Imre Törö & Carlo Carolis

1. Structural studies of Holliday junction resolvases from yeast and archaea

Holliday junctions (four-way junctions) are universal intermediates in repair and reorganization of DNA by homologous recombination. They are mobile links between two homologous DNA duplexes and generate new segments of heteroduplex DNA by branch-migration. A crucial step is the final resolution of the junction without loss of nucleotides. Holliday junction-resolving enzymes mediate the termination process by recognizing DNA four-way junctions and introducing symmetrical nicks. Members of this ubiquitous family of structure-specific endonucleases function as dimers and require divalent cations for cleavage. We have solved the crystal structures of cruciform cutting enzyme 1 (CCE1) from *Candida glabrata* (341 aa, 39kDa) and Holliday junction cutting enzyme (Hjc) from the hyperthermophile archaeon *Archaeoglobus fulgidus* (136 aa, 15.5kDa) at 2.8 and 1.7Å resolution, respectively. They represent two structurally distinct resolvase families with the same biological function, but exhibiting clearly different substrate specificities. Recently, we have solved the complex of Hjc with a synthetic junction at 3.3Å resolution.

2. All the proteins were expressed in E. coli (BL21(DE3), Bl21(DE3)pLysS, CodonPlus RIL, Rosetta) as protease cleavable fusions with solubility/affinity tags using standard technics. Neither Hjc nor Cce1 present any biological risk. All data collection at ESRF were done 100K. Crystals and datasets:

a) Hjc wild type (data collected on 27.02.07, ID14-3):

1.7Å resolution, SG=$P3_221$, cell parameters: a=37.56Å c=272.47Å, 2 mol./ASU

b) Hjc K90A+K91A surface mutants (data collected on 30.06.07, ID23-1):

1.55Å resolution, SG=$P2_1$, cell parameters: a=57.50Å b=75.48Å c=60.92, β =115.34°, 4 mol./ASU

c) Cce1 ammonium sulfate based condition (data collected on 27.02.07, ID14-3):

2.8Å resolution, SG=$I4_122$, cell parameters: a=206.22Å c=87.11Å, 2 mol./ASU

d) Hjc-junction complex (data collected on 30.06.07, ID23-1):

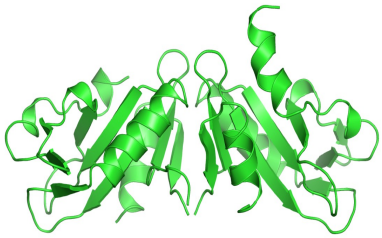
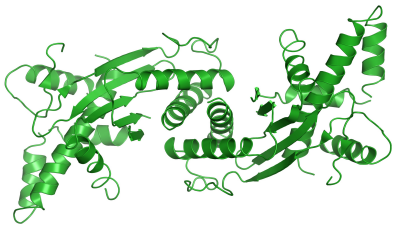
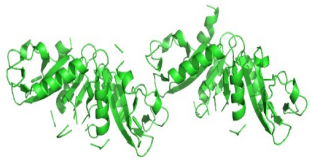
3.3Å resolution, SG=$P6_222$, cell parameters: a=123.18Å b=125.15Å

a-b) are refined structures, c) is partially built, the modelling of d) is in progress.

3. For the next BAG we would probably need 3 shifts on undulator beam lines, due to the weak diffraction of our DNA-protein complex crystals. No special technics would be involved, only cryo-cooling is a requirement for our projects.

4. We expect to improve resolution of partially built resolvase (Cce1) structures in order to reach publication quality. The crystallization of various resolvase/Holliday-junction complexes is under way. These complex structures might allow us to better understand the molecular mechanism of the last step of homologous recombination: the resolution of the Holliday junction. However, the notoriously weak diffraction of such

crystals necessitates the application of high brilliance synchrotron radiation.

		
HJC wt:	CCE1	HJC surface mutant

Fariborz Nasertorabi

Sirv2 Hjc is an archaeal virus Holliday junction resolvase infecting the extremely thermophilic archaeon Sulfolobus islandicus. As all resolvases it functions by introducing symmetrical nicks in equivalent strands of the 4-way junction without loosing any nucleotides.

We collected a data set at 3.8Å resolution at beam line BM 30A.
(Cell parameters: a=80.45Å b=87.56Å, c= 59.45 Å, β=96.87^0).

With a few optimization steps we have produced better crystals that may diffract in a reasonable range in a stronger beam line. We have also obtained crystals from this protein in complex with a 4-way DNA junction, that will be tested.

Esther Lenherr

γ-glutamate-cysteine-ligase (GCL)

1. Biological impact

Glutathione (GSH) is an important cellular redox buffer which by dimerization helps the cell to cope with different external stress stimuli. Further GSH can post-translationally modify proteins and serves as a major storage form of reduced sulfur. GCL catalyzes the first and rate-limiting step of GSH synthesis.

2a. Previous work

We described the structure of plant *Arabidopsis thaliana* GCL (Hothorn, JBC, 2006) and found an interesting new feature, which might serve as an additional activity-regulating element that is not present in bacterial homologues. In the oxidized state a disulfide bridge fixes a b-hairpin into a position, which allows free diffusion of substrates into the active site. A further disulfide bond is found near the dimer interface. We propose that the two disulfide bridges together allow a precise, redox-dependent regulcatio of the enzyme activity.

Structures of AtGCL with in vivo relevant ligands have been solved recently. The unpublished structure with ADP and ihbibitor shows that the nucleotide binds similar as it has been seen in the *E.coli* structure. Further the inhibitor, which mimics the reaction product and is very similar to GSH, suggested GSH as a feedback inhibitor. The structure with the GSH-analog has been solved with data collected at ESRF at BM16.

Exp host; E.coli (Arabidopsis DNA)

Resolution; 1.7A (GSH-structure), 1.8A (ADP-BSO-structure)

Unit cell&angles; 58.961 108.713 83.490 90.00 97.01 90.00

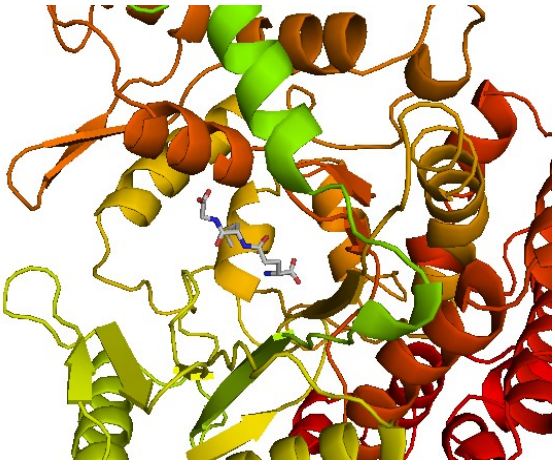
Cryo20% Ethyleneglycol

2b. Current status

We would like to further investigate the reaction mechanism and the regulation of GCL by the suggested redox-dependent mechanism. Crystals under denaturing conditions using new constructs concerning protein length and cysteine mutants will be used to see the expected shift of the b-hairpin under reducing conditions.

3. Technique

Crystals are grown by the hanging-drop vapor diffusion technique. 3 more shifts needed.



4. Expected benefits/significance of data collection

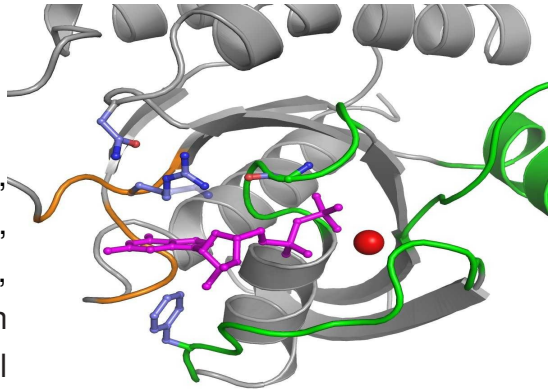
We would like to further investigate the reaction mechanism and the regulation of AtGCL. The suggested redox-dependent mechanism to regulate GCL activity might help to elaborate plant GCL regulation. Crystals under denaturing conditions using new constructs concerning protein length and cysteine mutants will be used to see the expected shift of the b-hairpin under reducing conditions.

Annabel Parret

1. Neurofibromatosis type I (NFI) is an autosomal dominant disease caused by mutations in the /NF/1 gene, which encodes the 320 kDa protein neurofibromin. Neurofibromin contains a central GAP-related domain (GRD) that acts as a Ras-specific GTPase activating protein (RasGAP) and a Sec14PH module. previously characterized in our group. Although crystal structures of this central portion of the protein (20%) have been determined, 80% of the protein has yet to be analyzed functionally and structurally. To this respect, we have developed a protocol to produce full lenght human NF1 protein in insect cells. This recombinant protein is currently included in crystallization trials.
beamtime: test case

2. **Characterization of activating Ras mutants involved in Costello syndrome**
Ras proteins are small GTPases encoded by proto-oncogenes that are mutated in ~30% of human tumors. In addition, several studies show the presence of *HRAS* mutations in approximately 85% of individuals with Costello syndrome. Costello syndrome is a mental retardation syndrome characterized by high birth weight, postnatal growth retardation, coarse face, loose skin, cardiovascular problems and tumor predisposition. In this project, we have characterized a missense mutation in codon 117 (K117R). The *HRAS* K117R mutation results in constitutive activation of the RAS/MAPK pathway similar to the typical G12S and G12A mutations. Recombinant HRAS K117R demonstrates normal intrinsic GTP hydrolysis and responsiveness to GTPase-activating proteins, but the nucleotide dissociation rate is increased 80-fold. To investigate the properties of the HRAS K117R mutant at a structural level, we determined the crystal structure of GDP-bound HRAS K117R (published in Denayer et al., 2007). The mutant protein was overexpressed in *E. coli*. For structure determination of the GDP-bound K117R mutant, crystals were grown at room temperature by the vapor diffusion technique in hanging or sitting drops by mixing 1 l of protein (15 mg/ml in TrisHCl, MgCl2, DTT, glycerol, pH 7.6) and 1 l from a total reservoir (PEG3350, TrisHCl pH 7.6). A data set to 1.49 Å resolution was collected on beamline ID29 (with cryo) (22/09/06). The structure was solved and protein coordinates and structure factors were deposited in the PDB database with code number 2QUZ. Our data indicate an altered interaction pattern of the side chain that is associated with unfavorable nucleotide binding properties further supporting our biochemical data. In future, we will analyze additional Ras mutants also involved in Costello syndrome in a similar way.
ESRF, ID29

Space group	R32
Unit cell (Å)	a=b=92.1, c=116.3
Resolution range (Å)	26-1.49



Reference: Denayer E., Parret A., Chmara M., Schubbert S., Vogels A., Devriendt K., Fryns J.-P., Rybin V., de Ravel T.J., Shannon K., Cools J., Scheffzek K., Legius E. (2007). Mutation Analysis in Costello Syndrome: Functional and Structural Characterization of the HRAS p.Lys117Arg Mutation. Human Mutation (in press).

Kanchan Anand

Structure determination of HIV CyclinT1-Tat-TAR complex

Background

The Human immunodeficiency virus type 1 (HIV-1) is the causative agent of Aquired immune Deficiency Syndrome. Transcription of the HIV-genome requires the interaction of the viral transcription activator Tat, a transactivating response element (TAR) present at the 5' end of nascent viral transcripts.

Destroying the CyclinT1-Tat-TAR interaction is thought to be of therapeutic impact in order to control AIDS pathogenesis. In this proposal we want to solve the structure of HIV Cyclin T1-Tat-TAR complexes. Crystals of biologically active Cyclin T1-Tat-TAR protein have been obtained, which diffracted to 3.3 A at ESRF ID23-1 beamline. Several experiment on different constructs have been done in order to optimize the crystal quality for better diffraction.

Experimental method; specific requirements

We would like to collect native data sets and for the case that Molecular Replacement attempts fail a MAD data set of SeMet labeled protein.

Results expected

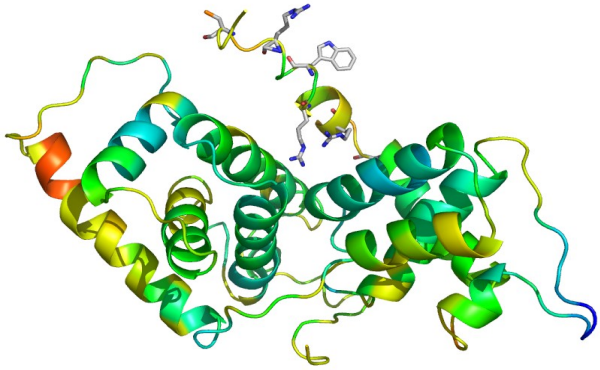
We expect to collect the high resolution data at 3rd generation synchrotron radiation source like SLS to solve the phase problem. In case the Molecular Replacement method doesn't work, we would like to collect the SeMet data of CyclinT1-Tat-TAR complex.

Estimate and justification of the beamtime

Due to limitations of crystal diffraction, we have performed many different experiments (e.g. different constructs, cryo-conditions, dehydration and soaking etc.) to optimize the better quality. As the optimal crystal-size is obtained in about 10 days and then they need about a week for dehydration experiment for few of the constructs, we would estimate at least **3 shifts** with the gap of 2-3 weeks among each shift.

Reference

Anand, K., Schulte, A., Fujinaga, K., Scheffzek, K., Geyer, M. (2007) Cyclin Box Structure of the P-TEFb Subunit Cyclin T1 Derived from a Fusion Complex with EIAV Tat. J. Mol. Biol. (2007) **370**: 826-836.



Stefan Welti

Structural analysis of Neurofibromins Sec14-PH domains in complex with ligands

1. Neurofibromatosis type I is a common inherited neurocutaneous disorder caused by alterations in the *NF1* gene encoding the Ras specific GTPase activating protein (GAP) Neurofibromin. Beside various other complications, the clinical manifestations include the formation of benign peripheral nerve sheet tumors called neurofibromas, which can progress to malignant forms. Recently we could solve the structure of a neurofibromin module including a Sec14- and a pleckstrin homology (PH) – like domain in complex with lipid ligands. To further characterize the lipid binding properties of the Sec14-like domain, we prepared protein bound to only one species of lipids. We prepared as well Sec14-PH fragments modified to resemble missense mutations found in patients to identify functional important parts of the domain architecture and the lipid biding site by comparison with the unmodified structure. This information will guide further biochemical characterization, help us to identify cellular functions of the module and finally give new insights into the mode of action of the disease.

2. All protein variants were expressed as His-Tag fusions in the *Escherichia coli* CodonPlus RIL cell line and purified using standard procedures. No one of the proteins present any biological risk. All datasets were collected under cryogenic conditions at ESRF:

a) Sec14-PH : Phospatidylcholine (14.07.2006, ID23-1)
   2.7Å; SG:72 $P4_12_12$; a=b=113.26, c=125.16

b) Sec14-PH : Phospatidylethanolamin (14.07.2006, ID23-1)
   2.7Å; SG:72 $P4_12_12$; a=b=113.64, c=125.26;

c) Sec14-PH TD1699-1713 (12.07.2007 BM30A)
   patient derived duplication of a linker region
   5Å; SG:181 $P6_422$; a=b=104.62, c=117.31; beta=120°

a,b) resolution not high enough to see the ligand binding mode in greater detail; c) resolution to low for precise comparison with the wild-type structure

3. For the next BAG we would need approximately 3 shifts on undulator beam lines to obtain high resolution data for detailed comparison of mutant and wild-type protein. Apart from cyro-cooling, no special techniques are required,

4. We expect to obtain high resolution data from further optimized crystals of the Sec14-PH TD1699-1713 and a newly crystallized patient derived mutant (Sec14-PH I1584V). Further constructs are already expressed and in the crystallization stage at the moment.
Comparison of high resolution structures of patient derived mutations with the wild-type structure would show us functionally important parts of the Sec14-PH module, guide our biochemical experiments to characterize this functions and give valuable insights into the mode of action of the disease.