Structural characterization of the antiapoptotic protein Bcl-xL and the NDH-1 complex subunit CupS by X-ray crystallography and NMR spectroscopy

Dissertation
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I declare that I have written this thesis on my own. I have not used other resources than those cited in this thesis.

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Structural biology provides insights into the function of proteins and into the molecular mechanisms in the cell. Moreover, the knowledge of a protein structure allows the design of specific drugs. However, not every protein is suitable for structure determination, because all methods have their limitations. While solution NMR spectroscopy is in general only appropriate for the structure elucidation of small molecules, X-Ray crystallography relies on the ability of proteins to crystallize. Furthermore, a high concentration of pure recombinant protein is required for both methods. The major objective of this research thesis is the structural characterization of the two proteins Bcl-xL and CupS.

The antiapoptotic protein Bcl-xL is overexpressed in some tumors and is thus an important target for drug design. It can be found both in the cytosol and at the mitochondrial membrane. For the localization to the mitochondrial membrane its C-terminus, which has been predicted to be a transmembrane domain, is important [1, 2]. Because it is difficult to purify high concentrations of the full-length protein, structural studies have only been performed on truncated forms lacking the C-terminal part. Based on these, Bcl-xL has been found to exhibit a ligand binding pocket that binds various BH3 peptides, and other species including the synthetic inhibitor ABT-263. It is also assumed to bind the C-terminus, which thereby leads to oligomerization of the full-length protein. It is not yet clear, whether Bcl-xL largely inserts into the mitochondrial membrane or whether it stays loosely attached. Upon membrane insertion, the ligand binding pocket, which is formed by central helices of the protein, is likely to be destroyed. However, due to the difficult and elaborate experimental requirements for protein structure elucidation in a membrane environment, the overall structure of membrane-bound or membrane-inserted Bcl-xL remains elusive.

This work now concentrates on the structural characterization of the full-length Bcl-xL protein. To enable this study, a novel purification protocol for the high-level expression of full-length Bcl-xL was developed. Using this, the proteins were obtained as homooligomers, which were characterized using size exclusion chromatography. Similar observations were reported previ-
ously [3] in studies on full-length Bcl-xL, which also revealed that the homooligomers could be split by a Bid BH3 peptide. This was also tested during this work by NMR titration experiments with the Bid BH3 peptide and a Bak BH3 peptide, but no changes were observed for full-length Bcl-xL. The truncated Bcl-xLΔLoopΔTM was used as a reference and displayed characteristic signal changes. The high-affinity inhibitor ABT-263, which is at the moment used in clinical studies, was tested as well. Addition of ABT-263 splits the full-length Bcl-xL homooligomers into monomers as was revealed by NMR spectroscopy and X-Ray crystallography. The crystal structure of full-length Bcl-xL complexed with the inhibitor shows ABT-263 in the canonical ligand binding pocket indicating that major structural changes do not occur upon oligomerization. Furthermore, these results support the model of the C-terminus binding into the ligand binding pocket, which is then replaced by the high-affinity inhibitor. Moreover, the obtained crystal structure reveals some features that were not discussed in the crystal structure of the truncated Bcl-xL in complex with ABT-737 [4]. These are the first X-Ray and NMR data of full-length Bcl-xL. To get a first impression of the structure of full-length Bcl-xL in a membrane environment, the protein was refolded into LDAO micelles. Those have been used successfully as membrane mimetics in other structural studies [5, 6]. The resulting LDAO micelles containing Bcl-xL were investigated using size exclusion chromatography as well as CD and NMR spectroscopy. Both spectroscopic techniques verify that Bcl-xL is folded in this environment. Because the $^{1}$H-$^{15}$N-HSQC spectrum of Bcl-xL in LDAO micelles shows severe differences to Bcl-xL in solution, it can be assumed that it undergoes a dramatic structural change. The structure elucidation of Bcl-xL integrated in the LDAO micelle could be possible in the future employing NMR studies in perdeuterated LDAO or crystallization.

The cyanobacterial protein CupS is one of the various subunits of the NAD(P)H dehydrogenase type 1 (NDH-1) complex. This complex is responsible for cyclic electron flow and, depending on its exact subunit composition, for respiration or CO$_2$ uptake. Although the crystal structures of the similar respiratory Complex I from Thermus thermophilus and Escherichia coli are known, no structural data are available for any part of the cyanobacterial NDH-1 complex. Especially the structures of those subunits that are not homologous to Complex I will help to understand their distinct functions. The 15.7 kDa protein CupS is a small subunit of the NDH-1MS complex, which is thought to be important for CO$_2$ acquisition. The concrete function of CupS in this complex is still unknown and might be better understood by structural information.

The scope of this work was the structural investigation of CupS from Thermosynechococcus elongatus by NMR spectroscopy. The backbone and side chain chemical shifts were determined using a combination of various NMR spectra and the solution structure of CupS was
calculated. This is the first structure of a cyanobacterial NDH-1 complex subunit. As such it might help to understand its role in CO$_2$ acquisition. Furthermore, the sequential and structural similarity to proteins of the Fasciclin superfamily is noted. By comparison with the protein sequences and NMR structures of human TGFB1p [7], MPB70 from *Mycobacterium bovis* [8] and Fdp from *Rhodobacter sphaeroides* [9] it was tried to get a first impression of the functional characteristics of CupS in the NDH-1MS complex.
Part I.

Bcl-\textsubscript{XL}
2 | Introduction

2.1. Bcl-\(x_L\) and its role in apoptosis

The different forms of cell death can be identified by various criteria and the Nomenclature Committee on Cell Death (NCCD) regularly updates their definitions. Apoptosis, often simply referred to as programmed cell death, was first described in 1972 as a means to remove damaged and infected cells in a controlled manner, without damaging adjacent cells [10]. It is often set in contrast to necrosis, a process that includes an immune response and was thought to be a form of accidental cell death for a long time. However, necrosis appears to be regulated as well and there are also many other variants of cell death [11]. Apoptosis can be divided into extrinsic apoptosis, which is induced by extracellular stress signals, and intrinsic apoptosis, which occurs upon intracellular stress levels (Fig. 2.1).

The extrinsic pathway of apoptosis is mediated by extracellular death ligands that bind to receptors on the cellular surface. The cytoplasmic tails of the CD95 (FAS) receptor form oligomers that, upon ligand binding, interact with FAS-associated proteins with a death domain (FADD) to form a death-inducing signaling complex (DISC) [13]. Pro-caspase-8 and (-10) as well as cellular FLICE inhibitory proteins (cFLIP) are also included in this complex [14, 15]. The activation of caspase-8 is regulated by various isoforms of cFLIP [15] and leads to the cleavage of procaspases-3 and -7 and thereby to the initiation of a caspase cascade [14]. The members of the caspase family of proteins are cysteine proteases that cleave after aspartic residues [16]. They can be directly inhibited by inhibitors of apoptosis proteins (IAP) [17].

Caspases are often referred to as the executioners of apoptosis, because they cleave a variety of cellular components. Moreover, caspase-8 can also cleave the proapoptotic BH3-interacting domain death agonist (Bid) to truncated Bid (tBid) [18]. tBid leads to mitochondrial outer membrane permeabilization (MOMP) by regulation of the oligomerization of the proapoptotic multidomain proteins Bcl-2 associated X-protein (Bax) and Bcl-2 antagonist/killer-1 (Bak) [19, 20].
CHAPTER 2. INTRODUCTION

Figure 2.1: The pathways of extrinsic apoptosis and intrinsic apoptosis. The extrinsic pathway is initiated by binding of lethal ligands, e.g. the CD95 (FAS) ligand, to the corresponding receptors. The cytoplasmic tails of the FAS receptors form a death-inducing signaling complex (DISC) together with the FAS-associated protein with a death domain (FADD), cellular FLICE-inhibitory proteins (cFLIPs) and procaspase-8 or procaspase-10. Lethal signals activate caspase 8 or procaspase-10, which directly triggers the caspase cascade (type I). Caspases can be inhibited by inhibitors of apoptosis proteins (IAPs, e.g. XIAP). Moreover, caspase 8 can cleave BH3-interacting domain death agonist (Bid) to truncated Bid (type II). This leads to mitochondrial outer membrane permeabilization (MOMP), which is mediated by Bcl-2 associated X-protein (Bax) and Bcl-2 antagonist/killer-1 (Bak). Besides this proapoptotic signalling, the assembly of the DISC can also activate NF-κB through IκB kinases (IKK) and thereby inhibit apoptosis. Both the extrinsic and the intrinsic apoptotic pathway lead to MOMP. Intrinsic apoptosis is mediated by intracellular stress levels like DNA damage or growth factor withdrawal. As a consequence, BH3-only proteins (e.g. Bcl-2 antagonist of cell death (Bad), p53-upregulated modulator of apoptosis (PUMA) and Bcl-2 interacting mediator of cell death (Bim)) can interact with Bax and Bak directly or indirectly via Bcl-2 or other antiapoptotic proteins. Upon MOMP, proteins from the intermembrane space like cytochrome c are released into the cytosol. Cytochrome c assembles with procaspase-9, dATP and apoptotic protease activating factor-1 (Apaf-1) to form the apoptosome, which activates caspase-9 and thereby the caspase cascade. [12]
The intrinsic pathway of apoptosis also leads to this crucial event. It occurs upon intracellular stress levels like DNA damage or growth factor withdrawal. As a result several BH3-only proteins (i.e. a subgroup of the Bcl-2 family that contains only the Bcl-2 homology (BH)3 domain) are activated to induce MOMP by direct interaction with Bak or Bax (direct activators) or by interaction with antiapoptotic proteins (derepressors/sensitizers, see Fig. 2.2) [21]. MOMP results in the release of proteins from the intermembrane space of the mitochondrion. Among them is cytochrome c, which assembles with apoptotic protease activating factor-1 (Apaf-1), dATP and procaspase-9 to form the apoptosome. This multimeric complex activates procaspase-9 and thus initiates the caspase cascade [22].

It has to be noted that this is just a short review of the basic mechanisms during apoptosis that involve Bcl-2 family proteins.

The members of the Bcl-2 (B-cell lymphoma 2) family of proteins play a crucial role in apoptosis, because, as outlined above, they are involved in the process leading to MOMP. Characteristic for the Bcl-2 family are the BH domains BH1-BH4. Most Bcl-2 proteins contain also a transmembrane domain (see Fig. 2.2). According to their domain topology and to their functions, the Bcl-2 proteins can be divided into subfamilies. Proteins belonging to the BH3-only subfamily possess just the BH3 domain and usually function proapoptotic. They can be divided into direct activators (e.g. Bid, Bcl-2 interacting mediator of cell death (Bim) or p53-upregulated modulator of apoptosis (PUMA)), which activate the proapoptotic effectors, and sensitizers/derepressors (e.g. Bcl-2 antagonist of cell death (Bad), Bcl-2 interacting killer (Bik), Bcl-2 modifying factor (BMF), harakiri (HRK), and Noxa), which inhibit antiapoptotic proteins. Indeed, some BH3-only proteins exhibit functions from both groups. Multidomain Bcl-2 proteins can be classified as pro- or antiapoptotic proteins and can possess up to four BH domains. The proapoptotic multidomain proteins (Bax, Bak and Bcl-2 related ovarian killer (Bok)) are often referred to as effectors, which form effector pores in the outer mitochondrial membrane (OMM) [23]. Yet, the role of Bok in pore formation is not clear. The antiapoptotic proteins (e.g. Bcl-2, B-cell lymphoma extra large (Bcl-xL), Bcl-w, myeloid cell leukemia 1 (Mcl-1) and Bcl-2-related gene A1 (A1)) inhibit MOMP by binding to the direct activator or effector proteins (Fig. 2.2).

The antiapoptotic protein Bcl-xL is only one of the splice variants of the Bcl-x gene. Another leads to the expression of B-cell lymphoma extra small (Bcl-xS), which is a proapoptotic protein. Bcl-xL can be found at the OMM, but also in the cytosol or at the membrane of the endoplasmic reticulum [24]. It acts antiapoptotic in all three compartments. At the ER it regulates the Ca\(^{2+}\) release channel inositol 1,4,5-triphosphate receptor (InsP\(_3\)R) and thereby leads to a more controlled Ca\(^{2+}\) release, which enhances mitochondrial bioenergetics and
Prevents apoptosis [25]. However, its more prominent function is the inhibition of MOMP by interaction with other Bcl-2 family proteins. Several models have been proposed for these interactions. Bcl-xL can bind to tBid, an activator of Bax and thus inhibit MOMP indirectly (MODE 1) or it binds to Bax and Bak and prevents the localization and integration to the mitochondria indirectly (MODE 2) (similar to Mcl-1 in Fig. 2.2, B). It was shown that MODE 1 can be more easily derepressed and that, due to this, MODE 2 is more efficient to prevent MOMP and apoptosis [26]. Another model, the ‘embedded together’ model, emphasizes the importance of the membrane for Bcl-2 protein interactions [27]. Because Bcl-2 proteins presumably change their conformation upon membrane insertion, binding affinities are assumed to be different in the cytosol and at the membrane. tBid was reported to interact with both Bax and Bcl-xL showing increased affinity at membranes [28, 29]. Therefore, studies using membrane mimetics have become rather important in the last years. All of these models for MOMP are reasonable and contribute to the overall picture.

Bcl-xL can exert its antiapoptotic function not only by the direct interaction with Bcl-2 family members, but also by the interaction with other proteins like the tumor suppressor pro-
tein p53 or the voltage-dependent anion channel 1 (VDAC-1) [30, 31]. Interestingly, Bcl-\(x_S\) interacts with the VDAC-2. The function of Bcl-\(x_S\) has not been studied as extensively as that of Bcl-\(x_L\), but it is known that it can induce the activation of Bak [32] through binding to the VDAC-2 and not by binding to Bak itself [33]. The VDAC-2 is known to interact with Bak and to prevent it from oligomerization [34]. The BH4 domain, which is located at the N-terminus of both Bcl-\(x_S\) and Bcl-\(x_L\) was shown to inhibit the VDAC activity [35].

2.2. The structure of Bcl-\(x_L\)

The structure of human Bcl-\(x_L\)\(\Delta C\) (AS 1-209) was determined by both X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy as the first structure of a Bcl-2 family member [36]. It contains eight \(\alpha\)-helices and no \(\beta\)-sheets. Two central hydrophobic helices, \(\alpha_5\) and \(\alpha_6\), are flanked by helices \(\alpha_3\) and \(\alpha_4\) on one side and by helices \(\alpha_1\), \(\alpha_2\), and \(\alpha_7\) on the other side (Fig. 2.3, A). The same characteristic fold could be observed for Bax, Bid and other Bcl-2 family members [37, 38, 39]. Moreover, there is an unstructured loop between the first two helices. In Bid and Bcl-\(x_L\) this loop is rather long and contains a caspase cleavage site [40, 41]. The importance of this loop for apoptosis has been discussed controversially [36, 42]. Nevertheless, loop deletion mutants were used in many structural studies. The BH1, BH2 and BH3 domain of Bcl-\(x_L\) form a hydrophobic pocket on the protein surface (Fig. 2.3, B), which can bind various ligands, but especially peptides derived from BH3 domains. The Bcl-\(x_L\)-Bak BH3 peptide structure was the first to reveal the residues important for binding (for more details see chapter 2.4) [43]. There is also a distinct binding site for the interaction of Bcl-\(x_L\) with the tumor suppressor protein p53. This is located close to the small loop between helices \(\alpha_3\) and \(\alpha_4\). Therefore, BH3 ligand binding to the hydrophobic pocket is still possible, but might affect p53 binding [44]. The interaction with the PUMA BH3 peptide for example induces partial unfolding of helix \(\alpha_3\) of Bcl-\(x_L\) and disrupts the binding to p53 [45]. Truncated Bcl-\(x_L\) shows the ability to homodimerize under alkaline conditions (pH 10) [46] or when heated above 50 °C [47]. These homodimer structures were characterized by X-ray crystallography and NMR experiments, respectively. The dimers, termed 'domain-swapped dimers' in the following, are formed by the rearrangement of the helices \(\alpha_5\) and \(\alpha_6\) to form one long \(\alpha\) helix with \(\alpha_6\) of one monomer taking the position of \(\alpha_6\) in the other monomer (Fig. 2.4). Because the core structure of Bcl-\(x_L\) has not changed, the hydrophobic ligand binding pocket is also retained and accessible for ligands [46, 47]. Recently, a similar domain-swapped dimer was reported for Bax [48].

The proapoptotic splice variant of Bcl-\(x_L\), Bcl-\(x_S\), lacks residues 126-188. Thus, the BH1 domain and a part of the BH2 domain are missing. Nevertheless, Bcl-\(x_S\) still contains the
same BH4 and BH3 domain as Bcl-xL. However, the structural integrity of the protein is likely to be disturbed, because the two central helices $\alpha_5$ and $\alpha_6$ are missing. Moreover, the domain-swapped dimers cannot be formed, either. At the moment, no structural information is available for Bcl-xS.

**Figure 2.3.** The structure of truncated Bcl-xL [39]. The ribbon structure (A) represents the four BH domains in different colors. BH1: magenta, BH2: red, BH3: green, BH4: yellow. The loop between $\alpha_1$ and $\alpha_2$ is omitted. The surface representation (B) highlights hydrophobic amino acids in yellow, arginine, histidine and lysine in blue and aspartate as well as glutamate in red. As a consequence the hydrophobic BH3 ligand binding pocket can be identified.

**Figure 2.4.** Homodimer of Bcl-xL induced at alkaline pH 10 (PDB: 2B48). Helices $\alpha_5$ (blue) and $\alpha_6$ (red) are swapped, but the overall core structure of Bcl-xL is retained.
2.3. Characteristics of full-length Bcl-xL

Structural studies on full-length Bcl-xL are difficult, because the purification of the full-length protein in sufficient concentrations is challenging. This is why most of the various structural studies on Bcl-xL in the past two decades have been performed on proteins with truncations of the C-terminus and the flexible loop region. Nevertheless, full-length Bcl-xL could be investigated by other methods. Co-immunoprecipitation demonstrated the presence of Bcl-xL homodimers in the cytosol of healthy cells, which were expressing the full-length form. The analysis of various mutants revealed the C-terminus and the hydrophobic BH3 ligand binding pocket to be important for this dimerization [49]. Thus, this dimer is distinct from the domain-swapped dimer found by O’Neill et al. [46] and Denisov et al. [47], because these groups investigated a C-terminal deletion mutant. Moreover, the binding of a Bad BH3 peptide has been shown to be able to split the full-length Bcl-xL homodimer. Based on these findings it was suggested that the C-terminus of one molecule binds to the hydrophobic pocket of another Bcl-xL molecule and vice versa [49]. Furthermore, the C-terminus is thought to adopt a helical fold (α9) upon binding (Fig. 2.5) [3]. This model is based on the NMR structure of full-length Bax, which shows the C-terminal domain bound to its hydrophobic ligand binding pocket [37]. The loop between helices α8 and α9 of Bcl-xL is longer than that of Bax, which enables the binding to the hydrophobic ligand binding pocket of another Bcl-xL molecule. Helix α9 of both Bcl-xL and Bax is predicted to be a transmembrane domain (TM) [1].

![Figure 2.5: Model of a full-length Bcl-xL dimer](image)
In addition to this, recombinant full-length Bcl-xL can form homooligomers that can be split by BH3 ligands [3]. Bhat et al. propose that this homooligomerization occurs either by self-association of the homodimers via an unknown interaction surface or by binding of the C-terminus to the hydrophobic pocket in a head-to-tail or "runaway" fashion (Fig. 2.6) [3]. In the latter case an additional interaction surface is also likely to exist. The function of such an oligomer is yet unclear, but it can be suggested to exert a regulating function controlling the amount of free and thus active Bcl-xL upon overexpression. In other words, the homooligomers are thought to be an autoinhibitory allosteric switch that is turned on upon induction of apoptosis and binding to BH3 peptides. This would release the C-terminus and lead to translocation of Bcl-xL monomers to the mitochondria [3]. There, they could execute their antiapoptotic function, like the retrotranslocation of mitochondrial Bax into the cytosol. Both the C-terminus and the hydrophobic ligand binding pocket of Bcl-xL are important for this action [50, 51].

Ligand binding to full-length Bcl-xL was shown to be diminished at acidic pH, while formation of large oligomers (megamers, \(\approx 34,000\) kDa) and membrane insertion appeared to be favored [52]. The acidification of the cytosol was shown to occur upon apoptosis, although it is controversial whether it occurs before or after the activation of the caspase cascade [53, 54]. It was suggested that the megamers found at acidic pH are molten globules that are intermediates primed for membrane insertion [52]. Similar observations were made for diphteria toxin [55], which shows structural similarity to Bcl-xL [36]. Indeed Bcl-xL can form pH-dependent
ion channels in synthetic lipid membranes like the bacterial toxins [56]. Structural models for the membrane-inserted protein employ the two central helices α5 and α6, as well as the TM α9. Because all Bcl-2 family proteins share the same core fold, similar mechanisms were suggested for Bax and Bid (Fig. 2.7) [1]. For dodecylphosphocholine (DPC) micelles, helix α1 of Bcl-xL was also reported to be located within the micelle [57]. At this moment, the functional principle of these structures remains enigmatic.

![Figure 2.7](image.png)

**Figure 2.7** Proposed models of membrane insertion of Bcl-xL (A), Bax (B) and Bid (C) as a two-step process. In C the caspase-8 cleavage site is indicated by an arrow. The experimental NMR structures in solution are shown in 1. The intermediate states (2) are hypothetical and the membrane-inserted species (3) are derived from experimental data like glycosylation mapping [1].

Bcl-xS has not been characterized structurally so far, but in comparison to Bcl-xL the two central helices α5 and α6 are missing. It is thus unlikely to insert into the membrane. However, localization to the membrane was shown to be essential for its proapoptotic activity [58]. By elimination of the hydrophobic core, the BH3 ligand binding pocket is also removed. Despite this, homodimerization of Bcl-xS and heterodimerization with Bcl-xL and Bax was observed, with a higher affinity for Bax [59]. While heterodimerization could be explained by the binding of the transmembrane domain of Bcl-xS (which is identical to that of Bcl-xL) into
the hydrophobic grooves of Bcl-xL or Bax, homodimerization remains unexplained due to the lack of structural information.

2.4. The interaction of Bcl-xL with BH3 ligands

The canonical BH3 ligand binding pocket of Bcl-xL is unoccupied in the C-terminally truncated protein. Thus, it is accessible to BH3 ligands. The structures of the Bcl-xL/Bak BH3 complex and the Bcl-xL/Bad BH3 complex were solved by solution NMR spectroscopy using truncated proteins [43, 60]. It was shown recently that the affinity of BH3 peptides to full-length Bcl-xL is lower than the affinity to the truncated protein, because the peptides have to compete with the C-terminal part to bind to the hydrophobic cleft [3]. In addition to the hydrophobic residues, the ligand binding pocket is flanked by charged amino acids that assist the binding of BH3 peptides such as the Bak BH3 peptide (Fig. 2.8). Although the BH3 peptides are unstructured in solution, they adopt a helical fold upon binding to the hydrophobic groove of Bcl-xL [43].

![Figure 2.8](image)

**Figure 2.8:** The Bcl-xL/Bak BH3 peptide complex structure. The Bak BH3 peptide is represented in green. The residues of Bcl-xL important for the interaction are emphasized by colors. Positively charged residues are depicted in blue, negatively charged residues in red and hydrophobic residues in yellow. [43]

Because Bcl-xL overexpression can be found in tumors [61], its binding site is an important target for drug design. Based on the knowledge about the interaction sites in the hydrophobic pocket, several artificial ligands were designed for Bcl-xL inhibition. The most prominent ones are ABT-737 [62], and its orally available analogue ABT-263 (Navitoclax) [63] (Fig. 2.9). Both show very high affinity (IC_{50} ≤ 1 nM) to the antiapoptotic proteins Bcl-w, Bcl-xL and Bcl-2. ABT-263 was successfully used in clinical phase I and phase II studies. However,
thrombocytopenia is a major side effect of the treatment [64, 65]. This is not very surprising, because Bcl-xL is important for platelet homeostasis by constraining the proapoptotic activity of Bak [66]. Since only Bcl-xL, and not Bcl-2, was shown to be able to inhibit Bak [67], the Bcl-2 selective inhibitor ABT-199 was developed recently to prevent thrombocytopenia in patients [68]. However, as Bcl-xL is the favored target for drug design due to its overexpression in tumors, new and specific Bcl-xL inhibitors were designed that are smaller and more efficient than ABT-737, and that show less thrombocytopenia \textit{in vivo} [69].

\begin{center}
\textbf{Figure 2.9.} The inhibitors ABT-737 (A) and ABT-263 (Navitoclax, B). Both show a high affinity (IC\textsubscript{50} \leq 1 \text{nM}) to the antiapoptotic proteins Bcl-w, Bcl-xL and Bcl-2.
\end{center}
3 | Results

3.1. Cloning, expression and purification of various Bcl-x constructs

The purification of full-length Bcl-2 proteins, in concentrations relevant for chemical studies, is a challenging task. Bcl-x\textsubscript{L}ΔTM and Bcl-x\textsubscript{L} ΔLoopΔTM are structurally well characterized monomeric proteins \cite{36, 43}, which can easily be purified with an N-terminal glutathione-S-transferase (GST)-tag. Expression of full-length Bcl-x\textsubscript{L} leads to the formation of inclusion bodies without the C-terminal intein/chitin binding domain (CBD)-tag and to only poor protein yield without the N-terminal GST-tag. Yet, when using a construct combining N-terminal GST-tag and C-terminal intein tag, these problems are not encountered. After the removal of both tags soluble homodimers and homooligomers can be purified.

Bcl-x\textsubscript{S}, which lacks the BH1 and most of the BH2 domain (Fig. 3.1) was also cloned into this expression system. However, it could not be purified successfully. After the removal of the GST-tag with thrombin, only GST was clearly visible on an SDS gel (data not shown). The Bcl-x\textsubscript{S} protein sequence itself does not contain a thrombin cleavage site, so there is no explanation for this at the moment. Therefore, the purification strategy will have to be further tested and optimized.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure3.png}
\caption{Schematic representation of Bcl-x\textsubscript{L}, its deletion mutants and Bcl-x\textsubscript{S}.}
\end{figure}
The purification strategy for Bcl-x\textsubscript{L}ΔTM has been described and documented previously [70]. The SDS-PAGE (Fig. 3.2) shows samples of all purification steps. Neither the pellet after cell disruption (lane 1) nor the lysate (lane 2) shows a significant overexpression band of 49 kDa (GST (26 kDa) + Bcl-x\textsubscript{L}ΔTM (23 kDa)). The eluate from the first GSH-column purification step (lane 4) clearly shows the overexpressed fusion protein GST-Bcl-x\textsubscript{L}ΔTM, which is then cleaved by thrombin (lane 5). The flowthrough of the second GSH-column purification step contains the isolated Bcl-x\textsubscript{L}ΔTM (lane 7). It runs at ≈ 30 kDa, despite its calculated molecular weight of 22.8 kDa. [70]

Bcl-x\textsubscript{L}ΔLoopΔTM was purified in the same way. The protein runs at 15 kDa on an SDS-PAGE (Fig. 3.3, right). This is slightly lower than the calculated molecular weight of 18.6 kDa. Full-length Bcl-x\textsubscript{L} and Bcl-x\textsubscript{L}ΔLoop were purified similarly, but 5 mM DTT was included in the resuspension buffer to cleave off the intein from the fusion protein. Monomeric full-length Bcl-x\textsubscript{L} (26.1 kDa) runs at 35 kDa on the SDS-PAGE (Fig. 3.3, left). Furthermore, the gel shows a rather intense band at ≈ 60 kDa, which is likely to belong to dimeric Bcl-x\textsubscript{L}. The sharp band slightly below 55 kDa and the weak band below 25 kDa might belong to uncut GST-Bcl-x\textsubscript{L} and GST, which were not bound to the GSH column for an unknown reason.

Bcl-x\textsubscript{L}ΔLoop runs at ≈ 20 kDa (Fig. 3.3, middle), which is quite close to its size of 21.8 kDa. Taken together, it can be stated that the Bcl-x\textsubscript{L} mutants, which contain the long loop between helices α1 and α2 exhibit a higher molecular weight on the SDS-PAGE, while the loop truncation mutants run at a lower molecular weight than that calculated. Thus, the presence or absence of the unstructured loop (44 aa, 4.6 kDa) extremely alters the retention behavior.
CHAPTER 3. RESULTS

Figure 3.3 Different purified Bcl-xL mutants. On an SDS-PAGE (15 %) monomeric full-length Bcl-xL (left) runs at 35 kDa, Bcl-xLΔLoop (middle) at ≈ 20 kDa and Bcl-xLΔLoopΔTM (right) at 15 kDa.

The difference between the Bcl-xL mutants with loop and the corresponding mutants without loop is 15 kDa.

Size exclusion chromatography (Superdex 75 10/300, GE Healthcare) shows the oligomeric states of the different Bcl-xL mutants (Fig. 3.4). Bcl-xLΔLoopΔTM occurs in a defined state with only a very small fraction at higher molecular weight. The protein elutes mainly at a volume of 11.6 mL and a very small amount elutes at 10.1 mL, which, according to our calibration curve, corresponds to molecular weights of 40 kDa or 90 kDa, respectively. This indicates, that Bcl-xLΔLoopΔTM with a molecular weight of 18.6 kDa exists mainly as a dimer. Yet, this is in disagreement with with the literature, which shows an almost identical curve shape, but characterizes Bcl-xLΔLoopΔTM to be monomeric [3]. This was also confirmed by an NMR structure of this mutant [71]. It is therefore assumed that Bcl-xLΔLoopΔTM is monomeric, although the chromatogram is contradictory for an unknown reason. Owing to this, the correct interpretation of the size exclusion chromatography of full-length Bcl-xL and Bcl-xLΔLoop is difficult. Basically it can be stated that the two proteins including the TM occur in three different forms. Full-length Bcl-xL elutes at 8.8 mL (196 kDa), 9.6 mL (121 kDa) and 11.0 mL (56 kDa). Referring to the size of Bcl-xL, which is 26.1 kDa these peaks refer to a hepta- or octamer, a tetra- or pentamer and a dimer. Bcl-xLΔLoop has a molecular weight of 21.8 kDa. The occurring peaks at 9.0 mL, 10.3 mL and 11.6 mL correspond to 171 kDa, 83 kDa and 40 kDa, respectively. These values suggest the existence of an octamer, a tetramer or a dimer. Interestingly, the different oligomeric states can be isolated and there is no equilibrium (Fig. 3.5). This is in contrast to the results of Bhat et al., who found a reversible monomer-dimer-multimer-polymer equilibrium [3]. The large hepta-
or octamer peak is likely to be distorted, because it is very close to the exclusion volume of the Superdex 75 10/300 column. Thus, another column (Superose 6 10/300, GE Healthcare) was tested to have a closer look at this region.

![Size exclusion chromatography (Superdex 75 10/300) of different Bcl-xL constructs.](image)

**Figure 3.4.** Size exclusion chromatography (Superdex 75 10/300) of different Bcl-xL constructs. While Bcl-xLΔLoopΔTM (green) only exists in one defined form (probably monomer), both Bcl-xLΔLoop (red) and full-length Bcl-xL (blue) occur at various sizes. The calculated molecular weights that correspond to the elution volume of the peaks are indicated. The large peak at 8.8 mL is related to a molecular weight of 196 kDa, but is very close to the exclusion volume of the column. Therefore, this value is likely to be misleading.

On Superose 6 10/300 (Fig. 3.6) the exclusion volume is clearly separated from a full-length Bcl-xL peak at 12.8 mL (1279 kDa). Moreover, there are peaks at 16.1 mL (129 kDa), 16.7 mL (84 kDa) and 17.4 mL (52 kDa), which basically resemble the peaks occurring on the Superdex column. Unfortunately, the three peaks are overlapping making size determination in this region rather difficult. The peaks indicate the presence of a pentamer, a trimer and a dimer and an additional multimer of about 50 molecules. Bcl-xLΔLoop occurs at 13.4 mL (868 kDa), 16.6 mL (92 kDa) and 17.3 mL (54 kDa) resembling a 40-mer, a tetramer and a dimer. The tetramer peak however might be a mixture with trimers and pentamers, because the resolution of the column is not high enough in this size region. The peaks of the 50-mer of full-length Bcl-xL and of the 40-mer of Bcl-xL are rather broad and might comprise of assemblies of different sizes as well. Importantly, the protein eluting from the column
The different oligomeric forms of Bcl-xLΔLoop can be isolated. Size exclusion chromatography (Superdex 75 10/300) of Bcl-xLΔLoop shows the isolated dimer (red) and oligomer (blue).

First is relatively small, meaning that the large polymers (3000 kDa) that were observed by Bhat et al. [3] are not apparent in our samples. Again, the similarities between full-length Bcl-xL and Bcl-xLΔLoop emphasize that the oligomerization of the proteins occurs in an organized fashion. Unspecific aggregates would tend to be larger and shape curves would not be reproducible.

Size exclusion chromatography (Superose 6 10/300) of full-length Bcl-xL (blue) and Bcl-xLΔLoop (red). The calculated molecular weights are indicated. The peak at ≈ 8 mL can be ascribed to the exclusion volume.
3.2. NMR spectroscopy of Bcl-x\textsubscript{L}

NMR experiments confirm the homooligomerization of full-length Bcl-x\textsubscript{L}. While TROSY-HSQC spectra of Bcl-x\textsubscript{L}ΔLoopΔTM (Fig. 3.7, blue) and Bcl-x\textsubscript{L}ΔTM (Fig. 3.8, pink) show intense, well-dispersed signals, the spectrum of full-length Bcl-x\textsubscript{L} differs dramatically in signal intensity. Only few signals are visible (Fig. 3.7 and 3.8, teal), which is accounted for by the size of the oligomer. Large molecules possess a slower tumbling rate than small molecules and this results in extensive line broadening. Therefore, most signals apparent in the spectrum of full-length Bcl-x\textsubscript{L} can be assumed to belong to a rather flexible region that is not subjected to line broadening, as is the long loop between helices \(\alpha1\) and \(\alpha2\). This was confirmed by a comparison between the spectrum of full-length Bcl-x\textsubscript{L} and the spectra of Bcl-x\textsubscript{L}ΔLoopΔTM and Bcl-x\textsubscript{L}ΔTM (Fig. 3.7 and 3.8). Most signals apparent in the spectrum of the full-length protein cannot be found in the spectrum of the loop deletion mutant, while there is an almost complete overlap with the signals of Bcl-x\textsubscript{L}ΔTM. Thus, the signals in the spectrum of full-length Bcl-x\textsubscript{L} belong to the long loop, which appears to be unaffected by oligomerization. This is in consensus with the results from size exclusion chromatography.

The 1D-\textsuperscript{1}H-spectra of the various Bcl-x\textsubscript{L} mutants do not show severe differences with regard to line broadening. Nevertheless, there are differences between the spectra of Bcl-x\textsubscript{L}ΔLoopΔTM and full-length Bcl-x\textsubscript{L} (Fig. 3.9). These are less pronounced in the overlay of the spectra of Bcl-x\textsubscript{L}ΔTM and full-length Bcl-x\textsubscript{L} (Fig. 3.10). The long unstructured loop between helices \(\alpha1\) and \(\alpha2\) is the reason for most differences between full-length Bcl-x\textsubscript{L} and Bcl-x\textsubscript{L}ΔLoopΔTM. The 1D-\textsuperscript{1}H-spectra were referenced with respect to the NMR standard DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid), which was added to the sample of Bcl-x\textsubscript{L}ΔLoopΔTM and leads to a signal at 0 ppm. The water signal is observed at 4.7 ppm. Aromatic and amide protons lead to signals between 6.5 ppm and 10.5 ppm. The tryptophan H\textsubscript{e} protons are visible in the Bcl-x\textsubscript{L} spectra downfield of 9.3 ppm. Bcl-x\textsubscript{L}ΔLoopΔTM contains five tryptophan residues, which can be clearly identified in the TROSY-HSQC spectrum. Bcl-x\textsubscript{L}ΔLoop and full-length Bcl-x\textsubscript{L} contain six or seven tryptophan residues, respectively, because one is located in the loop region and one in the TM. The tryptophan from the loop region can be clearly identified by comparison of the TROSY-HSQC spectra, but that from the TM cannot be seen. This supports the assumption that the C-terminus is not flexible, but bound to a specific part of the protein such as the hydrophobic ligand binding pocket. The amide protons of Bcl-x\textsubscript{L} are located mainly upfield of 8.5 ppm, which indicates an \(\alpha\)-helical fold. This is further supported by the position of the H\textsubscript{\alpha} signals in the 1D-\textsuperscript{1}H-spectra, which are usually found between 3.5 and 5.5 ppm. In \(\alpha\)-helical regions they are predominantly found between 3.5 and 4.5 and in \(\beta\)-sheet regions between 4.5 and 5.5 ppm [72]. The aliphatic side chain protons
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Figure 3.7. $^1$H-$^{15}$N-TROSY-HSQC spectra of Bcl-x$_L$ΔLoopΔTM (blue) and full-length Bcl-x$_L$ (teal).

Figure 3.8. $^1$H-$^{15}$N-TROSY-HSQC spectra of Bcl-x$_L$ΔTM (pink) and full-length Bcl-x$_L$ (teal).
are observed upfield of 3.5 ppm. Distinct signals between 1 and -1 ppm, which appear in all Bcl-xL 1D-\textsuperscript{1}H-spectra, are an indication for a folded protein [73].

\textbf{Figure 3.9}: 1D-\textsuperscript{1}H-spectra of Bcl-xLΔLoopΔTM (blue) and full-length Bcl-xL (teal).

\textbf{Figure 3.10}: 1D-\textsuperscript{1}H-spectra of Bcl-xLΔTM (pink) and full-length Bcl-xL (teal).
3.3. NMR interaction studies of Bcl-xL with various peptides

The full-length Bcl-xL homooligomers were reported to be split by BH3 peptides that bind to the ligand binding pocket occupied by the C-terminal TM [49, 3]. To confirm these results full-length Bcl-xL was titrated with the BH3 peptides of Bak and Bid and investigated by NMR spectroscopy. Bcl-xLΔLoopΔTM was used as a reference, because its ligand binding pocket is freely accessible. Moreover, a peptide with the sequence of the C-terminal helix, which is omitted in Bcl-xLΔTM (referred to as 'TM peptide') was added to Bcl-xLΔLoopΔTM to probe its binding affinity.

The addition of the Bak BH3 peptide to Bcl-xLΔLoopΔTM led to a lot of clear signal changes (Fig. 3.11). The localization of the binding site is difficult, because many signals shift slightly and especially in crowded regions an unambiguous assignment is impossible. However, the corresponding NMR complex structure was solved in 1997 and showed the Bak BH3 peptide in the ligand binding pocket. The affinity was determined to be 0.34 μM [43]. The interaction seems to be slow on the NMR time scale. Upon the addition of the Bak BH3 peptide the signals of the free protein disappear, while those of the bound protein appear at other chemical shifts values. At a protein-peptide ratio of 1:1 there is a similar amount of protein in the free and in the bound form. This is concluded from the occurrence of signals from both forms with a similar intensity. At a ratio of 1:3 most protein molecules have bound the Bak BH3 peptide. The tryptophan side chain signals are shown as an example (Fig. 3.11, B). If the interaction was fast on the NMR time scale, both the free and the bound form would be detected at the same time, which would lead to the observation of averaged signals. The addition of an increasing amount of ligand leads to shifts from the signals of the free form to the signal position of the bound form. In that case the interaction site can be easily detected.

Despite the high affinity of the Bak BH3 peptide the full-length Bcl-xL homooligomer was not affected by its addition. No changes could be observed in the NMR spectrum.

The Bid BH3 peptide was reported to have an affinity of 0.79 μM to Bcl-xLΔLoopΔTM and of 9.97 μM to full-length Bcl-xL. Moreover, it was shown to split the homooligomers [3]. In conformity with the lower affinity, the spectrum of the truncated form with the Bid BH3 peptide showed fewer signal changes, which appeared at higher concentrations, than the corresponding spectrum with the Bak BH3 peptide. The spectrum of the full-length protein did not show any signal changes upon addition of the Bid BH3 peptide.

The addition of the 'TM peptide' showed only very small signal changes in the spectrum of Bcl-xLΔLoopΔTM. The changing signals do not belong to a specific area of the protein, but scatter over the whole protein structure. Moreover, precipitation could be observed in the NMR tube upon peptide addition. The low solubility of the peptide could have diminished
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Figure 3.11: NMR titration of Bcl-xL ΔLoopΔTM with the Bak BH3 peptide. A: $^1$H-$^{15}$N-TROSY-HSQC spectra in absence (blue) and presence (purple) of the peptide (ratio 1:3). B: Resonance shifts of the five tryptophans in Bcl-xL ΔLoopΔTM upon addition of the Bak BH3 peptide. Extract from the $^1$H-$^{15}$N-TROSY-HSQC spectra of Bcl-xL ΔLoopΔTM in absence (blue) and presence of the Bak BH3 peptide (yellow: ratio 1:1, purple: ratio 1:3).
the observed interaction between the peptide and the protein. Nevertheless, a low binding affinity is in conformity with the assumption that other BH3 peptides can replace the TM from the ligand binding pocket. The insolubility of the peptide is also reasonable, because as a sequence coding for a predicted TM it is rather hydrophobic.

The hydrophobicity of the TM peptide becomes also clear in the helical wheel representation, which was used to compare the various peptides according to their amphipathicity. For the TM peptide (29 aa) only the 21 C-terminal amino acids were plotted, which are homologous to the C-terminal residues of Bax that are bound in a helical conformation. While both, the Bak BH3 peptide and the Bid BH3 peptide appear to form amphipathic helices, the TM peptide is largely hydrophobic and contains only two charged amino acids, which are the two C-terminal residues. In general, the solubility, which was observed for the different peptides is in consensus with the number of charged residues in their peptide sequence. Consequently, the Bid BH3 peptide was highly soluble in NMR buffer, while the Bak BH3 peptide showed a decreased solubility and the TM peptide was rather insoluble in NMR buffer.

The affinity of the peptides to Bcl-xL can also be explained by their sequence. Based on the complex structure of Bcl-xL with the Bak BH3 peptide [43], three charged residues of the peptide (R76, D83 and D84, emphasized by an asterisk in Fig. 3.12) could be identified, which are responsible for binding to the ligand binding pocket (see also Fig. 2.8). The sequence of the Bak BH3 peptide can be easily aligned to the sequence of the Bid BH3 peptide. Here, one of the aspartate residues (D84) is substituted by an uncharged serine residue, which results in the lower binding affinity of the Bid BH3 peptide. The TM peptide does not show any charged residues except for the C-terminal arginine and lysine residues, which cannot be

![Figure 3.12: Helical wheel representations of the three peptides used in the interaction studies. Positively charged residues are colored in blue, negatively charged residues in red, polar, but uncharged residues in green and unpolar hydrophobic residues in yellow. The figure was produced manually with the help of the EMBOSS pepwheel tool (http://emboss.bioinformatics.nl/cgi-bin/emboss/pepwheel).](image-url)
related to the BH3 peptide sequences. Therefore, binding of the TM peptide is unfavored and there has to be a yet unknown mechanism, which allows binding in the full-length Bcl-xL oligomer (discussed in chapter 4.2).

3.4. NMR titrations of Bcl-xL with ABT-263

The synthetic inhibitor ABT-263 (Navitoclax) shows a very high affinity ($IC_{50} \leq 1$ nM) to the antiapoptotic proteins Bcl-w, Bcl-2 and Bcl-xL. Therefore, its ability to split the full-length Bcl-xL homooligomers was also tested by NMR spectroscopy. It has to be noted that ABT-263 appears to be almost insoluble in water and precipitated immediately in the NMR sample tube. Nevertheless, the NMR spectra of both Bcl-xL ∆Loop∆TM and full-length Bcl-xL change dra-

![Figure 3.13](image_url)

**Figure 3.13**: $^1$H-$^{15}$N-TROSY-HSQC spectra of Bcl-xL ∆Loop∆TM in absence (light blue) and presence (blue) of ABT-263 (ratio 1:3).
matically upon addition of ABT-263 (Fig. 3.13 and 3.14). The truncated form shows severe differences of almost every signal, which makes an assignment only based on the previously published chemical shift assignment for the unliganded, truncated Bcl-xL [74] impossible. However, ABT-263 was designed for specifically binding to the hydrophobic ligand binding pocket based on the inhibitor ABT-737 [63], for which a crystal complex structure is available [4]. Moreover, a published HSQC spectrum of truncated Bcl-xL in complex with ABT-737 [75] shows the same characteristic signal changes observed here for Bcl-xL ΔLoopΔTM in complex with ABT-263, especially in the glycine and tryptophan region. Nevertheless, the latter shows more distinct signal changes in the central region of the spectrum. One reason might be that the TROSY-HSQC pulse sequence that was used instead of a conventional HSQC experiment leads to a higher spectral resolution. Based on the high amount of signal

Figure 3.14: $^{1}$H-$^{15}$N-TROSY-HSQC spectra of full-length Bcl-xL in absence (teal) of ABT-263 and incubated with ABT-263 for five days (ratio 1:3, red). The encircled signals are likely to correspond to the C-terminal residues, which are freed upon addition of the inhibitor.
changes, ABT-263 binding to the BH3-binding pocket was considered to affect the overall protein structure.

In the spectrum of full-length Bcl-x\textsubscript{L} slight signal changes are visible upon direct addition of ABT-263 (ratio 1:3), but, more important, after five days of incubation new signals appear (Fig. 3.14, red). This indicates a dramatic change in the homooligomer assembly leading to higher flexibility and thus suggests splitting of the oligomer. The comparison with the spectrum of the Bcl-x\textsubscript{L}ΔLoopΔTM-ABT-263 complex shows an overlap of most of the new signals (Fig. 3.15). Consequently, it can be assumed that full-length Bcl-x\textsubscript{L} also binds to ABT-263 and that the binding mode of ABT-263 is the same in both molecules. Some signals visible in the spectrum of the full-length form refer to the long loop between helices α1 and α2 and are thus not apparent in the spectrum of the deletion mutant. Furthermore, some strong

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_15.png}
\caption{\textsuperscript{1}H-\textsuperscript{15}N-TROSY-HSQC spectra of Bcl-x\textsubscript{L}ΔLoopΔTM (blue) and full-length Bcl-x\textsubscript{L} (red) in presence of ABT-263 (ratio 1:3). The encircled signals are likely to correspond to the C-terminal residues, which are freed upon addition of the inhibitor.}
\end{figure}
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Signals appear in the spectrum with inhibitor, which are detected neither in any spectrum of the truncated form nor in the full-length reference spectrum (encircled in Fig. 3.14 and 3.15). These are likely to belong to the C-terminus, which is freed upon inhibitor binding. Taken together, the results support the model of the C-terminal part binding to the hydrophobic BH3 ligand binding pocket in the homooligomer, which is then replaced by the high-affinity inhibitor.

3.5. Crystal structure of Bcl-xL in complex with its inhibitor ABT-263

To verify the results obtained by NMR spectroscopy, crystallization screens of full-length Bcl-xL with and without ABT-263 were performed. Only in the presence of the inhibitor crystals could be grown. The Bcl-xL homooligomer did not crystallize. This is not surprising, because, besides purity, homogeneity is a factor influencing the ability of a sample to crystallize. In solution, full-length Bcl-xL does not adopt a defined oligomeric state, but exists as dimer, tetramer or even as a larger multimer (see chapter 3.1). Later it was found that these different forms can be isolated, so a crystallization screen of only one of these species might be more successful.

The crystals of Bcl-xL in complex with ABT-263 are triclinic, space group P1, a = 78.14 Å, b = 85.81 Å, c = 93.64 Å, α = 72.936 °, β = 67.424 °, γ = 69.382 °, and contain twelve molecules in the asymmetric unit (Mol A-L). R_work and R_free were 19.48 % and 24.22 %, respectively. More crystallographic details for the Bcl-xL-ABT-263 complex are reported in Table 3.1. The ramachandran plot showed 96.9 % of residues in favored regions, 2.9 % in allowed regions and only 0.2 % in disallowed regions. Residues in disallowed regions were found at the termini and before or after the unstructured loop. Some residues had to be replaced by alanine residues due to missing electron density of the side chains. Especially the electron density of helix 3 (aa 106-113) was poorly defined in most molecules. Molecule D showed the best electron density and has thus been selected for presentation in most figures.

Each of the twelve molecules in the asymmetric unit binds one inhibitor molecule in its hydrophobic ligand binding pocket. This result confirms the NMR results. The homooligomers can be split by the inhibitor, which binds to the ligand binding pocket and thereby replaces the C-terminal helix. Unfortunately, no electron density could be observed for the loop region between helices α1 and α2 (aa 25-81) and the C-terminal part (aa 197-233) indicating flexibility of these parts. Despite the very low calculated water content of 27.5 % the free space in the lattice is still big enough to accommodate the missing residues. The electron density of helix α3 showed differences in quality and this region also exhibits the highest B-factors.
(Fig. 3.16, A). The structure of the molecule with the best electron density fit (MoID) is shown in Fig. 3.16 and Fig. 3.18.

**Data collection**

- **Space group**: P1

**Cell dimensions**

- \( a, b, c \) (Å): 78.1, 85.8, 93.6
- \( \alpha, \beta, \gamma \) (°): 72.9, 67.4, 69.4

**Completeness (%)**: 97.49

**Water content (%)**: 27.5

**Refinement**

- **Resolution (Å)**: 46.3 - 2.3
- **No. reflections**: 89478
- **R<sub>work</sub>/R<sub>free</sub>**: 0.197 / 0.242
- **No. atoms**: 14703
- **Non-solvent**: 14512
- **Solvent**: 191

**Ramachandran statistics (%)**

- **Outliers**: 0.24
- **Allowed**: 2.86
- **Favored**: 96.91
- **Rotamer outliers**: 0.28
- **C-beta outliers**: 0

**R.m.s. deviations**

- **Bond lengths (Å)**: 0.010
- **Bond angles (°)**: 1.311

**Table 3.1**: Data collection and refinement statistics for the crystal structure of full-length Bcl-x<sub>L</sub> in complex with ABT-263.

The orientation of ABT-263 resembles very closely that of ABT-737 in the corresponding crystal structure of truncated Bcl-x<sub>L</sub> in complex with ABT-737 (Fig. 3.16, B) and involves two hydrophobic pockets termed P2 and P4 [4] (Fig. 3.16, C). The thiophenyl group binds to the P4 pocket and is stabilized by \( \pi \)-stacking with the phenyl group that is substituted with the trifluoromethanesulfonyl group. This orientation was also observed for the co-crystal structures of Bcl-x<sub>L</sub> with ABT-737 and of Bcl-2 in complex with ABT-263 [68]. Similar to Bcl-2, the pocket P2 is occupied by the 1-chloro-4-(4,4-dimethylcyclohex-1-enyl)-benzene
Figure 3.16: The crystal structure of full-length Bcl-xL in complex with ABT-263. A: Representation as a B-factor putty. Line thickness and colors indicate the B-factor values. Thin lines and blue color imply a low B-factor, thick lines and red color indicates high B-factors. Therefore, the depiction emphasizes the structurally destabilized region of helix α3. B: Comparison of the crystal structure of full-length Bcl-xL in complex with ABT-263 (grey, green) and the crystal structure of truncated Bcl-xL with ABT-737 (light magenta, purple, PDB: 2YXJ). C: Vacuum electrostatic surface representation of the crystal structure of full-length Bcl-xL in complex with ABT-263. Red color indicates a negative, blue color a positive electrostatic potential. The long loop between helices α1 and α2 and the C-terminal part are not visible in the electron density. The inhibitor occupies the hydrophobic BH3 ligand binding pocket. Arrows indicate the pockets P2 and P4, which are essential for ligand binding. D: The synthetic inhibitor ABT-263.

moiety of ABT-263. However, the here presented crystal structure provides additional details regarding the stabilization of the ligand through hydrogen bonds. As reported for ABT-737, the acylsulfonamide of ABT-263 forms a hydrogen bond to the backbone amide of residue
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G138 and in some molecules to the side chain of N136. Moreover, the morpholine group of ABT-263 (substituted by a 2-dimethylaminoethyl group in ABT-737) is involved in a hydrogen bond to residue E96 only in some of the molecules as observed in the crystal structure with ABT-737. The introduction of a trifluoromethanesulfonyl group leads to a sterical clash with residue Y195, which is thereby oriented differently. More important, the inhibitor also shows interaction with another Bcl-xL molecule. Residue Y120 of the other molecule and the phenyl group attached to the trifluoromethanesulfonyl group interact via $\pi$-stacking. Furthermore, the acylsulfonamide group is connected by a hydrogen bond network via water molecules to P116 and R165 and - depending on the quality of the electron density - to adjacent residues of the other Bcl-xL molecule (Fig. 3.17, A).

Thus, ABT-263 is always buried between two Bcl-xL molecules that are oriented in a head-to-tail fashion (Fig. 3.17, C). This leads to a continuous chain that extends throughout the crystal with the same interface between every two molecules. In total there are four chains, which are oriented parallel and perpendicular to each other (Fig. 3.17, D). The twelve molecules in the asymmetric unit resemble the three different conformations of four chains. However, the corresponding interface areas were determined by PISA [76] to be quite small (between 270 Å and 370 Å) and thermodynamically unfavored. Thus, they are probably not of biochemical relevance in this inhibitor-bound form. The PISA analysis also revealed the interfaces between ABT-263 and the respective Bcl-xL molecule (between 650 Å and 690 Å) and interfaces between different Bcl-xL molecules, which predominantly occurred due to parts of the rather flexible termini (the largest being 716 Å). Therefore, there are no oligomers in the crystal. This is consistent with the observation that the inhibitor splits the oligomers formed by full-length Bcl-xL into monomers as suggested due to the NMR experiments.

The high organizational level of the crystal with the low water content, which occurs despite the flexibility of the loop and C-terminus might be explained by a pre-orientation of the full-length Bcl-xL homooligomer, which is then split by ABT-263. In the homooligomer, the TM domain is bound instead of the inhibitor, so the interface areas and thermodynamics are different from the results obtained by PISA. Hence, the crystal orientation might be an indication for a further interface involved in Bcl-xL oligomerization (discussed in chp. 4.2).

The new interface on the side, which is not directly bound to the inhibitor is located between helices $\alpha 4$ and $\alpha 6$ (Fig. 3.17, B). The orientation of these helices is more parallel to each other than in unliganded Bcl-xL. Binding of ABT-263 induces structural changes in helices $\alpha 3$ and $\alpha 4$ (Fig. 3.18, A). Crystal structures of Bcl-xL in complex with BH3 peptides of Bad [77] and Bim [78] showed the same shift of these helices, but the orientation of $\alpha 3$ differs slightly (Fig. 3.18, B). This is in agreement with our data, because the electron density around helix $\alpha 3$ exhibits differences in quality indicating structural destabilization. In
In the crystal, ABT-263 is always buried between two Bcl-xL molecules. A: Interface of two molecules in the crystal of full-length Bcl-xL with ABT-263. One molecule binds ABT-263 in its BH3 ligand binding pocket (raspberry), while another molecule (cyan, blue) interacts with ABT-263 via π-stacking and a hydrogen bond network. Residues P116 to Q121, G138 and R165 are shown as sticks. B: ABT-263 is bound to the hydrophobic pocket of one Bcl-xL molecule. The interface of the other Bcl-xL molecule is located between helices α4 and α6. C: Representation of three Bcl-xL molecules in the asymmetric unit (from left to right: C (blue), G (raspberry), E (cyan)). The inhibitor ABT-263 is buried between two Bcl-xL molecules that are thus oriented in a head-to-tail fashion and form chain-like structures. D: Representation of 12 adjacent unit cells, which reveals that the 12 molecules in the asymmetric unit belong to four different chain-like structures (indicated by colors) in the crystal.

The crystal structure of the domain-swapped dimer [46], helix α3 appears to be completely unstructured (Fig. 2.4). This was also observed in solution NMR structures of the Bak or PUMA BH3 peptide complexes (Fig. 3.18, C) [43, 45].
Figure 3.18: A: The crystal structure of full-length Bcl-xL in complex with ABT-263 (grey) and the crystal structure of Bcl-xL without any ligand (blue, PDB: 1MAZ). B: The crystal structure of full-length Bcl-xL in complex with ABT-263 (grey) and the crystal structures of truncated Bcl-xL in complex with a Bad BH3 peptide (yellow, PDB: 2BZW) and a Bim BH3 peptide (pink, PDB: 1PQ1). C: The NMR structures of truncated Bcl-xL in complex with a Bak BH3 peptide (red, PDB: 1BXL) and PUMA BH3 peptide (green, PDB: 2M04).

3.6. Bcl-xL in LDAO micelles

The X-ray and NMR data of full-length Bcl-xL do not show structural differences to the data obtained for the truncated proteins. The C-terminus and the loop between helices α1 and α2 are flexible. Nevertheless, the C-terminus is thought to be important for membrane insertion and to trigger the insertion of helices α5 and α6 of Bcl-xL [1] (see chapter 2.3). To study structural changes upon membrane association, N,N-dimethyldodecylamine-N-oxide (LDAO) micelles were utilized. Perdeuterated LDAO has been used successfully for the solution of the NMR structure of the integral mitochondrial outer membrane protein VDAC-1 [6]. In this context, its interaction with Bcl-xL in the LDAO micelle was also investigated and the putative membrane-inserted helices α5 and α6 were found to be responsible for binding to the VDAC-1 [5].

As a first test, LDAO was added to purified samples of both Bcl-xLΔLoopΔTM and full-length Bcl-xL in a protein:detergent molar ratio of 1:10. Size exclusion chromatography (SEC) was used to investigate the effect of the detergent micelles on Bcl-xL [Fig. 3.19]. The molecular size of the LDAO micelle itself was determined to be 17 kDa [79] or 21.5 kDa [80]. Both Bcl-xLΔLoopΔTM and full-length Bcl-xL were shown to interact with the LDAO micelles. SEC of the truncated protein with LDAO showed the appearance of two peaks at 276 kDa and 110 kDa. The peak at 276 kDa is probably distorted due to its proximity to the exclusion volume. Interestingly, the intense peak of 41 kDa, corresponding to Bcl-xLΔLoopΔTM in
solution, completely disappeared and the very weak 90 kDa peak might be shifted to 110 kDa due to the size of the LDAO micelle of ≈ 20 kDa. Therefore, it can be assumed that the oligomeric, probably tetrameric, formation, which was present without LDAO only in a very small fraction, seems to be one of the preferred states in the LDAO micelle. The addition of LDAO to the full-length protein showed changes in the chromatogram as well. Here, the peak, which is likely to belong to a tetramer, was increased in intensity and shifted from 121 kDa to 148 kDa, which might be due to the size of the LDAO micelle. Thus, as observed for Bcl-xL∆Loop∆TM, the tetrameric state is more preferred than without LDAO. The large peak at 196 kDa shifted to 225 kDa, which might also be related to the size of the micelle, but concrete statements about the nature of the corresponding oligomer cannot be made, because this peak is so close to the exclusion volume. The peak at 53 kDa, which is thought to be dimeric full-length Bcl-xL, shifts only slightly, to 57 kDa, and is now likely to belong to a Bcl-xL monomer in an LDAO micelle.

Figure 3.19: The addition of LDAO leads to changes in the SEC chromatogram (Superdex 75 10/300). Comparison of full-length Bcl-xL and Bcl-xLΔLoopΔTM without LDAO (blue and green) and upon addition of LDAO (ratio 1:10; purple and teal). The calculated molecular weights are indicated.

Refolding in LDAO leads to the formation of two main peaks for both Bcl-xLΔLoopΔTM and full-length Bcl-xL. For Bcl-xLΔLoopΔTM they are found at 121 kDa and 50 kDa and for full-length Bcl-xL at 138 kDa and 56 kDa. The first peak is likely to belong to a tetrameric
Figure 3.20: Refolding into LDAO micelles leads to defined peaks for both full-length Bcl-xL (red) and Bcl-xL \( \Delta \)Loop\( \Delta \)TM (green). The chromatogram (Superdex 75 10/300) of full-length Bcl-xL without LDAO (blue) is shown as a reference. The calculated molecular weights are indicated.

Bcl-xL in an LDAO micelle, which was also found upon addition of LDAO and - without the micelle - for Bcl-xL and Bcl-xL \( \Delta \)Loop in absence of LDAO (Fig. 3.4). The peaks at 50 kDa and 56 kDa might refer to a monomeric Bcl-xL molecule in an LDAO micelle. For Bcl-xL \( \Delta \)Loop\( \Delta \)TM, with its molecular weight of 18.6 kDa it could also be a dimer in an LDAO micelle. Nevertheless, due to the similar overall appearance of the chromatograms, it was suggested that the two different mutants adopt the same oligomeric states. Interestingly, the large peaks close to the exclusion volume, which were apparent upon the simple addition of LDAO, are not visible, when the proteins are refolded into LDAO. This indicates that Bcl-xL preferably forms smaller oligomeric states, i.e. tetramers or monomers, in LDAO and most likely in the mitochondrial membrane and that the observed higher oligomers might occur due to the head-to-tail oligomerization of full-length Bcl-xL in solution, which was discussed before. Upon the addition of LDAO these homooligomers might simply be included in a micelle without structural rearrangement. Yet, the occurrence of the higher oligomers for Bcl-xL \( \Delta \)Loop\( \Delta \)TM cannot be explained at the moment. The smaller and partially overlapping peaks that are observed around 20-25 kDa in the SEC chromatograms were not characterized.
Figure 3.21: The full-length Bcl-xL monomer (blue) and tetramer (red) in the LDAO micelle can easily be separated by size exclusion chromatography (Superdex 75 10/300). Freezing and thawing does not have an effect on the chromatogram, either.

but might refer to protein fragments or monomers, which were not included in the LDAO micelle. The two main peaks could easily be separated (Fig. 3.21) and investigated. Circular dichroism (CD) spectroscopy was used to determine the potential structural changes of Bcl-xL in the LDAO environment. The mean residue ellipticity $[\theta]_{mrw}$ was calculated for each wavelength and the resulting spectra were uploaded to the DICHROWEB server [81]. Three different algorithms (SELCON3 [82, 83], CONTINLL [84, 85] and K2D [86]) were used to analyze the secondary structure of the CD samples. The results are depicted in Table 3.2. For all samples the CONTINLL algorithm showed the best NRMSD (normalized root mean square deviation) and the best overlap of the calculated spectra with the experimental spectra. Thus, the resulting values are likely to be most relevant. SELCON3 showed a similar secondary structure content. Both algorithms distinguish between regular and distorted helices and sheets. It is assumed that four residues at the termini of an \( \alpha \)-helical segment and two residues per \( \beta \)-strand are distorted, i.e. they deviate from the ideal conformational backbone dihedral angles \( \phi \) and \( \psi \) [83]. The K2D algorithm does not make this difference. Moreover, it tends to calculate a larger \( \alpha \)-helical content than SELCON3 and CONTINLL. As a reference, the structurally well-characterized Bcl-xL Loop\( \Delta \)TM was used (Fig. 3.22, blue). The first crystal structure of Bcl-xL (PDB: 1MAZ) [36] exhibits an \( \alpha \)-helical content of 66 % and no \( \beta \)-sheets. Here, the CD spectrum also shows the minima at 208 nm and 222 nm, which are characteristic for a high \( \alpha \)-helical content [87]. SELCON3 and CONTINLL
### Table 3.2: Analysis of the secondary structure content of different Bcl-xL mutants in the absence and presence of LDAO by CD spectroscopy. Values are given in %.

<table>
<thead>
<tr>
<th>Method</th>
<th>Bcl-xL ΔLoopΔTM in solution</th>
<th>Bcl-xL ΔLoopΔTM monomer in LDAO</th>
<th>Bcl-xL ΔLoop dimer</th>
<th>Bcl-xL ΔLoop tetramer</th>
<th>full-length Bcl-xL monomer in LDAO</th>
<th>full-length Bcl-xL tetramer in LDAO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Helix regular</td>
<td>Helix distorted</td>
<td>Strand regular</td>
<td>Strand distorted</td>
<td>Turns</td>
<td>Other</td>
</tr>
<tr>
<td>SELCON3</td>
<td>38.9</td>
<td>14.6</td>
<td>6.0</td>
<td>4.7</td>
<td>12.6</td>
<td>23.9</td>
</tr>
<tr>
<td>CONTINLL</td>
<td>38.8</td>
<td>14.7</td>
<td>6.2</td>
<td>4.5</td>
<td>10.9</td>
<td>24.9</td>
</tr>
<tr>
<td>K 2D</td>
<td>61</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SELCON3</td>
<td>36.7</td>
<td>18.5</td>
<td>3.7</td>
<td>3.8</td>
<td>12.6</td>
<td>23.1</td>
</tr>
<tr>
<td>CONTINLL</td>
<td>35.1</td>
<td>19.5</td>
<td>3.2</td>
<td>4.4</td>
<td>15.1</td>
<td>22.6</td>
</tr>
<tr>
<td>K 2D</td>
<td>60</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SELCON3</td>
<td>29.3</td>
<td>14.3</td>
<td>8.0</td>
<td>6.0</td>
<td>14.4</td>
<td>26.6</td>
</tr>
<tr>
<td>CONTINLL</td>
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<td>13.6</td>
<td>7.9</td>
<td>5.6</td>
<td>15.0</td>
<td>27.3</td>
</tr>
<tr>
<td>K 2D</td>
<td>59</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SELCON3</td>
<td>6.4</td>
<td>5.5</td>
<td>20.8</td>
<td>8.4</td>
<td>21.1</td>
<td>33.2</td>
</tr>
<tr>
<td>CONTINLL</td>
<td>9.5</td>
<td>10.5</td>
<td>16.1</td>
<td>9.9</td>
<td>21.1</td>
<td>32.8</td>
</tr>
<tr>
<td>K 2D</td>
<td>23</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: Values are given in %.*
determined it to be 53.5 %, so that in this case the value calculated by the K2D algorithm (61 %) was closer to the real value. However, all methods also recognize a rather small β-strand fraction, which is not apparent in the structure. The Bcl-xL ΔLoopΔTM monomer shows a similar CD spectrum in LDAO (Fig. 3.22, red) and therefore leads to similar results, so that it can be assumed that the LDAO micelle does not have a vast effect on the overall Bcl-xL structure.

In the next step, Bcl-xL ΔLoop was investigated. So all structural changes can be accounted for by the C-terminal helix and the resulting oligomerization. CD spectra of Bcl-xL ΔLoop dimers and tetramers, which had been separated by SEC, were obtained. Interestingly, the CD spectra of both oligomeric species differ (Fig. 3.23). While the spectrum of the dimer reveals similar characteristics as the spectra of Bcl-xL ΔLoopΔTM, the spectrum of the tetramer shows an overall lower mean residue ellipticity and the minimum at 222 nm is not visible. The secondary structure analysis of the dimer indicates an α-helical content of ≈ 43 % (SELCON3 and CONTINLL) or 59 % (K2D). The fit calculated with K2D showed only a poor overlap with the experimental spectrum, so this result has to be treated carefully. The tetramer shows a much lower helical content of about 20 % and a rather high strand content between 21 and 27 % (CONTINLL and K2D). The result from SELCON3 indicates an even
lower α-helical content of 12 % and a strand content of about 30 %. More important, the amount of turns and unstructured elements is increased to \( \approx 54 \% \). The NMRSD of 0.186 is rather high compared to those of the other methods (0.074 for CONTIN and 0.066 for K2D), so here the SELCON3 algorithm is not recommended. Taken together, these results indicate, that the Bcl-xL ΔLoop tetramer has another structure containing less structural content than the Bcl-xL ΔLoop dimer.

Finally, full-length Bcl-xL was investigated in an LDAO environment. Here the two prominent peaks observed in the SEC chromatogram, which are thought to belong to a monomer and a tetramer included in an LDAO micelle, were measured. The spectra (Fig. 3.24) both show the minima at 208 nm and 222 nm characteristic for an α-helical content. However, the secondary structure analysis also shows a decrease of helices and a slight increase of sheets compared to the reference Bcl-xL ΔLoopΔTM. The CONTINLL method indicates an α-helical content of 28 % for the monomer and 41 % for the tetramer and a sheet content of 21 % and 14 %, respectively. SELCON3 and K2D lead to similar results. The monomer shows a higher amount of turns and unstructured elements (\( \approx 51 \% \)) than the tetramer (\( \approx 44 \% \)). Thus, the full-length Bcl-xL tetramer in LDAO appears to have an increased secondary structure content compared to the monomer, which is in contrast to the results for the Bcl-xL ΔLoop dimer.
tetramer in solution. As a consequence, it can be suggested that the tetramer is structurally stabilized in LDAO, which is in consensus with the observation of the increased tetramer peaks in LDAO (Fig. 3.19 and 3.20). The structural difference between the full-length Bcl-xL monomer and tetramer in LDAO could be explained by the emergence of other structural elements through monomer interaction and the related environment change.

NMR spectroscopy of proteins in LDAO micelles is demanding due to the large molecular weight. TROSY-HSQC spectra were obtained for the two oligomeric states (tetramer and monomer) of both full-length Bcl-xL and Bcl-xL ΔLoopΔTM in LDAO. Neither of the spectra of Bcl-xL ΔLoopΔTM in LDAO shows any detectable NMR signals. Full-length Bcl-xL, however, shows signals in LDAO (Fig. 3.25, maroon) that differ dramatically from those obtained in NMR buffer. Nevertheless, few signals (especially around 8.3 ppm) can be recognized by comparison with the spectrum of full-length Bcl-xL in NMR buffer (Fig. 3.25, teal). These cannot be found in the spectrum of Bcl-xL ΔLoopΔTM in NMR buffer and can thus be suggested to belong to the long unstructured loop. Yet, the number of signals from the loop that can be identified in the spectrum of full-length Bcl-xL in LDAO is very low. Most signals in the LDAO spectrum cannot be identified in any of the Bcl-xL spectra obtained during this work. The depicted spectrum belongs to monomeric full-length Bcl-xL in LDAO, which was
acquired with 960 scans. Tetrameric full-length Bcl-x\textsubscript{L} in LDAO was also investigated and showed the same, but much weaker peaks, which were very close to the noise level. This was basically due to the drastically lower number of scans (64). Interestingly, the addition of ABT-263 to full-length Bcl-x\textsubscript{L} in LDAO does not lead to any signal changes. Taken together, these data support the assumption that Bcl-x\textsubscript{L} can insert into an LDAO micelle as a model for the mitochondrial membrane and that it rearranges structurally upon insertion. It is not able to bind to BH3 ligands anymore, so the basic core structure including the hydrophobic ligand binding pocket is lost.

\textbf{Figure 3.25}: $^1\text{H}-^{15}\text{N}$-TROSY-HSQC spectra of full-length Bcl-x\textsubscript{L} (monomer) refolded in LDAO micelles (maroon) and of the full-length Bcl-x\textsubscript{L} homooligomers without LDAO (teal).
4 | Discussion

4.1. The different Bcl-x constructs

Bcl-x\textsubscript{L} is located both in the cytosol and at the mitochondrial membrane [2]. Upon insertion into the mitochondrial membrane Bcl-x\textsubscript{L} is suggested to undergo structural changes [1]. However, it is unclear how BH3 ligands are supposed to bind, as the hydrophobic pocket is likely to get lost upon membrane insertion. To address this question, structural studies on full-length proteins are important.

Bcl-x\textsubscript{L}\textsubscript{ΔLoop ΔTM} and Bcl-x\textsubscript{L}\textsubscript{ΔTM} are structurally well-characterized monomeric proteins. They can be easily purified using an N-terminal GST-tag. First attempts to purify full-length Bcl-x\textsubscript{L} in the same way were not successful due to the formation of inclusion bodies, which could not be refolded easily [70]. The Bax protein, which has a high structural similarity to Bcl-x\textsubscript{L}, was purified using a C-terminal intein/CBD-tag [37]. Full-length Bcl-x\textsubscript{L} was also cloned into this expression system, but the protein yield was rather low [70]. Therefore, during this work a combination of N-terminal GST-tag and C-terminal intein/CBD-tag was used. This novel strategy results in high protein concentrations that can be used for structural studies. While the GST-tag is removed by thrombin-cleavage, the intein/CBD-tag is cleaved off by 5 mM DTT, which is present in all buffers during the purification. Thereby, no additional amino acids are present at the C-terminus, which is important to investigate the homooligomerization mediated by the C-terminal TM. The same purification strategy was used for Bcl-x\textsubscript{L}\textsubscript{ΔLoop}.

Both full-length Bcl-x\textsubscript{L} and Bcl-x\textsubscript{L}\textsubscript{ΔLoop} form homooligomers of varying size, which was shown by SEC. Similar results were reported previously [3]. The TM appears to be responsible for this oligomerization by binding into the ligand binding pocket of another Bcl-x\textsubscript{L} molecule. SEC results show distinct peaks that are similar for both full-length Bcl-x\textsubscript{L} and Bcl-x\textsubscript{L}\textsubscript{ΔLoop} oligomers. Thus, the oligomerization appears to be nonarbitrary. This is further confirmed by the surprising fact that the different forms of oligomers can be separated from each other and therefore do not exist in a reversible equilibrium as was previously reported.
CHAPTER 4. DISCUSSION

[3]. The fact that full-length Bcl-xL did not crystallize without ABT-263 is in consensus with the finding that it adopts different homooligomeric states, because inhomogeneous protein samples do not form crystals easily. It might be possible to perform a crystallization screen with one of the separated oligomeric species. The concrete determination of the oligomer size is difficult by SEC, because Bcl-xL ΔLoopΔTM, which is well-characterized and known to be a monomer, runs as a dimer on the SEC column.

The flexible loop between helices α1 and α2 that was omitted in Bcl-xL ΔLoop is not involved in the oligomerization process. The chromatogram shows the same characteristic signals as full-length Bcl-xL. Furthermore, the loop is the only region, which is visible in the NMR spectrum of full-length Bcl-xL. This can be clearly shown by the overlap of the spectrum of full-length Bcl-xL and the spectra of the truncation mutants Bcl-xL ΔLoopΔTM and Bcl-xL ΔTM. Thus, the absence or presence of the TM is sufficient to influence the oligomerization state of the protein.

4.2. Implications for the structure of the Bcl-xL oligomer

Bcl-xL forms homooligomers in solution that largely prevent structural investigations by NMR spectroscopy or X-Ray crystallography. The NMR spectra show severe signal loss due to the molecular mass of the oligomer and only resonances assigned to the flexible loop region were detected. The crystallization screen of Bcl-xL without inhibitor was not successful to obtain any structural data of the oligomer, either. However, the obtained crystal structure of the full-length Bcl-xL monomer in complex with ABT-263 supports the hypothesis that the helical core structure with the ligand binding pocket is retained and accessible to high-affinity BH3 ligands, as was suggested earlier [3]. Hence, the full-length Bcl-xL oligomer is not an unspecific aggregate and it does not adopt a completely new fold. Moreover, domain-swapping as reported by O’Neill et al. [46] was not observed. Two models for Bcl-xL oligomerization modulated by the C-terminal TM have been described recently. One involves the formation of dimers - binding each other’s TM in the hydrophobic BH3 ligand binding pocket - that assemble into an oligomer by further surface interactions [3]. Another describes a 'head-to-tail' or 'run-away' mechanism, in which a chain of Bcl-xL molecules is formed by binding the TM of one Bcl-xL molecule in the BH3 ligand binding pocket of another Bcl-xL, which is bound to yet another Bcl-xL molecule and so on [52].

For both models, there has to exist an interaction surface distinct from the BH3 ligand binding pocket, which remains elusive to date. Importantly, in the crystal of the Bcl-xL-ABT-263 complex, Bcl-xL forms a chain-like arrangement in a head-to-tail fashion. Yet, the molecules in the crystal are monomeric, because their interfaces are too small to be
relevant as determined by PISA [76]. Moreover, NMR spectroscopy indicates that the large Bcl-xL oligomers are split by ABT-263. Nevertheless, the position of the interface found in the crystal (helices α4 and α6) is quite reasonable for being the other interaction surface involved in oligomerization. Helix α4 is modified upon ligand binding and probably upon binding of the TM. This might act as an allosteric switch leading to an ordered and specific oligomerization: Upon binding of the TM, helix α4 becomes more parallel to helix α6 leading to a new surface, which would then be exposed for directed oligomerization. Figure 4.1 shows the surface between helices α4 and α6 and its changes upon ligand binding. The free form of the protein (A) shows helix α4 sticking out from the surface. Upon ligand binding (B) this helix tilts towards the protein core and new cavities for binding are created on the protein surface. At the same time, the sequence of the TM, which is likely to adopt a helical fold [3], would have to be selective for binding to the BH3 ligand binding pocket on the one side and on the other side to the cleft between helices α4 and α6. This has to be proven, though. However, the sequence of the TM helix is rather hydrophobic and not amphipathic (see helical wheel representation in Fig. 3.12). This is an indication that it binds to another protein and that it is not exposed to the solvent. Consequently, these data can serve as a blueprint for future experiments to elucidate the structure of the full-length Bcl-xL oligomer in solution.
4.3. The consequences of peptide / inhibitor binding

Full-length Bcl-x\textsubscript{L} is split by ABT-263, but not by BH3 peptides as shown by NMR spectroscopy. The very high affinity of the inhibitor (IC\textsubscript{50} ≤ 1 nM) is likely to be the most important reason for this. However, in the cell, other Bcl-2 proteins are supposed to bind to Bcl-x\textsubscript{L} via their BH3 domain. Thus, either there are no oligomers in the cell or the affinity has to be increased by other factors, such as the presence of a membrane or interaction partners, which bind to a region of Bcl-x\textsubscript{L} still accessible in the oligomer. It can be also suggested that the concentrations in the cell are not as immense as in the NMR sample tube. Thus, it is possible that Bcl-x\textsubscript{L} exists mainly as a dimer and not as a higher order oligomer in the cell. This was also observed by Jeong \textit{et al.} [49].

The crystal structure of truncated Bcl-x\textsubscript{L} in complex with ABT-737 was already reported and discussed with respect to its similarity to the complex with the Bim BH3 peptide [4]. In the following years the related inhibitor ABT-263 was designed, clinical studies were performed and thrombocytopenia was found to be a major side effect. Now, we can interpret the crystal structure of full-length Bcl-x\textsubscript{L} in complex with the pharmacologically relevant ABT-263. Assuming that Bcl-x\textsubscript{L} exists as a dimer or oligomer in the cytosol, it can be split into monomers by BH3-only proteins like Bad upon apoptotic stimuli and translocates to the mitochondrial membrane [49]. There it can sequester Bak, which is located at the mitochondrial membrane [20] and has a higher affinity for Bcl-x\textsubscript{L} than Bad [60]. In the cell, ABT-263 splits the homooligomers as well, but it cannot be replaced by Bak due to its high affinity to Bcl-x\textsubscript{L}. Thus Bak-mediated apoptosis is not restrained in ABT-263-treated cells, which would be important for platelet survival [66]. This leads to the major side effect observed in clinical trials, which is thrombocytopenia [64, 65].

Moreover, Bax retrotranslocation from the mitochondria to the cytosol was shown to be hampered by the related inhibitor ABT-737. More important, the inhibitor increases the retrotranslocation of Bcl-x\textsubscript{L} itself, for yet unknown reasons [51]. ABT-263 can be assumed to have the same effect due to its similarity. Especially when compared to the complex structure with the Bad BH3 peptide [60] (Fig. 3.18, B) that is thought to translocate Bcl-x\textsubscript{L} to the membrane [49], there is no significant structural difference that could explain this contradictory behavior. The only slight structural changes are observed in helix \(\alpha3\). The remaining unknown quantity here is the membrane and the related potential structural changes. Bcl-x\textsubscript{L} is thought to undergo conformational changes upon mitochondrial membrane translocation due to the insertion of helices \(\alpha5\) and \(\alpha6\) [1]. Because the TM is important for membrane translocation [50], it can be suggested that the binding or insertion of this promotes the integration of helices \(\alpha5\) and \(\alpha6\). Obviously, this is not possible when bound tightly to ABT-263.
or -737, because the ligand binding pocket would have to be disrupted. This might be one reason for Bcl-xL retrotranslocation. Yet, also smaller structural changes of the protein core occurring upon insertion of the TM might be prevented by the bound inhibitor, which thereby hindered membrane translocation.

The structural changes of Bcl-xL occurring at the membrane and the inability to bind to ABT-263 or -737 in this stage is further supported by results of Llambi et al. [26], although they related their results neither to the structural biology of the proteins nor to the knowledge on affinities at the membrane. They investigated the effect of the addition of the inhibitor to cells overexpressing Bcl-xL and GFP-tBid. The amount of tBid could be measured by the GFP intensity. Moreover, the onset of MOMP was monitored by live-cell imaging. The correlation between the amount of tBid and MOMP was surprising, because cells that expressed lower levels of tBid occurred to induce MOMP faster than cells that expressed higher tBid concentrations. It was argued that at low tBid concentrations MODE 2 inhibition (i.e. the binding of Bcl-xL to effectors like Bax and Bak) is more prominent than MODE 1 inhibition (i.e. the binding of Bcl-xL to direct activators like tBid), and this relation is reverted at high tBid concentrations. Llambi et al. concluded that MODE 1 inhibition is more easily derepressed than MODE 2. However, taking into account the knowledge on binding affinities of Bcl-xL and tBid, another explanation for this correlation between tBid concentration and MOMP onset can be found. tBid interacts with Bcl-xL predominantly at the membrane [29], thus probably a large amount of Bcl-xL is located at the membrane at high tBid concentrations. The structural changes of Bcl-xL occurring at the membrane are likely to prevent its derepression by ABT-737. Therefore, MOMP occurs more easily. At low tBid concentrations there is still a lot of Bcl-xL in the cytosol, which is accessible to ABT-737.

4.4. Bcl-xL in membraneous systems

Bcl-xL changes its structural conformation upon membrane insertion. LDAO micelles were employed as a membrane model system. They were also used for the structural characterization of the VDAC-1 [6], VDAC-2 [88] and to study the interaction of VDAC-1 with a loop and TM deletion mutant of Bcl-xL [5]. In this context, NMR spectra of the truncated Bcl-xL in LDAO micelles were also obtained and about 60% of the resonances could be assigned [5]. Furthermore, the protein was investigated in DPC micelles. Here, more than 90% of the backbone resonances could be assigned [57]. In both works, deuterated protein samples and detergents were used. During this work no signals could be observed in the $^{1}$H-$^{15}$N-HSQC spectrum of Bcl-xLΔLoopΔTM. The reason is probably that the sample was not deuterated and no deuterated LDAO was used. Deuteration helps to prevent signal loss caused by the
large size of the protein-micelle-complex. One additional reason might be the rather low concentration of the sample occurring because of the long purification procedure.

In contrast, full-length Bcl-xL showed signals in the $^{1}$H-$^{15}$N-HSQC spectrum. These signals are not similar to any spectrum of Bcl-xL in micelles from the literature. The reported spectra all show a narrow distribution of NMR signals between 7 ppm and 9 ppm. Most signals overlap in the region around 8 ppm. Only few residues of the loop were included in these constructs and those were shown to exhibit a better amide chemical shift resolution than the rest of the protein [57]. The high signal dispersion, the number of signals and the fact that no signals were observed for Bcl-xL∆Loop∆TM in LDAO in this work indicate that the signals in the spectrum of full-length Bcl-xL in LDAO might refer to the flexible loop between helices α1 and α2, which was largely omitted in the reported studies. However, the characteristic tryptophan residue from the loop, which could be recognized easily in the spectrum of full-length Bcl-xL in solution is not visible - at least not at the same position - in the LDAO micelle spectrum. Furthermore, because of the high signal dispersion, the loop, which is unstructured in solution, appears to contain secondary structure elements in the micelle environment. The signals downfield of 9 ppm might indicate β-sheet structures, which would be in consensus with the increased β-sheet content calculated from the CD spectra.

Upon refolding of Bcl-xL, two main peaks (corresponding to a tetramer and a monomer in LDAO) were observed on the SEC column for both Bcl-xL∆Loop∆TM and full-length Bcl-xL. In contrast, Malia et al. found truncated Bcl-xL only to be monomeric in the LDAO micelle. Yet, they did not refold the protein into the micelle, but simply added LDAO [5]. This was also tried in this work, but resulted in the formation of higher oligomers of Bcl-xL∆Loop∆TM only. In contrast, full-length Bcl-xL was also found as a monomer upon LDAO addition.

CD spectra of truncated Bcl-xL, missing both the C-terminus and the long loop, displayed an increase of the α-helical content in the DPC micelles compared to that in solution [57]. However, the here obtained CD spectra of Bcl-xL∆Loop∆TM showed a high α-helical amount, which was comparable in buffer and in the LDAO micelle. Full-length Bcl-xL, however, has a decreased α-helical portion in the LDAO environment.

Taken together, all these data indicate a conformational change of full-length Bcl-xL in the LDAO micelle. The tetrameric state, which is also apparent in solution, seems to be advantaged in this system. Compared to the structurally well-characterized monomeric Bcl-xL∆Loop∆TM in solution, the α-helical amount is decreased and the β-sheet amount is increased. The 2D NMR spectrum of monomeric full-length Bcl-xL in LDAO supports the assumption that the structure is changed dramatically.
This work presents the successful purification as well as first NMR and X-Ray data of full-length Bcl-xL, and in addition, first spectroscopic data of full-length Bcl-xL in LDAO micelles. The availability of recombinant full-length Bcl-xL at a larger scale is a prerequisite for obtaining structural data of the membrane bound form of the protein. The C-terminus omitted in most studies is thought to be a transmembrane domain, which is crucial for membrane translocation and insertion. Yet, proteins including this domain are difficult to purify. This limitation was overcome by using an innovative vector containing an N-terminal GST and a C-terminal intein tag. Using this purification strategy, soluble homooligomers are obtained, which can be split by the inhibitor ABT-263 binding to the hydrophobic ligand binding pocket on the protein surface. The crystal structure of monomeric full-length Bcl-xL in complex with ABT-263 was solved and it confirms that the Bcl-xL oligomer has basically retained the core structure of truncated Bcl-xL. Moreover, the protein forms chain-like structures in the crystal, which might give clues on the orientation in the homooligomers in solution. Thus, the data might also serve as a template for the design of future experiments to elucidate the oligomer structure. The BH3 ligand binding pocket is likely to be occupied by its C-terminus, which is freed upon binding of BH3 ligands. Yet, the addition of BH3 peptides derived from Bak and Bid were not able to split the full-length Bcl-xL homooligomers as shown by NMR titration experiments, although these peptides interact with Bcl-xLΔLoopΔTM. The addition of a 'helix' peptide, resembling the C-terminal TM of Bcl-xL to the truncated protein did not induce significant signal changes.

Refolding of both Bcl-xLΔLoopΔTM and full-length Bcl-xL into LDAO micelles leads to the formation of two different membrane-protein complexes. CD spectroscopy emphasizes, although decreased, mainly α-helical structural elements and a slightly increased β-sheet content compared to Bcl-xLΔLoopΔTM in solution. Moreover, the 1H-15N-HSQC spectrum shows other signals than those of all investigated Bcl-xL mutants in solution and thereby indicates severe structural differences of the membrane-embedded protein. Future experiments using deuterated proteins and deuterated LDAO might help to investigate Bcl-xL in the mem-
brane environment by NMR spectroscopy. Having established the experimental setup for the NMR studies of Bcl-xL in LDAO micelles, interaction studies would become possible. These might help to explain open questions, e.g. how tBid interacts with Bcl-xL at the membrane, when the ligand binding pocket is destroyed due to the structural rearrangement.
Part II.

CupS
6 | Introduction

6.1. The cyanobacterial NDH-1 complex

The cyanobacterial NAD(P)H dehydrogenase type 1 (NDH-1) complex is located at the thylakoid membrane and probably at the plasma membrane. It is structurally and functionally very similar to the Complex I of the respiratory chain. However, while Complex I is only involved in respiration, the NDH-1 complex of cyanobacteria is also responsible for cyclic electron flow and CO₂ uptake [89]. Complex I is the first enzyme of the respiratory chain and catalyzes the transfer of two electrons from NADH to ubiquinone via flavin mononucleotide (FMN) and nine Fe-S clusters. At the same time it translocates four protons across the membrane, which contributes to the proton gradient needed for ATP synthesis [90, 91]. 11 out of the 14 subunits of Complex I from *E. coli* (NuoA-NuoN) or of the 15 subunits of Complex I from *Thermus thermophilus* (Nqo1-Nqo15) are homologous to the subunits of the cyanobacterial NDH-1 complex (NdhA-NdhK). Yet, the other three subunits (NuoE, NuoF and NuoG or Nqo1, Nqo2, Nqo3, respectively), for which no homologues exist in the NDH-1 complex, contain the NADH and FMN binding sites as well as six of the nine Fe-S clusters and are therefore important for the enzymatic function of Complex I [92].

During the last years, different NDH-1 complexes with distinct functions were identified. Depending on their size, when they were first discovered in the cyanobacterium *Synechocystis* sp. PCC 6803, they were termed NDH-1L (large), NDH-1M (medium) and NDH-1S (small) [93]. Later, studies of the cyanobacterium *Thermosynechococcus elongatus* showed that NDH-1M and NDH-1S form the complex NDH-1MS in vivo, which is rather fragile in the presence of detergents used in most studies. Thus, NDH-1M and NDH-1S are no entire functional NDH-1 complexes, but subcomplexes thereof [94]. NDH-1M is a common element, which is also the major part of the NDH-1L complex. It contains most of the subunits homologous to Complex I (all except for NdhD and NdhF) and four subunits NdhL-NdhO, which form the oxygenic photosynthesis-specific (OPS) domain [89]. The genes for the subunits NdhD and NdhF are found in several copies in cyanobacteria and the products were
Figure 6.1: Functionally distinct NDH-1 complexes. The subcomplex NDH-1M contains a hydrophilic domain (purple), a membrane core domain (yellow) and the oxygenic photosynthesis-specific (OPS) domain (green). The NdhD and NdhF subunits are colored in grey (NdhD1,NdhF1), blue (NdhD2), red (NdhD3, NdhF3) and brown (NdhD4, NdhF4) to emphasize their different variants. The subunits CupA, CupS (red) and CupB (brown) are characteristic for the NDH-1MS and NDH-1MS' complex, respectively. The question mark indicates a possible unknown activity domain (Act-NDH). ([89], modified according to [96])

shown to exert diverse functions [95]. Consequently, the different variants of NdhD and NdhF form functionally distinct complexes with NDH-1M (Fig. 6.1): NDH-1L and NDH-1L', which are responsible for respiration and NDH-1MS and NDH-1MS', thought to be involved in CO₂ uptake. NDH-1MS and NDH-1MS' additionally contain the subunits CupA, CupS and CupB. All four complexes were shown to be involved in cyclic electron flow [96].

6.2. The subunits of the NDH-1 complex and their functions

The subunits in the NDH-1 complex that resemble those of respiratory Complex I are believed to function similarly. There have been suggestions that they use electrons of NADPH as a substrate instead of NADH and plastoquinone as an electron acceptor instead of ubiquinone [97]. However, the subunits responsible for electron uptake could not be found in any of the complexes isolated so far (indicated by question mark in Fig. 6.1).
The NDH-1 complexes contain more than the subunits, which are homologous to the respiratory Complex I. Four additional subunits (NdhL-NdhO) are included in the NDH-1M complex, which are correlated with the ability to perform oxygenic photosynthesis [89]. Moreover, two novel subunits (NdhP and NdhQ) were recently discovered in the NDH-1L complex [98]. Another subunit, NdhS, was found in both the NDH-1L and NDH-1MS complex [99]. The NDH-1MS complex further comprises the proteins CupA and CupS, which are replaced by CupB in the NDH-1MS' complex. CupB is important for constitutive CO\textsubscript{2} uptake, while CupA is involved in high-affinity CO\textsubscript{2} uptake at low CO\textsubscript{2} concentrations [100]. The physiological role of CupS, however, still remains unknown. No changes in phenotype could be observed in a deletion mutant of Synechococcus sp. PCC7002 [101]. The CupS protein contains 133 amino acids in Synechocystis sp. PCC 6803 (Sll1734, Uniprot: P73392) and 149 amino acids in T. elongatus (Tll0220, Uniprot: Q8DMA1). It is homologous to the secreted bacterial protein MPB70 [93] and shows sequence similarity to the Fasciclin family of proteins, which is involved in cell adhesion. These are extracellular proteins and thus cannot directly be related to CupS and its function.

6.3. The structure of the NDH-1 complex

To gain insight into the concrete functions and reaction mechanisms of the NDH-1 subunits, structural information is necessary. Electron microscopy studies revealed an L-shaped structure for the NDH-1L complex and a U-shaped structure for the NDH-1MS complex (Fig. 6.2) [102, 103]. The difference is caused by the additional CupA and CupS proteins, which are attached to the subunits NdhD3 and NdhF3. The CupS subunit is too small to be distinguished, but could be located next to CupA at the tip of the membrane arm by the use of a YFP-fusion system [104].

Figure 6.2 The NDH-1L complex is L-shaped (A), while the NDH-1MS complex is U-shaped due to the CupA protein that is attached to the subunits NdhD3 and NdhF3. The CupS subunit cannot be distinguished. [103]
The crystal structures of the partly homologous respiratory Complex I from *E. coli* [105] and *T. thermophilus* [106] give further indications for the NDH-1 complex structure and also for the subunits. Complex I consists of a hydrophilic cytoplasmic domain and a membrane domain, which are arranged in an L-shaped manner. The ubiquinone binding site was located close to the conjunction of these two domains. Electrons are transferred from NADH to a non-covalently bound FMN and then along a chain of Fe-S clusters in the cytoplasmic domain to the bound ubiquinone [106]. A similar mechanism can be assumed for the cyanobacterial NDH-1 complex and plastoquinone, although an electron acceptors such as FMN is unknown at the moment and a dehydrogenase activity of NADH or NADPH has not been detected so far [89]. The subunits NuoL-N (*E. coli*) or Nqo12-14 (*T. thermophilus*) of Complex I and the homologous NDH-1 complex subunits NdhB, NdhD and NdhF are antiporter-like subunits, which are likely to be involved in proton or ion transport [107]. The structural changes introduced by the electron transfer in the cytoplasmic domain are coupled to these antiporters and believed to introduce the necessary changes for the proton transfer. The fourth proton is suggested to be translocated at the interface of the cytoplasmic and the membrane domain [108](Fig. 6.3). However, this model is incomplete due to the missing unknown electron donor and the missing CupA, CupS and CupB subunits. The latter is involved in carbon uptake, but the related mechanism remains unclear. Consequently, to understand the function of the NDH-1 complexes, which differs from that of Complex I, structural data on the NDH-1 complexes themselves are necessary. Especially interesting are the structures of the subunits that are not homologous to any respiratory Complex I subunits (e.g. CupS).
Figure 6.3: Hypothetical model for the electron and proton transfer of the cyanobacterial NDH-1 complex including the common element NDH-1M (colors as in Fig. 6.1) and unspecified subunits NdhD and NdhF (white). The electrons are transferred from an unknown donor via Fe-S clusters (red-yellow spheres) in the NdhI and NdhK subunits to plastoquinone (PQ). The PQ binding site is located at the conjunction between the cytoplasmic and the membrane domain (green dashed line). The electron transfer leads to structural changes in the cytoplasmic domain, which are coupled to the antiporters NdhB, NdhD and NdhF (transmission chain in red). The C-terminal helix of NdhF is important for this crosstalk. A fourth proton is likely to be translocated at the interface of the two main domains. ([89], according to [108])
Results

7.1. Backbone and sidechain assignment of CupS

CupS from *Thermosynechococcus elongatus* was cloned, expressed and purified by Hannes Wulfhorst (Group Cyanobacterial Membrane Protein Complexes, Plant Biochemistry, RUB) [109]. The 149 amino acid protein is connected to a C-terminal Strep-tag via a two amino acid linker (Fig. 7.1). The obtained chemical shift assignments for CupS including the tag and linker are almost complete (Tbl. 7.1). The spectra show a good signal dispersion, although there are also some rather crowded regions as can be seen in the $^1$H-$^15$N-HSQC spectrum (Fig. 7.2). Here only few residues (M1, L17, D51 and G52) could not be assigned. The sidechain resonances of all proline residues were found. Alanine 79 showed an unusual amide proton chemical shift of 11.667 ppm. It appears in a region typical for tryptophan sidechain amides. However, A79 was unambiguously assigned to its resonances using the triple resonance spectra HNCA CB and CBCA(CO)NH (Fig. 7.3). The 3D $^1$H-$^15$N-NOESY confirmed the residue assignment. The chemical shifts were deposited in the BioMagResBank under accession number 19971.

The very high backbone assignment level was achieved by a combination of the HNCA CB and CBCA(CO)NH spectra. Extreme signal overlaps in the $^1$H-$^15$N-HSQC spectrum, e.g. of Y77 and V111, could be identified in the HNCA CB spectrum (Fig. 7.3). Due to the chemical shift differences of tyrosine and valine carbon atoms and because of the connectivity to other signals, the amino acids could be assigned unambiguously. The C$\alpha$ and C$\beta$ resonances were directly correlated to the signals in the CBCA(CO)NH spectrum. Especially the C$\beta$ resonances

**Figure 7.1:** Amino acid sequence of the CupS-Linker-StreplI construct.
CHAPTER 7. RESULTS

Figure 7.2 2D $^1$H-$^1$5N-HSQC spectrum of CupS from *Thermosynechococcus elongatus*. The central region (red) and the side chain amide region (blue) are expanded in B and C, respectively.
Figure 7.3. Strips of the HNCA CB spectrum emphasizing the route for the backbone assignment of residue A79. The corresponding signals in the different amide planes are connected by dashed lines (blue for $C_\beta$ and green for $C_\alpha$). The $C_\alpha$ resonances of Y77-A79 and D81 are very close to each other. The $C_\beta$ resonances help to assign the signals unambiguously.
Table 7.1: Assignment completeness for the CupS-Linker-Strepl sequence.

<table>
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<th>Type</th>
<th>% assigned</th>
<th>Type</th>
<th>% assigned</th>
<th>Type</th>
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</tr>
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<td>Cζ</td>
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<td>Nζ</td>
<td>0</td>
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</tbody>
</table>

are of great value, because they help to assign the amino acid type. Based on the backbone assignment, most Hα and Hβ resonances could be assigned by use of the HBHA(CO)NH spectrum. These were then utilized to assign the signals in the H13C-HSQC spectrum and the correlating diagonal peaks in the 13C-edited NOESY spectrum and in the HCCH-TOCSY spectrum. The latter exhibited a large signal dispersion and a good signal-to-noise ratio. Thus, it was largely used to assign the side chain resonances (for assignment details see chapter 11.8).

7.2. Solution structure of CupS

The CupS structure family was determined using 2089 distance restraints (929 intra-residual, 448 sequential, 281 medium-range, and 431 long-range) derived from the 15N-edited NOESY spectrum and the aliphatic 13C-edited NOESY spectrum. Moreover, 221 dihedral angle restraints were used, which were derived from the Cα and Cβ chemical shifts using TALOS+ [110]. The dihedral angles of Lys107 were added manually to minimize the variation of these values and improve the quality of the ramachandran statistics. The amino acid residues 130-159 were omitted from the structure calculation, because they showed only intraresidual NOEs and first calculations including the C-terminal residues indicated their structural flexibility. The overall protein fold was found by using UNIO/CYANA [111, 112] without intraresidual and sequential restraints. The resulting assigned peaklists were then used for structure calculation with ARIA/CNS [113, 114, 115]. Additionally, the complete peak list including all distance restraints was used. After each structure calculation run, the restraints were sorted and major violations were removed. ARIA uses a final step of water refinement, which led to a structure family with very good RMSD (root-mean-square deviation) values and ramachandran statistics as indicated by the iCING validation tool [116] (Tbl. 7.2).

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Figure 7.4: Cartoon representation of the NMR solution structure of CupS. A: The energetically most favored structure (0 noe viol. >0.5 Å, 2 dihedral viol. > 5°). B: The structure with the lowest violation values (0 noe viol. >0.5 Å, 1 dihedral viol. > 5°)

The protein CupS consists of seven α-helices (α1 4-10, α2 16-24, α3 26-31, α4 43-51, α5 53-59, α6 62-70, α7 79-85) and seven β-sheets (β1 36-41, β2 72-74, β3 87-90, β4 93-100, β5 104-106, β6 112-117, β7 122-126). Therefore, the 129 N-terminal residues of CupS adopt a rather complex fold. All structural elements are solvent-exposed, because none of them is completely buried in a hydrophobic core. Moreover, CupS does not possess any cysteine residues and therefore no disulfide bonds. The illustration in Fig. 7.4 emphasizes that the α-helical regions cluster at the one side of the protein (with the exception of helix α7), while the β-sheets cluster at the other side. All β-sheets except β1 and β7 are antiparallel to each other. The β1-β2-β6-β7 sheet is orthogonal to the β3-β4-β5 sheet. The three N-terminal helices α1, α2 and α3 are loosely packed against α6 and β2. The strands β1 and β2 are connected by the three α-helices α4, α5 and α6. Furthermore, strands β2 and β3 are connected by a rather long loop containing the helix α7. The α1-α2-α3 and α4-α5-α6 segments are each arranged in a U-shaped manner. The structure family of CupS (Fig. 7.5) shows low RMSD values. The α-helical region shows some structural variability among the members of the structure family. Especially helix α4 displays a high variability (see comparison of Fig. 7.4 A and B). Moreover, the length of helix α3 is not recognizable in all structures. The β-sheet region is very well-defined, only β2 is more pronounced in some structures than in others.
CHAPTER 7. RESULTS

Distance restraints
Total 2089
Intraresidual 929
Sequential 448
Medium-range 281
Long-range 431
Restraints per residue 16.2
Long-range restraints per residue 3.3

Dihedral angle restraints
$\phi, \psi$ 221

RMSD
Backbone 0.72 ± 0.18
Heavy atoms 1.04 ± 0.18

Ramachandran analysis
Most favored region (%) 83.6
Additionally allowed region (%) 15.3
Generously allowed region (%) 0.5
Disallowed region (%) 0.6

Table 7.2: NMR statistics for the CupS structure family, calculated from an ensemble of the 10 lowest energy structures from 40 after water refinement in ARIA.

Figure 7.5: The structure family of CupS. The superposition of the protein backbone is shown in blue. $\alpha$-helical regions are emphasized in red, $\beta$-sheets in green.
8.1. CupS and related proteins

The NMR solution structure of CupS from *T. elongatus* comprises seven α-helices and seven β-sheets and is thus very complex. The protein consists of 149 amino acids (Tll0220, Uniprot: Q8DMA1), 20 of which have been omitted in the structure calculation, because they showed neither long-range nor medium-range NOEs. The same observation was made for the protein MPB70 from *Mycobacterium bovis*, which shows sequence similarity to CupS (discussed in chapter 8.2). Interestingly, the homologous CupS protein from *Synechocystis* sp. PCC 6803 (Sll1734, Uniprot: P73392) contains only 133 amino acids. Therefore, the C-terminal part does not seem to be important for the CupS function. The sequence identity between the two proteins is 68% as calculated by PSI-BLAST.

The CupS protein is part of the NDH-1 complex, which is located at the thylakoid membrane and probably at the plasma membrane of cyanobacteria [89]. This is supported by the results of the PSORTb v3.0 tool [117], which predicts CupS to be located at the cytoplasmic membrane. Nevertheless, CupS shows high sequence similarity with the Fasciclin superfamily as determined by PSI-BLAST (http://blast.ncbi.nlm.nih.gov). These are often extracellular proteins, which are involved in cell adhesion. Among them are Fasciclin I, transforming growth factor-beta-induced protein (TGFBIp, also known as βig-h3), MPB70 and the Fasciclin I domain protein (Fdp). The sequence identity to CupS from *T. elongatus* and the corresponding proteins is shown in Table 8.1:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence identity</th>
<th>Z-score</th>
<th>RMSD</th>
</tr>
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<tr>
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<tr>
<td>TGFBIp</td>
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<td>6.8</td>
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<tr>
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</tr>
<tr>
<td>Fdp</td>
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</tr>
</tbody>
</table>

*Table 8.1: PSI-BLAST and DALI results for CupS from *T. elongatus*
CHAPTER 8. DISCUSSION

Figure 8.1: Sequence alignment of Fasciclin superfamily members. CupS from Thermosynechococcus elongatus, CupS from Synechocystis sp. PCC 6803, the Fas1-4 domain of human TGFBIp, the secreted protein MPB70 from Mycobacterium bovis and Fdp from Rhodobacter sphaeroides. The conserved regions H1 and H2 (according to Kawamoto et al. [121]) are emphasized in blue. Residues conserved among all aligned sequences are printed in red.

results from the DALI server [118], which compares protein structures, are shown in Table 8.1. Fasciclin I is an insect cell adhesion molecule, which contains four homologous domains of ≈ 150 residues, called Fas1 domains 1-4. Attempts to crystallize full-length Fasciclin I were unsuccessful, but the crystal structure of Fas1 domains 3-4 could be solved [119]. According to PSI-BLAST the sequence identity is rather low for both Fas1 domain 3 (22 %) and Fas1 domain 4 (14 %). However, this is not unusual, because other FAS1 domains also exhibit a sequence identity < 20 % [119]. Moreover, this was the best match when using DALI (see Tbl. 8.1). TGFBIp contains also four Fas1 domains. This extracellular protein is found in several human tissues, but especially in the cornea [120] and several mutations are involved in corneal dystrophy. For the Fas1-4 domain of TGFBIp the NMR structure was solved recently [7]. NMR structures are also available for MPB70 (a protein that is secreted from Mycobacterium bovis, which causes tuberculosis in animals, and that might bind to cell surface proteins of the host organism) [8] and Fdp (another protein with cell adhesion properties) from Rhodobacter sphaeroides [9].

8.2. Structural comparison with the Fasciclin superfamily

The CupS sequences of T. elongatus and Syn. sp. PCC 6803 were aligned with the Fas1-4 domain of human TGFBIp, MPB70 from Mycobacterium bovis and Fdp from Rhodobacter sphaeroides (Fig. 8.1). Fasciclin I was not included due to the low sequence identity. Two highly conserved regions H1 and H2 of unknown function were reported previously [121] and can be recognized in the alignment.

The crystal structure of the Fas1-3 domain of Fasciclin I and the NMR structures of the Fas1-4 domain of TGFBIp, MPB70 and Fdp all show a homologous core fold consisting of
seven β-sheets and five to eight α-helices. The β-sheet region is very similar between the structures of the different Fasciclin superfamily members. However, the α-helical regions are structurally less conserved. The NMR structure of CupS, which was solved in this work, exhibits a topology very close to that of the Fas1-4 of TGFBIp (Fig. 8.2, A&B). Three N-terminal α-helices are followed by the sheet β1 and another three α-helices. A rather short β-sheet β2, which is oriented antiparallel to β1, connects these helices with the seventh α-helix preceding the rest of the rather conserved β-sheet arrangement, which was described in more detail in chapter 7.2. The Fas1 domain 3 of Fasciclin I shows only five α-helices, two at the N-terminus, two between β1 and β2 and one between β2 and β3. MPB70 possesses eight α-helices with five of them being located at the N-terminus and the residual three between β1 and β2. In contrast to the other structures described here, MPB70 does not contain an α-helix between β2 and β3. Fdp does not comprise this helix, either, but has a helical turn at this position.

**Figure 8.2.** Structural comparison of CupS and the Fas1-4 domain of TGFBIp. The topology of both proteins is very similar (A: CupS, B: TGFBIp [7]), but the NMR structures of CupS (C) and Fas1-4 of TGFBIp (D, PDB: 2LTB) show distinct surfaces. Regions H1 and H2 are colored blue.
Despite the similar topology, there is a major structural difference between CupS and all other proteins described here, which is the position of the three N-terminal α-helices. For CupS, these are oriented away from the protein core, so that the residues of the conserved regions H1 and H2 are exposed on the surface (Fig. 8.2, C). The other protein structures (e.g. TGFBIp) show the N-terminal helices attached to this surface (Fig. 8.2, D). Therefore, it is not directly accessible for the interaction with any other proteins or ligands. Due to the distinct arrangements of these helices in comparison to the other fasciclin superfamily members, CupS shows a very different protein surface and is thus likely to function in another way. Interestingly, the three N-terminal helices and especially the residues involved in binding to the protein core in TGFBIp do not show a variety high enough to explain the discrepancies between the three-dimensional structures. Because of this, the 3D NOESY spectra were explicitly searched for NOEs between the N-terminal residues and the surface formed by H1 and H2, but none were found. Nevertheless, the structural difference is reasonable, because CupS, as a subunit of a membrane protein complex, is not likely to function in the same way as the extracellular cell adhesion proteins.

For the cell adhesion function of TGFBIp via integrin, an Asp-Ile sequence close to the H1 and H2 regions was shown to be important [122]. These amino acids are also conserved in CupS (Asp115-Ile116), but in Fasciclin I and Fdp the isoleucine residue is substituted by a valine residue. Because these residues are buried in the interface to the N-terminal helices (in all described protein structures except for CupS), it has been speculated that ligand (e.g. integrin) binding competes with the intramolecular binding of the N-terminal helices in an autoinhibitory mechanism [9]. This is further supported by the fact that the multiple mutations of TGFBIp (also known as βig-h3) causing corneal dystrophy are located in the protein core, which is not accessible with the N-terminus attached [8]. Although the concrete function of CupS remains elusive to date, as a part of an intracellular membrane protein complex in cyanobacteria it is not involved in cell adhesion and does not have the same ligands as Fdp, TGFBIp or related proteins. So the autoinhibitory function of the N-terminal helices might not be necessary for CupS. Mutational interaction studies with CupA or the NdhF subunit might help to locate the interaction surface between these NDH-1 complex subunits and to explain the structural and functional differences to the other members of the Fasciclin superfamily.
The structure of the cyanobacterial NDH-1 complex subunit CupS was solved using NMR spectroscopy. Both the backbone and sidechain chemical shift assignments were obtained almost completely. The calculated structure consists of seven $\alpha$-helices and seven $\beta$-sheets and the structure family shows good RMSD values and ramachandran statistics. The 30 C-terminal amino acids of the construct were omitted in the calculation due to missing structural restraints, which indicate flexibility.

CupS is sequentially and structurally similar to the members of the Fasciclin superfamily. However, these proteins are usually located extracellularly and are involved in cell adhesion. Consequently, the relation to the Fasciclin superfamily does not help to understand the function of CupS, which is part of a multiprotein complex at the membrane. Moreover, Fasciclin domains have a similar topology as the determined NMR structure of CupS, but there is also a major structural difference: the N-terminal helices are oriented away from the protein core in CupS, but attached to the core in familiar proteins such as TGFBIp, Fdp or MBP70. Yet, just like the Fasciclin superfamily members, CupS might function as an interaction domain via the conserved regions H1 and H2, but obviously has distinct ligands. It has been hypothesized for Fdp that the orientation of the N-terminal helices is related to an autoinhibitory function. This might not be necessary for CupS, which is probably not a regulatory protein, but simply a component of the cyanobacterial NDH-1 complex. To prove that the H1 and H2 region or the Asp-Ile sequence are important, interaction studies have to be performed. These could include CupA or NdhF3, two subunits of the NDH-1 complex, which are thought to be attached to CupS. Furthermore, crystallization of the whole NDH-1 complexes is still a major goal of the actual research.
Part III.

Materials & Methods
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**Bacterial strains**

*E. coli* BL21(DE3)pLysS  
genotype: F−, ompT, hsdS B, (rB−mB−)  
Novagen
gal, dcm, lon, λ(DE3) pLysS(cmR)

*E. coli* NovaBlue  
genotype: endA1, hsdR17, (rK12m+K12),  
Novagen
supE44, thi-1, recA1, gyrA96, relA1, lac,  
F'[proA+B+ lacIqZΔM15::Tn10] (TetR)

**Buffer**

CD-buffer, pH 8.0
10 mM  sodium hydrogen phosphate buffer, pH 8.0

CD-LDAO-buffer, pH 8.0
10 mM  sodium hydrogen phosphate buffer, pH 8.0
0.1 %  LDAO

Denaturing buffer, pH 7.8
50 mM  Tris
6 M  guanidine hydrochloride

Dialysis buffer, pH 7.8
50 mM  Tris
5 mM  DTT
### Dilution buffer, pH 7.8

- 50 mM Tris
- 5 mM DTT
- 1% LDAO

### Elution buffer (Bcl-x\textsubscript{L}ΔLoopΔTM and Bcl-x\textsubscript{L}ΔTM), pH 7.8

- 1x PBS
- 2 mM DTT
- 10 mM glutathione

### Elution buffer (Bcl-x\textsubscript{L}ΔLoop and Bcl-x\textsubscript{L}), pH 7.8

- 50 mM Tris
- 5 mM DTT
- 10 mM glutathione

### LDAO-SEC buffer, pH 7.8

- 50 mM Tris
- 5 mM DTT
- 0.1% LDAO

### NMR buffer, pH 8.0

- 20 mM sodium hydrogen phosphate buffer, pH 8.0
- 5 mM magnesium chloride
- 5 mM sodium chloride
- 5 mM DTT

### Resuspension buffer (Bcl-x\textsubscript{L}ΔLoopΔTM and Bcl-x\textsubscript{L}ΔTM), pH 7.8

- 1x PBS
- 2 mM DTT

### Resuspension buffer (Bcl-x\textsubscript{L}ΔLoop and Bcl-x\textsubscript{L}), pH 7.8

- 50 mM Tris
- 5 mM DTT
PBS (phosphate buffered saline), pH 7.4
137 mM sodium chloride
2.7 mM potassium chloride
10 mM disodium hydrogen phosphate
2 mM potassium dihydrogen phosphate

Sodium hydrogen phosphate buffer, 20 mM, pH 8.0
0.19 g/L sodium dihydrogen phosphate monohydrate
4.99 g/L disodium hydrogen phosphate heptahydrate

Tris buffer, pH 7.8
50 mM Tris

Chemicals
ABT-263 Selleckchem
Ammonium chloride J. T. Baker
Ammonium chloride (\(^{15}\text{N}, 99\%\)) Cambridge Isotope Laboratories
Ampicillin Roth
Biotin Sigma-Aldrich
Boric acid Sigma-Aldrich
Bradford reagent Roti®-Nanoquant, Roth
Calcium chloride J. T. Baker
Chloramphenicol AppliChem
Cobalt (II) chloride hexahydrate Sigma-Aldrich
Copper (II) chloride dihydrate Sigma-Aldrich
Deuterium oxide Merck
Dimethylsulfoxide (DMSO)-d\(_6\) Merck
Disodium hydrogen phosphate heptahydrate J. T. Baker
Dithiothreitol (DTT) AppliChem
Ethanol Sigma-Aldrich
Glucose AppliChem
Glucose (\(^{13}\text{C}, 99\%\)) Cambridge Isotope Laboratories
Glutathione AppliChem
Glutathione(GSH)-beads Protino® Glutathione Agarose 4B Macherey-Nagel
Guanidine hydrochloride Roth
CHAPTER 10. MATERIALS

Iron (III) chloride hexahydrate
Isopropyl-β-D-thiogalactopyranoside (IPTG)
LB medium (pre-mixed powder)
Magnesium chloride
Magnesium sulfate
Manganese (II) chloride tetrahydrate
N,N-dimethyldodecylamine-N-oxide (LDAO)
Potassium chloride
Potassium dihydrogen phosphate
Protease inhibitor
Sodium dihydrogen phosphate
Sodium chloride
Thiamin
Tris
Zinc chloride

Sigma-Aldrich
AppliChem
Roth
J. T. Baker
J. T. Baker
Sigma-Aldrich
Sigma
J. T. Baker
J. T. Baker
complete mini, EDTA free, Roche
J. T. Baker
Sigma-Aldrich

Consumables
Concentrators
Gravity-flow columns
NMR tubes
PD10 desalting columns
Quartz cuvette, 0.5 mm
Amicon® Centrifugal Filter Units 10,000 MWCO, Merck Millipore
Protino® Columns 35 mL, Macherey-Nagel
Norell, 3mm
GE Healthcare
Hellma

Enzymes
FastDigest® restriction enzymes
Thrombin
ThermoScientific
Merck

Instruments
CD spectrometer
Centrifuges
J-815, Jasco
Table Centrifuge 5415 R, Eppendorf
(Group Protein interactions, Prof. Dr. Herrmann, Physical Chemistry I, RUB)
5810 R, Eppendorf
Sorvall RC5B
Sorvall RC5C
CHAPTER 10. MATERIALS

Chromatography columns
- Sephadex 75 10/300 (analytical), GE Healthcare
- Sephadex 75 16/60 (preparative), GE Healthcare
- Superose 6 10/300 (analytical), GE Healthcare

FPLC pumps
- ÄKTAprime plus, GE healthcare
- ÄKTApurifier, GE healthcare
- GradiFracSystem, Pharmacia

Incubator
- Minitron, Infors HT

Microfluidizer
- Microfluidics Corporation

NMR spectrometer
- DRX 600, Bruker (for Bcl-xL)

Probe head
- 5 mm PATXI 1H/D-13C-15N Z-GRD, Bruker
  (for Bcl-xL)

Sonifier
- Bandelin Sonopuls HD2200
  (Group Protein interactions, Prof. Dr. Herrmann,
  Physical Chemistry I, RUB)

Kits
- Cloning kit: CloneJet PCR Cloning Kit, ThermoScientific
- Crystallization screening kit: JCSG Core Suites I-IV, Qiagen

Marker and loading dyes
- PageRuler Prestained Protein Ladder, ThermoScientific

Media
- LB medium (pre-mixed powder)
  - 25 g/L
  - (100 mg/L ampicillin)
  - (34 mg/L chloramphenicol)
Mineral medium

1 x M9 salt solution (10x)
1 x Trace element solution (100x)
0.3 mM calcium chloride
1 mM magnesium sulfate
1 mg/L biotin
1 mg/L thiamin
2-4 g/L glucose or $^{13}$C-glucose
0.5 g/L ammonium chloride or $^{15}$N-ammonium chloride
(100 mg/L ampicillin)
(34 mg/L chloramphenicol)

Peptides
All peptides were synthesized by China Peptides with a purity > 95%.

Bak BH3 peptide  Ac-GQVGRQLAIIGDDINR-NH₂
Bid BH3 peptide  Ac-DIIRNIARHLAQVGDMSDRS-NH₂
TM peptide       Ac-KGQERFNRWFLTMVAGVLLGSLFSRK

Solutions

M9 salt solution (10x)
60 g/L disodium hydrogen phosphate
30 g/L potassium dihydrogen phosphate
5 g/L sodium chloride

Trace element solution (100x)
0.83 g/L iron (III) chloride hexahydrate
84 mg/L zinc chloride
13 mg/L copper (II) chloride dihydrate
10 mg/L cobalt (II) chloride hexahydrate
10 mg/L boric acid
1.6 mg/L manganese (II) chloride tetrahydrate

Vectors
pGEX-4T-1 GE Healthcare
pTXB1 New England Biolabs
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11.1. Cloning of Bcl-x constructs

The sequences encoding for four different Bcl-xL mutants (full-length Bcl-xL, Bcl-xL ΔLoop, Bcl-xL ΔTM and Bcl-xL ΔLoopΔTM, Fig. 11.1) were cloned into two different expression systems. The CloneJET PCR Cloning Kit and E. coli NovaBlue cells were utilized for high-efficiency cloning.

For the full-length construct, cDNA of Bcl-xL (rattus norvegicus) was amplified with NdeI and SapI recognition sequences and subcloned into the vector pTXB1 to attach a C-terminal intein/CBD-tag. The use of the SapI restriction site allows cloning without any additional C-terminal amino acids and the intein tag can easily be removed by DTT (for details see the manufacturer’s instructions or [70]). Together with the sequence for the intein tag, the Bcl-xL gene was finally cloned into the vector pGEX-4T-1, which contains an N-terminal GST-tag using the restriction enzymes SmaI and XhoI. This leads to the seven-residue N-terminal cloning artifact GSPEFPG. For Bcl-xL ΔLoop, the loop region (aa 35-78) was substituted with four alanine residues by making use of the restriction enzyme PstI. The same method was reported for Bcl-2 [123].

The truncated forms Bcl-xL ΔTM (aa 1-204) and Bcl-xL ΔLoopΔTM (aa 1-204, Δ35-78) were directly cloned into the expression vector pGEX-4T-1, which includes an N-terminal GST-tag. For this the restriction enzymes EcoRI and SmaI were used. Thereby, the five amino acids GSPEF were added to the N-terminus as a cloning artifact.

The cloning of Bcl-xS was performed by amplifying the two fragments Bcl-xL(1-125) and Bcl-xL(189-233)-intein by PCR using the Bcl-xL-intein construct as a template. The recognition sequences for SmaI/SapI and SapI/XhoI were attached for the final cloning step into the pGEX-4T-1 vector. SapI is a special restriction enzyme that cleaves next to its recognition sequence. Therefore, cloning is possible without artifacts, which is necessary for the cloning of a functional Bcl-xS construct without any additional amino acids in the middle of the sequence.
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Figure 11.1: Protein sequences of the different Bcl-x mutants. The N-terminal cloning artifact is printed in red, the alanine-linker substituting the loop region in blue.

11.2. Expression and purification of Bcl-x constructs

Recombinant proteins were expressed in E. coli BL21 (DE3) pLysS. For \(^{15}\text{N}\)-isotopically enriched proteins, cells were grown at 37 °C to an OD\(_{600}\) > 1, collected by centrifugation (2700 x g), washed in 1 x M9 medium and resuspended in minimal medium containing 0.5 mg/L \(^{15}\text{N}\)-ammonium chloride. Before induction, the cells were incubated for 1 h at the appropriate expression temperature. Expression was induced with 1 mM IPTG at 37 °C (Bcl-xL (\(\Delta\text{Loop}\)\(\Delta\text{TM}\)) or 18 °C (full-length Bcl-xL, Bcl-xL \(\Delta\text{Loop}\) and Bcl-xS). Cells were harvested after 4 hours (Bcl-xL (\(\Delta\text{Loop}\)\(\Delta\text{TM}\)) or 24 hours (full-length Bcl-xL, Bcl-xL \(\Delta\text{Loop}\) and Bcl-xS).

The cells were resuspended in resuspension buffer including protease inhibitor and disrupted by using a microfluidizer or sonifier. The lysate was purified using GSH-beads. A self-made gravity-flow column was connected to an FPLC-pump system (GradiFrac System or ÄK-TAprime plus) and equilibrated with resuspension buffer. The lysate was applied, in the case of full-length Bcl-xL, Bcl-xL \(\Delta\text{Loop}\) and Bcl-xS at a very low flow rate (\(\approx 0.1 \text{ mL/min}\)), so that the DTT was able to separate the intein from the GST-fusion protein. The column was then washed with resuspension buffer to remove other proteins of the lysate and GST-Bcl-xL/S was eluted with elution buffer. The eluate was concentrated and the GST was cut off by thrombin (\(\approx 75 \text{ u}\)) for 16 h at 20 °C. Glutathione was removed and the buffer was changed to the appropriate buffer (NMR buffer for NMR titrations and Tris buffer for crystallization or size exclusion chromatography) using centrifugal filter units or PD\(_{10}\) desalting columns.

The same buffer was now used to equilibrate the GSH column. The protein solution was then applied to this, so that the GST was bound to the column and Bcl-xL/S was collected in the flowthrough. To regenerate the column, GST was eluted with elution buffer and the column...
was washed with water and 20% ethanol for storage. Protein samples were applied to 15% SDS gels and the protein concentration was measured using Roti®-Nanoquant, a modified Bradford reagent, according to the manufacturer’s instructions.

11.3. Refolding of Bcl-xL into LDAO micelles

Full-length Bcl-xL and Bcl-xL ΔLoopΔTM were refolded into LDAO micelles to investigate these proteins in a membrane-like environment. 10 µL of the purified, concentrated proteins were dissolved in 1 mL denaturing buffer and incubated for 1-2 h. The denatured proteins were then refolded by dropwise dilution with stirring into 10 mL dilution buffer. The solution was stirred overnight at 4 °C. Finally, the sample was dialyzed overnight against 4 L of dialysis buffer to remove excess LDAO.

11.4. Size exclusion chromatography

Size exclusion chromatography was used to characterize the different oligomeric states of the purified proteins and to isolate these forms for further studies. The investigation of the oligomers was carried out on the analytical size exclusion chromatography column Superdex 75 10/300. To determine the amount of protein running in the exclusion volume, the analytical column Superose 6 10/300 was used additionally for full-length Bcl-xL. For the isolation of the proteins at a large scale, the preparative column Superdex 16/60 was used. All columns were connected to the ÄKTApurifier system at 4 °C. The experiments were performed in Tris buffer or, when working with LDAO micelles, in LDAO-SEC buffer. The samples were applied by use of a 500 µL loop (analytical columns) or a 2 mL loop (preparative column) and the flow rates were 0.5 mL/min and 2 mL/min, respectively.

11.5. NMR spectroscopy of Bcl-xL

All NMR experiments of Bcl-xL were performed at 298 K on a Bruker DRX-600 spectrometer using standard Bruker pulse sequences (1D-1H-spectra: p3919gp; 1H,15N-TROSY-HSQC spectra: trosyetf3gpsi). Transversal relaxation optimized spectroscopy (TROSY) spectra are used to lower the transverse relaxation rate, which allows the study of large proteins [124]. Thus, these are useful for the investigation of the oligomeric full-length Bcl-xL. Moreover, it was shown earlier that even the probably monomeric Bcl-xL ΔTM is subjected to transverse relaxation. This leads to line broadening, especially when recording triple resonance spectra [70].
The protein concentrations were 0.6 mM (Bcl-xL\(\Delta\)Loop\(\Delta\)TM) or 0.75 mM (full-length Bcl-xL) for the reference and ABT-263 measurements and 0.2 mM (Bcl-xL\(\Delta\)Loop\(\Delta\)TM) or 0.5 mM (full-length Bcl-xL) for the NMR studies with the peptides. The sample of Bcl-xL\(\Delta\)TM was concentrated to 0.8 mM. The peptides were dissolved in NMR buffer and added to ratios of 1:1, 1:2 and 1:3. ABT-263 was added from a 100 mM stock solution in DMSO-\(d_6\) to a ratio of 1:3. Five days after the addition of ABT-263 to the full-length protein another spectrum was recorded.

The NMR samples of Bcl-xL in LDAO were refolded into the micelles as described in chapter 11.3, but the buffers were prepared with NMR buffer instead of 50 mM Tris, 5 mM DTT. The two different oligomeric states in the LDAO micelle were isolated by SEC using LDAO-SEC buffer, which was also prepared with NMR buffer instead of 50 mM Tris, 5 mM DTT. All \(^1\)H-\(^{15}\)N-TROSY-HSQC spectra of Bcl-xL were usually recorded with 64 scans, but 960 scans were acquired for monomeric full-length Bcl-xL in LDAO.

All NMR spectra were processed with NMRPipe 2009 [125] and analyzed with CcpNmr Analysis 2.3.1 [126].

### 11.6. X-Ray crystallography

Full-length Bcl-xL was concentrated to 26 mg/mL (≈ 1 mM) and ABT-263 was added to a molar ratio of 1:1 from a 100 mM stock solution in DMSO-\(d_6\). The crystallization screen was realized at the Max Planck Institute of molecular physiology in Dortmund, Germany with the assistance of Dr. Ingrid Vetter. 4 x 96 conditions (JCSG core suites I to IV) were tested for Bcl-xL in absence and presence of ABT-263. The crystals of the Bcl-xL-ABT-263 complex were grown at 20 °C by the hanging-drop vapor diffusion method using 0.2 M calcium chloride and 20 % (w/v) PEG3350 as precipitant. Data collection, processing and molecular replacement, which are described in the following, were performed by Dr. Ingrid Vetter. Diffraction data were collected at the PXII beamline of the SLS synchrotron in Villigen, Switzerland at 100 K using a Pilatus 6M detector. The data were processed with XDS and XSCALE [127]. Molecular replacement was performed with a crystal structure of Bcl-xL (PDB: 1R2D) using the program PHASER in the CCP4 program suite [128]. The AutoBuild function [129] implemented in the PHENIX program suite [130] was used for model building. Refinement was performed by iterative use of manual refinement in COOT [131] and phenix.refine [132]. The tool MolProbity within the PHENIX program suite was used for data validation [133]. The structural figures were generated with PyMOL [134].
11.7. CD spectroscopy

CD spectra were obtained at a Jasco J-815 spectrometer at 20 °C in a 0.5 mm cuvette. The sample was scanned from 250 nm to 190 nm in 0.5 nm intervals with 100nm/min. Five scans were accumulated for each spectrum. The baseline was corrected by substraction of a spectrum of the appropriate buffer solution. Samples of Bcl-xL ΔLoopΔTM and Bcl-xL ΔLoop were measured in CD-buffer and the samples of Bcl-xL ΔLoopΔTM and full-length Bcl-xL refolded in LDAO were measured in CD-LDAO buffer.

The protein concentration was calculated by use of the recorded absorption at 205 nm. The absorbance at 280 nm was not measured. According to Scopes the extinction coefficient at 205 nm can be calculated by using the absorbance values at 205 nm and at 280 nm. If the absorbance at 280 nm is not known, as in this case, ε_{205} can estimated to be 31 [135]. However, latest studies enable the calculation of ε_{205} based on the protein sequence, for which an online tool is available (http://spin.niddk.nih.gov/clore) [136]. This was used for the determination of the extinction coefficient of the proteins. To calculate the protein concentration, the extinction coefficient was included in the Beer-Lambert law:

\[ A_\lambda = \epsilon_\lambda c d \] (11.1)

This means that at a certain wavelength \( \lambda \), the absorption \( A \) is proportional to the concentration \( c \) and to the path length \( l \). \( \epsilon \) is the extinction coefficient.

The mean residue weight (MRW) was calculated by dividing the molecular mass of the protein (M) by the number of peptide bonds (N-1 with N being the number of amino acids). Using the MRW and the concentration of the protein, the mean residue ellipticity \([\theta]_{mrw}\) at each wavelength \( \lambda \) could be calculated by the following equation:

\[ [\theta]_{mrw,\lambda} = \frac{MRW \cdot \theta_\lambda}{(d \cdot c)} \] (11.2)

Here, \( \theta_\lambda \) is the measured ellipticity (mdeg) at wavelength \( \lambda \), MRW is the mean residue weight (g/mol), \( d \) is the cuvette path length (mm) and \( c \) is the concentration (mg/mL). The resulting normalized CD spectra were uploaded to the DICHROWEB online server [81] to analyze the secondary structure of the protein samples. The three different algorithms SELCON3 [82, 83], CONTINLL [84, 85] and K2D [86] were used and compared. Reference set 4 [137], which is optimized for the wavelength range from 190 nm to 240 nm, was employed for the SELCON3 and CONTINLL methods. K2D does not require a reference set.
11.8. NMR spectroscopy of CupS

The CupS protein sample was prepared by Hannes Wulforst (Group Cyanobacterial Membrane Protein Complexes, Dr. Marc Nowaczyk, Plant Biochemistry, RUB). CupS was expressed with a C-terminal Strep-tag and a two amino acid linker in minimal medium containing $^{15}$N-ammonium chloride and $^{13}$C-glucose. It was purified using StrepTactin affinity chromatography and size exclusion chromatography. The CupS sample was finally concentrated to 0.5 mM in 50 mM Tris, 50 mM NaCl, pH 8.0. The cloning and purification strategies are explained in detail in the Ph. D. thesis of Hannes Wulforst [109].

All spectra were measured and processed by Dr. Takahisa Ikeyama from the Institute of Protein Research at the Osaka University, Japan. The final NMR sample contained 0.5 mM CupS in 50 mM Tris, 50 mM NaCl, 10 mM deuterated DTT and 10% D$_2$O. The spectra were obtained at 298 K at BrukerBioSpin Avance-III 950 or Avance-I 800 spectrometers, both equipped with a z-axis gradient, triple resonance cryogenic probe, or on DRX-600 or DRX-500 spectrometers equipped with a xyz-axis gradient, triple resonance probe at the Osaka University. The spectra were processed with NMRPipe [125].

The analysis of the spectra was performed using CcpNmr Analysis 2.3.1 [126]. For the backbone assignment $^1$H-$^{15}$N-HSQC [138], HNCA CB [139], CBCA(CO)NH [140] and HNCO [141] spectra were employed in combination with the $^{15}$N-NOESY-HSQC [142] spectrum. The HNCA CB, CBCACONH and HNCO spectra are three-dimensional ($^1$H-, $^{15}$N- and $^{13}$C-dimension). The amide proton and amide nitrogen chemical shifts are correlated to the $\alpha$ and $\beta$ shifts of the preceding amino acid i-1 (CBCACONH), to the $\alpha$ and $\beta$ shifts of the preceeding amino acid i-1 and those of the own amino acid i (HNCA CB) or to the CO shift (HNCO). The backbone assignment is started with the comparison of the HNCA CB and CBCACONH spectra. These spectra are projected in the $^1$H-$^{13}$C-plane and the $^{15}$N-dimension can be varied. Like this, four signals belonging to $\alpha_i$, $\alpha_{i-1}$, $\beta_i$ and $\beta_{i-1}$ can easily be distinguished in the HNCA CB spectrum in one NH strip, i.e. at the same amide proton and nitrogen chemical shift, but at distinct carbon resonances. These signals can be overlapped with the corresponding signals from the CBCACONH spectrum, which are the two signals belonging to $\alpha_{i-1}$ and $\beta_{i-1}$. These can also be identified, because they are weaker than those of the own amino acid i. $\alpha_{i-1}$ and $\beta_{i-1}$ can be found in the HNCA CB spectrum in another NH strip belonging to amino acid i-1 itself. Here, signals of $\alpha_{i-1}$, $\alpha_{i-2}$, $\beta_{i-1}$ and $\beta_{i-2}$ can be found. The nitrogen dimension is scanned again to find the $^{15}$N-dimension of amino acid i-2 and so on. Like this the connectivity between the amino acids can be determined. Due to characteristic $\alpha$ and $\beta$ chemical shift values, some amino acid types can be identified and by comparison with the sequence, the residues are assigned. Having assigned
the amide proton and nitrogen chemical shifts, the CO shifts could be easily determined by looking at the appropriate strip in the HNCO spectrum. There is only one signal and the carbon shift can be assigned to the CO resonance.

The spectra of CupS were of high quality and showed a very good signal dispersion. Nevertheless, there were some difficulties with overlapping or missing signals. The 3D $^{15}$N-edited NOESY helped to support the backbone assignment. It is usually projected in the $^1$H-$^1$H-plane and the $^{15}$N-dimension can be varied. This spectrum shows cross peaks (NOEs) for two protons that are in spatial proximity of $\leq 5 \text{ Å}$. Because sequential amide protons are usually in close proximity, NOEs can be observed for sequential amide protons.

HBHA(CO)NH [143], H(CCO)NH, (CCCO)NH [144], HCCH-TOCSY [145] and HCCH-COSY [141] spectra were used as well as the 2D $^{13}$C-HSQC spectrum and the 3D aliphatic and aromatic $^{13}$C-edited NOESY [146] spectra to obtain sidechain resonance assignments. At first the HBHA(CO)NH, H(CCO)NH and (CCCO)NH spectra were used to obtain $\text{H}^{\alpha}$ and $\text{H}^{\beta}$, $\text{H}^{\gamma}$ or $\text{C}^{\gamma}$ chemical shifts, respectively. These shifts are directly correlated to the amide proton and nitrogen chemical shifts of the subsequent amino acid, which are known from the backbone assignment. The combination of the carbon atom shifts with the appropriate hydrogen atom shifts allowed the assignment of the 2D $^{13}$C-HSQC spectrum and the corresponding CH strips in the HCCH-TOCSY and HCCH-COSY. The HCCH-COSY reveals the protons of directly neighboring carbon atoms, while the HCCH-TOCSY shows signals from all protons of one residue in the related CH strip. The aromatic $^{13}$C-edited NOESY spectrum was used for the assignment of the residues with aromatic side chains. Moreover, the aliphatic $^{13}$C-NOESY could also help to find missing resonances such as $\text{H}^{\gamma}1$ of threonine, which is attached to an oxygen atom and can thus be neither observed in the HCCH-TOCSY nor in the HCCH-COSY spectrum.

11.9. Structure calculation

For the structure calculation of CupS, distance restraints from the $^{15}$N- and $^{13}$C-edited NOESY spectra were used. NOEs were picked manually and obvious intraresidual and sequential NOEs were assigned. Dihedral angle restraints were obtained from TALOS+ [110], which employs chemical shift values. The dihedral angles of Lys107 were added manually to minimize the variation of these values. For structure calculation UNIO (CANDID) [147]/CYANA 3.0. [112, 148] and ARIA 2.3 [113]/CNS 1.2.1 [114] were used. Both programs assign the picked peaks automatically before each structure calculation cycle.

UNIO combines algorithms for the automated backbone assignment (MATCH) [149], automated sidechain assignment (ASCAN) [150], peak picking (ATNOS) [111] and NOE assign-
ment (CANDID) [147]. For the data analysis of CupS only the ATNOS/CANDID functions were used due to the lack of APSY (Automated projection spectroscopy) spectra, which are usually needed for MATCH and ASCAN, and to ensure the high assignment level needed for the automated NOE assignment. For the automated peak picking with ATNOS, the $^{15}$N- and $^{13}$C-edited NOESY spectra were converted into CARA [151] format. These peaks were assigned by CANDID, which is also implemented in the structure calculation program CYANA. Because not all NOEs can initially be assigned unambiguously, the assignment is an iterative process of cycles consisting of NOE assignment and structure calculation with torsion angle dynamics. In the initial cycle, assignment decisions are based on several criteria such as the self-consistency of the NOE network (also known as network anchoring) or the agreement between the shift list and the peak list. After the first cycle, the output structures are used to assist the NOE assignment for the next cycle. For CupS, the peaks picked with ATNOS did not lead to good results. Thus, the manually picked peak lists were used for the structure calculation. The initial run with UNIO was performed without intraresidual and sequential NOEs, which resulted in a well-folded structure family. ARIA (Ambiguous Restraints for Iterative Assignment) also assigns NOE peaks iteratively. Yet, it was used without network-anchoring. The assigned output peak lists from UNIO were used for further structure calculations in ARIA with torsion angle dynamics. These assignments were denoted to be trusted, so that they are definitely used in the structure calculation. In addition, the manually picked peak lists were added, but the assignments were not to be trusted. This helped ARIA to quickly find the core fold of the CupS protein. The amount of violations was minimized iteratively by removal of assignment errors, highly overlapping or violated NOEs and by manual assignment corrections. After a few structure calculation runs the peak lists were merged (trust assignments: no) and the amino acids 130-159 were removed. Finally, 100 structures were calculated in the last iteration and the 40 best structures were refined in water. The 10 lowest-energy structures were validated using the iCING validation package [116].
Abbreviations

A1 Bcl-2-related gene A1
Apaf-1 apoptotic protease activating factor-1
APSY automated projection spectroscopy
ARIA Ambiguous Restraints for Iterative Assignment
Bad Bcl-2 antagonist of cell death
Bak Bcl-2 antagonist/killer-1
Bax Bcl-2 associated X-protein
Bcl-2 B-cell lymphoma 2
Bcl-xL B-cell lymphoma extra large
Bcl-xS B-cell lymphoma extra short
BH domain Bcl-2 homology domain
Bid BH3-interacting domain against death agonist
Bik Bcl-2 interacting killer
Bim Bcl-2 interacting mediator of cell death
BMF Bcl-2 modifying factor
Bok Bcl-2-related ovarian killer
CBD chitin binding domain
CD circular dichroism
cFLIP cellular FLICE-inhibitory protein
DISC death-inducing signaling complex
DMSO dimethylsulfoxide
DPC dodecylphosphocholine
DSS 4,4-dimethyl-4-silapentane-1-sulfonic acid
DTT dithiothreitol
FADD FAS-associated protein with a death domain
Fdp Fasciclin I domain protein
FMN flavin mononucleotide
GFP green fluorescent protein
GSH glutathione
GST glutathione-S-transferase
HRK harakiri
HSQC heteronuclear single quantum coherence
IAP inhibitor of apoptosis protein
IKK IκB kinase
ABBREVIATIONS

InsP$_3$R  inositol 1,4,5-triphosphate receptor
IPTG  isopropyl-β-D-thiogalactopyranoside
LDAO  N,N-dimethyldodecylamine-N-oxide
Mcl-1  myeloid cell leukemia 1
MOMP  mitochondrial outer membrane permeabilization
MRW  mean residue weight
NCCD  Nomenclature Committee on Cell Death
NDH-1 complex  NAD(P)H dehydrogenase type I complex
NMR  nuclear magnetic resonance
NOE  nuclear Overhauser effect
NOESY  nuclear Overhauser effect spectroscopy
NRMSD  normalized root mean square deviation
OMM  outer mitochondrial membrane
OPS  oxygenic photosynthesis-specific
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PQ  plastoquinone
PUMA  p53-upregulated modulator of apoptosis
RMSD  root-mean-square deviation
SDS  sodium dodecyl sulfate
SEC  size exclusion chromatography
tBid  truncated Bid
TGFBIp  transforming growth factor-beta-induced protein
TROSY  transversal relaxation optimized spectroscopy
VDAC  voltage-dependent anion channel
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