Examining the Molecular Mechanism of Bcl-2 Family Proteins at Membranes by Fluorescence Spectroscopy

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Abstract

The Bcl-2 family proteins control apoptosis by regulation of outer mitochondrial membrane permeabilization. Studying the Bcl-2 family is particularly difficult because the functional interactions that regulate apoptosis occur at or within intracellular membranes. Compared to other biophysical methods, fluorescence spectroscopy is well suited to study membrane-bound proteins as experiments can be performed with intact membranes and at protein concentrations similar to those found in cells. For these reasons, fluorescence spectroscopy has been particularly useful in studying the

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regulation of membrane permeabilization by Bcl-2 family proteins. Here, we discuss four fluorescence-based assays used to study protein dynamics at membranes, with a focus on how these techniques can be used to study the Bcl-2 family proteins.
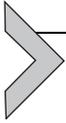
1. INTRODUCTION

The Bcl–2 family of proteins regulates permeabilization of the outer mitochondrial membrane (OMM). In most cell types, once the OMM is permeabilized, the cell is committed to undergoing programmed cell death (Budd, Tenneti, Lishnak, & Lipton, 2000). The sequence of events leading to permeabilization of the OMM begins with prodeath signals triggering posttranslational modifications of activator BH3-only proteins, such as the cleavage of Bid to cBid (comprised of a p7 and p15 fragment, the latter also referred to as tBid), that target them to the OMM where they bind to and activate the pore-forming proteins Bax and Bak. Activation of Bax and Bak results in their oligomerization within the OMM followed by permeabilization of the OMM and release of intermembrane space proteins such as cytochrome c and SMAC that act in downstream apoptotic pathways, culminating in cellular apoptosis (Shamas–Din, Kale, Leber, & Andrews, 2013). The antiapoptotic proteins, such as Bcl–2 and Bcl–X_L, inhibit apoptosis by binding to and sequestering both BH3-only activators and Bax/Bak (Bogner, Leber, & Andrews, 2010).

Significant research has been focused on determining the structure of Bcl-2 family proteins. X–ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have revealed that the Bcl-2 family proteins share a highly conserved core structure (Petros, Olejniczak, & Fesik, 2004). These studies have provided insight into how the Bcl–2 family proteins bind to each other and suggest how they may interact with membranes. However, the current relatively static structures for the Bcl-2 family are for proteins without the lipid bilayer required for functional interactions of several of the Bcl-2 family proteins (Leber, Lin, & Andrews, 2007). Determining the structures of proteins within a membrane mimetic environment using X–ray crystallography and NMR spectroscopy is particularly difficult. These techniques require a large amount of protein in a sample environment that mimics but differs significantly from that of the cell and typically includes detergents that can alter the functions of the Bcl-2 family proteins (Hsu & Youle, 1997). As an example, unlike native Bax, detergent–treated

Bax can cause permeabilization of the OMM when added to isolated mitochondria (Antonsson, Montessuit, Lauper, Eskes, & Martinou, 2000), can form oligomers that can be cross-linked in the absence of membranes (Zhang et al., 2010) and has undergone a conformational change that is a prerequisite for Bax activation (Yethon, Epand, Leber, Epand, & Andrews, 2003). Additionally, the zwitterionic detergent CHAPS can prevent the authentic interaction of Bax and tBid (Lovell et al., 2008), further reinforcing the need to study the Bcl-2 family proteins in the absence of detergents.

Fluorescence-based techniques are well suited to study protein dynamics at membranes under physiological conditions in the absence of detergents (Kale, Liu, Leber, & Andrews, 2012). Fluorescence spectroscopy allows observation of protein:protein- and protein:membrane-binding dynamics in real time, while gathering information about the kinetics and affinities of these interactions that cannot be measured using typical structural techniques due to complications from the membrane (Perez-Lara, Egea-Jimenez, Ausili, Corbalan-Garcia, & Gomez-Fernandez, 2012; Satsoura et al., 2012). Additionally, by using an environment-sensitive probe, it is possible to determine the environment of specific residues as they undergo conformational changes within membranes (Malhotra, Sathappa, Landin, Johnson, & Alder, 2013; Shamas-Din, Bindner, et al., 2013). Initial fluorescence-based studies of the Bcl-2 family proteins have used a simple *in vitro* system to study the dynamic interactions that occur at, on, and within membranes.
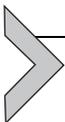
2. AN *IN VITRO* FLUORESCENCE-BASED LIPOSOME SYSTEM

The functional interactions of the Bcl-2 family proteins occur in membranes. Interaction of cBid with the membrane causes the p7 and p15 fragments of cBid to dissociate, whereupon the p15 fragment (tBid) undergoes a conformational change, that does not occur in solution and permits binding between tBid and Bax within the membrane (Shamas-Din, Bindner, et al., 2013; Shamas-Din, Kale, et al., 2013). Binding between Bax and cBid or Bax and Bcl-X_L requires membranes for an interaction to occur as an interaction is not detected in solution (Billen, Kokoski, Lovell, Leber, & Andrews, 2008; Lovell et al., 2008). Therefore, to study the function of these proteins, a biochemical system is required that includes a phospholipid bilayer that separates two distinct aqueous compartments

mimicking that of the cytoplasm and the interior of cellular organelles. We and others (Bleicken et al., 2010; Landeta et al., 2011; Ren et al., 2010; Shamas-Din, Bindner, et al., 2013; Shamas-Din, Kale, et al., 2013) have used different variations of liposome or proteoliposome-based systems to study the core mechanism of Bcl-2 family protein regulation of membrane permeabilization. All of these systems lack the detergents typically required for biochemical and structural studies of membrane proteins. For our studies, fluorescently labeled purified full-length recombinant proteins and artificial membranes in the form of liposomes with a composition mimicking that of the OMM are used. This system is free of any other complicating factors such as unknown binding partners that may be present at the OMM or within the cytoplasm.

To use fluorescence to study proteins at membranes, it is essential to make judicious choices of fluorophore, type of measurement, and instrument. Fluorescence measurements require excitation of the fluorophore by illuminating the sample with a specific wavelength of light and then recording the emission from the fluorophore. Upon excitation, after some period of time, termed the fluorescence lifetime (typically 1–10 ns), the fluorophore returns to the ground electronic state via emission of a photon at a lower energy, and thus longer wavelength, than the illuminating (excitation) light. Because the emitted fluorescence is of much lower intensity than the excitation light, the system must be free of molecules that absorb the emitted light, and fluorescence contaminants that may interfere with the emission signal from the fluorophore. Molecules such as quenchers that provoke non-radiative decay of the fluorophore must also be avoided as they change the fluorescence properties of the dyes. If these conditions are met, changes in both fluorescence lifetime and emission intensity can provide specific information about the underlying biochemical properties of the protein the fluorophore is attached to (Lakowicz, 2006).

2.1. Expression and purification of Bcl-2 family proteins

2.1.1 Expression of Bax, Bcl-X$_L$, and Bid

1. *Escherichia coli* are transformed (BL21-AI, New England Biolabs for cBid and Bax; DH5α, New England Biolabs, for Bcl-X$_L$) with the full-length Bax, Bcl-X$_L$, or Bid expression plasmid, plated on LB-ampicillin agar, and then incubated overnight at 37 °C. Bax and Bcl-X$_L$ are expressed with a carboxy-terminal intein-chitin binding domain (IMPACT expression system, New England Biolabs) and Bid is expressed with an amino terminal 6× histidine tag.

2. The next day, a single colony is picked and used to inoculate 100 mL of LB-ampicillin and grown overnight at 30 °C with shaking. Then 1–3 L of LB-ampicillin is inoculated with the overnight culture (20 mL for each liter of LB-ampicillin) and grown at 37 °C with shaking until the bacterial growth is in log phase (OD_{600} is typically between 0.6 and 0.8) at which point protein expression is induced with either arabinose (0.2%, w/v, BL21–AI cells) or IPTG (1 mM, DH5α cells). Bacteria are then incubated for 3–5 h at 30 °C with shaking, harvested using centrifugation, and stored at −20 °C. We find that, for both Bax and Bcl-X_L, a longer expression time (5 h) yields more recombinant protein.

2.1.2 Purification of Bax and Bcl-X_L

1. The bacterial pellet is resuspended in either Bax or Bcl-X_L lysis buffer (10 mL for each 2.5 g of bacterial pellet) and lysed via French press. Lysed cells are centrifuged at $20,000 \times g$ and the cell lysate is incubated with 1.5 mL of chitin bead slurry (New England Biolabs) for 2 h at 4 °C while rotating.

2. The cell lysate and resin slurry is then loaded into an Econo-Pac chromatography column (BioRad, Cat. #: 732-1010EDU) and the lysate passed through (three to four times) before the resin is washed with 50 mL of either Bax or Bcl-X_L wash buffer. The column is equilibrated with either Bax or Bcl-X_L cleavage buffer (10 mL) and capped with ∼1 mL of cleavage buffer remaining on top of the chitin beads followed by incubation for 24–36 h at 4 °C. The cleavage buffer contains hydroxylamine, which causes cleavage of the intein–chitin binding domain allowing full-length Bax and Bcl-X_L to be eluted. Bax or Bcl-X_L is then eluted with cleavage buffer (4×1 mL fractions). The majority of the protein is typically within fractions 1 and 2.

3. **a.** Bax: A 0.2-mL bed volume DEAE–Sepharose column is prepared and equilibrated with 2.5 mL of Bax-cleavage buffer (without hydroxylamine). Bax elution fraction 1 and 2 are pooled and passed through the column three times which removes additional contaminants that bind to the column.

   b. Bcl-X_L: A 0.3-mL bed volume high–performance phenyl Sepharose column is equilibrated with 3 mL of Bcl-X_L wash buffer. Bcl-X_L elution fractions 1–4 are pooled and applied to the column where Bcl-X_L binds and the column is washed with 5 mL of Bcl-X_L wash buffer (no PMSF in the buffer is needed). Bcl-X_L is eluted (3×1 mL

fractions) with Bcl-X$_L$ wash buffer that does not contain NaCl or PMSF.

4. Both Bax and Bcl-X$_L$ are dialyzed against 3×1 L of dialysis buffer (4 °C with stirring). After dialysis, the protein can be aliquoted and stored at −80 °C or can be labeled with fluorescent dyes (see Section 2.2).

2.1.3 Purification of cBid

1. The bacterial pellet is resuspended in Bid-lysis buffer (10 mL for each 2.5 g of bacterial pellet) and lysed via French Press. Lysed cells are centrifuged at $20,000 \times g$, and the cell lysate is incubated with 0.8 mL Ni–NTA agarose slurry (Qiagen) for 1.5 h at 4 °C while rotating.

2. The cell lysate and resin slurry is then added to a Poly–Prep column (BioRad, Cat. #: #731–1550EDU) and the lysate passed through three times. The column is washed with 50 mL of Bid-wash buffer, and Bid is eluted with 10 mL of Bid-elution buffer (collecting 5×1 mL fractions). The first two fractions typically contain the highest concentration of Bid and are pooled together. At this point, Bid can be cleaved to cBid (see below, step 3), or if labeling Bid with a fluorescent dye, the pooled fractions are first dialyzed 3×1 L against dialysis buffer at 4 °C with stirring and then labeled (see Section 2.2), followed by Bid cleavage and a final dialysis step.

3. To produce cBid, the pooled Bid elutions are adjusted to contain 40 mM HEPES, 1 mM EDTA, 10 mM DTT and incubated with 500 U of recombinant human caspase-8 (Enzo Life Sciences, Cat. #: BML-SE172-5000), and incubated for 48 h with rotating in the dark at room temperature. The cBid sample is next dialyzed against 3×1 L of dialysis buffer (4 °C with stirring) and then aliquoted and stored at −80 °C.

2.1.4 Buffer recipes

2.1.4.1 Bax

Bax-lysis buffer: 10 mM HEPES pH 7.0, 100 mM NaCl, 0.2% (w/v) CHAPS, 1 mM PMSF, DNase, RNase

Bax-wash buffer: 10 mM HEPES pH 7.0, 500 mM NaCl, 0.5% (w/v) CHAPS

Bax-cleavage buffer: 10 mM HEPES pH 7.0, 200 mM NaCl, 0.1% (w/v) CHAPS, 100 mM Hydroxylamine. It is important to check the pH of the cleavage buffer after adding hydroxylamine as it decreases the pH, which will lead to insufficient yield of protein.

2.1.4.2 Bcl-X$_L$

Bcl-X$_L$-lysis buffer: 20 mM Tris pH 8.0, 500 mM NaCl, 1% (w/v) CHAPS, 1 mM PMSF, DNase, RNase

Bcl-X$_L$-wash buffer: 20 mM Tris pH 8.0, 200 mM NaCl, 0.2% (w/v) CHAPS, 20% (v/v) glycerol, 1 mM PMSF

Bcl-X$_L$-cleavage buffer: 20 mM Tris pH 8.0, 200 mM NaCl, 0.2% (w/v) CHAPS, 20% (v/v) glycerol, 1 mM PMSF, 100 mM Hydroxylamine. It is important to check the pH of the cleavage buffer after adding hydroxylamine as it decreases the pH, which will lead to insufficient yield of protein.

2.1.4.3 Bid

Bid-lysis buffer: 10 mM HEPES pH 7.0, 100 mM NaCl, 10 mM imidazole, 1 mM PMSF, DNase, RNase

Bid-wash buffer: 10 mM HEPES pH 7.0, 300 mM NaCl, 10 mM imidazole, 1% (w/v) CHAPS

Bid-elution buffer: 10 mM HEPES pH 7.0, 100 mM NaCl, 200 mM imidazole, 0.1% (w/v) CHAPS, 10% (v/v) glycerol

2.1.4.4 Dialysis

Dialysis buffer (for Bax, cBid, and Bcl-X$_L$): 10 mM HEPES pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol

Extensive dialysis is needed to remove CHAPS which can alter the function and binding interactions of Bcl–2 family proteins. We typically dialyze our purified protein samples for a minimum of 4 h against 1 L of buffer, followed by dialysis overnight (∼12–16 h) against 1 L of buffer and a final dialysis against 1 L of buffer for a minimum of 4 h. The use of spin–concentrator columns should be avoided, in our experience, as they severely attenuate the function of the Bcl–2 family proteins.

2.2. Site-specific protein labeling

The fluorescence–based techniques we use to study the Bcl–2 family require purified recombinant proteins labeled with a fluorophore at a specific location. There are two main options for labeling proteins, thiol or primary amine labeling. Cysteine residues are less abundant than lysines in most protein sequences, thus we most frequently create single-cysteine mutants to label the protein as this approach minimizes the number of mutations required. There is a full spectrum of fluorescent probes available for purchase, which have different spectral properties that can be ordered with

attached thiol reactive moieties such as iodoacetamide or maleimide derivatives. Dyes must be chosen that are compatible not only with your protein of choice but also with the system and equipment available.

In the methods reported below, the proteins were labeled with the low-molecular weight fluorescent probes DAC (N-(7-dimethylamino-4-methylcoumarin-3-yl) maleimide; Anaspec, Cat. #: 81403) and NBD (N,N'-dimethyl–N-(Iodoacetyl)-N'-(7–nitrobenz-2–oxa-1,3–diazol-4–yl) ethylenediamine; Molecular Probes, Cat. #: D–2004). The small size of these dyes is a distinct advantage as they rarely perturb protein function; however, measurements of NBD fluorescence require a sensitive instrument as the quantum yield (ratio of photons emitted to photons absorbed) is low. Moreover, excitation of DAC requires an ultraviolet light source and both the excitation and emission of this dye overlap endogenous fluorophores in cells typically limiting its use to liposome-based systems. Many brighter (higher quantum yields and extinction coefficients) fluorescent dyes have molecular weights above 1 kDa, and in our experience these larger dyes frequently change the function of the protein they are attached to.

Initially, it is best to follow the labeling protocol included by the manufacturer when labeling your protein of interest; however, it is often necessary to deviate from these conditions to get labeling that is both specific and efficient.

1. For Bid, Bax, and Bcl–X_L, the protein–labeling reaction is performed in a HEPES-based buffer at pH 7.0–7.5 (10 mM HEPES, 200 mM NaCl, 0.4%, w/v CHAPS). This pH range allows the cysteines to be most–reactive while decreasing the reactivity of primary amines. A 10–20 × M excess of dye is added to the sample tube dropwise, to prevent protein denaturation as dyes are typically dissolved in DMSO, and the labeling reaction is rotated at room temperature for approximately 2 h in the dark. A reducing agent (5 mM DTT) is then added to quench the reaction.

2. **a.** Bax and Bcl–X_L: Free dye is removed via gel filtration over a G–25 fine Sephadex column (10 mL bed volume, equilibrated with dialysis buffer). Bax- and Bcl–X_L–labeling reactions are applied to the column and approximately 12 0.5 mL fractions are collected.

   b. Bid: The Bid-labeling reaction is applied to a Ni–NTA column (0.2 mL bed volume) and passed through three to four times allowing labeled Bid to bind to the column. The column is washed with 50 mL of Bid-wash buffer and eluted with 5 mL of Bid-elution buffer, collecting 4 × 0.5 mL fractions.

3. Absorbance spectroscopy is used to determine protein containing fractions. Absorbance at both 280 nm (to detect protein) and at the absorbance peak of the dye used is determined. The fractions containing the highest amount of protein are pooled. Bid can now be cleaved (see Section 2.1.3, step 3). Bax, Bcl-X_L, and cBid are then dialyzed against 3×1 L dialysis buffer at $4\,°C$ with stirring to remove any remaining free dye and detergent, and the protein is aliquoted and stored for later use.

4. After dialysis, labeling efficiency is calculated by first determining the concentration of your protein via absorbance at 280 (Bax), BCA assay (Bcl-X_L), or Bradford assay (cBid). Then the concentration of the dye in the protein sample is determined by the OD of the sample at the peak absorbance wavelength of the label, as outlined in the protocol supplied by the manufacturer. Assuming that the protein is only labeled at the single-cysteine residue and that there is no free dye in the sample, the concentration of the dye should equal that of the labeled protein. Labeling efficiency is the fraction of labeled protein to that of total protein.

Single-cysteine mutants of the purified recombinant protein need to be assayed functionally before and after labeling to determine if the mutation or the addition of the dye alters protein function. Ideally, we begin using mutants where one of the endogenous cysteines is present to minimize the amount of mutations introduced into the protein. If the protein does not contain any cysteines, choosing which residue to mutate to cysteine for efficient labeling and proper protein function is largely empirical. Typically, if the structure is known, one begins using solvent exposed residues, since those located in hydrophobic regions are difficult to label. Algorithms used to predict solvent exposure or antigenicity (antigenic sites tend to be both structured and solvent exposed) can often be useful in selecting a location if the structure of your protein is unknown.

2.3. Production of mitochondria-like liposomes

Large unilamellar vesicles (LUVs) are liposomes that have with a mean diameter of 120–140 nm and one lipid bilayer (Hope, Bally, Webb, & Cullis, 1985). OMM-like LUVs have been established as a valid biochemical model for membrane permeablization by Bcl-2 family members (Kuwana et al., 2002). These liposomes are assembled from lipids in fixed molar ratio similar to that of the OMM, based upon lipid composition studies from solvent extracted Xenopus mitochondria (Kuwana et al., 2002). Such liposome-based systems allow the analysis of Bcl-2 family proteins in a simple context

while preserving their authentic functions. It is possible to more directly explore Bcl-2 family function in this kind of system because the protein and lipid components are well defined and tractable, unlike isolated mitochondria or proteoliposomes prepared from membranes.

2.3.1 Preparing lipid films and generating liposomes

1. Chloroform solublized lipids are added to a glass test tube to make a lipid mixture of a defined composition (Table 1.1) to a total of 1 mg lipid mass. The chloroform is evaporated off with nitrogen gas while rotating the tube to ensure an even distribution of lipids on the wall and then put under vacuum for 2 h at room temperature to remove any remaining chloroform. The dry lipid film is then either used immediately or can be stored for up to 2 weeks at $-20\,°C$. To reduce lipid oxidation by atmospheric oxygen during storage, it is advisable to layer nitrogen or argon gas on top of the lipid film and seal the tube with parafilm.

2. The dry 1 mg lipid film is hydrated with 1 mL of assay buffer (10 mM HEPES, 200 mM KCl, 5 mM MgCl$_2$, 0.2 mM EDTA, pH 7). The lipids become suspended and spontaneously form lipid bilayer vesicles due to

Table 1.1 Mitochondria-like lipid film composition

Name	Company	Catalog #	Molar (%)	Molecular weight (g/mol)	Amount needed for 1 mg lipid film (mg)
"PC": L–α–phosphatidylcholine (egg, chicken)	Avanti	840051C	48	770.123	0.4596
"PE": L–α–phosphatidylethanolamine (egg, chicken)	Avanti	841118C	28	726.076	0.2528
"PI": L–α–phosphatidylinositol (liver, bovine)	Avanti	840042C	10	902.133	0.1122
"DOPS": 1,2-dioleoyl–sn-glycero–3-phospho-L–serine	Avanti	840035C	10	810.025	0.1007
"TOCL": 1,1′,2,2′–tetra-(9Z-octadecenoyl) cardiolipin	Avanti	710335C	4	1501.959	0.0747

the association of the hydrophobic tails, forming the center of the bilayer, and the grouping of the hydrophilic heads of the phospholipids, forming the edges of the bilayer. However, these vesicles are multilamellar as they contain more than one lipid bilayer and their size distribution is not homogeneous. To generate unilamellar liposomes, the lipid mixture is subjected to 8–10 freeze/thaw cycles by alternately placing the sample vial in liquid nitrogen and a warm water bath (Hope et al., 1985). The unilamellar liposomes are extruded 11 times through a filter with 0.1 μm pore size to produce liposomes of a uniform size, at a final concentration of 1 mg/mL lipid.
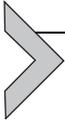
3. MEMBRANE PERMEABILIZATION ASSAY

The Bcl–2 family proteins play a pivotal role in regulating apoptosis by controlling the permeabilization of the OMM through the activation of Bax/Bak. Thus, a membrane permeablization assay is one crucial functional assay for the Bcl–2 family proteins. To assay liposome permeabilization, the liposomes are encapsulated with a polyanionic fluorophore, ANTS (8-aminonaphthalene–1,3,6–trisulfonic acid; Molecular Probes, Cat. #: A350), and cationic quencher, DPX (p-xylene-bis-pyridinium bromide; Molecular Probes, Cat. #: X1525). Due to the high local concentration of DPX, ANTS fluorescence is quenched when liposomes are still intact. Recombinant Bax and/or other Bcl–2 family proteins and/or reagents are added to the system in order to assay permeabilization. As the liposomes permeabilize, ANTS and DPX are released from the liposomes, greatly decreasing the local concentration of the quencher resulting in a gain of ANTS fluorescence. The kinetics and extent of membrane permeabilization can reveal crucial information for studying relationships between Bcl-2 family members and how they regulate membrane permeabilization.

3.1. ANTS/DPX release assay

1. A dry 1 mg lipid film is hydrated with 1 mL of assay buffer with the addition of ANTS (12.5 mM) and DPX (45 mM). The lipid suspension is vortexed until the ANTS and DPX dissolve, and liposomes are created as above via 10 freeze/thaw cycles and extrusion through a 0.1 μm pore size membrane.

2. Excess ANTS and DPX are removed by applying the extruded liposomes onto a CL2B size-exclusion column (10 mL bed volume), that separates the ANTS/DPX encapsulated liposomes from the free

ANTS/DPX in solution (Billen et al., 2008; Yethon et al., 2003). Fractions (1 mL each) are collected in glass tubes and the liposome containing fractions (typically fractions 3 and 4) are identified by an increase in cloudiness of the sample which occurs due to light scattering by the liposomes. The two liposome containing fractions are combined resulting in a final ANTS/DPX liposome concentration of approximately 0.5 mg/mL lipid. These liposomes can now be used to test the regulation of membrane permeabilization by the Bcl–2 family proteins.

3. The assay is set up in a low protein binding 96–well plate (Corning; Cat. #: 3686) and in each well to be measured, 8 μL of ANTS/DPX liposomes are added to 92 μL of assay buffer. Background measurements (F_0) are recorded at 30 °C using a fluorescence plate reader (Tecan M1000 pro) set to excite the sample at 355 nm (5 nm bandwidth) and collect emission at 520 nm (12 nm bandwidth).

4. Proteins are added to the desired concentrations and combinations in each well and fluorescence emission of ANTS (F) is recorded every minute for 3 h at 30 °C. Any increase in fluorescence emission is directly related to membrane permeabilization.

5. To normalize the data, 100% ANTS release is determined by the addition of Triton to each well at a final concentration of 0.2% (w/v) causing permeabilization of all liposomes and ANTS fluorescence is measured (F_{100}). This results in a slight overestimation of the intensity of 100% release due to the dye becoming trapped in detergent micelles. Nevertheless, the release percentage generally does not take this into account and is calculated as follows:

$$\text{ANTS release}\,(\%) = \frac{F - F_0}{F_{100} - F_0} \times 100\%$$

The ANTS/DPX release assay can be used to dissect exactly how the different classes of Bcl–2 family proteins affect permeabilization of the OMM. When cBid (20 nM), Bax (100 nM), or Bcl–X$_L$ (40 nM) are added individually to liposomes they do not cause membrane permeabilization (Fig. 1.1A). Incubation of liposomes with cBid and Bax results in membrane permeabilization due to cBid binding to membranes causing separation of the two fragments of cBid with the p15 (tBid) fragment remaining membrane-bound and -activating Bax. Bcl–X$_L$ inhibits this process by binding to and inhibiting both tBid and Bax (Billen et al., 2008; Lovell et al., 2008). Obviously, other techniques are needed to discern exactly how these
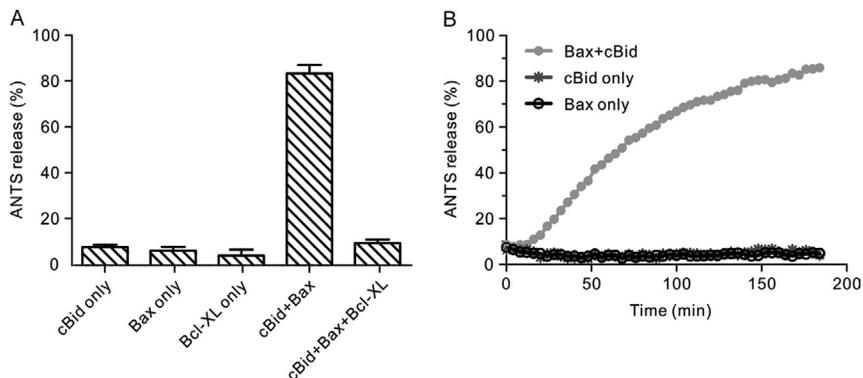
Figure 1.1 (A) Endpoint values of ANTS assay with 100 nM Bax, 20 nM cBid, 40 nM Bcl-X$_L$ or both, or with 100 nM Bax, 20 nM tBid, and 40 nM Bcl-X$_L$. ($n = 3$). (B) Liposomes encapsulated with ANTS and DPX were incubated with 100 nM Bax, 20 nM cBid, or both. Membrane permeabilization was assayed by an increase of ANTS fluorescence.

interactions occur (see Section 4); however, this dye release assay allows the functional consequence of the addition of any number of various combinations of Bcl–2 family members or small molecule effectors of the proteins to be determined. Furthermore, it provides information on how changes in relative concentrations of the proteins can vary the extent of permeabilization or how alterations in the parameters of the assay affect membrane permeabilization. For example, it is possible quantify how changes in liposome composition affect Bcl–2 family proteins functions to permeabilize membranes or test specific mutations that may inhibit/activate the protein of interest. Additionally, the kinetics of pore formation can be studied allowing the comparison of kinetics for Bax–mediated membrane permeabilization in response to various BH3-only activators (Fig. 1.1B).
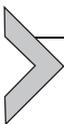
4. FLUORESCENCE RESONANCE ENERGY TRANSFER

Here, fluorescence resonance energy transfer (FRET) will be used to detect binding between cBid and Bax, and Bax oligomerization. FRET is possible between fluorophores when the emission spectra of one fluorescent molecule, termed the donor, overlaps the excitation spectra of another fluorophore, the acceptor. When a donor fluorophore is excited by light, an electron moves to a higher energy state and, in the presence of an acceptor, the energy is transferred nonradiatively to the acceptor fluorophore via dipole–dipole interactions between the two probes. This transfer of energy

results in a decrease of the donor emission, and it is the change in the light emitted by the donor that we track to measure FRET between two proteins.

One of the main advantages of FRET is that it requires both the donor and acceptor fluorophores to be in close proximity for the required dipole coupling to occur. As a result, FRET efficiency decreases to the sixth power of distance according to the formula for FRET efficiency (E) at a fixed donor acceptor distance:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

where R_0 is the Förster distance, the distance between a donor acceptor pair at which a 50% FRET efficiency is observed and r is the distance between the donor and acceptor. The distance dependence of FRET is illustrated in Fig. 1.2A where FRET efficiency is calculated for distances between a donor
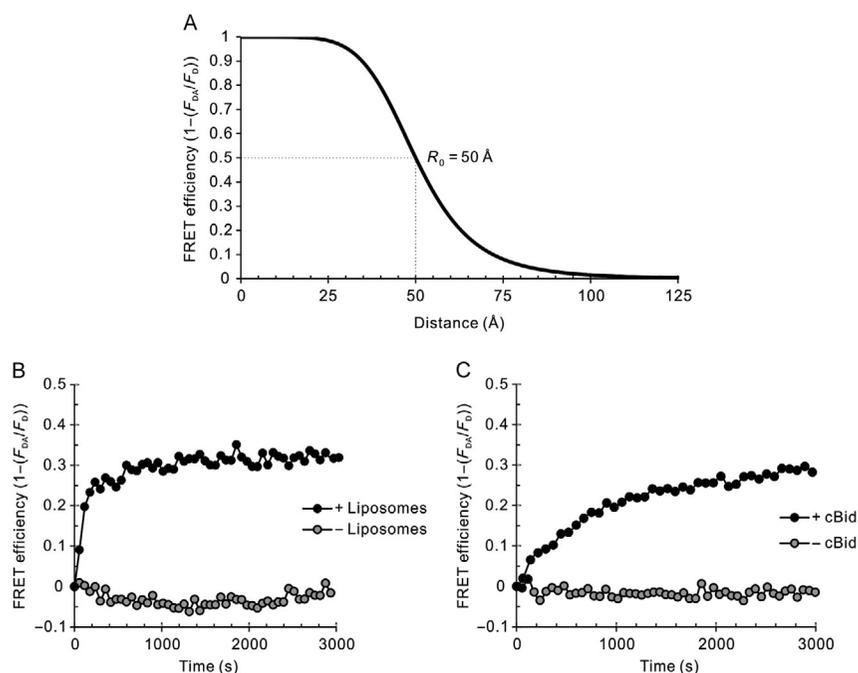
Figure 1.2 (A) FRET efficiency as a function of distance between a dye pair with a theoretical Förster distance of 50 Å. (B) FRET between cBid-DAC (20 nM) and Bax-NBD (100 nM) in the presence (black circles) and absence (gray circles) of liposomes (0.2 mg/mL). (C) FRET between Bax-DAC (20 nM) and Bax-NBD (100 nM) in samples containing liposomes (0.2 mg/mL) with (black circles) or without (gray circles) 20 nM cBid.

and acceptor pair with an R_0 of 50 Å (Lakowicz, 2006). For this dye pair, FRET will only be detected if the distance between the two fluorophores is 70 Å or less. Typical R_0 values for a donor and acceptor pair are between 30 and 60 Å, similar to the size of proteins; thus, if FRET between donor- and acceptor-labeled proteins is detected then they are bound to each other.

4.1. Detecting the interaction between two proteins using FRET

As mentioned in Section 1, the BH3-only protein cBid targets to and embeds within the OMM where it recruits and activates cytosolic Bax (Leber et al., 2007; Lovell et al., 2008). Active membrane-bound Bax oligomerizes within the OMM resulting in membrane permeabilization. Here, we are using DAC and NBD as the donor and acceptor molecules, respectively. We will be using FRET to detect (1) the binding between cBid and Bax and (2) the binding between Bax molecules during oligomerization.

1. Liposomes are made as above (Section 2.3.1) resulting in liposomes at a concentration of 1 mg/mL lipid.
2. The fluorimeter (Photon Technology International) is set to record the fluorescence of DAC (380 nm excitation, 2 nm slit width; 460 nm emission, 10 nm slit width) with stirring for 1 h at 37 °C. Either 200 μL of liposomes and 800 μL of assay buffer, or as a control, 1 mL of assay buffer is added to a quartz cuvette and the signal is read until it remains stable (∼5 min). Two reactions are required to detect FRET. One that contains both the donor- and acceptor-labeled proteins and a control that contains the donor-labeled protein and unlabeled acceptor protein. This control accounts for any changes in the donor protein that occur due to binding interactions, conformational changes, or environment changes that may affect the spectral properties of the donor dye.
3. The donor-labeled protein is added to the cuvette at a concentration of 20 nM and DAC fluorescence is read until the signal is stable. At this point, the acceptor protein that is either labeled with NBD or unlabeled is added to the system at a concentration of 100 nM. It is important to keep the amount of acceptor higher (5–10×) than that of the donor. Keeping the donor protein in excess ensures, it will be saturated by the acceptor.
4. The DAC signal is recorded for 1 h at 37 °C. FRET efficiency (E) is measured by comparing the relative intensity of the donor in the presence of labeled (F_{DA}) and unlabeled (F_D) acceptor and is calculated by:

$$E = 1 - \frac{F_{\mathrm{DA}}}{F_{\mathrm{D}}}$$

Figure 1.2 illustrates two binding interactions between the Bcl-2 family proteins cBid and Bax. Donor (DAC)-labeled cBid (20 nM) is incubated with acceptor (NBD)-labeled Bax (100 nM), and only in the presence of liposomes do the two proteins interact (Fig. 1.2B). This underlines the point that many functional interactions of the Bcl-2 family proteins only occur in the presence of a lipid bilayer. Additionally, the activator protein cBid is required for Bax to oligomerize, since FRET between donor (DAC) and acceptor (NBD)-labeled Bax is only observed when cBid is added to the system (Fig. 1.2C). As we observe the interactions of two proteins in real time, kinetics of the reactions can be determined. Indeed, it is clear from the data shown that the cBid–Bax interaction occurs faster than Bax oligomerization, suggesting that cBid first binds to and activates Bax followed by Bax oligomerization. Additionally, it is possible to generate a binding curve where an affinity for the interaction can be determined as was done for the binding between cBid and Bax (Lovell et al., 2008). To do this multiple FRET measurements are obtained by titrating the amount of acceptor, while keeping the donor concentration fixed.

5. TRACKING THE CONFORMATION CHANGES OF A PROTEIN

NBD is an environment-sensitive low-molecular weight fluorescent dye that has been used to track environment changes of specific residues of proteins (Dattelbaum et al., 2005; Lin, Jongsma, Pool, & Johnson, 2011). The emission intensity and fluorescence lifetime increases and the emission peak of NBD blueshifts from 570 nm, in an aqueous environment, to 530 nm when it is in a hydrophobic environment due to a decrease in fluorescence quenching by water (Crowley, Reinhart, & Johnson, 1993). The small size of NBD allows specific labeling of single-cysteine mutants of proteins, with less potential perturbation of wild-type function. Importantly, NBD is uncharged but has sufficient polar characteristics that it remains stable in both polar and nonpolar environments such that it is less likely than other environment-sensitive dyes to change the membrane-binding characteristics and/or conformation of the protein being studied (Shepard et al., 1998). These properties of NBD make it particularly useful to study membrane-binding proteins such as Bax and cBid that transition

from the aqueous environment and embed into a membrane bilayer (Lovell et al., 2008; Shamas-Din, Bindner, et al., 2013; Shamas-Din, Kale, et al., 2013).

5.1. NBD-emission assay

Real-time changes in the fluorescence of NBD can be measured to determine whether and when specific regions of Bax (labeled with NBD) insert into the membrane during the activation of Bax. It is known from chemical-labeling studies that Bax inserts helices 5, 6, and 9 into the membrane (Annis et al., 2005). By labeling Bax at residue 175 (helix 9), it is possible to track the conformational change of Bax as it transitions from a soluble monomer to membrane embedded oligomer.

1. The fluorimeter is set to record NBD fluorescence (475 nm excitation, 2 nm slit width; 530 nm emission, 10 nm slit width), and as in the FRET experiment above, 200 μL of 1 mg/mL liposomes are added to 800 μL of assay buffer in a quartz cuvette. Background signal (Bg) is recorded with stirring until stable at 37 °C.

2. NBD-labeled Bax (100 nM) is added to the cuvette. Since Bax does not insert into membranes in the absence of an activator (Hsu & Youle, 1998), Bax-NBD has a stable signal when incubated with liposomes and an initial fluorescence value can be recorded (F_0). Alternatively, the very first point upon addition of the protein can be used as the F_0 value if the protein insert into lipids too rapidly. This approach is useful for proteins that are unstable in the assay solution such as cBid which spontaneously targets to membranes (Shamas-Din, Bindner, et al., 2013; Shamas-Din, Kale, et al., 2013). In the absence of membranes, cBid has sufficient exposed hydrophobicity that it tends to aggregate and to stick to the walls of the cuvette.

3. In our example, the change in emission over time (ΔF) of the dye labeled Bax is collected once an activator, cBid, is added. Fluorescence intensity plateaus after the protein comes to equilibrium (1 h endpoint). By calculating the ΔF value, one can track the relative change in emission intensity of the labeled residue in real time:

$$\Delta F = \frac{F - \text{Bg}}{F_0 - \text{Bg}}$$

Both residues 3 and 175 of Bax transition to a more hydrophobic environment as indicated (Fig. 1.3A) by the relative change in emission (ΔF). As the

environment change of the residue can be tracked over time, kinetics of membrane binding can be measured. Tracking the kinetics of the environment changes of various residues as a protein undergoes a conformational change has been used to order specific structural changes of the protein (Shamas-Din, Bindner, et al., 2013; Shamas-Din, Kale, et al., 2013). Here, the carboxyl terminus of Bax (residue 175) has slower kinetics compared to that of the amino terminus of Bax (residue 3) suggesting that Bax undergoes a conformational change at residue 3 before that of 175. Additionally, residue 175 moves to a more hydrophobic environment since Bax 175C–NBD has a larger change in NBD emission compared to Bax 3C–NBD. This
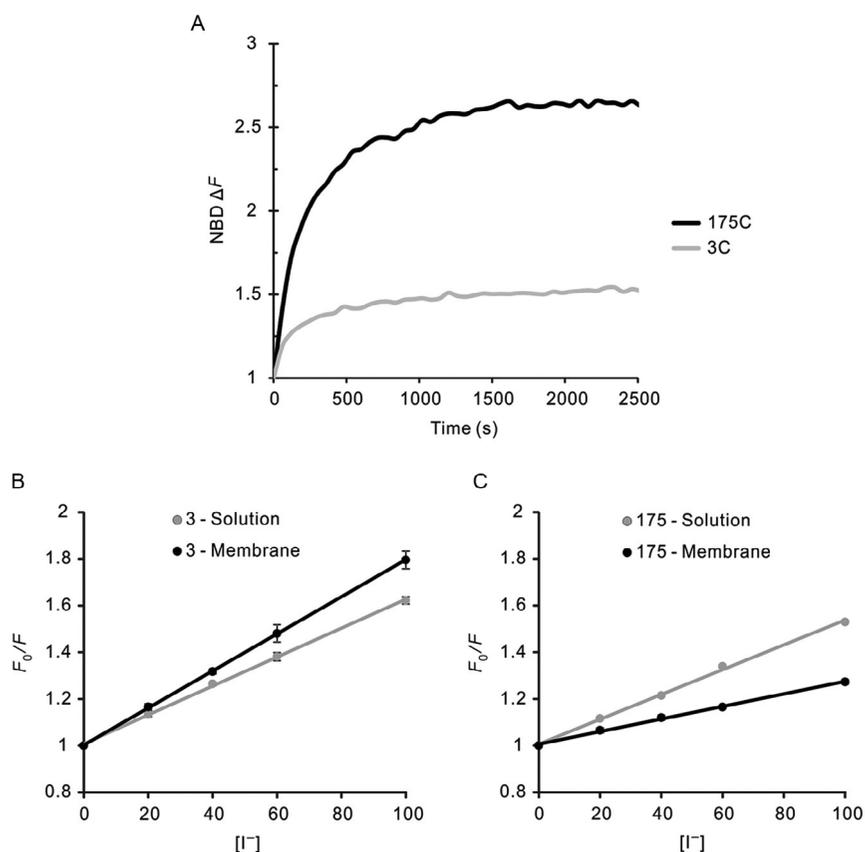
Figure 1.3 (A) NBD emission change for Bax 175C-NBD (100 nM) and Bax 3C-NBD (100 nM) upon addition of cBid (20 nM) in the presence of liposomes (0.2 mg/mL) (B and C) Iodide quenching data of 100 nM Bax 3C-NBD (B) or 175C-NBD (C) in solution (gray) or in the presence of liposomes (0.2 mg/mL) and cBid (20 nM) (black).

paired with the quenching data discussed below, suggests that residue 175 of Bax inserts into the phospholipid bilayer. As this residue is part of a larger hydrophobic sequence believed to span the bilayer, the kinetics for this residue likely represent insertion of the Bax carboxyl–terminal tail into membranes. This is in accordance with data that shows residue 175C is embedded within the mitochondrial membrane in cells (Annis et al., 2005). By using various activators of Bax or mutations known to perturb Bax function, it is possible to determine whether these changes affect the extent of or rate at which Bax helix 9 inserts into phospholipid bilayers.

6. DETERMINING THE TOPOLOGY OF PROTEINS WITHIN MEMBRANES

Fluorescence quenching by heavy atoms such as iodide can be used to determine how exposed a fluorescently tagged residue is to the solvent. This is due to collisional quenching that occurs when I^- collides with an excited fluorophore, resulting in a loss of energy back to ground state without emission of a photon. Typically, collisional quenching requires direct molecular interaction with the fluorophore such that the distance of quenching is <2 Å giving a very high resolution to detect solvent accessibility (Lakowicz, 2006).

Since I^- quenches NBD fluorescence (Crowley et al., 1993; Lin, Jongsma, Liao, & Johnson, 2011), this technique would be advantageous to look at the difference of residue solvent accessibility between soluble monomeric Bax, in the absence of activator, and membrane-bound oligomeric Bax, in the presence of an activator.

6.1. Iodide quenching of NBD-labeled Bax

1. As in the method for NBD emission change, the fluorimeter is set to record NBD fluorescence, and 200 µL of 1 mg/mL liposomes are added to 800 µL of assay buffer in a quartz cuvette. Background signal is read with stirring until stable at 37 °C.

2. NBD-labeled Bax (100 nM) and cBid (20 nM) are added to cuvettes containing either liposomes (200 µL liposomes, 800 µL assay buffer) for quenching of membrane bound Bax, or assay buffer only (1 mL assay buffer), for quenching of solution Bax. NBD emission (F_0) is recorded after incubation of the sample at 37 °C for 1 h.

3. Multiple quenching reactions are set up where aliquots of potassium iodide (2 M, supplemented with 2 mM sodium thiosulfate to prevent

oxidation) and potassium chloride (2 M) stock solutions are added to each sample so that the total ion concentration, and thus ionic strength, in samples is the same (typically 100 mM) (Table 1.2). The NBD emission for each concentration of KI is determined (F) and collisional quenching is calculated by the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{sv}[Q]$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity at a specific quencher concentration [Q] and K_{sv} is the Stern–Volmer quenching constant.

As the concentration of I$^-$ increases, so does the extent of quenching as determined by the Stern–Volmer equation, allowing the titration curve to be fit with a line where the slope is the Stern–Volmer constant (K_{sv}). The smaller the Stern–Volmer constant the more protected a residue is from the solvent. Quenching can then be used to compare the change in exposure of Bax residues to solvent upon the addition of an activator. Residue 3 shows a slight decrease in protection from quenching upon the addition of cBid (Fig. 1.3B), whereas residue 175C of Bax becomes more protected from quenching, in agreement with this region of Bax inserting into the bilayer (Fig. 1.3C) (Annis et al., 2005).

7. CONCLUSION

Here, four techniques have been highlighted to show how membrane proteins can be studied by fluorescence spectroscopy. The high sensitivity of fluorescence-based assays along with the ability to probe the dynamics of protein:protein and protein:membrane interactions in real time lends itself well to study a complex regulatory system such as the Bcl-2 family of proteins. The methods outlined use a simplified liposome system but can be extended to study Bcl-2 family regulation in isolated mitochondria (Shamas-Din, Bindner, et al., 2013; Shamas-Din, Kale, et al., 2013). Additionally, interactions between proteins can be detected via FRET in live cells using fluorescence lifetime imaging microscopy that recapitulate what we see *in vitro* (Aranovich et al., 2012). Thus, fluorescence is a very powerful technique that not only allows us to determine the core mechanism of Bcl-2 family regulation *in vitro* but also allows us to extend these studies to live cells and *in vivo*, bridging the gap between simplified and complex systems (Kale et al., 2012).

Table 1.2 Iodide quenching values of NBD-labeled Bax

KI (mM)	KCl (mM)	3C[a]		175C[a]	
		Solution (F_0/F)	Membrane (F_0/F)	Solution (F_0/F)	Membrane (F_0/F)
0	100	1	1	1	1
20	80	1.1351 ± 0.0105	1.1657 ± 0.0136	1.1158 ± 0.0002	1.0650 ± 0.0134
40	60	1.2645 ± 0.0068	1.3176 ± 0.0101	1.2140 ± 0.0029	1.1110 ± 0.0112
60	40	1.3812 ± 0.0180	1.4807 ± 0.0373	1.3397 ± 0.0019	1.1647 ± 0.0020
100	0	1.6224 ± 0.0156	1.7954 ± 0.0376	1.5294 ± 0.0002	1.2730 ± 0.0093

[a]Values for solution and membrane quenching are plotted in Fig. 1.3C.

ACKNOWLEDGMENTS

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