The role of the mitochondrial membrane system in apoptosis:

The influence of oxidative stress on membranes and their interactions with apoptosis-regulating Bcl-2 proteins

Martin Lidman
To my family
Table of contents

1 List of publications iii
2 Abstract iv
3 List of abbreviations v
4 Introduction 1
   4.1 Biological background 1
      4.1.1. Programmed cell death 1
   4.1.2. Mitochondrion 4
   4.1.3. Lipids 7
      4.1.3.1. What are lipids? 7
   4.1.3.2. Molecular properties influencing the membranes 9
   4.1.3.3. Heterogeneity of biomembranes 12
   4.1.4. Oxidation of mitochondrial membranes 13
   4.1.5. Regulation of the mitochondrial apoptosis by the Bcl-2 protein family 16
   4.2 Aim of this thesis 22
5 Materials and methods 23
   5.1 Differential Scanning Calorimetry (DSC) 23
   5.2 Nuclear Magnetic Resonance (NMR) spectroscopy 26
      5.2.1 Solid state NMR: static and MAS NMR approaches 30
   5.3 Circular Dichroism (CD) spectroscopy 33
      5.3.1 Some properties of waves 33
   5.3.2 Secondary structure of proteins 35
   5.4 Fluorescence spectroscopy 37
   5.5 Cell free protein synthesis 39
6 Summary of papers 41
   6.1 Paper I – Impact of oxidized phospholipids on the structural and dynamic organization of phospholipid membranes: a combined DSC and solid state NMR study 41
   6.2 Paper II – The oxidized phospholipid PazePC modulates interactions between Bax and mitochondrial membranes 44
   6.3 Paper III – Reconstitution of the anti-apoptotic Bcl-2 protein into lipid membranes and biophysical evidence of its detergent-driven association with the pro-apoptotic Bax protein 47
   6.4 Paper IV – Membranes and their lipids: A molecular insight into their organization and function 51
   6.5 Paper V – The oxidized phospholipid PazePC promotes the formation of Bax pores in mitochondrial membranes 53
7 Main findings and outlook 57
8 Acknowledgements 59
9 References 62
1 List of publications

This thesis is based on the following papers:


IV: **Lidman, M.**, Wallgren, M., Gröbner, G. ”Membranes and their lipids: a molecular insight into their organization and function” New Developments in NMR No. 3; Advances in Biological Solid-State NMR: Proteins and Membrane-Active Peptides, Edited by Frances Separovic and Akira Naito; Published by the Royal Society of Chemistry February 2014, Print ISBN: 978-1-84973-910-8.

2 Abstract

Apoptosis is a crucial process in multicellular organisms in sculpting them, especially during embryogenesis. In addition, apoptosis is responsible for the clearance of harmful or damaged cells which can otherwise be detrimental to the organism. The Bcl-2 family proteins are key players in the regulation of the intrinsic pathway of the apoptotic machinery. This family consists of three subfamilies with B-cell CLL/lymphoma 2 (Bcl-2) protein itself representing anti-apoptotic members, the Bcl-2-associated X protein (Bax), and pro-apoptotic BH3-only signaling proteins. The interplay between pro- and anti-apoptotic proteins on the mitochondrial membranes is central to the balance between the life and death decision of whether the membrane should be permeabilized or not. The cytosolic Bax protein can upon cellular stress translocate to the mitochondrial membrane where it can either carry out its action of forming homo-oligomers that cause outer membrane permeabilization or be inhibited there by the anti-apoptotic membrane protein Bcl-2. Upon mitochondrial outer membrane permeabilization (MOMP) apoptogenic factors leak out from the intermembrane space (IMS) of the mitochondria, leading to caspase activation and ultimately cell death. A common stress signal initiating apoptosis is an increased formation of reactive oxygen species (ROS in the mitochondria, who can cause oxidative damage to lipid membranes. This membrane damage presumably influences the lipid landscape and the membrane features and hence the interactions of the Bcl-2 family proteins with each other and the mitochondrial outer membrane (MOM). To investigate the significance of membrane oxidation on the behavior of the Bcl-2 family proteins, especially Bax, synthetically produced oxidized phospholipids (OxPls) were incorporated in MOM-mimicking vesicles. Differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD) spectroscopy revealed a major perturbation in membrane organization in the presence of OxPls. These changes in membrane properties increase the affinity of Bax to its target membrane and enable its partial penetration and formation of pores, as fluorescence leakage assays confirmed. However, in the absence of BH3-only proteins these pores are not sufficiently large for the release of apopototic factors such as cytochrome C (CytC). To understand the inhibition of Bax by the full-length Bcl-2 protein, suitable detergent solubilizing conditions were carefully chosen to enable the measurement of their direct binding to each other outside the membrane, by an antimycin A: fluorescence assay. The observed protein-protein interaction was confirmed by surface plasmon resonance (SPR). An established protocol for the reconstitution of Bcl-2 into stable proteoliposomes now paves the way for structural studies of this key protein, in its membrane environment near physiological conditions; information essential for understanding its function, on a molecular level, and its potential as a cancer drug target.
# 3 List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>Brij-35</td>
<td>Polyoxyethylene-(23)-lauryl-ether</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>C_p</td>
<td>Heat capacity under constant pressure</td>
</tr>
<tr>
<td>CP</td>
<td>Cross polarization</td>
</tr>
<tr>
<td>CS</td>
<td>Contact sites</td>
</tr>
<tr>
<td>CSA</td>
<td>Chemical shift anisotropy</td>
</tr>
<tr>
<td>CytC</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>ΔH</td>
<td>Change in enthalpy</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ENDOG</td>
<td>Endonuclease G</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FLIP</td>
<td>Fluorescence loss in photobleaching</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GUVs</td>
<td>Giant unilamellar vesicles</td>
</tr>
<tr>
<td>HAZpC</td>
<td>1-hexadecyl-2-azelaoyl-(sn)-3-phosphatidylcholine</td>
</tr>
<tr>
<td>hr</td>
<td>Hot rod</td>
</tr>
<tr>
<td>IDPs</td>
<td>Intrinsically disordered proteins</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
</tr>
<tr>
<td>INEPT</td>
<td>Insensitive nuclei enhanced by polarization transfer</td>
</tr>
<tr>
<td>LUVs</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>MAS</td>
<td>Magic angle spinning</td>
</tr>
<tr>
<td>mGPx4</td>
<td>Mitochondrial glutathione peroxidase 4</td>
</tr>
<tr>
<td>MLVs</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>MOM</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OxPls</td>
<td>Oxidized phospholipids</td>
</tr>
<tr>
<td>Pa</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PazePC</td>
<td>1-palmitoyl-2-azelaoyl-(sn)-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PLA2s</td>
<td>Phospholipases A2</td>
</tr>
<tr>
<td>PGPC</td>
<td>1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPE</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>POPVC</td>
<td>1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PoxnoPC</td>
<td>1-palmitoyl-2-(9'-oxononanoyl)-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SUVs</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>tBid</td>
<td>Truncated Bid</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TMCL</td>
<td>1,1',2,2'-tetra-myristoyl-cardiolipin</td>
</tr>
</tbody>
</table>
4 Introduction

4.1 Biological background

4.1.1. Programmed cell death

Apoptosis is a form of programmed cell death (PCD) and is important in embryogenesis and normal development as well as in aging and removal of diseased or damaged cells. In the absence of apoptosis people would develop skin between the fingers and toes as well as sets of both male and female genitalia. By removing damaged and harmful cells apoptosis protects us from diseases. Therefore, any malfunctioning of the apoptotic machinery can cause severe conditions such as cancer, autoimmune and neurodegenerative disorders [1].

Multicellular organisms are complicated structures which have gained much of their functionality due to the complexity of their individual cells. These eukaryotic cells possess a range of intracellular membrane systems, which compartmentalize regions of the cells into organelles with highly specific functions. This level of cellular complexity has opened up possibilities to develop different types of tissues and organs in the bodies of animals over the course of evolution. Some structures developed early in the evolution and became obsolete at later stages. This is still apparent today since rudimentary structural features often are still visible during earlier stages of embryogenesis but removed at later stages. Similarly, we can see deletions of structures during the metamorphosis of insects and amphibians.

In the 1960’s and 70’s it gradually become clear that cells can die in a controlled fashion which could be called programmed cell death, as opposed to the more traumatic and uncontrolled necrosis. Kerr et al. suggested the term “apoptosis” in 1972 for one form of PCD, which was characterized by a blebbing of the membranes analogous to the dropping of petals or leaves [2]. In 1973 Schweichel and Merker classified three morphological forms of PCD as shown in Figure 1 [3, 4]. According to this classification apoptosis was a type 1 PCD. While other forms of PCDs exist, apoptosis and autophagy are by far the most common ones. The main distinction between these two types is the fragmentation process (or blebbing) and early heterophagocytosis of the dying cell in apoptosis (type 1 PCD), while in autophagy (type 2 PCD) the dying cell is mainly breaking down itself by encapsulating whole organelles in vacuoles which then fuse with lysosomes [3].
Figure 1. There are three main types of morphological modes in which a healthy cell can undergo programmed cell death (PCD). Type 1 and type 2 are by far the most common, and in many cases they can inhibit each other. (adapted from Clarke 1990 [3]; and from Chiara Maiuri 2007 [5]).

The third type of PCD is called non-lysosomal vesiculate degradation, or cytoplasmic degeneration. This type shares some characteristics with necrotic cell death, such as swelling of the mitochondria and the endoplasmic reticulum, but this PCD type does not form blebs or internal vacuoles. Type 2 and type 3 PCDs are also accompanied by late heterophagocytosis [3].

Variations of PCDs can share aspects from either one of these different types of PCD, but in general cells will fall into one of these morphological modes [3]. However, which mode is used in different contexts is not really obvious. For example there are cell types which can be deleted by type 1 PCD during early development but upon aging only by type 2 PCD; in other tissues it can be the other way around [3]. Exactly which factors determine the specific mode of dying is exceedingly complex and involves an abundance of different interaction partners [1,
Apoptosis and autophagy are to some extent exerting crosstalk between each other, which often result in mutual inhibition with the consequence that one of the two processes will be predominant [5]. This can be important for cell survival because autophagy can also sometimes act in a cytoprotective way [5].

Figure 2. There are at least three pathways which can be involved in the onset of apoptosis; the death receptor pathway, the intrinsic pathway and the perforin/granzyme pathway (adapted from Elmore 2007 [1], and Chiara Maiuri 2007 [5]).

To further underline the complexity of PCD, at least three different pathways for apoptosis are known so far as summarized in Figure 2; and they are presumably all interconnected to some extent [1]. Both, the death-receptor pathway (often referred to as the extrinsic pathway) and the perforin/granzyme pathway are receiving death-stimuli from the outside of the cell, while the intrinsic or mitochondrial pathway – the main topic of the research presented in this thesis – predominantly receive death-signals originating from within the cell. All three pathways are ultimately stimulating both caspase dependent and caspase independent lysis of the cell, but are also to different extent converging at the mitochondrion to activate BH3-only proteins at an even earlier time point.
This thesis is not aiming to elucidate exactly every twist and turn of the interplays of all these processes. The focus of this thesis is at the mitochondrion where the outer membrane is the target for pro- and anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) family proteins, which are the gate keepers in the regulation of mitochondrial apoptosis [7-11]. The main function of the Bcl-2 family is to control the integrity of the mitochondrial outer membrane (MOM) and consequently the release of IMS proteins acting as apoptogenic factors when released into the cytosol [7-11]. The Bcl-2 protein family itself consists of anti-apoptotic multidomain proteins such as Bcl-2 itself, pro-apoptotic multidomain proteins (e.g. Bcl-2 associated X protein (Bax) and pro-apoptotic BH3-only proteins such as Bid (for an overview see Figure 3)).

Figure 3. The Bcl-2 protein family. Overview of the various subclasses: anti-apoptotic (pro-survival) multidomain proteins such as Bcl-2 itself; pro-apoptotic (anti-survival) multidomain proteins such as Bax (focus in this thesis) and pro-apoptotic BH3-only proteins. Transmembrane regions are denoted as TM and conserved Bcl-2 homology domains are denoted as BH in each subclass (adapted from J.C Martinou 2011 [7]).

Bcl-2 was the first identified mammalian oncogene whose gene product was able to block the intrinsic pathway of apoptosis [6]. The Bcl-2 protein is permanently anchored into the MOM where it is protecting the cell from committing suicide by preventing pro-apoptotic multidomain members like Bax and Bak, from oligomerizing on and permeabilizing the membrane. The pro-apoptotic multidomain proteins do in turn get their kill-signal upstream from the signaling BH3-only proteins.

4.1.2. Mitochondrion

Mitochondria are essential organelles of diverse functionality in eukaryotic cells. They are not only the cell’s ATP generating powerhouses but also the sites where various metabolic processes occur, such as reactions belonging to the fatty acid and the amino acid metabolism. However, in addition to obviously beneficial functions they do also have a second, darker function, namely to execute cells via programmed cell death [1, 3, 5, 7, 12, 13].
The mitochondrion itself is an organelle which may, according to the endosymbiotic theory, have been introduced into eukaryotic cells at an early point in evolution where a single celled organism engulfed another one [14]. This type of reasoning has been applied to many other organelles, but in the case of mitochondria it is most certainly true after analysis of the mitochondrial genome, which is of different origin from the nuclear origin [14]. The events that led to the assimilation of mitochondria into another cell happened very early in evolution, and it is not clear if the nucleus or the mitochondrion was first introduced into eukaryotes [14].

In mammals there are about 1500 different proteins associated with mitochondria of which only a handful (mostly respiratory chain polypeptides) are encoded in the mitochondrial DNA [15, 16]. Mitochondria provide a very effective way to produce energy, and it might seem as this would be the most important aspect of mitochondria, although it has been questioned that increased ATP-production would have been a reason for the original incorporation into eukaryotes [14]. Mitochondria also have other important roles in metabolism of lipids and amino acids, as well as calcium signaling, cell growth and apoptosis [16]. Mitochondria usually display approximately similar sizes as bacteria, ca 0.5-1 µm in diameter. However, in some cases they can form very large interconnected webs consisting of large numbers of mitochondria [7, 17-21]. Mitochondria can also be transported to distinct regions of a cell, notably in neurons were mitochondria mainly locate to the synaptic regions of the axons [17, 20]. The number of mitochondria in a cell does of course also differ depending on the needs of that specific tissue. Muscles can for instance accommodate several thousand mitochondria.

**Figure 4.** Schematic picture of a mitochondrion. The mitochondrial outer membrane and inner membrane are separated by the intermembrane space. The matrix indicates the interior of the mitochondrion, containing granules, ribosomes and DNA. Cristae describe the many invaginations of the inner membrane (adapted from "Animal mitochondrion diagram" by Mariana Ruiz Villarreal).
Adjustment of the amount of mitochondria, mitochondrial morphology and subcellular distribution via fusion and fission events is a complete research area in itself, but have also been suggested to play roles in apoptosis [7, 18-20, 22].

Mitochondria have an outer and an inner membrane (see Figure 4). The MOM is the interface to the rest of the cell and the intermembrane space (IMS), and the inner mitochondrial membrane (IMM) is the interface between the IMS and the matrix. The inner membrane has deep folds called cristae which considerably increase the surface area of the inner membrane. The two membranes also meet at contact sites (CS).

In the matrix we can find the mitochondrial DNA and it is also the location where metabolic processes like the Krebs cycle and β-oxidation of fatty acids take place. The inner mitochondrial membrane is incorporated with the electron transport chain proteins, where Cytochrome C (CytC) plays a role as an electron carrier. CytC is an IMS protein which is loosely associated to the inner mitochondrial membrane particularly due to its interaction with the mitochondria-specific phospholipid cardiolipin (CL) [23], which is enriched at the IMM and especially at the CSs [24]. CytC functions as an apoptogenic factor when it leaks out into the cytosol. And this step is of particular importance for apoptosis since it is one of the building blocks in the apoptosome complex. The IMS also contains other apoptogenic factors like apoptosis-inducing factor (AIF), endonuclease G (ENDOG), HtrA2/Omi, SMAC/Diablo, with all of them propagating apoptotic signals upon release into the cytosol [1, 5].

CytC actually facilitates its own dissociation from the IMM in the early stages of apoptosis and in addition to function as a component of the apoptosome, it also assists in the recruitment of members of the Bcl-2 family to the mitochondrial surface, as will be discussed in forthcoming section about peroxidation of lipids.

The main players in the mitochondrial pathway of apoptosis are the proteins of the Bcl-2 family. Their main function is the tight regulation of the integrity of the MOM and their function will be discussed in detail further below. In addition there are also other mitochondria-associated proteins which are involved in apoptotic functions, such as the mitochondrial permeability transition pore (PTP) complex; a complex which also resides at the MOM, and most likely can also interact with the Bcl-2 protein [12].
4.1.3. Lipids

4.1.3.1. What are lipids?

In general, lipids are hydrophobic substances which are defined by their solubility in a non-polar solvent [25]. Lipids thus includes fabulous fractions of different classes of molecules with subclasses such as long chain monohydroxy alcohols, free fatty acids, phospholipids, glycolipids, triglycerides and steroids, which can form different structures such as lipid bilayers (see Figure 5).

![Figure 5](image)

**Figure 5.** Schematic picture of a biological membrane with its typical two-dimensional fluid-like leaflet features and constituents. The basic structure is a highly dynamic environment built by lipids (grey) and sterols like cholesterol (yellow) which undergo fast intra- and intermolecular motional processes. This environment has embedded integral membrane proteins (dark red) and is surface associated with peripheral proteins (light red), which provide the membrane with its main biological functionality (diagram adapted with permission from an original drawing by Vanessa Kunkel).

Besides acting as structural elements there are numerous other functions of lipids. Lipids are due to their inherent reduced state well suited for energy storage (triglycerides), and some lipids are functioning as signaling molecules (steroidal hormones), or even antioxidants (vitamin A, D). Lipidomics have set out to study the diversity of structure and function of lipids in living organisms; not a small undertaking given that due to combinatorics of ‘building blocks’. For example, by varying the different acyl chains and head groups of phospholipids, it could be possible to produce up to 100 000 different lipids [26], and around 5% of eukaryotes genomes are dedicated to the synthesis of thousands of different lipids [27].
Biomembranes found in our cells are two-dimensional leaflets, called bilayers, which to a large extent are built up by amphiphilic lipid molecules (typical structure see Figure 6). These lipids possess long hydrophobic chains which cluster together in the hydrophobic interior of the membrane and their hydrophilic headgroups form a polar interface on both sides of the bilayer and face out towards the aqueous biological environment.

Some additional components of the membranes, like cholesterol and steroidal hormones are incorporated mainly in the membrane’s hydrophobic core. Apart from the purely lipid component most biomembranes are occupied by large amounts of proteins, which can be approximately a 1:1 lipid-to-protein ratio [27, 28]. These protein are either transmembrane integral proteins or peripheral proteins, the latter which are only attached to the membrane interfaces, and some proteins are even bearing post-translationally modified fatty acid anchors [29].

The most abundant lipids in biomembranes are glycerophospholipids, as seen in Figure 5. These molecules have two fatty acids linked by ester bonds to a glycerol molecule at the sn-1 and sn-2 positions, whereas at the sn-3 position of the glycerol there is a phosphate group with varying moieties, which defines the type of phospholipid (see Figure 5). The most common headgroups among phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylerine (PS), phosphatidylinositol (PI) and phosphatidylic acid (PA). If no phosphate is attached to the sn-3 position, the molecule is simply called diacylglycerol (DAG) [27]. Depending on the headgroup and pH of the medium the headgroup may have a neutral, negative or zwitterionic charge. In some cases, the sn-1 position has an ether-linkage to the hydrophobic chain, as typical for the lipid class of plasmalogens. The ‘sn’ (for “stereospecifically numbered”) naming convention of glycerolipids comes from the fact that glycerol is a prochiral molecule, and that other rules like L/D or R/S can result in mixed up names which do not reflect the common nature of glycerophospholipid.

Cardiolipin (CL) is another phospholipid which is quite common in bacteria, but among eukaryotes it is only found in the mitochondria. The CL lipids (IUPAC name "1,3-bis(sn-3’-phosphatidyl)-sn-glycerol") are very special, since they contains four acyl chains and three glycerols in their headgroup region; one glycerol is linked via phosphates at its sn-1 and sn-3 positions to the sn-3 position of two separate DAG “building blocks”, as can be seen in Figure 6 that shows tetramyristoyl-cardiolipin (TMCL) as an example.

Most PC molecules carry mixed fatty acids, often with a saturated fatty acid at the sn-1 position and one cis-unsaturated acyl-chain at its sn-2 position, which enable the PC lipids to form bilayers which are in their biologically active fluid (liquid-crystalline) state at room temperature [27, 30]. It has been suggested that unsaturations in the sn-2 chain have a larger disordering (more fluid-like) effect on the membrane than any unsaturations present in the sn-1 chain [30].
In the papers included in this thesis, the MOM close to the mitochondrial CS have been mimicked by liposomes consisting of synthetic lipids at a composition that reflect the types and the ratio as found in naturally occurring mitochondria [24]. Lipid components used in these liposomes where 1-palmitoyl-2-oleoyl-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-3-phosphoethanolamine (POPE) and TMCL, as well as the OxPl 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazePC). The liposomes used in the papers typically contained molar ratios of 43/36/21 POPC/POPE/TMCL to represent mitochondria prior to oxidative stress (see Figure 6). The effect of oxidative stress on the mitochondrial membranes was simulated by exchanging the most common lipid component POPC against its oxidized derivative PazePC giving lipid ratios of 43-x/36/21/x POPC/POPE/TMCL/PazePC (see Figure 7).

**Figure 6.** In this study an *in vitro* liposome system was used to mimicking the MOM near their CS. The phospholipid species and their ratios used in liposomes reflect the main lipid composition *in vivo*. Following model lipid species were used (molecular structure depicted; Avanti Lipids, Inc.): (POPE, POPC and TMCL).

### 4.1.3.2. Molecular properties influencing the membranes

Individual lipids are experiencing relatively large motional freedom in the plane of the bilayer since mainly non-covalent weak forces holding the lipids in the membrane together. As long as the hydrophilic headgroup is oriented towards the polar exterior, the amphiphilic lipids can rapidly rotate along an axis perpendicular to the plane of the membrane, and they can diffuse laterally along the membrane.
Also intramolecular reorientations of long unsaturated carbon chains with the chain segments moving between \textit{trans-gauche} configurations on a fast timescale [31].

Bilayers built up from phospholipids are, in physico-chemical terms, liquid-crystalline systems which possess a complex phase behaviour. The most important variables for their phase behavior are their dependence on temperature (thermotropic phase behaviour) and hydration (lyotropic phase behaviours). Since membranes \textit{in vivo} are sufficiently hydrated, most physico-chemical studies to characterize the basic features of lipid based membranes investigate phase transitions as a function of temperature [32]. As seen in the differential scanning calorimetry (DSC) derived thermogram in Figure 7, two distinct phase transition events are visible for fully hydrated multilamellar vesicles formed by a single phosphatidylcholine species.

![DSC Thermogram](image)

**Figure 7.** Typical phase transition events of lipid bilayers as visible in a DSC profile (thermogram) of vesicles made of a single phosphatidylcholine species. The thermal event at lower temperature (T\textsubscript{p}) indicates a phase change of the membrane from its gel-phase into a typical ripple gel phase; a phenomenon called the pretransition event. The main thermal event occurring at higher temperature (T\textsubscript{M}) indicates the melting of the fatty acid chains. This main transition process converts the lipid bilayers into their liquid-crystalline state which is the typical one for membranes \textit{in vivo} (adapted with courtesy from A. Dingeldein).
The lamellar liquid crystalline ($L_\alpha$) phase, which is arguably of greatest importance in biology, exists at a higher temperature. At lower temperatures a there is a shift towards the lamellar gel phase with tilted fatty acid chains ($L_{\beta'}$) and at intermediate temperatures a rippled gel phase ($P_{\beta'}$) predominates. The main transition event is the one into the liquid-crystalline phase, which is caused by the melting of the fatty acid chains. The transition at low temperatures into the ripple phase is connected with an increase in rotational freedom, mainly the headgroup region [33]. Depending on the overall shapes of phospholipids, namely the lengths and saturation degree of their chains, and the type, size and charge of their headgroups as well as the lipid composition, a variety of further phases can also exist. Those include the liquid ordered phases (often in the presence of sterols) and non-tilted crystalline gel phase [32]. The thermotropic phase behavior of the complex model membranes used in this thesis has been probed by nuclear magnetic resonance (NMR) spectroscopy and DSC, as described in their respective method sections.

PC lipids are perfect for forming bilayers owing to the roughly equal cross-sectional areas of headgroup and hydrophobic acyl chains, respectively [34]. For lyso-lipids, with only one acyl chain, there may be a comparatively more narrow cross-sectional area on the lipid side, which imposes a positive surface curvature and a tendency to form micelles instead of bilayers. By comparison, the presence of phospholipids like PE and CL, which have relatively smaller headgroup to acyl chain cross-sectional areas, leads to a negative curvature on the membrane, which can result in an inverted hexagonal ($H_{II}$) phase. These observations are true for isolated lipids, but it should be noted that most biological membranes, including bilayers, contain high amounts of non-bilayer lipids [34].

As can be seen in Figure 6 the headgroup of PE is smaller than PC, and can therefore impose a negative curvature on membranes, but it is still the second most common membrane lipid after PC. CL has four acyl chains instead of two and a rather small headgroup. The CL headgroup is however negatively charged, providing headgroup repulsion which allows it to form a lamellar $L_\alpha$ phase, though in the presence of divalent cations, which screens off the charge, CL has a high tendency to impose negative curvature and also form inverted hexagonal phases [34]. In lipid mixtures which have formed a bilayer with lipids preferring a certain curvature in both leaflets, the membrane is said to be frustrated [34]. A frustrated bilayer can be said to store energy in the form of curvature stress, which can be released for example by the formation of inverted hexagonal phases, incorporation of peripheral membrane proteins or be used as a source of energy for membrane proteins undergoing conformational changes [34]. The curvature stress of a bilayer can be increased by a reduction in lipid headgroup size or by increasing the acyl chain unsaturation, and vice versa [34, 35]. It has been suggested that when lipids are peroxidized, this can result in the complete reversal of the oxidized sidechain towards the headgroup region of the membrane. This could be viewed as an increase
in the size of the headgroup leading to a modulation of curvature stress [36]. A more general model for how proteins sense lipid membranes is by the so-called lateral pressure profiles, which is a parameter that is dependent on the depth into the membrane. In this model, an increased pressure in the acyl chain region of the membranes is correlating with an elevated curvature stress [34].

4.1.3.3. Heterogeneity of biomembranes

Throughout the cell exists a great heterogeneity in the distribution of lipids, with the membranes of the various organelles have different composition of lipids [27]. The main location of lipid synthesis in the cell is the endoplasmic reticulum (ER), producing a majority of the cells’ phospholipids and cholesterol, as well as many other structural and signaling lipids [27]. Some lipids are also generated in the Golgi apparatus (e.g. sphingolipids), and mitochondria (e.g. CL). Lipids are also transported around in the cell, for example cholesterol and ceramide produced in the ER, which are precursors to other sterols and complex sphingolipids, respectively. These other sterols and complex sphingolipid are however not found at high levels in the ER [27].

Within a particular biomembrane there can also be heterogeneity, both across the leaflets and laterally in the plane of the membrane. The two leaflets of the membrane do not necessarily need to have the same composition of lipids, and indeed it can be of great biological importance that they are actually dissimilar. For example, PS is only found in the inner leaflet of the plasma membrane in healthy cells, but during apoptosis this lipid is translocated to the outer leaflet where it is acting as a recognition signal for heterophagocytosis, and as a propagation signal in blood coagulation [27, 37]. The lipid composition at the leaflets of the ER is symmetric, while at the Golgi and plasma membrane P4 ATPases translocate PS and PE to the cytosolic faces [27].

Heterogeneity of the lipid composition in the lateral dimensions of the membrane, producing distinct lipid domains suspended in a sea of other lipid molecules, has been the subject for a lot of attention in recent years. The lipid raft hypothesis suggests that there are preferential interactions between groups of lipids which bring them together at localized ‘rafts’ in the lipid membrane [27, 38]. Such interactions can include hydrophobic mismatch; as long as the lipid is dispersed it will expose more hydrophobic surface towards the hydrophilic surrounding compared to if it was surrounded by lipids which obscure the hydrophobic surfaces [38]. Rafts containing large quantities of sphingolipids and cholesterol, which preferentially interacts with each other, have a phase which is more ordered than the surrounding membrane [38]. Since there are proteins which have preferential interactions to lipid membranes in a certain phase it has been suggested that these proteins would also selectively target distinct domains in lipid membranes, and
indeed some proteins like caveolin have been shown to have a lipid ordering effect [27].

4.1.4. Oxidation of mitochondrial membranes

Due to the various metabolic processes at the mitochondrion, particularly the oxidative phosphorylation coupled to mitochondrial electron transport chain, with the accompanying leakage of electrons, mitochondria are an important source for intracellular reactive oxygen species (ROS) [39]. ROS is not a single species, and one important subclass is derived from O$_2$ by its stepwise single electron reduction firstly to a superoxide anion (O$_2^-$), and secondly to hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ may then undergo Fenton-chemistry, which in a reductive step produces a hydroxyl radical (OH$^-$), which is especially important for lipid peroxidation, as well as the formation of a hydroperoxyl radical (HOO$^-$) which is produced in an oxidative step [39-41]. ROS can damage DNA, proteins and lipids, and cells are naturally trying to counteract these detrimental effects with radical scavenging enzymes like superoxide dismutases and peroxidases. Antioxidants also help keeping ROS under control, and vitamin E is an example of an hydrophobic molecule that can counteract radical reactions in lipid membranes [40]. Once oxidative damage has been done on the mitochondrial membranes there are some countermeasures to undo the damage, such as the actions of the mitochondrial glutathione peroxidase 4 (mGPx4) which reduces fatty acid hydroperoxides [42], and phospholipases A2 (PLA2s) which release free fatty acids from the sn-2 carbon of glycerophospholipids. PLA2s have also been suggested to promote permeabilization of mitochondrial membranes by altering the lipid composition [43]. Increasing intracellular levels of ROS has long been known to be a hallmark of apoptosis, and modifications of ROS levels have been linked to resistance to cancer chemotherapy [11, 12, 44].

Peroxidation of CL has been identified as an early apoptotic event, preceding the release of CytC [41, 45, 46]. During apoptosis the CytC/CL complexes can be formed and exert peroxidase activity on CL [41, 47]. The oxidation of CL has been suggested as an explanation for dissociation of CytC from the IMM as well as for the redistribution of CL from the IMM to the CS. CL can then start to interact with truncated Bid (tBid) and other Bcl-2 proteins [41]. Although many OxPls are produced enzymatically, a significant amount can also be produced non-enzymatically [48]. CL constitutes a significant part of the lipids in the mitochondria, still the most abundant lipid in the MOM, CS and IMM is PC [24], and also other lipids account for a significant fraction of the mitochondrial lipids. It should therefore come as no surprise that other lipids than CL can be oxidized and potentially play a role in apoptosis.

Oxidation of CL has attracted a significant amount of attention with regards to its interaction with CytC, which also facilitates the oxidation of CL. Recently there has been an increased interest for the effects that other OxPls such as PazePC may
have on CytC’s structure and affinity for lipid bilayers [49]. Furthermore, it has been recognized that many other oxidized lipids besides CL may be involved in apoptosis [48]. Indeed, it has been shown that oxidation of PC, PE and PS is associated with apoptosis in endothelial cells [50]. In the 90’s it was shown that the PS is transported from the inner leaflet to the surface of the plasma membrane in apoptotic cells, where it serves as an “eat-me” signal, and more recently it has been demonstrated that some fraction of PS may be oxidatively damaged [51]. Furthermore, the presence of oxidized PC lipids at the surface of apoptotic cell has been proposed to play a role in the recognition by macrophages [48].

Apoptosis can be induced in pulmonary epithelial-like A549 cells by treatment with the truncated phospholipid 1-palmitoyl-2-(9’-oxononanoyl)-sn-glycero-3-phosphocholine (PoxnoPC), which carries an aldehyde on its sn-2 chain [52]. The slightly more oxidized PazePC instead carries a carboxylic acid moiety on its truncated chain. The nine carbon atom long azelaoyl fragment is not a product of β-oxidation, and has for a long time been a known marker for oxidative damage [53]. The azelaoyl fragment on the sn-2 chain is very common in diacyl glycerophospholipids due to the fact that the linoleoyl (C_{18:2}) and linolenoyl (C_{18:3}) are the most common esterified polyunsaturated fatty acids [53]. Linoleoyl and linolenoyl, both preferentially cleaved at the double bond between carbon 9 and 10, can produce both carboxylic acid species and aldehyde species [54]. In papers I, II and IV the OxPl PoxnoPC and PazePC, as seen in Figure 8, have been used to simulate the oxidation of mitochondria by replacing a fraction x of POPC with either OxPl species, where the double bond between carbon 9 and 10 has been oxidized to either an aldehyde or carboxylic acid, respectively.

**Figures**

**Figure 8.** MOM mimicking liposome system doped with OxPls to imitate the situation under intracellular oxidative stress conditions. Either the POPC-derived PazePC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine or PoxnoPC (1-palmitoyl-2-(9’-oxo-nonanoyl)-sn-glycero-3-phosphocholine) was used and incorporated into the POPC/POPE/TMCL based lipid bilayers up to 10 mol%. Structures for both OxPls are depicted (Avanti Polar Lipids, Inc.).
A truncated phospholipid which is very closely related to PazePC is 1-hexadecyl-2-azelaoyl-sn-3-phosphatidylcholine (HAzPC), which only differs from PazePC in that it carries an ether functionality on its sn-1 chain instead of an ester bond. It has been shown to promote apoptosis via the intrinsic mitochondrial pathway both in cells and in isolated mitochondria [53]. Noteworthy, it has been observed that HAzPC is the most effective among several truncated phospholipids, including PazePC, and lyso-lipids tested [53]. Among alkyl glycerophospholipids which occurs in oxidized low density lipoprotein, HAzPC is one of the most common [55] and can be transported within the body, though it should be noted that diacly species are much more common than glycerophospholipids carrying alkyl on the sn-1 position. Exogenous HAzPC localizes to mitochondria, induces mitochondrial swelling and expulsion of apoptogenic factors; a process which can be counteracted by overexpression of the anti-apoptotic protein Bcl-xL and aided by tBid [53, 56].

Similarly, there exist also shorter analogues of truncated phospholipids with a terminal aldehyde group, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), or a terminal carboxylic acid group, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), that have been show to inhibit growth and induce apoptosis in isolated cells [57]. The fact that OxPls with a range of different chain lengths, carrying either aldehyde or carboxylic acid moieties, have an apoptotic effect indicates that they may similarly affect mitochondria. A common characteristic among these lipids may very well be that they are affecting membrane properties of mitochondria.
4.1.5. Regulation of the mitochondrial apoptosis by the Bcl-2 protein family

The intrinsic or mitochondrial pathway of apoptosis is of great importance in PCD. Not only does it end a cellular life but is also communicates with other pathways of apoptosis as discussed in section 6.1.1. A crucial event in the intrinsic apoptotic pathway is the perforation of the outer mitochondrial membrane whereupon apoptotic factors, most prominently CytC, leak out from the IMS to the cytosol. This event occurs when the balance of pro- and anti-apoptotic Bcl-2 family proteins are shifted toward the pro-apoptotic ones with the consequence of a cellular death decision.

The Bcl-2 family can be divided into various sub-classes, as seen in Figure 3. There are anti-apoptotic multidomain members (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1/Bfl-1), pro-apoptotic multidomain members (e.g. Bax, Bak, Bok), and pro-apoptotic BH3-only proteins (e.g. Bid, Bim/Bod, Bad, Bmf, Bik/Nbk, Blk, Noxa, Puma/Bbc3, and Hrk/DP5) [7]. Most of the multidomain anti-apoptotic proteins as well as the pro-apoptotic Bak are localized at the MOM [58]. Although the primary function of all of these proteins is to control the integrity of the MOM, some of these proteins, including the pro-apoptotic Bax, are usually cytosolic with a small mitochondria associated population in healthy cells, while the anti-apoptotic Bcl-xL can be found in both the cytosol and at the mitochondrial membrane [59]. Although Bax is usually cytosolic, it has to translocate to mitochondria during apoptosis, where it is carrying out its action by creating pores through the membrane. The exact nature of these pores (e.g. what is the oligomeric structure of Bax? If the pore is proteinatious, or lipidic and toroidal [60], is still an open question, although suggestions has been put forward, such as a hypothetical model where dimeric Bax act as clamp stabilizing a toroidal pore (see Figure 9 to the right) [61]).

Some studies have shown that both Bcl-xL and Bax are repartitioning towards the mitochondrial membrane upon apoptotic stimuli [59], and that tBid recruits both proteins to synthetic liposomes [62]. Recent studies using fluorescence loss in photobleaching (FLIP) in combination with fluorescence recovery after photobleaching (FRAP), have examined the rates at which Bax and Bcl-xL are translocating back and forth between the cytosol and the MOM [63, 64]. It was suggested that Bax was in a dynamic equilibrium between the cytosol and the MOM. And this equilibrium could be modulated by Bcl-2, Mcl-1 and Bcl-xL expression levels, which increased Bax retranslocation (notably Bcl-xL, which was suggested to leave the mitochondria together with Bax). This study was performed in HCT116 colon carcinoma cells [63]. However, a similar study suggested that, although Bax is in a dynamic equilibrium between the cytosol and the membrane, Bax dissociate from the MOM independently of anti-apoptotic Bcl-2 family proteins or BH3-only proteins [64]. Indeed, their results also indicated that overexpression of fluorescent mutants of either Bcl-xL or Mcl-1 inhibited apoptosis at the same time as it reduced Bax retranslocation into the cytosol. Their results also showed that expression of fluorescent Bax in Bax<sup>−/−</sup> Bak<sup>−/−</sup> cells stabilized a population of Bcl-xL
on the MOM. Trying to reconcile these very different observations they suggested that there may exist two populations of membrane associated Bax; one loosely associated, which is still in equilibrium with the cytosol (possibly by the help of Bcl-xL), and a membrane inserted population inhibited by anti-apoptotic proteins. The membrane insertion, via the TM-domain in helix α9, for the more tightly bound population would be an important distinction since Bax was not membrane inserted when retranslocated by Bcl-xL in the HCT116 cells [64, 65]. Some of the authors were participating in both of the studies with the HCT116 cells. The removal of the α9 helix from the hydrophobic core has been suggested to be irreversible since it would be too energetically demanding [66].

The results that the membrane association/disassociation of Bax is independent of protein activity are in line with the embedded together model [64]; a model which implies that tBid is not interacting with Bax in the cytosol to mediate its membrane translocation, but that both proteins must be present on the membrane for the protein-protein interaction [64, 67, 68]. The embedded model will be discussed later in this section, and is central to the perspectives of this thesis.

The secondary structures of most multidomain Bcl-2 family proteins are mainly α-helical, as seen in Figure 9 [69]. The tertiary fold, which is shared by both the pro- and anti-apoptotic multidomain proteins, is typically two central hydrophobic helices surrounded by either six or seven amphipathic helices [8, 69]. Although the sequence identity of the multi domain protein is not particularly high they have quite similar folds and low root mean square deviations [69, 70]. By comparison, the BH3-only proteins have such little sequence and structural similarity to the other Bcl-2 family members that it is an open question if they truly belong to the Bcl-2 family, and it has been suggested that they have separately evolved by convergent and divergent evolution [71]. All BH3-only proteins, with Bid as a singular exception, are intrinsically disordered proteins (IDPs), meaning that they lack secondary and higher order structure. Bid is also not active until it has been proteolytically cleaved to tBid, and gone through an unfolding step on a membrane to release the BH3-motif [68, 71]. Only upon interaction with multidomain proteins do BH3-only proteins fold into a helical structure that binds into the hydrophobic pocket of the target protein.
Figure 9. On the left side a schematic picture of the structure of non-active, cytosolic Bax monomer (1F16) with the flexible 12 aa N-terminal part omitted for better clarity; orange for α-helices with regions in green contributing to the hydrophobic groove. Two of the BH-regions are highlighted, namely the BH3 domain in red and the BH4 domain in purple (adapted from D. Westphal et al. 2011 [60]). On the right side a hypothetical model proposed by Bleicken et al. (adapted from Mol. Cell 2014 [61]) for Bax induced pore formation at the MOM upon apoptotic activation of Bax.

All Bcl-2 family proteins contain motifs called Bcl-2 homology (BH) domains, numbered 1-4, and in many cases an additional transmembrane domain (TM-domain) [7]. The BH3-only proteins have, as one would expect, only one of the homology domains but may have the additional TM-domain.

In the multidomain proteins BH1, BH2 and BH3 together form a hydrophobic groove [69], which may either be occupied by the proteins’ own C-terminal TM-domain, or serve as a surface for intermolecular interactions where other Bcl-2 family proteins can insert their BH3-domain, located in their α2-helix [69, 70]. As will be discussed below, this general mode of interaction is central to all suggested models for activation of pro-apoptotic effector proteins. BH3-only proteins have been suggested to propagate apoptotic signal by either directly activating the effector proteins or indirectly activating the effectors by inhibition of the anti-apoptotic Bcl-2 proteins. The current consensus is that aspects of both models are valid in many cases, and unified models (see Figure 10) have been presented, as well as the embedded together model, the latter suggesting an active and strong participation from the mitochondrial membrane where the proteins actually are exerting their action [67, 72-75].

In the direct activation model some BH3-only proteins (tBid, Bim and Puma) are considered to function as activators by interacting directly with the effector proteins, while others (Bad, Bmf, Bik, Blk, Noxa, Hrk) are considered to act only as sensitizers by interacting with anti-apoptotic family members. In the direct activation model anti-apoptotic proteins are acting by binding to and inhibiting the activators by sequestering them from the mitochondrial. In the indirect activation
model the effectors are supposedly already populating an active state independently from any activation from BH3-only proteins, and their membrane permeabilizing action is continuously suppressed by anti-apoptotic proteins. In this context BH3-only proteins are propagating apoptotic signals not by interaction with the effectors *per se* but by competitively interacting with the anti-apoptotic proteins. The more potent propagators of apoptotic signal (tBid, Bim and Puma) have, in the indirect activation model, been suggested to act promiscuously by interacting with all of the different anti-apoptotic proteins, while the rest (Bad, Bmf, Bik, Blk, Noxa, Hrk) are more selective and are only able to interact with specific anti-apoptotic proteins. Besides bringing the different interactions between Bcl-2 family members together in a unified model, Andrew’s group proposed the embedded together model where the MOM is of importance in itself, supported by observations such as that tBid interacting with Bax and Bcl-xL on membranes but not in the cytosol, and that Bax exposes the 6A7 epitope upon membrane interaction and in the absence of other proteins [62, 67, 68, 75-79].

**Apoptotic switch models**

![Diagram of apoptotic switch models](image)

**Figure 10.** Different models explaining the regulation of the apoptotic switch by the interplay of Bcl-2 protein family members. The direct activation model suggests that activators such as the BH3-only proteins Bim or tBid can directly interact and activate the pro-apoptotic Bax protein, whereas pro-survival proteins such as Bcl-2 sequesters these BH3-only proteins. In the indirect activation model proteins like Bcl-2 sequester also Bax molecules which are activated to expose their BH3 domain. Upon neutralization of all pro-survival proteins, further Bax molecules can then progress to oligomerize in the MOM into pores to release CytC. The unified model includes the first model with pro-survival proteins sequestering BH3-only proteins but simultaneously also the second model with these Bcl-2 like proteins also sequestering activated Bax (adapted from P.E. Czabotar et al. 2014 [72]).
Activation of pro-apoptotic Bax/Bak is accompanied by major conformational changes, which may be initiated from either the N-terminus, the C-terminus or both [60]. The transient exposure of the 6A7 epitope (Bax residues 13-19), caused by displacement of the N-terminal region of the Bax protein has long been known to be an early event in apoptosis [79]. A natural variant of Bax, called Bax .psi., which does not include the first 19 residues of the N-terminus, as well as a truncated variant of Bax lacking the first 20 residues of Bax displayed increased mitochondrial localization [60].

The TM-domain of Bcl-2 family proteins are typically ca 20 residues long, located in helix α9, and flanked with charged residues on both the cytosolic and the luminal side [58]. A notable exception to this rule is that Bax does not possess charged residues on the cytosolic side of the TM-domain, but a proline which allows it to turn the TM-domain in towards it hydrophobic pocket [58]. The displacement of the C-terminal TM-domain of Bax from the hydrophobic groove and its subsequent insertion of into the MOM, was suggested to be caused by the insertion of another protein’s BH3-domain into the hydrophobic groove, and that this process would also drive the localization of the usually cytosolic Bax towards the mitochondrial membrane [58, 70].

By contrast, the interactions between the BH3 domains of anti-apoptotic proteins with the hydrophobic pocket of Bax have been suggested to control the sequestration or retranslocation of Bax from the MOM out to the cytosol [62, 63]. Dimerization of pro-apoptotic pore-forming multidomain proteins by the symmetrical insertion of the BH3 domains into the hydrophobic pockets of the opposing partner has been suggested as a likely first step toward the formation of oligomeric pores [60, 61, 80-83]. This would effectively mean that each monomer in the pore-complex would have two faces, one being the BH3:groove while the other side is less well defined, with interactions between α6:α6 being one candidate for forming extended oligomers [60, 81, 84]. The suggestion of an α6:α6 interface would leave the question open if this interface could exist at the cytosolic side of the membrane or occurring in the hydrophobic core, as would be the case if helices α5 and α6 would bury together in the membrane [60]. However, recent results have implied that the α5 and α6 helices do not, as previously suggested, insert through the membrane as a hairpin loop, but may instead only insert shallowly in the membrane [80].

Several structural studies have been made with truncated multidomain Bcl-2 family members in solution bound to peptides from the BH3-region of other members via their hydrophobic pocket [58, 69, 70]. Through such studies it has been possible to identify hydrophobic patches lining the binding groove which align with conserved hydrophobic regions in the BH3-only proteins [69].

To acquire well resolved structural data of these multidomain proteins of the Bcl-2 family one has to use modified proteins, typically with a deleted TM and/or deletion of unstructured loop, either to make it soluble for liquid state NMR
experiments or to enable crystallization of the protein of interest [66, 69, 85]. The disordered loops connecting the α1 and α2 helices vary greatly in length between different Bcl-2 family members [69], and are in many cases not modeled into acquired electron densities [69] or are not present in the truncated variants of the proteins [66]. The region spanning the α1 and α2 helices may be important in membrane interaction among anti-apoptotic Bcl-2 family proteins. The α1 helix of Bcl-xL lacking its C-terminal TM-domain has been proposed to insert into the hydrophobic core based on NMR studies performed on detergent micelles [86]. It has been demonstrated with truncated Bcl-xL lacking the majority of the α1α2 loop, that the protein insert its C-terminal TM-domain at a 25° angle compared to the membranes normal vector and thereby orients the rest of the globular head in such a way that the α1α2 loop comes in close proximity to the membrane [66]. In that study it was not possible to see any big conformational differences between the cytosolic form and the membrane bound form of Bcl-xL but it was noted that this may be an instantaneous view, or a snapshot, of Bcl-xL on the membrane and that further conformational changes may very well be dependent on factors that could not be accommodated, such as specific membrane lipids [66, 85]. It was also noted in this context [85] that other studies of anti-apoptotic MCL-1 linked the intrinsically disordered α1α2 loop region to membrane insertion of the soluble part of the protein [87, 88]. The first two helices of Bax have also been mutated to form cysteine-links (disulfide-bonds), thereby preventing them from undergoing conformational changes. This led to inhibited apoptosis (but did not prevent 6A7 epitope exposure), decreased interaction with Bcl-xL and, surprisingly, an increased mitochondrial population, which was interpreted as an effect of decreased Bax retranslocation by Bcl-xL [63].

Still much more can be said about all the interesting work which is currently carried out, but to this day one thing is certain –that we do not know enough about the interplay between the many interaction-partners, proteins and lipids alike, which throughout our lives decides what part of us may live, and which parts have to be killed. And until we do, we will be left vulnerable to the unfortunate cases where these intricate systems do not work.
4.2 Aim of this thesis

Apoptosis is very interesting from a medical point of view. A balance between survival and death must be maintained. If the balance is shifted towards too much death we may suffer from atrophy. If the balance is shifted towards too much survival we may develop cancer. Bcl-2 family proteins have been subjected to the attention of numerous research groups all over the world, trying different approaches to gain a better understanding of their functions. Regardless of all effort put into this field many questions still remain, and as a whole it still remains a mystery exactly how the Bcl-2 family proteins are interacting with each other, and with the membrane, to decide whether to kill or save the life of a cell. The whole problem lies in the intricacy of the whole system. There can be incredibly many underlying reasons for an organism to either gain or lose from the survival of a cell, and the undertaking of deconstructing a whole cell in a manner which is as safe as possible is non-trivial. Consequently, both the regulation of apoptosis and the steps required to carry out apoptosis are complex and require a large number of proteins affecting each other and the cellular membranes. Biophysical studies of Bcl-2 family proteins have mainly been limited to soluble proteins even in the case of natively membrane bound members like Bcl-2. Because of this there is only limited research on how the membranes are actually taking part in the action of Bcl-2 family proteins.

The aim of this thesis has therefore been to elucidate the behavior of the Bcl-2 family members at the mitochondrial membrane system by biophysical means to provide new knowledge about the molecular mechanism by which Blc-2 proteins regulate apoptotic events at the mitochondrion.
5 Materials and methods

5.1 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry is a method used to characterize phase transitions in various types of material and especially in biological systems [89, 90]. The basic principle of DSC is very simple, as schematically shown in Figure 11. The temperature in a sample cell and a reference cell is controlled by transferring heat to or from the cells. The two cells reside in an adiabatic jacket ensuring that heat is not ‘leaking out’ to the surrounding in an uncontrolled manner, and the pressure is kept constant by a spring-loaded seal screwed in place over the cells. As the temperature of both cells will be varied in a systematically way, the heat transfers to the two cells are reported as a difference in heat capacity under constant pressure ($C_p$). If more heat is required in the sample cell than in the reference cell, then the difference is said to be positive, and vice versa.

![Figure 11](image_url)

**Figure 11.** Principle of differential scanning calorimetry (DSC) applied to lipid vesicles: Schematic drawing of a DSC device with sample and reference cell and heating device. Heating of both cells is carried out at a constant temperature rate and the difference in the power to heat both cells at the same rate is recorded. The respective heat capacity difference is then plotted as a function of temperature as visualized in the DSC profile (or thermogram) on the right side (top). The shown thermogram is typical for a lipid bilayer made of a single Phosphatidylcholine species (DMPC here). The recorded thermal events correspond to two major phase transitions occurring in these systems with a pre-transition a lower temperature with a small enthalpy change, as well as a main transition at higher temperature with a larger enthalpy change. A sketch of these phase transitions is plotted at the bottom for PC lipids and PE lipids (bottom trace, only one transition).
If the two cells have identical content then you would expect a difference close to zero. Usually this is not perfectly true even for identical samples, and the raw data is routinely baseline corrected. In case the sample cell contains a sample which undergoes a temperature dependent process, such a phase transition, then you will see this as an increase (or decrease) in heat transfer to the sample cell relative to the reference cell (i.e. the difference is non-zero). When the process is finished then the difference will return to zero. Since \( C_p \) values are collected over a temperature range they can be integrated to yield the calorimetric enthalpy change \( \Delta H_{cal} \) associated with the transition. Also if the transition was taking place over a sufficiently small temperature range, such that we can assume it to take place at the temperature \( T_m \) (the subscript \( m \) denoting melting), then we can also calculate the entropy change associated \( \Delta S \) with the transition.

\[
\Delta H_{cal} = \int_{T_1}^{T_2} C_p \\
\Delta S = \frac{\Delta H_{cal}}{T_m}
\]

In biological systems DSC is can be used to study thermotropic processes such as the unfolding of proteins or the melting of lipid bilayers into the biologically relevant liquid-crystalline \( L_n \)-phase [90]. In the context of this thesis, the DSC approach has been used for studying phase behavior of mixed lipid membrane systems mimicking the CS at the MOM, and modulation of their phase behavior due to the presence of oxidized lipids at various concentrations and the presence of the Bax protein. DSC was also used to obtain thermograms for basic studies of the impact of OxPls on the organization of pure model membranes made of 1,2-dimyristoyl-\( sn \)-glycero-3-phosphocholine (DMPC) lipids only. In addition, DSC was also used to monitor the thermal denaturation of Bax protein itself in solution and upon the presence of oxidized MOM-mimicking membrane vesicles.

DSC experiments and related findings are mentioned in papers I, II, and IV. In all cases where the phase behavior of lipid membranes was the focus, experiments were performed in essentially the same way; the degassed samples and reference buffer solution were equilibrated at a low temperature of 5 °C. Thereafter three temperature scans were performed. The first two scans, called pre-scans, heating up to 45 °C at a fast scan rate (60 °C/h) and then cooling down to 5 °C. The main reason was to relieve possible metastable, irreversible phase states that can occur in lyotropic lipid bilayer systems. These pre-scans were typically not used for further analysis. The third up-scan was then performed at a slow scanning rate of either 2 °C/h (paper I and II) or at 5 °C/h (paper IV) and then used for further analysis. In the case of thermal denaturation of Bax (paper II) the temperature was increased at a
high scan rate of 90 °C/h and continued up to 95 °C. The analyzed data for the protein samples were acquired from the first up scans due to the irreversible melting behavior of many proteins including Bax.
5.2 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectroscopy is a technique which relies on the spin properties of atoms nuclei and is widely used in biological sciences to determine structure and dynamics of proteins, membranes and small molecules [91-95]. The presence and size of a magnet nuclear moment is coupled to the nuclear spin which a nucleus possesses. The nuclear spin is defined by the quantum number I, which can take integer or half-integer values (eg. 0, 1/2, 1, 3/2, …) and a magnetic quantum number m\textsubscript{I} which depends on I and comes in the interval I, I-1,… -I+1, -I, (giving a total number of values 2I+1). The magnetic momentum \( \mu \) of a nucleus depends on the magnetic quantum number and the gyromagnetic ratio which is characteristic for a specific nucleus (e.g. \( ^1\text{H} \) or \( ^{13}\text{C} \)). In an NMR instrument the nucleus which resides in a molecule, is subjected to an external magnetic field, B\textsubscript{0}, which results in a number of possible magnetically aligned stationary states according to its magnetic quantum number. These different states are a consequence of the interaction of the magnetic moment with the static magnetic field yield different energy levels, called Zeeman levels as indicated in Figure 11. In a sample with myriads of spins, these different energy levels are populated according to the Boltzmann distribution. At zero field strength these energy-levels are degenerate and no state is preferentially populated. The size of the separation, called Zeeman splitting, between these energy-levels is dependent on the size of the magnetic moment and the size of the externally applied field strength as shown in Figure 12 (right part). For spin \( \frac{1}{2} \) nuclei (such as \( ^1\text{H} \)) only two levels are generated, and the lower state is denoted \( \alpha \) while the higher is denoted \( \beta \) as visualized in Figure 12. The energy difference is small and as a consequence, even at high field strengths, the lower energy state is only slightly more populated than the higher level at equilibrium. Therefore NMR is intrinsically an insensitive spectroscopic technique. Since slightly more nuclei are spinning in one direction this causes a net-magnetization aligned with the magnetic field (the z-direction of the laboratory frame). Although the net-magnetization is in z-direction of the laboratory frame the individual nuclear moments are precessing around the z-vector in the laboratory frame.
Figure 12. Principle of NMR spectroscopy: A nucleus with spin ½ (e.g. $^1$H) can possess two stationary states in a strong outer magnetic field. The states are characterized by the magnetic spin number +1/2 ($\alpha$-state) and -1/2 ($\beta$-state). Because of the corresponding orientations of their magnetic moment in z-direction $\mu_z$ (related to their respective angular momentum $P_z$ and magnetic quantum number), both states are characterized by different energy levels due to the interaction of the magnetic moment with the outer magnetic field. These energy levels correlate linearly with the magnetic field strength. The difference between both levels corresponds to the Larmor frequency at which the nuclei precess around the magnetic field. (adapted from T. Polenova 2011 [96] left, and courtesy by T. Sparrman right).

The ‘typical’ resonance frequency of a nucleus with a magnetic moment under these conditions in a magnetic field is called the Larmor frequency, and is directly connected to the difference in energy levels. Although the SI unit for the magnetic field strength $B$ is Tesla, in NMR instruments the field strength is typically expressed in the Larmor-frequency of $^1$H nuclei (e.g. 500 MHz) which corresponds to the radiofrequency pulse required to excite between $\beta$ and $\alpha$ state.

The effective magnetic fields sensed by the nuclei are modulated by the surrounding electrons. When the electrons exist in the external magnetic field, they will induce local magnetic fields which counteract external field. This has the effect that the nuclei are shielded from the external field and senses an effective field which is less strong than the applied field. Because of different chemical surroundings, the effective magnetic fields can be different even for nuclei or the same isotope. Therefore the energy differences between states and therefore also the resonance frequencies become different.

For a proton spectrum at a 500 MHz instrument such local differences can give rise to chemical shifts variations up to several kHz [94]. Normally, chemical shifts are normalized against a known sample (e.g. tetramethylsilane) and converted to ppm which is a scale which is independent of the instruments field strength. These shifts, are in the interest of the NMR spectroscopist, and not the Larmor-frequency.
When the system is in equilibrium the nuclear spins are aligned with the magnetic field, and there is population difference between the energy levels. By applying radio frequency pulses at these transition energies (at Larmor frequencies), this equilibrium is perturbed and the response to this perturbation is measured as the free induction decay (FID) in the NMR coil and converted via a Fourier transformation into a NMR spectrum. To perturb the equilibrium one applies a radio frequency pulse (see Figure 13) which has strength $B_1$ and is perpendicular to the $B_0$-field. When the resonance condition is satisfied with the radiofrequency equal to the Larmor frequency, the population difference between the energy states will be lost as a consequence of simultaneous absorption from the lower energy level and emission from the higher energy level. If continually irradiated this process eventually leads to saturation when the energy levels are equally populated. The $B_1$ field is rotating at the Larmor-frequency of the laboratory frame. By transferring this system into a rotating frame at this frequency, the magnetization vector can be considered stationary. This allows the use of a simple vector model to understand the effect of pulse sequences on the magnetization vector [92, 94].

The effect of applying the $B_1$-field will be that the net magnetization is rotated, away from the $z$-direction, according to the right hand rule about the direction of the applied $B_1$-field. By applying a $90^\circ$ pulse in the $x$-direction, the net-magnetization will then be rotated into the $-y$-direction as indicated in Figure 13. It should be noted that for nuclei carrying a positive value for $\gamma$, which is the more common case, the sign of the Larmor frequency is negative, and an applied $90^\circ$-pulse will have a nutation frequency, $\omega_1$, which will also be negative when following a right-hand rule. In other cases (negative $\gamma$) a left-hand rule is used instead, using positive frequencies. See Levitt 1997 for a detailed discussion [97].
Figure 13. Principle of pulsed NMR spectroscopy: A sample in the static magnetic field $B_0$ has a macroscopic magnetization $M_0$ parallel to the outer magnetic field which also defines the $z$-direction in the vector model used here. Applying a 90° radiofrequency pulse (perpendicular to the outer magnetic field) at the resonance frequency of the nuclei of interest in the sample will precess the magnetization vector away from the $z$-direction into the $x$-$y$ plane. By applying the pulse parallel to the $x$-direction the magnetization vector will then be moved to the $-y$ axis. This vector will then process around the $z$-axis in the $x$-$y$ plane at the Larmor frequency of the nuclei excited and the rotating vector will induce an oscillating electric signal into the NMR detection coil. This signal will decrease over time with the spin-spin relaxation time $T_2$ and is called free induction decay or FID.

Radio-frequency pulses are applied as so-called pulse sequences which make it possible to probe various NMR parameters and nuclear interactions. These pulses sequences are implemented in so called pulse-programs controlling the frequencies, timings, durations and amplitudes of the applied $B_1$-fields. Once the pulse program is finalized the net magnetization of the reporter nuclei are typically in the transverse plane for signal detection and is required to relax back into $z$-direction characterized by the spin-lattice time $T_1$.

Upon applying a so-called 90° radio frequency pulse as seen in Figure 13, net magnetization moves into the transverse-plane (the $xy$-plane), and in this plane the magnetization vector will precess around $z$-axis at the Larmor-frequency and induce an oscillating electric current in the detection coil. The detected signal is called Free induction decay (FID) and will decrease to zero over time due to the spin-spin relaxation. The oscillation is detected in the time domain, but is visualized in the frequency domain most often by processing the signal by Fourier transformation. For perfect sinusoidal-waves, the Fourier transformation would yield discreet (infinitely sharp) signals in the frequency domain; this is however never seen in practice.
Even a sharp NMR signal has a defined width and some of the signal is detected slightly off-center, a characteristic which is usually reported as the width at half-height. Broadening of the lines is inversely proportional to the spin-spin relaxation $T_2$ of the sample. The reason for this is the loss of net magnetization in the transverse plane (as seen in the decrease of intensity of the FID) due to spins of the same type losing coherence to each other while precessing in the x-y plane. The $T_2$ (also called spin-spin, or transverse) relaxation is loss of net magnetization (in the transverse plane) as a result of destroying the phase coherence [94]. In addition $T_2$ arises due to “flip-flop” between nuclei in a particular spin-system. It is related to slower dynamics, up to milliseconds, and is affected by molecular interactions and inhomogeneity in the external magnetic field. Simultaneously another relaxation mechanism exists, called $T_1$ (or spin-lattice, or longitudinal relaxation), which corresponds to re-establishing the Boltzmann population distribution of $\alpha$ and $\beta$ states again [94]. This can be viewed as the net magnetization vector rotating up towards the z-direction to return to equilibrium after being disturbed by the application of a radiofrequency pulse. Over the course of this process a projection of the vector on to the z-axis is extending, at the cost of the projection on the x-axis which gets shorter. $T_1$ is related to fast vibrational and translational motions on the Larmor frequency timescale, and energy from the nucleus is lost to its surrounding (including the molecule containing the nuclei itself). $T_1$ relaxation depends on the molecules structure and size as well as the molecular environment, e.g. viscosity and temperature. To illustrate this in a vector model it is important to note that the net magnetization vector is the result of a large number of smaller vectors which represent single spins at their respective orientations with respect to the external magnetic field.

For small molecules which experience fast isotropic (directionally independent) tumbling conditions $T_1$ and $T_2$ are often similar, whereas $T_2$ drops dramatically for larger molecules, as well as membranes, which experience slow tumbling on the NMR timescale, and thereby contribute to a broadening of the signal. Large systems like multilamellar vesicles used in this thesis, are practically immobile at the NMR-timescale which should be kept in mind.

5.2.1 Solid state NMR: static and MAS NMR approaches

The main feature of samples suitable for solid state NMR is their immobility on the NMR timescale which generates NMR signals which are dependent on the orientation of individual molecules with respect to the outer magnetic field. Nuclei experience a shielding by the surrounding electrons which generally differs in different directions around the nucleus; it is anisotropic or direction dependent. Due to this anisotropy the NMR signal frequency becomes dependent on the orientation of the molecule. This effect is called chemical shift anisotropy (CSA) [91, 93]. Therefore solid state spectra are normally very wide without any well-resolved NMR peaks, since the spectra contain many resonances that all have different
frequencies (originating from the sum of all possible orientations a single molecule can have in the magnetic field). A typical powder $^{31}$P NMR spectrum of multilamellar lipid bilayers is shown in Figure 14. A detailed analysis of these lineshapes can be found elsewhere [91, 98].

In addition, anisotropy can also originate from dipolar couplings between different nuclei that possess a nuclear magnetic moment [93, 94]. However if a molecule is randomly rotating fast with respect to the NMR timescale, this anisotropic effects are negligible. Under such conditions the chemical shift anisotropy (CSA) of a nucleus is averaged to an isotropic value, and dipolar interactions are averaged to zero.

Large molecules such as proteins with a molecular weight over 30 kDa, protein aggregates or membranes tumble quite slow or not at all so that they display anisotropic effects even if in solution or if suspended, and solid state NMR approaches have to be used [93, 98, 99].

![Static NMR and MAS NMR spectra of lipid bilayers](image)

**Figure 14.** Typical $^{31}$P NMR spectra of lipid bilayers composed of phosphatidylcholine and phosphatidylglycerol lipids at a 2:1 molar ratio. Top: static wideline NMR spectrum which represents a powder-type envelope over all resonance lines who vary with the orientation of the individual lipids in the magnetic field. The width of the spectrum expressed as chemical shift anisotropy $\Delta \sigma$ reflect the liquid-crystalline state of the bilayer; Bottom: MAS NMR spectrum of the same bilayers under magic angle conditions (6 kHz spinning speed). The isotropic chemical shifts ($\sigma_i$) for both resonances are indicative for the respective lipid component, while their integrals reflect their relative fraction in the lipid bilayer.
By a process called Magic Angle Spinning (MAS) it is possible to average CSA to zero and even any anisotropic dipolar couplings [93, 95, 99]. This averaging process produces solution-like spectra with resonance lines that are characterized by their isotropic chemical shift values (as indicated in Figure 14). Practically, MAS NMR is done by orienting a ceramic rotor which contains the sample, at a specific angle relative to the external magnetic field and spinning it at a rate $v_R$ which exceeds the frequency of the anisotropic linewidth $\Delta \sigma$). The angle corresponds to the space diagonal going between opposing corners in a cube, and one of the edges and this 54.7° are called the “magic angle”. At a rotating speed $v_R$, rotation-sidebands (or rotor echoes in the FID) will appear in the spectra at either side of the isotropic resonances; these side-bands appear at $v_R$ away from the isotropic line. At higher $v_R$-speed these sidebands move far away from the isotropic line, decrease in intensity and disappear finally. If heteronuclei, like $^{31}$P are observed; usually dipolar coupling to protons is normally removed by decoupling [93].

For high-γ nuclei like $^1$H, $^{19}$F and $^{31}$P the magnetization can be transferred to low-γ nuclei like $^{13}$C and $^{15}$N by cross polarization (CP) experiments to enhance signal to noise for the observation of these low-abundance nuclei. However this requires that these nuclei are coupled by dipolar interactions to – in the most cases – the protons. The main nuclei used for enhancing signals of biological material by CP are the $^{13}$C and $^{15}$N nuclei [95, 99].

In paper I, static $^{31}$P NMR and static $^2$H NMR were used to study phase behavior of DMPC vesicles doped with OxP1 PazePC and PoxnoPC at various temperatures. In Paper II $^{31}$P MAS NMR and $^1$H MAS NMR have been deployed to investigate vesicles mimicking mitochondrial contact sites under oxidative stress (containing 10 mol% PazePC) with and without the presence of the apoptotic Bax protein. In publication IV different NMR-techniques were discussed and $^1$H MAS and $^{13}$C CP MAS NMR spectra were used to exemplify means of studying thermotropic phase behavior. In paper V and $^1$H MAS and $^{13}$C CP MAS NMR were used to study the thermotropic phase behavior of vesicles mimicking mitochondrial contact site with and without 10mol% PazePC. Also in paper V, in solution a two-dimensional $^1$H-$^{15}$N TROSY experiment was performed on Bax protein to confirm correct fold, and the protein was incubated with membrane mimicking mitochondrial contact sites. Solid state $^{15}$N CP and INEPT MAS NMR was then performed on the membrane bound protein to observe dynamically restricted regions (membrane bound) and flexible regions (outside the membrane).
5.3 Circular Dichroism (CD) spectroscopy

Chiral molecules do exist as pairs of mirror-image isomers which are not superimposable on each other. Physical and chemical properties are identical with the exceptions that they interact differently with other chiral molecules and that they interact differently with light polarization. Since proteins (and many other biological molecules like DNA) are chiral molecules, these light interacting properties can be used to study secondary protein structures (α-helices, β-sheets, random-coil). These secondary structures can be studied by circular dichroism [100, 101].

Any light wave consists of a combination of left-hand and right-hand circular polarized light. This even applies to planar polarized light, due to the ratios of left- and right-hand rotations being equal, resulting in zero net rotation. If plane polarized light passes through an optically active sample, the two components will absorb by different amounts. The resulting difference is called Circular Dichroism (CD) and is given by:

\[
CD = \Delta A(\lambda) = A(\lambda)_L - A(\lambda)_R
\]

where \( \lambda \) is the observed wavelength and \( A(\lambda)_L \) and \( A(\lambda)_R \) are the absorption in left- and right-hand circular polarized light respectively. However, it may not be immediately obvious what this mean.

5.3.1 Some properties of waves

Maybe the easiest way to understand what circular polarized light is to first consider plane polarized light. For instance two plane polarized waves of equal wavelength, intensity and phase, but rotated away from each other by 90º around the propagation axis, can be additively combined to produce a resulting plane polarized wave rotated away by 45º (around the propagating axis) from either wave. The resulting wave will still have nodes at the same places as the original waves had (because we were adding two zero-vectors).

If we would however combine two plane polarized waves of equal wavelengths and intensity, but out of phase by 90º, the two original waves do not cut each other in common nodes (when one is zero the other one is at maximum intensity), and additively combining them will result in a circulating wave which can rotate either left- or right-hand rotating. If both of them had the same max intensity, the resulting wave will always be at that same distance from the rotation-axis, describing a round helix as it propagates. Note however that if one of the plane waves had a lower peak-intensity, the resulting wave would surely rotate with the same wavelength, but the distance from the center would fluctuate with time, and the rotation would describe an ellipse along the propagation axis.

Analogously one can combine two oppositely rotating waves of the same intensity and wavelength to produce a planar wave. The resulting wave will
fluctuate in the plane in-between the component waves, and have peak intensity where the two rotating components cut each other. The resulting wave will always be planar regardless of phase, but how the plane is rotated with respect to the coordinate space is dependent on the phase.

This last observation is also used by another laboratory technique called optical rotatory dispersion (ORD) spectroscopy. Chiral molecules have a property of being birefringent (have two different refractive indices for left- and right-hand rotating light), which effectively means that left- and right-hand rotating light are going at different speed while interacting with the molecule. In ORD-spectroscopy an incident wave of planar light passes through a birefringent sample, and while doing so the phase between the two rotating components will shift so that while the light is in the sample it will be rotating. After exiting the sample the two components will however be going back to the same speed and the light will be planar-polarized again, however now it will be fluctuating in a plane which is rotated relative to the incident wave. Measuring the optical rotation as a function of wavelength is ORD-spectroscopy.

However, if the wavelength of the incident light moves into the wavelength-region where the chiral molecule absorbs light, the chiral molecule will absorb the two rotating components differently. For proteins this is typically below 250 nm in the far-UV region, where peptide bonds absorb, as well as in aromatic side-chains and disulfide bonds which absorb from 260 nm and above in the near-UV region. Imagine a plane wave, composed of left- and right-rotating light. If one of those components becomes completely absorbed you will be left with perfectly circularly rotating light. But if it is not completely absorbed, one is left with something in-between a planar wave and a rotating wave; it will be elliptical. This ellipse characterized by its lowest and strongest intensities which relates to the intensities of the components $I_L$ and $I_R$. For example if left-hand rotated light was absorbed to a higher extent; then the lowest intensity on the ellipse will correspond to $I_{\text{low}}=I_R-I_L$ and the highest intensity will correspond to $I_{\text{high}}=I_R+I_L$. The $I_{\text{low}}$ and $I_{\text{high}}$ always come at 90º separations, and CD-signal is traditionally reported as millidegrees (mdeg) of ellipticity ($\theta$) which is the tangent of the ratio of the minor to major elliptical axis (as seen in Figure 15).

However, what are actually being measured are the differences in absorption $\Delta A$, which relates to ellipticity according to:

$$\theta = 32.98 \left( A_L - A_R \right)$$

Which in turn can be converted to molar ellipticity which is reported in units of degrees*cm²*dmol⁻¹, according to

$$[\theta] = \frac{\theta}{c \times l}$$

where c is the concentration in mol and l is the pathlength in cm.
Figure 15. When left- and right-rotational light components of plane-polarized light get absorbed unevenly by chiral molecules, the resulting light wave after exiting will be elliptical, and have maximum- and minimum-intensities which relates to the maximum intensities of the left- and right- components. The ellipticity is reported in millidegrees (mdeg) and is the angle (θ) in the picture.

5.3.2 Secondary structure of proteins

As mentioned in the previous section, CD signals can be detected in proteins predominantly in the far-UV region below 250 nm, where the peptide bonds absorb. The amide bonds in proteins are not rotating (because of resonance giving the peptide bond partial double bond character), and conformational changes in proteins are taking place by changing the rotation around the bonds to either side (denoted as φ and ψ) of the peptide bonds. These rotations are however affecting the peptide bonds ability to absorb circularized light. The φ and ψ angles do typically only take certain angles, and proteins adapt typical secondary structures like α-helices, and β-sheets. Since absorption spectra of individual peptide bonds extensively overlap with each other, studying proteins by CD-spectroscopy is inherently tapping into global properties of the proteins, and it is not possible to get atomic resolution like in NMR. As signals from different types of secondary structures overlap it is necessary to deconvolute these spectra. This can for instance be done by describing the signal as a linear combination of pure α-helix, β-sheet and random coil spectra as seen in Figure 16, or by comparison within databases of lots of CD-spectra from proteins with known structures. Proteins in these databases have structures which have been studied by other methods like NMR and X-ray crystallography.
Figure 16. Different secondary structures in proteins give rise to CD-profiles with typical characteristics. Spectra such as these are derived from proteins which structures are already known (by means of more exact techniques). It is however not to be expected that only a deconvolution of a CD-spectra will give a perfect representation of the secondary structure of any given protein. (adapted from Czarnik-Matusewicz, B. and Pilorz, 2006 [100]).

In paper II and III far-UV CD spectroscopy was used to study changes in secondary structure of Bcl-2 family proteins upon intermolecular interaction as well as protein-lipid interaction. In paper II small unilamellar vesicles (SUVs) mimicking mitochondrial contact sites with or without 10 mol% PazePC (to simulate oxidative stress) was incubated together with Bax at a 1:200 protein-to-lipid ratio. SUVs were employed because larger vesicles would have caused more significant scattering, and prevented good signal at shorter wavelengths. CD spectra were recorded at 10 ºC, 20 ºC, and 37 ºC for Bax alone and in the presence of SUVs. Spectra were deconvoluted with the CDNN v2.1 software. In paper III full length Bcl-2 protein had been produced by cell-free synthesis (as will be described in the section regarding this technique), where the detergent Brij-35 had been employed to keep the otherwise membrane bound Bcl-2 in solution. Spectra were collected of Bax and Bcl-2 separately as well as in presence of each other. Spectra were also collected at different concentrations of Brij-35 below (0.0028 %w/v) and above (0.05 %w/v) the critical micellar concentration. By comparing the theoretical averaging of the separate of Bax and Bcl-2 with the experimental spectra with Bax and Bcl-2 in presence of each other we could observe small changes in secondary structure, indicating protein-protein interactions below the CMC of Brij-35.
5.4 Fluorescence spectroscopy

If a molecule have been electronically exited by absorbing electromagnetic light, one way for the molecule to relax back to a lower energy level is by fluorescence. Fluorescence spectroscopy has been extensively used in biological science with applications ranging from measuring intermolecular distances to imaging of cellular compartments [102]. So, before fluorescence occurs, we assume that absorption has already occurred. This means that the molecule has gone through a transition of being in the lowest vibrational level of its electronic ground-state ($S_0$, where $S$ denotes a singlet state) to being ‘pushed up’ to a higher electronic level (typically $S_1$ as for many molecules the energy difference $S_0$-$S_2$ could also be enough to break bonds). Electronic absorption is always accompanied with increase in vibrational levels, which can be seen as a broad spectra tapering off when going to higher frequencies. If a molecule is in a high vibrational state, it will ‘quickly’ ($10^{-14}$-$10^{-11}$ seconds) relax down to the lowest vibrational energy level within $S_1$ through internal conversion. For many molecules the relaxation back to electronic level $S_0$ is also quick and occurs without release of a photon. But fluorescent molecules will stay in $S_1$ for a ‘long’ time ($10^{-9}$-$10^{-7}$ seconds) before releasing a photon to relax down to one of the vibrational states in $S_0$. On a side note fluorescence can also be ‘delayed’ by spending some time in a triplet state ($T_1$) before either going back to the $S_1$ singlet state or releasing a photon to go back to the $S_0$ state by phosphorescence. The fluorescence emission will always be of longer wavelength (lower energy) than the excitation wavelength, and the line shape will roughly mirror the absorption spectra tapering off towards longer wavelengths.

Non-radiative electronic relaxation can also occur through interaction between molecules, in a process known as quenching. This means that if the observed fluorescing molecule is in close proximity to a quenching molecule, it will absorb the emission, but the fluorescence emission will be decreased. Emission quenching has been used in paper III to show that the fluorescent molecule antimycin A2, which is known to bind in the hydrophobic pocket of Bcl-2, is displaced by Bax when below CMC of the detergent Brij-35. In this experimental setup, antimycin A2 will give a stronger fluorescence signal when sequestered in the hydrophobic pocket, but is quenched by the water when displaced by Bax.

Calcein is a molecule which experience significant self-quenching at concentrations above 1 mM. This property was employed in paper V where large unilamellar vesicles (LUVs) containing concentrated calcein was used to study Bax induced leakage. In intact vesicles there was no fluorescence, but upon leakage the calcein was diluted and started to fluoresce. The effects of varying Bax concentration and the PazePC-content in the membranes were studied.

Since it was also interesting to know whether Bax was forming pores large enough to release apoptotic factors like CytC in the IMS, experiments with gigantic unilamellar vesicles (GUVs) containing either a small molecule Atto-488, or dextran
of different size (ca 3 kDa and 10 kDa) labeled with AlexaFluor®488 was performed.

The lipid mixtures also contained a small amount of the fluorescent probe DID, which was used to determine the lipid concentration.
5.5 Cell free protein synthesis

Expressing and purifying membrane proteins are more difficult than handling other proteins because they are often inherently toxic to ordinary expression organisms (yeast, bacteria and insect cells), they are not soluble in ordinary buffer systems, and they have a high tendency to aggregate due high hydrophobicity. The inherent toxicity of membrane proteins are often related to an overload in the host cells translocon machinery, effectively preventing the host cell from expressing membrane proteins that it needs to function properly. Of course, other types of proteins can be toxic as well. This reduces the number of cells which are able to express proteins. If low expression levels were bad to begin with it gets even worse for hydrophobic proteins which clump together to inclusion bodies, making careful extraction with detergents necessary.

There is an inherent problem of handling living things which have to be kept alive and well if you want to express proteins which are toxic and/or hydrophobic. In both cases it may be desirable to get rid of “the living part” and only use the machinery inside of the cell which is used for protein production. In a cell-free protein synthesis (CFPS) system you run no risk of killing the host cells, which is something which done already in the preparations for the expression, so toxicity is basically not an issue. Moreover there is easier to optimize expression conditions, and it is for instance possible to express membrane proteins in the presence of detergent micelles, lipodiscs, or nanodiscs, in which membrane-interacting hydrophobic regions of the proteins can insert.

The basic idea of a cell-free protein expression system is that cells from a suitable expression organism (often bacteria, wheat germs, and rabbit reticulocytes) are lysed and the protein expression machinery is extracted. This is done by subjecting the lysate to differential centrifugations and other separation techniques to separate desirable components, like ribosomes which are needed for proteins expression, from cell-components that can interfere with protein expression (notably DNA and RNA). However, in this process other important components are also removed, and have to be replaced in situ. Additives should cover the energy demand of protein production (e.g. by adding high energy phosphate compounds and in some cases kinases which can couple these to ATP production), NTP’s and RNA-polymerase for RNA-production, free-amino acids which are consumed, and of course homologous DNA. Several other additives are also present, but will not be discussed in detail.

There are two basic modes of CFPE, namely batch mode and continuous exchange mode. In batch mode all components are mixed in the same reaction vessel, and are always in contact. Batch mode is an especially good choice when expressing proteins which are isotope-labeled for NMR. The reason for this is that isotope labeled amino acids are expensive, and that the alternative continuous exchange method typically do not consume all of the amino-acids provided in the feeding mix. The second method is called continuous exchange (see Figure 17),
where two compartments are containing a reaction mix and a feeding mix respectively. Cell extract, enzymes, RNAse inhibitor, and DNA in particular are part of the reaction mix which is in contact with the outer compartment called the feeding mix via a semi permeable membrane. This allows nutrients to be feed into the reaction mix from the feeding mixture, at the same time as by-products from the reaction mix is diluted out into the feeding mix.

In paper III full-length human Bcl-2 protein was expressed by batch mode CFPE (without isotope labeling) in presence of micelles of the non-ionic detergent Brij-35. Bcl-2 was reconstituted into DMPC multilamellar vesicles. Bcl-2 was also shown to interact with Bax in presence of sub-cmc concentrations of Brij-35 as described in the sections regarding CD spectroscopy- and fluorescence. Surface plasmon resonance (SPR) spectroscopy was also used to show this interaction, but this technique will not be further described here.

**Figure 17.** In the continuous exchange method of CFPE a reaction mixture and feeding mixture is separated by a semipermeable membrane, which let smaller molecules exchange between compartments. This allows removal of byproducts and a continuous feed of amino-acids and high energy phosphates (courtesy of David Staunton).
6 Summary of papers

6.1 Paper I – Impact of oxidized phospholipids on the structural and dynamic organization of phospholipid membranes: a combined DSC and solid state NMR study

Aim of the study

In a cellular environment elevated oxidative stress is associated with the generation of OxPls. Due to their unusual molecular features their presence presumably has dramatic effects on membrane properties with profound consequences on membrane associated protein function and regulation, especially the Bcl-2 family acting at the MOM. Here, we used a combination of DSC and solid state NMR approaches to obtain a macroscopic and a molecular picture of the impact of two OxPls species on the three-dimensional organization of basic lipid bilayers; as visible in their phase behavior and the organization of individual lipid species in these membranes. To understand the complex thermotropic phase behavior of OxPls containing membranes a binary lipid mixture was chosen, where either of the OxPls PazePC or PoxnoPC was present at different molar ratios in DMPC vesicles.

Results

In general, the presence of OxPls species changed the phase behavior of DMPC based membranes, as visible in a broadening of the main transition peak at 23 °C, the disappearance of the pretransition event at 12 °C (not shown here but the data is presented in table I of paper I) and the occurrence of multicomponent profiles at elevated concentrations of OxPls. Deconvolution of these complex thermal events was carried out for different concentrations of OxPls. This analysis revealed three different components (see Figure 18) which could be assigned to two partially overlapping thermal events at higher temperatures and a third one at low temperature.

The overall trend as visible in the thermograms and their analysis could be summarized as follows: With increasing OxPl-content, a high-temperature component (indicated as I) widens and become more intense with increasing amount of OxPl present. Simultaneously, the sharp component (indicated as II) is becoming smaller. This component which reflects the main transition (corresponding to sharp cooperative melting of a phase containing much DMPC) for OxPl-free bilayers moves towards lower temperatures with increasing OxPl-content. These two events were therefore assigned to the presence of two different domains at this temperature region, namely an OxPl-rich DMPC domain (I) and OxPl-poor DMPC domain (II). As the static solid state $^{31}$P NMR experiments revealed, these two events were also
characterized by two overlapping liquid-crystalline power lineshapes. The one with a reduced CSA reflects the OxPl-rich phase with an increase disorder or motional freedom of the lipid headgroups, whereas a domain with a larger CSA mirrors the less perturbed DMPC domain with a lower amount of OxPl present. These overlapping NMR spectra were observed in the same temperature region as the corresponding DSC profiles. Deconvolution of the static $^{31}$P NMR spectra obtained at various temperatures, also showed an unusual temperature behavior of the CSA, which increased with temperature; this behavior can be seen below and above the two-phase region, but eventually the CSA starts to decrease as typical for an L$_{α}$-phase.

The third DSC visible (but NMR invisible) component at low temperature (indicated as III) might reflect a mixing/demixing effect as previously described for ergosterol containing lipid bilayers [103].

Figure 18. Illustration of the results obtained in peak-fitting deconvolution analyses of the DSC thermograms exhibited by DMPC bilayers containing 2 mol% (A) and 10 mol% (B) of the OxPl PazePC, respectively.

As the DSC profiles clearly showed, the presence of OxPls leads to a disordering of the membrane structure. This disordering effect could potentially give rise to an increased penetration of water molecules across the membrane interface towards its hydrophobic region. This was studied by performing DSC experiments with both H$_2$O and D$_2$O. The DSC thermograms did not reveal any unexpected alterations. To
study water penetration in more molecular detail, wide-line $^2$H-NMR was used. At a hydration level of 12-15:1 D$_2$O:lipid molar hydration ratios, all water molecules are bound to the interface, and free water does not give rise to isotropic signals. In pure DMPC two populations were present; one near to the headgroup, and the other closer to the glycerol and the outermost part of the fatty acid region. However, due to the fast exchange between the pools only an averaged NMR signal was visible. In the PoxnoPC-containing membranes, both populations were visible as well as a third spectral component which appeared at elevated temperatures. PazePC-containing samples were special in the sense that it experienced almost no variation, hence retaining a narrow lineshape until a slight broadening upon the transition into the L$_{α-}$ phase.

**Conclusion**

The obtained DSC thermograms suggest the formation of lipid domains of different lipid composition. The three observed components resemble previously reported binary mixtures containing ergosterol; and the data can be interpreted as two coexisting lipid domains with low and high OxPl concentrations respectively and a mixing/demixing event for OxPl/DMPC at low temperatures. However, as inferred from CSA analysis of $^{31}$P solid state NMR spectra only the first two populations are visible and can be connected to the respective thermal events at higher temperatures. An NMR-invisible weakly endothermic process occurring at lower temperatures (immediately preceding the main melting of the DMPC) may be reflecting a reversible solid-phase mixing/demixing.

Both, the DSC profiles and observed reduced $^{31}$P CSA values are pointing toward a large disordering effect of incorporation of OxPl. This conclusion is in line with computational modeling of aldehyde and carboxylic acid bearing sn-2 side chains, displaying chain-inversion out towards either the interfacial region or completely out into the aqueous-phase [36]. These inversions both leave more free volume for the sn-1 chain to move around, and can disturb the packing of the headgroup region.

When comparing the DSC profiles of PoxnoPC and PazePC, the greatest effect on the temperature shift for the sharp phase transition was seen for PoxnoPC which could be caused by an intercalation of the sn-2 in the headgroup region which disturbs the interactions between molecules. However, PazePC decreased the enthalpy change to a higher extent, which may be due to a smaller area per lipid in these membranes. This can lead to lower levels of hydration on these membranes, which can explain the less complex $^2$H NMR spectra. Structured water surrounding the membranes is contributing to the enthalpy of melting, and when the hydration levels decrease so does the enthalpy change.
6.2 Paper II – The oxidized phospholipid PazePC modulates interactions between Bax and mitochondrial membranes

Aim of the study

Initiation of the mitochondrial apoptosis pathway upon intracellular oxidative stress subsequently causes the translocation of pro-apoptotic Bax protein to the MOM. There, it can insert into and permeabilize the membrane, a process characterized by conformational changes of the protein. All these effects are closely connected but a detailed understanding of the mechanism behind these membrane associated events and their regulation is missing. In addition to trigger the onset of apoptosis, oxidative stress generates OxPls whose impact on the mitochondrial membrane integrity and the activity of membrane associated Bax is unclear. It is therefore of great importance to obtain information about how these lipid are involved the function of the Bax protein at the MOM, and how they modulate protein affinity, membrane-induced conformational changes as well as protein incorporation and membrane perforation. To this end, liposomes which mimicked mitochondrial outer membranes near their contact sites with the inner membrane were used, in the absence and presence of Bax. By replacing 10 mol% of ordinary PC lipids by the OxPl PazePC, the effect of the presence of OxPls on the membrane associated behavior of Bax protein was studied. Membrane-induced conformational changes in the protein itself were investigated by CD spectroscopy, while occurring membrane-protein interactions were characterized by DSC measurements and the behavior of the membranes prior and upon the presence of Bax protein by solid state NMR spectroscopy.

Results

The DSC experiments on vesicles without or with PazePC present revealed that Bax induced significant changes in the thermograms only if PazePC was present. In the absence of Bax, the insertion of OxPls in these vesicles led to a severe broadening of the phase transition profile of the membrane and a higher melting temperature. Upon addition of the Bax protein to these membranes the phase transition became more cooperative, as seen by a narrowing of the temperature range where the melting took place at a lower temperature, accompanied with an increase in the melting enthalpy. This indication of an intimate membrane association of Bax in the presence of PazePC lipid was further investigated by DSC studies with focus on the Bax protein melting behavior as well as using a simple membrane-binding assay. In the latter, membrane-bound Bax and Bax freely dissolved in solution were separated by ultracentrifugation, which indicated that only a small fraction was freely dissolved. DSC traces for Bax in buffer revealed a melting covering over a wide range of temperatures (65 °C to 95 °C) with the peak maximum at around 82 °C to 83 °C). Bax protein in the presence of PazePC-
containing vesicles was much more thermostable, with the melting process starting sharply near 90 °C. The melting or unfolding process was not completed within the temperature range of the experiment. Also, no indication of any membrane-free Bax in this system was visible.

CD-experiments revealed that at the physiological temperature of 37 °C, Bax in solution is mainly α-helical as clearly indicated by two pronounced minima at 208 nm and 222 nm (left side in Figure 19). Upon incubation with PazePC-free lipid vesicles, these minima became more pronounced with a larger change in intensity seen for the 222 nm band. These changes became even more pronounced in the presence of vesicles doped with 10 mol% PazePC. There the minimum at 222 nm was deeper than the one at 208 nm while in the other cases 208 nm was always more pronounced (right side in Figure 19). While the increase in intensity indicates an increase in helical contents, the relative changes in their ratios suggest local reorientations of the helical coils relative to each other upon contact of Bax with the PazePC-containing membrane [104].

Figure 19. Far-UV CD spectra of 5 µM Bax protein in buffer without the presence of lipid vesicles at various temperatures as indicated (A); and in the presence of POPC/POPE/TMCL vesicles doped with 10 mol% of PazePC lipid (B).

In PazePC-containing membranes, modulation of secondary structure was dependent on temperature, and the minimum at 208 nm became more intense than the signal at 222 nm upon a drop in temperature from 37 °C to 10 °C. This temperature dependence was however not seen in the absence of PazePC. When no membrane was present, there was only slight reduction in CD signal, and the overall lineshape of the CD spectra were basically the same.

The behavior of the lipids in these PazePC-containing vesicles was characterized on a molecular level by applying $^{31}$P- and $^1$H-MAS NMR at 37 °C. In the corresponding NMR spectra the presence of Bax and its interaction with the
membranes were clearly visible. In $^{31}$P-NMR, each type of headgroup (PC, PE, CL) gave rise to individually resolved signals (noting that PazePC carries the same headgroup as POPC). Upon incubation with Bax protein, all lipid resonances which indicate the different lipid headgroup species, exhibited a downfield shift and became narrower. Also in the glycerol region of the $^1$H-NMR spectra effects of Bax-membrane interaction could be observed, as the signal broadened upon incubation with Bax. In the acyl-region of the $^1$H NMR spectra a new component appeared slightly downfield from the main acyl-peak when incubated with Bax. The main acyl-peak could by multi-component analysis be determined to be composed by two peaks, which upon Bax incubation became sharper without changing the chemical shifts.

**Conclusion**

In healthy cells, the Bax protein is mainly cytosolic and only a small population is present at the mitochondrial membranes. A higher population of Bax would only translocate to the mitochondria upon upstream apoptotic signalling. In this paper we showed that Bax protein can repartition towards PazePC-containing liposomes, without first being activated by the BH3-only protein tBid. In the presence of these liposomes Bax undergoes conformational changes as shown by CD spectroscopy. These changes indicate not only an increase in helical features in the Bax protein upon membrane association but also a partial penetration into the membrane; an assumption supported by DSC and $^1$H-MAS NMR results.

From these observations one can suggest that the occurrence of OxPls *in vivo* facilitates an increase in the affinity of Bax to membranes (resulting in an increase of in membrane associated population), followed by its partial insertion into mitochondrial membranes; a first crucial step prior further activation of Bax by tBid protein for inducing pores for the release of apoptotic factors such as CytC.
6.3 Paper III – Reconstitution of the anti-apoptotic Bcl-2 protein into lipid membranes and biophysical evidence of its detergent-driven association with the pro-apoptotic Bax protein

Aim of the study

The anti-apoptotic Bcl-2 protein is inhibiting apoptosis by counteracting the effects of pro-apoptotic effectors proteins such as Bax. However, it is not known in detail, how the action of effector proteins is regulated by anti-apoptotic proteins at the MOM. Earlier models have either been suggesting that Bcl-2 could indirectly control the action of Bax by preventing BH3-only proteins to activate Bax, or that Bcl-2 can directly bind to and sequester Bax, thereby blocking its pro-apoptotic function.

Direct binding between Bcl-2 and Bax has been suggested by indirect methods such as photo-cross-linking and co-immunoprecipitation assays, but no direct biophysical evidence has been reported yet for this direct complex formation. Therefore we used full length Bcl-2 protein and Bax protein to probe their mode of interactions by CD spectroscopy, fluorescence and SPR methods. In addition, we also incorporated Bcl-2 into its native-like membrane environment; a prerequisite for further detailed study on the Bcl-2 action at the membrane in the presence of Bax.

Results

Full-length human Bcl-2 protein was produced by cell-free protein expression in the presence of the detergent Brij-35, and Bax was overexpressed in E.coli. Since Bcl-2 has to be solubilized by a detergent, if not present in its native membrane environment, the interaction studies were performed in presence of detergent. Although Bcl-2 in buffer in the absence of detergent aggregates, we found that at a detergent concentration of Brij-35 even below the CMC was sufficient to keep Bcl-2 in solution. The presence of Brij-35 could possibly also activate Bax protein, as was reported for other non-ionic detergents. Although it has previously been observed that Brij-35 is not activating Bax, and that no homo- or hetero-dimerization (with Bcl-xl) occur, we observed an increased α-helical content for Bax in the presence of Brij-35, as determined by CD-spectroscopy. Such differences have previously been reported for detergent-activated Bax. The previous result indicating no activation had however been performed by co-immunoprecipitation in cell-lysate, which could very well have affected the result. Hence we found it appropriate not to exclude the possibility that the purified Bax in our system was indeed adopting a slightly different conformation and that it might have been activated.

Far-UV CD spectroscopy was first performed on either 5μM Bax or 5μM Bcl-2 in presence of 0.05% w/v Brij-35 (well above the CMC = 0.011% w/v). The sum of these spectra was then compared against the CD-spectra of a sample containing both
of 5µM Bax and 5µM Bcl-2. The summed spectra did not differ significantly from
the spectrum with both proteins present. Likewise, thermal denaturation of the
proteins was performed while monitoring the CD-signal at 222 nm (corresponding
to the greatest difference between α-helical and random coil secondary structure).
Neither of these results showed deviations which could suggest that Bax and Bcl-2
would induce changes in each other’s secondary structure or modulate each other’s
melting in presence of Brij-35 above the CMC.

**Figure 20.** Principle of fluorescence based binding assay to probe direct Bcl-2 interactions
with Bax; left: putative antimycin A$_2$ binding site to a Bcl-2 like protein (adapted from Kim
K. et al., 2001 [105] with Bcl-xL as template); right: Change in fluorescence intensity at 500
nm upon binding of antimycin A$_2$ to hydrophobic groove of the Bcl-2 protein (courtesy of M.
Wallgren).

Antimycin A$_2$ is a fluorescent inhibitor of anti-apoptotic Bcl-2 family proteins. In buffer the fluorescent signal is quenched, but the signal becomes more intense
upon the binding of the antimycin A$_2$-ligand to the putative hydrophobic pocket of
Bcl-2 (see Figure 20). This has previously been shown to occur for recombinant
truncated Bcl-2 and the ligand can be displaced by BH3-peptides. However, in our
experimental setup no ligand displacement was seen when Brij-35 concentration was
above the CMC. Also, antimycin A$_2$ did not bind to Bax.

Neither CD-spectroscopy nor the ligand displacement assay seemed to indicate
any direct interaction between Bax and Bcl-2. One possible explanation was that
Brij-35 may block protein-protein interaction by protecting Bcl-2 due to the
formation of micelles. Therefore, the experiments were repeated with Brij-35
concentrations of 0.0028% which is well below the CMC for the detergent. At this
Brij-35 concentration, no changes in the secondary structures and thermal unfolding
of Bax alone (compare to buffer without detergent) nor Bcl-2 alone (compared to
buffer with Brij-35 above CMC) were detected.
The sum of the individual CD-spectra for Bax and Bcl-2, obtained at low detergent concentration, were compared with the corresponding spectra when Bax and Bcl-2 were added together. No significant alterations of secondary structure were apparent. However, when comparing data for thermal unfolding it seemed like Bax and Bcl-2 in the presence of each other were melting in a less cooperative fashion. At this low detergent concentration Bcl-2, but not Bax, was again able to bind antimycin A₂. But unlike the case seen at high detergent concentrations, Bax was now able to displace the antimycin A₂-ligand, due to the reduction in fluorescence signal when both proteins were present. To ascertain a dissociation constant for the interaction between Bax and Bcl-2, under low detergent concentration conditions, SPR-experiments were performed. Bax was immobilized by amine coupling to a CM-5 Biacore chip, and solubilized Bcl-2 was injected at different concentrations. Analysis of these experiments provided a binding constant of 35.8 nMol for this complex.

To achieve incorporation of Bcl-2 protein into a lipid environment, mixed micelles of DMPC with Triton X-100 were produced prior to addition of Bcl-2 protein in Brij-35 micelles. Subsequently, the detergents were slowly removed by incubating the mixture together with Bio-Beads SM-2 which selectively adsorbs the detergents. The resulting DMPC proteoliposomes were visible due to their large size by the naked eye. These proteoliposomes were layered upon a 30% w/v sucrose barrier and isolated by ultracentrifugation. SDS-PAGE confirmed that the Bcl-2 protein had successfully been incorporated into the DMPC membrane by this reconstitution procedure.

**Conclusion**

How anti-apoptotic Bcl-2 proteins inhibit their pro-apoptotic counterparts is central in understanding the mechanism behind the regulation of apoptosis in cells and in particular the regulation in tumor cells. Here, we could show that Bcl-2 and Bax can interplay directly using a soluble test system based on a Bcl-2 solubilizing Brij-35 detergent system. At high Brij-35 concentrations a direct interaction was not observed either due to structural changes in the proteins or the prevention of physical contact due to the size of the protein bearing micelles. At sub-CMC concentrations of Brij-35, Bax competitively binds to Bcl-2 by displacing antimycin A₂. SPR showed a strong interaction between Bax and Bcl-2 at sub-CMC concentrations of Brij-35. CD-spectroscopy indicated that this binding occurs even without any large changes in the secondary structure of Bax. This is the first time where biophysical data have been presented for the direct interaction between the full length Bax and Bcl-2 proteins.

Full length Bcl-2 was expressed in the presence of Brij-35 to remain soluble, and although it is possible to perform some experiments while retaining the protein in this state, it is desirable to study the actions of the proteins in a milieu that more
closely resembles the biological conditions. Therefore, a protocol for the incorporation of the full length Bcl-2 into proteoliposomes was developed. This protocol can either be used as it is for further biophysical studies, or it may be improved upon by changing lipid composition to more closely resemble the native mitochondrial membrane.
6.4 Paper IV – Membranes and their lipids: A molecular insight into their organization and function

Aim of the study

The seventh chapter in the book *New Development in NMR Nm.3 Advances in Biological Solid-State NMR: Protein and Membrane-Active Peptides* an introduction to biomembrane systems is provided. This chapter covers a detailed overview of the investigation of membrane properties, including their organization and functions by advanced biological solid state NMR approaches and calorimetric methods. As a current research topic the role of OxPls in membranes and their impact on lipid-lipid and lipid-protein interactions are investigated in detail and new results are presented. The overview and results are used to develop a better understanding of membrane-protein interactions, especially in the context of mitochondrial apoptosis. In addition, specific techniques like lipid deuteration and observation of lipid headgroups as natural voltmeters via NMR are highlighted. Finally, there is also a short excursion to the analysis of NMR data and how they can benefit from combination with other methods like DSC and CD-spectroscopy as well as multivariate analysis.

Results

Since the book is aimed to a broad audience of researchers a general introduction is given regarding the diversity and importance of lipids membranes as well as their properties. A significant part of this publication is describing earlier work, carried out by us and colleagues with particular interest in the field. Such earlier works describes specific techniques and what type of information is thereby possible to attain. In particular, examples are given as to how NMR is applied in our studies of oxidative stress of mitochondrial membranes. The second part of the chapter presents new insight into biomembranes upon oxidative stress.

In particular the paper exemplified how solid-state NMR can be used in combination with DSC to study the thermotropic phase behavior of vesicles mimicking the MOM near their contact sites, prior to and after oxidative stress. DSC thermograms for MLVs prepared from pure DMPC as well as mixtures of POPC:POPE:TMCL (43:36:21 molar ratio) prior and upon doping with oxidized lipids at 10 mol% PazePC (POPC:POPE:TMCL:PazePC at a 33:36:21:10 molar ratio), is presented. The DMPC profile is typical for PC-lipids, with a small pretransition corresponding to an increase in rotational freedom for the headgroup when going from gel-phase to ripple-phase, and a large sharp transition corresponding to a highly cooperative melting of the acyl chains.

By contrast, the multi-component lipid vesicles are significantly less cooperative, displaying a broad melting behavior over a 25 °C range. The DSC-
profiles are then used for identification of temperatures point for subsequent NMR studies to monitor the membranes in their different phase states. For this purpose $^1$H- and $^{13}$C- MAS-NMR spectra are acquired at three different temperatures. As reference sample DMPC bilayers are used and compared with MOM-mimicking vesicles prior and after the incorporation of the oxidized lipid PazePC. Comparison of the NMR spectra at 308 K clearly reveals narrow lines typical for membranes in their disordered liquidcrystalline-phase. At 288 K the PazePC-containing vesicles produce a $^{13}$C NMR spectrum which has a mainly gel-like character but has a second more mobile subcomponent as visible as a 30 ppm peak for aliphatic chain carbons (gel-like signal is 32 ppm). The corresponding spectrum at 298 K displays quite narrow lines already for the aliphatic region, which is unusual and might either indicate the formation of smaller fast-tumbling vesicular structures or a very complex meta-stable phase state. Comparing the different $^1$H spectra at 308 K some differences are pointed out. For instance, it is easily seen that there is significantly less PC-headgroups in the complex membranes compared to DMPC. Also, there are specific differences in the complex membranes which originate from unsaturation on the oleoyl-chains in POPC and POPE, and more complex headgroup regions.

In the $^{13}$C spectra for the different temperatures it is possible to follow the melting of the membrane at a molecular level, and it is possible to see how the phase transition is affecting the different regions of the molecules.

**Conclusion**

Summarizing the current knowledge about membrane organization and methods to probe them, the results provided show a clear disorganization of lipid membranes upon the generation of OxPls. As shown earlier by us, this disordering enhances the affinity of Bax protein towards MOM membranes and enables them an easier partial penetration into the membrane. Based on this recent work and our earlier findings (Paper I, II, III) we have developed a working model for interplay of Bax and Bcl-2 at the MOM and the active role that the membrane plays in this process. This model is also the basis for our further research in this area.
6.5 Paper V – The oxidized phospholipid PazePC promotes the formation of Bax pores in mitochondrial membranes

Aim of the study

Recent research has suggested that tBid is activating Bax at the MOM and not in the cytosol, in contrast to what was inferred from some earlier work. Therefore, this process requires the translocation of the Bax protein to its target membrane before the tBid mediated pore formation by Bax molecules can take place. We have previously shown that Bax is in a dynamic equilibrium between the cytosol and the MOM, and that this equilibrium is shifted towards the membrane upon oxidative stress. In this paper three main questions have been raised: 1) to what extent is it possible for Bax to form pores without first becoming activated by the BH3-only protein tBid? 2) Is there a threshold concentration of OxPls for Bax to spontaneously form pores in membranes? 3) How does the OxPl PazePC affect the membrane to facilitate its interaction with Bax?

Results

For basic characterization of membrane organization and its interplay with the Bax protein DSC and NMR experiments were carried out. For PazePC-free membranes a broad melting profile was observed in the DSC thermograms, reflecting the melting of several overlapping lipid components. The introduction of increasing amounts of PazePC into the model membranes furthered the uncooperative behavior of the melting, severely broadened the melting profile as well as reduced the enthalpy required for the melting. Initially, the overall melting-temperature of the melting profiles was shifted to higher temperatures with higher PazePC content, but increased no further after reaching 5 mol% PazePC.

As previously shown by us, Bax protein will translocate towards PazePC-containing membranes without the need of BH3-only proteins. Changes induced by Bax could be seen already at 2 mol% PazePC in the DSC experiments. Bax increased the excess heat capacity and shifted the melting towards higher temperatures when the PazePC-content was increased. In general, Bax made the transition more cooperative in a PazePC dependent way.

MLVs either without PazePC or with 10% PazePC were used in NMR experiments to give atomic resolved snapshots of the membranes at selected temperatures as indicated by DSC corresponding to 1) where both membranes were in gel-phase (278 K), 2) where PazePC-free membranes were already completely melted, but PazePC-containing membranes were only partially melted (298 K), and 3) where PazePC-containing membranes were completely melted as well (308 K).

At 308 K the effects of introducing PazePC, as seen by $^1$H NMR, were two new signals in the same region where the glycerol 2-CH signal as well as the hydrogens at the oleoyl unsaturation are usually found. At the different temperatures studied,
peaks corresponding to the acyl-region increased with temperature in the \(^{1}\text{H}\) spectra, and while this also apply to the signals originating from protons in alpha-position relative to the unsaturation in the oleoyl-chains they did not increase as fast as other signals in the acyl-region.

From \(^{13}\text{C}\) CP MAS at 308 K narrow signals were observed, typical for liquid crystalline-membranes experiencing fast molecular dynamics and low spin-spin relaxation rates. As the temperature was decreased lines broadened as a consequence of slower dynamic and higher relaxation rates. At 298 K the PazePC-free vesicles were still clearly in their liquid crystalline-state while the PazePC-containing sample was already partly in the gel-phase and liquid crystalline-phase as indicated by two coexisting peaks at 30 ppm and 32 ppm, respectively. At 278 K, both samples were displaying spectra indicative of both liquid crystalline- and gel-phases, and two distinct carbonyl-signals could be seen in both spectra. The carbonyl signals were reduced in the spectra corresponding to liquid crystalline-phase, and were not even visible in the PazePC-containing MLVs at 308 K.

Fluorescence leakage assays were performed using either LUVs or GUVs with MOM-micking vesicles with different PazePC concentrations to probe Bax induced leakage at varying Bax-to-lipid ratios. Leakage from the LUVs was monitored by following the de-quenching of the fluorescent probe calcein, which is self-quenching at concentrations exceeding 1 mM. With increasing Bax concentration it was seen that the time for leakage onset, \(t_0\), happened earlier, the amount of released dye increased linearly, and the flux across the membrane increased in a non-linear fashion.

With varying PazePC-concentration it was observed that the flux across the membrane was amplified nearly four-fold as the PazePC concentration in the membrane was elevated from 0 to 10 mol\%. This behavior was the result of an increase in the total pore area at the membranes. However, at this constant Bax concentration it seemed that a similar amount of calcein was leaked from the different lipid mixes. The variation of these two parameters would suggest either that the pores only became slightly larger, or that when new pores were formed they typically appeared at vesicles where pores already exist.

Leakage experiments with GUVs made it possible to study individual vesicles, since they are so large that they can be observed even under a conventional microscope. In the GUVs experiments the fluorescent molecules were located outside the vesicles before induction of leakage (see Figure 21). When observing GUVs in the presence of the small molecule Atto 488, the distribution of the flux across membranes was fairly broad, but was shifted towards higher values for PazePC-containing membranes. As expected from the fairly constant levels of the final fluorescence leakage from LUVs (ca 80\%), GUVs with different PazePC-concentrations did also leak at comparable levels. Importantly it was observed that GUVs typically leaked all of their content or not at all, which indicated long-lived pores.
To determine the size of the pores in the GUVs, leakage experiments were performed with fluorescently labeled dextran molecules of molecular sizes 3 kDa or 10 kDa (which can be compared against 12 kDa for CytC). In experiments monitoring the smaller dextran molecules, 50% of the GUVs leaked (compared to ca 80% of the GUVs in the present of the small Atto 488 dye). No leakage was observed for the larger dextran molecules. The proportions of molecular weights of the large and small dextran molecules suggested that the pore size radius cannot be larger than 1.5 times of what was required for leakage of the smaller dextran. Importantly, without the presence of tBid, the pore size was not sufficient for the release of CytC; however, this protein has to be released in vivo for the final execution of cellular death (a kind of “point of no return”).

Figure 21. Principle of Atto 488 influx assay to monitor Bax induced leakage of GUV: An image of GUVs containing 8 mol% of PazePC 60 minutes after addition of Bax. Black vesicles indicate no influx of dye.

The protein-membrane interaction was also studied from the protein side by solid-state NMR. Homogeneously $^{15}$N-labeled Bax was confirmed to be correctly folded in solution by a two-dimensional $^1$H-$^{15}$N TROSY experiment. One dimensional $^{15}$N solid-state CP and INEPT experiments were performed to get information about flexible and rigid parts of the protein and confirmed the partial penetration of Bax into the lipid bilayer.

Conclusion

By using fluorescence leakage approaches we could show that the presence of the OxPl PazePC facilitates pore formation in MOM-mimicking unilamellar vesicles by Bax; a behaviour which depends strongly on the Bax concentration and the fraction of OxPls being present, and that there are no required threshold-
concentrations for either Bax or PazePC. Leakage studies for GUVs also indicated that the formed pores are too small to facilitate leakage of CytC.

For Bax to generate pores in the membranes it needs to modulate the physical behavior of the membrane, which was studied by a combined DSC-NMR approach. DSC and solid state NMR experiments revealed that PazePC has a dis-ordering effect on the membrane, which facilitates at least partial penetration of Bax as suggested by DSC thermograms where Bax have an ordering effect on the PazePC-containing membranes. The solid state $^{15}$N-CP and INEPT NMR spectra using labelled Bax in complex with the membrane, confirmed the penetration of various segments into the membranes. These protein regions are therefore dynamically restricted and CP visible, while other parts remain flexible (visible in INEPT NMR) and at or outside the membrane surface; an observation in agreement with recent models [61].

However, it should be emphasized that our system without Bax-activating tBid produced pores which were not sufficiently large to release CytC. In our system the situation may reflect a cellular state where it may still be possible to avoid irreversible steps toward MOMP and ultimately cell death.
7 Main findings and outlook

The main objective of this thesis was to provide a better understanding of the role which the mitochondrial membrane system plays in apoptosis under intracellular oxidative stress conditions. It could be demonstrated that OxPIs which are generated during oxidative stress have profound disorganizing effects on in vitro lipid membranes which faithfully mimic the MOM at its contact sites with the IMM. This disordering effect serves to shift a pre-existing equilibrium of the pro-apoptotic effector protein Bax towards the mitochondrial membrane, where it can form pores which facilitate leakage. Both of these separate processes occur without the requirement of the presence of Bax activating pro-apoptotic BH3-only proteins or the anti-apoptotic Bcl-2 protein to which Bax has a high affinity as shown in this thesis. However in the absence of BH3-only proteins the pores are not big enough to release the apoptotic factor CytC, seen as the final trigger for cell death. The summary of our findings clearly supports the active role which the mitochondrial membrane, under oxidative stress conditions, takes in the progression of events leading up to irreversible cell death via interactions with Bcl-2 family proteins. All these main findings can be summed up in our concept model of the membrane as an active partner in the apoptotic regulation by the Bcl-2 protein family (see Figure 22).

Figure 22. Our concept: Membrane-mediated recognition of Bcl-2 by Bax and its consequences is based on the “embedded together model” which includes the membrane as an active partner [67]; In a normal cell Bcl-2 inhibits the few primed Bax molecules upon membrane binding (left); ROS induced production of OxPIs (green; increases population of primed Bax (middle), which can overcome the inhibitory Bcl-2 effect by direct binding, followed by membrane permeabilization and finally cell death (right).
The point of no return is clearly connected to the formation of large pores by Bax and the release of apoptogenic factors from the IMS. It was shown in this work that Bax on its own can already form pores (although small), and that this process requires the protein to be at least partially inserted, as observed by NMR experiments of membrane-associated Bax. Our results therefore suggest that Bax has to undergo further conformational changes upon the activation by BH3-only proteins at the mitochondrial membrane to increase the size of the pores to enable CytC release. Therefore, the cell might be able to escape certain cell death by regulating the pore size of Bax in earlier stages of the apoptotic pathway.

The behavior of Bax at oxidized mitochondrial membranes underscores the importance of studying the interactions between different Bcl-2 family proteins at their native membrane environment, e.g. prior and upon onset of oxidative stress (as also induced by many cancer drugs).

It has become quite clear that the membrane’s lipid composition can have a large impact on the functions and interplay of the Bcl-2 family members, and that it is desirable to study how lipids can modulate these proteins structures and affinity towards each other at membranes; all these are still open questions. Importantly, we have provided biophysical evidence for the putative interaction between the full length anti-apoptotic Bcl-2 and the pro-apoptotic Bax in the presence of low detergent concentrations. Also, the full-length Bcl-2 was reconstituted into DMPC proteoliposomes. The next steps would obviously be to obtain the structure of the Bcl-2 protein in its membrane environment, followed by the structural characterization of its membrane-bound complex with Bax. The information from these studies would not only provide a detailed picture of the membrane-assisted conformational changes which both proteins undergo when the arbitrate life or death decision at the mitochondrial membrane, but it would also generate a wealth of information urgently needed in the development of novel cancer treatments.
8 Acknowledgements

I could not have wished for a better supervisor than Gerhard. The love and support you are showing for all people around you are simply fantastic. Your consideration for other people stretches far beyond duty or what is simply courteous. Both your personal and professional guidance have been crucial during the time I have spent as a PhD student. It is hard to put into word that would give justice to how awesome you are.

My co-supervisor Magnus who most excellently communicates his love for science both as a teacher, and as a group-leader. Although the times which I have turned to you for personal guidance have been few, your advices and mental cues have been as nuggets of gold.

Papa-bear, Tobias for always going above and beyond for those who need it.

Ander Pedersen and Göran Karlsson at Swedish NMR Centre, for all the effort you put into our collaboration with the Bcl-2 project; both in producing the Bcl-2 protein, and for receiving me in Gothenburg to let me work with batch-CFPE myself.

Radek, Sarka and Martin Hof for a close, although geographically distant, collaboration studying Bax in oxidized membranes. I am sure that you have a fantastic gang down there in Prague, and that your work is most of the time very fun.

Kristoffer for running SPR-experiments, and for all the good company during the Friday AW at MedChem.

The fantastic people who have previously and currently worked in our group have made life fun, and when the work is giving rise to frustration, doubt and woe they are the people who can really keep you going. Marcus understands my work, my thoughts and my feelings about it better than anyone else. Again there are no words to give justice to how important you have been for me, both professionally and personally. Your friendship above all is something that I value deeply. Johan - We have had interesting discussions about high and low, especially about TV-series and movies. You taught me how to brew beer, and I hope that we will expand upon that in the future. Artur, you are a very nice guy with cool interests like playing music with people from across the world and playing tabletop RPGs with you friends in Germany. You introduced me to Firefly, and have otherwise provided much appreciated geekiness to my everyday life. I am confident that you will be
doing fantastic things in your life. **Ilona**, it is always nice to talk to you, although it is sometimes hard to get you over to the fika-room. You should calm down a bit;) **Dat** and **Konrad** who although staying for some shorter time in our group still made impressions on us all to be wonderful people. I wish to see you again sometime soon.

Marcus did also especially invite me over to the excellent **After-Works** regularly organized at both **Organic Chemistry** and **MedChem**, where I have met many very nice people.

Everybody should have a couple of goto-guys when different questions arise. Although such people may be different depending on the questions at hand, two people have often presented themselves as first-hand options. **Patrik** and **Jörgen** have been of great help due to their helpful and open nature, as well as a sense for practical solutions. Also both of you are fantastic people.

Throughout the years at the department a number of research-groups have participated in inhouse seminars variously named after the PIs of the groups (**Gerhard**, **Magnus**, **Pernilla**, **Kwangho**, and **Ronnie**). Besides the seminars there have also been nice group activities and everyday socializing that have greatly contributed to the work-experience. Also all the people from the third and fourth floor participating (or not) in the Thursday-fika tradition throughout the years, have been great companies during this journey.


**The UPSC-gang.** You know who you are. Work would have been much more boring without you.

**Ett flertal vänner** som jag antingen har fått med mig från Arvindsjaur eller haft förmånen att få inkluderade i min bekantskapskrets efter att jag kom till Umeå. Även en del som är lite spridda över världen, men ändå är mina människor. Ni vet ungefär vilka ni är. Ni är alla fantastiska människor, och ni är alla speciella för mig. =)

Alla mina lärare som jag haft genom tiderna. I synnerhet **Lars-Håkan** på Sandbackaskolan, som inspirerade mig till att utbilda mig till kemist.
Veckosluten spenderade med Ximena “Hime-chan” och Tomas “T-Bone ConraDargo” för att titta på anime har varit ovärderliga.

Christian min kära barndomsvän, du kommer alltid ha en speciell plats i mitt hjärta.

Peter och Rikard, -ni är mina bästa vänner, och ingen förutom min närmaste familj har haft så stort inflytande över mitt liv som det ni har haft. Jag älskar er och båda era familjer.

Till min stärka Mamma och till min Pappa, vars knä jag kröp upp i för att se på Vetenskapens Värld och Nova när jag var liten. Ni lade grunden för allt som jag är.

Till Marina, Mikael och Mattias som mer än någon annan har influerat mig genom hela mitt liv. Ni lärde mig att tänka. Tack vare er så får jag lära känna Linus, Isak, och Hannes som är de mest fantastiska människor som finns.
9 References

17. Koutsopoulos, O.S., et al., Human Miltons associate with mitochondria and induce microtubule-dependent remodeling of


31. Weisz, K., et al., Deuteron Nuclear-Magnetic-Resonance Study of the Dynamic Organization of Phospholipid Cholesterol Bilayer-


