4. Biochemical and Molecular Biological Methods

4.1. Cloning of Expression Constructs

Biochemical protein studies and crystallographic analyses in particular depend on large amounts of protein. Most proteins cannot be purified in suitable quantities from their natural source. Instead, there are various methods to overproduce the recombinant protein in a foreign host (i.e. *E. coli*, yeast or virus-infected higher eukaryotic cells). Here, overproduction in *E. coli* was employed. For this purpose, the gene of interest was PCR amplified and flanked with restriction sites. It was inserted into an expression vector downstream of a strong, inducible promoter. Expression constructs used here are based on the pET system (Novagen, Madison, WI) with thrombin-cleavable N-terminal His_6-tags to simplify the purification procedure.

Different portions of the cloned cDNA coding for human ADAR1 (Genbank accession number U10439) were PCR-amplified and inserted into the expression vector pET28a (Novagen) resulting in N-terminal His_6-tagged fusion proteins. In detail, Za131 (residues 96-226), Za77 (133-209) and Zab236 (133-368) were PCR amplified using complementary primers flanked with restriction sites at their termini (chapter 4.2). The PCR setups were desalted (4.3) and the DNA ends digested with the appropriate restriction enzymes (4.5). The entire setup was electrophoresed on an agarose gel (4.6); bands of the correct size were extracted and subcloned into the NdeI-HindIII sites (Za131 and Za77) or the NheI-HindIII sites (Zab236) of the multiple cloning site of pET28a, resulting in the vectors pZa131, pZa77 and pZa236, respectively (4.7). The ligation reactions were used to transform competent *E. coli* cells (4.8; 4.9). Colonies grown on selective agar plates were screened for the presence of the desired plasmid construct via colony PCR (4.10). Another construct, ZabΔl, missing one of the two 49 aa linker modules separating the $Z\alpha$ and $Z\beta$ motifs, was created from pZab236 as follows: the 1.1 kb SphI-HindIII restriction fragment was digested with the restriction enzyme DrdI, resulting in two cleavage sites at identical locations at nucleotide positions 789 and 936 (numbers according to GenBank accession number U10439). The resulting DNA fragments were deproteinized and precipitated (Sambrook *et al.*, 1989). After incubation with T4 DNA Ligase (25° C, 4 h) the reaction mixture was analyzed on an agarose gel.

The 930bp ligation product was isolated and subcloned in the 5 kb SphI-HindIII restriction fragment of pET28a, resulting in the vector pZabΔl. To ensure that the plasmids were correct, they were analyzed by restriction digestion and the coding regions were sequenced following the dideoxy chain termination reaction (4.11).

4.2. PCR Amplification of DNA Fragments

PCR reaction (50 µl)

Template DNA (50 ng / µl)	4 µl
Primer I (1 µM)	5 µl
Primer II (1 µM)	5 µl
10x PCR buffer	5 µl
10x dNTP (2.5 mM / each)	5 µl
dH_2O	add 50 µl
Pfu DNA Polymerase	2.5 U

After mixing the sample, the template was denatured at 95 °C for 2 minutes. The following temperature program was used for 30 cycles:

denaturation	95 °C	45 seconds
primer annealing	55 °C	45 seconds
primer extension	72 °C	2 minutes / kb
final extension	72 °C	5 minutes

4.3. Desalting PCR Products

PCR products have to be purified before restriction digestion.
For this purpose, PCR reactions were desalted on MicroSpin G-25 columns (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

4.4. Plasmid Preparation

Plasmid preparations were carried out using Mini-/Maxi-prep kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

4.5. Restriction Digestion

Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and reaction conditions applied as recommended by the supplier.

4.6. Gel Purification of DNA

DNA fragments were purified after restriction digestion on horizontal agarose gels in 1x TAE buffer medium. The bands were visualized under UV light after staining the gel in an ethidium bromide solution. Appropriate bands were cut out from the gel and the DNA isolated using Qiaex gel extraction kits (Qiagen, Hilden, Germany).

4.7. Ligation

Reaction mix:

restriction digested vector	100 ng
restriction digested PCR fragment	30-300 ng
10x ligase buffer	1.5 µl
dH_2O	add 14 µl
T4 DNA ligase	1 µl

Three ligation reactions were set up in parallel, with varying amounts of PCR fragment and a constant amount of digested vector. In addition, a negative control without any PCR fragment was set up. Reactions were incubated at 24 °C for 2 h and sometimes for additional 12 h at 16 °C.

4.8. Preparation of Competent *E. coli* Cells

Cells were made competent following the RbCl method (Hanahan, 1983). A single *E. coli* colony was picked from a LB-plate and transferred to 10 ml of LB liquid medium. After incubation at 37 °C overnight, 500 ml of LB liquid medium were inoculated with the preculture and grown to an optical density at 600 nm of 0.5-0.7. The culture was quickly chilled on ice and the cells were spun down at 4 °C and 3000 g for 5 min. All subsequent steps were strictly done at 4 °C with prechilled solutions. The cell pellet was resuspended in 10 ml TFB I, diluted with 140 ml TFB I and incubated for 2 hours. Following another centrifugation step (4 °C, 3000 g, 5 min), cells were resuspended in 20 ml TFB II and aliquoted in 100 µl samples in 1.5 ml sample tubes. The aliquots were shock frozen in liquid nitrogen and stored at –70 °C.

4.9. Transformation

Frozen aliquots of competent *E. coli* cells were thawed on ice and 3 µl of a ligation reaction added. Gently mixed, the cells were kept on ice for 30 min and then incubated at 42 °C for 45 sec. The cells were incubated another 2 min on ice, then 1 ml of LB medium / 2 % glucose were added and the culture incubated at 37 °C. Cells were spun down in a tabletop centrifuge (10.000 rpm, 2 min), resuspended in 50 µl LB medium and plated on LB selection plates. Plates were incubated at 37 °C until colonies appeared.

4.10. Colony PCR

Transformation of ligated vectors is not an error-proof process. Therefore, transformed colonies must be analyzed to insure that the vector sequence is correct. A fast method to ensure the presence of the ligated DNA insert in the vector is colony PCR. A small amount of the total bacterial DNA is used as template. The DNA is amplified with primers, which are chosen such that correct inserts can be detected afterwards on agarose gels.

Cells from a single *E. coli* colony were picked with a sterile toothpick, transferred first to a PCR reaction (25 µl / 1U Taq DNA Polymerase) and then onto a master LB plate. This procedure was repeated typically for 12-24 colonies. A 30-cycle PCR reaction was carried out and the products were

analyzed on agarose gels. 2-3 clones with correct vector inserts were picked from the master plate and 5 ml LB liquid cultures grown over night. Plasmid DNA from these cultures was isolated and further analyzed by DNA sequencing.

4.11. DNA Sequencing

Recombinant genes have to be sequenced to detect possible frameshifts and point mutations as a result of the applied PCR and ligation techniques. Here, the dideoxy chain termination reaction using the Sequenase V2.0 kit (Amersham, Uppsala, Sweden) was employed. The reactions were analyzed autoradiographically using α-^{35}S-dATP for labeling.

4.12. Denaturing Double-Stranded DNA

To obtain single-stranded DNA, 5 µg of plasmid DNA was diluted with dH_2O to a volume of 100 µl. 10 µl of 2 M NaOH and 1 µl of 0.5 M EDTA were added and the solution incubated for 10 min at 24 °C. The DNA was ethanol precipitated according to standard protocols (Sambrook *et al.*, 1989).

4.13. Sequencing Reaction

The sequencing reaction was carried out according to the manufacturer's guidelines in the Sequenase V2.0 kit (Amersham, Uppsala, Sweden).

4.14. Gel Electrophoresis

A 6 % denaturing urea polyacrylamide gel was cast using premade Sequagel solutions (National Diagnostics, Atlanta, GA). Electrophoresis was carried out on a Sequencing Gel Electrophoresis System 2 (GibcoBRL, Rockville, MD) device. TBE long-range was used as running buffer. The gel was run at 95 W heating the gel to around 50 °C. After electrophoresis, the gel was blotted on Whatman paper, covered with plastic film, dried on a gel dryer and exposed at –70 °C for 8 to 12 h on a Kodak BioMax MR film.

4.15. Site-Directed Mutagenesis

For heavy atom derivatisation of protein crystals accessible cysteine residues are the preferred binding sites for many metal ions. Cysteine residues can be introduced into a protein by site-directed mutagenesis of the encoding gene. For this purpose, forward and reverse mutagenesis primers, which are complementary to the coding region except for the mutation site of interest, were annealed on an expression vector. The primers were extended using the PCR technique. The newly synthesized vectors differ from the original template propagated in bacteria, by having nicks at the 5'-end of the primers and, more importantly, by not being methylated. This difference is employed by digesting the reaction mixture with the restriction enzyme Dpn I, which cuts only methylated DNA. Subsequent transformation of bacteria with the digested reaction mixture results in bacteria harboring plasmids with the mutated gene. The practical details of the described method are described in the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

4.16. Overproduction of Recombinant Proteins

NovaBlue (DE) bacterial cells harboring the pET-28a-derived expression vector were scraped off a frozen aliquot and used to inoculate 25 ml of LB medium / 100 µg / ml kanamycin. After incubation overnight at 37 °C, the preculture was added to 1 l LB medium supplemented with 2 % (w / v) glucose and 100 µg / ml kanamycin. Expression of the recombinant gene was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an optical density at 600 nm of 0.7-0.9. 2 h later, cells were harvested by centrifugation (5000 rpm, GS-3 rotor, 10 min). Cell pellets were stored at –70 °C.

4.17. Protein Purification

Protein purification was carried out under non-denaturing conditions.

A cell pellet obtained from a 1 l culture was resuspended in 15 ml buffer A (50 mM Tris / HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 5 mM beta-mercaptoethanol (β-ME), 20 µg / ml RNase A, 100 µM phenylmethylsulfonyl fluoride (PMSF)) and the cells lysed using a French press. The lysate was centrifuged 30 min at 25,000 x g, the clear supernatant separated and incubated with 2 ml Ni-nitrilotriacetic acid metal affinity resin (Qiagen, Hilden, Germany) for 1 h. The resin was washed 3 times with 20 ml buffer A in a batch, and then washed with 40 ml buffer B (50 mM Tris / HCl pH 8.0, 1 M NaCl, 10 mM imidazole, 5 mM β-ME) in a column. Overproduced His_6-tagged fusion protein was eluted with an imidazole step gradient in buffer C (50 mM Tris / HCl pH 8.0, 300 mM NaCl, 5 mM β-ME). Steps were 30 mM, 50 mM and 200 mM imidazole, respectively. Fractions were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) on 15 % or 18 % gels. Fractions containing protein were pooled and dialyzed against buffer D (20 mM Tris / HCl pH 8.0, 150 mM NaCl, 2 mM dithiothreitol (DTT)). After 1 h of dialysis, 15 U of thrombin (Calbiochem, San Diego, CA) were added to cleave the N-terminal His_6-tag. 12 h later, the cleaved protein was dialyzed against buffer E (20 mM HEPES pH 7.5, 20 mM NaCl, 2 mM DTT) and finally purified using cation exchange chromatography on a Mono S HR 5 / 5 column (Pharmacia, Uppsala, Sweden). Proteins were eluted with a linear 30 ml gradient of NaCl (0.05 M - 0.3 M) in 20 mM HEPES pH 7.5, 1 mM DTT at a flow rate of 0.7 ml / min, resulting in sharp peak profiles.

4.18. Controlled Proteolysis

Protease digestion was performed by treating 50 µg protein (0.5 µg / µl) with trypsin, chymotrypsin, thermolysin or *Staphylococcus aureus* endoproteinase Glu-C (V8) in 50 mM Tris / HCl [pH 8.0], 150 mM NaCl, 2 mM DTT at a protein-to-protease mass ratio in the range of 50:1 to 1000:1 for various times at 24 °C. Reactions were usually stopped by heat denaturation at 100 °C for 5 min. To examine the effect of various DNA conformers on Zab digestion, the reaction was performed in 10 mM HEPES pH 7.5, 20 mM NaCl, 5 mM DTT and 10 mM $MgCl_2$. DNA was used in a base-pair-to-protein molar ratio of 5:1. Poly [d($^{5\text{-Me}}$C-G)] was used as substrate DNA, and as unspecific DNA poly [d(A-G)] • poly [d(C-T)]. The digests were separated by SDS-PAGE on 18 % gels, followed by staining with Coomassie Brilliant Blue G-250. In the case of protein digested for the experiment shown in Fig. 6.4.1 (lanes 10–13), the reactions were stopped by adding PMSF (1 mM) instead of heat inactivation, to ensure non-denatured protein.

4.19. Mass Spectrometric Analysis of Proteolytically Obtained Protein Fragments

The proteolytic fragments were analyzed by mass spectrometry on a Voyager DE Workstation (PerSeptive, Framingham, MA) using matrix assisted laser desorption ionization – time of flight (MALDI-TOF) technology. As a matrix, sinapinic acid (10 µg / µl) in acetonitrile / H_2O / trifluoroacetic acid (70:29.9:0.1) was used. Alternatively, for fragments smaller than 10 kD the matrix was prepared with α-cyano-cinnamic acid (10 µg / µl) instead of sinapinic acid. Various fragments were further analyzed by amino-terminal sequencing on an Applied Biosystems 475 / 477A protein sequencer (Perkin Elmer, Norwalk, CT).

4.20. Functional Z-DNA Binding Assays

4.20.1. Electrophoretic Mobility Shift Assay

DNA binding was assayed by native PAGE (Lane *et al.*, 1992). The assay was carried out using d($^{5\text{-Br}}$CG)$_{20}$ as the substrate, which is stable in the left-handed Z-DNA conformation under the applied conditions (Malfoy *et al.*, 1982). The substrate was end-labeled with ^{32}P and purified on native PAGE prior to the experiment. A reaction mixture of 10 µl containing the ADAR1 fragment (4 nM – 500 nM) with less than 1 pM substrate in 10 mM Tris / HCl pH 7.8, 20 mM NaCl, 5 mM DTT, 5 % glycerol, 100 µg / ml bovine serum albumin, and 50 µg / ml poly [d(AG)] • poly [d(TC)] (Pharmacia) as an unspecific competitor, was incubated for 30 min at 24 °C. The mixture was analyzed on a 6 % native polyacrylamide gel using 0.5 x TB (22.5 mM Tris borate) as the running buffer. After electrophoresis (10 V / cm, 90 min) the gel was dried and autoradiographed at –70 °C on Kodak X-OMAT Blue film with intensifying screens.

4.20.2. Circular Dichroic Spectroscopy

CD spectra were recorded at 24 °C on an Aviv model 62DS spectrometer (Aviv Instruments, Lakewood, NJ). Conformational change in DNA-oligomers was monitored between 235 nm and 305 nm. DNA samples used were annealed prior to the experiment. For this purpose a concentrated solution of the self-complementary sequence d(CG)$_6$ or an equimolar amount of d(CA)$_7$ and d(TG)$_7$ were heated to 85 °C for 10 min, and then slowly cooled to less than 20 °C over 1h. Measurements were carried out in 10 mM Na phosphate pH 7.0, 10 mM NaF, 1 mM EDTA, 2 mM DTT, using a DNA concentration of 30.0 µM base pairs and an optical path length of 5 mm. Spectra were recorded in 1 nm steps and averaged over 4 sec. Protein was added to the sample from a concentrated stock solution, in aliquots never exceeding 5 % of the total volume. The mixture was equilibrated 5 min before each measurement. The spectra were corrected for buffer baseline and smoothed using software provided by Aviv. Protein spectra were recorded between 190 nm and 250 nm. Zα was measured at a

concentration of 10.0 μM, ZabΔl and Zab at 5.0 μM and an optical path length of 1 mm. Spectra were measured in 1 nm steps and averaged over 10 sec.
For comparisons of the spectra of Zab between 190 and 250 nm in the presence and absence of substrate, poly [d($^{5\text{-Me}}$C-G)] was used as substrate. A 2:1 base-pair-to-protein molar ratio was used.

4.21. DNA Purification

Synthetic DNA oligomers (DNAgency, Malvern, PA) were dissolved in 10 mM Tris/HCl pH 8.0, 50 mM NaCl. The solution was incubated for 10 min at 80 ºC and then slowly cooled to less than 20 ºC within 1 hour. The annealed double stranded oligomer was purified using FPLC on a Mono Q HR5/5 (Pharmacia, Uppsala, Sweden) anion exchange column. A 30 ml salt gradient from 50 mM NaCl to 400 mM NaCl in 50 mM Tris/HCl pH 8.0, 2 mM EDTA at a flow rate of 1 ml/min was applied.