Chapter 5

Summary and Final Remarks

5.1 Summary

Comparative sequence analysis, carried out by Peer Bork (EMBL, Heidelberg) and co-workers, identified BRCT domains as novel domain family present in the C-terminal region of BRCA1 and other DNA-damage responsive cell-cycle checkpoint proteins. Functional inactivation of the BRCA1 BRCT region is highly correlated with the development of breast and ovarian cancer. Cell biological and biochemical studies revealed that BRCT repeats may activate transcription and bind phosphorylated peptide motifs present in target proteins upon activation of DNA-damage signaling pathways. As mutations in BRCT domains are frequently correlated with genomic instability and cancer development, the key function of BRCT proteins is likely consist in the assembly of protein complexes in response to DNA damage, thus eventually activating cell-cycle checkpoints.

To elucidate the molecular basis for the observed cellular functions, one project of this research work was concerned with structural studies on the BRCT region of human BRCA1. Firstly, the physical existence of the BRCT domains was verified by proteolytic dissection of the BRCA1 BRCT region, yielding the tandem BRCT repeat as the most stable fragment. The double BRCT repeat was very resistant to proteolytic degradation without being further cleaved to yield the predicted individual BRCT domains (BRCT-n and BRCT-c). However, when recombinantly expressed with the GroEL/GroES chaperone system, the isolated second BRCT domain (BRCT-c) was equally stable toward proteolytic degradation, thus indicating for the first time that single BRCT domains may fold properly even in the absence of an intramolecular BRCT tandem partner. Co-expression with the chaperonins GroEL and GroES significantly increased the fraction of soluble BRCT constructs, and protein (re)folding after cell lysis was further improved by adding ATP as well as Mg^{2+}, K^{+} and Cl^{-} ions to drive the GroEL/GroES reaction cycle *in vitro*. Analytical UCF and SEC showed that BRCT-c exists primarily as a monomer in solution.

NMR spectroscopy, first used to identify BRCT constructs suitable for structural analysis, turned out to be the method of choice to determine the three-dimensional structure of the second BRCT domain (BRCT-c). This also represents the first reported structure of a single, isolated BRCT domain from a BRCT tandem repeat. Comparison with the crystal structure of the complete BRCT double repeat showed that helices α1 and α3 of BRCT-c slightly reorient in the absence of the first BRCT domain (BRCT-n). This structural deviation suggests, together with previous functional and mutagenesis data, that BRCT-n may possibly modulate the transcriptional activation function of BRCT-c. This raises the intriguing possibility of allosteric cross-talk between the two BRCT domains and, by that, the precise fine-tuning of structure and function through direct interactions at the BRCT interface.

In a second project of this thesis work the catalytically impaired variant of a bacterial 1,3-1,4-β-glucanase, H(A16-M)$^{E105Q/E109Q}$, was used for co-crystallization experiments with a natural hexasaccharide substrate. 1,3-1,4-β-glucanases are highly specific enzymes that cleave β-1,4 glycosidic bonds in 3-O-substituted glucopyranosyl units within polysaccharides with mixed linkage. Crystals were obtained by sparse matrix crystallization screening and diffracted beyond 1.6 Å resolution. Structure solution by molecular replacement showed four enzyme-carbohydrate complexes in the asymmetric unit and unambiguously revealed a *tetra*-saccharide moiety (Glcβ4Glcβ4Glcβ3Glc) bound in the substrate binding cleft. The carbohydrate ligand, covering the glucosyl-binding subsites –IV to –I, clearly had a β-1,3 glycosidic linkage between the glucosyl residues located in subsites –II and –I as expected for natural ligands of 1,3-1,4-β-glucanases. Subsites +I/II were occupied by a network of well-ordered water molecules, suggesting that the hexasaccharide substrate used for crystallization had been cleaved, possibly by trace impurities of wild-type enzyme resulting from partial deamidation of Gln105 and Gln109. The crystal structure thus represents the non-covalent enzyme-product (E·P) complex. The high resolution diffraction data allowed unambiguous determination of sugar configuration, conformation and linkage-type. Compared to the Michaelis complex model, the tetrasaccharide ligand showed previously unanticipated strong stacking interactions with Phe92 in the catalytic subsite –I. The crystal structure of the E·P complex, together with structural information from the apoenzyme H(A16-M) and a covalent enzyme-inhibitor (E·I) complex, and kinetic and mutagenesis data from previous studies, yielded new insights into the structural basis of carbohydrate binding and processing, and allowed conclusions about the molecular basis for product release.

In summary, both NMR spectroscopy and X-ray crystallography have been successfully applied for the structure determination of two molecular targets:

(i) The isolated second BRCT domain (BRCT-c) from the human tumor suppressor protein BRCA1, and

(ii) The non-covalent E·P complex between the bacterial 1,3-1,4-β-glucanase variant H(A16-M)$^{E105Q/E109Q}$ and Glcβ4Glcβ4Glcβ3Glc, a natural tetrasaccharide ligand.

5.2 Concluding Remarks

Structural studies on biological macromolecules are usually highly demanding with respect to target selection, sample preparation and data acquisition. Thus, the field of structural biology has witnessed different approaches to combat the experimental challenges, such as the enormous time investment and the need for large amounts of biological material for screening purposes. In recent years, much effort has been made to establish industrial-like assembly lines for standardized large scale high-throughput structure determination. In fact this kind of shot-gun method may prove very efficient for easy-to-get targets, typically well soluble, stable cytosolic proteins or protein domains. However, the steadily increasing knowledge about structural and functional interactions in living cells demands for a rational, target-specific, and in a way more scientific strategy, thereby using the methods of structural biology beyond others as a means to unravel the molecular features of complex cellular networks. High-throughput structural biology methods, however, may become very valuable for screening purposes targeted at the structure-function relationship of small proteins or isolated protein domains, and the identification of novel protein folds. The high-throughput approach on the one hand, and target-specific structure determination of macromolecular complexes on the other may therefore considered complementary means to study the molecular basics of cell biology.

5.3 Overview of Experimental Techniques

Molecular Biology and Microbiology

- Polymerase Chain Reaction (PCR), molecular cloning
- Transformation and cultivation of *E. coli* cells

Protein Expression and Purification

- Co-expression of proteins with chaperonins (GroEL/GroES) and $tRNA^{Arg/Ile/Leu}$
- Improved protein folding *in vitro* using the GroEL/GroES system and Mg^{2+}-ATP
- Affinity purification, size exclusion chromatography (SEC), ion-exchange (IEX) and hydrophobic interaction chromatography (HIC)

X-ray Crystallography

- Crystallization, data collection, model building and refinement, structure analysis
- Software: DENZO, XPLOR, CNS, REFMAC, CCP4 suite

NMR Spectroscopy

- ^{13}C and ^{15}N isotopic labeling, resonance and NOE assignment, structure calculation
- Interaction mapping with binding partners
- Software: XWIN-NMR, AZARA, ANSIG, XPLOR, CNS, CYANA

5.4 List of Personal Publications

1. Gaiser O. J., Oschkinat H., Heinemann U. & Ball L. J. (2004). ^{1}H, ^{13}C and ^{15}N resonance assignments of the C-terminal BRCT domain from human BRCA1. J. Biomol. NMR **30** (2), 221-222.

2. Gaiser O. J., Ball L. J., Schmieder P., Leitner D., Strauss H., Wahl M., Kühne R., Oschkinat H. & Heinemann U. (2004). Solution structure, backbone dynamics, and association behavior of the C-terminal BRCT domain from the breast cancer-associated protein BRCA1. Biochemistry **43** (51), 15983-15995.

3. Gaiser O. J., Piotukh K., Ponnuswamy M. N., Planas A., Borriss R. & Heinemann U. (2006). Structural basis for the substrate specificity of a *Bacillus* 1,3-1,4-β-glucanase. J. Mol. Biol. **357** (4), 1211-1225.