Preface

Biological membranes have long been identified as key elements in a wide variety of cellular processes, including cell defence, communication, photosynthesis, signal transduction and motility, and thus they emerge as primary targets in both basic and applied research. In order to fully appreciate the role that biological membranes play in these essential biological processes it becomes crucial to get a molecular-level picture of the structure of their main components (i.e. lipids and membrane proteins) as well as the nature of their cross-interactions in order to decipher how these molecular recognition processes determine the modulation of membrane-related functions. In this respect, the high-resolution structure of quite a few membrane proteins determined in recent years, many of them of prokaryotic origin, are at centre stage in modern membrane research as they allow for the rational design and interpretation of experiments, which were unthinkable not too long ago.

This book is an attempt to assemble in a single volume the most recent views of some of the experts in the area of protein–lipid interactions which draw a general picture of the advances that this field has achieved in recent years, from very basic aspects to specialized technological applications. The topics in this book include the application of X-ray and neutron diffraction, infrared and fluorescence spectroscopies, as well as high-resolution NMR methodologies that improve our understanding of the specific interactions established between lipids and proteins within biological membranes, their structural relationships, and the implications to the biological functions they mediate. In addition, the insertion of proteins and peptides into the membrane and the concomitant formation of definite lipid domains within the membrane, are other topics covered in this volume. We hope that the selection of subjects is timely and stimulating both for the dedicated scientist and for the general reader.

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NMR of Membrane Proteins in Lipid Environments: the Bcl-2 Family of Apoptosis Regulators

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2.1 Introduction

Many fundamental cellular functions are regulated by proteins that are integral to membranes, or that associate peripherally with their surfaces. Understanding the structures of these molecules is a major goal of structural biology; however, despite their importance, only a few structures of membrane proteins have been deposited in the Protein Data Bank (PDB), compared to the thousands of coordinates deposited for globular proteins (www.rcsb.org/pdb/). This deficit reflects the lipophilic character of membrane proteins, which makes them difficult to over-express and purify, complicates crystallization for X-ray analysis, and results in highly overlapped and broadened solution NMR spectral lines. NMR spectroscopy offers two complementary approaches to membrane protein structure determination: solution NMR with samples of membrane proteins dissolved in lipid micelles, and solid-state NMR with samples of proteins incorporated in lipid bilayers, a method that is particularly attractive because it enables structures to be determined in an environment that closely mimics the biological membrane.

High-quality solution NMR spectra can be obtained for some large, helical, membrane proteins in micelles, but it is very difficult to measure and assign a sufficient number of long-range nuclear Overhauser effects (NOEs) for structure determination (Klein-Seetharaman et al. 2002; Oxenoid et al. 2004). This limitation can be overcome by preparing weakly aligned micelle samples for the measurement of residual dipolar couplings (RDCs) and residual chemical shift anisotropies (RCSAs) (Bax et al. 2001; Prestegard and Kishore 2001), as demonstrated for the structure of the bacterial mercury detoxification membrane protein MerF (Howell et al. 2005).

High-resolution solid-state NMR spectra can be obtained for membrane proteins that are expressed, isotopically labeled, and reconstituted in uniaxially oriented planar lipid bilayers. The spectra have characteristic resonance patterns that directly reflect protein structure and topology, and this direct relationship between spectrum and structure provides the basis for methods that enable the simultaneous sequential assignment of resonances and the measurement of orientation restraints for protein structure determination (Marassi and Opella 2000; Wang et al. 2000; Marassi 2001). Recent developments in sample preparation, recombinant bacterial expression systems for the preparation of isotopically labeled membrane proteins, pulse sequences for high-resolution spectroscopy, and

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structural indices that guide the structure assembly process, have greatly extended the capabilities of the technique. The structures of a variety of membrane peptides and proteins have been examined using this approach, and several atomic-resolution structures have been determined and deposited in the PDB (Ketchem et al. 1993; Opella et al. 1999; Valentine et al. 2001; Wang et al. 2001; Marassi and Opella 2003; Park et al. 2003).

In this chapter, the methods are illustrated with examples from the Bcl-2 family proteins, which regulate a major mechanism for commitment to programmed cell death (apoptosis), and play critical roles in tissue development, differentiation, and homeostasis.

2.2 The Bcl-2 Family Proteins and Programmed Cell Death

Programmed cell death is initiated when death signals activate the caspases, a family of otherwise dormant cysteine proteases. External stress stimuli trigger the ligation of cell surface death receptors, thereby activating the upstream initiator caspases, which in turn process and activate the downstream cell death executioner caspases (Denault and Salvesen 2002). In addition, caspases can be activated when stress or developmental cues within the cell induce the release of cytotoxic proteins from mitochondria. This intrinsic mitochondrial pathway for cell death is regulated by the relative ratios of the pro- and anti-apoptotic members of the Bcl-2 protein family.

Several Bcl-2 family members have been identified in humans, including both anti-apoptotic (cytoprotective) and pro-apoptotic (death-promoting) proteins (Green and Reed 1998; Kroemer and Reed 2000; Cory and Adams 2002; Danial and Korsmeyer 2004). The relative ratios of the pro- and anti-apoptotic proteins determine the ultimate sensitivity and resistance of cells to diverse death-inducing stimuli, including chemotherapeutic drugs, radiation, growth factor deprivation, loss of cell attachment to extracellular matrix, hypoxia, infection, and lysis by cytolytic T cells. Imbalances in their relative expression levels and activities are associated with major human diseases, characterized by either insufficient (cancer, autoimmunity) or excessive (AIDS, Alzheimer‘s disease) cell death.

The Bcl-2 proteins span approximately 200 amino acids, and share sequence homology in four evolutionarily conserved domains (BH1–BH4), of which the BH3 domain is highly conserved and essential for both cell killing and oligomerization among Bcl-2 family members (Fig. 2.1). The anti-apoptotic family members have all four domains, while all of the pro-apoptotic members lack BH4, and some others only have BH3. These BH3-only proteins are activated by upstream death signals, which trigger their transcriptional induction or post-translational modification, providing a key link between the extrinsic death receptor and intrinsic mitochondrial pathways to cell death. Most family members also have a hydrophobic C-terminus (C) which is sufficiently long to span the membrane, and is essential for membrane targeting.

The apoptosis regulatory activities of the Bcl-2 family proteins are exerted through binding with other Bcl-2 family members, binding with other non-ho-

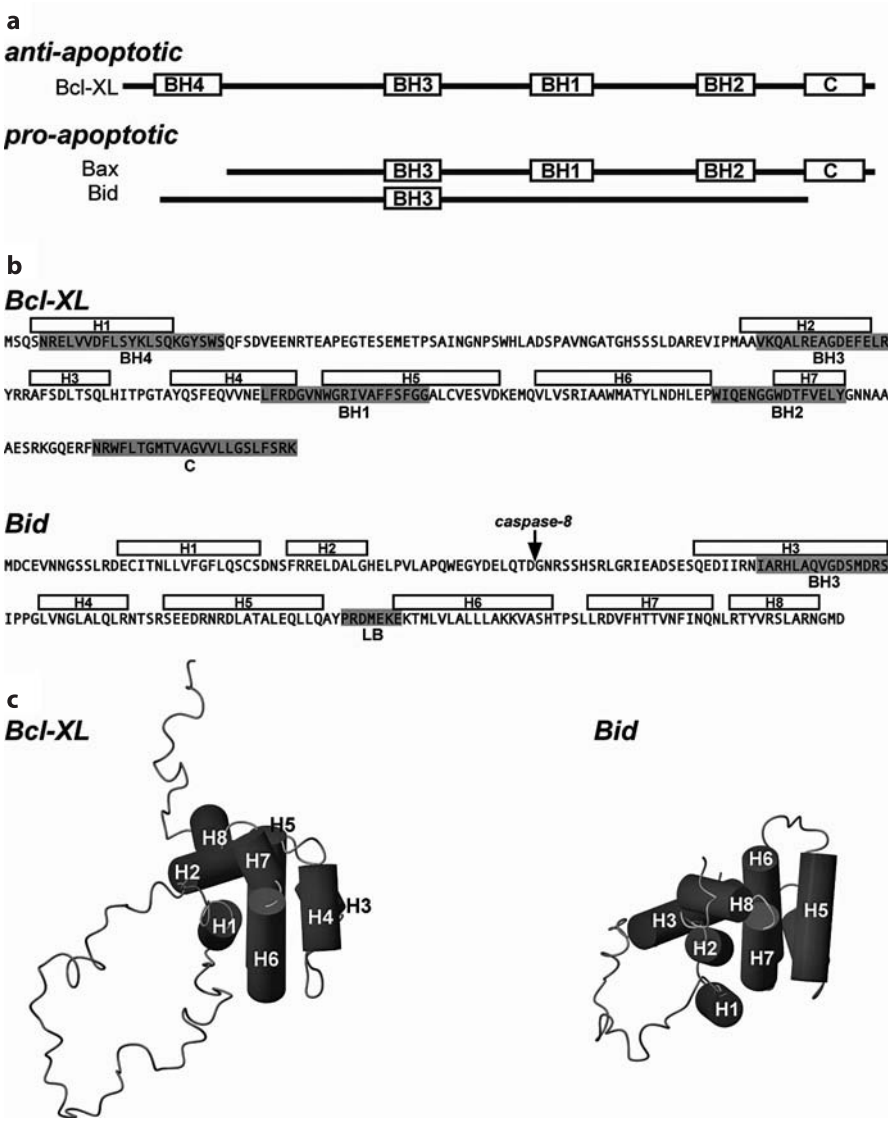

Fig. 2.1. Domain organization (**a**) and amino acid sequences (**b**) of human Bcl-XL and Bid. The helices (H1 to H8) are those identified in the solution NMR structures of Bcl-XL (Muchmore et al. 1996; Aritomi et al. 1997) and Bid (Chou et al. 1999; McDonnell et al. 1999) shown in (**c**). The central core helices are H5 and H6 in Bcl-XL and H6 and H7 in Bid. The C-terminal hydrophobic segment of Bcl-XL is denoted as C. The putative lipid binding motif of Bid is denoted as LB. The sequence of tBid starts at Gly61, and the *arrow* marks the caspase-8 cleavage site at Asp60

mologous proteins, and through the modulation of ion-conducting pores that are thought to influence cell fate by regulating mitochondrial physiology. Their functions are also regulated by subcellular location, as the proteins cycle between soluble and membrane-bound forms. For example, some family members, like anti-apoptotic Bcl-XL, localize to mitochondrial, endoplasmic reticulum, or nuclear membranes, while others, like pro-apoptotic Bid, are found in the cytosol, but are stimulated by death signals to target the mitochondrial outer membrane, where they participate in cytochrome-c release and apoptosis.

The structures of Bcl-XL and Bid, in solution, are very similar. They consist of seven (Bcl-XL) or eight (Bid) α-helices arranged with two central somewhat more hydrophobic helices which form the core of the molecule (Fig. 2.1). In Bcl-XL, the third helix spans the BH3 domain, and is connected to the first helix by a long flexible loop, while helices 5 and 6 form the central hydrophobic hairpin (Muchmore et al. 1996; Aritomi et al. 1997). The structure was determined for a truncated form of the protein lacking the hydrophobic C-terminus. In Bid, the third helix contains the BH3 domain and is connected to the first two helices by a long flexible loop, which includes Asp60, the caspase-8 cleavage site. The hydrophobic hairpin is formed by helices 6 and 7 (Chou et al. 1999; McDonnell et al. 1999). Despite the lack of sequence homology, the structures of Bcl-XL and Bid are strikingly similar to each other, and those of other pro- and anti-apoptotic Bcl-2 family proteins (Suzuki et al. 2000; Petros et al. 2001; Huang et al. 2002; Denisov et al. 2003; Hinds et al. 2003; Huang et al. 2003; Day et al. 2004; Day et al. 2005). Interestingly, they are also similar to the structure of the pore-forming domains of bacterial toxins, and, like the toxins and other Bcl-2 family members, they also form ion-conducting pores in lipid bilayers (Cramer et al. 1995; Schendel et al. 1998; Schendel et al. 1999).

The structural basis for Bcl-2 pore formation is not known, since the structures that have been determined are for the soluble forms of the proteins, and pore formation by the Bcl-2 family proteins has not been established in vivo. Nevertheless, by analogy to the bacterial toxins, the Bcl-2 pores are thought to form by a rearrangement of their compactly folded helices upon contact with the mitochondrial membrane. One model proposes membrane insertion of the core helical hairpin with the other helices folding up to rest on the membrane surface, while an alternative model envisions the helices rearranging to bind the membrane surface without insertion. A third possible mechanism for the regulation of mitochondrial physiology by the Bcl-2 proteins is through their interaction with other mitochondrial channels.

2.3 Protein Expression and Purification

NMR structural studies require milligram quantities of isotopically labeled proteins, and the most versatile and widely used method for obtaining recombinant proteins is by expression in *E. coli*, since this enables a wide variety of isotopic labeling schemes to be incorporated in the NMR experimental strategy. Smaller peptides can be prepared by solid phase peptide synthesis; however, this

is impractical for larger proteins and for the preparation of uniformly labeled samples, where efficient expression systems are essential. The ability to produce milligram quantities of pure proteins also facilitates functional studies that, together with structure determination, can provide important structure–activity correlations.

Some polypeptides, however, are toxic to the bacterial hosts that express them. For example, some membrane proteins and peptides, including some of bacterial origin, congest the cell membranes when they are over-expressed, and act as toxic, antibacterial agents, regardless of their actual biological functions. For these difficult polypeptides, solid-phase synthesis is not a practical alternative, because it is typically limited to sequences shorter than 50 amino acids, and while this size limit can be extended through the use of chemical ligation methods (Dawson et al. 1994; Kochendoerfer 2001) that can also be applied to membrane proteins (Kochendoerfer et al. 1999; Kochendoerfer et al. 2004), NMR studies still require bacterial expression of the polypeptide precursors for the practical introduction of various isotopic labels.

Several *E. coli* cell strains and expression strategies have been developed to address this problem (Miroux and Walker 1996; Rogl et al. 1998; Jones et al. 2000; Majerle et al. 2000; Opella et al. 2001; Sharon et al. 2002; Bannwarth and Schulz 2003; Booth 2003; Kiefer 2003; Lindhout et al. 2003; Smith and Walker 2003; Wiener 2004), and more recently, cell-free expression has been used to obtain milligram quantities of isotopically labeled membrane proteins (Klammt et al. 2004). Many strategies rely on the use of fusion protein tags to improve expression and facilitate purification, and many involve protein expression in inclusion bodies, to keep the hydrophobic polypeptide away from the bacterial membranes, and thus increase the level of expression. The formation of inclusion bodies also limits proteolytic degradation, and simplifies protein purification, which can be further assisted by the incorporation of an engineered His tag for metal affinity chromatography.

The TLE (a portion of the Trp ΔLE 1413 polypeptide) (Miozzari and Yanofsky 1978; Kleid et al. 1981; Staley and Kim 1994), and KSI (ketosteroid isomerase) (Kuliopulos et al. 1994) fusion partners promote the accumulation of expressed proteins as inclusion bodies, and have been used to express several membrane peptides and proteins ranging in size from 20 to 200 amino acids (Opella et al. 2001; Opella and Marassi 2004).

Recently, we developed a fusion protein expression vector, pBCL, that directs the expression of a target polypeptide fused to the C-terminus of a mutant form of the Bcl-2 family protein Bcl-XL, where the hydrophobic C-terminus has been deleted, and Methionine residues have been mutated to Leucine, to facilitate CNBr cleavage after a single Methionine inserted at the beginning of the target polypeptide sequence (Thai et al. 2005). As shown in Fig. 2.2, this fusion partner yields the high-level expression of membrane proteins belonging to the FXYD family of Na,K-ATPase regulators, including some that had resisted expression with TLE and KSI.

The pTLE and pBCL vectors may be generally useful for the high-level expression of other membrane-associated proteins that are difficult to express because of their toxic properties. The use of chemical cleavage eliminates the difficulties,

poor specificity and enzyme inactivation, often encountered with protease treatment of insoluble proteins. However, in cases where Met mutation is not feasible, protein cleavage from the fusion partner can be obtained enzymatically, by engineering specific protease cleavage sites for the commonly used enzymes thrombin, Fxa, enterokinase, and tobacco etch virus (TEV) protease. Thrombin and TEV retain activity in the presence of detergents, including low mM concentrations of SDS.

The pro-apoptotic Bcl-2 family protein, Bid, is activated upon cleavage by caspase-8, to release the 15 kD C-terminal fragment tBid, which translocates from the cytosol to the outer mitochondrial membrane inducing massive cytochrome-c release and cell death (Scorrano and Korsmeyer 2003). Full-length Bid can be expressed at high levels, in *E. coli*, as a soluble protein, however tBid is toxic for bacterial cells. To produce milligram quantities of ^{15}N-labeled tBid for NMR studies we used the TLE fusion protein vector (Fig. 2.2). tBid was separated from the fusion partner by means of CNBr cleavage at the engineered N-terminal Met residue, and this method yields approximately 10 mg of purified ^{15}N-labeled tBid from 1 L of culture. To avoid cleavage within the tBid segment, the four Met residues in the tBid amino acid sequence were mutated to Leu, and therefore, it was important to demonstrate that the recombinant protein retained its biological activity. Recombinant tBid, isolated from inclusion bodies, was fully active in its ability to induce cytochrome-c and SMAC release from isolated mitochondria, and retained its capacity to bind anti-apoptotic Bcl-X_L through its BH3 domain despite the M97L mutation in its sequence (Gong et al. 2004).

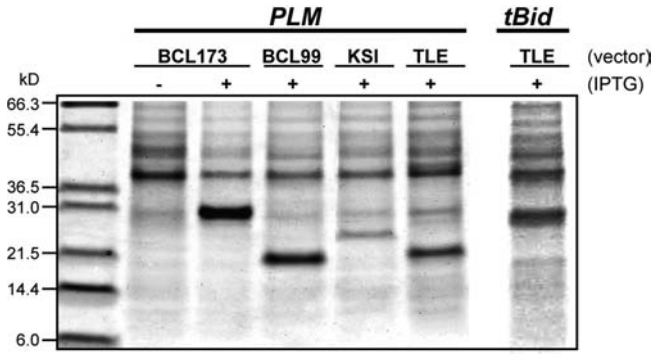

Fig. 2.2. Expression of the membrane protein phospholemman (PLM) and of tBid with four different fusion protein plasmid vectors: pBCL173, pBCL99, pKSI, and pTLE. The gel shows total lysates from cells, transformed with each plasmid, and harvested before (–) or after (+) induction with IPTG. Fusion protein over-expression is marked by the appearance of a distinct band at the molecular weight of the corresponding fusion protein: BCL173-PLM (29.8 kD), BCL99-PLM (21.2 kD), KSI-PLM (23.4 kD), TLE-PLM (22.1 kD), and TLE-tBid (30.0 kD) (Gong et al. 2004; Thai et al. 2005)

2.4 NMR in Micelles

Solution NMR methods rely on rapid molecular reorientation for line narrowing, and can be successfully applied to membrane proteins in micelles (Henry and Sykes 1994; Williams et al. 1996; Almeida and Opella 1997; Gesell et al. 1997; MacKenzie et al. 1997; Arora et al. 2001; Fernandez et al. 2001; Hwang et al. 2002; Ma et al. 2002; Mascioni et al. 2002; Oxenoid et al. 2002; Sorgen et al. 2002; Crowell et al. 2003; Krueger-Koplin et al. 2004; Howell et al. 2005). The size limitation is substantially more severe than for globular proteins, because the many lipid molecules associated with each polypeptide slow its overall reorientation rate. Micelles afford rapid and effectively isotropic reorientation of the protein, and their amphipathic nature simulates that of membranes, offering a realistic alternative to organic solvents for studying membrane proteins. Moreover, for the proteins examined by both solution and solid-state NMR, similar structural features have been found in micelle and bilayer samples (Lee et al. 2003; Mesleh et al. 2003).

The first step in solution NMR studies of proteins is the preparation of folded, homogeneous, and well-behaved samples, and several lipids are available for membrane protein solubilization (Krueger-Koplin et al. 2004). For membrane-bound proteins, small micelles containing approximately 60 lipids and one protein provide a generally effective model membrane environment, without the damaging effects of organic solvents. The primary goal in micelle preparation is to reduce the effective rotational correlation time of the protein so that resonances will have the narrowest possible linewidths. Careful handling of the protein throughout the purification is essential, since subtle changes in the protocol can have a significant impact on the quality of the resulting spectra. It is essential to optimize the protein concentration, lipid nature and concentration, counter ions, pH and temperature, in order to obtain well-resolved NMR spectra, with narrow ^{1}H and ^{15}N resonance linewidths.

Although high-quality solution NMR spectra can be obtained even for some large helical membrane proteins in micelles (Krueger-Koplin et al. 2004; Oxenoid et al. 2004; Howell et al. 2005), there are only very few cases where it has been possible to measure and assign sufficiently long-range NOEs for structure determination. This limitation can be overcome by preparing weakly aligned micelle samples for the measurement of RDCs (Bax et al. 2001; Prestegard and Kishore 2001) from the backbone amide sites, and the analysis of these orientation restraints in terms of dipolar waves (Mesleh et al. 2002; Lee et al. 2003; Mesleh et al. 2003; Mesleh and Opella 2003). Stressed polyacrylamide gels provide an ideal orientable medium for membrane proteins in micelles, because they do not suffer from the drawbacks of bicelles, which bind tightly to membrane proteins, or phage particles, which are destroyed by micelles (Sass et al. 2000; Tycko et al. 2000; Chou et al. 2001; Meier et al. 2002; Howell et al. 2005). Another useful approach to compensate for insufficient NOEs involves the combination of site-directed spin labeling and NMR (Battiste and Wagner 2000), where distances derived from paramagnetic broadening of NMR resonances are used to determine global fold. In addition, spin label probes and metal ions can be incorporated within the micelles in order to probe protein insertion (Papavoine et al. 1994; Van Den Hooven

et al. 1996; Jarvet et al. 1997; Damberg et al. 2001; Sorgen et al. 2002; Kutateladze et al. 2004).

2.4.1 Determining the Structures of Proteins in Micelles

The measurements of as many homonuclear $^1H/^1H$ NOEs as possible among the assigned resonances provide the short-range and long-range distance restraints required for structure determination (Clore and Gronenborn 1989, Wuthrich 1989, Ferentz and Wagner 2000). These are supplemented by other structural restraints, such as spin–spin coupling constants, chemical shift correlations, deuterium exchange data, and RDCs in order to assign resonances and to characterize the secondary structure of the protein. The HSQC (heteronuclear single quantum coherence) spectra of samples in D_2O solutions identify the most stable helical residues, and can provide useful information on the topology of membrane proteins in micelles (Czerski et al. 2000). In addition, hydrogen–deuterium fractionation experiments extend the range of exchange rates that can be monitored to identify more subtle structural features (Veglia et al. 2002).

The two-dimensional HSQC spectra also serve as the basis for the measurement of the ^1H and ^{15}N relaxation parameters of protein backbone amide sites, which are useful for describing protein dynamics. The heteronuclear $^1H–^{15}N$ NOEs of the backbone amide sites provide remarkably direct and sensitive information on local protein dynamics (Gust et al. 1975; Boguski et al. 1987; Bogusky et al. 1988). They can be measured with and without ^1H irradiation to saturate the ^1H magnetization (Farrow et al. 1994).

RDCs are extremely useful both for structure refinement, and for the *de novo* determination of protein folds (Tolman et al. 1995; Clore and Gronenborn 1998; Delaglio et al. 2000; Fowler et al. 2000; Hus et al. 2000; Mueller et al. 2000). During refinement, these measurements supplement an already large number of chemical shifts, approximate distance measurements, and dihedral angle restraints. Among the principal advantages of anisotropic spectral parameters in solution NMR spectroscopy is that they can report on the global orientations of separate domains of a protein and of individual bonds relative to a reference frame, which reflects the preferred alignment of the molecule in the magnetic field. This does not preclude their utility in characterizing the local backbone structure of a protein molecule.

The RDCs and RCSAs measured in solution NMR experiments provide direct angular restraints with respect to a molecule-fixed reference frame (Bax et al. 2001; Prestegard and Kishore 2001; Lee et al. 2003). They are analogous to the non-averaged dipolar couplings and chemical shift anisotropies measured in solid-state NMR experiments (Marassi and Opella 2000; Wang et al. 2000; Marassi 2001). These orientation restraints are the principal mechanism for overcoming the limitations resulting from having few reliable long-range NOEs available as distance restraints, often encountered with samples of membrane-bound proteins in micelles.

Dipolar waves are very effective at identifying the helical residues in membrane-bound proteins and the relative orientations of the helical segments, and also serve as indices of the helix regularity in proteins (Mesleh et al. 2002). The magnitudes of the RDCs are plotted as a function of residue number and fitted to a sine wave with a period of 3.6 residues (Mesleh et al. 2002; Mesleh et al. 2003; Mesleh and Opella 2003). The quality of fit is monitored by a scoring function in a four-residue sliding window and the phase of the fit. Dipolar waves from solution NMR data give relative orientations of helices in a common molecular frame. On the other hand, Dipolar waves from solid-state NMR data give absolute measurements of helix orientations because the polypeptides are immobile and the samples have a known alignment in the magnetic field.

2.4.2 tBid in Micelles

The cleavage of Bid by caspase-8 results in a C-terminal product, tBid, which targets mitochondria and induces apoptosis with strikingly enhanced activity. To characterize the conformation of tBid in lipid environments, we obtained its CD (circular dichroism) and solution NMR $^{1}H/^{15}N$ HSQC spectra in the absence or in the presence of lipid micelles (Fig. 2.3) (Gong et al. 2004). The HSQC spectra of proteins are the starting point for additional multidimensional NMR experiments that lead to structure determination. In these spectra, each ^{15}N-labeled protein site gives rise to a single peak, characterized by ^{1}H and ^{15}N chemical shift frequencies that reflect the local environment. In addition, the peak linewidths and lineshapes, and their dispersion in the ^{1}H and ^{15}N frequency dimensions, are sensitive indicators of protein conformational stability and aggregation state.

In the absence of lipids, the CD spectrum of tBid displays minima at 202 nm and 222 nm, characteristic of predominantly helical proteins (Fig. 2.3a, solid line). However, while tBid retains its helical conformation even when it is separated from the 60-residue N-terminal segment, many of the resonances in its HSQC spectrum cannot be detected (Fig. 2.3b), suggesting that the protein aggregates in solution, adopts multiple conformations, or undergoes dynamic conformational exchange on the NMR time-scales. This is consistent with the dramatic changes in the physical properties of the protein that result from caspase-8 cleavage.

When tBid is dissolved in lipid micelles its HSQC spectrum changes dramatically, and single, well-defined $^{1}H/^{15}N$ resonances are observed for each ^{15}N-labeled NH site, indicating that it adopts a unique conformation in this environment (Figs. 2.3c, d). Several lipids are available for protein solubilization, and we tested both SDS and LPPG for their ability to yield high-quality HSQC spectra of tBid for structure determination. Both gave excellent spectra where most of the 130 amide resonances of tBid could be resolved; for example the resonances from the five Gly amide sites are resolved in SDS (Fig. 2.3c), and four out of five are resolved in LPPG (Fig. 2.3d). Both SDS and LPPG are negatively charged but they differ in the lengths of their hydrocarbon chains (C12 for SDS; C16 for LPPG), and their polar headgroups (sulfate for SDS; phosphatidylglycerol for LPPG), thus the differences in the ^{1}H and ^{15}N chemical shifts between the two HSQC spectra most

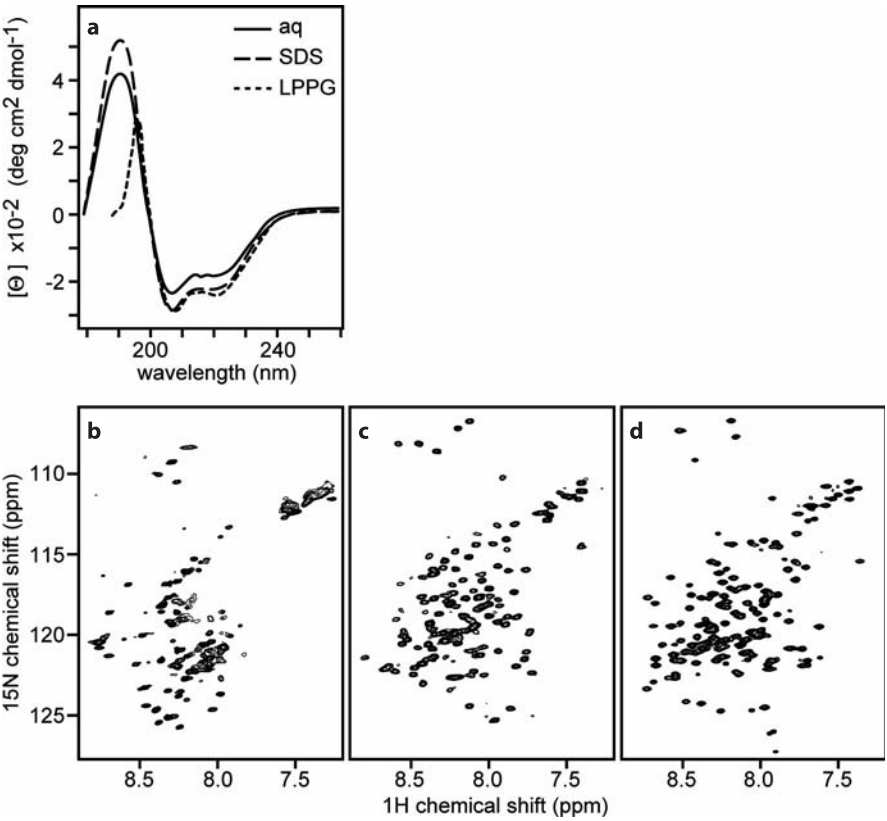

Fig. 2.3. tBid adopt well-defined helical folds in lipid micelles. The CD spectra in (**a**) were obtained at 25 °C for tBid in aqueous solution (*solid line*), SDS micelles (*broken line*), or LPPG micelles (*dotted line*). The $^{1}H/^{15}N$ HSQC NMR spectra in (**b, c, d**) were obtained at 40 °C for uniformly ^{15}N-labeled tBid in (**a**) aqueous solution, (**b**) SDS micelles, or (**c**) LPPG micelles. Aqueous samples were in 20 mM sodium phosphate, pH 5; SDS micelle samples were in 20 mM sodium phosphate, pH 7, 500 mM SDS; and LPPG micelle samples were in 20 mM sodium phosphate, pH 7, 100 mM LPPG

likely reflect the different lipid environments. The spectrum in LPPG has exceptionally well-dispersed resonances with homogeneous intensities and linewidths. LPPG was recently identified as a superior lipid for NMR studies of several membrane proteins (Krueger-Koplin et al. 2004), and is particularly interesting for this study because it is a close analog of cardiolipin and monolysocardiolipin, the major components of mitochondrial membranes that bind tBid. The limited chemical shift dispersion in the two spectra is typical of helical proteins in micelles, and this is confirmed by the corresponding CD spectra, which are dominated by minima at 202 nm and 222 nm, and thus show that tBid retains a predominantly helical fold in both SDS and LPPG (Fig. 2.3a, broken and dashed lines).

2.5 NMR in Bilayer Membranes

When the lipid bilayers are oriented with their surface perpendicular to the magnetic field, the solid-state NMR spectra of the membrane-associated proteins trace out maps of their structure and orientation within the membrane, and thus provide very useful structural information prior to complete structure determination (Marassi and Opella 2000; Wang et al. 2000; Marassi 2001). For example, helices give characteristic solid-state NMR spectra where the resonances from amide sites in the protein trace-out helical wheels that contain information regarding helix tilt and rotation within the membrane. Typically, trans-membrane helices have PISEMA spectra with ^{15}N chemical shifts between 150 and 200 ppm, and ^{1}H–^{15}N dipolar couplings between 2 and 10 kHz, while helices that bind parallel to the membrane surface have spectra with shifts between 70 and 100 ppm and couplings between 0 and 5 kHz. We refer to these as the trans-membrane and in-plane regions of the PISEMA spectrum, respectively.

Glass-supported oriented phospholipid bilayers containing membrane proteins accomplish the principal requirements of immobilizing and orienting the protein for solid-state NMR structure determination. The planar lipid bilayers are supported on glass slides, and are oriented in the NMR probe so that the bilayer normal is parallel to the field of the magnet, as shown in Fig. 2.4a. The choice of lipid can be used to control the lateral spacing between neighboring phospholipid molecules as well as the vertical spacing between bilayers. The use of phospholipids with unsaturated chains leads to more expanded and fluid bilayers, and the addition of negatively charged lipids increases inter-bilayer repulsions leading to larger interstitial water layers between bilayer leaflets.

Samples of membrane proteins in lipid bilayers oriented on glass slides can be prepared by deposition from organic solvents followed by evaporation and lipid hydration, or by fusion of reconstituted unilamellar lipid vesicles with the glass surface (Marassi 2002). The choice of solvents in the first method, and of detergents in the second, is critical for obtaining highly oriented lipid bilayer preparations. In all cases the thinnest available glass slides are utilized to obtain the best filling factor in the coil of the probe. With carefully prepared samples it is possible to obtain ^{15}N resonance linewidths of less than 3 ppm (Marassi et al. 1997). Notably, these linewidths are less than those typically observed in single crystals of peptides, demonstrating that the proteins in the bilayers are very highly oriented, with mosaic spreads of less than about 2°.

2.5.1 Bcl-XL and tBid in Bilayers

To examine the conformations of Bcl-XL and tBid associated with membranes, we obtained one-dimensional ^{15}N chemical shift and two-dimensional ^{1}H/^{15}N PISEMA solid-state NMR spectra of the ^{15}N-labeled proteins reconstituted in lipid bilayers (Franzin et al. 2004; Gong et al. 2004). In these samples, the lipid composition of 60% DOPC and 40% DOPG was chosen to mimic the highly nega-

tive charge of mitochondrial membranes. This lipid composition is identical to that of the liposomes used for the measurement of the ion channel activities of Bcl-XL, Bid, and tBid (Minn et al. 1997; Schendel et al. 1999), which were prepared in the same way as the oriented lipid bilayers used in the NMR study.

The samples of Bcl-XL and tBid in bilayers were prepared by spreading lipid vesicles, reconstituted with ^{15}N-labeled protein, on the surface of the glass slides, allowing bulk water to evaporate, and incubating the sample in a water-saturated atmosphere (Franzin et al. 2004; Gong et al. 2004). Each sample was wrapped in parafilm and then sealed in thin polyethylene film prior to insertion in the NMR probe. The degree of phospholipid bilayer alignment can be assessed with solid-state ^{31}P NMR spectroscopy of the lipid phosphate headgroup. The ^{31}P NMR spectra obtained for lipid bilayers with Bcl-XL are characteristic of a liquid-crystalline bilayer arrangement, in both oriented (Fig. 2.4b) and unoriented samples (Fig. 2.4c). The spectrum from the oriented sample has a single peak near 30 ppm, as expected for highly oriented bilayers.

2.5.1.1 Membrane-Associated Bcl-xL

The spectra in Fig. 2.4 were obtained from samples of uniformly ^{15}N-labeled Bcl-xL in oriented and unoriented lipid bilayers (Franzin et al. 2004). The spectrum obtained from oriented Bcl-xL (Fig. 2.4d) is separated into discernable resonances with distinct intensities near 80 and 170 ppm. These spectral features reflect a structural model where the helices of Bcl-XL associate with the membrane surface with limited transmembrane helix insertion. The spectrum from unoriented bilayers (Fig. 2.4e) provides no resolution among resonances, but it provides an indication of protein dynamics, because of the pronounced effects of motional averaging on such spectra. Most of the backbone sites are structured and immobile on the time-scale of the ^{15}N chemical shift interaction (10 kHz), contributing to the characteristic amide powder pattern between 220 and 60 ppm. Some of the Bcl-XL backbone sites, probably near the termini and loop regions, are mobile, and give rise to the resonance band centered near 120 ppm. Therefore, while certain resonances near 120 ppm, in the spectrum of oriented Bcl-XL, may reflect specific orientations of their corresponding sites, some others arise from mobile backbone sites. The intensity near 35 ppm, also present in the spectrum from the oriented sample, is from the protein amino groups, which have a considerably narrower ^{15}N chemical shift anisotropy. Taken together, the ^{15}N and ^{31}P spectra provide evidence that Bcl-xL, an anti-apoptotic Bcl-2 family protein, associates predominantly with the membrane surface, without disruption of the membrane integrity.

2.5.1.2 Membrane-Associated tBid

The ^{15}N chemical shift spectrum of tBid in spherical lipid bilayer vesicles is a powder pattern (Fig. 2.5a, solid line) that spans the full range (60–220 ppm) of the

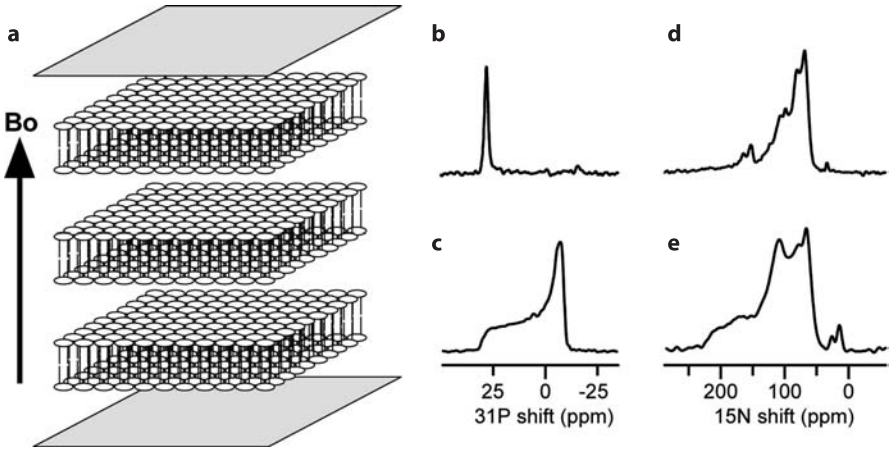

Fig. 2.4. Effect of sample orientation on the solid-state NMR spectra of isotopically labeled proteins. (**A**) The glass-supported phospholipid bilayer samples are oriented in the NMR probe so that the bilayer normal is parallel to the direction of the magnetic field (Bo). (**B**) Oriented phospholipid bilayers give single-line one-dimensional ^{31}P chemical shift NMR spectra, while (**C**) spherical lipid bilayer vesicles give powder patterns. (**D**) The one-dimensional ^{15}N chemical shift NMR spectrum of uniformly ^{15}N-labeled Bcl-XL in oriented lipid bilayers displays multiple resonances, compared to (**E**) the powder pattern that is obtained for the same protein in unoriented lipid bilayer vesicles. The ^{15}N chemical shifts are referenced to 0 ppm for liquid ammonia

amide ^{15}N chemical shift interaction (Fig. 2.5a, dashed line). The absence of additional intensity at the isotropic resonance frequencies (100–130 ppm) demonstrates that the majority of amino acid sites are immobile on the time-scale of the ^{15}N chemical shift interaction, although it is possible that some mobile unstructured residues could not be observed by cross-polarization. The peak at 35 ppm is from the amino groups at the N-terminus and sidechains of the protein. The spectrum of tBid in planar oriented lipid bilayers is very different (Fig. 2.5c). All of the amide resonances are centered at a frequency associated with NH bonds in helices parallel to the membrane surface (80 ppm), while no intensity is observed at frequencies associated with NH bonds in trans-membrane helices (200 ppm). The NMR data show no evidence of conformational exchange on the millisecond to second time-scales of the channel opening and closing events, thus eliminating the possibility of transient insertion of tBid in the membrane. Thus tBid binds strongly to the membrane surface and adopts a unique conformation and orientation in the presence of phospholipids (Gong et al. 2004).

Amide hydrogen exchange rates are useful for identifying residues that are involved in hydrogen bonding, and that are exposed to water. Typically, the amide hydrogens in trans-membrane helices have very slow exchange rates due to their

strong hydrogen bonds in the low dielectric of the lipid bilayer environment, and their ^{15}N chemical shift NMR signals persist for days after exposure to D_2O (Franzin et al. 2004). Trans-membrane helices that are in contact with water because they participate in channel pore formation, and other water-exposed helical region proteins, have faster exchange rates, and their NMR signals disappear on the order of hours (Tian et al. 2003). To examine the amide hydrogen exchange rates for membrane-bound tBid, we obtained solid-state NMR spectra after exposing the oriented lipid bilayer sample to D_2O for 2 h, 5 h, and finally for 7 h. The majority of resonances in the ^{15}N chemical shift spectrum of tBid disappeared within 8 h, indicating that the amide hydrogens exchange and hence are in contact with the bilayer interstitial water.

The tBid amino acid sequence has four Lys residues (Lys144, Lys146, Lys157, and Lys158) all located in or near helix-6, one of the two helices thought to insert in the membrane and form the tBid ion-conducting pore. The spectrum of ^{15}N-Lys labeled tBid in bilayers is notable because its amide resonances all have chemical shifts near 80 ppm, in the in-plane region of the spectrum, and this cannot be reconciled with membrane insertion (Fig. 2.5b). Since tBid maintains a helical fold in lipid micelles and it is reasonable to assume that the helix boundaries are not changed from those of full-length Bid, the solid-state NMR data demonstrate that helix-6 does not insert through the membrane but associates parallel to its surface. This is also supported by a recent EPR study (Oh et al. 2004).

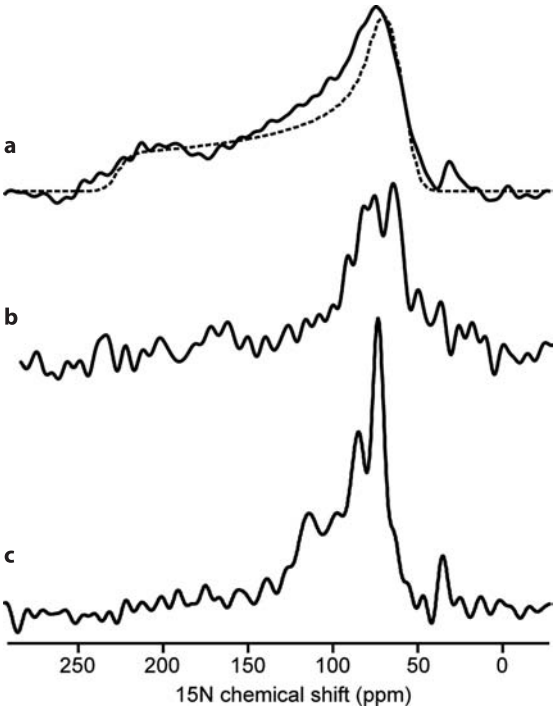

Fig. 2.5. One-dimensional ^{15}N chemical shift spectra of tBid in lipid bilayers. (**a**) Uniformly ^{15}N-labeled tBid in unoriented lipid bilayer vesicles (*solid line*), and powder pattern calculated for a rigid ^{15}N amide site (*dotted line*). (**b**) One-dimensional ^{15}N spectrum of selectively ^{15}N-Lys-labeled tBid in oriented lipid bilayers. (**c**) One-dimensional ^{15}N spectrum of uniformly ^{15}N-labeled tBid in oriented lipid bilayers

2.5.2 Determining the Structures of Proteins in Bilayers

When membrane proteins are incorporated in planar lipid bilayers that are oriented in the field of the NMR magnet, the frequencies measured in their multidimensional solid-state NMR spectra contain orientation-dependent information that can be used for structure determination (Marassi 2002). The PISEMA (polarization inversion with spin exchange at the magic angle) experiment gives high-resolution, two-dimensional, $^{1}H–^{15}N$ dipolar coupling / ^{15}N chemical shift correlation spectra of oriented membrane proteins where the individual resonances contain orientation restraints for structure determination (Wu et al. 1994). PISEMA spectra of membrane proteins in oriented lipid bilayers also provide sensitive indices of protein secondary structure and topology because they exhibit characteristic wheel-like patterns of resonances, called Pisa wheels, that reflect helical wheel projections (Schiffer and Edmunson 1967) of residues in both α-helices and β-sheets (Marassi and Opella 2000; Wang et al. 2000; Marassi 2001). When a Pisa wheel is observed, no assignments are needed to determine the tilt of a helix, and a single resonance assignment is sufficient to determine the helix rotation in the membrane. This information is extremely useful for determining the supramolecular architectures of membrane proteins and their assemblies.

The shape and position of the Pisa wheel in the spectrum depends on the protein secondary structure and its orientation relative to the lipid bilayer surface, as well as the amide N–H bond length and the magnitudes and orientations of the principal elements of the amide ^{15}N chemical shift tensor. This direct relationship between spectrum and structure makes it possible to calculate solid-state NMR spectra for specific structural models of proteins, and provides the basis for a method of backbone structure determination from a limited set of uniformly and selectively ^{15}N-labeled samples (Marassi and Opella 2002; Marassi and Opella 2003).

The Pisa wheels calculated for single helices or strands, oriented at varying degrees in a lipid bilayer, are shown in Fig. 2.6. When the helices or strands cross the membrane with their long axes exactly parallel to the lipid bilayer normal and to the magnetic field direction (0°), all of the amide sites in each structure have an identical orientation relative to the direction of the applied magnetic field, and therefore all of the resonances overlap with the same dipolar coupling and chemical shift frequencies. Tilting the helix or strand away from the membrane normal introduces variations in the orientations of the amide NH bond vectors in the magnetic field, and leads to dispersion of the $^{1}H–^{15}N$ dipolar coupling and ^{15}N chemical shift frequencies, manifest in the appearance of Pisa wheel resonance patterns in the spectra. Since helices and strands yield clearly different resonance patterns, with circular wheels for helices and twisted wheels for strands, these spectra represent signatures of secondary structure (Marassi 2001). The spectra also demonstrate that it is possible to determine the tilt of a helix or strand in lipid bilayers without resonance assignments. Pisa wheels have been observed in the PISEMA spectra of many uniformly ^{15}N labeled α-helical membrane proteins (Opella et al. 1999, Marassi et al. 2000; Wang et al. 2001; Marassi and Opella 2003; Park et al. 2003; Zeri et al. 2003).

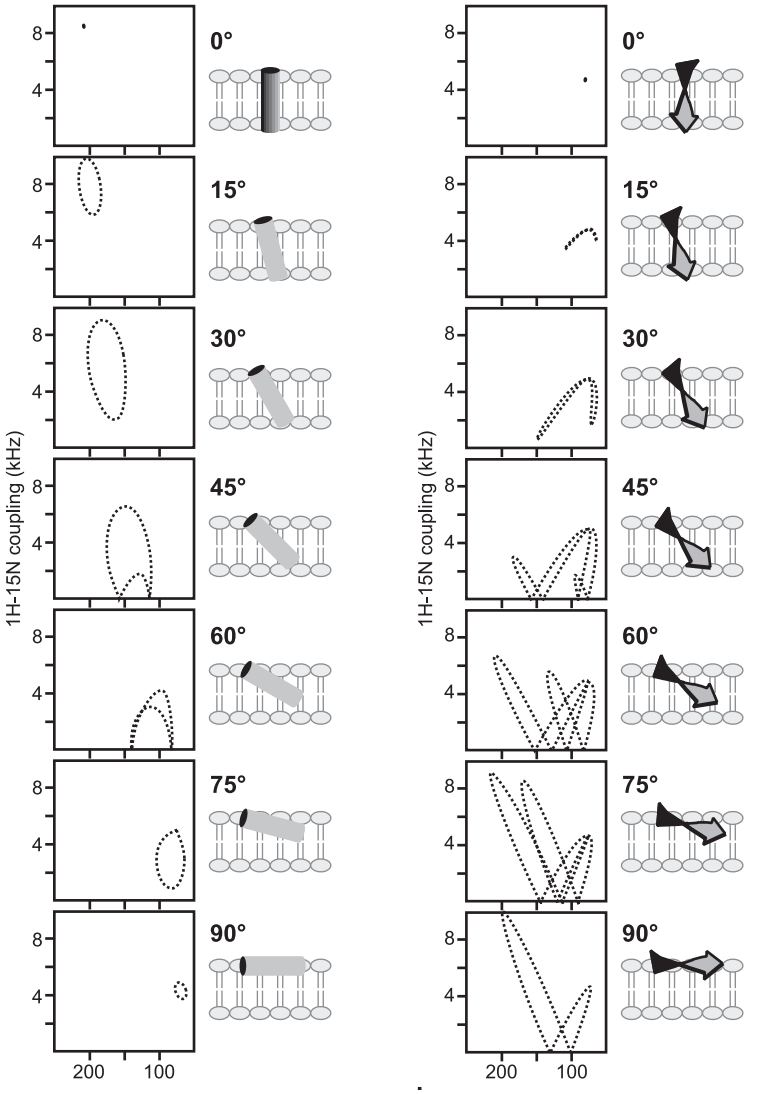

Fig. 2.6. Helices and strands in oriented planar lipid bilayers give characteristic solid-state NMR spectra called Pisa wheels. The ^{1}H–^{15}N dipolar coupling /^{15}N chemical shift PISEMA spectra were calculated for (**a**) an ideal α-helix with uniform dihedral angles (ϕ/ψ = –65/–40°), and (**b**) an ideal β-strand with uniform dihedral angles (ϕ/ψ = –135/140°), at different tilts relative to the magnetic field direction and the membrane normal. The ^{15}N chemical shifts are referenced to 0 ppm for liquid ammonia. Spectra were calculated as described by Marassi 2001

2.5.3 Conformation of tBid in Lipid Bilayers

The two-dimensional $^{1}H/^{15}N$ PISEMA spectrum of tBid in bilayers is shown in Fig. 2.7 (Gong et al. 2004). Each amide site in the protein contributes one correlation peak, characterized by $^{1}H–^{15}N$ dipolar coupling and ^{15}N chemical shift frequencies that reflect the NH bond orientation relative to the membrane. For tBid, the circular wheel-like pattern of resonances in the spectral region bounded by 0–5 kHz and 70–90 ppm, provides definitive evidence that tBid associates with the membrane as surface-bound helices without trans-membrane insertion. The substantial peak overlap reflects a similar orientation of the tBid helices parallel to the membrane, and spectral resolution in this region requires three-dimensional correlation spectroscopy and selective isotopic labeling (Marassi et al. 2000).

As shown in Fig. 2.6, the NMR frequencies directly reflect the angles between individual bonds and the direction of the applied magnetic field, and, therefore, it is possible to calculate solid-state NMR spectra for specific models of proteins in oriented samples. A comparison of the calculated and experimental spectra then provides useful structural information prior to complete structure determination, which requires sequential assignment of the resonances. The spectra calculated for several orientations of an ideal 18-residue helix, with 3.6 residues per turn and identical backbone dihedral angles for all residues (ϕ, ψ = –57°, –47°), are shown in Fig. 2.8. This analysis demonstrates that trans-membrane helices, with orientations between 90° and 45°, have wheel-like spectra in a completely unpopulated region of the tBid spectrum. Based on a comparison of the calculated spectra with the PISEMA spectrum of tBid in lipid bilayers we place the helices of tBid nearly parallel to the lipid bilayer plane (0° orientation), with a tilt of no more than 20° from the membrane surface.

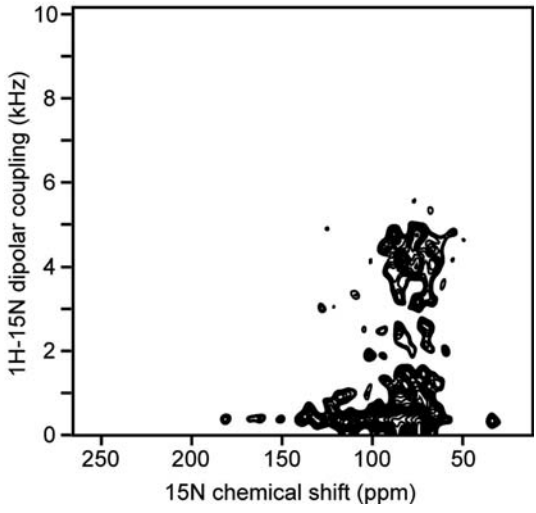

Fig. 2.7. Two-dimensional $^{1}H/^{15}N$ PISEMA spectrum of uniformly ^{15}N-labeled tBid in oriented lipid bilayers

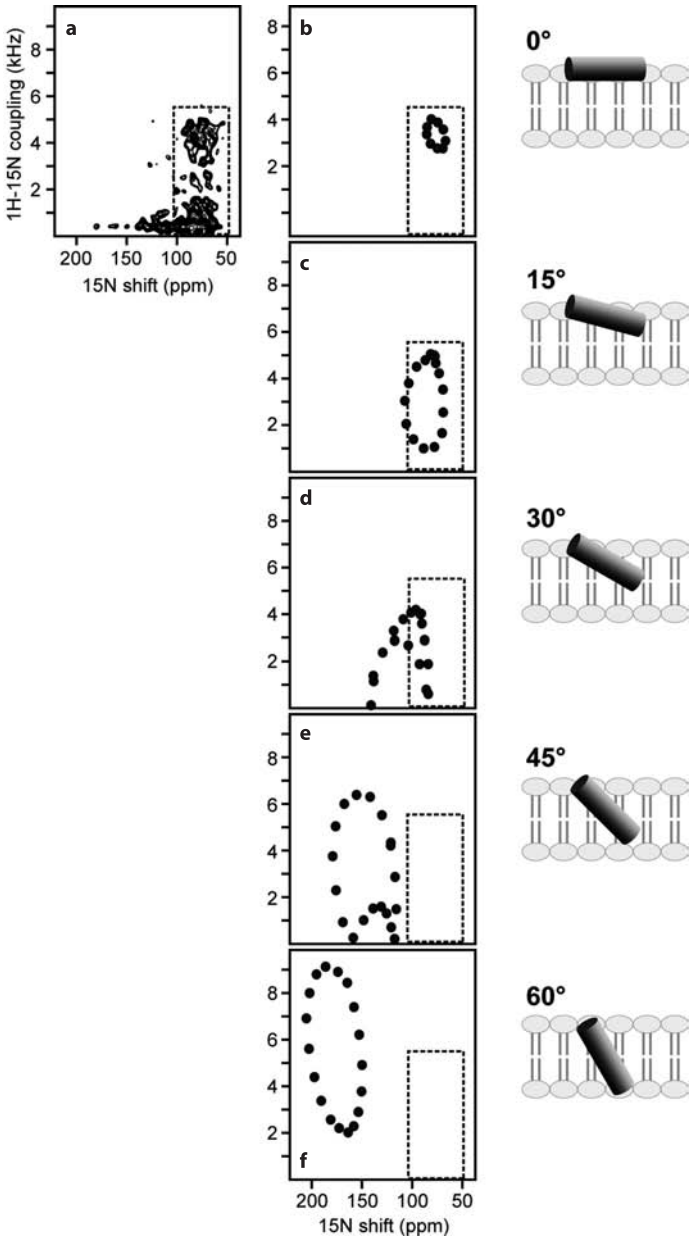

Fig. 2.8. Two-dimensional solid-state NMR $^{1}H/^{15}N$ PISEMA spectrum of uniformly ^{15}N-labeled tBid in oriented lipid bilayers. The experimental spectrum (*inset box*) is compared with the spectra calculated for an 18-residue α-helix, with uniform backbone dihedral angles ($\phi = -57°$; $\psi = -47°$), and different helix tilts (0 to 75°) relative to the membrane, depicted in the cartoon above the spectra. The 0° orientation is for a helix parallel to the membrane surface

Solution and solid-state NMR studies demonstrate that tBid adopts a unique helical fold in lipid environments, and that it binds the membrane without insertion of its helices. Solid-state NMR studies of the anti-apoptotic Bcl-2 family member, Bcl-X_L, also indicate that membrane insertion of the Bcl-X_L helices is only partial (Franzin et al. 2004), and solution NMR studies show that Bcl-X_L adopts an extended helical conformation in lipid micelles (Losonczi et al. 2000). Both tBid and Bcl-X_L form ion-conductive pores that are thought to play a role in apoptosis through their regulation of mitochondrial physiology, and it is important to note that, since the samples in both the solid-state NMR and ion channel activity studies of Bcl-XL and tBid were identical in their lipid composition and the manner of sample preparation, the membrane surface association of Bcl-XL and tBid, observed by solid-state NMR, represents the channel-active conformation of the proteins.

A model for the mode of membrane association by tBid is shown in Fig. 2.9. Cleavage by caspase-8 in the flexible loop of the soluble Bid structure (Fig. 2.9a), generates the C-terminal product tBid (Fig. 2.9b), which undergoes a conformational change and binds the surface of mitochondrial membranes (Fig. 2.9c). It is possible that the structure of tBid, destabilized by dissociation from the N-terminal fragment after caspase-8 cleavage, undergoes a conformational change, whereby it opens about the flexible loops that connect its helical segments, to an extended helical conformation which binds to the membrane surface. This would be similar to the mechanism proposed for the lipoprotein apolipophorin-III, which adopts a marginally stable helix bundle topology that allows for concerted opening of the bundle about hinged loops (Wang et al. 2002). It is notable that the Bid amino acid sequence (P_{141}RDMEKE$_{147}$), at the beginning of helix-6, is similar to the conserved sequence (P_{95}DVEKE$_{100}$) that forms a short lipid recognition helix in apolipophorin-III. In Bid, this sequence forms a short loop that is perpendicular to the axis of helix-6 and solvent-exposed, while in apolipophorin-III it forms a short helix that is perpendicular to the helix bundle and at one solvent-exposed end of the molecule. This short motif is conserved in the Bid sequences from various species, suggesting that it plays a role in the protein biological function, and may constitute a lipid recognition domain for Bid similar to that of apolipophorin-III.

Pore formation by the Bcl-2 family proteins has been thought to involve translocation of the central core helices through the membrane, and the helices of both Bid and Bcl-X_L are sufficiently long to span the lipid bilayer. However, their amphipathic character is also compatible with membrane surface association, in a manner that is reminiscent of the antimicrobial polypeptides where binding of the polypeptide helices to the bacterial membrane surface is thought to transiently destabilize the membrane and change its morphology, inducing leakage of the cell contents, disruption of the electrical potential, and ultimately cell death (Boman 1995; Marassi et al. 1999, Marassi et al. 2000). It is notable that bacterial and mitochondrial membranes have very similar structures and surface charge, and that tBid is both capable of altering bilayer curvature, and of remodeling the mitochondrial membrane, which would be sufficient to cause the release of mitochondrial cytotoxic molecules. Thus, the BH3-independent mechanism of pore-formation and mitochondrial cytochrome-c release by tBid, may be similar to that of the an-

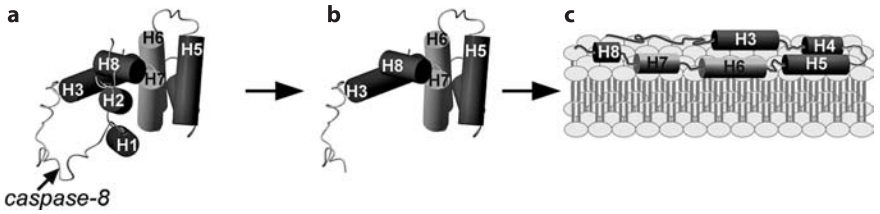

Fig. 2.9. Model for the association of tBid with the membrane surface. (**a**) Cleavage by caspase-8 in the flexible loop of the soluble Bid structure (Chou et al. 1999; McDonnell et al. 1999), generates the C-terminal product tBid (**b**), which undergoes a conformational change and binds the surface of mitochondrial membranes (**c**)

timicrobial polypeptides. In addition, the membrane surface association of tBid may serve to display the BH3 domain on the mitochondrial membrane surface, making it accessible for binding by other Bcl-2 family members. Although tBid does not insert in DOPC/DOPG lipid bilayers, it is possible that trans-membrane insertion may be driven by the presence of natural mitochondrial lipids, such as cardiolipin and monolysocardiolipin. It is also possible that the interactions with other Bcl-2 family proteins such as Bak and Bax, or with other non-homologous proteins such as the mitochondrial voltage-dependent anion channel, may promote insertion of the tBid helices through the mitochondrial membrane.

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