondrial apoptosis

Studies have shown that VDAC interacts with pro- and anti- apoptotic Bcl-2 family proteins, regulating apoptosis, VDAC channel gating, and mitochondrial calcium

homeostasis. Interaction of Bcl-xL with VDAC has been extensively investigated by various methods, including co-immunoprecipitation and GST pull down (Shimizu et al., 1999). Bcl-xL could inhibit the uptake of ^{14}C sucrose uptake in VDAC-reconstituted liposomes in a concentration-dependent manner (Shimizu et al., 1999). Consistent with this, Monaco Giovanni and his coworkers have argued that BH4 domain of Bcl-xL inhibits VDAC1-mediated calcium ion uptake into the mitochondria in order to protect cells against mitochondrial apoptosis (Monaco et al., 2015). Varda Shoshan-Barmatz and her coworkers also showed that Bcl-xL induces channel closure of VDAC by examining channel activity of bilayer-reconstituted VDAC (Arbel et al., 2012). However, Marco Colombini and his coworkers have suggested that recombinant Bcl-xL promotes open state of VDAC proteins to maintain metabolite exchange through the outer mitochondrial membrane (Vander Heiden et al., 2001). Thus, it remains to be determined whether Bcl-xL regulates the VDAC positively or negatively.

VDAC was also shown to interact with pro-apoptotic Bcl-2 family proteins. Shimizu Shigeomi and his coworkers showed that VDAC liposomes bind to recombinant Bak and Bax proteins by immunoprecipitation and western blot (Shimizu et al., 1999). Also, Bak and Bax enhanced the uptake of ^{14}C sucrose in VDAC-reconstituted liposomes. VDAC was required in Bax- or Bak-mediated loss of membrane potential and cytochrome c release from the isolated yeast mitochondria. Taken together, Shimizu and his coworkers have suggested that both pro- and anti- apoptotic Bcl-2 family proteins regulate VDAC activity to modulate mitochondrial changes that are related to apoptosis.

More recently Cheng et al., using cross-linking and mass spectrometry, showed that Bak interacts with VDAC2 in mouse embryonic fibroblast cells (Cheng et al., 2003). Stanley J. Korsmeyer and his coworkers have shown that VDAC2 regulates apoptosis in Bak-dependent manner. When overexpressed, VDAC2 could successfully inhibit apoptosis in Bax deficient MEF cells in response to STS, but not in Bak deficient MEF cells, which was shown by Annexin-V apoptosis assays. This indicates that VDAC2 possesses an inhibitory role on regulation of apoptosis by interacting with Bak protein.

Taken together, despite the conflicting results, it is clear that interaction of the VDACs and Bcl-2 family proteins is important in regulating mitochondrial apoptosis.

1.4.4 Bcl-2 family proteins interact with the N-terminal domain of VDAC

Numerous reports have suggested that VDAC interacts with Bcl-2 family proteins, especially Bcl-xL, through its N-terminal region. Surface plasmon resonance (SPR) experiments showed that synthetic peptides of the VDAC1 N-terminal region bound to immobilized Bcl-2 and Bcl-xL in a concentration-dependent manner (Abu-Hamad et al., 2009; Arbel et al., 2012). Similarly, binding of VDAC N-terminal peptide to Bcl-2 has also been demonstrated by cellulose-bound peptide arrays (Arbel and Shoshan-Barmatz, 2010). Addition of peptide derived from VDAC1 N-terminal region could disrupt binding of Bcl-xL to VDAC1, which resulted in inhibition of Bcl-xL pulldown by GST-fused VDAC1 (Huang et al., 2013). As mentioned previously, it has been reported that Bcl-xL could induce the closure of VDAC channel, maintaining low conducting state. However, addition of Bcl-xL to N-terminally truncated VDAC1 ($\Delta(26)$ VDAC1) did not have any effect (Arbel et al., 2012). This demonstrates that Bcl-xL requires N-terminal region of VDAC1 to modulate its channel activity. Also, it has been shown that addition of cell-permeant peptide corresponding to VDAC1 N-terminal region could reduce mitochondrial calcium uptake of wild type MEF cells, whereas no change was observed when the peptide was added to Bcl-xL knockout MEF cells (Huang et al., 2013). This result indicates that mitochondrial calcium uptake is regulated by interaction of Bcl-xL with VDAC N-terminal region.

Furthermore, immunostaining experiments on T-REx293 cells revealed that anti-apoptotic effect of Bcl-2 and Bcl-xL against STS was inhibited by expression of VDAC1 N-terminal peptide of 26 amino acids, which demonstrates that VDAC N-terminal region interacts with Bcl-2 proteins to prevent their anti-apoptotic activity (Abu-Hamad et al., 2009; Arbel and Shoshan-Barmatz, 2010; Arbel et al., 2012).

In summary, it has been suggested by several reports that the interaction between Bcl-2 family proteins and VDAC is essential in voltage-dependent channel gating of VDAC at OMM, as well as in the regulation of mitochondrial apoptotic pathway. Also, numerous experimental data and evidences have been indicating the N-terminal region of VDAC as the target for Bcl-2 family proteins. Hence, studying the molecular basis of interaction of the N-terminal region of VDAC with Bcl-2 family proteins will advance our understanding of the mechanism underlying the regulation of mitochondrial homeostasis and apoptosis.

1.5 Objective of this project

In previous chapters, the importance of Bcl-2 family in the regulation of mitochondrial changes associated with apoptosis has been discussed. It has been suggested by many scientific reports that pro- and anti- apoptotic Bcl-2 family proteins control MOMP by forming homo- and hetero- dimers and oligomers. In addition, oligomerization of Bcl-2 family proteins and their three-dimensional structures have been introduced, together with their functional implication in apoptosis. Compared to Bak/Bax oligomers which have been extensively studied due to its formation of pore during MOMP, oligomerization of anti-apoptotic Bcl-2 family proteins and their physiological functions remains to be elucidated. Anti-apoptotic Bcl-xL has been shown to undergo conformational changes to form three-dimensional domain swapped (3DDS) dimers in the presence of certain physico-chemical environmental changes. However, it has not been investigated whether this induced-dimerization is a common behavior among anti-apoptotic Bcl-2 family proteins, and if so, it is intriguing to find out what could be the functional implication of such structural transition. To this end, we have over-expressed and purified recombinant anti-apoptotic Bcl-2 family proteins, and performed screening of several physico-chemical stimuli which can induce structural transition of these proteins. Also, functional implication of such structural transitions has been investigated by biochemical methods.

Secondly, mitochondrial porin VDAC protein and its interaction with Bcl-2 family proteins has been discussed. To date, various experimental data from immunoprecipitation and cell-based functional assays have revealed that Bcl-xL interacts with N-terminal region of VDAC to regulate mitochondrial calcium homeostasis and mitochondrial changes associated with apoptosis. However, only little information is available on the structural basis of this interaction. Earlier, full length VDAC1 interacting with Bcl-xL has been studied using NMR spectroscopy in LDAO micelles (Malia and Wagner, 2007). Here, to gain further molecular insight into this protein -protein interaction, we have used VDAC1/2/3 N-terminal peptides to understand their interaction with Bcl-xL using NMR spectroscopy. The aim of this dissertation is to gain a better understanding of the protein-protein interaction which is important in regulation of mitochondrial apoptosis.

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Antibodies against Bcl-xL(54H6) and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against 6XHis-tag was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Carbenicillin disodium salt, Isopropyl β -D-1-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were purchased from Gold Biotechnology (St. Louis, Missouri, USA). Kanamycin sulfate, NativePAGE™ Novex™ 4-16% Bis-Tris Protein Gels, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) and bismaleimido-hexane (BMH) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The plasmid purification kit, PCR purification kit, and gel extraction kit were purchased from Axygen Scientific (Waltham, Massachusetts, USA). ECL western blotting substrate was from Advanta (Menlo Park, CA, USA). HyClone™ Dulbecco's Modified Eagles Medium (DMEM), HyClone™ Penicillin-Streptomycin 100× solution, HiLoad 16/600 Superdex 75 and 200 prep grade, Superdex 200 10/300 GL, Superdex 75 5/150 GL, and Resource Q column were purchased from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK). Gibco Fetal bovine serum (FBS) was from Thermo Fisher Scientific. Ni²⁺-NTA agarose resin was from Qiagen (Hilden, Germany). Protein molecular weight markers were purchased from Bio-Rad (Hercules, CA, USA). T4 ligase, Phenylmethylsulfonyl fluoride (PMSF), Annexin-V-FLOUS Staining kit, and EDTA-free protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, Missouri, USA). *Pfu* and *Taq* DNA polymerases and standard buffers were from Thermo Fisher Scientific. All the restriction enzymes, reaction buffers, Gel Loading Dye (purple, 6×) for agarose gel, and dNTP mixtures were purchased from New England Biolabs (Ipswich, Massachusetts, USA). GelRed™ Nucleic Acid Gel Stain was purchased from Biotium (Fremont, CA, USA). ¹⁵N labelled ammonium chloride (15N, 99%) and deuterium labelled water (D₂O) for NMR experiments were from Cambridge Isotope Laboratories (Tewksbury, MA USA). All other chemicals were purchased either from Sigma-Aldrich or Bio-Rad.

2.1.2 Bacterial strain and mammalian cell lines

E.coli (Escherichia coli) DH5 α was used for cloning experiments, site-directed mutagenesis, and plasmid DNA amplification by miniprep. *E.coli* BL21(DE3) was used for protein expression. Mouse embryonic fibroblast (MEF) cells stably expressing wild type or mutant Bcl-xL (E158P and M159P), a kind gift from Carl White group, were used for annexin V apoptosis assay.

2.1.3 Plasmid DNA constructs

The Bcl-xL cDNA lacking flexible loop (28-80) and transmembrane domain (197-233) (Bcl-xL Δ Loop Δ TM) was cloned into the pET-SUMO vector. Bcl-xL Δ Loop Δ TM with a point mutation at Glu158 or Met159 (E158P and M159P) were prepared by Dr. Nguyen Quoc Toan. For expression of Bcl-xL, Bcl-2, and Bak, a 6 $\times$ His-small ubiquitin-related modifier (SUMO) tag fusion was used to enhance the solubility of protein expressed in *E.coli*. In addition, six histidine residues in the 6 $\times$ His-SUMO tag enabled the purification of expressed proteins by Ni²⁺-NTA affinity chromatography.

The cDNA of Bcl-2 hybrid construct without transmembrane domain (Bcl-2 hybrid Δ TM) was cloned into the pET-SUMO vector. Wild type Bcl-2 with intact loop has poor solubility in solution, and deletion of the flexible loop region also results in insoluble protein (Anderson et al., 1999). To get a soluble protein sample, DNA construct of Bcl-2 of which the loop was replaced with negatively charged Bcl-xL loop (Petros et al., 2001) was cloned into pET-SUMO vector and transformed into BL21(DE3) *E.coli* strain.

Mcl-1 with a truncation of transmembrane domain and N-terminal 170 residues (1-170) was used as residues 171-327 generate Bcl-2 like region (BLR) (Thomas et al., 2010). To enhance the protein stability and solubility of Mcl-1, cDNA coding BLR region of Mcl-1 with 9 chimeric mutation (Czabotar et al., 2007b) was cloned in pET28a and kindly gifted by Seung-Wook Chi from Korea Research Institute of Bioscience and Biotechnology (KRIBB). The cDNA of Bcl-w with a deletion of the transmembrane domain (Bcl-w Δ TM) cloned in pET21a vector was also a kind gift from Dr. Chi. The 6 $\times$ His tag fused to the C-terminus of Bcl-w Δ TM and the N-terminus of Mcl-1 Δ N170 Δ C21 allowed the purification of expressed protein by Ni²⁺-NTA affinity chromatography.

The cDNA of Bak with N- and C-terminal deletion (Bak Δ N20 Δ C21) was also cloned into pET SUMO vector. **Table 2** shows the list of plasmid constructs that have been used for the research.

Table 2 List of the plasmid vectors used in this study.

cDNA	Truncation	Vector	Antibiotic resistance	mutation	Tag	Expression host
Bcl-xL	Δ 28-80 Δ 197-233	pET-SUMO	Kanamycin	Wild type	6 $\times$ His-SUMO (N-ter)	BL21(DE3)
				E158P		
				M159P		
Bcl-2	Δ 208-239	pET-SUMO	Kanamycin	Flexible loop region was replaced with a portion of Bcl-xL (29D-44E)	6 $\times$ His-SUMO (N-ter)	BL21(DE3)
Bcl-w	Δ 158-193(Denisov et al., 2003a)	pET21a	Ampicillin	Wild type	6 $\times$ His (C-ter)	BL21(DE3)
Mcl-1	Δ N170 Δ C21	pET28a	Kanamycin	chimera of mouse and human Mcl-1 ^{BLR} (at nine N-terminal amino acids) (Czabotar et al., 2007a)	6 $\times$ His (N-ter)	BL21(DE3)
Bak	Δ N20 Δ C21	pET-SUMO	Kanamycin	Wild type	6 $\times$ His-SUMO (N-ter)	BL21(DE3), BLR(DE3), BLR(DE3)pLysS

2.1.4 Medium

2.1.4.1 Antibody stocks

Kanamycin (30 mg/ml in ddH₂O stock, 30 µg/ml in working solution), Carbenicillin (100 mg/ml in dd ddH₂O stock, 100 µg/ml in working solution), Chloramphenicol (25 mg/ml in ethanol stock, 25 µg/ml in working solution), Penicillin-Streptomycin (10,000 U/ml in stock from HyClone™, 100U/ml in working solution).

2.1.4.2 Lysogeny Broth (LB) medium (Sambrook, 2001)

In 1 L ddH₂O, 5 g NaCl, 5 g bacto-yeast extract, and 10 g bacto-tryptone were dissolved and autoclaved.

2.1.4.3 M9 medium

M9 medium was used for isotopic labeling for protein NMR spectroscopy. To prepare 1 L of M9 media, the following buffer solutions and chemicals were autoclaved/filtered and added into an autoclaved flask.

: 105.2 ml 0.5 M Na₂HPO₄, 26.6 ml 1 M KH₂PO₄, 2 ml 5 M NaCl, 1.2 ml 1 M MgSO₄, 1.2 ml 0.1 M CaCl₂, 1.2 ml 0.5% Thiamine, 22.6 ml 20% D-(+)-glucose, 1 g NH₄Cl, and 840 ml autoclaved ddH₂O.

For ¹⁵N labeling, NH₄Cl was replaced with ¹⁵NH₄Cl. For deuteration of proteins, all the buffer solutions were prepared in D₂O and autoclaved ddH₂O was replaced with D₂O.

2.1.4.4 Mammalian cell culture medium

Filtered 50 ml FBS and 5 ml Penicillin-Streptomycin stock were added to 500 ml DMEM and mixed by gentle shaking.

2.1.5 Buffers and solutions

2.1.5.1 Solution and buffer for agarose gel electrophoresis

Tris-acetate-EDTA (TAE) buffer

50× stock solution was prepared in 1 L ddH₂O (242 g Tris, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA pH 8.0).

Agarose gel solution

1% (w/v) agarose solution was prepared in 1× TAE buffer. Agarose powder was dissolved in TAE buffer by microwaving for several minutes. 1× GelRed stain was added to the dissolved agarose solution and mixed.

2.1.5.2 Buffer for protein purification

Lysis buffer

50 mM Tris, 150 mM NaCl, 0.5 mM TCEP·HCl, pH 7.0

50 mM Tris, 150 mM NaCl, 0.5 mM TCEP·HCl, pH 7.5

50 mM Tris, 150 mM NaCl, 1 mM PMSF, 1 mM BME, pH 8.0

Wash buffer

Wash buffer 1: 20 mM Tris, 150 mM NaCl, 0.5 mM TCEP·HCl, 25 mM Imidazole, pH 7.5

Wash buffer 2: 20 mM Tris, 1 M NaCl, 0.5 mM TCEP·HCl, 25 mM Imidazole, pH 7.0

Elution buffer

20 mM Tris, 150 mM NaCl, 0.5 mM TCEP·HCl, 500 mM Imidazole, pH 6.5

20 mM Tris, 150 mM NaCl, 0.5 mM TCEP·HCl, 500 mM Imidazole, pH 7.0

Gel filtration chromatography buffer

20 mM Tris, 150 mM NaCl, 0.01% NaN₃, pH 7.0

20 mM Tris, 150 mM NaCl, 0.01% NaN₃, pH 7.5

20 mM Tris, 150 mM NaCl, 0.01% NaN₃, pH 8.0

20 mM phosphate, 50 mM NaCl, 0.01% NaN₃, pH 6.5

Anion exchange chromatography buffer

Buffer A: 50 mM Tris, 1 mM EDTA, 2 mM BME, pH 8.0

Buffer B: 50 mM Tris, 1 mM EDTA, 1M NaCl, pH 8.0

2.1.5.3 Buffer and solution for SDS-PAGE

Acrylamide gel solution

Resolving gel: 12% acrylamide mix, 0.39 mM Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED

Stacking gel: 5.1% acrylamide mix, 0.13 mM Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.1% TEMED

5× SDS-PAGE running buffer

1.5% (w/v) Tris, 7.5% (w/v) glycine, 0.5% (w/v) SDS

: A mixture of 75 g Tris, 360 g glycine, and 25 g SDS was dissolved in 5 L distilled water.

SDS gel staining solution

40% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.3% (w/v) Coomassie Brilliant Blue G-250

: 1.5 g Coomassie Brilliant Blue G-250 was dissolved in a mixture of 200 ml methanol, 50 ml glacial acetic acid, and 250 ml distilled water.

SDS gel destaining solution

45% (v/v) methanol, 15% (v/v) glacial acetic acid

: A mixture of 900 ml methanol, 300 ml glacial acetic acid, 800 ml distilled water.

6× SDS-PAGE loading buffer

300 mM Tris-Cl, 12% SDS, 60% glycerol, 600 mM DTT, 0.2% Bromophenol blue, pH 6.8

2.1.5.4 Buffer and solution for Blue-Native PAGE

20× Cathode buffer

0.4% (w/v) Coomassie Brilliant Blue G-250 solution was made by dissolving 1 g Coomassie Brilliant Blue G-250 in 250 ml distilled water.

20× Anode buffer

50 mM Bis-Tris, 50 mM Tricine, pH 6.8

Fix solution

40% (v/v) methanol, 10% (v/v) glacial acetic acid

: A mixture of 40 ml methanol, 10 ml glacial acetic acid, 50 ml distilled water.

Destaining solution

8 % (v/v) glacial acetic acid

: A mixture of 8 ml glacial acetic acid and 92 ml distilled water.

2.1.5.5 Buffer and solution for Western blot**Membrane transfer buffer**

1× SDS-PAGE running buffer, 20% (v/v) methanol

: A mixture of 200 ml 5× SDS-PAGE running buffer, 200 ml methanol, and 600 ml distilled water.

20× TBS stock buffer

500 mM Tris-Cl, 60 mM KCl, 2.8M NaCl, pH 7.4

Membrane wash solution (TBST buffer)

1× TBS stock buffer, 0.1% Tween-20

: A mixture of 50 ml 20× TBS stock buffer, 10 ml 10% Tween-20, and 940 ml distilled water.

Blocking solution

5% (w/v) non-fat dry milk in TBST buffer was made by dissolving 2.5 g non-fat dry milk in 50 ml TBST buffer.

2.1.5.6 Buffers for protein dimerization study

Acidic buffer: 50 mM sodium acetate, 150 mM NaCl, 0.01% NaN₃, pH 4.0

Basic buffer: 20 mM Tris, 150 mM NaCl, pH 10.0

2.1.6 Peptides

All the peptides below corresponding to the indicated sequences of VDAC proteins were chemically synthesized by GL Biochem (Shanghai, China).

hVDAC1 (1-25): MAVPPTYADLGKSARDVFTKGYGFG

hVDAC2 (12-36): MCIPPSYADLGKAARDIFNKGFGFG

hVDAC2-SG20 (17-36): SYADLGKAARDIFNKGFGFG

hVDAC2-SF17 (17-33): SYADLGKAARDIFNKGFG

hVDAC3 (1-25): MCNTPTYCDLGKAAKDVFNKGFGFG

2.2 Methods

2.2.1 Preparation of competent cells

Bacterial competent cells were made for *E.coli* transformation. Briefly, bacterial cells were grown by streak-plate method on the LB agar plate overnight in 37°C. On the next day, a single colony was inoculated into 5 ml of LB media and cultured overnight in an incubator at 37°C, while shaking at 200RPM. The overnight culture was transferred to 100 ml LB media and shaken at 200RPM at 37°C. Once OD₆₀₀ reached 0.4~0.5, the cell culture was transferred to a centrifuge tube and chilled on ice for 10 minutes. Bacterial cells were harvested by centrifugation at 2,850×g for 15 minutes at 4°C. The cell pellet was re-suspended in 50 ml of autoclaved, ice-cold 0.1 M CaCl₂, and incubated on ice for 2 hours. Cells were harvested by centrifugation at 2,850×g for 15 minutes at 4°C, and the pellet was re-suspended in 2 ml of ice-cold buffer containing 0.1 M CaCl₂ and 20 % glycerol. Bacterial resuspension was kept in 50 µl aliquots in sterile Eppendorf tubes. The aliquots were flash-frozen in liquid nitrogen and stored at -80°C.

2.2.2 Transformation

Competent cells were thawed on ice for 20 minutes. To a volume of 50 µl of competent cells in a sterile Eppendorf tube, 1 µl of plasmid vector was added, and mixed by flicking the Eppendorf tube several times. The mixture was incubated on ice for 20 minutes to allow the plasmid to bind onto the surface of the competent cells. The competent cells were then exposed to heat shock at 42°C for 45 seconds, and incubated on ice for 1 minute for the bacterial cells to recover. A volume of 300 µl of fresh LB media was added to the cells and they were incubated at 37°C by shaking at 200RPM for an hour. After this, the LB bacterial culture was spread evenly onto LB agar plate with appropriate antibiotic and incubated overnight at 37°C.

2.2.3 Isolation of plasmid DNA

A single colony from the LB agar plate was inoculated into 5 ml LB medium and shaken overnight at 200RPM at 37°C, and cultured overnight in a shaker. AxyPrep Plasmid miniprep kit from Axygen Scientific was used to isolate plasmid DNA from the bacteria.

2.2.4 Agarose gel electrophoresis

An 1% agarose gel containing 1× GelRed stain was prepared. For loading samples, a volume of 4 µl of DNA sample was mixed with 1 µl of 5× DNA loading dye. The electrophoresis was performed at 100V for 30 to 50 minutes, depending on the length of DNA sample. After the electrophoresis, the DNA bands were visualized using T: Genius Gel Documentation System from Syngene Bioimaging Private Ltd (Haryana, India).

2.2.5 Extraction of DNA from agarose gel

DNA samples were loaded into 1% agarose gel and run at 100V for 40 minutes for separation. DNA bands were visualized by exposing the agarose gel to UV light. The agarose gel containing the DNA band was excised using utility cutter while illuminating the DNA with UV light. DNA was extracted from the gel fragment using AxyPrep DNA Gen Extraction Kit from Axygen Scientific.

2.2.6 Polymerase Chain Reaction (PCR) amplification

PCR experiments were performed to amplify DNA fragments for cloning or to introduce a mutation in DNA. *Pfu* polymerase was used for general PCR experiments, whereas *Taq* polymerase was used for colony PCR screening. Annealing temperature was calculated based on the melting temperature of the forward and reverse primers. A typical PCR mixture contains the following components in **Table 3**.

Table 3 List of the components and their volume used for PCR experiment.

Component	Volume
Forward primer	1 μ l
Reverse primer	1 μ l
Template DNA	1 μ l
dNTP mix (10mM)	0.5 μ l
Autoclaved water	18.5 μ l
10x <i>pfu</i> buffer	2.5 μ l
<i>Pfu</i> DNA polymerase	0.5 μ l
Total	25 μ l

After all the components were added into a PCR tube, the PCR reaction was performed on a PCR thermal cycler from Applied Biosystems (Foster City, CA, US) using the temperature and time setup shown in **Figure 2.1**.

Figure. 2.1 Thermal cycling condition set for PCR experiment.

For figure legend, see next page.

Before the thermal cycling, sample was heated at 95°C for 5 minutes to completely denature the double stranded DNA. Annealing temperature was determined based on the characteristic of the forward/reverse primers. Extension time was set according to the rate of DNA polymerases (*Pfu*: 500 bp/minute, *Taq*: 1 kbp/minute) and also to the length of template DNA. After running the 30 cycles of extension, the sample was cooled down and kept at 4°C for further experiments.

2.2.7 Site-directed mutagenesis

To generate a point mutation or a deletion mutation, PCR was performed using a set of mutagenic primers. The PCR product was incubated with 1 µl of *DpnI* at 37 °C for an hour to digest the methylated template plasmid DNA. After this, the DH5α competent cells were transformed with the PCR product and plated on agar plates containing appropriate antibiotics. On the following day, a single colony was inoculated into fresh LB with an antibiotic and grown overnight at 37°C, while shaking at 200RPM. Plasmid DNA was purified from the bacterial culture by miniprep and analyzed by DNA sequencing at the Axil Scientific (41 Science Park Rd, Singapore).

2.2.8 Protein over-expression

For protein over-expression, a plasmid vector containing target gene of interest was transformed into BL21(DE3). Other bacterial strains such as BLR(DE3), BLR(DE3)pLysS were also used to optimize the condition of protein over-expression. A single colony obtained from the transformation reaction was inoculated into 10 ml LB with antibiotic and grown overnight at 37°C, while shaking at 200RPM. On the next day, the culture was transferred into 1 L fresh LB with antibiotic and cultured for 3 hours at 37°C, by shaking at 200RPM. OD₆₀₀ of the resulting culture was measured using BioSpec-mini from Shimadzu (Kyoto, Japan). If OD₆₀₀ reached 0.6-0.8, 1 ml of 1 M IPTG was added to the culture and shaken at 150RPM to induce protein over-expression, for 4 hours at 25°C. The bacterial cells were harvested at 4°C by centrifugation at 12,000×g for 30 minutes using Beckman Coulter centrifuge (Brea, CA, USA). The cell pellet was transferred to a 50 ml centrifuge tube and stored in -80°C prior to bacterial cell lysis.

2.2.9 Bacterial cell lysis

The bacterial cell pellet above was thawed on ice. A volume of 30 ml of lysis buffer was added to the bacterial cell pellet harvested from 1 L culture. The cell pellet was resuspended in lysis buffer using FINEPCR vortex mixer (Gyeonggi-do, Korea). The resuspended cells were lysed on ice by sonication for 10 minutes with 4sec/1sec ON/OFF pulser using Sonics Vibracell VCX750 from Sonics & Materials (Newtown, CT, USA). Sonication was repeated two to three times until the opaque cell resuspension turned into a translucent solution. Bacterial cell lysate was obtained by centrifuging the sonicated cell resuspension at 40,000×g for 30 minutes at 4°C.

2.2.10 Ni²⁺-NTA affinity column chromatography

Prior to protein purification, Ni²⁺-NTA agarose beads were loaded onto chromatography column (Bio-Rad) and washed with three column volume (CV) of lysis buffer. A volume of 2 ml of the beads were used for protein purification from 1 L bacterial culture. Cell lysate was transferred to the chromatography column and mixed with the beads, and incubated on a GyroMini nutating mixer from Labnet (Edison, NJ, USA) in the cold room (4°C) for an hour. The chromatography column was then taken out from cold room and fixed onto a clamp stand. Flow-through from the column was collected, and the beads were washed with three CVs of wash buffer 1 and 2, respectively. After the wash fractions were collected, SUMO-tagged or His-tagged proteins bound to the beads were eluted with the elution buffer. Protein elution from the column was checked by adding a fraction of the eluted sample into Bradford reagent (Bio-Rad) and observing the color change of the mixture.

2.2.11 Protein concentration

Purified recombinant proteins were transferred to Amicon™ Ultra centrifugal filter from Merck Millipore (Billerica, Massachusetts, USA) and concentrated by centrifugation at 2,850×g at 4°C until a desired volume was reached.

2.2.12 Buffer exchange

High concentration of imidazole in elution buffer can interfere with SUMO protease reaction when removing the SUMO tag from the recombinant protein. Therefore, imidazole in the eluted protein sample was removed by exchanging the sample buffer with a lysis buffer using PD-10 desalting column (GE Healthcare). PD-10 column was equilibrated with 5CV (25 ml) of lysis buffer, and a volume of 2.5 ml of concentrated protein sample from purification was loaded onto the column. Protein samples in the PD-10 desalting column was then eluted with an additional volume of 3.5 ml of lysis buffer.

2.2.13 Cleavage of SUMO protein tag

After the buffer exchange of the sample, recombinant proteins with the SUMO tag were incubated with the SUMO proteases at a concentration ratio of 25:1 (protein:SUMO protease), at 4°C or 18°C overnight to remove SUMO tag from the protein.

2.2.14 Size exclusion chromatography

HiLoad 16/60 Superdex 75 PG, Superdex 200 PG, and Superdex 200 10/300 GL were used for gel filtration chromatography. The column was equilibrated with 150 ml gel filtration buffer for 16/60 columns and 30 ml for 10/300 column. Concentrated protein sample obtained from the Ni²⁺-NTA purification after the cleavage of SUMO tag was injected to the column, and protein elution from the column was detected by measuring absorbance at 280 nm. Gel filtration conditions that have been used for each column is described in **Table 4**.

Table 4 List of size exclusion column and their specifications.

	HiLoad 16/60 Superdex 75 PG	HiLoad 16/60 Superdex 200 PG	Superdex 200 10/300 GL
Column Volume	130 ml	130 ml	25 ml
Flow rate	0.5 ml/min	0.5 ml/min	0.2 ml/min
Sample volume	Up to 2 ml	Up to 2 ml	Up to 0.5 ml
Separation range (globular protein)	3 to 70 kDa	10 to 600 kDa	3 to 70 kDa
Max. Pressure	0.5 Mpa	0.5 Mpa	1.8 Mpa

2.2.15 Determination of protein concentration

Concentration of the protein sample was measured to calculate protein purification yield as well as to prepare for experiments such as crosslinking, NMR titration, and so on. ND-1000 spectrophotometer (Thermo Fisher Scientific) was used to measure protein concentration (Desjardins et al., 2009). Basically, protein concentration was calculated from the sample absorbance at 280nm using molar absorptivity coefficient (or extinction coefficient value) calculated by ExPASy Protparam tool (Gasteiger et al., 2005). Extinction coefficient value and molecular weight of the proteins that have been used in this study are listed in **Table 5**.

Table 5 List of extinction coefficient value and MW of the proteins in this study.

Protein	Ext. coefficient ($M^{-1} cm^{-1}$)	Molecular weight (kDa)
Bcl-xLΔLoopΔTM	36440	19.3
Bcl-2ΔTM	37930	19.3
Bcl-wΔ158-193	28085	20.0
Mcl-1ΔN170ΔC21	20970	20.3
BakΔN20ΔC21	26930	19.1

2.2.16 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to analyze the purity of protein sample. As the majority of the proteins in this research are about 20 kDa in size, 12% resolving gel have been used. SDS-PAGE loading samples were prepared by mixing the protein sample with 1 $\times$ SDS-PAGE loading buffer and heating the sample at 95°C for 5 minutes (protein), or for 10 minutes (cell pellet). Gel electrophoresis was conducted under constant voltage (120V) for 95 to 105 minutes. After the electrophoresis, the gel was stained in SDS gel staining

solution on a shaker (The Belly Dancer (Thermo Fisher Scientific)) for 1 hour at room temperature. The gel was destained in the destaining solution until the background cleared. The gel was rinsed in distilled water before scanning with the GS-800 Calibrated Densitometer (Bio-Rad).

2.2.17 Induction of protein dimerization

To study structural plasticity of the Bcl-2 family proteins, various conditions in the following were applied to the protein and the protein structural transition was observed.

- ① Low pH: protein sample buffer was exchanged with sodium 50 mM sodium acetate buffer (refer to **2.1.5.6**)
- ② High pH: protein sample buffer was exchanged with 20 mM Tris buffer. (refer to **2.1.5.6**)
- ③ Heat: protein was heated at 55°C for 2 hours.
- ④ Detergent: OM detergent was added to protein sample till a final concentration of 2% and the sample was incubated at room temperature for 2 hours.

2.2.18 Crosslinking by BMH

Chemical crosslinking of protein was performed to examine the existence of protein dimer or higher order oligomer in the sample. BMH was added to the protein sample to a final concentration of 1-2 mM, and the sample was incubated at room temperature for 1 hour. Crosslinking reaction was quenched by adding DTT to a final concentration of 4 mM and incubating for 15 minutes.

2.2.19 Western blot analysis

Western blot experiments were performed to identify the protein in the sample. Proteins were separated on a thick SDS polyacrylamide gel (1.5 mm), and transferred to the nitrocellulose membrane, using an assembly as shown in **Figure 2.2**, while cooling the apparatus with ice for 1 hour at 100V.

Figure. 2.2 Assembly of transfer sandwich for wet transfer.

All the components for transfer sandwich were kept in ice-cold transfer buffer prior to transfer sandwich assembly. Plastic roller was used during the assembly to prevent air bubble formation which adversely affects protein band transfer to the membrane.

The nitrocellulose membrane was removed from the sandwich and incubated with blocking solution on a shaker for an hour at room temperature. Blocking solution was removed and the membrane was incubated with primary antibody overnight using the GyroMini nutating mixer in cold room. On the next day, the membrane was washed three times by repeating 10-minutes incubations in membrane wash solution at room temperature. The membrane was then incubated with HRP-conjugated secondary antibody on a shaker for an hour at room temperature. To remove unbound antibodies and nonspecifically bound antibodies, the membrane was washed three times in the wash solution by 10-minutes incubation for each at room temperature. Enhanced chemiluminescence (ECL) solution was added dropwise onto the membrane and incubated for 2 minutes at room temperature. Finally, the membrane was placed in the X-ray film cassette and imaged onto an X-ray film in the dark.

2.2.20 Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE was performed to check protein dimerization and oligomerization without denaturing or crosslinking the protein samples. NativePAGE™ Novex® 4-16% Bis-Tris Protein Gel was placed in XCell SureLock® Mini-Cell (Thermo Fisher Scientific).

Approximately 200 ml cathode buffer and 600 ml anode buffer were poured into the upper and lower chambers, respectively. To prepare BN-PAGE loading samples, 2.5 μ l of NativePAGE™ Sample Buffer (4 $\times$) and 0.5 μ l of NativePAGE™ 5% G-250 Sample Additive (Thermo Fisher Scientific) were mixed with 7 μ l of the protein sample. 5 μ l of the loading samples were loaded into each well and the electrophoresis was performed at a constant voltage (150V) for 135 minutes. After the electrophoresis, the gel was placed in 100 ml fixing solution, heated by microwave on high for 45 seconds, and shaken on The Belly Dancer for 15 minutes. For destaining, the fixing solution was replaced with 100ml of destaining solution, microwaved on high for 45 seconds, and shaken at room temperature overnight.

2.2.21 Analytical size exclusion chromatography

Analytical size exclusion chromatography was conducted to detect the protein dimerization and oligomerization in the sample using a Superdex 75 5/150 GL column. First, the column was equilibrated with 30 ml of gel filtration buffer at 0.2 ml/min flow rate. A volume of 150 μ l of protein sample was centrifuged at 4°C, 16,000 $\times$ g for 10 minutes to remove precipitated particles, and was injected onto the column. The chromatography was performed at 0.2 ml/min flow rate with 1.5 MPa as the maximum pressure limit for 25 minutes (end volume=5 ml), monitoring the protein peaks with the absorbance at 280 nm.

2.2.22 Anion exchange chromatography

Before connecting the column to the FPLC system, all the loops and the system were washed with 70% ethanol and ddH₂O at a high flow rate (10 ml/min, 200 psi as maximum pressure limit). After washing the system, Resource Q column was connected and equilibrated with both Buffer A and Buffer B (2 ml/min). About 1.5 ml of concentrated protein sample was injected to the column. Proteins bound to the column were eluted by applying a salt gradient from 20% to 100 % buffer B containing 1 M NaCl. Absorbance at 280 nm was used to detect protein elution. Eluted protein fractions were kept at 4°C for further experiments.

2.2.23 Annexin V apoptosis assay

Annexin V apoptosis assay was performed to detect apoptosis in drug-treated cells. Cells in early apoptotic stage expose phosphatidylserine (PS) on the surface of cell membrane. Annexin V binds specifically to PS, which can be visualized by FITC conjugated to annexin V. For the assay, cells are stained with both FITC-conjugated annexin V and Propidium Iodide (PI). PI staining distinguishes the necrotic cells from the early apoptotic cells. Unlike the cells in early apoptotic stage that have the intact membrane, necrotic cell loses its membrane integrity, which allows PI to enter the cell and bind to DNA. Therefore, it is possible to differentiate healthy cells, apoptotic cells, and necrotic cells by their fluorescence signals (**Figure 2.3**).

Figure. 2.3 Principle of annexin V apoptosis assay

Both PI and FITC cannot stain the viable cells. When the cells are undergoing apoptosis, PS molecules located in the inner leaflet of cell membrane translocate to the outer leaflet of the plasma membrane. Annexin V binds to PS exposed on the membrane surface, resulting in FITC positive staining of the cells. On the other hand, necrotic cells have both FITC and PI positive staining as they lose the integrity of plasma membrane during necrosis.

Mouse embryonic fibroblast (MEF) cells were used for the assay. First, 1×10^6 cells were seeded onto a set of four Corning plastic culture dishes (Sigma-Aldrich), in which three were for controls (Unstained, Annexin V only, Propidium Iodide only). On the next day, cells were treated with a drug or DMSO as a control. A few hours after the drug treatment, cells floating in the culture media were collected by centrifugation at $670 \times g$ for 5 minutes. Cells that were attached on the plates were trypsinized and collected by centrifugation. Both cell pellets were put together in a test tube, and washed twice with phosphate buffer saline (PBS). After the second wash, the cell pellets were

re-suspended in 400 μ l PBS, and staining reagents were added to the cells as in **Table 6**.

Table 6 Sample composition for annexin V apoptosis assay staining.

	Cells	IB*	Annexin V	PI
Experimental cells	400 μ l	100 μ l	2 μ l	2 μ l
Control 1 (unstained)	400 μ l	100 μ l	-	-
Control 2 (Annexin V only)	400 μ l	100 μ l	2 μ l	-
Control 3 (PI only)	400 μ l	100 μ l	-	2 μ l

*IB=Incubation buffer

The cells were incubated in a dark room for 15 minutes for staining, and analyzed with a 3-Laser Fortessa X-20 cell analyzer from BD Biosciences (San Jose, CA, USA). All the experiments were performed in triplicate for each condition and repeated at least twice.

2.2.26 Circular dichroism (CD)

Circular dichroism (CD) experiments were performed to analyze the secondary structure of the peptides, using an AVIV Circular Dichroism Spectrometer 420 from AVIV Biomedical (Lakewood, NJ, USA). Peptide samples were prepared in 0.7 mM in 10 mM sodium phosphate (pH 7.4) buffer. A volume of 80 μ l of the sample was added into a quartz cuvette with a 0.2 mm light path length (Hellma Analytics, Müllheim, Germany). The CD spectra were obtained at room temperature, using the spectral range from 190 nm to 260 nm with 0.5 nm wavelength step. The spectra of the sodium phosphate buffer were measured in duplicate as the background, and the peptide samples were measured in triplicate. Molar residue ellipticity (MRE) values of the peptides were calculated using the software provided by AVIV Biomedical.

2.2.27 Preparation of peptide samples for NMR experiments

Peptide NMR experiments were conducted to study the structural characteristic of VDAC N-terminal peptides. NMR samples were prepared by diluting the peptide stock solution (20 mM in the NMR buffer) to a desired concentration. D₂O was added to a final concentration of 5% to the sample prior to experiment.

2.2.28 Preparation of the ^{15}N -labelled protein sample for NMR experiment

^{15}N -labelled protein samples were prepared for ^1H - ^{15}N 2D HSQC experiment. Bacterial cells were cultured in M9 media instead of LB, and protein over-expression was induced overnight at 16°C , while shaking the culture at 160RPM. Protein purification was performed as usual, except for the tris-gel filtration buffer being replaced with the phosphate-gel filtration buffer. Protein samples collected from size exclusion chromatography were diluted to 0.2 mM and kept in aliquots of 171 μl in Eppendorf tubes. A volume of 9 μl of D_2O was added to the aliquot freshly before the NMR experiments to a final concentration of 5%.

2.2.29 NMR experiment for structural investigation of peptide and protein

All NMR experiments were performed on a Bruker Advance AV600 or AV700 equipped with a cryoprobe (Bruker, Fällanden, Switzerland) with the help of Dr. Ye Hong and Dr. Shin Joon. NMR data were collected and processed using NMRpipe or Bruker Topspin version 2.1, and 2D NMR spectra were analyzed and assigned using Sparky software (Delaglio et al., 1995).

2.2.29.1 ^1H 1D NMR experiment

A ^1H 1D NMR experiment was performed with the help of Dr. Ye Hong. For 1D NMR experiment for peptides, peptide samples were prepared in NMR buffer with 5% D_2O at 0.2 mM in a volume of 550 μl . The 1D NMR spectra were recorded at 298K using the pulse program 'zgesgp', with 128 scans.

2.2.29.2 ^1H - ^{15}N 2D HSQC NMR experiment

^1H - ^{15}N 2D HSQC NMR data were acquired at 298K using the pulse program 'hsqcetf3gpsi' with the help of Dr. Ye Hong. Protein samples were uniformly labelled with ^{15}N and concentrated to 0.2 mM in NMR buffer with 5% D_2O . Chemical shift perturbations were recorded upon addition of ligands, and the chemical shift

perturbation plots (CSPs) were generated by calculating the weighted chemical shift perturbation (ΔCS) using the equation below:

$$\Delta CS = \sqrt{0.154 \times (\Delta N)^2 + (\Delta H)^2}$$

2.2.29.3 NMR experiments for peptide structure determination

NMR experiments for structure determination of SG20 peptide bound to Bcl-xL were performed on a Bruker Advance AV700 equipped with a cryoprobe at 298K with the help of Dr. Shin Joon. NMR sample was prepared by dissolving 2.1 mg of lyophilized peptide in 350 μ l NMR buffer first. Then a volume of 100 μ l of 1 mM ^{15}N -labelled Bcl-xL was added to the peptide solution. Finally, 50 μ l D_2O was added to the sample to a final concentration of 10%. The final concentration of SG20 peptide in NMR sample was 2 mM, and the ratio of protein to peptide was 1:10. 2D- F_1 , F_2 - ^{15}N -filtered Nuclear Overhauser Effect Spectroscopy (NOESY) experiment for SG20 peptide in a bound state with Bcl-xL was performed with a mixing time of 200 ms and the NOE spectra of the peptide were collected. Also, 2D- F_1 , F_2 - ^{15}N -filtered Total Correlation Spectroscopy (TOCSY) experiments were performed with a mixing time of 70 ms for the same sample. All 2D NMR data were recorded in the phase sensitive model using the time-proportional phase increment (TPPI) method, with 2048 data points in the T_2 domain and 256 points in the T_1 domain (Wüthrich et al., 1983). Homonuclear NOESY (mixing time 400ms) and TOCSY spectra (mixing time 70ms) were collected using the free SG20 peptide solution as a reference for assignment. Resonance of solvent was suppressed by water-gated pulse sequences or excitation sculpting, combined with the pulsed field gradients (Hwang and Shaka, 1995; Liu et al., 1998). Prior to Fourier transformation in the T_1 dimension, the first row was half-weighted to suppress the T_1 ridge (Otting et al., 1986). The proton chemical shifts were referenced to the internal sodium 4,4-dimethyl-4 silapentane 1-sulfonate (DSS).

2.2.29.4 Structure calculation of SG20 peptide bound to Bcl-xL

TOCSY spectra of SG20 in a Bcl-xL bound state was assigned manually to identify individual spin systems, and assignment of 2D NOESY spectra to connect amino acids in sequence was made. A NOE table was generated for structural calculation, and the

NOE distance restraints were classified as strong (1.8–3.0 Å), medium (1.8–4.0 Å), weak (1.8–5.0 Å), or very weak (1.8– 6.0 Å), based on the intensities of NOE derived from NOESY spectra recorded in 10% D₂O. The distance geometry simulated annealing (SA) protocol with the CNS 1.2 was used to calculate the solution structure of SG20 bound to Bcl-xL (Brünger et al., 1998). Simulated annealing refinement was performed with the CNS to generate 15 refined structures, and the Ramachandran analysis was performed using PROCHECK-NMR program to check the quality of calculated structure (Laskowski et al., 1993). The average root-mean-square-deviation (RMSD) value of the structure segment from Tyr18 to Asp30 for final 15 REM structures was calculated using the Molmol software, and the average structure calculated from final 15 REM structures was visualized using Pymol (DeLano, 2002; Koradi et al., 1996).

2.2.29.5 Docking modeling by HADDOCK

The model structure of the Bcl-xL/SG20 complex was generated using HADDOCK webserver (De Vries et al., 2010; Dominguez et al., 2003). For restraint definition, the active site residues for structural modelling were selected using the result of NMR titration experiment result as a reference. Residues showing Δ CS above 0.12 ppm at the ‘protein: peptide’ ratio of 1:4 were selected as the active site residues. Passive residues for both Bcl-xL and SG20 were automatically picked by HADDOCK webserver. Total 200 complex fit pdb files were generated using published crystal structure of Bcl-xL (PDB ID: 1MAZ) as the first molecule and the structure of SG20 peptide calculated in the bound state as the second molecule. Finally, the best model structure from several clusters was selected as the final model of Bcl-xL/SG20 complex, based on the HADDOCK score and total energy.

2.2.30 Crystallization

Apart from the structural investigation of the interaction between VDAC2 N-terminal peptide and Bcl-xL using NMR spectroscopy, we have also tried to study the interaction of VDAC N-terminal segment with Bcl-xL and Bak using X-ray crystallography. To that end, we have attempted to obtain protein crystals of Bcl-xL or Bak in complex with VDAC N-terminal peptides by screening conditions for protein crystallization. Firstly,

recombinant Bak or Bcl-xL was over-expressed and purified from *E.coli*. Purified protein was concentrated to 10 mg/ml, and the N-terminal peptides of three VDAC isoforms were added at a molar ratio of 1:4 (protein:peptide), and the samples were incubated overnight in 4°C. On the following day, the protein/peptide mixtures were subjected to crystal screening. Wizard Classic crystallization screen series 1&2 from Rigaku Reagents (Bainbridge Island, WA, USA) and Hampton Research IndexTM HR2-144 crystallization screens from Hampton Research (Aliso Viejo, CA, USA) were used for protein crystallization screening. An equal volume of protein sample and the reservoir solution were mixed in a 4 µl drop for crystallization, using the hanging drop vapor diffusion method (McPherson, 2006). Plates containing reservoir solutions and protein sample drops were incubated in 18°C. Formation of protein crystal was monitored using a Nikon SMZ zoom stereomicroscope (Minato, Tokyo, Japan).

Results and Discussions

3.1 Protein expression and purification

3.1.1 Bcl-xL

Protein expression was confirmed by the appearance of a thick protein band at ~30 kDa in the SDS-PAGE after IPTG addition to the BL21(DE) cells harboring a pET-SUMO vector containing the DNA of Bcl-xL with a truncation of the transmembrane domain and the flexible loop (Bcl-xL Δ Loop Δ TM) (**lane 3, Figure 3.1.A**). The fusion protein was successfully purified by the Ni²⁺-NTA affinity (**lane 6, Figure 3.1.A**). The truncated Bcl-xL was successfully purified after cleavage of the fusion protein with SUMO protease (**lanes 7-8, Figure 3.1.A**). After size exclusion chromatography of the resulting protein, the protein appeared as a single band (**lane 10, Figure 3.1.A and Figure 3.1.B**).

Figure 3.1 Purification of Bcl-xL by Ni²⁺-NTA affinity chromatography and gel filtration.

A) Bcl-xL was expressed in *E.coli* BL21(DE3) and purified by Ni²⁺-NTA column and gel filtration. The indicated fractions were analyzed by SDS-PAGE. The red arrow indicates SUMO-tagged Bcl-xL and the blue arrow indicates Bcl-xL after SUMO tag cleavage. B) Gel filtration chromatography of Bcl-xL truncation mutant (Bcl-xL Δ Loop Δ TM). HiLoad 16/600 Superdex 75 pg was used for fast performance liquid chromatography (FPLC).

3.1.2 Bcl-2

Protein expression of Bcl-2 fused to 6×His-SUMO tag was confirmed by the appearance of a protein band at ~30 kDa in the SDS-PAGE after IPTG addition to the BL21(DE) cells harboring a pET-SUMO vector containing the DNA of Bcl-2 hybrid with a truncation of the transmembrane domain (Bcl-2ΔTM) (**lane 3, Figure 3.2.A**) (Petros et al., 2001). The fusion protein was successfully purified by the Ni²⁺-NTA affinity (**lane 6, Figure 3.2.A**). Bcl-2ΔTM was successfully purified after cleavage of the fusion protein with SUMO protease (**lanes 7-8, Figure 3.2.A**). After size exclusion chromatography of the resulting protein, the protein appeared as a single band (**lane 11, Figure 3.2.A and Figure 3.2.B**).

Figure 3.2 Purification of Bcl-2 by Ni²⁺-NTA affinity chromatography and gel filtration.

A) Bcl-2 was expressed in *E.coli* BL21(DE3) and purified by Ni²⁺-NTA chromatography and gel filtration. The indicated fractions were analyzed by SDS-PAGE. The red arrow indicates SUMO-tagged Bcl-2 and the blue arrow indicates Bcl-2 after SUMO tag cleavage. B) Gel filtration chromatography of Bcl-2 hybrid ΔTM. HiLoad 16/600 Superdex 200 pg was used for FPLC.

3.1.3 Mcl-1

Protein expression of Mcl-1 fused to 6×His tag was confirmed by the appearance of a protein band at ~20 kDa in the SDS-PAGE after IPTG addition to the BL21(DE) cells

harboring a pET28a vector containing the DNA of Mcl-1 BLR with 9 chimeric mutations and a truncation of transmembrane domain (Mcl-1 Δ N170 Δ C21) (**lane 3, Figure 3.3.A**) (Czabotar et al., 2007b). The fusion protein was successfully purified by the Ni²⁺-NTA affinity (**lane 6, Figure 3.3.A**). After size exclusion chromatography of the resulting protein, the protein appeared as a single band (**lane 8, Figure 3.3.A and Figure 3.3.B**).

Figure 3.3 Purification of 6 $\times$ His-tagged Mcl-1 BLR with 9 chimeric mutations by Ni²⁺-NTA affinity chromatography and gel filtration.

A) Mcl-1 fused to 6 $\times$ His tag was expressed in *E.coli* BL21(DE3) and purified by Ni²⁺-NTA chromatography and gel filtration. The indicated fractions were analyzed by SDS-PAGE. The blue arrow indicates 6 $\times$ His-tagged Mcl-1. B) Gel filtration chromatography of Mcl-1 BLR with 9 chimeric mutations. HiLoad 16/600 Superdex 200 pg was used for FPLC. The red arrow indicates the eluted fraction of 6 $\times$ His tagged Mcl-1.

3.1.4 Bcl-w

Protein expression of Bcl-w fused to 6 $\times$ His tag was confirmed by the appearance of a protein band at ~20 kDa in the SDS-PAGE after IPTG addition to the BL21(DE) cells harboring a pET21a vector containing the DNA of Bcl-w with a truncation of the transmembrane domain (Bcl-w Δ 158-193) (**lane 3, Figure 3.4.A**). The fusion protein was successfully purified by the Ni²⁺-NTA affinity (**lane 6, Figure 3.4.A**). After size

exclusion chromatography of the resulting protein, the protein appeared as a single band (lane 7, Figure 3.4.A and Figure 3.4.B).

Figure. 3.4 Purification of 6×His-tagged Bcl-wΔ158-193 by Ni²⁺-NTA affinity chromatography and gel filtration.

A) Bcl-w fused to 6×His tag was expressed in *E.coli* BL21(DE3) and purified by Ni²⁺-NTA chromatography and gel filtration. The indicated fractions were analyzed by SDS-PAGE. The blue arrow indicates 6×His-tagged Bcl-w. B) Gel filtration chromatography of Bcl-wΔ158-193. HiLoad 16/600 Superdex 200 pg was used for FPLC.

3.1.5 BakΔN20ΔC21

Protein expression of of Bak with a truncation of the transmembrane domain (BakΔC21) in *E.coli* BLR(DE3) pLysS resulted in a low protein expression efficiency after the addition of IPTG to the cells (lane 3 and 4, Figure. 3.5.A). Moreover, protein aggregation of BakΔC21 was observed after size exclusion chromatography (Figure. 3.5.B and C).

Figure. 3.5 Protein expression and purification of Bak Δ C20.

A) Bak Δ C20 fused to SUMO tag was expressed in *E.coli* BLR(DE3) pLysS. B) Gel filtration chromatography of Bak Δ C20. Superdex 200 10/300 GL chromatography column was used for FPLC. C) The indicated fractions were analyzed by SDS-PAGE.

To improve the poor protein stability and solubility, all protein purification procedures were conducted at 4°C. As Bak structural studies showed previously (Moldoveanu et al., 2006; Moldoveanu et al., 2013; Wang et al., 2009), to enhance the formation of Bak crystal, the cDNA of Bak with a truncation of N-terminal residues has been engineered and the resulting Bak Δ N20 Δ C21 cloned in pET-SUMO vector has been used for protein expression and purification (**Figure. 3.6**). Bak Δ N20 Δ C21 fused to SUMO tag was expressed in *E.coli* BLR(DE3) and BL21(DE3), and the resulting protein was analyzed by western blot (**Figure. 3.7**).

```

Bak_N20C21      -----SASEEQVAQDTEEVFRSYVFYRHQQEQEAEAGVAAPADPEM 40
Bak_FL          MASGQGP GPPRQECGE PALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEAGVAAPADPEM 60
Bak_2IMT        -----EPALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEAGVAAPADPEM 45
Bak_2M5B        -----ALPSASEEQVAQDTEEVFRSYVFYRHQQEQEAEAGVAAPADPEM 43
Bak_2YV6        -----SEEQVAQDTEEVFRSYVFYRHQQEQEAEAGVAAPADPEM 38
                *****

Bak_N20C21      VTLPLQPSSTMGQVGRQLAIIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFE 100
Bak_FL          VTLPLQPSSTMGQVGRQLAIIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFE 120
Bak_2IMT        VTLPLQPSSTMGQVGRQLAIIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFE 105
Bak_2M5B        VTLPLQPSSTMGQVGRQLAIIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFE 103
Bak_2YV6        VTLPLQPSSTMGQVGRQLAIIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFE 98
                *****

Bak_N20C21      SGINWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVD FMLHHCIARWIAQRGGWVAA 160
Bak_FL          SGINWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVD FMLHHCIARWIAQRGGWVAA 180
Bak_2IMT        SGINWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVD FMLHHCIARWIAQRGGWVAA 165
Bak_2M5B        SGINWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVD FMLHHCIARWIAQRGGWVAA 163
Bak_2YV6        SGINWGRVVALLGFGYRLALHVVYQHGLTGFLGQVTRFVVD FMLHHCIARWIAQRGGWVAA 158
                *****

Bak_N20C21      LNLGNGPILN----- 170
Bak_FL          LNLGNGPILNVLVVLGVVLLGQFVVRFFKS 211
Bak_2IMT        LNLGNG----- 171
Bak_2M5B        LNLGNG----- 169
Bak_2YV6        LNLGN----- 163
                *****

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Figure. 3.6 Amino acid sequence alignment of the Bak construct (Bak Δ N20 Δ C21) with other published Bak structures.

Full length Bak sequence is labelled as ‘FL’, and the sequences of Bak protein used in previous structural studies of Bak (Moldoveanu et al., 2006; Moldoveanu et al., 2013; Wang et al., 2009) were labelled with its PDB ID, respectively (2IMT, 2M5B, and 2YV6). The sequence of Bak Δ N20 Δ C21 was labelled as ‘N20C21’. Protein sequence alignment was conducted using Clustal Omega (Chenna et al., 2003) from EMBL website. (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)

Results of the western blot experiments indicated that protein was over-expressed with a better yield in BL21(DE3) than in BLR(DE3) pLysS, and overnight (20 hours) incubation at 25°C was the best condition in protein expression compared to other conditions. Therefore, to obtain sufficient amount of protein for setting up protein crystallization screens, large-scale Bak Δ N20 Δ C21 protein over-expression was performed using the same condition. The fusion protein was successfully purified by the Ni²⁺-NTA affinity (lane 6 and 7, Figure 3.8.A). After size exclusion chromatography of the resulting protein, the protein appeared as a single band (lane 10, Figure. 3.8.A and Figure 3.8.B). Protein purification yield after SEC was 8 mg/ml in 1 ml.

Figure. 3.7 Western blot analysis of Bak Δ N20 Δ C21 expression under various conditions.

pET-SUMO Bak Δ N20 Δ C21 proteins were detected by anti-6 $\times$ His tag antibodies. A) Protein over-expression in BL21(DE3) bacterial strain. Bacterial culture incubated at 37°C after IPTG addition showed much lower protein expression compared to other temperature, probably due to cell overgrowth and/or cell death. B) Protein over-expression in BLR(DE3) pLysS bacterial strain. In general, BLR(DE3) pLysS had lower protein expression than BL21(DE3) bacterial strain in all induction conditions. C) The conditions for protein over-expression of the Bak Δ N20 Δ C21 protein corresponding to the indicated lanes are shown.

Figure. 3.8 Purification of Bak Δ N20 Δ C21 by Ni²⁺-NTA affinity chromatography and gel filtration.

A) Bak Δ N20 Δ C21 was expressed in *E.coli* BL21(DE3) for 20 hours at 37°C, and resulting protein was purified by Ni²⁺-NTA chromatography and gel filtration chromatography. The indicated fractions were analyzed by SDS-PAGE. The blue arrow indicates the Bak Δ N20 Δ band. B) Gel filtration chromatography of Bak Δ N20 Δ C21. Superdex 200 10/300 GL was used for FPLC.

3.2 Structural transition and dimerization of Bcl-2 family proteins

3.2.1 Dimerization of Bcl-xL in OM

Bcl-xL proteins subjected to various conditions as described in section 2.2.17 were cross-linked with BMH as described in section 2.2.18. The SDS- or BN- PAGE analysis of these showed that OM detergent was the best environment to induce protein dimerization, showing the thickest protein dimer band at ~40 kDa (**Figure 3.9 and Figure 3.10**). Protein dimerization was also observed in heated sample, but majority of the proteins remained as monomers in the sample. On the other hand, pH adjustment could not induce any changes in the sample (**Figure 3.9 and Figure 3.10**). Clearly, BMH did not cause dimerization in the absence of OM (**lane 3, Figure 3.9.B**), further confirming our findings.

Figure. 3.9 Analysis of Bcl-xL dimerization by crosslinking and SDS-PAGE.

A) SDS-PAGE analysis of Bcl-xL after cross-linking. 0.5 mM Bcl-xL proteins incubated in various conditions as described in section 2.2.17 were cross-linked, and 2.5 μ g of each sample was loaded onto the gel for analysis. Bcl-xL monomer and dimer protein bands are indicated by red arrows. B) SDS-PAGE analysis of Bcl-xL dimerization in 2% OM detergent. 1 μ g of each protein sample was loaded onto the gel for analysis. ‘OM only’ and ‘BMH only’ samples were added as negative control.

Figure. 3.10 Analysis of Bcl-xL dimerization by BN PAGE.

BN-PAGE was performed to analyze Bcl-xL samples from different conditions. 0.5 mM Bcl-xL proteins were incubated in various conditions as described in section 2.2.17 were cross-linked, and 2.5 μ g of each sample was loaded onto the gel for analysis. Only samples from heating and incubating with the detergent showed dimeric protein.

Gel filtration analysis of the treated Bcl-xL samples was also consistent with that of crosslinking experiment (**Figure. 3.11**). Bcl-xL conversion ratio from monomer to dimer was the highest in OM detergent, and other conditions could not induce dimerization of Bcl-xL (**Figure. 3.11**).

Overall, it was shown that Bcl-xL undergoes structural transition and dimerization in the presence of 2% OM detergent using three different analytical methods. Other conditions including acidic and basic conditions, or heating resulted in no significant changes in the protein homogeneity.

Figure. 3.11 Analysis of Bcl-xL dimerization by analytical SEC.

Bcl-xL samples after incubation in various conditions described in section 2.2.17 were analyzed by analytical size exclusion chromatography (SEC). Each sample of 10 mg/ml, 150 μ l was centrifuged to remove precipitated proteins before injecting to Superdex 75 5/150 GL column. ‘M’ indicates protein monomer peak at $\sim$ 1.8 ml, and ‘D’ indicates protein dimer peak at $\sim$ 1.5 ml.

3.2.2 Dimerization of other Bcl-2 family proteins

From experiments on Bcl-xL, it was revealed that OM detergent environment was the most potent condition to induce structural transition and dimerization of the protein. The same experiments were repeated for other members of anti-apoptotic Bcl-2 family proteins to examine if they behave the same as Bcl-xL in various conditions.

Protein samples were cross-linked by BMH using the same method used for Bcl-xL and analyzed by SDS-PAGE. (**Figure. 3.12**) Like Bcl-xL, other members of anti-apoptotic Bcl-2 family also possess a single cysteine residue (Bcl-2: Cys160, Bcl-w: Cys109, and Mcl-1: Cys286) in the α 5: α 6 interface. If these Bcl-2 family members also form 3DDS dimer, BMH will be able to cross-link these cysteine residues similar to Bcl-xL scenario. Mcl-1, Bcl-w, and Bcl-2 could form dimers without any changes in the environment as shown in **Figure 3.12.A**. Previously, it has been reported that Bcl-2 and Bcl-w can dimerize in the absence of any physico-chemical inducement (Lee et al., 2011;

Zhang et al., 2004), and this dimerization is more likely to be a property of the amino acid sequence of the proteins. Hence, it seems reasonable that these two proteins could be cross-linked without any treatment. On the other hand, there has been no dimerization reported for Mcl-1 so far, but in our cross-linking experiment, protein band with a size of ~40 KDa was observed in SDS-PAGE (**Figure 3.12.A**). This may be due to a formation of Mcl-1 dimer, or it may be due to Cys286 residues, exposed to the surface of the Mcl-1 molecules and cross-linked to one another. We may perform another set of cross-linking experiments, using different type of crosslinker such as glutaraldehyde, to confirm that the 40 KDa protein band was not an artifact of cross-linking resulted from a disulfide bond between Mcl-1 molecules

Protein stability in heat or pH change varied among the family members. Bcl-2 was the most vulnerable protein to heat and acidic pH, as nearly 100% of the protein sample was precipitated after buffer exchange as well as heating incubation. As a result, there was no protein band observed in the gel after SDS-PAGE. Mcl-1 and Bcl-w were also prone to precipitation when heated. Bcl-w protein aggregated in acidic pH, which resulted in non-specific crosslinking between aggregated proteins leading to multiple protein bands with higher molecular weight in the gel. In terms of protein dimerization, both heating and acidic pH could hardly induce dimerization for any of the protein except for Bcl-2 (**Figure. 3.12.B and D**). Bcl-xL dimer band was observed when heated but the amount of dimer protein was insignificant. Mcl-1, Bcl-w, and Bcl-2 could form dimers at pH 10 (**Figure 3.12C**) without any significant increase compared to 'control'. Hence, the basic pH does not appear to induce the proteins to dimerize. In detergent OM, the conversion ratio from monomer to dimer was the highest for Bcl-xL and Bcl-2 compared to any other conditions. On the contrary, Mcl-1 dimer dissociated into monomers in the presence of 2% OM, and Bcl-w remained unchanged in OM compared to the control sample. (**Figure 3.12E**)

Figure. 3.12 Analysis of Bcl-2 family protein dimerization by crosslinking and SDS-PAGE.

Bcl-2 family proteins (Bcl-xL, Bcl-2, Bcl-w, and Mcl-1) were purified and concentrated to 10 mg/ml. Concentrated protein samples were incubated under various conditions and crosslinked by BMH. Protein dimerization and oligomerization were analyzed by SDS-PAGE.

We were unable to resolve Mcl-1 protein using BN PAGE because the protein formed smeared band in the gel. Therefore, only analytical SEC was performed to analyze all four anti-apoptotic proteins.

Figure. 3.13 Analysis of Bcl-2 family protein dimerization by analytical SEC.

Protein samples were prepared the same way as in section 3.13 and analyzed by analytical size exclusion chromatography (SEC). Each sample of 10 mg/ml, 150 μ l was centrifuged to remove precipitated proteins before injecting to Superdex 75 5/150 GL column. 'M' indicates protein monomer peak and 'D' indicates protein dimer peak.

Analytical size exclusion chromatography of the Mcl-1 showed that it readily formed protein dimer in the absence of any environmental changes (**Figure. 3.13A**). When the protein was placed in low pH, heat, or detergent environment, protein dimer was dissociated to monomers. Mcl-1 dimer peak (D) was only found in control and basic pH condition, which was consistent with crosslinking result in **Figure. 3.12**. In contrast, Bcl-w had inconsistent result from analytical SEC compared with chemical crosslinking

experiments. In chemical cross-linking, Bcl-w from control, pH 10, or OM detergent contained both monomer and dimer proteins, whereas no dimer protein peak was observed in any conditions from analytical SEC (**Figure 3.13.B**). During sample preparation, Bcl-w was extremely vulnerable in acidic pH or heating condition and severe protein precipitation was observed, which resulted in very low absorbance at ~1.8 ml. Analytical SEC experiment was also conducted for Bcl-xL, and the result was consistent with the crosslinking experiment (**Figure 3.13.C**). About 30% of the protein formed dimer in 2% OM detergent (**Figure 3.13.C**). Heating at 55°C also induced protein dimerization to a certain extent, but OM was still the best condition for inducing protein dimerization. Bcl-2 precipitated heavily in pH 4, resulting in the absence of a peak in absorbance curve from sample in pH 4 (**Figure 3.13.D**). Protein dimerization only occurred in 2% OM environment, which did not coincide with crosslinking experiment result (**Figure 3.12**). In SDS-PAGE after crosslinking, Bcl-2 dimer was observed in control, pH 10, and OM condition (**Figure 3.12**). However, it was still consistent that dimerization of Bcl-2 was more evident in OM compared to any other conditions from both experiments. Overall summary of the two experiments on anti-apoptotic Bcl-2 family proteins were made in the **Table 7**.

Table 7 Summary of results from crosslinking and analytical SEC experiments.

Protein	Method	Control	pH 4	pH 10	Heat	OM
Bcl-xL	Crosslinking	No dimer	No dimer	Negligible dimer	Negligible dimer	~30% dimer
	Analytical SEC	No dimer	No dimer	No dimer	~15% dimer	~30% dimer
Bcl-2	Crosslinking	~15% dimer	Precipitated	No dimer	Precipitated	~40% dimer
	Analytical SEC	No dimer	Precipitated	No dimer	Precipitated	~15% dimer
Bcl-w	Crosslinking	~20% dimer	Aggregated	~20% dimer	Precipitated	~20% dimer
	Analytical SEC	No dimer	Precipitated	No dimer	Precipitated	No dimer
Mcl-1	Crosslinking	~30% dimer	No dimer	~30% dimer	Precipitated	Negligible dimer
	Analytical SEC	~40% dimer	No dimer	~30% dimer	Precipitated	No dimer

3.2.3 Discussion

In this chapter, dimerization of anti-apoptotic Bcl-2 family proteins in various conditions was examined using chemical crosslinking and analytical SEC experiments. Results revealed that dimerization of Bcl-2 family proteins occurred most frequently in OM detergent environment, with one exception of Mcl-1. While conducting both crosslinking and analytical SEC experiments, inconsistent results were obtained from Bcl-2 and Bcl-w proteins, as shown in **Table 7**. One possible explanation to this discrepancy is that the dimeric proteins might have higher affinity to the size exclusion gel than the monomeric proteins, causing less elution of dimers from gel filtration. Another possible explanation is that protein dimerization of anti-apoptotic Bcl-2 family proteins might be transient and the dimeric proteins could dissociate back to monomer. By crosslinking the dimers and forming covalent bond between monomer subunits, proteins could remain as dimers without dissociation. This might explain why protein dimers were observed only in chemical crosslinking experiments.

Dimerization of Bcl-2 family proteins in nonionic detergent has already been reported several times, but these studies were mainly focused on pro-apoptotic Bcl-2 family proteins such as Bak or Bax. For instance, it was reported that Bax homodimerizes in the presence of nonionic detergent Nonidet P-40, Triton X-100 (Hsu and Youle, 1997),

or OM (Czabotar et al., 2013). There have also been other reports about conditions that could induce Bcl-2 family proteins to homodimerize. Dimerization of Bcl-xL could be induced by performing anion exchange chromatography in pH 10 (O'Neill et al., 2006), or heating the protein at 55°C (Denisov et al., 2007). However, these conditions like acidic pH or temperature at 55°C are hardly considered to be physiological environment. OM detergent, on the other hand, might be able to make a more suitable condition for studying protein dimerization in physiological conditions, as detergents can form membrane-mimicking environment. In addition, from our experiment results, protein dimerization was most frequently observed in OM detergent compared to other conditions. For these reasons, we have decided to use OM detergent micelles as a way of inducing the structural transition and dimerization of Bcl-2 family proteins, and to conduct studies for structural and functional characterization of such protein dimers. However, it would be difficult to work on several anti-apoptotic Bcl-2 family proteins at the same time. Hence, we chose to conduct our research focused on Bcl-xL, as it showed the highest yield of purification and conversion ratio from monomer to dimer.

3.3 Molecular basis of $\alpha 5$ - $\alpha 6$ hairpin for Bcl-xL dimerization

3.3.1 Purification and characterization of Bcl-xL domain swapping dimer induced by OM detergent

In order to study structural characteristic of Bcl-xL dimer, it was necessary to get homogeneous protein dimer sample. To obtain a pure dimer sample, protein dimerization was induced in the presence of 2% OM detergent, and the dimer was purified by anion exchange column chromatography. Samples eluted from the anion exchange chromatography were analyzed by BN PAGE. (**Figure 3.14.A**) Fractions 1 to 5 were pooled together and concentrated, and further purified by SEC. Purity of the protein dimer sample was examined by BN PAGE and analytical SEC (**Figure 3.14.B and C**). From the result of BN PAGE and analytical SEC, it was confirmed that the final protein sample contains pure dimer.

Figure. 3.14 Purification of Bcl-xL dimer after incubation in OM detergent.

A) Bcl-xL after incubation with OM detergent was injected into Resource Q column for anion exchange chromatography, and all the fractions obtained from the column were analyzed by BN PAGE. B) After SEC, the purified dimer sample was examined for its purity by BN PAGE (lane 2). In lane 3, monomer Bcl-xL was run as a control to compare the protein size. C) Purified dimer Bcl-xL was also analyzed by analytical SEC. ‘M’ and ‘D’ indicates the monomer and dimer peak of Bcl-xL, respectively.

We conducted structural studies on the purified Bcl-xL dimer by X-ray crystallography. The crystal structure of the Bcl-xL dimer was solved by Dr. Sreekanth, one of our colleagues (Rajan et al., 2015b) (**Figure 3.15**). In the crystal structure, the Bcl-xL molecules form a three-dimensional domain swapped (3DDS) dimer, which is identical to the structure solved by David M. Hockenbery and his colleagues (O'Neill et al., 2006) (PDB ID: 2B48). During conformational change of Bcl-xL to form domain swapped dimer, one subunit of Bcl-xL exchanges its helices $\alpha 6$ - $\alpha 8$ with $\alpha 6'$ - $\alpha 8'$ helices of another

Bcl-xL molecule. Three-dimensional folding of the domain swapped dimer in solution was supported by Small angle X-ray scattering (SAXS) by our collaborator, Professor Gerhard Grüber and his colleagues, and this low-resolution solution structure was consistent with the X-ray crystal structure of Bcl-xL dimer with an RMSD of 0.93 Å (Rajan et al., 2015b).

Figure. 3.15 Three-dimensional structure of Bcl-xL monomer and 3DDS dimer solved by X-ray crystallography.

A) Ribbon representation of Bcl-xL monomer crystal structure (PDB ID:1MAZ) is shown as a reference, with all alpha helices numbered. B) A cartoon representation of crystallographic structure of the Bcl-xL 3DDS dimer is presented. The axis dividing the two monomeric folds is represented by dashed line.

3.3.2 Characterization of Bcl-xL mutants by analytical size exclusion chromatography

With the Bcl-xL 3DDS dimer solved, our next aim was to understand the functional aspect of Bcl-xL dimerization in regulation of apoptosis. For this, we attempted to

generate a mutant Bcl-xL incapable of structural transition during domain swapping and to compare its anti-apoptotic capacity with the wild-type Bcl-xL. From the structure of Bcl-xL 3DDS dimer, we reasoned that the flexibility of the hairpin region $\alpha 5$ - $\alpha 6$ would be crucial in the structural rearrangement of Bcl-xL in domain swapping, as the Bcl-xL molecule needs to stretch this hairpin region for reciprocal exchange of the helices $\alpha 6$ - $\alpha 8$. While Bcl-xL undergoes structural transition to form 3DDS dimer, opening of $\alpha 5$ - $\alpha 6$ hairpin results in formation of a single extended alpha helix made up of $\alpha 5$ and $\alpha 6$ helices (**Figure 3.16.B**).

Figure. 3.16 Structure of Bcl-xL monomer and dimer subunit solved by X-ray crystallography, and a closer view at the loop connecting $\alpha 5$ - $\alpha 6$ helices.

A) Ribbon representation of the Bcl-xL monomer crystal structure (PDB ID:1MAZ) is shown. Residues located at the loop region connecting the two alpha helices, $\alpha 5$ - $\alpha 6$, are presented as sticks in a closer view. Alpha helices 5 and 6 are numbered. B) Cartoon representation of one Bcl-xL subunit from the 3DDS dimer is presented. An extended large helix made up of $\alpha 5$ and $\alpha 6$ is colored yellow and orange.

Using this feature of Bcl-xL 3DDS dimer, we designed a mutation to prevent the dimerization of Bcl-xL by substituting Glu158 or Met159 (**Figure 3.16.A**) at the loop connecting $\alpha 5$ - $\alpha 6$ helices with Proline, a well-known helix breaker. We hypothesized

that the conformational rigidity of proline will stop Bcl-xL from undergoing structural transition at the loop region, which in turn will prevent the $\alpha 5$ - $\alpha 6$ hairpin from stretching for domain-swapping. Site-directed mutagenesis for point mutation was performed by Dr. Toan from our group, and the Bcl-xL mutant proteins were over-expressed and purified using the same condition for Bcl-xL wild type protein. The capacity of these Bcl-xL mutant proteins (E158P and M159P) in undergoing structural transition and dimerization was analyzed by analytical SEC. First, Bcl-xL wild type and two mutants were concentrated to 10 mg/ml and incubated in 2% OM condition for 2 hours at room temperature to induce protein dimerization. After the incubation, 150 μ l of each sample was centrifuged to remove precipitated proteins and injected to the column for FPLC. (**Figure 3.17**)

Figure. 3.17 Analysis of mutant and wild type Bcl-xL dimerization by analytical SEC.

Gel filtration chromatography of recombinant Bcl-xL wild type and two mutants (E158P and M159P) after incubation in 2% OM for 2 hours at room temperature. ‘M’ indicates protein monomer peak and ‘D’ indicates protein dimer peak. Superdex 75 5/150 GL column was used for analysis.

The chromatography from the gel filtration analysis showed that these Bcl-xL mutants failed to dimerize in the presence of 2% OM, as only the monomer peak (M) at ~1.8 ml was observed for both E158P and M159P, whereas the wild type Bcl-xL displayed both

the monomer and dimer peaks (D) at ~1.5 ml. These results demonstrate that the dynamic conformational change at the residues (Glu158 or Met159) in the loop region of $\alpha 5$ - $\alpha 6$ hairpin is required for Bcl-xL to form domain swapped dimer.

3.3.3 Discussion

From the crystallographic structure of the OM-induced Bcl-xL dimer, it was clear that the Bcl-xL molecule undergoes a dynamic conformational change and forms the 3DDS dimer in a membrane-mimicking environment. The site-directed mutagenesis and analytical SEC experiments, revealed that the flexibility at the loop region in the $\alpha 5$ - $\alpha 6$ hairpin is essential for OM-induced domain-swapping by Bcl-xL. As the point mutations E158P or M159P could successfully inhibit the protein dimerization induced in membrane-mimicking environment, these mutants are not expected to form the 3DDS dimer in membrane environment when expressed in tissue culture cells. Considering that Bcl-xL is an anti-apoptotic member of Bcl-2 family located at the mitochondrial outer membrane, its inability to dimerize in membrane environment might affect its anti-apoptotic function in cells. To test this possibility, a series of experiments were conducted to compare the anti-apoptotic activity of the mammalian cells expressing a wild type Bcl-xL with those expressing the mutant proteins (E158P or M159P Bcl-xL).

3.4 Functional implications of the structural transition of Bcl-xL in tissue culture cells

3.4.1 Comparison of the anti-apoptotic capacity between wild type and mutant Bcl-xL

The anti-apoptotic capacity of the tissue culture cells expressing wild type or mutant Bcl-xL was examined by annexin V apoptosis assay, with Bcl-xL knockout mouse embryonic fibroblast (MEF) cells stably expressing the empty vector, wild type (WT), or mutant Bcl-xL (E158P or M159P), kind gifts by our collaborator, Professor Carl White. MEF cells were cultured and treated with 10 μ M of gossypol (Oliver et al., 2005) to induce mitochondrial-mediated apoptosis. Distribution of viable cells, apoptotic cells, and necrotic cells were analyzed by FACS (**Figure 3.18**).

A **B** **C**

Figure. 3.18 Effect of the point mutation (E158P, M159P) on anti-apoptotic activity of Bcl-xL.

For figure legend, see next page.

Percentage distribution of healthy, apoptotic, and necrotic MEF cells in the presence or absence of gossypol treatment were analyzed by annexin V apoptosis assay (KO=knockout, WT=wild type). The mean and standard error are shown. A) Percentage distribution of MEF cells before gossypol (10 μ M) treatment. To keep the condition for control the same as for the cells treated with the drug, DMSO was added to the control samples at a final concentration of 0.1%. B) Percentage distribution of MEF cells after gossypol (10 μ M) treatment. C) Percentage distribution of apoptotic cells before and after gossypol (10 μ M) treatment.

Overall cell viability among the Bcl-xL KO MEF cells was similar before and after addition of gossypol, regardless of the expression vectors (**Figure 3.18.A and B**). About 50% of all MEF cells were viable after gossypol treatment. However, a clear difference was observed when the percentage distribution of cells at apoptotic stage was compared. When the cells were treated with gossypol, Bcl-xL KO MEF cells carrying the empty vector (KO) or expressing two mutants (E158P, M159P) resulted in about 25%-30% of apoptotic cells, whereas cells expressing wild type Bcl-xL (WT) had a lower percentage (15%) of apoptotic cells (**Figure 3.18.C**). The fact that cells expressing mutant Bcl-xL have similar anti-apoptotic capacity with Bcl-xL KO cells indicates that mutant Bcl-xL lost certain degree of anti-apoptotic activity. This result suggests that impaired dimerization of Bcl-xL is correlated with the loss of its anti-apoptotic function in the cells.

3.4.2 Discussion

From the result of apoptosis assay, it was shown that Bcl-xL KO MEF cells stably expressing mutant Bcl-xL (E158P, M159P) have higher percentage of apoptotic cells than the cells expressing wild type Bcl-xL, after gossypol treatment. However, this result was not sufficient to substantiate the idea that the structural plasticity of Bcl-xL allowing to form 3DDS dimer might control cell fate, as there was no difference in overall cell viability among the cells expressing wild type or mutant Bcl-xL. In addition, even though the wild type and mutant Bcl-xL showed different anti-apoptotic capacity, it is still not clear how exactly the impaired structural plasticity of Bcl-xL affected anti-apoptotic mechanism in the cells.

Meanwhile, our collaborators Carl White and his coworkers compared the mitochondrial calcium uptake in Bcl-xL KO MEF cells expressing WT, E158P, M159P, and empty vector. Interestingly, when the external $[Ca^{2+}]$ was increased from 0 to 10 μ M, MEF cells expressing mutant Bcl-xL showed a decreased calcium uptake in mitochondria compared to the cells expressing wild type Bcl-xL (Rajan et al., 2015b). This indicates that the above point mutations in Bcl-xL affects the regulation of calcium uptake in mitochondrial outer membrane. This led us to put our focus on studying the relation of Bcl-xL to regulation of mitochondrial calcium homeostasis, as described in Part II.

3.5 Structural characterization of VDAC N-terminal peptides in solution

We first performed a series of experiments on peptides alone which are derived from N-terminal region of VDAC isoforms, in order to gain a better understanding of structural characteristic of VDAC N-terminal moiety. For this purpose, we designed four peptides derived from the N-terminal region of VDAC isoforms (see section 2.1.6). In an amino acid sequence alignment of the N-terminal region of VDAC isoforms, it was observed that 25 amino acids in VDAC1 (1-25), VDAC2 (12-36), and VDAC3 (1-25) were conserved among the VDAC isoforms (**Figure 3.19**). VDAC2 has an additional 11 residues at the N-terminus, which were excluded from our studies. In addition, a shorter version of VDAC2 N-terminal peptide was added to the series of experiments (see section 2.1.6), for reasons as described in **chapter 3.5.1**.

Figure. 3.19 Amino acid sequence alignment of the N-terminal region of three VDAC isoforms

The N-terminal region of Human VDAC isoforms were aligned using Clustal Omega (Chenna et al., 2003) from EMBL website. (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)

3.5.1 Secondary structure characterization of VDAC N-terminal peptides by Circular Dichroism

Circular dichroism (CD) experiment was performed to get the secondary structure information of VDAC N-terminal peptides. The CD spectra of the peptide samples were measured in triplicate, and the average molar residue ellipticity (MRE, θ) are shown in **Figure. 3.20.A**. Also, the alpha helical contents of the peptides are shown in **Figure 3.20.B**.

Figure. 3.20 CD spectra of VDAC 1,2,3 N-terminal peptides and their helical contents.

CD spectrum of VDAC 1, 2, 3, and shorter VDAC 2 (SF17) N-terminal peptides recorded at pH 7.4. A) The average MRE of each peptide was calculated from 3 independent experiments for 190-260 nm. B) Alpha helical content percentage of each peptide was obtained from CD deconvolution software provided by AVIV Biomedical.

Our CD result demonstrates that the secondary structure of the free peptides are less structured and more unfolded, showing strong negative dichroism at around 200 nm (Fasman, 2013). VDAC2 N-terminal peptide (12-36) possesses an alpha helical content of 14.7%, while VDAC1 and VDAC3 possess 12.9% and 12.6%, respectively (**Figure 3.20.B**). This result is corresponding to what Scorciapino and his colleagues reported by CD and NMR experiments (Guardiani et al., 2015). VDAC2 (12-36) peptide has almost the same α -helical content as the SF17 (14.3%) in which the five N-terminal residues (¹²MCIPP¹⁶) and the three C-terminal residues (³⁴GFG³⁶) of VDAC2 are removed. This

indicates that neither the N- nor the C- terminal residues affect the secondary structure of VDAC2 N-terminal region (**Figure 3.20.B**). This is consistent with Schredelseker et al (Schredelseker et al., 2014), in which only residues 17-33 form an alpha helix.

3.5.2 Discussion

The low helical contents of the peptides are contrary to the 3D structures of VDAC isoforms in which the N-terminal region of the protein is described as an amphipathic helix (Bayrhuber et al., 2008; Schredelseker et al., 2014). One possible explanation for this discrepancy is the lack of membrane-mimicking detergent molecules in our study. As VDAC is a membrane protein, published structures of full length VDAC proteins were all solved in membrane-mimicking environment using detergent micelles (Hiller et al., 2008) or bicelles (Ujwal et al., 2008), which might have helped in stabilization of alpha-helical conformation of N-terminal segment. According to De Pinto and his coworkers, negatively charged micelles could enhance the alpha helical conformation of VDAC1 N-terminal peptide (De Pinto et al., 2007). An alternative explanation is that the N-terminal region of VDAC might require interaction with the beta barrel moiety of VDAC embedded in membrane to stably form alpha helical conformation.

3.6 NMR study on the interaction of Bcl-xL with VDAC N-terminal peptides in solution

3.6.1 NMR titration of Bcl-xL with VDAC N-terminal peptides

We conducted a series of NMR titration experiments using ^{15}N -labelled Bcl-xL titrated with VDAC 1,2,3 N-terminal peptides to determine the binding affinity of different peptides to Bcl-xL. Two shorter peptides derived from VDAC2 N-terminal region (SF17 and SG20) were also included in the NMR experiments to test whether $^{12}\text{MCIPP}^{16}$ or $^{34}\text{GFG}^{36}$ residues affect the interaction of the peptide with Bcl-xL.

3.6.1.1 ^1H - ^{15}N 2D HSQC experiment of Bcl-xL titrated with VDAC 1,2,3 N-terminal peptides

^{15}N -labelled Bcl-xL was titrated with VDAC N-terminal peptides (NP) at two different molar ratios, 1:1 and 1:4, to monitor the Bcl-xL residues interacting with the peptides

by ^1H - ^{15}N 2D HSQC experiment. Chemical shift changes between free Bcl-xL and Bcl-xL titrated with VDAC NPs at 1:4 molar ratio were calculated to generate the chemical shift perturbation (CSP) plot. During titration of Bcl-xL with VDAC NPs, prominent chemical shift changes were observed among the residues located in the hydrophobic pocket of Bcl-xL. When the values of chemical shift change ($\Delta\delta$) were compared using CSP plot from all spectra, VDAC2 NP (**Figure 3.22.B**) showed considerably larger $\Delta\delta$ than VDAC1 (**Figure 3.21.B**) or VDAC3 (**Figure 3.23.B**) NPs. Hence, the threshold of CSP for Bcl-xL with VDAC2 NP was set higher (0.12 ppm) than others (0.07 ppm). Despite the higher threshold value, Bcl-xL titrated with VDAC2 NP showed more residues that have CSP above the threshold and located at the hydrophobic groove (**Figure 3.22**).

Figure. 3.21 NMR titration experiment on Bcl-xL interacting with VDAC1 (1-25).

A) ^1H - ^{15}N HSQC spectrum showing chemical shifts of Bcl-xL (0.2 mM) residues measured from titration with VDAC1 N-terminal peptide (0.2 mM, 0.8 mM) in the NMR buffer (20 mM phosphate, 50 mM NaCl, 0.01% NaN_3 , pH 6.5). B) CSP plot representing the chemical shift changes ($\Delta\delta$) between free Bcl-xL and Bcl-xL titrated with VDAC1 NP at 1:4 molar ratio. Red line indicates an arbitrary threshold at 0.07 ppm. C) Residues showing CSP above the threshold were mapped on the surface representation of Bcl-xL (PDB ID:1MAZ).

Figure. 3.22 NMR titration experiment on Bcl-xL interacting with VDAC2 (12-36).

A) ^1H - ^{15}N HSQC spectrum showing chemical shifts of Bcl-xL (0.2 mM) residues measured from titration with VDAC2 N-terminal peptide (0.2 mM, 0.8 mM) in the NMR buffer (20 mM phosphate, 50 mM NaCl, 0.01% NaN_3 , pH 6.5). B) CSP plot representing the chemical shift changes ($\Delta\delta$) between free Bcl-xL and Bcl-xL titrated with VDAC2 NP at 1:4 molar ratio. Red line indicates an arbitrary threshold at 0.12 ppm. C) Residues showing CSP above the threshold were mapped on the surface representation of Bcl-xL (PDB ID:1MAZ).

Figure. 3.23 NMR titration experiment on Bcl-xL interacting with VDAC3 (1-25).

A) ^1H - ^{15}N HSQC spectrum showing chemical shifts of Bcl-xL (0.2 mM) residues measured from titration with VDAC3 N-terminal peptide (0.2 mM, 0.8 mM) in the NMR buffer (20 mM phosphate, 50 mM NaCl, 0.01% NaN_3 , pH 6.5). B) CSP plot representing the chemical shift changes ($\Delta\delta$) between free Bcl-xL and Bcl-xL titrated with VDAC3 NP at 1:4 molar ratio. Red line indicates an arbitrary threshold at 0.07 ppm. C) Residues showing CSP above the threshold were mapped on the surface representation of Bcl-xL (PDB ID: 1MAZ).

In Bcl-xL, there were 15 amino residues which consistently have CSP value above the threshold during titration with various VDAC NPs. Out of 15 residues, 10 amino acid residues (F97, Y101, D107, L108, E129, L130, R139, A142, Y195, and G196) were located in the hydrophobic pocket. These residues are mapped on the surface representation of Bcl-xL (**Figure 3.24**). These results clearly show that VDAC N-terminal peptides interact with the Bcl-xL at the canonical hydrophobic pocket of the protein, suggesting that this hydrophobic groove is a binding site for VDAC NPs.

VDAC2 NP showed larger $\Delta\delta$ upon interacting with Bcl-xL than VDAC1 or VDAC3 NP. This led us to our further studies on VDAC2 N-terminal region interaction with Bcl-xL.

Figure. 3.24 Bcl-xL residues adjacent to hydrophobic pocket and interacting with N-terminal peptides of three VDAC isoforms.

The 10 amino acid residues of Bcl-xL, which consistently have CSP above the threshold in all titration experiments, were mapped on the surface representation of Bcl-xL (PDB ID:1MAZ). Five other residues (L8, G94, L112, I114, L150), not contiguous to the hydrophobic groove are not shown.

3.6.1.2 ^1H - ^{15}N 2D HSQC experiment on Bcl-xL titrated with SF17 and SG20

Interaction of Bcl-xL with two shorter VDAC2 NPs (SF17 and SG20) were monitored by ^1H - ^{15}N 2D HSQC experiment in order to check if $^{12}\text{MCIPP}^{16}$ or $^{34}\text{GFG}^{36}$ residues affect the interaction of the peptide with Bcl-xL.

Figure. 3.25 NMR titration experiment on Bcl-xL interacting with SF17.

A) ^1H - ^{15}N 2D HSQC spectrum showing chemical shifts of Bcl-xL (0.2 mM) residues measured from titration with SF17 peptide (0.2 mM, 0.8 mM) in the NMR buffer (20 mM phosphate, 50 mM NaCl, 0.01% NaN_3 , pH 6.5). B) CSP plot representing the chemical shift changes ($\Delta\delta$) between free Bcl-xL and Bcl-xL titrated with SF17 at 1:4 molar ratio. Red line indicates an arbitrary threshold at 0.12 ppm. C) Residues showing CSP above the threshold were mapped on the surface representation of Bcl-xL (PDB ID:1MAZ).

Figure. 3.26 NMR titration experiment on Bcl-xL interacting with SG20.

A) ^1H - ^{15}N HSQC spectrum showing chemical shifts of Bcl-xL (0.2 mM) residues measured from titration with SG20 peptide (0.2 mM, 0.8 mM) in the NMR buffer (20 mM phosphate, 50 mM NaCl, 0.01% NaN_3 , pH 6.5). B) CSP plot representing the chemical shift changes ($\Delta\delta$) between free Bcl-xL and Bcl-xL titrated with SG20 at 1:4 molar ratio. Red line indicates an arbitrary threshold at 0.12 ppm. C) Residues showing CSP above the threshold were mapped on the surface representation of Bcl-xL (PDB ID: 1MAZ).

Overall, CSPs of Bcl-xL interacting with the shorter VDAC2 NPs (SF17 and SG20) were similar or slightly larger than CSPs of Bcl-xL interacting with the original VDAC2 NP. However, SF17 had fewer amino acid residues involved in interaction with Bcl-xL, excluding F97, R103, L108, and R139. (**Figure 3.25.C**) On the contrary, SG20 maintained the interaction with Bcl-xL for all the same residues as VDAC2 NP did (**Figure 3.26.C**). This indicates that truncation of $^{12}\text{MCIPP}^{16}$ does not affect the interaction of VDAC2 NP with Bcl-xL, while that of $^{34}\text{GFG}^{36}$ did affect the interaction between the peptide and the protein. Due to this reason, SF17 was not selected for further studies.

When 1D proton NMR of VDAC2 NP with various length was performed, the resolution of the spectrum for the 25 a.a peptide spectrum was rather poor and several chemical shifts were overlapping, making the peak assignment difficult for structural studies. (**Figure 3.27**) Compared to the longer peptide, 1D ^1H spectra of 17 and 20 a.a length peptides (SF17, SG20) showed better resolution with well-separated peaks observed in the spectra. Thus, we decided to use SG20 (see section 2.1.6) for our further studies of VDAC N-terminal region with Bcl-xL on structural perspective.

Figure 3.27 1D ^1H NMR spectra of VDAC2 N-terminal peptides.

An overlay of the amide region of the spectra are shown. (Top: SF17 (17-33), colored black. Middle: SG20 (17-36), colored red. Bottom: VDAC2 NP (12-36), colored blue)

3.6.2 Structural modelling of VDAC2 N-terminal peptide (17-36) in complex with Bcl-xL

We conducted NMR experiments to calculate SG20 peptide structure which is in a Bcl-xL-bound state. Based on the calculated structure of SG20 in a bound state with Bcl-xL, structural model of Bcl-xL/SG20 complex was generated using the HADDOCK webserver.

3.6.2.1 Structural calculation of VDAC2 N-terminal peptide (17-36) in a bound state with Bcl-xL

Isotope-filtered 2D TOCSY and NOESY experiments were performed using unlabeled SG20 incubated with ^{15}N -labelled Bcl-xL. TOCSY spectrum of SG20 in a bound state with Bcl-xL was assigned manually to identify the spin systems (**Figure. 3.28.A** and **Table 8**). Then, the NOESY spectra were used to connect the individual spin systems. Finally, the sequential and medium range NOEs were assigned to the NOESY spectrum.

A

B

Figure. 3.28 2D TOCSY and NOESY spectra of SG20 in a bound state with Bcl-xL.

A) Characteristic fingerprint region of SG20 TOCSY spectrum. Spin systems of each amino residue are connected with a line. B) The backbone amide proton region of SG20 NOESY spectrum. Sequential and medium range NOEs of amide-amide protons are indicated.

Table 8 Proton NMR chemical shift assignments for SG20.

Residues	NH	C α H	C β H	Others
Ser17	-	4.28	3.80	-
Tyr18	-	4.43	2.94, 2.89	C δ H 7.02 C ϵ H 6.72
Ala19	8.12	4.09	1.19	-
Asp20	7.91	4.44	2.64, 2.53	-
Leu21	8.05	4.14	1.57	C δ H 0.80, 0.74 C γ H 1.49
Gly22	8.33	3.81	-	-
Lys23	7.90	4.09	1.73, 1.66	C δ H 1.56 C ϵ H 2.85 C γ H 1.3
Ala24	8.12	4.10	1.29	-
Ala25	8.03	4.14	1.29	-
Arg26	8.06	4.12	1.73, 1.56	C δ H 3.06 C γ H 1.48 NH ϵ 7.12
Asp27	8.14	4.47	2.65, 2.56	-
Ile28	7.72	3.87	1.63	C δ H 0.60 C γ H ¹ 1.05, 0.91 C γ H ² 0.62
Phe29	8.05	4.46	3.06, 2.89	C δ H 7.11 C ϵ H 7.19 C ζ H 7.27
Asn30	8.11	4.50	2.68, 2.63	NH δ ² 6.72, 7.56
Lys31	8.14	4.13	1.72, 1.65	C δ H 1.56 C ϵ H 2.88 C γ H 1.30
Gly32	8.18	3.77, 3.73	-	-
Phe33	7.98	4.45	3.00, 2.85	-
Gly34	8.14	3.76, 3.58	-	-
Phe35	7.92	4.55	3.12, 2.84	C ϵ H 7.21
Gly36	7.94	3.68, 3.59	-	-

Sequential NOEs between backbone amide protons ($d_{NN(i,i+1)}$) were observed from Ala19 to Lys31, and the sequential NOEs between the alpha protons of residue i and the amide protons of residue $i+1$ ($d_{\alpha N(i,i+1)}$) were detected from Ser18 to Ile28. The NOE patterns for an alpha helical secondary structure ($d_{\alpha N(i,i+2)}$, $d_{\alpha N(i,i+3)}$, $d_{NN(i,i+2)}$, and $d_{NN(i,i+3)}$) were also observed, which is summarized in a diagram in **Figure 3.29.A**. Overall, the NOE patterns of an alpha helical conformation were observed from Tyr18 to Asn30, which indicated that this segment of the peptide adopts an alpha helical structure. These results are consistent with the X-ray structure of VDAC2 from zebrafish (Schredelseker et al., 2014) (PDB ID: 4BUM), which showed N-terminal region from Tyr7 to Asn20 as an alpha helix. The solution structure of SG20 bound to Bcl-xL was calculated from 256 distance restraints derived from NMR experiments (**Figure 3.29.B**). The average RMSD values of probable structural segment (Tyr18 to Asn30) for the final 15 structures were $0.25 \pm 0.06\text{\AA}$ and $1.43 \pm 0.25\text{\AA}$ for the backbone and the heavy atoms, respectively (**Table 9**).

Table 9 Structural statistics of NMR structures of SG20.

Number of NOE constraints	
All	256
Intra residues $ i-j =0$	9
Sequential, $ i-j =1$	136
Medium-range, $1< i-j <5$	111
Long-range, $ i-j \geq 5$	0
Number of Hydrogen Bond Constraints	0
Number of Dihedral Angle Constraints	0
Number of Constraint Violations ($>0.5\text{\AA}$)	0
Number of Angle Violations ($>5^\circ$)	0
Energies (kcal/mol)	
E_{total}	16.57 ± 0.24
E_{NOE}	1.17 ± 0.12
$E_{\text{bond}}+E_{\text{angle}}+E_{\text{improper}}$	12.88 ± 0.059
E_{VDW}	2.85 ± 1.26
RMS Deviation of the structural segment(Tyr18-Asn30) for final 15 structures to REM structure	
Backbone (N, C α , C')	$0.25 \pm 0.06\text{\AA}$
Heavy Atoms	$1.43 \pm 0.25\text{\AA}$
Ramachandran Plot	
Most Favored Regions	49.8%
Additionally Allowed Regions	42.2%
Generously Allowed Regions	8.0%
Disallowed Regions	0.0%

3.6.2.2 Docking model structure of Bcl-xL/SG20 by HADDOCK webserver

HADDOCK2.2 webserver was used to generate a docking model of Bcl-xL/SG20 complex. HADDOCK clustered 191 structures in 7 clusters, which represented 95.5 % of the water-refined models by generated HADDOCK. The best model structure from the second cluster was selected, based on the HADDOCK score and total energy. The statistic information of the final four best structures from cluster 2 is listed in the **Table 10**, and the selected model structure is shown in **Figure 3.30**.

Table 10 Statistics of the final four best energy water-refined structures.

HADDOCK score	-144.0 $\pm$ 6.2
Energies (kcal/mol)	
Electrostatic	-284.1 $\pm$ 50.4
Van der Waals	-66.3 $\pm$ 3.0
Desolvation energy	-32.3 $\pm$ 2.2
Restraints violation energy	114.5 $\pm$ 42.29
Buried surface area	1506.7 $\pm$ 65.2
Z-score	-1.9
RMSD from the overall lowest-energy structure	-0.4 $\pm$ 0.2

From the docking model structure of Bcl-xL/SG20 complex, it was shown that SG20 behaves like a BH3-mimetic docking into the hydrophobic groove of Bcl-xL (**Figure 3.30.A**). For comparison, the crystal structures of Bcl-xL/Bim BH3 complex (**Figure 3.30.B**) (PDB ID: 4QVF) and Bcl-xL/Bid BH3 complex (**Figure 3.30.C**) (PDB ID: 4QVE) (Rajan et al., 2015a) are presented, respectively.

Figure. 3.30 Haddock docking model structure of Bcl-xL/SG20 complex and its comparison with Bcl-xL/BimBH3 or Bcl-xL/BidBH3 complex structure

Structural model of Bcl-xL/SG20 generated from HADDOCK docking (A) is presented with the crystal structures of Bcl-xL/Bim BH3 (B) and Bcl-xL/Bid BH3 (C) complex for comparison. Bcl-xL is shown in surface representation (white), and peptide ligands bound to the hydrophobic pocket of Bcl-xL are shown in cartoon representation (magenta). The hydrophobic residues of Bcl-xL interacting with the peptide ligand are in yellow, and positively or negatively charged amino acid residues located near the hydrophobic pocket are colored in blue and red, respectively.

3.6.2.3 Discussion

In this chapter, the three-dimensional structure of SG20 peptide in a Bcl-xL-bound state was determined by NMR experiments. From the determined structure of peptide was in an alpha helical conformation bound to the hydrophobic pocket of Bcl-xL. This is in contrast to the CD results (**Chapter 3.5**), where VDAC N-terminal peptides were largely unstructured. Bim, Bad, and Bmf BH3 peptides, which are known to bind to the hydrophobic groove of anti-apoptotic Bcl-2 family proteins, are reported to be intrinsically disordered in solution. Upon binding to anti-apoptotic Bcl-2 proteins, these BH3 peptides undergo structural transition from a random coil to an alpha helical conformation (Hinds et al., 2007). However, this is not the case for SG20 binding to Bcl-xL. When NOESY spectrum of SG20 peptide in free state was compared with that of SG20 in a bound state with Bcl-xL, appearance of the additional NOE peaks was rarely observed, indicating that there was no dramatic change in conformation of SG20 peptide induced by binding to Bcl-xL. Based on our NMR data, it seems rather likely that SG20 peptide itself has helical propensity in solution, and its helical conformation is not induced by binding to Bcl-xL.

Bcl-xL/SG20 complex model structure generated from HADDOCK docking has shown that the N-terminal segment of VDAC2 sits on the hydrophobic pocket of Bcl-xL. This hydrophobic groove is known to be the canonical binding site for BH3 domain of pro-apoptotic Bcl-2 family proteins, such as Bim and Bid. Both Bim and Bid BH3 have been reported to bind to Bcl-xL via hydrophobic interaction between the peptide and the hydrophobic groove of the protein (Liu et al., 2003; Rajan et al., 2015a). BH3 regions of pro-apoptotic Bcl-2 family protein have four conserved hydrophobic residues, which are facing the hydrophobic cleft of anti-apoptotic Bcl-2 family protein, thereby promoting BH3 peptide binding to the anti-apoptotic protein via hydrophobic interaction. HSQC NMR titration experiments showed that VDAC N-terminal segments also interact with Bcl-xL at the canonical hydrophobic pocket like BH3-only peptide does. Hence, we compared the VDAC N-terminal peptides with BH3 only peptides, in terms of sequences as well as their binding pattern to Bcl-xL. To that end, we have aligned amino acid sequences of VDAC 1,2,3, N-terminal regions, together with Bim and Bid BH3 regions. (**Figure 3.31.A**) Amino acid sequence alignment revealed that surprisingly, VDAC 1,2,3, N-terminal region also have conserved hydrophobic residues,

which are aligned well with the conserved canonical hydrophobic residues in BH3 domain of Bim and Bid (labelled as H1 to H4 in **Figure 3.31.A**). In addition, VDAC N-terminal segment has three additional conserved amino residues facing the interface between Bcl-xL and the peptide, indicated with dashed arrows (**Figure 3.31.A**).

Next, the N-terminal region of three-dimensional structure of VDAC isoforms were aligned with the structure of BimLOCK BH3 peptide in a bound state with Bcl-xL (Okamoto et al., 2012) (**Figure 3.31.B**). As three-dimensional structure of VDAC3 isoform has not been determined yet, the structure of the VDAC3 N-terminal region was modelled, based on the VDAC1 structure. The structure alignment clearly showed that the conserved hydrophobic residues of both Bim BH3 and VDAC N-terminal peptides were well aligned, facing the hydrophobic cleft of Bcl-xL. This result implies that the N-terminal peptide of VDAC isoforms might interact with Bcl-xL through hydrophobic interactions between the hydrophobic pocket of Bcl-xL and the conserved hydrophobic residues of the peptide, similar to the BH3 domains. Lastly, helical wheels of the VDAC N-terminal segment and the Bim BH3 peptide were generated using MPEx software (Snider et al., 2009). The result showed that both VDAC N-terminal and Bim BH3 regions are amphipathic helices, with the charged amino acids and the aliphatic and aromatic residues localized on the opposite sides of the wheel. Taken all together, it is clear that the structural and biochemical characteristics of the VDAC N-terminal peptides and BH3 peptides of pro-apoptotic Bcl-2 family proteins share similar structural and biochemical characteristics.

Figure. 3.31 Comparison of VDAC N-terminal segment with BH3 domain of Bim.

A) Sequence alignment of the VDAC 1,2,3, N-terminal peptides with Bim and Bid BH3 domains. Four conserved hydrophobic residues are labeled with grey or black boxes. Acidic and basic amino residues which are involved in charge-charge interaction with Bcl-xL are colored in red and blue, respectively. B) Structural superposition of VDAC 1,2,3, N-terminal regions on BimLOCK peptide structure that is bound to Bcl-xL. (PDB ID: 2YQ7) The four conserved hydrophobic residues are presented as sticks. C) Helical wheels of VDAC 1,2,3, N-terminal segments and Bim BH3 generated from Membrane Protein Explorer (MPEx) software (Snider et al., 2009).

As a striking similarity has been observed for VDAC N-terminal segments and the BH3 domains of Bim or Bid in both the sequence and structure, we investigated whether the molecular basis underlying the interaction between Bcl-xL and these peptides are also similar to each other. Hence, we have compared our HADDOCK docking model structure of Bcl-xL/SG20 with Bcl-xL/Bim BH3 and Bcl-xL/Bid BH3 complex structure solved by our group. (PDB ID: 4QVE and 4QVF) Binding of BH3 domains of Bim/Bid to Bcl-xL not only involves hydrophobic interaction mediated by the conserved hydrophobic residues of BH3 domain, but also involves the formation of salt bridges. In Bid BH3:Bcl-xL interface (PDB ID: 4QVE) (Rajan et al., 2015a), a salt bridge is formed between Arg139 in Bcl-xL and the highly conserved BH3 residue, Asp95 (**Figure 3.32.B**). Whereas Bid BH3:Bcl-xL interface only involves a single pair of salt bridge interaction, BH3 regions of Bim and other pro-apoptotic Bcl-2 family have two or more pairs of salt bridge formation in BH3:Bcl-xL interface (PDB ID: 4QVF) (Rajan et al., 2015a). Bim BH3 forms a salt bridge by Asp99 with Arg139 of Bcl-xL, and additional salt bridge by Arg95 with Glu129 of Bcl-xL, which is not found in Bid BH3 (**Figure 3.32.C**).

Ribbon and surface representations of Bcl-xL/peptide ligand complex structures. Bcl-xL is colored in cyan, and the peptide bound to Bcl-xL is colored in magenta. The basic and acidic residues are shown as sticks, and the salt bridge interactions are indicated with yellow dashed lines. The electrostatic potential surface of Bcl-xL is displayed on the right, and the region where the salt bridges are formed between the peptide and Bcl-xL is indicated with dashed circles. A) Salt bridge formation observed in the HADDOCK docking model structure of Bcl-xL/SG20 complex. B) Salt bridge formation observed in the crystal structure of Bcl-xL/Bid BH3 complex. (PDB ID: 4QVE) C) Salt bridge formation observed from the crystal structure of Bcl-xL/Bim BH3 complex. (PDB ID: 4QVF) D) A closer view of the superposed Bcl-xL/peptide ligand complex structures. The inter-atom distances are indicated in Å units for each salt bridge.

In the docking model structure of Bcl-xL/SG20 complex, a similar pattern of salt bridge formation was observed (**Figure 3.32.A**). Both Glu129 and Arg139 of Bcl-xL, which were shown to form salt bridge interaction with Bim BH3, were also shown to interact with Arg26 and Asp27 of SG20, respectively. However, the orientation of the salt bridges formed between Bcl-xL and the ligand peptides are peptide-dependent, as shown in **Figure 3.32.D**. Further experiments including the site-directed mutagenesis and the HSQC NMR titration are required to validate the importance of these salt bridge interaction in the binding of SG20 to Bcl-xL. For example, Glu129 or Arg139 could be substituted with hydrophobic Ala by site-directed mutagenesis, and the binding of SG20 peptide to this mutant Bcl-xL could be compared with that of wild type Bcl-xL by NMR titration experiment.

In addition, a unique feature of Bcl-xL/SG20 interaction was observed from our docking model structure, which was not observed in Bcl-xL/Bim BH3 and Bcl-xL/Bid BH3 complex structure (**Figure 3.33**). The unique residue Phe29, which is highly conserved among VDAC isoforms as shown in **Figure 3.31.A**, interacts with Tyr101 of Bcl-xL via formation of parallel-displaced π -stacking interaction (McGaughey et al., 1998) (**Figure 3.33.A**). On the other hand, both Bim and Bid BH3 do not have such aromatic residues in proper orientation and at a distance from Tyr101 of Bcl-xL to form a π - π stacking interaction. (**Figure 3.33.B and C**) Thus, this π - π stacking interaction might contribute to the stability of Bcl-xL/SG20 complex uniquely.

Figure. 3.33 π - π stacking interaction is a unique feature of Bcl-xL/SG20 complex.

Ribbon and surface representations of Bcl-xL/peptide ligand complex structures. Bcl-xL is colored in cyan, and the peptide bound to Bcl-xL is colored in magenta. A) A π - π stacking interaction observed from the HADDOCK docking model structure of Bcl-xL/SG20 complex. The π - π stacking interaction between Phe29 of SG20 and Tyr101 of Bcl-xL is indicated with a yellow dashed line. B) and C) A closer view of Tyr101 in Bcl-xL which could not form π - π stacking interaction with the indicated peptide ligands.

In this chapter, we have determined the structure of SG20 peptide in a state bound to Bcl-xL by NMR spectroscopy. Also, a model structure of Bcl-xL/SG20 complex was generated using HADDOCK docking webserver. As VDAC N-terminal region and BH3 domain of Bim and Bid showed sequence and structure similarities, we have compared

the interaction involved in the Bcl-xL/SG20 binding interface with that of Bcl-xL/Bim BH3 and Bcl-xL/Bid BH3. This revealed that interactions of SG20 peptide with Bcl-xL not only involve the hydrophobic interactions, but also salt bridges between Arg/Glu129, and Asp/Arg139, which were similar to those conserved in Bcl-xL/BH3 complexes. In addition, our model structure of Bcl-xL/SG20 complex identified a π - π stacking interaction that is unique to the Bcl-xL/SG20 complex. Based on these finding, mutagenesis experiments could be designed to test whether these salt bridge formations and/or π - π stacking interaction affect the binding of SG20 to Bcl-xL, thereby determining whether the hydrophobic interaction between the hydrophobic groove of Bcl-xL and the four conserved hydrophobic residues of SG20 are sufficient for SG20/Bcl-xL complex formation. In addition, as the Bcl-xL/SG20 complex presented here is a structural model generated from HADDOCK docking, it would be desirable to determine a full structure of Bcl-xL/SG20 complex by NMR spectroscopy or X-ray crystallography to validate the interactions mentioned above.

3.7 Structural characterization of VDAC2 N-terminal peptide in membrane-mimicking environment

Considering that VDAC is a mitochondrial outer membrane protein, the N-terminal region of VDAC could interact with surrounding membrane environment. According to De Pinto and his colleagues (De Pinto et al., 2007), VDAC1 N-terminal peptide interacts with the detergent micelles and undergoes a structural transition, forming an alpha helix in the presence of negatively charged detergent micelles. Also, Varda et al. (Geula et al., 2012) suggested that if the N-terminal region of VDAC1 loses its alpha helical conformation, it gets translocated out of the barrel, losing the voltage-dependence of the channel. This observation demonstrates that the structural plasticity of VDAC1 N-terminal region could control the channel activity. Currently, no information is available for VDAC2 NP in such environment. Thus, we extended our studies on the structural investigation of SG20 and its interaction with Bcl-xL in membrane environment using CD and NMR approaches. If SG20 undergoes structural transition in the membrane-mimicking environment, such structural plasticity of SG20 in membrane could affect its interaction with Bcl-xL.

3.7.1 CD experiments on the VDAC2 N-terminal peptide in a membrane-mimicking environment

The 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent, a zwitterionic, non-denaturing, mild detergent, was used to generate membrane-mimicking environments. CHAPS is VDAC 1,2,3 NPs were incubated with 30 mM or 80 mM CHAPS solution overnight at 4°C, and the samples were subjected to CD measurement in triplicate (**Figure 3.34.A**). From the CD spectra, the MRE at 222 nm and the helical content of each VDAC NPs were estimated (**Figure 3.34.B and C**). An increase in $-\left[\theta\right]_{222\text{nm}}$ indicates an increase in the helicity of the peptide (Hirst and Brooks, 1994).

Figure. 3.34 CD spectra of VDAC 1,2,3 NPs recorded in the presence of CHAPS.

A) CD spectra of VDAC 1,2,3 NPs in phosphate buffer containing 0 mM (black), 30 mM (red), and 80 mM (blue) CHAPS. B) $-[\theta]_{222\text{nm}} \cdot 10^{-3}$ of each peptide at various CHAPS concentration. (VDAC1 NP: black line with square symbol. VDAC2 NP: red line with circle symbol. VDAC3 NP: blue line with triangle symbol.) C) Alpha helical content (%) of each sample was obtained using the CD deconvolution software provided by AVIV Biomedical.

Figure 3.34.B shows that the CHAPS detergent could induce only a slight increase in helicity of VDAC 1,2,3, NPs, corresponding to an increase in the alpha helical content (**Figure 3.34.C**) less than 2%. Given that the critical micelle concentration (CMC) of CHAPS is 8 mM, 80 mM used here is considerably a high concentration. Despite the excessive amount of the detergent, all VDAC NPs showed only a modest increase in helicity, which led us to screen other reagents as described in the following. Due to time and resource constraints, the following CD experiments were performed on VDAC2 N-terminal peptide only.

Trifluoroethanol (TFE) is a solvent which is well known to mimic the membrane environment and induce and stabilize the alpha helical structure of small peptides (Jasanoff and Fersht, 1994; Sonnichsen et al., 1992). VDAC2 NP was thus incubated with TFE at various concentrations from 0% to 40% overnight in 4°C, and the samples were measured in triplicate (**Figure 3.35**). **Figure 3.35.A** clearly shows that MRE at 208 nm and 222 nm were lowered as the TFE concentration increased, demonstrating that addition of TFE solvent successfully stabilizes alpha helical secondary structure of VDAC2 NP. Increase in alpha helical conformation of VDAC2 NP by TFE is more clearly shown by $-\left[\theta\right]_{222\text{nm}}$ plot in **Figure 3.35.B**, as well as helical content bar graph in **Figure 3.35.C**. Our results are consistent with the literature (De Pinto et al., 2007; Guo et al., 1995), in which the alpha helical conformation of VDAC1 N-terminal peptide was enhanced by TFE. In conclusion, VDAC2 N-terminal peptide could undergo structural transition to form a stable alpha helical structure in the presence of membrane-mimicking environment made by TFE solvent. However, TFE might not be the best option to study the interaction of VDAC2 NP with Bcl-xL in membrane environment, as TFE is known to denature 3D structure of proteins at high concentration (Gast et al., 1999; Povey et al., 2007).

Figure 3.35 CD spectra of VDAC2 NP recorded in the presence of TFE.

A) CD spectra of VDAC2 NP incubated with 0% (black), 10% (red), 20% (blue), 30% (purple), and 40% (green) TFE concentration (v/v). B) $-[\theta]_{222\text{nm}} \cdot 10^{-3}$ of each sample at different TFE concentration. C) Alpha helical content percentage of each sample was obtained using the CD deconvolution software provided by AVIV Biomedical.

Finally, the structural transition of VDAC2 NP in the OM detergent micelles was examined by CD experiments. Previously, it was shown that Bcl-xL undergoes structural transition and form the 3DDS dimer in the presence of 2% OM (Rajan et al., 2015b). To check whether VDAC2 NP can also undergo conformational changes in the OM detergent, VDAC2 NP was incubated with 0%, 0.5%, 1%, 1.5%, and 2% OM overnight at 4°C, and the CD of the samples were measured in triplicate. As the concentration of OM increased, the two minima at 208 nm and 222 nm were lowered, indicating improved helicity of peptides (**Figure 3.36.A**). However, unlike TFE which induced a gradual increase in helical conformation of the peptide, OM at 2% induced a sudden increase in the helicity of peptide (**Figure 3.36.B and C**). Considering that CMC of OM is 0.9%, it appears that VDAC2 NP can only undergo conformational change in OM concentration above the CMC. Indeed, when the concentration is above 1 CMC, VDAC2 NP started to undergo structural transition, and its helicity sharply increases in 2 CMC OM. From these results, OM detergent micelles appears to provide the necessary

membrane mimicking environment for the VDAC2 N-terminal peptide to exist in an alpha helical conformation.

Figure. 3.36 CD spectra of VDAC2 NP recorded in the presence of OM.

A) CD spectra of VDAC2 NP incubated with 0% (black), 0.5% (red), 1.0% (blue), 1.5% (purple), and 2.0% (green) OM concentration (w/v). B) $-\theta_{222nm} \cdot 10^{-3}$ of each sample as a function of the OM concentration. C) Alpha helical content (%) of VDAC2 NP, obtained using the CD deconvolution software provided by AVIV Biomedical.

Despite the success of identifying OM by CD, too much materials (peptides) are needed for screening many reagents with CD, making this approach impractical. Hence, we decided to employ the 1D ¹H NMR titration experiments for enable screening of a large number of detergents and lipids. In addition, we also performed our structural studies on the interaction of VDAC2 NP with Bcl-xL by NMR spectroscopy.

3.7.2 1D ¹H NMR experiments of VDAC2 N-terminal peptide titrated with detergents or lipids

SG20, 20 a.a length VDAC2 NP, was mixed with several detergents and lipids, and incubated overnight at 4°C., as described in **Table 11**. In **Table 11**, lipids and detergents that were used in this study are listed with their chemical structures and CMC values.

The LDAO has been extensively used in studying the 3D structure of VDAC proteins (Bayrhuber et al., 2008; Hiller et al., 2008; Jaremko et al., 2016; Schredelseker et al., 2014; Ujwal et al., 2008). DPC and LMPC lipids are commonly used to mimic membrane environment for structural studies. OG is a carbohydrate based detergent with non-ionic hydrophilic head group, similar to OM.

Table 11 List of detergents used in 1D ^1H NMR titration experiments.

Detergent	CMC (mM)	CMC (%)	Structure
OG (Octyl β -D-glucopyranoside)	20	0.53
Triton X-100	0.24	0.02
DPC (n-Dodecylphosphocholine)	1.1	0.04
LMPC (Lysomyristoylphosphatidylcholine)	0.09	0.004
LDAO (n-Dodecyl-N,N-Dimethylamine-N-Oxide)	1.5	0.02
OM (n-Octyl- β -D-Maltopyranoside)	19.5	0.89
CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate)	11/36 (1 st /2 nd)	0.68

The Secondary structures of the peptide samples were analyzed by 1D ^1H NMR experiments (**Figure 3.37**). According to the statistical studies conducted by Wishart

and his colleagues, chemical shift values of amide proton of residues from unstructured peptide tend to be concentrated narrowly in amide region, whereas chemical shift values of amide proton from alpha helical or beta strands have tendency to spread more widely in the amide region of spectra (Wishart et al., 1991). Using this fact, we interpreted the capacity of surfactants in improving secondary structure of peptide by observing the chemical shift values in the amide region.

CHAPS, unlike any other surfactants used in this study, has an amide bond in its chemical structure (**Figure 3.37.A, indicated with red arrow**). Hence, its signal appears in the amide region of 1D proton NMR spectrum, which resulted in a strong chemical shift observed near 8 ppm in **Figure 3.37.A**. Other than that, no significant change in chemical shift at the amide region was found during titration with CHAPS, indicating that it did not enhance the alpha helicity of SG20. In order to compare the effect of membrane-mimicking detergents and lipids on developing secondary structure of SG20 peptide, 1D proton NMR spectra of all samples except CHAPS-titrated sample are presented in an overlay in **Figure 3.38**. Only DPC showed the highest degree of chemical shift perturbation with proton chemical shifts spreading widely when titrated with SG20 (**Figure 3.37.G and Figure 3.38**). All the other surfactants except DPC were not able to induce dispersion of proton chemical shifts in the amide region of the SG20 peptide. Unlike the result by CD in the previous chapter, OM made the distribution of the chemical shift of the amide region of sG20 become narrower, indicating that it does not enhance the alpha helicity under the condition of NMR (**Figure 3.37.B**). Summarizing, DPC micelles were the only reagents that enhances the alpha helicity of SG20.

Figure. 3.37 1D ^1H spectra of SG20 titrated with several surfactants.

For figure legend, see next page.

SG20 peptides (0.2 mM) titrated with various surfactants were analyzed by 1D ^1H NMR spectroscopy. All surfactants were incubated with SG20 peptide at two different concentrations, and the amide and aromatic region of all 1D proton spectra of samples with the same surfactant are presented in overlay. A) SG20 peptide in the presence of 2.2% (red) and 4.4% (green) CHAPS. B) SG20 peptide in the presence of 2% (red) and 4% (green) OM. C) SG20 peptide in the presence of 0.5% (red) and 5% (green) OG. D) SG20 peptide in the presence of 0.2% (red) and 1% (green) Triton X-100. E) SG20 peptide in the presence of 0.04% (red) and 0.4% (green) LMPC. F) SG20 peptide in the presence of 0.1% (red) and 0.2% (green) LDAO. G) SG20 peptide in the presence of 0.4% (red) and 2% (green) DPC.

Figure. 3.38 Overlay of 1D ^1H spectra of SG20 titrated with several surfactants.

Amide and aromatic region of the spectra obtained from all titration are presented in an overlay. (black: free SG20 in solution, red: SG20 in 10 CMC DPC, green: SG20 in 10 CMC OG, purple: SG20 in 4 CMC OM, yellow: SG20 in 10 CMC LDAO, orange: SG20 in 50 CMC Triton X-100, light green: SG20 in 100 CMC LMPC)

Previously, one of our colleagues reported in his thesis that Bcl-xL was denatured and lost its original three-dimensional structure in the presence of 10 CMC DPC. As shown in **Figure 3.37.G**, SG20 was already saturated with DPC at 10 CMC (0.4%), as there

was no difference in the 1D spectrum between 10 CMC and 50 CMC DPC. Therefore, we performed 1D proton NMR experiments on SG20 titrated with lower concentrations of DPC to identify the optimal DPC concentration which would enhance SG20's secondary structure without denaturing Bcl-xL (**Figure 3.39**). Subsequently, selected concentrations of DPC were applied to Bcl-xL, followed by ^1H - ^{15}N 2D HSQC experiments to analyze the effect of DPC on Bcl-xL. Firstly, for titration of SG20 with DPC micelles, 1, 2, and 3 CMC of DPC were added to the peptide and conformational change of the sample was analyzed by 1D proton NMR. (**Figure 3.39**) From the overlay of 1D spectra, no difference was observed between 1D proton NMR spectrum of free SG20 (black) and SG20 with 1 CMC DPC (red). On the contrary, it was observed that the amide proton chemical shifts of SG20, which were clustered in a narrow region near 8.0 – 8.2 ppm, started to spread widely in 2 CMC DPC (green), indicating the enhancement of secondary structure in the peptide. Hence, we assumed that 2 CMC (0.08%) is the minimum concentration of DPC required to interact with SG20 peptide to induce structural transition. Next, we examined the effect of 2 CMC DPC on Bcl-xL, as well as its interaction with SG20 peptide in DPC micelles by ^1H - ^{15}N 2D HSQC experiments.

Figure. 3.39 1D ^1H spectra of SG20 titrated with DPC micelles.

The amide and aromatic region of the spectra obtained from DPC titration at lower concentration are presented in an overlay. (black: free SG20 in solution, red: 1 CMC DPC, green: 2 CMC DPC, purple: 3 CMC DPC) Peaks separated after the addition of DPC are indicated with dashed arrows.

3.7.3 ^1H - ^{15}N 2D HSQC experiment of Bcl-xL titrated with VDAC N-terminal peptide in DPC micelles

^{15}N -labelled Bcl-xL was titrated with DPC at two different concentrations, 2CMC and 4CMC, and Bcl-xL residues interacting with DPC micelles were monitored by ^1H - ^{15}N 2D HSQC experiment. (**Figure 3.40**) The superimposed HSQC spectra revealed that Bcl-xL interacts with DPC micelles quite strongly. When 2 CMC of DPC was added to Bcl-xL, many peaks moved significantly, indicating strong interaction of the protein with DPC. In the presence of 4 CMC DPC, several new peaks appeared (indicated in **Figure 3.40** with red arrows). This result suggested that Bcl-xL might undergo a dramatic conformational change to lose its original three-dimensional structure, or form complex with DPC micelles in the presence of 4 CMC DPC. Therefore, we decided to

use DPC at 2 CMC, which was the minimum concentration required for inducing structural transition of SG20 peptide without affecting the Bcl-xL structure.

Figure. 3.40 NMR titration experiment on Bcl-xL interacting with DPC micelles.

^1H - ^{15}N HSQC spectrum showing chemical shifts of Bcl-xL residues measured from titration with DPC micelles. Red arrows in the box indicate the appearance of new peaks in the presence of 4 CMC DPC.

Next, 0.2 mM of ^{15}N -labelled Bcl-xL was incubated with 2 CMC DPC in the presence or absence of SG20 equimolar to Bcl-xL. The 2D ^1H - ^{15}N HSQC experiment of each sample was performed and acquired spectra are presented in an overlay as in **Figure 3.41**. The chemical shift perturbations of G196, G147, and N136 were prominent upon addition of 2 CMC DPC into free Bcl-xL (red dashed arrows in **Figure 3.41**). Small chemical shifts perturbations were observed after adding SG20 peptide to the protein in detergent micelles (blue dashed arrows in **Figure 3.41**). **Figure 3.42 A and B** show the 2D HSQC NMR spectra of ^{15}N -labeled Bcl-xL titrated with an equimolar SG20 in the presence or absence of 2 CMC DPC, respectively. Clearly, many chemical shifts of Bcl-xL residues were perturbed to a considerable degree upon addition of SG20 in solution only in the absence of DPC micelles (**Figure 3.42.B**), indicating that the affinity of Bcl-

xL binding to SG20 in solution was much stronger than in membrane mimicking environment.

Figure. 3.41 2D HSQC spectra of Bcl-xL with DPC micelles and SG20 peptide.

^1H - ^{15}N HSQC spectrum showing chemical shifts of ^{15}N -labeled Bcl-xL residues measured before and after addition of 2 CMC DPC and SG20 at the final concentration of 0.2 mM. Resonances with significant chemical shift perturbation were presented with residue number in zoom-in.

Figure. 3.42 2D HSQC spectra of Bcl-xL with DPC micelles and SG20 peptide.

A) An overlay of ${}^1\text{H}$ - ${}^{15}\text{N}$ HSQC spectra showing chemical shifts of ${}^{15}\text{N}$ -labelled Bcl-xL residues in 2CMC DPC micelles, measured before and after addition of SG20 at the final concentration of 0.2 mM. B) An overlay of ${}^1\text{H}$ - ${}^{15}\text{N}$ HSQC spectra showing chemical shifts of ${}^{15}\text{N}$ -labelled Bcl-xL residues in solution, measured before and after addition of SG20 at the final concentration of 0.2 mM.

To investigate this phenomenon further, an overlay of the spectra showing chemical shifts of free Bcl-xL in solution (red), Bcl-xL in DPC micelles (blue), and Bcl-xL with SG20 at 1:1 ratio in solution (green) is presented (**Figure 3.43**). In this overlay, it is clear that the chemical shifts of Bcl-xL residues that were perturbed by DPC micelles were also perturbed by SG20 peptides. In other words, most of the residues on Bcl-xL which showed interaction with DPC micelles were also interacting with SG20 peptide. This indicates the competitive binding of DPC lipid molecules to the hydrophobic pocket of Bcl-xL, which is also a putative binding site of SG20 to Bcl-xL. DPC, as a lipid, has a hydrophilic head group and a long hydrophobic tail in its chemical structure. Due to its hydrophobicity, the hydrophobic tail has a high chance of interacting with the hydrophobic pocket on the surface of Bcl-xL via hydrophobic interaction, which might have resulted in a significant chemical shift perturbation of Bcl-xL residues upon addition of 2 CMC DPC. Therefore, hydrophobic pocket of Bcl-xL might have been already occupied by DPC molecules, which makes it unavailable for SG20 peptides to interact with the protein. This could explain the reason of reduced binding of Bcl-xL with SG20 in the presence of DPC micelles (**Figure 3.42.A**).

Figure. 3.43 Overlay of 2D HSQC spectra on Bcl-xL with DPC micelles or SG20 peptide.

^1H - ^{15}N HSQC spectrum showing chemical shifts of ^{15}N -labelled Bcl-xL residues measured before and after addition of 2 CMC DPC or SG20 at the final concentration of 0.2 mM.

3.7.4 Discussion

In this chapter, the effect of membrane mimicking environment on structure of VDAC NPs has been examined. By performing a series of CD experiments, we examined the capacity of several detergents for mimicking membrane environment which can interact with VDAC NPs (especially focused on VDAC2 NP) to induce structural transition. CHAPS, a zwitterionic detergent, failed to form proper membrane environment for inducing such conformational changes in VDAC NPs. TFE, on the other hand, could mimic membrane environment which interacts with VDAC NP2 to enhance its alpha helical conformation. Our result is consistent with previous CD studies on peptides derived from N-terminal region of VDAC1 incubated in hydrophobic solvent such as TFE and methanol (De Pinto et al., 2007; Guo et al., 1995). However, we could also find inconsistency from what De Pinto and his colleagues proposed in the literature (De Pinto et al., 2007). In their CD studies, peptide derived from N-terminal 2-20 region of VDAC1 failed to form alpha helical structure in the presence of dodecyl β -maltoside

(D β M), whereas SDS could successfully enable the peptide to form alpha helical secondary structure. Thus, they suggested that hydrophobic environment alone might not be enough to induce structural transition of the peptide. As negatively charged SDS micelle could induce conformational change of the peptide, they proposed that electrostatics might play a key role in the interaction of VDAC1 NP with the membrane environment and its structural transition. From our CD studies, VDAC2 NP could interact with non-ionic OM detergent micelle to undergo conformational changes. This result indicates that unlike VDAC1 NP, VDAC2 NP might not require negative charges on the surface of membrane for interaction and structural transition.

Secondly, several surfactants have been screened for their interactions with SG20 peptide by NMR experiments. Among the surfactants tested, DPC micelles have shown the strongest interaction with SG20, which was indicated by the significant chemical shift perturbation observed from 1D proton NMR spectra compared to any other detergent micelles. Based on our observation, we have predicted that DPC micelles could interact with SG20 to induce structural transition of the peptide, improving its secondary structure. As a next step, we have attempted to examine the interaction between Bcl-xL and SG20 in the presence of DPC micelles. We tested if the conformational change of SG20 induced by membrane environment has affected its interaction with Bcl-xL by 2D HSQC NMR experiment. It turned out that DPC molecules not only interact with SG20, but also interact with Bcl-xL, as the hydrophobic tail of the lipid could bind to the hydrophobic groove of Bcl-xL via hydrophobic interaction. Hence, the hydrophobic pocket on the surface of Bcl-xL was occupied by DPC lipids, preventing the access of SG20. As a result, we were not able to study the interaction between Bcl-xL and SG20 in the presence of DPC micelles. It seems that simple addition of DPC into the sample might not be a proper way for studying the Bcl-xL/SG20 complex in membrane environment, as free DPC lipids which were not incorporated into lipid micelles could interfere the binding of SG20 to Bcl-xL. Instead, using a lipid bilayer made of DPC would be a better way to investigate the effect of conformational change of SG20 on its interaction with Bcl-xL in membrane environment. During preparation of lipid bilayer by vesicle fusion method, free lipids which were not integrated into the bilayer could be washed away (Cremer and Boxer, 1999). Hence, by using this way, it would be possible to prevent the free lipid molecules

from binding to the hydrophobic pocket, while maintaining the membrane environment for our study. In addition, compared to detergent micelles, the lipid bilayers can generate a membrane-mimicking environment that is more similar with biological membrane, which makes it a better choice for studying protein-protein interaction in membrane. Unfortunately, using the lipid bilayer for NMR structural studies would be challenging, due to large size of the bilayer.

3.8 Attempts to crystallize Bcl-2 family protein in complex with VDAC 1,2,3, N-terminal peptides

To better understand the molecular basis of the interaction of these NP's with the Bcl-2 family members, we also attempted to utilize X-ray crystallography. Thus, for the structural investigation of Bcl-2 family proteins interacting with N-terminal region of VDACs, protein crystallization of Bcl-xL/VDAC NPs or Bak/VDAC NPs complex (incubated at molar ratio of 1:5) was attempted by using commercially available protein crystallization screens. The combination of Bcl-xL/VDAC NPs did not yield any crystal hits. In contrast, protein crystals were observed from Bak/VDAC1 and VDAC2 NP protein samples. Unfortunately, data collection and processing revealed the presence of only free Bak proteins, lacking the electron density of the peptide ligands. Hence, we were not able to obtain crystal structure of Bcl-2 family protein/VDAC N-terminal peptide complex. List of protein crystallization screens and the outcome of the protein crystallization are summarized in **Table 12**.

Table 12 List of protein crystallization screens used for protein crystallization attempts.

Protein	peptide	Screens	Remarks
Bcl-xL	VDAC1 N-terminal peptide (1-25)	1) Wizard Classic crystallization screen series 1&2 2) Hampton Research Index™ HR2-144 crystallization screens	No hits
Bcl-xL	VDAC2 N-terminal peptide (12-36)	1) Wizard Classic crystallization screen series 1&2 2) Hampton Research Index™ HR2-144 crystallization screens	No hits
Bcl-xL	VDAC3 N-terminal peptide (1-25)	1) Wizard Classic crystallization screen series 1&2 2) Hampton Research Index™ HR2-144 crystallization screens	No hits
Bak	VDAC1 N-terminal peptide (1-25)	1) Wizard Classic crystallization screen series 1&2 2) Hampton Research Index™ HR2-144	Crystal growth in 0.2M Ammonium sulfate, 0.1M BIS-TRIS, pH 5.5, 27% w/v Polyethylene glycol 3,350

		crystallization screens	<p>: X-ray diffraction results revealed that the obtained protein crystals lack the electron density of the peptide ligand, indicating that these protein crystals contain only Bak protein.</p>
Bak	VDAC2 N-terminal peptide (12-36)	1) Wizard Classic crystallization screen series 1&2 2) Hampton Research Index™ HR2-144 crystallization screens	Crystal growth in HR Index 2, 66: 0.2M Ammonium sulfate, 0.1 M BIS- TRIS pH5.5, 25% w/v Polyethylene glycol 3,350. <p>: X-ray diffraction results revealed that the obtained protein crystals lack the electron density of the peptide ligand, indicating that these protein crystals contain only Bak protein.</p>
Bak	VDAC3 N-terminal peptide (1-25)	1) Wizard Classic crystallization screen series 1&2 2) Hampton Research Index™ HR2-144	No hits

		crystallization screens	
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Conclusion

Proper regulation of apoptosis is essential to maintain normal development and functioning of multicellular organisms. Abnormal pattern of apoptosis has been observed in diseases such as cancer and autoimmune disorders. Due to its significance associated with pathogenesis, apoptosis has been extensively studied over decades. Several mechanisms have been suggested to explain how apoptotic pathways are regulated, and it has been established that mitochondrial pathway of apoptosis is tightly regulated by a number of proteins located at the outer mitochondrial membrane. Proteins of the Bcl-2 family play a major role in regulating the mitochondrial apoptotic pathway deciding the fate of a cell. The Bcl-2 family proteins are further divided into subfamilies – pro-apoptotic, anti-apoptotic, and pro-apoptotic BH3 only proteins. The regulation of mitochondrial homeostasis takes place by molecular interactions and formation of homo- and hetero dimers between pro- and anti- apoptotic Bcl-2 family proteins. To date, the structural transition of the Bcl-2 family proteins during homo- and hetero-dimerization has been reported in many literatures, and many attempts have been made to understand the functional implications of such structural plasticity of the Bcl-2 proteins in the modulation of mitochondrial homeostasis.

In the first part of this thesis, we have examined conformational change of several anti-apoptotic Bcl-2 family proteins and formation of dimers induced by a number of physico-chemical environmental changes. Various conditions including pH changes, heat, and detergent micelles have been used to induce structural transition and dimerization of the Bcl-2 family proteins including Bcl-xL, Bcl-2, Bcl-w, and Mcl-1. By X-ray crystallography, Bcl-xL was shown to undergo a structural change to form 3D domain swapped dimer in the presence of OM detergent micelles. Together with our collaborator's work, it has been suggested that such structural plasticity of Bcl-xL might have its functional implication in the regulation of mitochondrial calcium homeostasis, as MEF cells expressing Bcl-xL mutants with impaired dimerization showed reduced mitochondrial calcium uptake.

In the second part of this thesis, structural investigation of the interaction between Bcl-xL and the N-terminal segment of Voltage dependent anionic channel (VDAC) was

conducted by NMR spectroscopy. The results showed that the N-terminal peptides of all VDAC isoforms interact with the residues of Bcl-xL, especially on the hydrophobic pocket which is the binding pocket of BH3-only peptides. In addition, we observed that the N-terminal segment of VDAC2 showed a stronger interaction with Bcl-xL than VDAC1 or VDAC3. Therefore, as a preliminary study to understand the structural aspect of interaction between Bcl-xL and VDAC N-terminal segment, we used a 20-amino acid long VDAC2 N-terminal peptide (SG20) for our structural investigation. We have determined the structure of SG20 in a state bound to Bcl-xL using NMR spectroscopy. From the structure, it was shown that SG20 adopts alpha helical conformation when bound to Bcl-xL. Using the structure, a model structure of Bcl-xL/SG20 complex was generated using the HADDOCK docking webserver. Based on the sequence and structure similarities between SG20 and BH3-only peptides of pro-apoptotic Bcl-2 proteins, published structures of Bcl-xL/Bim BH3 and Bcl-xL/Bid BH3 complex have been compared with our complex model structure. The comparison revealed that a salt bridge formed in the Bcl-xL/SG20 complex interface is consistent with a similar well conserved interaction among the BH3 domains of pro-apoptotic Bcl-2 family proteins with Bcl-xL. Furthermore, Bcl-xL/SG20 complex model structure was shown to have a unique π - π stacking interaction, which could contribute to the stability of Bcl-xL/SG20 complex, which is absent in other Bcl-xL/BH3-only complexes. Further studies including mutagenesis and NMR titration experiments would be required to show the significance of these interactions on the binding of SG20 to Bcl-xL.

Lastly, attempts have been made to study the interaction between SG20 with membrane mimicking environment, as VDAC is localized in the mitochondrial outer membrane. Several surfactants were screened to check which detergent or lipid could interact with SG20 to induce structural transition of the peptide. DPC was shown to interact with SG20, enhancing its secondary structure formation. Using DPC micelles as the membrane mimetic system, we tried to study the interaction of Bcl-xL with SG20 in the presence of membrane environment. However, it turned out that due to the hydrophobicity of lipid tail, DPC lipid molecules which were not incorporated into lipid micelles could also interact with the hydrophobic groove of Bcl-xL, thus preventing the binding of SG20 to Bcl-xL. Hence, we concluded that simple addition of lipid micelles

to the sample might not be a proper way to study the interaction between the protein and the peptide in the presence of membrane. For structural investigation of Bcl-xL interacting with SG20 in membrane environment, it is necessary to develop other ways of generating membrane mimicking environment which would not interrupt the binding of peptide to the hydrophobic pocket of Bcl-xL.

In summary, we have investigated the structural transition of Bcl-xL in the presence of OM detergent micelles, and its functional implication in the regulation of mitochondrial calcium homeostasis. Structural plasticity of Bcl-xL in the membrane mimicking environment might have a regulatory role in calcium homeostasis of mitochondria, even though the exact mechanism of such regulation is not clearly understood. Also, we have conducted the structural investigation of Bcl-xL binding to VDAC2 N-terminal peptide using NMR spectroscopy. Over the decades, it has been well established that BH3 domains of pro-apoptotic Bcl-2 family proteins interact with Bcl-xL to play its pro-apoptotic function. Compared to Bcl-xL/BH3 interaction, it is still unclear whether the interaction between VDAC N-terminal region and Bcl-xL has a positive or negative regulatory function on the channel activity of VDAC, as well as on the mitochondrial apoptotic pathway. In addition, even though VDAC N-terminal region has been shown to interact with Bcl-xL, the binding mechanism of the VDAC N-terminal segment and Bcl-xL at molecular level remains elusive. Our preliminary studies on the structural investigation of the interaction between Bcl-xL and VDAC2 N-terminal region provide insights into the structural and molecular mechanism of how Bcl-xL and VDAC regulate each other to modulate the mitochondrial homeostasis and cell fate.

Future work

First of all, as we have been focusing on the structural characteristic of VDAC2 N-terminal region, it would be interesting to conduct structural investigation on the N-terminal segment of other isoforms of VDAC – VDAC1 and VDAC3. By calculating the structure of VDAC1 and VDAC3 N-terminal peptide bound to Bcl-xL, we would be able to compare the complex structure of Bcl-xL/VDAC 1,2,3 N-terminal peptides. It would be also meaningful to find out whether the interactions involved in the binding of SG20 to Bcl-xL, such as salt bridge formation and π - π stacking interaction, are conserved in the N-terminal regions of other VDAC isoforms.

Additionally, mutagenesis studies could be performed to validate the interactions between Bcl-xL and SG20 observed from our model structure. For example, Bcl-xL constructs with amino acid substitutions such as E129A or R139A which could inhibit the salt bridge formation, or Y101A to interrupt π - π stacking interaction, could be generated to examine its binding with SG20 peptide. By performing such mutagenesis experiments, we could find out whether these interactions are crucial in binding of SG20 to Bcl-xL. Alternatively, if the full structure of Bcl-xL/SG20 (and possibly N-terminal peptides of other VDAC isoforms) complex could be determined by NMR spectroscopy in the future, it would validate the interaction between Bcl-xL and the VDAC N-terminal region more strongly than a structural model does.

Lastly, we could investigate more to design a proper platform to study the interaction between Bcl-xL and the VDAC N-terminal peptide in the presence of a membrane mimicking environment. We have tried to use DPC micelles to generate such an environment, but due to the lipid molecules binding to the hydrophobic pocket of Bcl-xL, binding of SG20 to Bcl-xL was interrupted. To avoid such incidence, we could use the lipid bilayers as a membrane mimicking environment. Even though using a lipid bilayer for NMR structural studies is challenging due to its large size, the lipid bilayers can maintain the interaction between Bcl-xL and VDAC N-terminal peptide while providing proper membrane environment.

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