III. Materials and Methods

General considerations

To minimize RNA degradation at maximum, samples were always handled with sterile latex gloves, and all plastic-ware used, was sterile and pyrogenic-free. RNAase traces were removed from the working bench, all non-disposable material and pipets with a commercial caustic solution, "RNAase AWAY" (Molecular BioProducts). Further, utensils were constantly exposed to ultraviolet (UV) light, prior to use. On a general basis, buffer solutions were prepared as 10X stocks, filtered with a sterile syringe filter unit, 0.22μm (Millipore) and stored at 4°C. If not stated otherwise, all enzymes were purchased from New England Biolabs or MBI Fermentas. Chemical reagents were purchased from Fluka, Merck or Sigma.

The concentration of nucleic acids (RNA and DNA) and protein was estimated as absorbance units (AU), at a wavelength of 260-280 nm. Calculations were based on the following proportions:
RNA, 1 AU=40 μg/ml 260 nm
DNA, 1 AU=50 μg/ml 260 nm
Protein, 1 AU= 1 mg/ml•(Cε) 280 nm -note: Cε= extintion coefficient

1) Preparation of RNA samples

1.1) CTE RNA constructs

By the beginning of this thesis, all CTE-encoding DNA oligonucleotides, had been inserted into the cloning plasmid pBKS^{+} (Stratagene), downstream the T7 promoter sequence and upstream a HindIII recognition site (figure 46). All CTE RNA constructs used during this study were transcribed *in vitro* from linearized plasmid templates, which contained the corresponding CTE-encoding DNA sequence. Three RNA constructs were used: CTE full-length (CTE), CTE B-loop (CTE-B initial) and CTE B-loop + two GG repeats (CTE-B2 optimized). Figure 46 is a diagram, which enumerates the complete process of RNA preparation. Each step on the process is described with details in the next subsections.

CTE full-length

5'-AGACCACCUCCCCUGCGAGCUAAGCUGGACAGCCAAUGACGGGUAAGAGA
GUGACAUUUUUCACUAACCUAAGACAGGAGGGCCGUCAGAGCUACUGCCUA
AUCCAAAGACGGGUAAAAGUGAUAAAAAUGUAUCACUCCAACCUAAGACAG
GCGCAGCUUCCGAGGGAUUG-3'

CTE-B initial

5'-UCACUAACCUAAGACAGGAGGGCCGUCAAAGCUACUGCCUAAUCCA
AAGACGGGUAAAUGUGA-3'

CTE-B optimized

5'-CCUCACUAACCUAAGACAGGAGGGCCGUCAAAGCUACUGCCUAAUCCA
AAGACGGGUAAAUGUGAGG-3'

1.2) Run-off *in vitro* transcription

Milligram amounts of CTE RNA were prepared, on the basis of a 10 ml *in vitro* transcription reaction. Generally, 1 to 1.5 mg of pure plasmid was linearized over-night or during 8 to 12 hours, with the enzyme HindIII (New England Biolabs). The linearized plasmid was further used as a template for the enzymatic transcription of the CTE RNA constructs, by means of the bacteriophage T7 RNA polymerase. The corresponding RNA

transcripts were finally subject to an exhaustive purification protocol, and used as soon as possible (figure 46).

1.2.1) Large-scale preparation of CTE-encoding pBKS$^+$ plasmids

Prior to linearization, milligram amounts of plasmid were obtained from large-scale bacterial cultures grown under selective antibiotic-based conditions. Chemically competent DH5α cells were transformed with a given CTE-encoding pBKS$^+$ plasmid and plated in solid Luria-Broth (LB) media that contained ampicillin. Normally, 3 Lts of LB media were inoculated with freshly plated cells and incubated during 8 to 12 hours, at 37°C and constant centripetal rotation (180 rev/min). Next, the cell suspension was subject to centrifugation, at 3500 rev/min and 4°C, with a Sorvall RC 3B *Plus* centrifuge and a H6000/HBB6 rotator (Sorvall Instruments). An alkaline lysis procedure was used to extract the plasmid from the cell debris, according to a commercial large-scale plasmid preparation kit (Qiagen). The pure plasmid was concentrated by precipitation with isopropanol at room temperature, and immediately separated by centrifugation. Centrifugation was performed in a refrigerated Sorvall RC 5C (Dupont), using an SS 34 rotor (Sorvall) and sterile "Corex" tubes. The conditions were 12,000 rpm at 4°C, for 30 minutes. The dry pellet was re-suspended with a minimal volume of sterile hot (37°C) water and kept at –20°C until use.

1.2.2) Plasmid linearization

1.0 to 1.5 mg of pure plasmid template were digested in a 5 ml reaction mixture, as follows:

1ml (1-1.5 mg/ml) of CTE-encoding pBKS$^+$

100μl Hind III enzyme

500μl 10X NEB2 (100mM Tris-HCl pH 7.9, 100mM $MgCl_2$, 500mM NaCl, 10 mM DTT) buffer

3.4 ml distillated H_2O

Total volume 5ml

Incubate 8-12 hours at 37°C

The linearized plasmid was purified by phenol extraction (see 1.2.5)

1.2.3) Run-off *in* vitro transcription reaction

1 to 1.5 mg of pure linearized plasmid template were subject to run-off *in vitro* transcription, in a 10 ml reaction mixture, as follows:
1ml (1-1.5 mg/ml) linearized CTE-encoding $pBKS^+$
500 μl of each 100mM NTP (ATP, CTP, GTP, UTP)
1 ml 10X transcription buffer (400 mM Tris pH 7.5, 160 mM $MgCl_2$, 10 mM spermidine, 50 mM DTT, 0.1% v/v triton X-100)
500-1000 μl T7 RNA polymerase
6.5-7.0 ml distillated H_2O
Total volume 10 ml
Incubate 6-8 hours at 37°C

1.2.4) RNA purification

Upon *in vitro* transcription, the reaction mixture was subject to phenol-extraction (see 1.2.5), from which the aqueous phase was kept and concentrated to 5 ml with a centrifugal filter device (Amicon Ultra, 10 KDa molecular weight cut-off). The CTE RNA transcript was separated from the side-reaction RNA products with a preparative denaturing urea-PAGE gel (see 3.1.2). Next, the elution band of interest was visualized with a UV-lamp and excised with a sterile cutter. The RNA was extracted from the polyacrilamide slice by electroelution, with an Elutrap (Schleider & Schuell) apparatus. To this point the RNA was pure, but contained traces of organic molecules, very probably xilene cyanol FF or bromophenol blue, which are used as molecular weight markers during denaturing electrophoresis (see section 3.1.2). For this reason, each CTE RNA construct was subject to a two-step precipitation protocol and extensive dialysis, as follows:

-RNA precipitation (twice)

On each step: 1 volume of RNA was mixed with cold (–20°C) 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH 5.3). The mixture was vortexed and incubated for 3 hours, at –80°C or over-night at –20°C. The precipitate was separated by centrifugation at

12000 rpm/min during 30 minutes, with a refrigerated (4°C) analytical centrifuge 5415 D (Eppendorf).

-RNA dialysis

The precipitated RNA was re-suspended in 3 to 4 ml of distillated H_20 and subject to several dialysis steps, each of them during at least 6 hours:

Step 1: against 1.0M NaCl (distillated H_20)

Step2: against 0.750M NaCl (distillated H_20)

Step2: against 0.500M NaCl (distillated H_20)

Step3: against 0.250M NaCl (distillated H_20)

Step4: against 0.100mM NaCl ($MgCl_2$ 5-10mM, MES/PO_4^{-3} buffer, pH 6.5)

1.2.5) Phenol/chloroform extraction protocol for DNA or RNA, from reaction mixtures

An equal volume of phenol/chloroform/isoamyl alcohol: 25/24/1 (AppliChem) was added to a given nucleic acid sample and homogenized with a vortex, until a stable phase-separation emulsion was formed. Next, the emulsion was separated by centrifugation in a refrigerated Sorvall RC 5C (Dupont), using an SS 34 rotor (Sorvall) and sterile "Corex" tubes, at 12,000 rpm and 4°C, during 30 minutes. The aqueous phase was recovered carefully, and the nucleic acid was subject to precipitation with cold (–20°C) 2.5 volumes of ethanol and 0.1 volumes of 3M sodium acetate (pH 5.3) or with one volume of room temperature isopropanol. The mixture was homogenized with a vortex and incubated at –20°C, during 20 minutes. The precipitated DNA/RNA was separated by centrifugation, as mentioned above in this subsection. The resulting pellet was washed with cold (–20°C) 70% v/v ethanol, and separated again by centrifugation, during 15 minutes. This final pellet was dried, until it was free of alcohol traces, re-dissolved with warm (37°C) H_2O and stored at –20°C, until use.

2) Preparation of protein samples

2.1) Subcloning: reaction conditions and primer olignucleotides used

All genes of interest were sub-cloned as PCR-amplified oligonucleotide inserts, into the vector pGEXcs (Parks *et al*., 1994) at 5'-NcoI and 3'-BamHI sites.

2.1.1) Insert amplification

Each corresponding oligonucleotide insert was amplified from a given DNA template by PCR (polymerase chain reaction), with the enzyme DeepVent DNA Polymerase (New England Biolabs), using standard procedures.
The PCR reaction protocol was generally as follows:

95°C, 3 min
95°C, (1 min) → 60°C, (1 min) → 72°C, (3 min), 30 cycles
72°C, (10 min)
4°C, ∞ (indefinite time)

Reaction mixture:
1 µl DNA plasmid template
1 µl Mg_2SO_4 (100 mM)
2 µl dNTPs mixture (10 mM)
1 µl 5' primer oligonucleotide
1 µl 3' primer oligonucleotide
1 µl DeepVent DNA polymerase 2.000 U/ml
5 µl 10X ThermoPol Buffer
38 µl distillated H_20
Total volume 50 µl

The oligonucleotide primer sequences used to PCR-amplify the gene-encoding inserts, were the following:

Forward (5'→ 3'), Reverse (3'→ 5')

-TAP118 Forward 5'-GGGGATCCCATGGCGGACGAGGGGAAGTCGTAC-3'

-TAP112 Forward 5'-GGGGATCCCATGGCGCCTCCTCCAGAGCGCGGA-3'

-TAP96 Forward

-5'-CATGCCATGGTGCGCCGCGACCGCGCTCCTCCAGAGCGCGGA-3'

-TAP372 Reverse 5'-CGGGATCCGGGCGGTAACGTCGTGGGGGCTTC -3'

2.1.2) Insert purification and ligation into pGEXcs

All PCR-derived DNA products were purified by electrophoresis, with a 1% agarose gel. The gel was stained with ethidium bromide and the band of interest visualized with an UV lamp, excised with a sterile cutter and recovered from the gel, using a commercial kit (MinElute Gel Extraction Kit, Qiagen). Pure DNA oligonucleotides were digested with the enzymes NcoI and BamHI (New England Biolabs) purified again and inserted into a pGEXcs template plasmid, using standard ligation protocols. Table 5 below, shows the protein constructs I have used for the expression of recombinant TAP-N constructs. All the recombinant REF constructs were kindly provided by Elisa Izaurralde.

2.2) Expression strategy: bacterial strains and plasmids

All recombinant proteins were prepared by heterologous expression in *E. coli* bacterial hosts, according to the T7 expression system (Studier *et al*., 1990). Plasmids containing the bacteriophage T7 promoter were used to transform chemically competent bacterial strains BL21 (DE3). When the protein prepared had to be used for protein/RNA crystallographic trials, a special RNAase free B strain, BL21 (DE3) pLysS RNAase$^-$ was used.

All the proteins prepared during this thesis were recombinant TEV-cleavable GST-fusions. For this reason, the large-scale expression and purification protocol was nearly the same for all the proteins prepared. This protocol is described below, stressing out differences in the preparation, according to the growth media used and/or spin-labeled isotopes required.

TABLE 5

Construct	Vector	Strain	Affinity tag	Cleavage site	Antibiotic
TAP96-372	pGEXcs	-BL21(DE3) -BL21(DE3) pLysS, RNAase $^{-}$	GST	TEV	Amp Amp/Cam
TAP112-372	pGEXcs	-BL21(DE3) -BL21(DE3) pLysS, RNAase $^{-}$	GST	TEV	Amp Amp/Cam
TAP118-372	pGEXcs	-BL21(DE3) -BL21(DE3) pLysS, RNAase $^{-}$	GST	TEV	Amp Amp/Cam
REF1-103	pGEXcs	BL21(DE3)	GST	TEV	Amp
REF1-128	pGEXcs	BL21(DE3)	GST	TEV	Amp
REF12-104	pGEXcs	BL21(DE3)	GST	TEV	Amp

2.2.1) Overexpression in LB media

Normally, a protein expression protocol took 3 days. A 100 ml volume of sterile LB media was inoculated with a single fresh colony of BL21 (DE3) transformants and incubated at 37°C overnight, under constant centripetal rotation (180 rev/min). By the morning of next day, this 100 ml pre-growth suspension was transferred into a 2 Lt sterile flask and diluted up to 800 ml with fresh LB. The diluted media was incubated once more under the same conditions, during 3 to 4 hours and then diluted 8-fold, again with fresh LB and distributed in 2 Lt sterile flasks, with approximately 800 ml of growth media each. On each LB-dilution step, 0.5 ml of ampicillin (100 mg/ml) were added per Lt of LB. A same amount of chloramphenicol was also added, when RNAase^{-} BL21 strains were used.

When the cells reached an optical density at 600 nm (OD_{600}) of 0.5-0.7, the temperature was fixed to 18°C and the expression of recombinant protein was induced with 0.5 mM IPTG. Cells were kept rotating at 18°C during 8-12 hours, to assure maximal expression yields. Finally, bacterial growth solids were separated by centrifugation at 3500 rev/min and 4°C, with a SORVALL RC 3B *Plus* centrifuge and a H6000/HBB6 rotator (Sorvall Instruments). The pellet was re-suspended with cold buffer (Tris 20 mM pH 8.5, 500 mM NaCl), which contained an appropriate amount of protease inhibitors. If not used immediately, the re-suspended pellet was flash frozen in liquid Nitrogen and stored at -80°C as 30 ml aliquots, in 50 ml falcon tubes (Greiner bio-one).

2.2.2) Overexpression in M9 minimal media

NMR experiments depend on the magnetic activity of ^{1}H-/^{2}H-, ^{15}N- and ^{13}C- elements, which, with the exception of ^{1}H- are not naturally abundant. For this reason, proteins prepared for NMR use, had to be spin-labeled depending on the type of experiment, to be performed. A ^{2}H-, ^{15}N-, ^{13}C-TAP-N sample was prepared for the chemical shift assignments, whereas ^{2}H-, ^{15}N- /^{15}N-TAP-N samples where prepared for the chemical shift perturbation experiments. In analogy, ^{15}N-protein samples were used in the case of REF constructs, for which only chemical shift perturbation experiments were recorded.

The growth conditions were the same as for LB, with the difference that only 200 ml of growth media were put into each 2 Lt flask. The reason for this was to promote optimal aeration, which was necessary for successful bacterial growth in minimal media. Table 6 below, describes the preparation of M9 media for each case. Given the relatively low yields obtained with M9 minimal media, only ^{2}H-, ^{15}N- and ^{15}N- samples were prepared this way. Another strategy was used for the over-expression of ^{2}H-, ^{15}N-, ^{13}C- TAP-N, which is described next in 2.2.3.

2.2.3) Overexpression of ^{2}H-, ^{15}N-, ^{13}C- TAP-N

In the case of ^{2}N-, ^{15}N-, ^{13}C- TAP-N, a commercially available yeast extract, known as *E.coli* OD1 (Silantes), was used to express the protein. This media is a concentrated extract rich in ^{2}N-, ^{15}N-, ^{13}C isotopes and suitable for growth of *E.coli* transformed cells.

Basically, the extract was diluted 10 times with LB and inoculated with a single colony of BL21 (DE3) transformants. Growth conditions and antibiotic addition were as described above for proteins expressed with LB.

2.3) Purification of recombinant proteins

Cells were lysed with an EmulsiFlex C5 high-pressure cell (Avestin, Canada). Membrane remnants and other hydrophobic particles where separated by centrifugation at 11,000 rev/min, during 30 minutes at 4°C. The supernatant was carefully separated and loaded onto a 20 ml of Glutathione resin, which had been uniformly packed in a XK 16 column, with a thermostat jacket (Pharmacia) or in batch, depending on the tendency of each protein to interact with the GST-fusion. After washing extensively with buffer, the enriched protein was eluted from the resin with a linear gradient (0-100% in 3 CV) of 20 mM reduced glutathion. The eluate was incubated with TEV protease at 4°C, until most of the fusion protein was cleaved. The GST fusion and other contaminants were cleaned away by ion exchange. Either a cation exchange HiS (Pharmacia) column or an anion exchange, HiQ column (Pharmacia) was used, depending on the protein construct. Finally, protein samples were homogenized by gel filtration, with a Hiload Superdex 75 or 200 (depending on the molecular weight of the protein), 16/60 chromatographic column (Pharmacia). All the chromatographic steps were performed on a fast flow liquid chromatographer (FPLC).

Buffers:

-Affinity (GST-based) chromatography

Loading buffer:

20 mM Tris pH 8.5

500 mM NaCl

Wash buffer:

20 mM Tris pH 8.5

100 mM NaCl

Elution buffer:

20 mM Tris pH 8.5

100 mM NaCl

20 mM reduced glutathione

-TEV cleavage

20 mM Tris pH 8.5

100 mM NaCl

2 mM DTT

-Cation exchange (HiS) chromatography

Loading buffer:

20 mM MES pH 6.5

100 mM NaCl

Elution buffer:

20 mM MES pH 6.5

1 M NaCl

-Anion exchange (HiQ) chromatography

Loading buffer:

20 mM Tris pH 8.5

100 mM NaCl

Elution buffer:

20 mM Tris pH 8.5

1 M NaCl

-Gel filtration chromatography

20 mM Tris pH 8.5/MES pH 6.5/PO^4 pH 6.5

100-250 mM NaCl

5-10 mM $MgCl_2$

TABLE 6

70% ^{2}H-, ^{15}N-	85% ^{2}H-, ^{15}N-	^{15}N-
100 ml M9 (10X)	100 ml M9 (10X) containing $^{15}NH_2Cl$	100 ml M9 (10X) containing $^{15}NH_2Cl$
10 ml trace elements (100X)	10 ml trace elements (100X)	10 ml trace elements (100X)
20 ml 20% v/w Glucose	20 ml 20% v/w Glucose	20 ml 20% v/w Glucose
1 ml 1M Mg_2SO_4	1 ml 1M Mg_2SO_4	1 ml 1M Mg_2SO_4
0.3 ml 1M $CaCl_2$	0.3 ml 1M $CaCl_2$	0.3 ml 1M $CaCl_2$
1 ml Biotin (1 mg/ml)	1 ml Biotin (1 mg/ml)	1 ml Biotin (1 mg/ml)
1 ml Thiamin (1 mg/ml)	1 ml Thiamin (1 mg/ml)	1 ml Thiamin (1 mg/ml)
0.5 ml Ampicillin 100mg/ml	0.5 ml Ampicillin 100mg/ml	0.5 ml Ampicillin 100mg/ml
700 ml $^{2}H_2O$	800 ml $^{2}H_2O$	----------
Up to 1Lt with sterile H_20	Up to 1Lt with sterile H_20	Up to 1Lt with sterile H_20

3) Analytical methods

3.1) Electrophoresis

All the electrophoretic experiments described here, were performed according to well-established standard protocols (Smith 1998).

3.1.1) Denaturing SDS polyacrilamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were used as a diagnostic tool during protein and RNA purification. A 5-10 μL aliquot was taken at each step and loaded onto an SDS-PAGE gel, to check for relative yields and integrity of the biopolymer being purified; 10, 15 and 18% gels were cast in-house using an electrophoretic MiniProteanII gel system (Biorad). During a typical SDS-PAGE experiment, each sample was mixed with at least 5 volumes of SDS-loading

buffer (50mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue), boiled at 95°C for 5 minutes, and loaded onto the gel. Gels were fixed and immersed in running buffer (25 mM Tris-glycine/0.1% SDS) at a constant 200 V electric field, for which an electrophoresis constant power supply ECPS 3000/15 (Pharmacia) was used (here and for the other electrophoretic techniques). To visualize the migration pattern, the gel system was de-assembled and the SDS-PAGE rinsed with water before staining. A coomasie-based staining solution (0.5mg/ml coomassie brilliant blue R-250, 25% v/v isopropanol, 10% v/v acetic acid) was used to visualize proteins and/or 0.1% v/v toluidine-based staining solution to visualize RNA. Optimal staining took around 20 minutes. Stained gels were rinsed with water and immersed in a de-staining solution (60% v/v H_20, 10% v/v ethanol, 30% v/v acetic acid) for about 3 hours. To keep record of the experiments, de-stained gels were rinsed with water and dried on Whatman paper. The drying system consisted of a gel-dryer (Zabona AG, Basel) connected to a cooling trap (UNICRYO MC 4L –80°C) and a vacuum pump (Vaccubrand GMBH).

3.1.2) Denaturing 8M urea polyacrilamide gel electrophoresis (urea-PAGE)

Preparative (20x40 cm) 8% urea-PAGE gels were used for the purification of large-scale *in-vitro* transcription reaction products. Gels were cast with a commercially available, ready-to-use ultrapure 8 % gel casting solution, SequaGel-8 (National Diagnostics). Glass plates, spacers and electrophoretic gel systems of appropriate dimensions, were built and provided in-house by the EMBL-Mechanical Workshop. Assembled urea-PAGE gels were fixed on a gel system, immersed in 0.5X TBE (0.45 M Tris pH 8.0, 0.45 M borate acid, 10 mM EDTA) buffer and pre-ran during 30 minutes. Electrophoretic migration was triggered with a constant 70 mA current, achieved with an electrophoresis constant power supply ECPS 3000/15 (Pharmacia). The temperature was kept between 50-60°C with a metallic cover leaf embracing most of the front glass plate. The RNA sample was mixed with a 1/10 volume of loading buffer (20% v/v glycerol, 10 mM EDTA, 1X TBE and trace amounts of xylen cyanol FF and bromophenol blue), heated for 15 minutes at 70°C to assure complete unfolding of all RNA moieties in the mixture, and slowly loaded onto the urea-PAGE. xylen cyanol FF and bromophenol blue are organic dyes that migrate on a urea-PAGE as if they were an RNA moiety with a specific nucleotide length (table 7) (Fritsch *et al*., 1989). This allowed a controlled monitoring of the sample migration.

TABLE 7

% Polyacrilamide/urea gel	Xylene cyanol FF (nucleotides)	Bromophenol blue (nucleotides)
4	155	30
6	110	25
8	75	20
10	55	10

Table taken from Fritsch *et al*., 1989.

3.1.3) Native polyacrylamide gel electrophoresis (native-PAGE)

Native-PAGE gels were used to estimate the binding activity and integrity of RNA and protein aliquots. A 20x20 cm 5% native-PAGE gel was cast at 4°C, as follows:
31 ml distilled H_20
5 ml 40% Acrylamide: N, N'- Methylenebisacrylamide 19:1 solution
4 ml 10x RNA running buffer (500 mM Tris-acetate, pH 7.5; 100 mM Mg-Acetate)
50 μL N, N, N, N', N'- Tetramtehylethylendiamine (TEMED)
250 μL Amonium persulfate (APS) 10% w/v
Total volume 40 ml

As a first step, the distilled H_20, the acrylamide solution and the 10X running buffer were mixed gently and degassed. Immediately before casting the gel, APS and TEMED were added. Assembled gels were pre-ran during 30 minutes at 4°C. The samples were loaded onto the gel and ran overnight or during 8-12 hours, at 4°C and 80 volts. Finally, the gel was de-assembled, gently rinsed with water and stained with 0.1% v/v toluidine. In keeping record of the experiments, native-PAGE gels were dried as described in section 3.1.1.

3.2) Analytical ultracentrifugation

Fresh protein and protein/RNA samples were prepared for analytical centrifugation experiments. To have an optimal signal, the optical density was adjusted to a value of 0.5-0.7 AU at 280 nm, with 25 mM Tris, pH 8.5, NaCl 100 mM. Data collection and analysis was kindly performed by Dr. Arie Geerlof at the in-house Protein Expression and Purification Unit.

3.3) Limited proteolysis

15 µl of TAP-N or TAP-N/CTE-B complex (1 mg/ml) were incubated with 4 µl of 100, 10 and 1(mM) of substilisine protease aliquots in Tris 20mM pH 7.0, 250mM NaCl and 10mM $MgCl_2$. Each reaction mixture was incubated at 37°C, during 30 minutes, after which the enzyme was inactivated with 1 µl of 100 mM PMSF and 5 µl of SDS loading buffer. Samples were heated at 95°C and analyzed with a 15% SDS-PAGE gel. Subtilisine protease was purchased from Boehringer and stored at –20°C in 1M stocks.

3.4) Protein/RNA binding reaction

Analytical protein/RNA binding reactions were set-up on the basis of a 10 µl total volume, and according to standard protocols (Smith 1998).

10 to 15 µl of RNA (approximately 10 mg/ml), were dissolved in sterile H_2O, annealed by heating the sample during 10 minutes at 80°C, and cooled-down slowly to room temperature. 1 to 3 µl of annealed RNA was added drop-wise to a cold 7 to 9 µl protein solution, which had a protein aliquot at a concentration of 0.1 to 0.5 mg/ml, in 20 mM Tris-acetate pH 8.5, 100mM NaCl. The reaction mixture was kept in ice for 30 to 60 minutes and analyzed with an 8% native-PAGE gel, as described in section 3.1.3.

4) Biophysical methods

4.1) NMR measurements

NMR experiments were recorded generally, at or 303°K on Brucker DRX spectrometers, operating at ^{1}H frequencies of 600 MHz with cryoprobe and 800 MHz without cryoprobe. Spectra were processed with NMRPIPE (Delaglio *et al*., 1995) and analysed using XEASY (Bartels *et al*., 1995). The chemical shift assignments of TAP-N were determined by multidimensional NMR, using a set of pulse sequences based on previously described methods (reviewed by Sattler *et al*., 1999). Both double ($^{1}H^{N}$-^{15}N HSQC-TROSY, ^{15}N-HSQC-NOESY) and triple (HNCA/HNCOCA, HNCACB/HNCOCACB) resonance experiments were recorded for TAP-N unbound in 10% v/v $^{2}H_20$, whereas only double resonance experiments were measured for TAP-N in complex with the CTE-B RNA, also in 10% v/v $^{2}H_20$. HNCA and HNCOCA experiments were used for the sequential assignment of the backbone NH, N, and C□ resonances, whereas HNCACB and HNCOCACB experiments were used to extend the correlations from NH to the C□ resonances. Finally, a ^{15}N NOESY-HSQC-TROSY was used, to complement the triple resonance experiments. All pulse sequences were optimized in order to improve the signal to noise ratio and enhance the sensitivity of the experiments.

Aim and justification.

To date, X-ray crystallography and NMR are the techniques available that give high-resolution images of biomolecules, at the atomic level. X-ray crystallography is limited to those cases where well-diffracting crystals can be obtained, whereas NMR spectroscopy is not dependent on this factor, but is only suitable for biomolecules of less than 40 kDa. Of special value, is the study of protein/protein or protein/RNA interactions by NMR spectroscopy. The reason being that many relevant protein/protein and protein/RNA complexes are unstable assemblages that normally do not crystallize easily. Multidimensional NMR protocols using high-field magnets, heteronuclear-based experiments, and specific protein/RNA labelling schemes, are now of common use, for mapping the surface of interaction of large molecular complexes. Provided that prior structural information for at least one of the individual components is available.

Chemical shift perturbation experiments

Chemical shift perturbation is by now, the most widely used NMR method to map the surface of interaction of protein/protein and protein/RNA complexes. Particularly useful for this means, are the $^{1}H^{N}$-^{15}N HSQC (heteronuclear single quantum coherence) and TROSY (transverse relaxation optimized spectroscopy) experiments, which are well developed two-dimensional (2D) NMR experiments. The TROSY experiment is an optimized $^{1}H^{N}$-^{15}N HSQC, where the lifetimes of NMR signals are substantially increased. This has a direct impact on the molecular size limitations. Thus, allowing for the investigation of biomolecules with a molecular weight beyond 40kDa (reviewed by Zuiderweg 2002).

During a typical chemical shift perturbation experiment, $^{1}H^{N}$-^{15}N HSQC/TROSY spectra are collected for a labelled sample on its unbound state (reference spectrum) and when titrated with a specific unlabelled ligand (e.g. protein/RNA). Chemical shifts for the free and bound form of a protein will be different, due to changes in the chemical environment of the binding site. Given a fully assigned reference spectrum, these perturbations are used to identify the location of binding sites. In a $^{1}H^{N}$-^{15}N HSQC or TROSY spectrum of a ^{15}N-labelled protein, all amino acids, except proline, give rise to sinlge peaks. With each peak representing a cross-correlation of the amide backbone ^{1}H and ^{15}N atoms for each amino acid. Using a variety of procedures, these individual ^{1}H-^{15}N cross-peaks can be assigned to specific backbone resonances in the protein, a process in NMR spectroscopy referred to as chemical shift assignment or backbone resonance assignment (Clarkson and Campbell 2003).

Backbone resonance assignments

The backbone resonance assignments refer to the resonance frequency of each spinning nuclei along the polypeptide backbone. They are normally thought as of an atomic signature, which identifies each site in the protein. The chemical shift resonances for each amino acid, include the values for $^{1}H/^{2}H$; ^{13}C; ^{15}N that correlate to each amino acid. They represent the resonance position on a spectrum for each residue, which reflects its chemical environment (Tugarinov *et al*., 2004).

Homo- ^{1}H-^{1}H (two-dimensional, 2D) and heteronuclear ^{1}H-, ^{13}C-, ^{15}N (three-dimensional, 3D)-based NMR protocols, are now well established, that allow the assignment of all the isotopically labelled atoms for each amino acid of a polypeptide. In the case of homonuclear-based assignment protocols, which are based on the correlation of a single common resonance frequency (^{1}H-^{1}H), the resonance overlap becomes so severe for proteins with more than 80 amino acids, that it is often impossible to identify adjacent spin systems. The use of ^{15}N-, ^{3}C- protein-labelling protocols and multidimensional NMR schemes improves the resolution among adjacent spin systems and overcomes the conformation dependence, inherent to $^{1}H_{N}$-, $^{1}H_{\alpha}$- based sequence assignment protocols. Thus, for bigger proteins, so-called heteronuclear (3D) NMR experiments are required for the backbone assignment. In a 3D assignment scheme, each cross peak is labelled by three frequencies (^{1}H-, ^{15}N- and ^{3}C-) that help resolve overlapping $^{1}H_{N}$ and $^{1}H_{\alpha}$ chemical shifts of each amino acid. The nomenclature for 3D NMR protocols reflects the magnetization transfer pathway of the experiment (figure 47). In other words, the nuclei that are involved in the magnetization transfer process, give the name to a particular experiment. For our studies we have used two sets of pair experiments: HNCA/HNCOCA and HNCACB/HNCOCA, which have been mentioned above (reviewed by Sattler *et al.*, 1999).

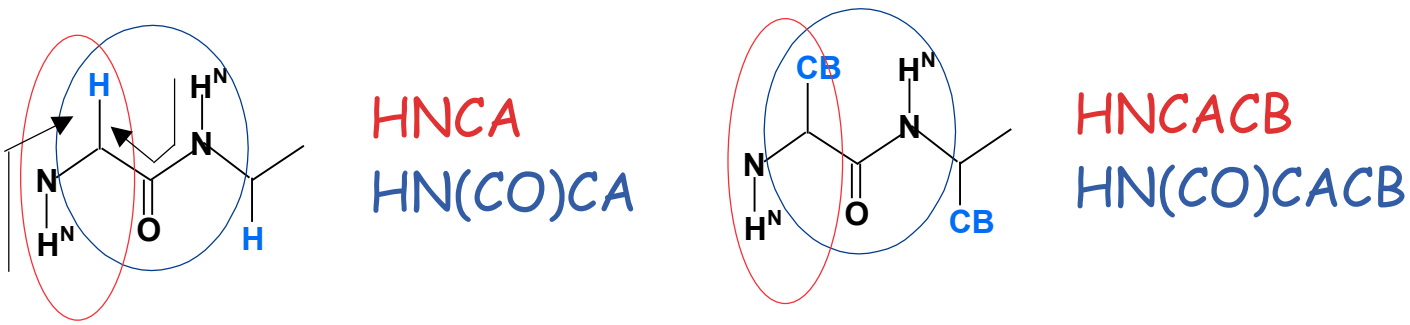

Figure 47. The multidimensional experiments used for the backbone chemical shift assignment.

4.2) X-ray data collection

Data on frozen crystals was collected on beamline PX1 at the Swiss Light Source (SLS). All datasets were collected at 100 °K (Oxford Cryosystems) with an oscillation range of 1°. In order to optimize the crystal freezing process, most crystals were transported to the synchrotron in the crystallization tray, and flash-frozen on-site in the cryosystem. For this particular case, the crystallization agents had cryogenic properties (PEGs and Hexanediol), the reason why no cryoprotectans were used. Finally, the data collected was indexed, integrated and processed with the programs MOSFLM (Leslie 1992) and SCALA (Collaborative Computational Project Number 4, 11994).

4.3) SAXS data collection

Solution small angle X-ray scattering (SAXS) experiments were performed at the EMBL-Hamburg X33 beamline of the Deutsches Elektronen Synchrotron (DESY) at a wavelength of 1.5 Å and a sample detector distance of 1.9 to 2.4 m, covering a total momentum transfer range of $0.01<0.5$ $Å^{-1}$. Further, the scattering curves were measured at several sample concentratins, ranging from 2 to 20 mg/ml. All samples were prepared as 20 mg/ml batch in 25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT. Data analysis was done by Dr. Dmitry Svergun, EMBL, Hamburg). The scattering curves were analyzed with the program SAPOKO (Svergun and Koch, unpublished results). The maximum dimension (Dmax), radius of gyration (Rg) and distance distribution p(r) of the particles was compared with the programs ORTOGNOM (Svergun 1993) and GNOM (Svergun *et al*., 1988). The molecular masses of the particles were estimated by comparison with a bovine serum albumina (BSA) solution of known concentration (usually 5-8 mg/ml) in 50 mM HEPES pH 7.5. The reference solution was was freshly prepared from lyophilized BSA (Sigma), spun for 10 minutes in a tabletop centrifuge and measured in the next few hours. Shape determination was done either using a bed approach implemented in the program DAMMIN (Svergun 1999) or using a dummy residues approach GASBOR (Svergun *et al*., 2001).