Production of Bcl-2 proteins involved in regulation of mitochondrial apoptosis

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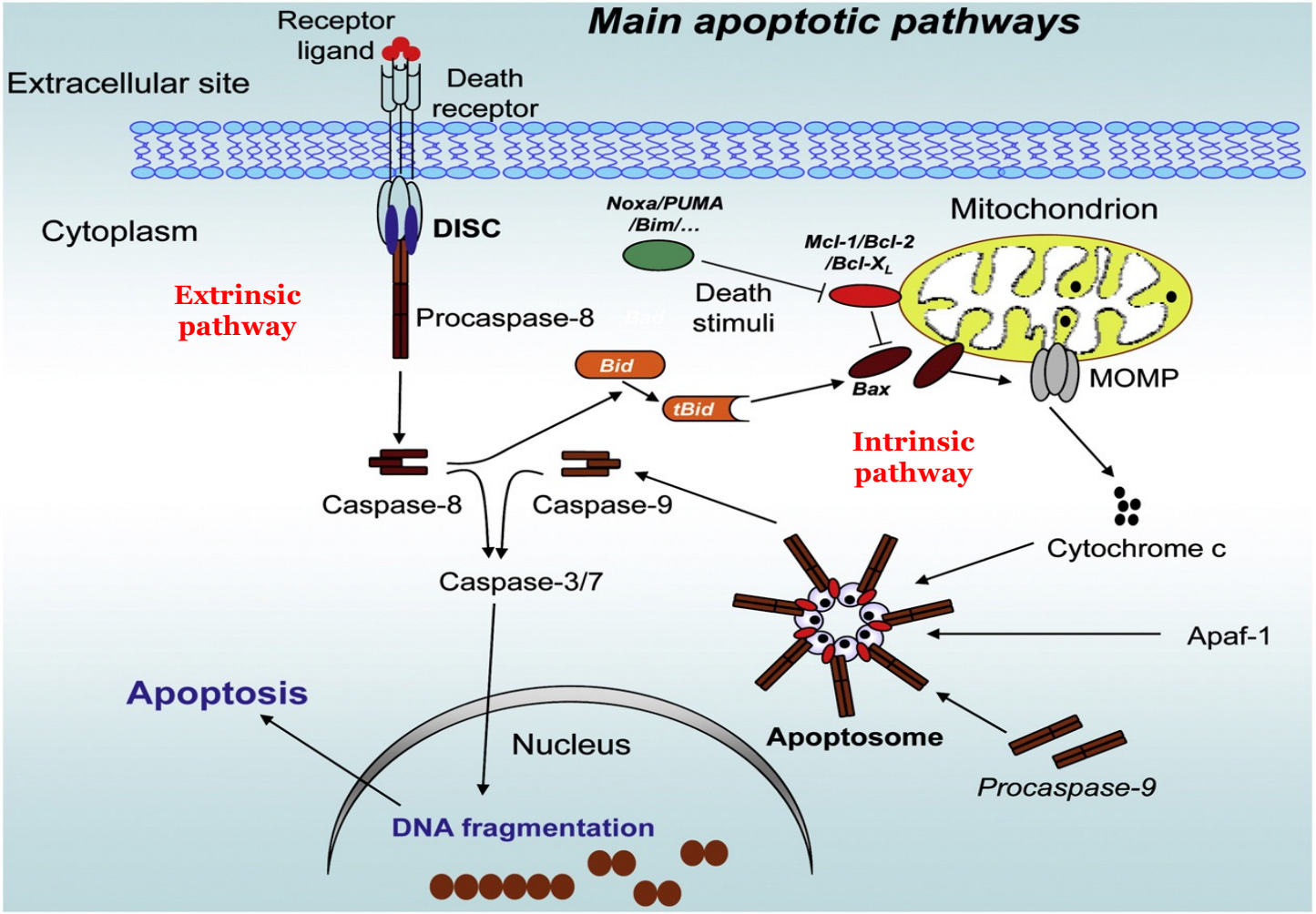
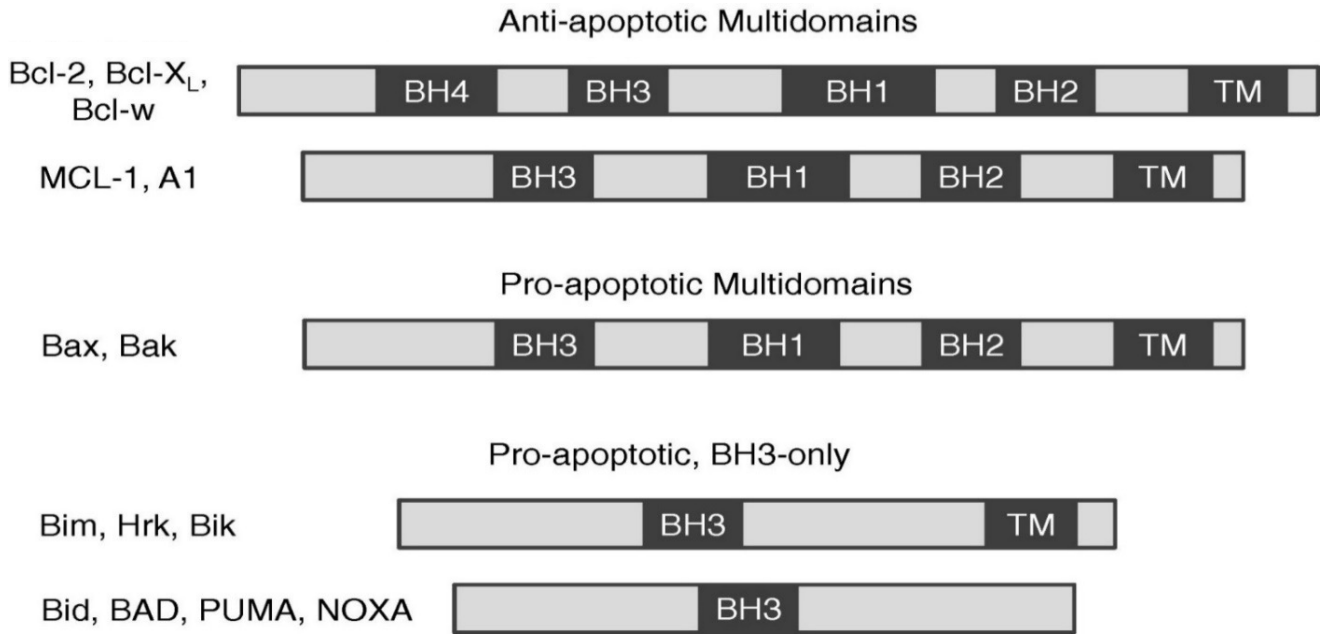
Apoptosis is key for controlled removal of cells



* Adapted from Nys K. *et al.*, Biochim Biophys Acta, 2011

The Bcl-2 protein family - regulators of the intrinsic pathway

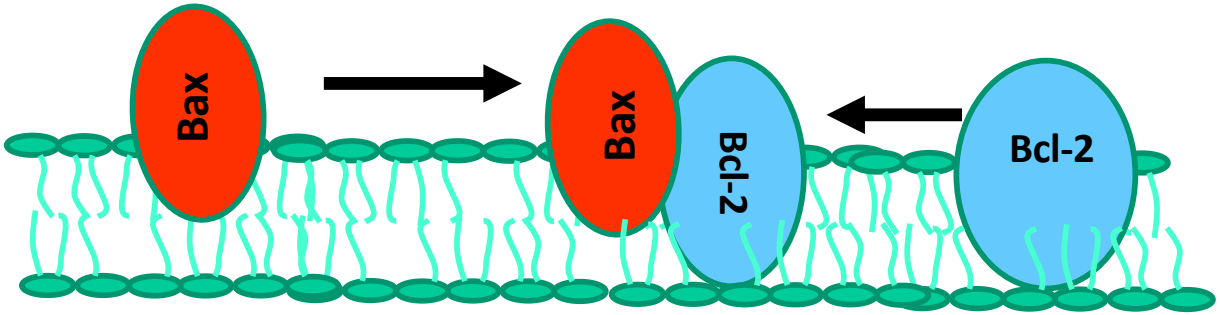
- **Mainly α-helical, with conserved BH-domains**
- **Anti- and pro-apoptotic classes, having opposing functions**
- **BH3-only proteins activate Bax and Bak**



*** Adapted from Simpson C. D. et al., Cancer Lett, 2008**

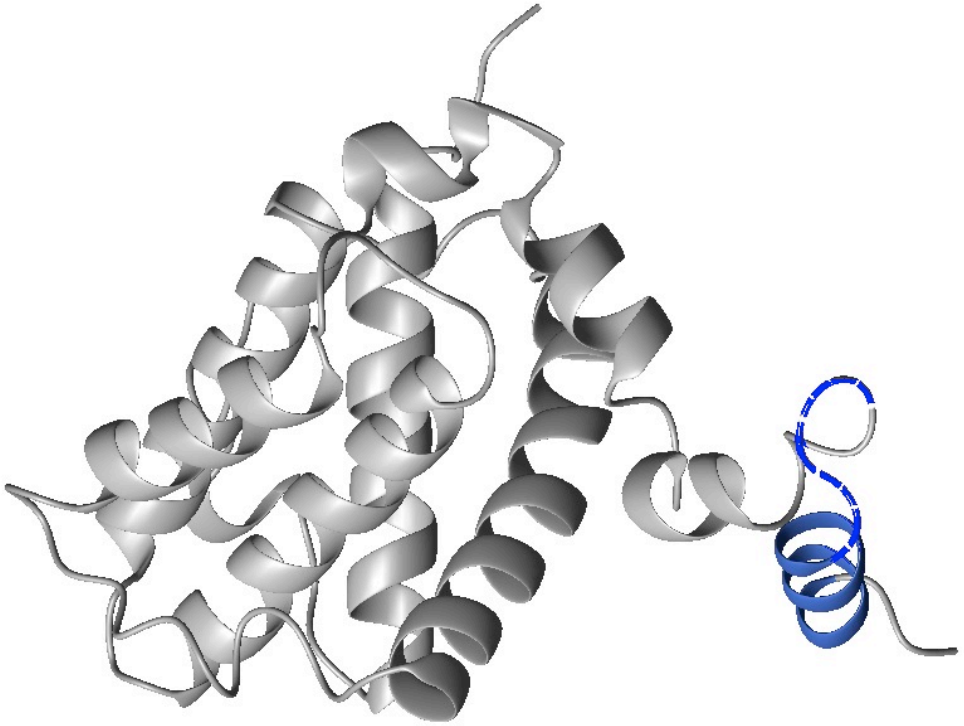
How does the anti-apoptotic Bcl-2 protein function at the membrane level?

Bcl-2 is a family of proteins that dictate the fate of the cell by a fine-tuned balance between anti- (Bcl-2) and pro-apoptotic (Bax) proteins that meet at the mitochondrial outer membrane.



- **Bcl-2 is an integral 26.5 kDa membrane protein**
- **Bcl-2 location not known**
- **Bcl-2 structure not known**
- **Bcl-2/Bax complex structure not known**

The anti-apoptotic membrane protein Bcl-2



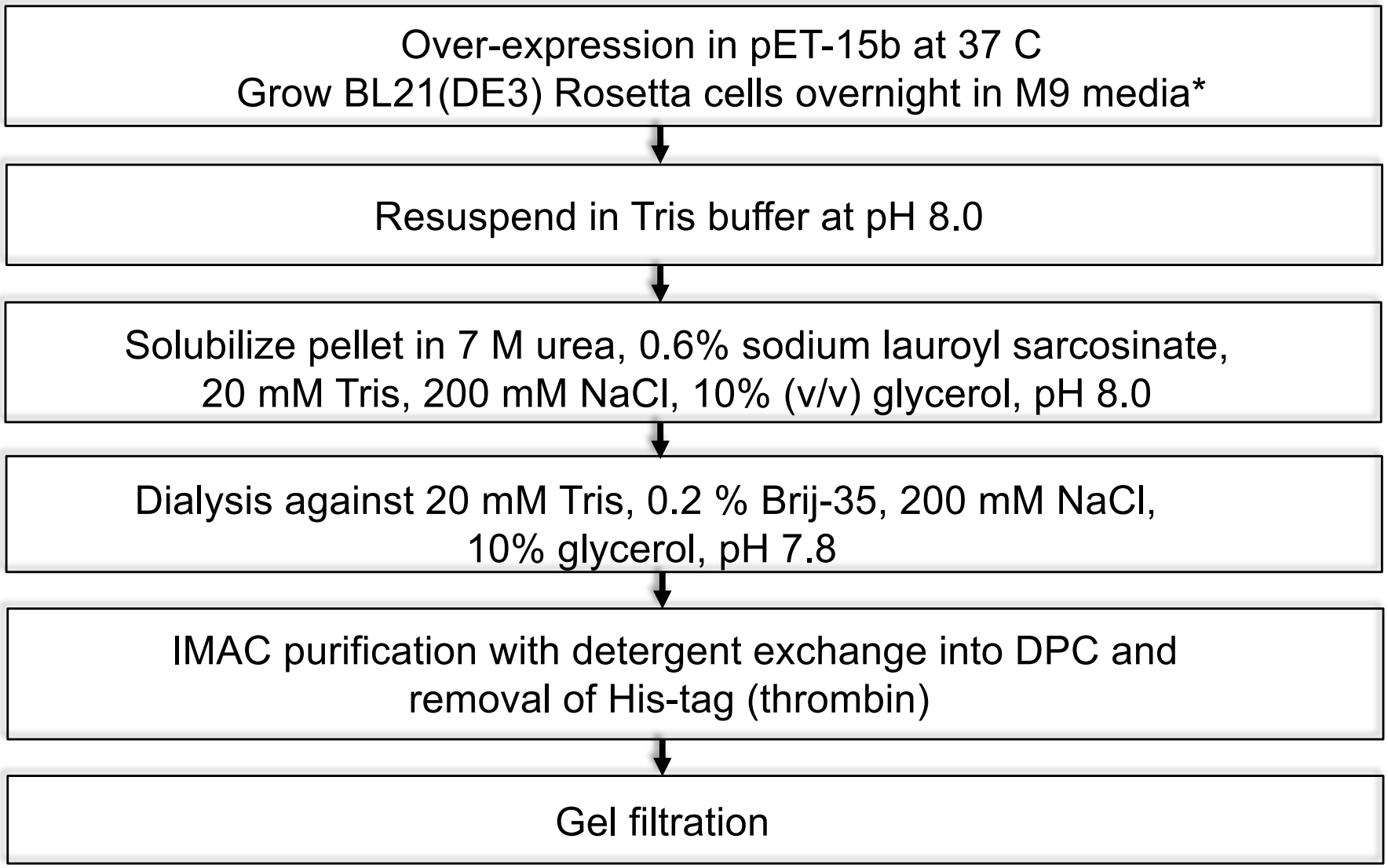
NMR structure of Bcl-xL. The C-terminal membrane anchor is shown in blue*

* A. Antignani, *et al.* J Mol Biol. 2015 Jul 3;427(13):2262-70, PDB ID: 1BXL

Our aims

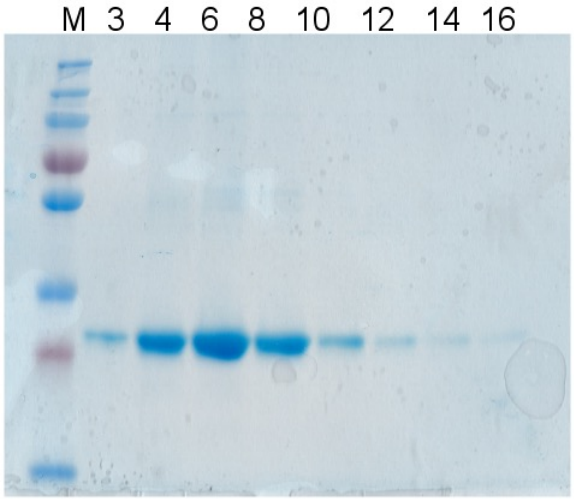
- **Solve the structure of the full-length anti-apoptotic membrane protein Bcl-2.**
- **Learn how the protein functions in the mitochondrial outer membrane.**
- **Study Bcl-2 together with Bax as it prevents it from forming membrane pores and trigger its cell-death inducing mechanism.**
- **Milligram amounts of protein is necessary when doing NMR and structural work.**

Development of an expression method for Bcl-2 in *E. coli*

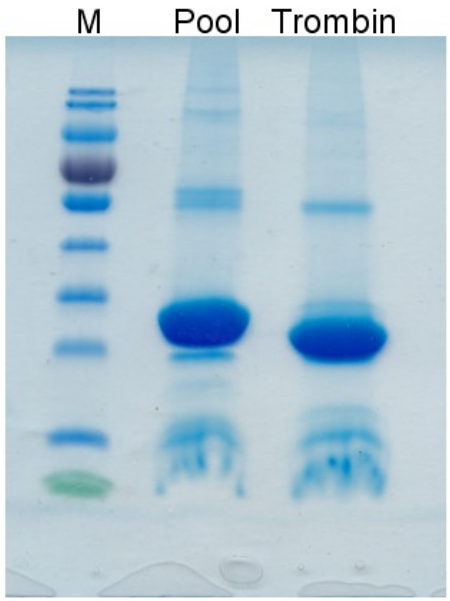


* *Protein Expression and Purification*, Elsevier 2020, Vol. 172 Ådén, Jörgen; Ul Mushtaq, Ameeq; Dingeldein, Artur P. G.; *et al.*

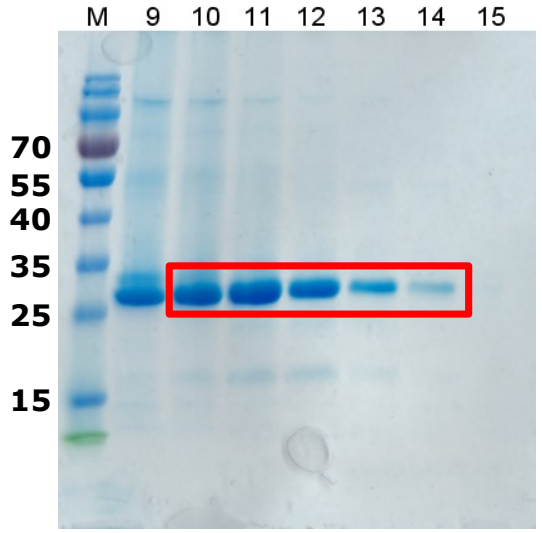
Expression and purification of Bcl-2 in *E. coli.*



Eluate from IMAC column



Separation of His-tag

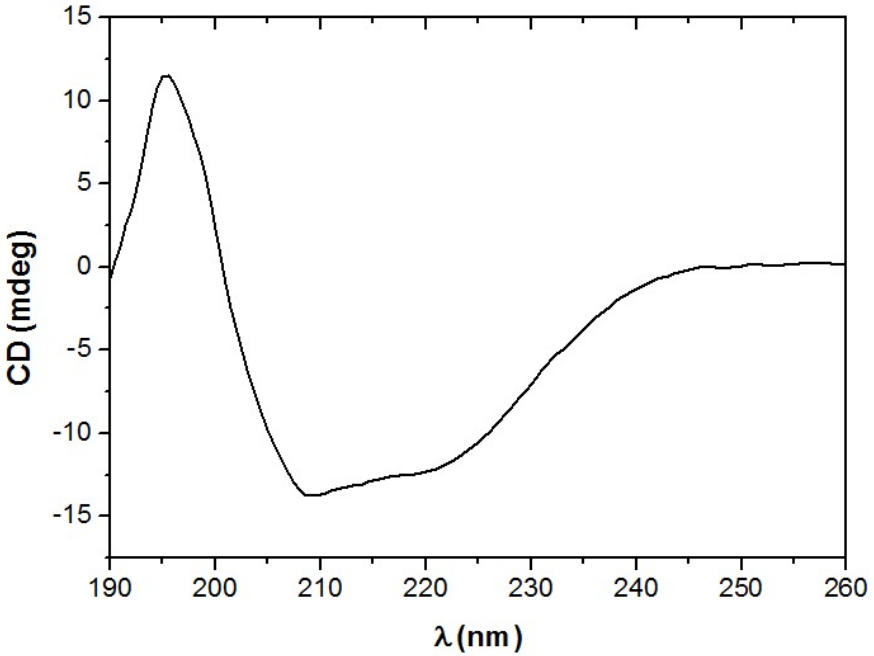


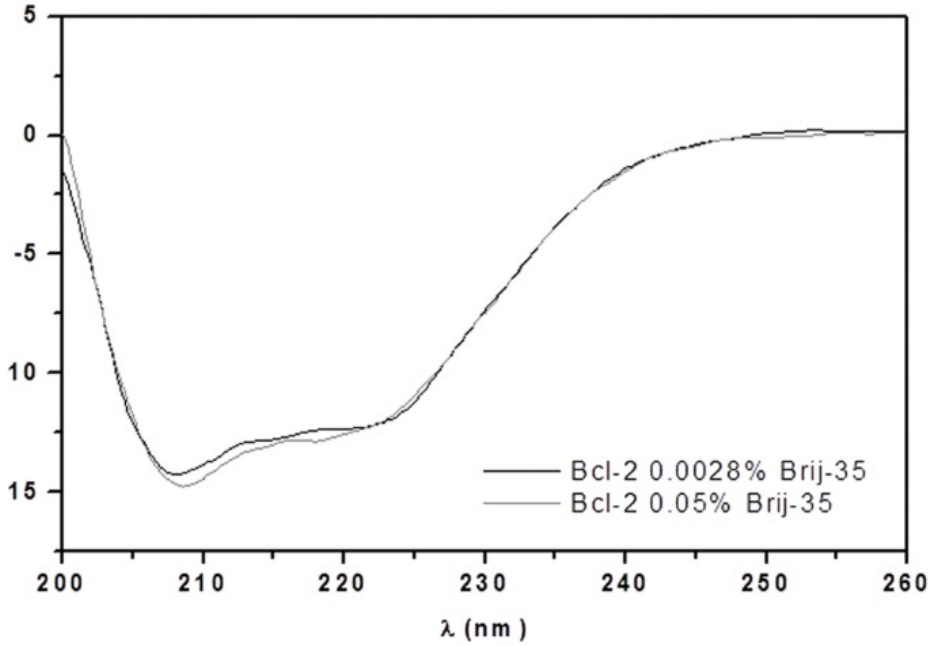
Gel filtration

Bcl-2

Expression of Bcl-2 can now be achieved in LB or M9 media to yield ^{2}H, ^{15}N or $^{13}C/^{15}N$-labeled protein

Far-UV spectroscopy confirms Bcl-2 fold



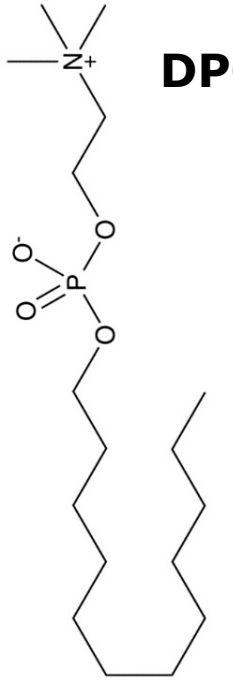


5 µM His-tagged Bcl-2 in 25 mM NaP, 20 mM KCl, 0.05 % Brij-35, 200 µM DTT, pH 7.4, 25 C
Expressed recombinantly in *E. coli*

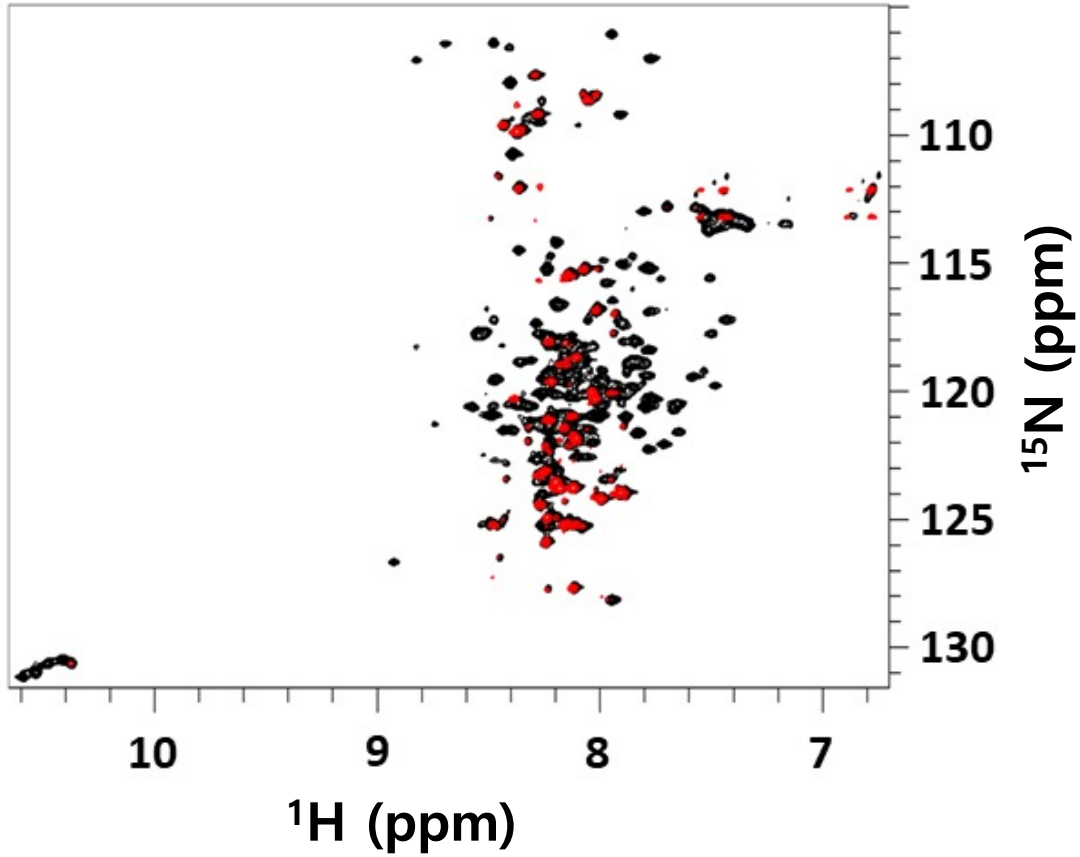
5 µM Bcl-2, in 25 mM NaP, pH 7.4, 50 mM NaCl, 25 C
Expressed previously using a cell-free expression system*

* G. Gröbner et al. **PLoS One. 2013; 8(4): e61452.**

Bcl-2 in DPC Micelles: Identification of flexible loop region residues

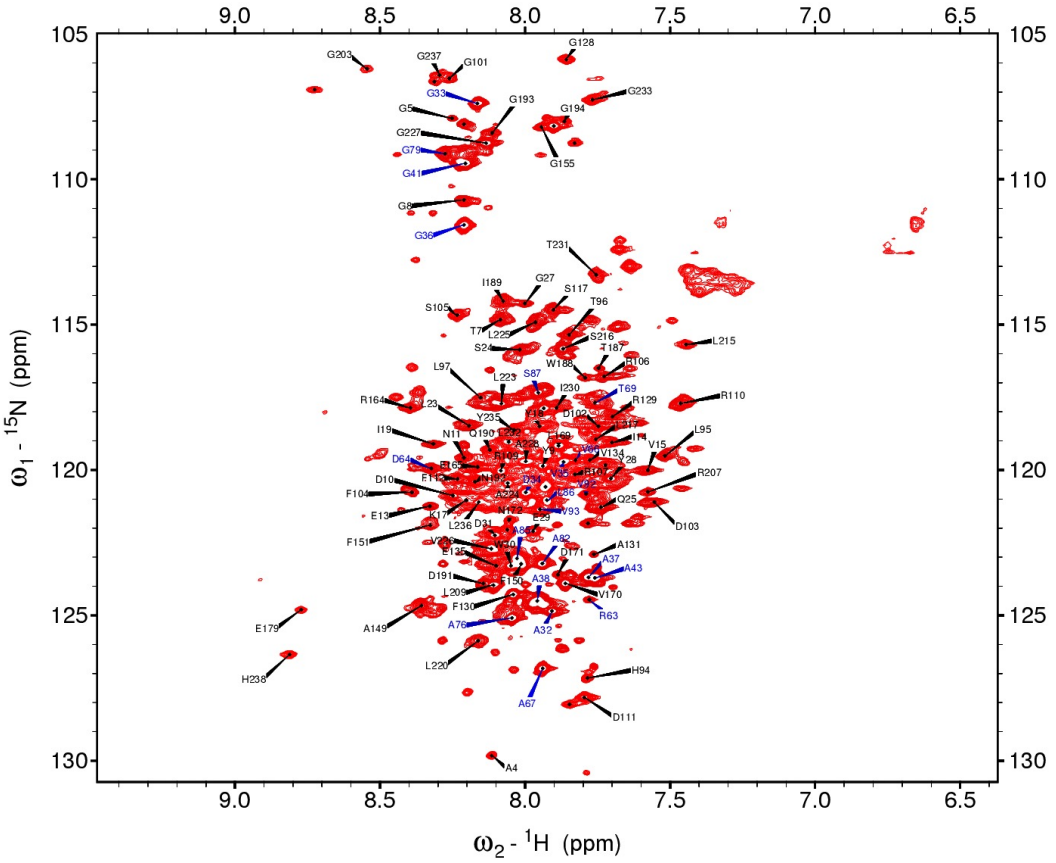


DPC



- We can identify the free loop region residues from T_2 experiments with longer T_2 delay ~ 410ms
- Those residues must be outside the membrane and micellar core region
- Which residues are involved?

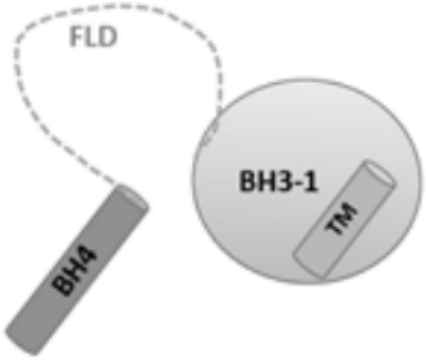
Sequence-specific assignment of flexible loop region residues by NMR



- All loop residues can be identified in the ^{1}H-^{15}N TROSY-HSQC spectrum.
- Residue-specific changes can be observed upon regulative activities such as monitoring of ligand/drug binding or interaction with other Bcl-2 proteins.
- Full-length spectrum of Bcl-2 is however crowded with several overlaps.

“Divide-and-conquer” strategy to improve the spectral resolution of Bcl-2

- Truncate the protein at various positions to overcome problems with overlap.
- Use several spectra together to help with residue assignments and/or to define functional regions within the protein.
- Use this strategy to aid structural determination.

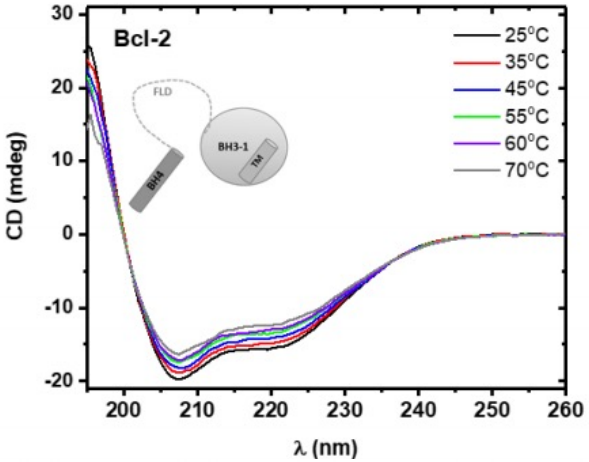


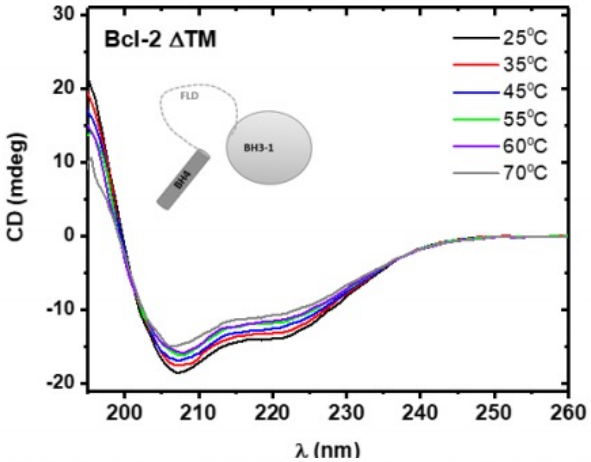
Strategy

- Make shorter Bcl-2 constructs by adding stop codons, terminating the sequence before its trans-membrane helix.
- Other constructs made to omit the BH4 domain and the FLD.

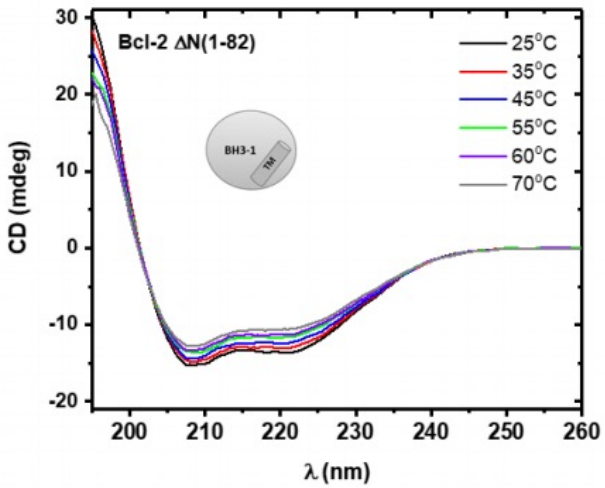
Far-UV CD of Bcl-2 and truncated variants in DPC micelles

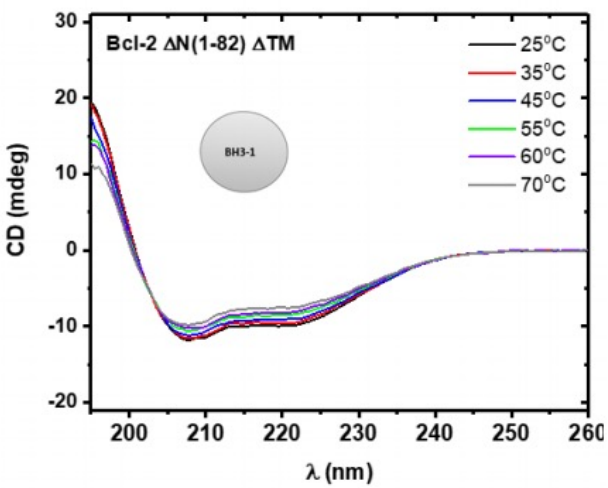
Partially soluble

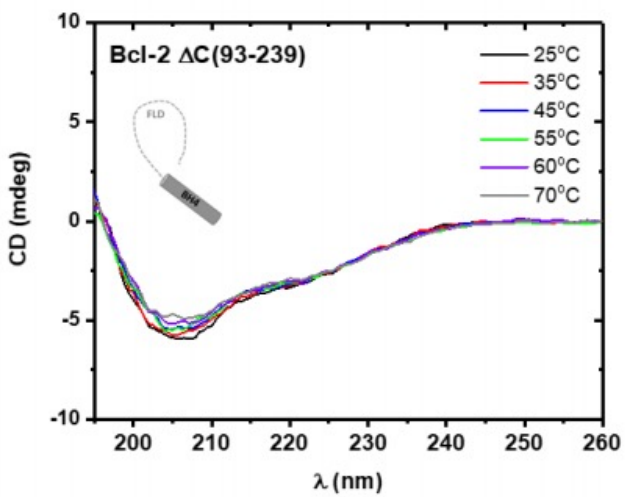




Recorded at pH 6.0 in DPC

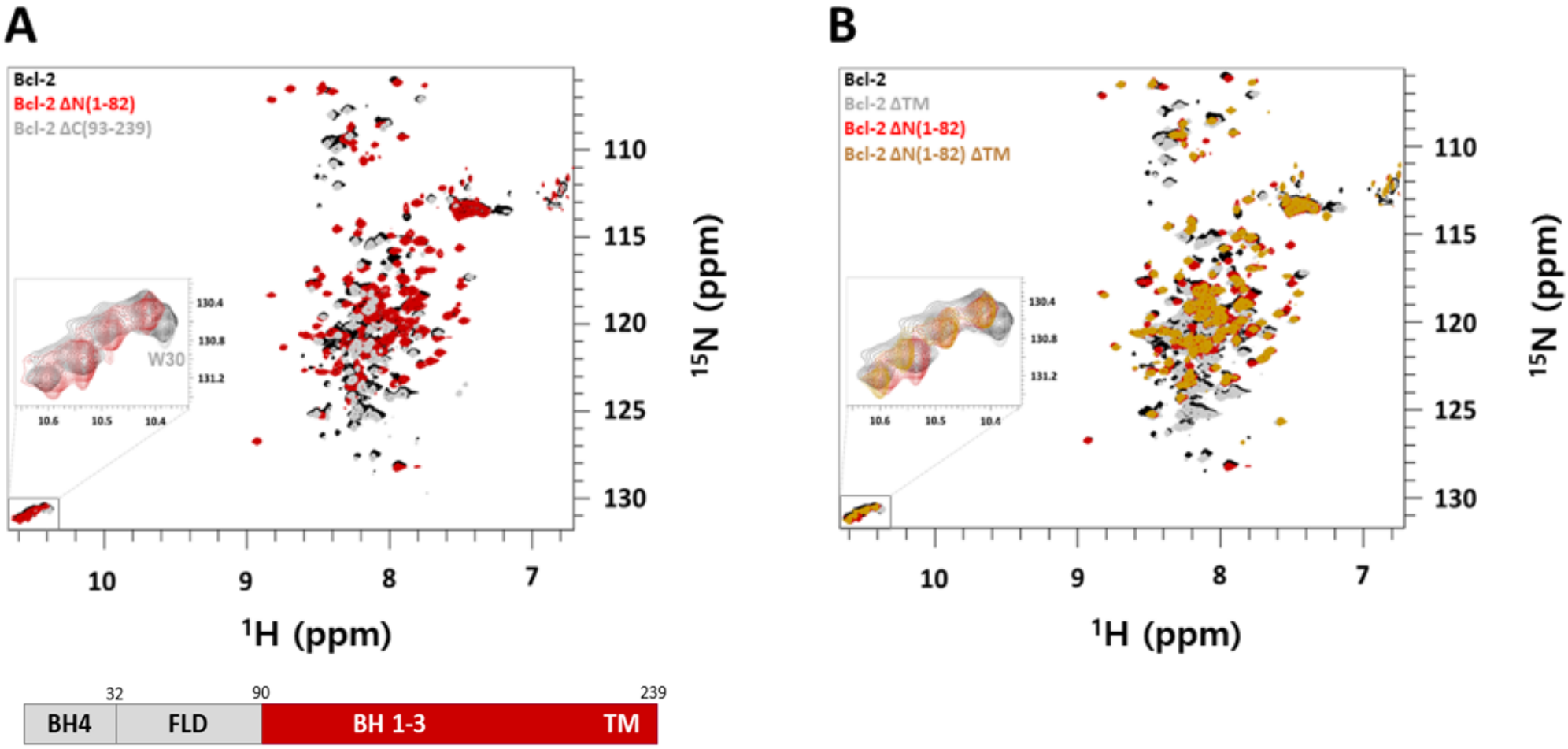






Molecules, MDPI, Vol. 26 2021, Ul Mushtaq, Ameeq; Ådén, Jörgen; Sparrman, Tobias; *et al.*

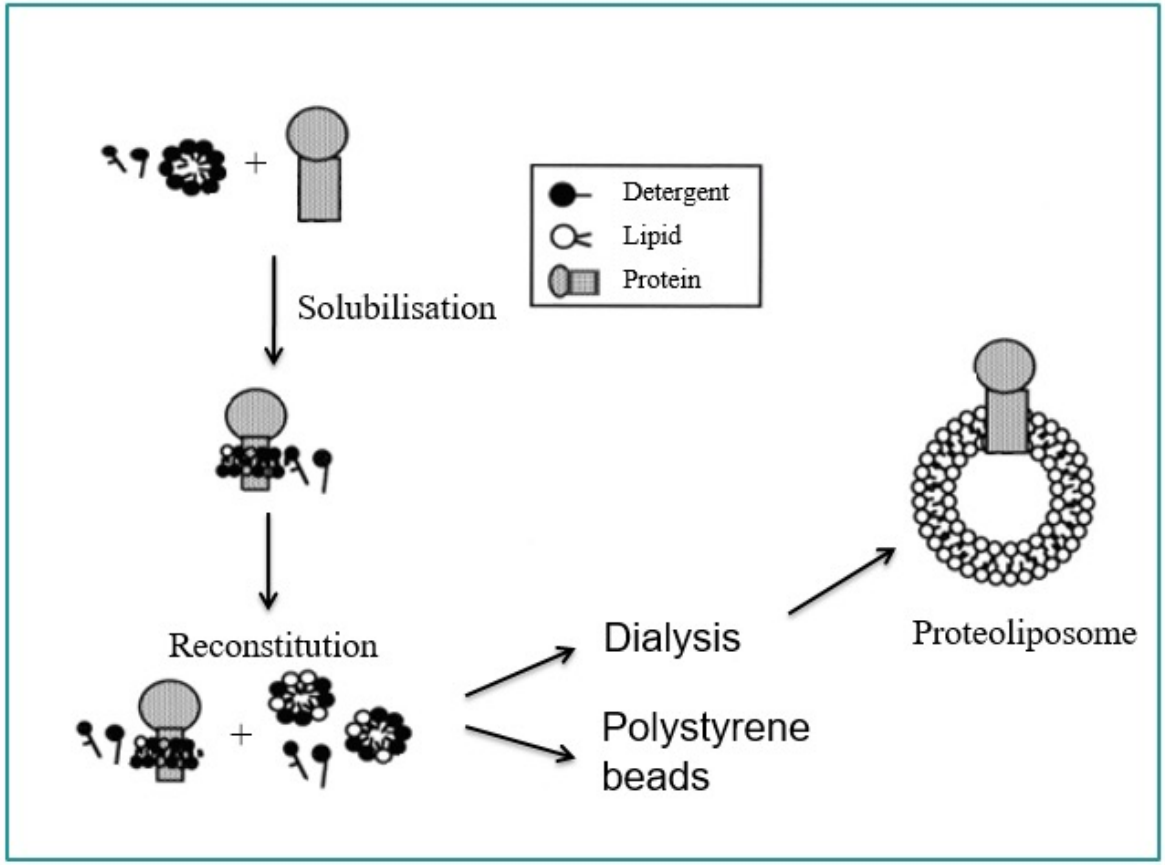
Characterization of truncated Bcl-2 constructs



- **Improved spectral resolution**
- **Identification of some residues by simple comparison**

Reconstitution of Bcl-2 into DMPC vesicles

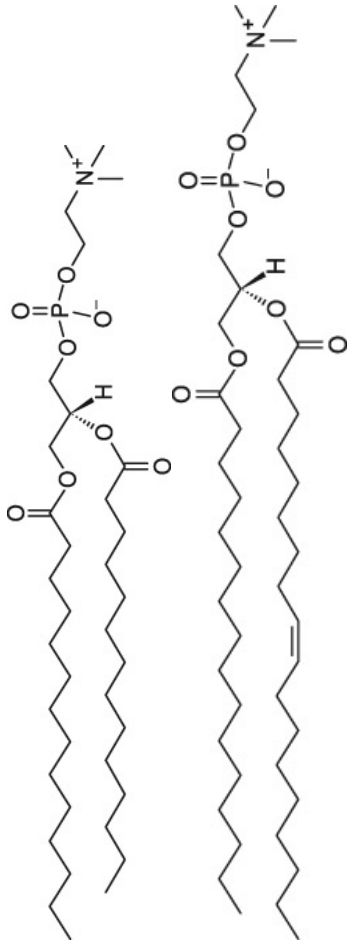
Incorporation of Bcl-2 into vesicles, for studies under native-like conditions

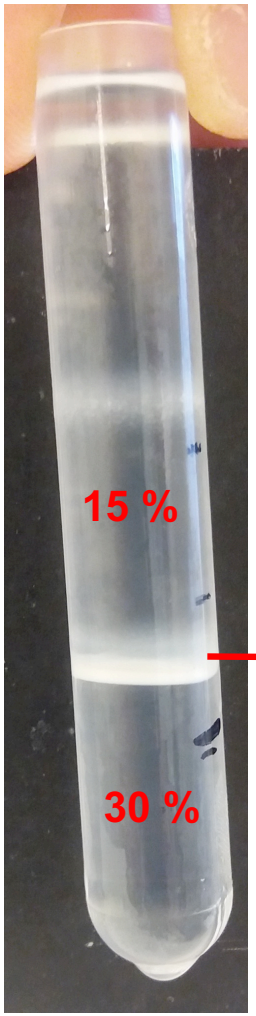


Adapted from Rigaud J.-L., Braz J Med Res, 2002

Reconstitution of Bcl-2 into proteoliposomes

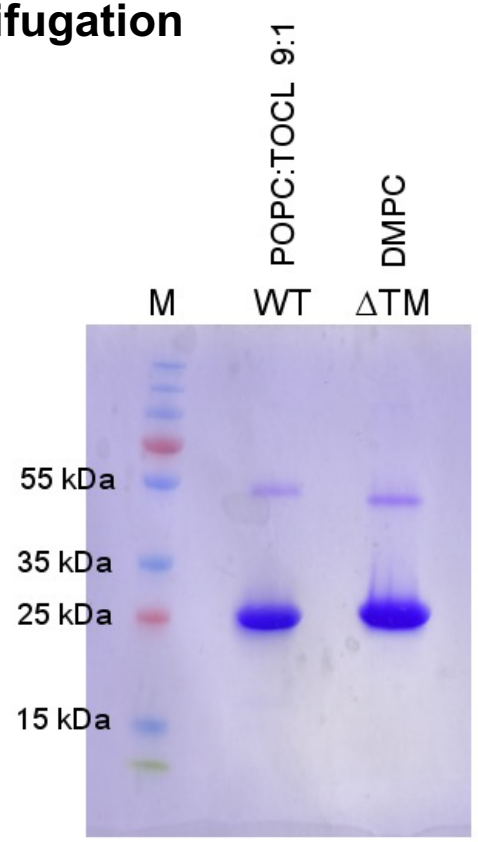
DMPC **POPC**





Bcl-2/DMPC

- **Removal of detergent with Bio-Beads SM-2 adsorbents at 4 C.**
- **After two days, proteoliposomes were obtained and isolated by ultracentrifugation**



SDS-PAGE of reconstituted Bcl-2 in DMPC and POPC/TOCL liposomes

Conclusions and future perspectives

- ✓ **An alternative expression method for the human Bcl-2 membrane protein has been established, yielding milligram amounts of protein.**
- ✓ **Bcl-2 has been successfully incorporated into DMPC and POPC/CL vesicles.**
- ✓ **DPC is a robust detergent for structural and functional studies.**
- ✓ **Different shorter Bcl-2 constructs can be used together to form a full picture of the intact Bcl-2 membrane protein.**

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