NMR Studies on Termination/-Antitermination Complexes of HIV-1 Transcription

Dissertation

zur Erlangung des Doktorgrades
der Fakultät Biologie, Chemie und Geowissenschaften
der Universität Bayreuth

vorgelegt von

Nageswara Rao Jampani
(aus Hyderabad, Indien)

Bayreuth 2006

Die vorliegende Arbeit wurde von September 2003 bis November 2006 am Lehrstuhl für Struktur und Chemie der Biopolymere der Universität Bayreuth unter der Leiutung von Prof. Dr. Paul Rösch angefertigt

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Diktor der Naturwissenschaften (Dr. rer. nat)

Promotionsgesuch eingereicht am : 25.07.2006
Tag des wissenschaftlichen Kolloquiums : 10.11.2006

Erster Gutachter : Prof. Dr. Paul Rösch
Zweiter Gutachter : Dr. Holger Dobbek

Dedicated to my grand parents.

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1 Introduction

1.1 The human immunodeficiency virus

The human immunodeficiency virus (HIV) is the etiological agent of the acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Coffin et al., 1986; Gallo et al., 1983; Levy et al., 1984). Two types of HIV are known. The most common HIV-1, which is responsible for the world-wide AIDS epidemic and the immunologically distinct HIV-2 (Clavel et al., 1986), which is less common and less virulent (Ariyoshi et al., 2000; Ariyoshi et al., 1999), but produces clinical findings similar to HIV-1 (Wilkins et al., 1993). By the end of 2004, around 40 million people had been infected with this virus globally, with 70% of these living in sub-Saharan Africa (According to UNAIDS). The basic pathology in AIDS is the loss of $CD4^+$ lymphocytes and a variety of disorders in immune function, leading to the onset of opportunistic infections (Levy et al., 1998).

HIV is a member of the lentivirus genus of the Retroviradae. HIV virons contain two identical copies of a single stranded RNA genome which are used as templates by the RNA and DNA dependent polymerase [Reverse Transcriptase (RT)] for production of DNA which is later integrated into the genome of the cell and serves as the basis for viral gene expression. Retroviruses are broadly divided into two categories simple and complex distinguishable by the organisation of their genomes (Coffin 1992; Murphy et al., 1994). Based on nucleotide sequence relationship and genome structure, retroviruses are further subdivided into seven genera. Five of these groups represent retroviruses with oncogenic potential (formerly referred to as oncoviruses), and the other two groups are the lentiviruses and the spumaviruses. All oncogenic members except the human T-cell leukemia virus-bovine leukemia virus (HTLV-BLV) genus are simple retroviruses. HTLV-BLV and the lentiviruses and spumaviruses are complex (Coffin et al., 1997).

1.1.1 Morphology of the mature viron

HIV is different in structure from previously described retroviruses. It is around 120 nm in diameter and is roughly spherical.

HIV-1 is composed of two copies of single-stranded RNA enclosed by a conical capsid comprising the viral protein p24. This is in turn surrounded by a plasma membrane of host-cell origin. The single-stranded RNA is tightly bound to the nucleocapsid protein, p7 and enzymes that are indispensable for the development of the virion, such as reverse transcriptase, protease and integrase. The nucleocapsid (p7) associates with the genomic RNA (one molecule per

hexamer) and protects the RNA from digestion by nucleases. A matrix composed of an association of the viral protein p17 surrounds the capsid, ensuring the integrity of the virion particle. Also enclosed within the virion particle are Vif, Vpr, Nef, p7 and viral protease (Fig 1.1). The envelope is formed when the capsid buds from the host, taking some of the host-cell membrane with it. The envelop includes the glycoproteins gp120 and gp41.

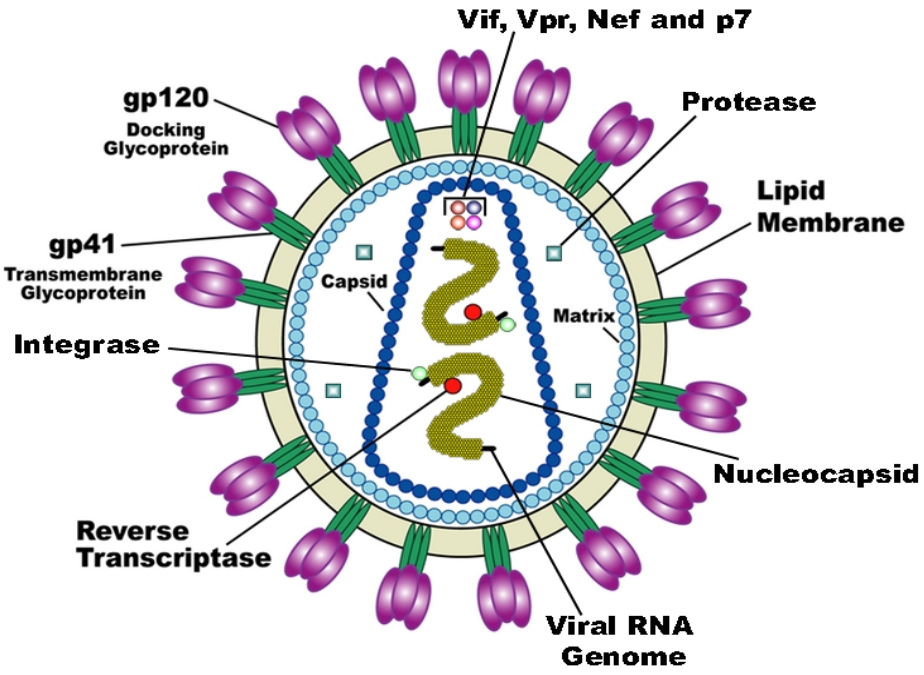


Fig. 1.1 Mature HIV-1 virion. Morphology of the mature HIV-1 virion. HIV-1 encoded proteins and genomic RNA are indicated (HIV structure and genome. *Wikipedia, The Free Encyclopedia*. 15 Jun 2006).

1.1.2 Genome organisation

The proviral DNA of HIV-1 (~9.2 kb) has three encoding regions *gag, pol*, and *env*, two long terminal repeats (LTRs) with transcriptional regulatory elements (Fig 1.1).

The RNA genome is flanked by two short redundant (R) sequences at both termini with adjacent unique sequences, U5 and U3, found at the 5' and 3' ends, respectively.

The *gag* gene encodes the large precursor protein p55 that is cleaved into four proteins: the matrix p17 (MA), the capsid p24 (CA), the nucleocapsid p7 (NC) and p6 (Freed, 1998).

The *pol* gene encodes three important enzymes that function at different time during the replicate cycle. The RT acts in the early steps of the virus replication to form a double-stranded

DNA of the virus RNA. The integrase p32 (IN) mediates integration of the proviral DNA into the host chromosomal DNA. The protease p10 (PR) is responsible for the cleavage of the viral Gag and Pol polyproteins during the maturation of the viral particle.

The *env* gene directs the production of an envelope precursor protein gp160, which undergoes cellular proteolytic cleavage into the outer envelope glycoprotein gp120, responsible for binding to $CD4^+$ receptors, and the transmembrane glycoprotein gp41, which catalyses the fusion of HIV to the target cell's membrane.

In addition, HIV-1 has at least six more genes encoding viral proteins with regulatory functions (*tat* and *rev*) or accessory functions (*nef*, *vif*, *vpr* and *vpu*), for reviews see (Cullen 1998, Emerman et al., 1998, Frankel et al., 1998, Kjems et al., 2000, Piguet et al., 1999, Pollard et al., 1998, Trono1995).

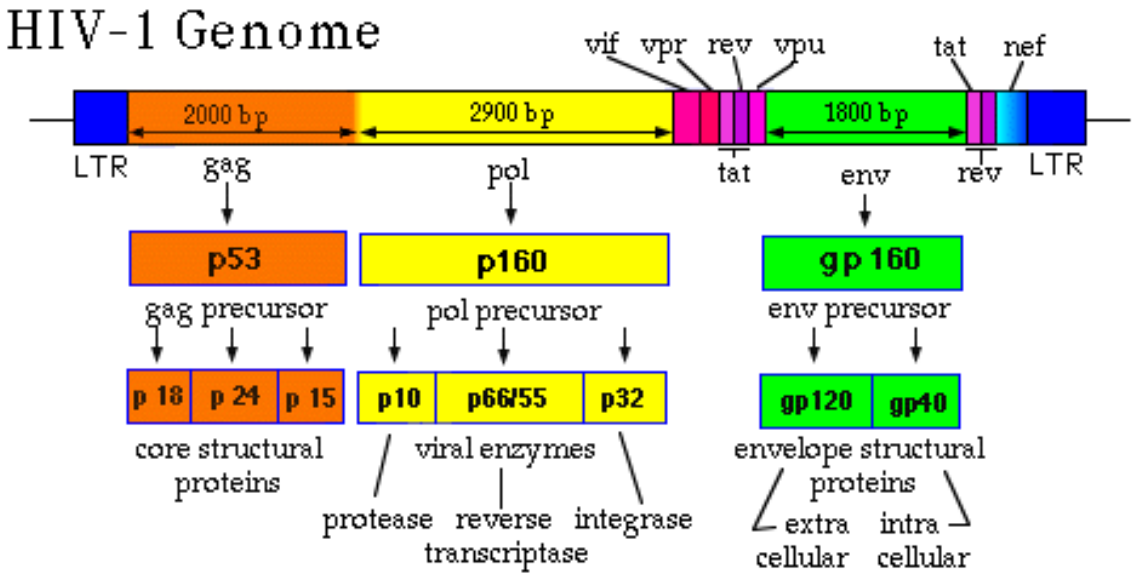


Fig. 1.2 HIV-1 genomic organisation. Like all other retroviruses, HIV-1 has three structural genes *gag*, *pol*, and *env* (shown in orange, yellow, and green colour), which are flanked by the long terminal repeats (blue). In addition, it has six more genes, include two regulatory genes *tat* and *rev*, and four accessory genes *nef*, *vif*, *vpr*, and *vpu* (shown in pink and purple).

1.1.3 Replication cycle

The interaction between the glycoprotein gp120 on the HIV virion and its receptor, CD4 on the target cell, provokes conformational changes in gp120. This exposes a region of gp120, the V3 loop, which binds to a cytokine receptor on the target cell. The change in gp120's shape also exposes a portion of the glycoprotein gp41 which was previously buried in the viral membrane and loosely bound to gp120. A fusion peptide within gp41 causes the fusion of the viral envelope and the host-cell envelope, allowing the capsid to enter the target cell (Chan et al., 1998; Wyatt et al., 1998). Once HIV has bound to the target cell, the HIV RNA and the viral enzymes, i.e. RT, integrase and protease are injected into the cell.

Once the viral capsid has entered the cell, the RT copies the viral RNA into a double stranded complementary DNA (cDNA) of 9 kb pairs (Fig 1.3). This process of reverse transcription is extremely error prone and it is during this step that mutations (such as drug resistance) are likely to arise. This new DNA is then transported into the cell nucleus. The integration of the proviral DNA into the host genome is carried out by another viral enzyme called integrase. This is called the latent stage of HIV infection (Zheng et al., 2005). To actively produce virus, certain transcription factors need to be present in the cell. The most important is NF-κB and is present once the T cell becomes activated.

Transcription is achieved by cellular RNA polymerase II (Rpol II) producing viral transcripts which are expressed from the promoter located in the 5'LTR with the viral transactivator protein (Tat) greatly enhancing the rate of transcription. Through Rev regulation a set of spliced and genomic RNAs are transported from the nucleus to the cytoplasm where they are translated. At this stage the structural proteins Gag and Pol are produced from the full-length mRNA and Env is produced from the singly spliced mRNA. The genomic RNA binds to the Gag protein and is packed into new virus particle.

The final stage of the viral life cycle, assembly of new HIV virions, begins at the plasma membrane of the host-cell. The env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host-cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host-cell. Maturation occurs in the immature virion after buds from the host-cell. During maturation HIV protease cleaves the polyproteins into indivudual functional HIV

proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Gelderblom, H. R 1997). The virus is then able to infect another cell.

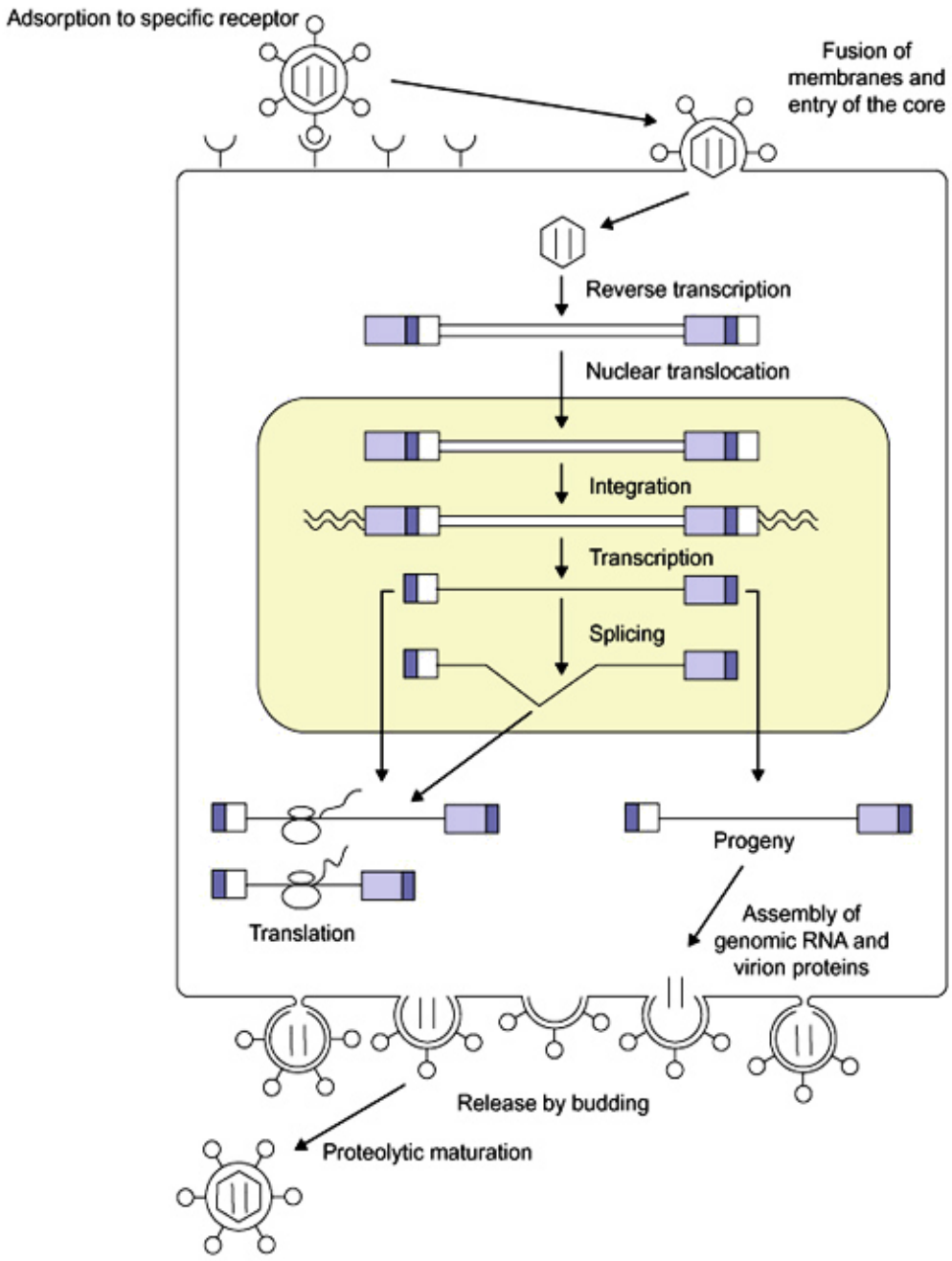


Fig. 1.3 HIV-1 life cycle. Each fundamental step is represented by a forward arrow, see text for details (Voisset and Andrawiss 2004).

1.2 Regulation of HIV-1 transcription at elongation

Transcription of proviral DNA to genomic viral RNA by Rpol II is regulated negatively and positively by several cellular factors and HIV-1 encoded proteins (Barboric and Peterlin, 2005).

1.2.1 HIV-1 TAR RNA

Unlike most DNA sequence-specific transcription activators, Tat stimulates RNA polymerase elongation by recognising the transactivation response (TAR) RNA stem-loop structure located at the 5' end of nascent viral transcripts. Tat activation of HIV-1 transcription is modulated by several cellular cofactors (Jones 1997; Jones et al., 1994). Only the apical portion of the stem loop from positions +17 to +45 is required for transcriptional activation *in vivo* (Jakobovits et al., 1988). Extensive mutagenesis studies have shown that the region of TAR important for binding, Tat, involves a set of nucleotides that surround a characteristic UUU nucleotide bulge; important residues include U23, the first bulged nucleotide, and the two base pairs that immediately precede and follow the bulge (Dingwall et al., 1989; Cordingley et al., 1990; Roy et al., 1990; Weeks et al., 1990). Although, Tat binds to TAR RNA around the bulge region a six nucleotide loop is required for the transactivation *in vivo* (Dingwall et al., 1989; Cordingley et al., 1990).

(a) **(b)**

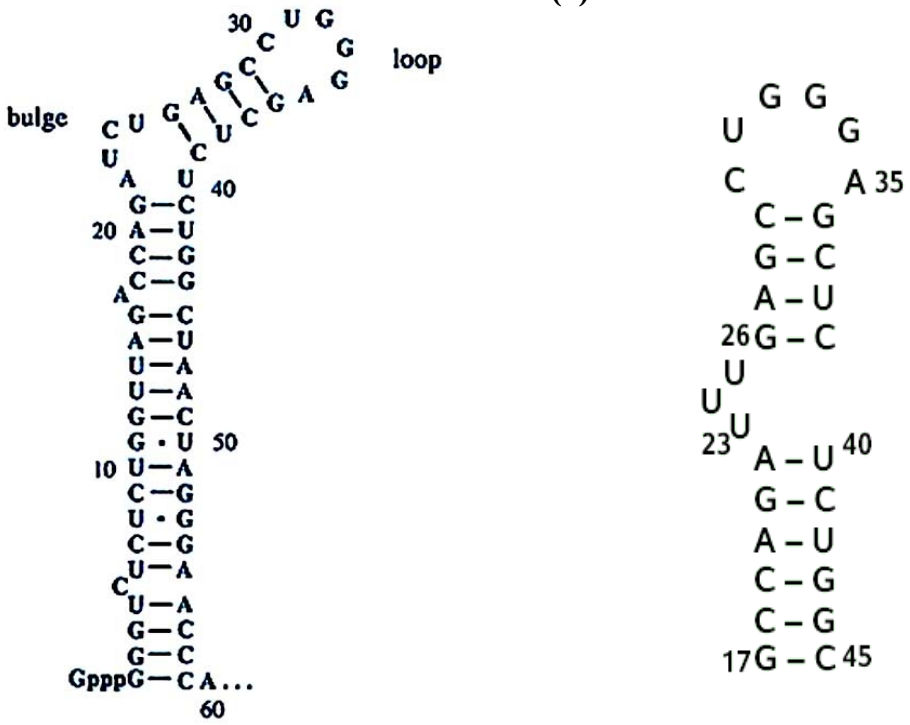


Fig. 1.5 HIV-1 TAR RNA. (a) Secondary structure of the full-length wild type HIV-1 TAR RNA. **(b)** HIV-1 TAR RNA analogue (TAR RNA spans the minimal sequence required for the transactivation *in vivo*) used for the binding studies with HIV-1 Tat protein.

1.2.2 HIV-1 Tat protein

Tat is a small viral regulatory protein, that is indispensable for viral replication. In the absence of Tat, transcription of HIV-1 mRNAs can be initiated but cannot be efficiently elongated to produce full-length viral RNA genome (Emerman et al., 1998). The presence of Tat, however results in a large increase in the level of transcripts that extend through the entire genome. Tat protein has a variable weight of 14-16 kDa. In fact, its constitution varies from 86 to 101 amino acids (AA). However, only 72 N-terminal amino acids are required for full activity (Cullen 1986). Tat protein consists of five different domains, or dominant functional regions: the N-terminal acidic domain, the cysteine-rich domain, the core domain, the arginine rich motif (ARM), and the glutamine rich domain (Fig. 1.4). The arginine rich region mediates the binding of HIV-1 Tat to TAR RNA. However, ARM alone is not sufficient to give full binding specificity and the addition sequence from the "core" region of the protein is required (Churcher et al., 1993).

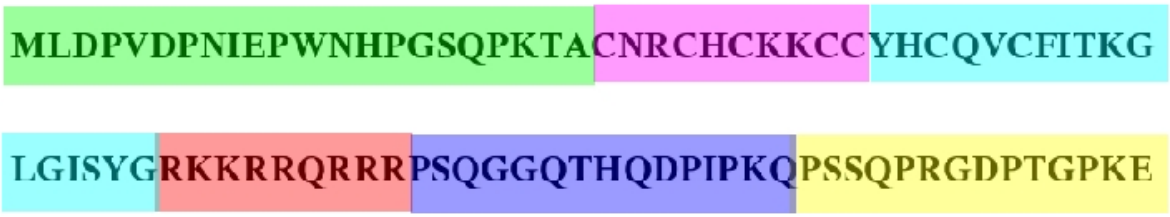


Fig. 1.4 Primary structure of HIV-1 Tat protein. The Tat protein functional regions are shown in different colours. The N-terminal acidic region (green), the cysteine rich region (magenta), the core region (cyan), the arginine rich region (red), the glutamine rich region (blue), and the exon (yellow).

1.2.3 Positive transcription elongation factor b

The positive transcription elongation factor b (p-TEFb), a general elongation factor, was first identified and purified from *Drosophila* extracts (Marshall et al., 1995). It acts to prevent RNA polymerase arrest and contains an associated kinase activity capable of hyperphosphorylating the C-terminal domain (CTD) of Rpol II (Marshall et al., 1996). p-TEFb is composed of two

subunits: the catalytic subunit cyclin-dependent kinase (CDK9) and the regulatory subunit cyclinT1 (Yang et al., 1997; Zhu et al., 1997; Wei et al., 1998). Complexes containing CDK9 and cyclinT1-related proteins, cyclinT2a or cyclinT2b, are also active for p-TEFb function (Peng et al., 1998). Tat interacts with cyclinT1 subunit of p-TEFb and recruits the kinase complex to the TAR RNA. Recruitment of p-TEFb to TAR has been proposed to be both necessary and sufficient for activation of transcription elongation from the HIV-1 LTR promoter (Bieniasz et al., 1999). The human cyclinT1 contains 726 amino acids (Wei et al., 1998; Peng et al., 1998). From position 1 to 250, 251 to 271, 370 to 430, 506 to530, and 709 to 726, two conserved cyclin boxes, a Tat-TAR recognition motif (TRM), a coiled-coil region, a histidine-rich region, and the C-terminal PEST sequence, respectively, are found (Wei et al., 1998). The first 272 residues of human cyclinT1 are sufficient to bind Tat and TAR *in vitro* and support Tat transactivation *in vivo* (Garber et al., 1998). In the TRM of human cyclinT1, the cysteine at position 261 is involved in a Zn^{2+} -dependent interaction with other cysteines and/or hisitidines from position 1 to 48 of Tat (Garber et al., 1998). Tat binding to human cyclinT1 induces binding of p-TEFb onto the TAR RNA loop (Richter et al., 2002) .

1.2.4 Negative transcription elongation factor

A new class of negative transcription elongation factors, including DRB (5,6-dichloro-1-β-D-ribifuranosylbenzimidazole) sensitivity inducing factor (DSIF) and negative elongation factor (NELF) has shed new light on the control of Rpol II elongation at TAR (Wada et al., 1998; Yamaguchi et al., 1999).

The purified DSIF from HeLa cell nuclear extracts is composed of two polypetides of 14 kDa (p14) and 160 kDa (p160) (Wada et al., 1998). Sequence analysis showed that p160 is a human homolog of the *Saccharomyces cervisiae* transcription factor Spt5 (Swanson et al., 1991). Furthermore, recombinant Supt4h protein, the human homolog of yeast Spt4 (Hartzog et al., 1996; Malone et al., 1993), is functionally equivalent to DSIF p14, strongly suggesting that DSIF is composed of the human homologs of Spt4 and Spt5. The sequence comparisons reveal that the central region of p160 has significant similarity to the bacterial transcription elongation factor NusG, which interacts with RNA polymerase and regulates its activity (Li et al., 1992; Sullivan et al., 1992). DSIF interacts directly with Rpol II and cooperates with NELF to represses Rpol II elongation (Yamaguchi et al., 1999). In addition to its negative effect on elongation, DSIF stimulates the rate of transcription elongation under limiting nucleotide conditions in a DRB-sensitive fashion (Wada et al., 1998). NELF is a heterotetrameric protein

consisting of NELF-A (66 kDa), NELF-B (61 kDa), alternatively spliced NELF-C (59 kDa) or NELF-D (58 kDa) and NELF-E (46 kDa), also called RD because of its internal repeats of the amino acids arginine (R) and aspartic acid (D) (Yamaguchi et al., 1999; Narita et al., 2003). NELF-A is encoded by *WHSC2*, a candidate gene for Wolf-Hirschhorn syndrome (Wright et al., 1999). Interestingly, its N-terminal segment shows sequence similarity to the hepatitis delta antigen (HDAg), the hepatitis delta virus protein that binds to Rpol II and activates transcriptional elongation (Yamaguchi et al., 2001). The sequence characterisation of NELF-E (Fig 1.6) indicated it contains an N-terminal leucine zipper motif, a central region rich in Arg-Asp dipeptide repeats (RD motif), and a C-terminal RNA recognition motif (RRM), its RNA binding activity is critical for NELF function (Yamaguchi et al., 1999; Yamaguchi et al., 2002). Furthermore, NELF-E interacts with the NELF-B subunit, probably *via* the leucine zipper motif (Narita et al., 2003). NELF-B is identical to COBRA1, reported to associate with the breast cancer susceptibility gene BRCA1 (Ye et al., 2001). NELF-C and NELF-D are highly related or identical to a protein called TH1, of unknown function (Bonthron et al., 2000). NELF-B and NELF-C or NELF-D are integral subunits that bring NELF-A and NELF-E together (Narita et al., 2003). NELF represses the transcription elongation through the binding to a nascent transcripts and preformed DSIF-Rpol II complex (Yamaguchi et al., 2002).

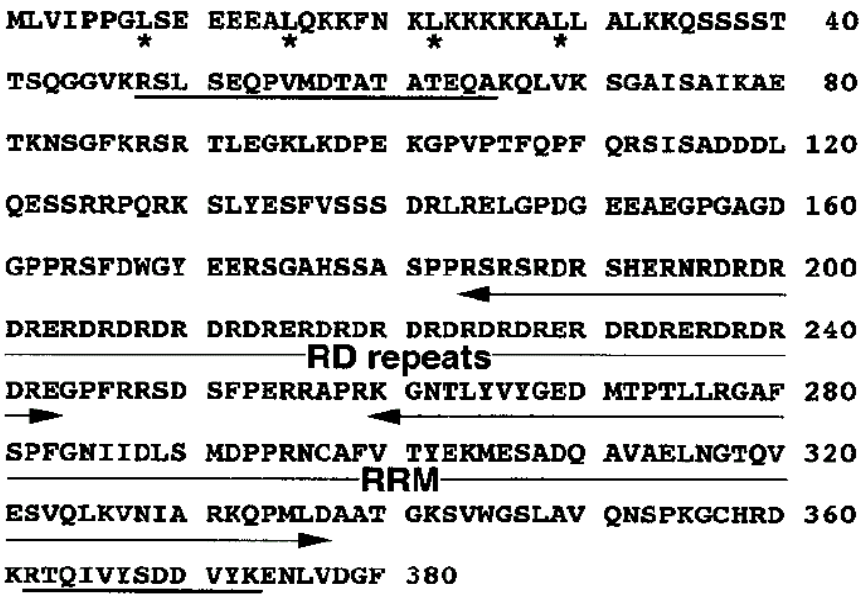


Fig. 1.6 Primary structure of NELF-E. The RD motif and the putative RRM are indicated by arrows. Asterisks denote leucine residues of the putative leucine zipper structure.

1.2.5 Termination of HIV-1 transcription

The Rpol II containing nonphosphorylated CTD of the largest subunit assembles on the HIV LTR promoter to form a preinitiation complex. TFIIH binds to nonphosphorylated Rpol II and plays a critical role in transcription initiation and promoter clearance (Goodrich et al., 1994; Lu et al., 1991; Kumar et al., 1998). TFIIH phosphorylates the CTD of the largest subunit of Rpol II and assists in promoter clearance. The TFIIH complex dissociates from transcription elongation complex (TEC) after initiation and is not part of the elongation complexes (Zawel et al., 1995). Both DSIF and NELF are found on the HIV-1 LTR after the initiation of viral transcription (Ping et al., 2001). Sequence-specific interactions between NELF-E RRM and nascent transcripts (in this case its HIV-1 TAR RNA) facilitate the formation of DSIF-NELF-Rpol II complex and represses the transcription elongation, which results in abortive termination of early elongation steps by the growing the transcripts (Fig 1.7).

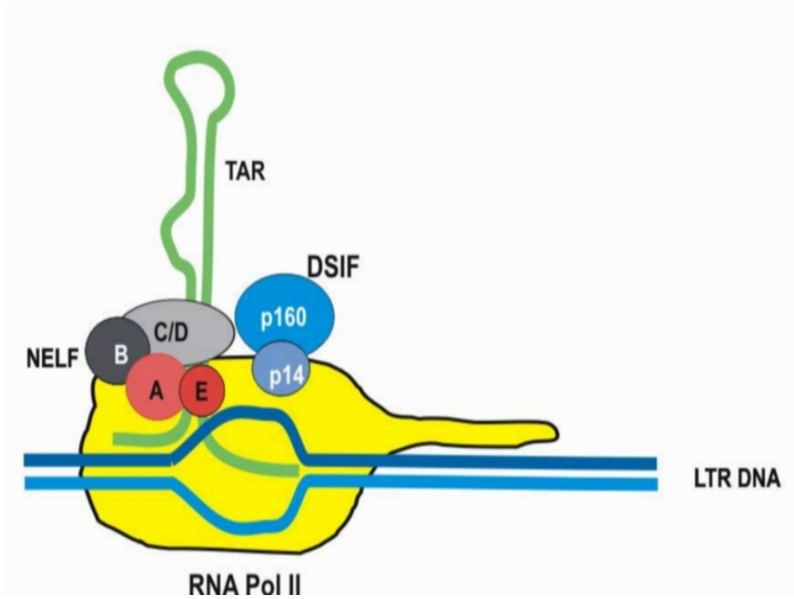


Fig. 1.7 Termination complex of HIV-1 transcription. Individual components in the termination complex and their function is described in text (Wöhrl, 2003).

1.2.6 Antitermination of HIV-1 transcription.

Tat appears to be required in order to overcome the arrest of Rpol II by the negative transcriptional elongation factors DSIF and NELF (Wada et al., 1998; Yamaguchi et al., 1999; Yamaguchi et al., 2002; Fujinaga et al., 2004). While Rpol II can associate with the proviral LTR and initiate transcription in the absence of Tat, these polymerase complexes are non-processive and dissociate from the template prematurely and produces very short transcripts

(Kao et al., 1987). Tat associates with cellular kinase complex p-TEFb (cyclinT1:CDK9) is believed to bring the catalytic subunit of this kinase complex (CDK9) in close proximity to Rpol II where it hyperphosphorylate the CTD of Rpol II (Zhou et al., 2000). The RD subunit of NELF and p160 subunit of DSIF, which associate through RD with the bottom stem of TAR, are also phosphorylated by p-TEFb (Yamaguchi et al., 2002; Fujinaga et al., 2004). Phosphorylation of RD results in its dissociation from TAR, which results in the productive elongation. Thus, Tat appear to facilitate elongation of HIV-1 transcript by hyperphophorylating the CTD of Rpol II and by removing the negative transcription elongation factors from TAR (Fig. 1.8).

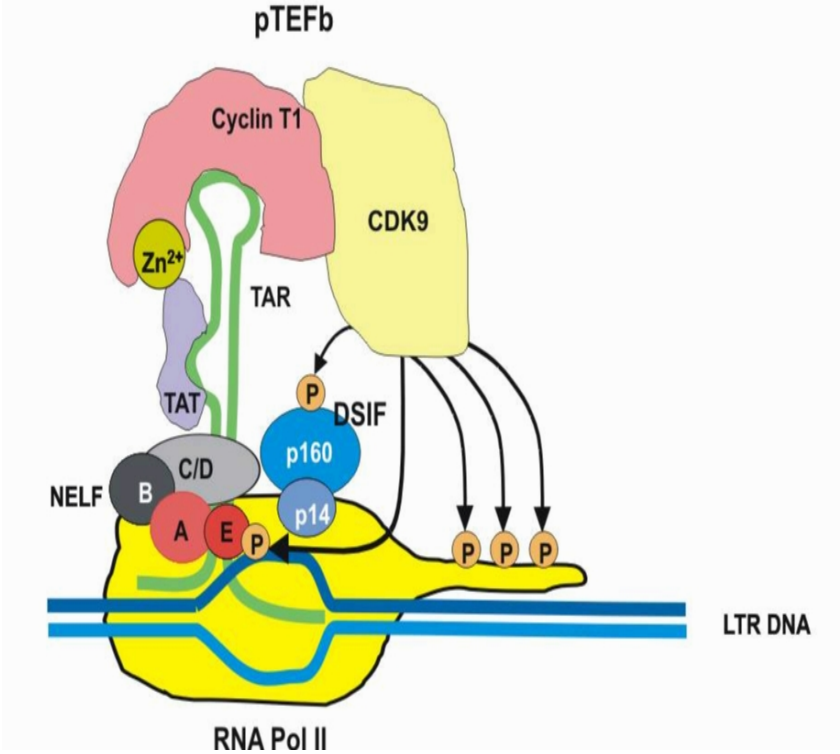


Fig. 1.8 Antitermination of HIV-1 transcription. Individual components in the antitermination complex and their function is described in text (Wöhrl, 2003).

2 Aim of this work

The regulation of HIV-1 transcription is a complex event that requires the cooperative action of both viral and cellular components. The aim of this work is to understand the mechanism of termination and antitermination of HIV-1 transcription. To understand this the structure and function of the contributing factors need to be analysed.

The HIV-1 Tat protein is a potent activator of transcription and is essential for viral replication. Tat protein activates and enhances the expression of all viral genes by binding to TAR RNA. The cellular factor NELF is involved in the termination of HIV-1 transcription and the RRM from subunit E plays a key role in arresting the elongation complex by binding to the stem region of TAR RNA. Thus, the present work is focussed on

1. Analyis of the interaction between Tat-Cys$^-$ and TAR RNA using CD and NMR spectroscopy.
2. Structural information of Tat-Cys$^-$ at atomic level in the complex with TAR RNA.
3. Structure determination of NELF-E RRM using multidimensional NMR spectroscopy.
4. Analysis of the interaction between NEFL-E RRM and TAR RNA.
5. Identification of RNA recognition site on NELF-E RRM by using TAR RNA oligonucleotides.

3 Materials and methods

3.1 Culture media

All the culture media were prepared using the ultra pure water (Filtering unit Milli-Q Biocel with Filter Millipak® Express 20, 0.22 µm, Millipore, Eschborn) and autoclaved for sterilisation (20 min, 1.2 bar, 120 °C, autoclave type 23, Melag, Berlin). To prepare the selective media, sterile-filtered (0.2 µM filter, Sartorius, Goettingen) ampicillin (final concentration 100 µg/ml) added to the media when the temperature was approximately 50 °C.

3.1.1 LB-medium (Luria Bertani)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
H_2O	ad 1000 ml

3.1.2 Minimal medium for uniform enrichment with ^{15}N

For the bacterial expression of protein in minimal medium, M9-minimal medium (Sambrook et al., 1989) with TS2- trace element solution (Meyer and Schlegel, 1983) was used.

5 x M9-medium stock solution:	$Na_2HPO_4.12H_2O$	85.5 g
	KH_2PO_4	15.0 g
	NaCl	2.5 g
	$^{15}NH_4Cl$	5.0 g
	H_2O	ad 1000 ml

Trace element solution-TS2	$ZnSO_4.7H_2O$	100 mg
	$MnCl_2.4H_2O$	30 mg
	H_3BO_3	300 mg
	$CoCl_2.6H_2O$	200 mg
	$NiCl_2.6H_2O$	20 mg

$CuCl_2.2H_2O$	10 mg
$Na_2MoO_4.2H_2O$	900 mg
Na_2SeO_3	20 mg
H_2O	ad 1000 ml

To prepare 1 l of 1 x M9 minimal medium, 200 ml 5 x M9 minimal medium was diluted with 800 ml of ultra pure water and autoclaved.

1 liter 1 x M9-Medium	1M $MgSO_4$	2 ml
	TS2-solution	2 ml
	10 mM Fe(III)-citrate	1 ml
	20 % (w/v) Glucose	20 ml
	1M $CaCl_2$	100 µl
	Vitamin B1	1 ml (40 mg/ml)

The above mentioned additives were added to the media when the temperature was approximately 50 °C.

3.1.3 IPTG stock solution

Isopropyl-β-D-thiogalactopyranoside (IPTG) was dissolved in water (2.38 g/10 ml) to a final concentration of 1 M. The stock solution was sterile filtered (0.22 µM) and stored at 4 °C. The final concentration of IPTG used in the culture was 1 mM.

3.2 Expression and purification of Tat-Cys$^-$

3.2.1 Expression of unlabeled Tat-Cys$^-$ protein

The recombinant cysteines free Tat (Tat-Cys$^-$) construct was expressed in the *Escherichia .coli (E.coli)* BL21(DE3) strain (Invitrogen). Cells were first grown in 10 ml LB medium with 1 µg/ml ampicillin (LB_{amp}) for 8 hours at 37 °C, 180 rpm, and then transferred into 200 ml LB_{amp}. The cells were grown overnight at 37 °C. 2.5 l LB_{amp} inoculated with the over night culture to final optical density at 600 nm (OD_{600}) of around 0.3 and the cells were grown at 37 °C until OD_{600} reached 0.8. The gene expression was induced for 4 hours with 1 mM IPTG. To

monitor the gene expression by sodium dodecyl sulphate-polyacrylamide gel electrophorysis (SDS-PAGE), samples were collected (samples corresponded to OD_{600} of 1/ml) every hour. Samples were analysed as described in Chapter 3.4. 4 h after induction, cells were harvested by centrifugation at 4 °C, 6000 rpm for 30 min and the cell pellet was stored at – 80 °C for further use.

3.2.2 Expression of ^{15}N labeled Tat-Cys$^-$

For the expression of ^{15}N labeled Tat-Cys$^-$ protein the cells were grown in 2.5 l LB_{amp} medium until OD_{600} reached 0.8, as it described in chapter 3.2.1. Cells were centrifuged at 15 °C, 6000 rpm for 15 min and the cell pellet was resuspended in 750 ml of 1 x M9 minimal medium lacking NH_4Cl and all the additives. The cell suspension was centrifuged at 15 °C, 6000 rpm for 15 min and the pellet was resuspended in 1xM9 minimal medium containing $^{15}NH_4Cl$ and all the additives. After 1h at 37 °C, 160 rpm gene expression was induced with 1 mM IPTG. Gene expression was monitored by SDS-PAGE. 4 h after induction cells were harvested as it described in Chapter 3.2.1.

3.2.3 Cell lysis

The cell pellet (Chapter 3.2.1/3.2.2) was resuspended in lysis buffer I (4 ml/g) and then stirred on on ice for 30 min.

Lysis buffer I : 50 mM Tris-HCl pH 8.0, 1 M $MgCl_2$, 2 mM phenylmethylsulfonylflouride (PMSF).

Freezing of the cell suspension was carried out in a dry ice/isopropanol solution followed by thawing in a water bath. This step was repeated three times and the suspension was stored at – 80 °C.

After thawing the cell suspension 0.2 mg/ml lysozyme, 0.2 mg/ml DNAse l, 4 mg/ml benzamidine, 1 protease inhibitor tablet EDTA-free (Roche) were added and the suspension was stirred on ice for 30 min. The cell suspension was sonified for 30 s with ultrasound (200 Watt, Sonifier Labsonic U, needle probe 40 T, B. Braun Biotech International, Melsungen) on ice to break up the cells and stirred on ice for 10 min in between each step. The cell extract was centrifuged for 45 min at 4 °C, 13000 rpm (19000 g) to separate the cell debris. Samples were collected before centrifugation and analysed by SDS-PAGE as described in chapter 3.4.

The supernatant was kept on ice while the cell pellet was again resuspended in lysis buffer II (4

ml/g) and stirred the cell suspension on ice for 30 min.

Lysis buffer II : 50 mM Tris-HCl pH 8.0, 1M $MgCl_2$, 2 mM PMSF, 0.2 mg/ml lysozyme, 0.2 mg/ml DNAse l, 4 mg/ml benzamidine, 1 protease inhibitor tablet EDTA-free.

The solution was sonified as described above. After centrifugation the supernatant was combined with the previous one and kept on ice for further use.

3.2.4 pH-precipitation

The supernatant (Chapter 3.2.3) was stirred on ice for 10 min. 1M HCl was added drop wise until pH = 3.0 was reached and then stirred on ice for 45 min. The suspension was centrifuged for 30 min at 4 °C, 13000 rpm. Tat-Cys^- protein should be in the supernatant (Chapter 4.1.2). Samples were taken before centrifugation and analysed by SDS-PAGE.

3.2.5 Ammonium sulfate precipitation

A solution of 1.5 M Tris-HCl, pH 9.0 was added to the supernatant of the pH precipitation until pH 8.0 was reached. The saturated ammonium sulfate solution was added until 50% v/v saturation reached and then the suspension was stirred on ice for 2 h. The suspension was centrifuged for 30 min at 4 °C, 13000 rpm. Tat-Cys^- protein should be in the pellet (Chapter 4.1.2). A sample was taken before centrifugation and analysed by SDS-PAGE. The pellet was resuspended in 10 mM potassium phosphate, pH 6.4, 200 mM NaCl (= buffer A) and dialysed against 2 x 2.5 l buffer A at 4 °C using a dialysis tube with a molecular weight cut off (MWCO) 3000 D (Roth, Karlsruhe)

3.2.6 Cation exchange chromatography

The recombinant overexpressed Tat-Cys^- was purified by employing cation exchange chromatography. The ÄKTA purifier 10 FPLC (*fast performance liquid chromatography*)-system (Amersham Biosciences, Freiburg) was used to purify the protein with a HiTrap™ Heparine HP-column (HiTrap™, Amersham Biosciences, Freiburg). This column was consisted of Heparine Sepharose™ high performance beads, which are negatively charged due to sulfate groups. The column was washed with five column volumes of sterile H_2O and followed by five column volumes of binding buffer (10 mM potassium phosphate, pH 6.4, 200 mM NaCl). The protein solution (Chapter 3.2.5) was loaded on column with a flow rate 1 ml/min. Flow through was collected in each step. The column was washed with buffer A for

five column volumes to remove unbound proteins. Bound protein was eluted by applying 10 mM potassium phosphate, pH 6.4, 2 M NaCl (buffer B) with a step of 0-0.2 M NaCl for 3 column volumes and then a constant gradient 0.2-2 M NaCl for 8 column volumes. Fractions collected during the elution were analysed by SDS-PAGE. After use, the column was washed with two column volumes of buffer B, five column volumes of sterile H_2O and 20% ethonal, respectively and kept in 20% ethanol at 4 °C until further use.

3.3 Determination of protein and nucleic acid concentration

Protein and nucleic acid concentrations were determined according to Beer-Lambert's law.

$$A = c.\, d.\, \varepsilon$$

A = absorption d = thickness of cuvette

c = concentration [M] ε = molar extinction coefficient [M^{-1} cm^{-1}]

For determination of the concentrations of protein and nucleic acid, the absorption was measured at a wavelength of 280 nm and 260 nm (A_{280} and A_{260}), respectively. Absorption of protein and nucleic acid was measured using a Heλios γ spectrophotometer (Thermo spectronic, Cambridge, UK) or a Uvikon 930 UV-VIS spectrophotometer (Kontron, Eching). Measurements were made in a black wall quartz cuvette (Hellma, Müllheim) with a thickness of 1 cm. As a reference, the appropriate buffer was used. Molar extinction coefficients of proteins and nucleic acids at 280 nm and 260 nm used for determination of the concentrations are shown below.

ε_{280} (Tat-Cys^-) = 8250 M^{-1} cm^{-1} (Boehm, 2000)

ε_{280} (NELF-E RRM) = 3840 M^{-1} cm^{-1} (Neumann, 2005)

ε_{260} (TAR from 17 to 45) = 194630 M^{-1} cm^{-1} (Metzger, 1997)

ε_{260} (TAR from 1 to 59) = 563100 M^{-1} cm^{-1} (Neumann, 2005)

3.4 SDS-PAGE

Results of overexpression tests, level of protein production as well as quality and purity of fractions obtained during successive steps purification were analysed by SDS-PAGE. Mini-gels (10 x 8 x 0.75 cm) were electrophoresed in Mighty small SE250/260 gel electrophoresis chambers (Hoefer, San Francisco, CA, USA). The Tris-Tricine SDS-PAGE was adapted from Schagger and von Jagow (Schagger et al., 1987).

The separation gel consisted of 16.5 % (w/v) acrylamide, 2 % (w/v) N,N-methylene-bisacrylamide, 0.1 % (w/v) SDS, 1M Tric-HCl, pH 8.45, 6 M urea and the stacking gel consisted of 10 % (w/v) acrylamide, 0.31 % N,N-methylene-bisacrylamide, 0.1 % SDS, 1M Tris-HCl, pH 8.45, 6 M urea. The polymerisation was initiated by addition of 10 µl TEMED and 100 µl 10 % (w/v) APS per 20 ml gel solution. Different buffers were used for anode and cathode.

Anode buffer (+) : 0.2 M Tris-HCl, pH 8.9. 24.22 g/l

Cathode buffer (-) : 0.1 M Tris-HCl, pH 8.25, 0.1 M Tricine, 0.1 % (w/v) SDS.

A constant voltage of 28 mA was applied until the samples reached the end of the stacking gel and then the current was increased to 40 mA in separation gel. As a molecular weight standard, for the estimation of protein molecular weight in gel BioRad low molecular weight standard (BioRad, München) was used.

Samples of cells to be analysed for protein expression were prepared by resuspending the bacterial pellet (chapter 3.2.1/3.2.2) in 50 µl of 2 x Roti-Load (Roth, Karlsruhe) and denatured by boiling at 95 °C for 5 min. For the samples collected during protein purification, 20 µl of sample was mixed with 30 µl of 2 x Roti-Load and denatured by boiling at 95 °C for 5 min. Depending on the protein concentration 5 to 20 µl sample was loaded onto the gel.

For visualisation of separated proteins, gels were incubated for 30 min in protein staining solution (0.05 % (w/v) Coomassie Brilliant Blue R-250, 45 % (v/v) methanol and 9.2 % (v/v) acetic acid). For destaining the gels were incubated 30 min in destaining solution I (25% (v/v) methonol, 1 % (v/v) acetic acid) and followed by 2 hours in destaining solution II (5 % (v/v) methanol, 7 % (v/v) acetic acid) on a horizontal shaker with 30 rpm. The destained gels were documented using gel-documentation system (Gel-Doc 2000, BioRad, Munich).

3.5 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a type of absorption spectroscopy that can provide information on the structures of many types of biological macromolecules. Circular dichroism is the difference between the absorption of left and right handed circularly-polarised light and is measured as a function of wavelength. Biological macromolecules such as proteins, polysaccharides and nucleic acids are composed of optically active elements and because they can adopt different types of three-dimensional structures, each type of molecule produces distinct CD spectra. CD spectroscopy has been used to monitor secondary structure, conformational changes, environmental effects, protein folding, protein denaturation, and dynamics.

The secondary structure formed by polypeptides and proteins have distinctive CD spectra. Typical for the α-helix is a negative band at about 222 nm due to nπ* transition, and a negative and positive couplet at about 208 and 190 nm due to the parallel and perpendicular components of the ππ* transition, respectively. The CD for a β-strand has a negative band at about 215 nm and positive band at about 198 nm. The more intense positive CD at 270 nm coupled with the negative CD at 210 nm, and the extremely intense positive CD at 185 nm are characteristic of the double stranded RNA (Van Holde K. E et al., 1998).

CD is measured as a quantity called mean residue ellipticity, whose units are degrees-cm^2/dmol.

$$[\Theta]_{MRW} = \frac{100\,\Theta}{c.d.N}$$

$[\Theta]_{MRW}$ = Mean residue molar ellipticity (deg. cm^2. $dmol^{-1}$) c = concentration of protein (M)

Θ = Ellipticity (deg) d = path length of cuvette (cm)

N = number of amino acids in the protein.

CD spectra were measured with a Jasco J-810S spectrometer (JASCO International, Tokyo, Japan). Samples were prepared in 10 mM potassium phosphate, pH 6.4, 150 mM NaCl and the temperature was maintained at 20 °C using temperature control unit CDF-426S (JASCO International, Tokyo, Japan). Concentrations of HIV-1 TAR RNA, Tat-Cys^- and of the 1:1 HIV-1 TAR RNA-Tat-Cys^- complex were 15 µM in a 1 cm path-length quartz cuvette (Helma, Muellheim). All the Spectra were measured 8 times at a scan rate of 50 nm/min averaged and corrected for contribution of the respective solvent.

3.6 RNA oligonucleotides

Single stranded RNA oligonucleotides used for the binding studies with NELF-E RRM were purchased from biomers.net. Sequence of the corresponding RNA oligonucleotides are shown below.

TAR1-10 : GGUCUCUCUG

TAR6-15 : CUCUGGUUAG

TAR11-21 : GUUAGACCAGA

TAR39-48 : UCUGGCUAAC

TAR44-53 : CUAACUAGGG

TAR49-57 : UAGGGAACC

3.7 NMR spectroscopy

3.7.1 Sample preparation

The expression and purification of the recombinant NELF-E RRM (244-343AA) was performed by Liane Neumann (Diplomarbeit, University of Bayreuth, 2005) and Katrin Weiss (Lab technician, Department of Biopolymers, University of Bayreuth). The purified protein was dialysed against 10 mM sodium phosphate, pH 6.9, 100 mM NaCl, 1 mM DTT, concentrated with Vivaspin concentrators (Vivascience, MWCO 5000 Da). Additionally 10% D_2O for the field frequency lock and 0.03% NaN_3 as an antimicrobial agent were added. 5 mm Ultra Precision NMR tubes (Norell, Landsville, NJ, USA) were used to measure the sample volume of at least 0.5 ml. Variable-restricted volume NMR tubes of the Shigemi company (Campro Scientific, Veendendaal, NL) were used to minimise the amount of material needed. For NMR structure determination of NELF-E RRM, uniformly labeled ^{13}C-^{15}N and ^{15}N protein samples were used with a sample concentration of 0.6 mM.

3.7.2 NMR spectrometers and measurement

All NMR experiments were performed at 298 K or 303 K on Bruker Avance 400, DRX600, Avance 700, and Avance 800 with proton resonance frequency of 400 MHz, 600 MHz, 700 MHz, and 800 MHz respectively, and equipped with inverse room temperature or cryogenic cooled $^{1}H/^{13}C/^{15}N$ triple-resonance probes with pulsed field gradient capabilities (Bruker Karlsruhe, D). The water suppression was performed by either 3-9-19 WATERGATE sequence (Sklenar et al., 1993) or *Water Flip-Back* (Grzesiek et al., 1993) or a coherence selection with pulsed field gradients (Schleucher et al., 1994). Quadrature detection in the indirectly detected dimensions was obtained by the States-TPPI (*time proportional phase incrementation*) method (States et al., 1982; Marion et al., 1989) or the echo/anti-echo method (Kay et al., 1992), if coherence selection with gradients was employed. The selective excitation of aliphatic and/or carbonyl region on the ^{13}C channel was achieved by using the appropriate combination of on/off resonance band-selective pulses such as Guassian cascades (Emsley et al., 1990) as well as proper calibrated rectangular pulses. A low-power GARP-I sequence (Shaka et al., 1985) was applied for heteronuclear broadband decoupling during the data acquisition. Proton broadband decoupling during the heteronuclear transfer steps in the triple resonance experiments was achieved by the WALTZ-16 sequence (Shaka et al., 1983). The

proton chemical shifts were referenced to external standard DSS (2,2-dimethyl-2-silapentanesulfonate). The chemical shifts of ^{13}C and ^{15}N resonances were referenced indirectly using the Ξ ratios of the zero-point frequencies at 298 K : 0.10132905 for $^{15}N/^{1}H$ and 0.25144952 for $^{13}C/^{1}H$ (Live et al., 1984).

3.7.3 NMR data processing and analysis

The NMR datasets were processed using in house written software (Schweimer, 2000) and analysed with the program packages NMRview (Johnson and Blevins, 1994) and NDEE (SpinUp Inc., Dortmund, Germany).

Data processing consists typically of SVD-Linear Prediction (Barkhuijsen et al., 1985) with root reflection (Press et al., 1992) in one heteronuclear dimension (normally the ^{15}N dimension of triple resonance experiments or the X-dimension in the X-edited spectra), apodization with 60°-90° shifted squared sinebell, one zero filling in all dimensions (Cavanagh et al., 1996) and Fourier transformation. For constant time evolution periods, mirror image linear prediction (Zhu and Bax, 1990) was employed. Finally baseline correction in the acquisition dimension was performed using a model free algorithm (Friedrich 1995).

3.7.4 NMR experiments

In order to obtain a sequence specific resonance assignments, distance restraints, and dihedral angle restraints a series of standard double and triple resonance experiments were recorded at 298 K with ^{15}N and/or ^{13}C-^{15}N labeled protein. All the NMR experiments recorded for the structure determination of NELF-E RRM and their observable correlations and parameter sets were shown in Table 3.1 and 3.2 respectively. The pulse programs used for the NMR experiments were optimised for the respective spectrometer based on the fundamental experiments described in the references (Dr. Kristian Schweimer, University of Bayreuth, 2000).

Experiment	Observable correlation
$^{1}H^{15}N$-HSQC	H (*i*), N (*i*)
ct-$^{1}H^{13}C$-HSQC	$H^{alipha}(i)$, $C^{alipha}(i)$
$^{1}H^{13}C$-HSQC (*Aromatic*)	$H^{aro}(i)$, $C^{aro}(i)$
HNCO	$H^{N}(i)$, N(*i*), CO(*i-1*)
HNCA	$H^{N}(i)$, N(*i*), C^{α} (*i-1*), C^{α} (*i*)
HNHA	$H^{N}(i)$, N(*i*), H^{α} (*i*)
CBCA(CO)NH	$H^{N}(i)$, N(*i*), C^{α} (*i-1*), C^{β} (*i-1*)
HBHA(CO)NH	$H^{N}(i)$, N(*i*), $H^{\alpha}(i-1)$, $H^{\beta}(i-1)$
HNCACB	$H^{N}(i)$, N(*i*), $C^{\alpha}(i-1)$, $C^{\beta}(i-1)$, $C^{\alpha}(i)$, $C^{\beta}(i)$
HCCH-TOCSY	H^{alipha} (*i*), C^{aliph} (*i*)
3D-^{1}H,$^{15}N^{1}H$-NOESY-HSQC	H(*i*) $\xrightarrow{NOE}$ H(*j*), N(*j*)
3D-^{1}H,$^{13}C^{1}H$-NOESY-HSQC	H(*i*) $\xrightarrow{NOE}$ H(*j*), C(*j*)
3D- ^{1}H,$^{13}C^{1}H$-NOESY-HSQC (*Aromatic*)	H(*i*) $\xrightarrow{NOE}$ $H^{aro}(j)$, $C^{aro}(j)$

Table 3.1 Observable correlations in the NMR experiments

The names of the triple resonance experiments sound very cryptic at first glance, but they are very descriptive. The name reflects the flow of magnetisation across the bonds or spatially neighboring protons in that experiment. The nucleus showed in parentheses was used only for magnetisation transfer and whose resonant frequency was not detected. C^{alipha}/H^{alipha} : aliphatic carbon/proton, C^{aro}/H^{aro} : Aromatic carbon/proton, and H^{N} : amide proton.

Dimension	resonance	SW(HZ)	TD	SF0(MHz)	reference
		$^{1}H^{15}N$-HSQC			
F1	^{15}N	1561.1	384		
F2 (32)	^{1}H	8389.6	1024	700	(Mori et al., 1995)
		ct-$^{1}H^{13}C$-HSQC			
F1	^{13}C	14084.5	720		
F2 (16)	^{1}H	11160.7	1024	800	(Vuister and Bax, 1992)
		$^{1}H^{13}C$-HSQC (Aromatic)			
F1	^{13}C	3621.8	256		
F2 (32)	^{1}H	8802.8	1024	800	(Vuister and Bax, 1992)
		HNCO			
F1	^{13}C	2817.1	96		
F2	^{15}N	1783.8	64		
F3 (16)	^{1}H	9615.3	1024	800	(Grzesiek and Bax, 1992b)
		HNCA			
F1	^{13}C	5634.3	96		
F2	^{15}N	1783.8	64		
F3 (16)	^{1}H	9615.3	1024	800	(Grzesiek and Bax, 1992b)
		HNHA			
F1	^{1}H	4901.4	160		
F2	^{15}N	1561.1	64		
F3 (16)	^{1}H	8389.2	1024	700	(Vuister and Bax, 1993)
		HNCACB			
F1	^{13}C	12072.0	128		
F2	^{15}N	1783.8	64		
F3 (16)	^{1}H	9615.3	1024	800	(Wittekind and Mueller, 1993)
		CBCA(CO)NH			
F1	^{13}C	12072.0	128		
F2	^{15}N	1783.8	64		
F3 (16)	^{1}H	9615.3	1024	800	(Grzesiek and Bax, 1992a)

Dimension	resonance	SW(HZ)	TD	SF0(MHz)	reference
		HBHA(CO)NH			
F1	^{1}H	5600.9	128		
F2	^{15}N	1783.8	64		
F3 (16)	^{1}H	9615.3	1024	800	(Grzesiek and Bax, 1993a)
		HCCH-TOCSY			
F1	^{1}H	5600.9	160		
F2	^{13}C	7042.2	64		
F3 (16)	^{1}H	10416.6	1024	800	(Wijmenga et al., 1997)
		3D-^{1}H,$^{15}N$$^{1}H$-NOESY-HSQC			
F1	^{1}H	7702.2	256		
F2	^{15}N	1561.1	72		
F3 (16)	^{1}H	8389.2	1024	700	(Talluri und Wagner, 1996)
		3D-^{1}H,$^{13}C$$^{1}H$-NOESY-HSQC			
F1	^{1}H	7702.2	256		
F2	^{13}C	6162.5	64		
F3 (16)	^{1}H	8389.2	1024	700	(Ikura et al., 1991b)
		3D-^{1}H,$^{13}C$$^{1}H$-NOESY-HSQC (Aromatic)			
F1	^{1}H	8801.4	256		
F2	^{13}C	3621.9	48		
F3 (16)	^{1}H	8802.8	1024	800	(Ikura et al., 1991b)

Table 3.2 Summary of all NMR experiments recorded for structure determination of NELF-E RRM and their parameter.

3.7.5 Secondary structure determination by NMR spectroscopy

The most commonly used method to determine the sequence specific secondary structure elements is based on the study of the chemical shift values. It has been known since the early days of protein spectroscopy that secondary structure and chemical shifts are closely linked (Wishart et al., 1994). It could be shown that C^{α} and C' resonate downfield when located in an α-helix and upfield when in β-strands; C^{β} and H^{α} behave contrarily. The random coil chemical shift values have been described in detail by Wishart & Sykes (Wishart et al., 1992; Wishart et al., 1994). They suggested an easy approach to define the secondary structure with high accuracy by defining the chemical shift index, CSI. There the values of the secondary chemical shift are classified in either +1, when the chemical shift is greater than the random coil value of the corresponding atom, or -1, when the chemical shift observed is less. The consensus CSI summarises the results of the independent approaches from the different nuclei. α-helices assume per definition the value -1, whereas β-strands are described by +1. The existence of an α-helix is suggested, when a dense grouping of at least four -1´s is not interrupted by a +1. For the formation of a β-strand three consecutive +1 s are sufficient.

3.7.5 Distance restraints

The nuclear Overhauser effect (NOE) arises from cross relaxation between dipolar coupled spins as a result of spin/spin interactions through space**.** Of special importance in this respect are proton-proton distances, which can be estimated from the signal intensities in the 3D-^{15}N NOESY-HSQC and ^{13}C NOESY-HSQC. Signal intensity depends on the distance r between two nuclei i and j, according to: $NOE_{ij} \sim 1/r_{ij}^{6}$. As the NOE build-up is time dependent, the magnetisation transfer from one nuclei to another such that after a while the magnetisation is equally distributed over the whole molecule. This process is called spin diffusion. In NOESY experiments, serving for the evaluation of distance restraints, mixing times have to be carefully chosen to ensure a sufficient time for the NOE build-up, but at the same time short enough to avoid spin diffusion. NOESY cross peaks were classified according to their relative intensities and converted to distance restraints with upper limits of 3.0 Å (strong), 4.0 Å (medium), 5.0 Å (weak), and 6.0 Å (very weak). For ambiguous distance restraints the r^{-6} summation over all assigned possibilities defined the upper limit (Nilges 1995). It is distinguished between cross peaks of protons not more than five amino acids apart in the protein sequence (medium range NOEs) and those which are more than five amino acids apart (long range NOEs). The former

are mainly indicative of the protein backbone conformation and are used for secondary structure determination, whereas the latter are an expression of the global structure of the protein and therefore contain the main information used for tertiary structure calculation.

3.7.6 Dihedral angle restraints

Scalar or J-couplings are mediated through chemical bonds connecting two spins. ^3J couplings are well-correlated with the central dihedral angle by an empirical correlation, the Karplus curve. For example $^3J(H^N,H^\alpha)$ defines the backbone angle ϕ in proteins.

$$^3J(H^NH^\alpha) = 6.4\ \text{Hz}\ \cos^2(\phi - 60°) - 1.4\ \text{Hz}\ \cos(\phi - 60°) + 1.9\ \text{Hz}$$

Taking into consideration that only certain combinations of ϕ and ψ angles are allowed in secondary structured elements, it follows that only certain coupling constants can be true for a certain conformation. These conclusions are condensed in the Ramachandran plot. The typical ϕ angle of a perfect α-helix is 57, this results in a $^3J(H^N,H^\alpha)$ coupling constant of 3.9 Hz. The values for an antiparallel β-sheet are ϕ= 139, and thus $^3J(H^N,H^\alpha)$ = 8.9 Hz (Evans et al., 1996)
Because of the large linewidth of the protein proton resonances the $^3J(H^N,H^\alpha)$ can not be determined from the signal splitting, instead of this a quantitative J-correlation (HNHA) is used for determining the coupling constants.

3.7.7 Hydrogen bonds

Hydrogen exchange rates can be measured with a NEWMEXICO (Measurement of EXchange rates in Isotopically labeled COmpounds) experiment (Koide et al., 1995). After excitation of all protons, ^{15}N-^1H bound magnetisation is filtered out. During the mixing time, only exchange of z magnetisation from water to the labile amide protons can occur. The result will be a 2D ^1H-^{15}N HSQC spectrum where only labile proton resonances in exchange with the water are visible. The signal buildup of these correlations during the mixing time reflects directly the hydrogen exchange rates when compared to the reference intensity of the standard HSQC.
Hydrogen bonds were included in the final structure calculation if the acceptor of a slowly exchanging amide proton characterised by a missing signal in a 150 ms NEWMEXICO experiment. Thus, a hydrogen bond was assumed if the distance of the carboxyl oxygen and the amide proton was below 2.6 Å, and the angle of the amide proton, the amide nitrogen and

the carboxyl oxygen was less than 60° in all accepted structures. For each hydrogen bond the distance between the amide proton and the acceptor was restrained to less than 2.3 Å, and the distance between the amide nitrogen and the acceptor was restrained to less than 3.3 Å (Schweimer et al., 2002).

3.7.8 Residual dipolar couplings

Residual dipolar couplings, arising from a weak alignment of a macromolecule in a magnetic field, represent a promising new source of restraints for macromolecular structure refinement. The introduction of dipolar couplings can greatly improve the accuracy and precision of NMR solution structures. The long-range information contained in the inter-nuclei angular and distance dependence of dipolar couplings provide restraints that are fundamentally different from the J-related torsion angle and distance-NOE-derived short inter-proton restraints, accessible by traditionally used NMR experiments (Tjandra et al., 1997).

The dipolar interaction between pairs of magnetically active spin ½ nuclei, do not average to zero when the molecules of interest have a preferred orientation. The net alignment of the molecules of interest, which can be introduced by a liquid crystalline medium, is in the order of 10^{-3} and is fundamental to the success of RDC based studies. The general expression for the residual dipolar coupling $D^{AB}(\theta,\phi)$ between two directly coupled nuclei A and B can be simplified to the following equation (Clore et al., 1998).

$$D^{AB}(\theta,\phi) = D_a\{(3\cos^2\theta - 1) + 1.5\ R\ (\sin^2\theta \cos 2\phi)\}$$

where, θ and ϕ are the polar angles that describe the orientation vector connecting between spin A and B with respect to principal alignment frame, D_a and R are the axial and rhombic components of the molecular alignment tensor, respectively. D_a and R can be estimated in a straightforward manner from the maximum, the minimum and the most frequently observed coupling values in the dipolar coupling histogram (Clore et al., 1998). In order to get a good estimate for D_a and R one would need to create a histogram that reflects the distribution of the measured dipolar couplings. The extremes of the histogram correspond to the alignment tensor components A_{xx}, A_{yy}, and A_{zz} ($|Azz| \geq |Ayy| \geq |Axx|$). The alignment tensor is traceless, that is $A_{xx} + A_{yy} + A_{zz} = 0$. D_a and R can be computed with the following equations:

$$A_{zz} = 2\ D_a$$

$$A_{yy} = -D_a\ (1 + 1.5\ R)$$

$$A_{xx} = -D_a\ (1 – 1.5\ R)$$

The larger the number of dipolar couplings used in creating the histogram the better the estimate is going to be.

$^1D(^1H^N, ^{15}N)$ residual dipolar couplings (RDCs) were determined by the IPAP method (Otting M, et al., 1998) using a weakly aligned sample of uniformly ^{15}N labeled NELF-E RRM in a mixture of monododecyl-pentaethylenglycol-ether (C12E5), hexanol and water (molar ratio C12E5:hexanol = 0.95, 3% (wt) C12E5 / water) (Otting et al., 2000). The tensor components of the alignment were optimised with a grid search by varying the axial component D_a and the rhombicity R in steps of 0.5 and 0.1, respectively. The initial values of D_a and R were estimated from the distribution of the $^1D(^1H^N, ^{15}N)$ (Clore, et al., 1998), and a molecular dynamics run was performed for each pair of D_a and R to yield the energetically most favorable combination of D_a and R.

3.7.9 Calculation of tertiary structure

The idea of computer-aided structure calculation is to convert distance- and torsion-angle-data (constraints) into a visible structure. However, the experimentally determined distances and torsion angles by themselves are not sufficient to fully characterise a protein structure, as they are based on a limited number of proton-proton distances. Only the knowledge of empirical input data, such as bond lengths of all covalently attached atoms and bond angles, enables a reasonably exact structure determination.

For this purpose, a randomly folded starting structure is calculated from the empirical data and the known amino acid sequence. The computer program then tries to fold the starting structure in such a way, that the experimentally determined inter-proton distances are satisfied by the calculated structures. In order to achieve this, each known parameter is assigned an energy potential, which will give minimal energy if the calculated distance or angle coincides with its input value. The computer program tries to calculate a structure with a possibly small overall energy.

Without the experimentally determined distance- and torsion angle-constraints from the NMR spectra, the protein molecule can adopt a huge number of conformations due to the free rotation around its chemical bonds (except for the peptide bond) the N-C^{alpha} bond and the C^{alpha}-CO bond. All these possible conformations are summed up in the so-called conformational space. Therefore, it is important to identify as many constraints as possible from the NMR spectra to restrict the conformational space as much as possible, thus getting close to the true structure of the protein. In fact, the number of constraints employed is more important than the accuracy of proton-proton distances. The simulated annealing (SA) method was employed for calculating a protein structure.

3.7.9.1 Energy potentials

A starting structure is needed for a molecular dynamics calculation, which is generated from all constraints for the molecular structure, such as bond-lengths and bond-angles. This starting structure may be any conformation such as an extended strand or an already folded protein. During the simulation, it develops in a potential field under the influence of various forces, in which all information about the protein is summarised. Two classes of energy terms are distinguished: $E_{empirical}$ and $E_{experimental}$.

$$V = E_{empirical} + E_{experimental}$$

$$E_{experimental} = E_{NOE} + E_{torsion}$$

$$E_{empirical} = E_{bond} + E_{angle} + E_{dihedral} + E_{vdw} + E_{electr}$$

$E_{empirical}$ contains all information about the primary structure of the protein and also data about topology and bonds in proteins in general. The contributions of covalent bonds, bond-angles and dihedral angles towards $E_{empirical}$ are approximated by a harmonic function. In contrast, non-covalent van-der-Waals forces and electrostatic interactions are simulated by an inharmonic Lennard-Jones potential and Coulomb potential, respectively. $E_{experimental}$ takes the experimentally determined constraints into account. Angle constraints are introduced by a harmonic function analogous to that for the dihedral angles. For distance constraints, the energy potential will be set to zero, if the corresponding distance is within the given limits. If it is outside these limits, a harmonic energy potential is used, which tries to push the value of the distance into the limits.

3.7.9.2 Simulated annealing protocol

At the beginning of the calculation, the starting structure is energy minimised by moving the atoms of the starting structure, until it reaches an energy minimum. As a result of this process, a structure is obtained which is normally trapped in a local minimum, without satisfying the constraints over vast regions. Such structures cannot reach the global energy minimum by further energy minimisation, as they cannot cross the energy barrier between local and global minimum.

However, this energy barrier can be overcome if the necessary energy is put into the system. This is achieved by simulating the heating of the system up to a few thousand Kelvin via a coupled temperature bath. At this temperature the system receives enough energy to cross the energy barrier. Now, the system once again develops in energyhyperspace under the influence of the potential field.

The atomic positions at the end of a simulations step are determined from their starting positions, as well as from their velocities and accelerations, which in turn are both derived from the starting positions. Velocities can be calculated from the Maxwell distribution at a given temperature and accelerations are determined by Newton's equation from the force field. After a simulation step the energy potential is recalculated for the new atomic positions and a further simulation step follows. This procedure is iterated, searching the energyhyperspace for a global minimum.

After a previously chosen number of simulation steps at high temperature (up to 6000 times), atomic velocities (i.e. temperature) are slowly reduced in many steps (usually 3000). At each temperature, the system is once again left to develop under the influence of the potential field. While the temperature of the system is reduced, simultaneously the force constants in the experimental constraints are raised in order to weigh them more strongly.

The result of the simulation is a minimum energy protein structure, but it cannot be excluded that this structure is stuck in a local minimum without ever reaching the global minimum, which is only marginally lower in energy. Therefore, about twenty different starting structures with random folds are used, which reach their final structure via different paths in energyhyperspace. These resulting structures are iteratively re-used as starting structures for another SA with slightly changed input protocols, until no further reduction in global energy is observed and the structures converge in conformational space.

After the structural calculations a family of structures is obtained instead of an exactly defined structure. This family spans out a relatively narrow conformational space, fulfilling the

experimental restraints. Therefore, the quality of a NMR structure can be defined by the mean deviation of each structure from this family (RMSD) from an energy minimised mean structure.

The structure calculations of NELF-E RRM were performed with the program XPLOR 3.8.5.1 using a three-step simulated annealing protocol (Nilges 1995; Nilges et al., 1988) with floating assignment of prochiral groups (Folmer et al., 1997). Initial conformational space sampling was carried out for 120 ps with a time step of 3 fs at a temperature of 2000 K, followed by a cooling period of 120 ps down to 1000 K, and 60 ps cooling to 100 K, both with a time step of 2 fs. A modified conformational database potential for backbone and side chain dihedral angles was applied (Kuszewski et al., 2000, Neudecker et al., 2001). The proline angles were modified according to Neudecker et al. (Neudecker et al., 2004). After simulated annealing, the structures were subjected to 1000 steps of Powell minimisation (Powell 1997), and the final 500 steps were minimised without conformational database potential. In a first step, 200 structures were calculated using 1926 distance, 24 hydrogen bond and 32 dihedral angle restraints. The 40 structures with the lowest total energy were then refined using 55 ^{1}D ($^{1}H^{N}$, ^{15}N) RDCs with a harmonic potential (Tjandra et al., 1997). Dipolar couplings of flexible residues showing a {^{1}H}-^{15}N NOE below 0.65 at 14.1 T were excluded from the calculations.

3.7.10 {^{1}H}-^{15}N heteronuclear NOE

Backbone motions of the protein on the picosecond to nanosecond timescale can be characterised using the {^{1}H}-^{15}N heteronuclear NOEs (Farrow et al., 1994). {^{1}H}-^{15}N NOE takes place when both, the nitrogen and the proton magnetisation are along z. A presaturation delay is applied on the amide protons, during which dipolar interactions occur between the saturated amide protons and their bound nitrogen. It follows a ^{1}H-^{15}N HSQC where the intensity of the NH peaks is directly correlated to the protein flexibility. Practically, motional parameters are determined with and without the presence of a ligand, enabling the recognition of the binding site as well as the regions that exhibit conformational changes due to ligand binding (Prasch et al., 2006), simply by data comparison.

{^{1}H}-^{15}N NOE values were determined using the pulse sequence of Dayie and Wagner (Dayie and Wagner 1994) with a relaxation delay of 6 s including the 3 s saturation period with 120° high power pulses for the saturated subspectrum. {^{1}H}-^{15}N NOE values were obtained from the ratios of peak intensities in the saturated spectrum to those in the unsaturated spectrum. Peak intensities were measured and analysed using the peak picking routine built into

NMRView (Johnson, B A, J.B.NMR 1994).

3.7.11 Chemical shift mapping

Since amide chemical shifts are very sensitive to variations in the local electronic environment, small changes in H^N and ^{15}N shifts upon titration of a weakly binding ligand into a ^{15}N-labeled protein can be used to map the binding interface. In cases where the assignments of the free protein are available, they can be readily transferred to the complex by tracking the changes that occur during the titration. Residues that do not participate in the interaction do not change chemical shift, while those resonances that shift are directly involved in the binding interface or affected by minor conformational rearrangements caused by the interaction. Due to the high sensitivity of the ^1H-^{15}N HSQC experiment, these investigations can be done even at µM concentrations and are frequently used in drug discovery-related ligand binding studies e.g. Structure Activity Relationships (SAR) by NMR and related techniques (Shuker et al., 1996).
The mapping of weak interactions is very useful as the crystallisation of such complexes is often impossible. When mapping the binding interface, many of the indirect effects on chemical shift can be screened out, by assessing the solvent exposure of the residues whose resonances experience a chemical shift change. In the absence of a major conformational change, those residues that are buried are unlikely to be directly contacting the ligand. In cases of moderately fast exchange, a significant exchange contribution to the signal linewidth can be present. This effect is likely to be largest for residues in the interface. In a strong binding situation the majority of the observed resonances in the binding interface are in slow exchange between those in the free and those in the bound form so that, at the NMR sample concentrations employed, the signals appear at their chemical shift position in the complex. The stoichiometry of the interaction can be determined in the course of a ^1H-^{15}N HSQC titration experiment. Transfer of existing assignments from the free form can be difficult as the interaction may lead to large changes in chemical shift. A complete reassignment may be required.

4 Results

4.1 Characterisation of Tat-Cys$^-$/-TAR complex by NMR and CD spectroscopy

4.1.1 Overexpression of recombinant Tat-Cys$^-$

The DNA coding sequence for the Tat-Cys$^-$ was cloned into the vector pET-11a and transferred into the *E.coli* bacterial strain BL21(DE3) (S. Gurka, Diplomarbiet, University of Bayreuth). The overexpression and purification of recombinant Tat-Cys$^-$ was performed as described in Chapters 3.2.1/3.2.2. The overexpression of Tat-Cys$^-$ was analysed by SDS-PAGE (Fig. 4.1).

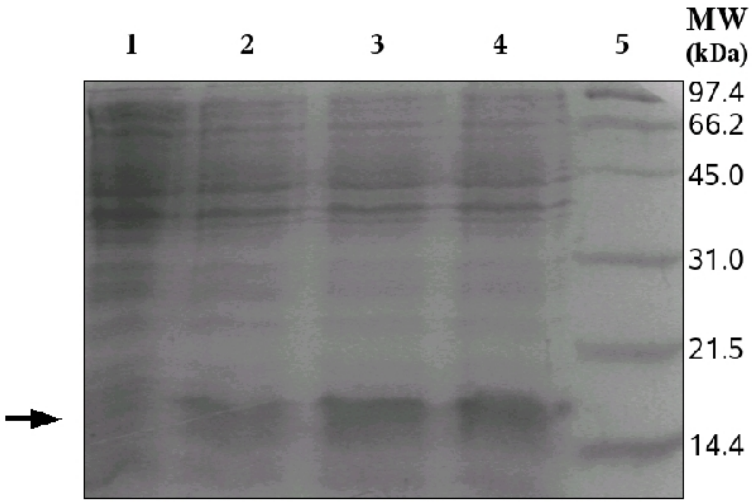


Fig. 4.1 Expression of Tat-Cys$^-$ in *E.coli* BL21(DE3). The over-expression of recombinant Tat-Cys$^-$ was induced by 1 mM IPTG. Samples were analysed by SDS-PAGE adapted from Schaegger and Jagow (Chapter 3.4). The molecular weights are assigned to the band of BioRad low molecular wight standard. The band representing Tat-Cys$^-$ is marked by an arrow. Band 1. before induction, band 2. 1 hour after induction, band 3. 2 hours after induction, band 4. 3 hours after induction, band 5. BioRad low molecular weight standard.

4.1.2 Cell lysis and purification

The cell growth, lysis and protein purification were performed as described in Chapters 3.2.3, 3.2.4 and 3.2.5. The results of the cell lysis, pH and ammonium sulfate precipitations were analysed by SDS-PAGE (Fig 4.2). After the cell lysis, most of the Tat-Cys$^-$ was in the supernatant (bands 2 and 3). During the pH precipitation step most of the *E.coli* proteins were precipitated, except Tat-Cys$^-$, which remained in the solution (band 5 and 6). Tat-Cys$^-$ was further purified by ammonium sulfate precipitation, where Tat-Cys$^-$ was detected in pellet (bands 7 and 8). The pellet from the ammonium sulfate precipitation was resuspended in 10 mM KPO_4, pH 6.4, 200 mM NaCl (buffer A) and dialysed against 2 x 2.5 l buffer A to remove the ammonium sulfate.

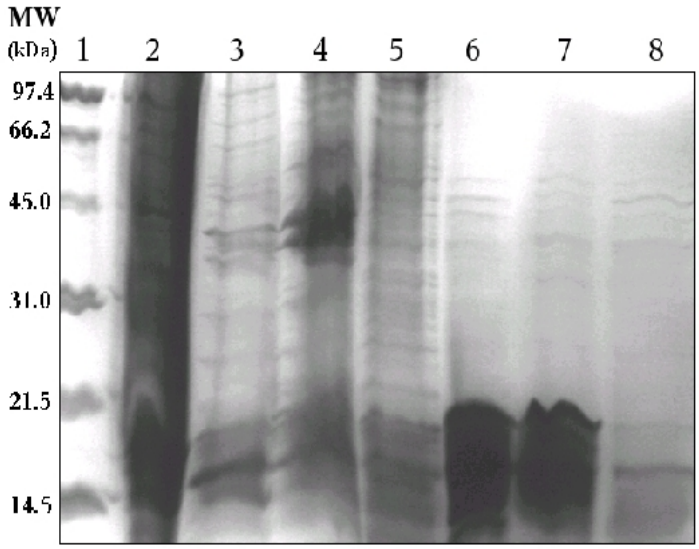
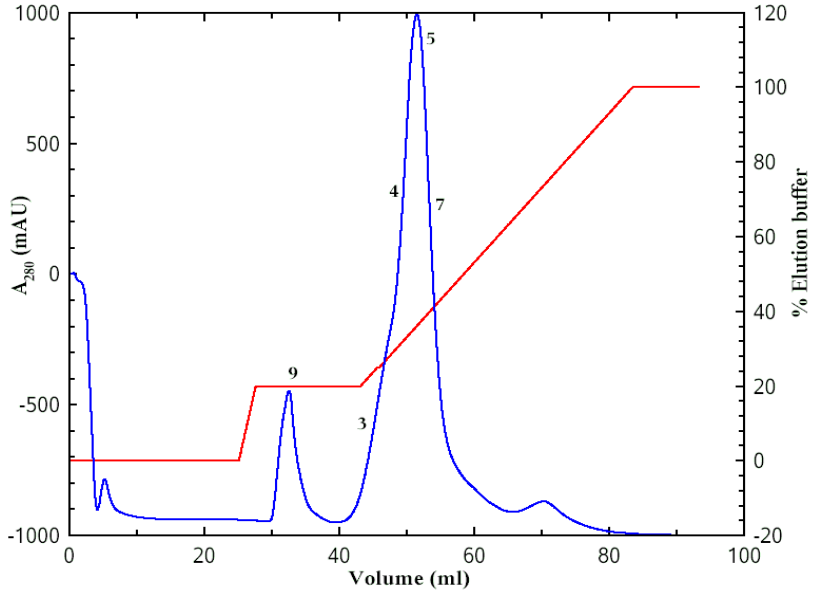


Fig. 4.2 Cell lysis, pH and ammonium sulfate precipitation. Band 1. BioRad low molecular weight standard, bands 2 and 3 supernatant after cell lysis, band 3. pellet after cell lysis, band 5. supernatant, pH precipitation, band 6. pellet, pH precipitation, band 7. pellet, ammonium sulfate precipitation, and band 8. supernatant, ammonium sulfate precipitation.

Further purification of Tat-Cys$^-$ was performed using cation exchange chromatography. The dialysed sample was loaded on to heparine column and purified as described in Chapter 3.2.6. Tat-Cys$^-$ was eluted from the column at 500 mM NaCl (Fig. 4.3.a). Fractions collected during the purification were analysed by SDS-PAGE (Fig. 4.3.b).

(a)



(b)

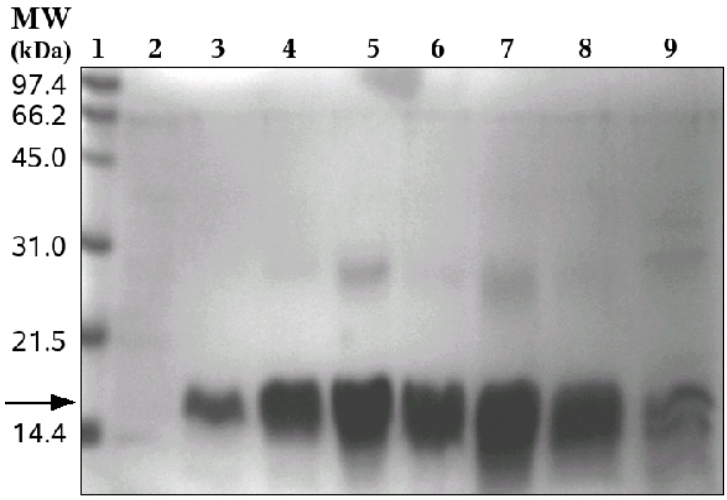


Fig. 4.3 Purification of HIV-1 Tat-Cys$^-$ using cation exchange chromatography. (a). chromatogram of the purification monitored at A_{280}. The numbers in the chromatogram correspond to the bands assigned in SDS-polyacrylamide gel. **(b)** Band 1. Rio-Rad low molecular weight standard, band 2. flow through, band 3-9 elution, bands 6 and 8 are two times diluted samples of 5 and 7, respectively.

4.1.3 CD Spectroscopic characterisation of Tat-Cys⁻:TAR complex

CD spectroscopy is a useful tool to analyse the secondary structure and conformational change of proteins and nucleic acids. The CD spectrum of TAR alone is characteristic of an A- form RNA with a long-wavelength band centred around 265 nm and a fairly intense negative band around 210 nm (Fig. 4.4.).

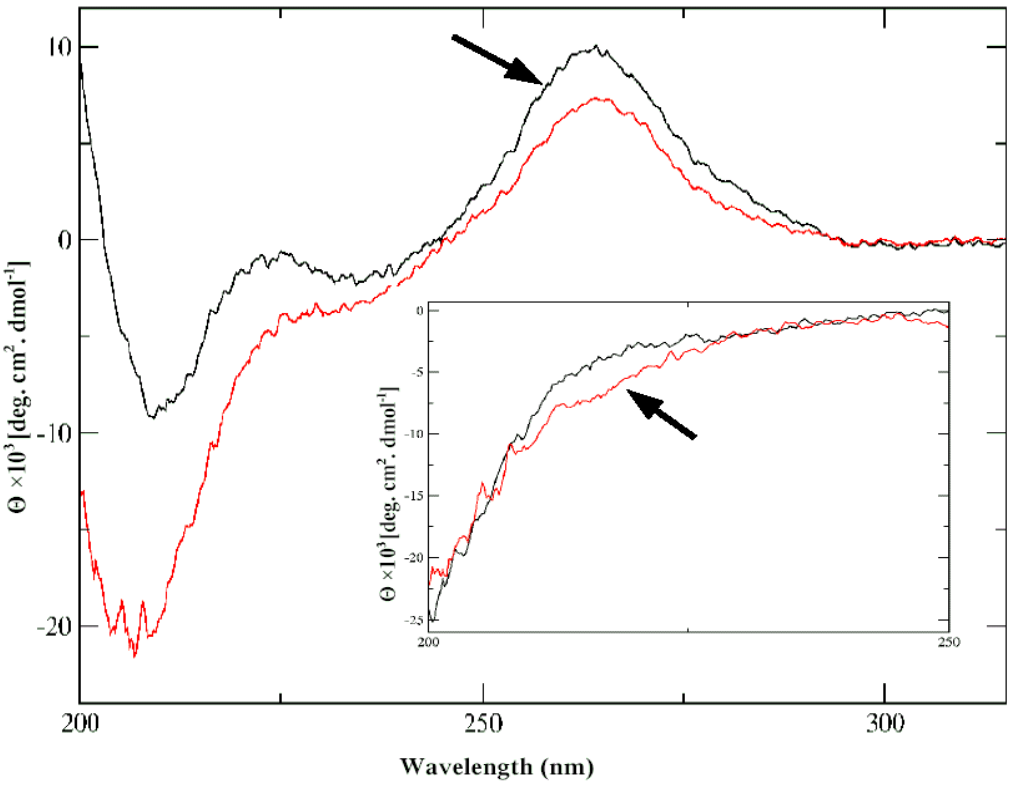


Fig. 4.4 CD spectroscopy of the Tat-Cys⁻:TAR complex. UV- CD spectra of TAR RNA (black) and its 1:1 complex with Tat-Cys⁻ (red). In the inset far UV-CD spectra of Tat-Cys⁻ (black) and the difference CD spectrum of 1:1 complex of TAR RNA:Tat-Cys⁻ minus TAR RNA (red). The spectra were measured at 20 °C in a buffer containing 10 mM potassium phosphate, pH 6.4, 150 mM NaCl at 20 °C. Arrows indicate the significant changes in the CD signal.

The CD spectrum of TAR RNA in the presence of Tat-Cys⁻ showed a significant change near 265 nm. This change is assigned to TAR RNA alone because no protein conformation will contribute at this wavelength. The CD of nucleic acids is generally very sensitive to change in base stacking (Aboul-ela et al., 1988). The change in the CD signal at 265 nm is attributed to the conformational change in TAR RNA upon binding to Tat-Cys⁻. The CD spectrum of free

Tat-Cys$^-$ with its minimum near to 200 nm indicated that Tat-Cys$^-$ is in random coil conformation. The difference CD spectrum derived from the Tat-Cys$^-$:TAR complex and free TAR RNA showed a significant change in the CD signal at 217 nm, which is approximately -3000 degrees, indicating some residual structure of Tat-Cys$^-$ in the complex with TAR RNA.

4.1.4 1D NMR of Tat-Cys$^-$:TAR complex

Binding of Tat-Cys$^-$ to TAR RNA was monitored by one dimensional NMR spectroscopy. Resonances indicating hydrogen bonding and formation of Watson-Crick base pairs are observed for all base pairs in the stem (Fig. 4.5), except for A22-U40, which is located directly below the triple-U bulge (Faber et al., 2000). In the free TAR RNA conformation, U40 stacks continuously on residue C41 of the lower helix and the bulge region is highly flexible, so the A22-U40 base pair does not appear to be in a typical Watson-Crick pairing geometry and the imino proton of U40 is rapidly exchanging with solvent (Aboul-ela et al., 1996).

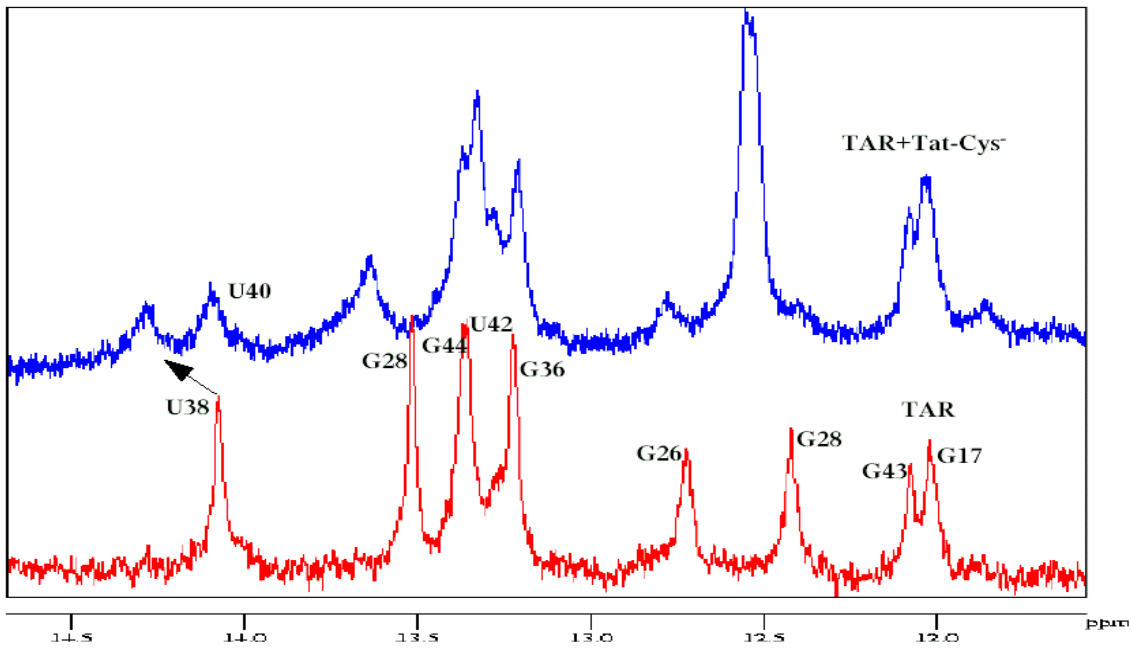


Fig. 4.5. One-dimensional NMR spectrum of the imino proton region of TAR RNA. Free TAR RNA (red) and 1:1 complex of TAR:Tat-Cys$^-$ (blue) in a buffer containing 10 mM potassium phosphate, pH 6.4, 150 mM NaCl, 90/10 % H_2O/D_2O. The assignment of the bases is indicated.

The one-dimensional imino proton resonances of TAR RNA showed significant chemical shift changes after the addition of Tat-Cys$^-$, indicating binding of Tat-Cys$^-$ to TAR RNA. The most significant change is the imino proton resonance of U40 observed in the complex with TAR RNA, which is not present in the free state.

4.1.5 ^{1}H-^{15}N HSQC of Tat-Cys$^-$ and Tat-Cys$^-$:TAR complex

In order to identify which amino acids residues of Tat-Cys$^-$ are involved in the complex formation with TAR, ^{1}H-^{15}N HSQC spectrums of Tat-Cys$^-$ were recorded, both in the absence and presence of TAR. ^{1}H-^{15}N HSQC of Tat-Cys$^-$ displayed a paucity of amide ^{15}N-^{1}H peaks and very narrow dispersion of resonances, which is the characteristic feature of an intrinsically unstructured protein. Due to the overlap and rapid exchange with solvent, only few amino acid resonances from the basic and the glutamine rich region could not be assigned unambiguously in the HSQC spectrum (E. Tapavicza, Diplomarbeit, University of Bayreuth). Biochemical analysis identified that the core and basic regions of HIV-1 Tat specifically interact with TAR RNA (Churcher et al., 1993). Tat-Cys$^-$:TAR complex formation is further supported by the observation of amide chemical shift changes or signals disappearing in the spectrum upon the addition of unlabeled RNA to ^{15}N- labeled Tat-Cys$^-$ (Fig. 4.6). The amino acid resonances from the core region showed a significant change in their amide chemical shifts and the resonances from the basic region disappeared in the spectrum due to the intermediate exchange on NMR time scale. The significant chemical shift changes observed for S31, Y33, V37, A38, F39, I40, T41, and I46, are located in the core region of Tat-Cys$^-$, which is known to be responsible for the specific TAR binding (Churcher et al., 1993). The resonances from the amino and carboxy terminal region remain unaffected upon the addition of TAR. These regions were so far not shown to be involved in Tat-TAR interactions.

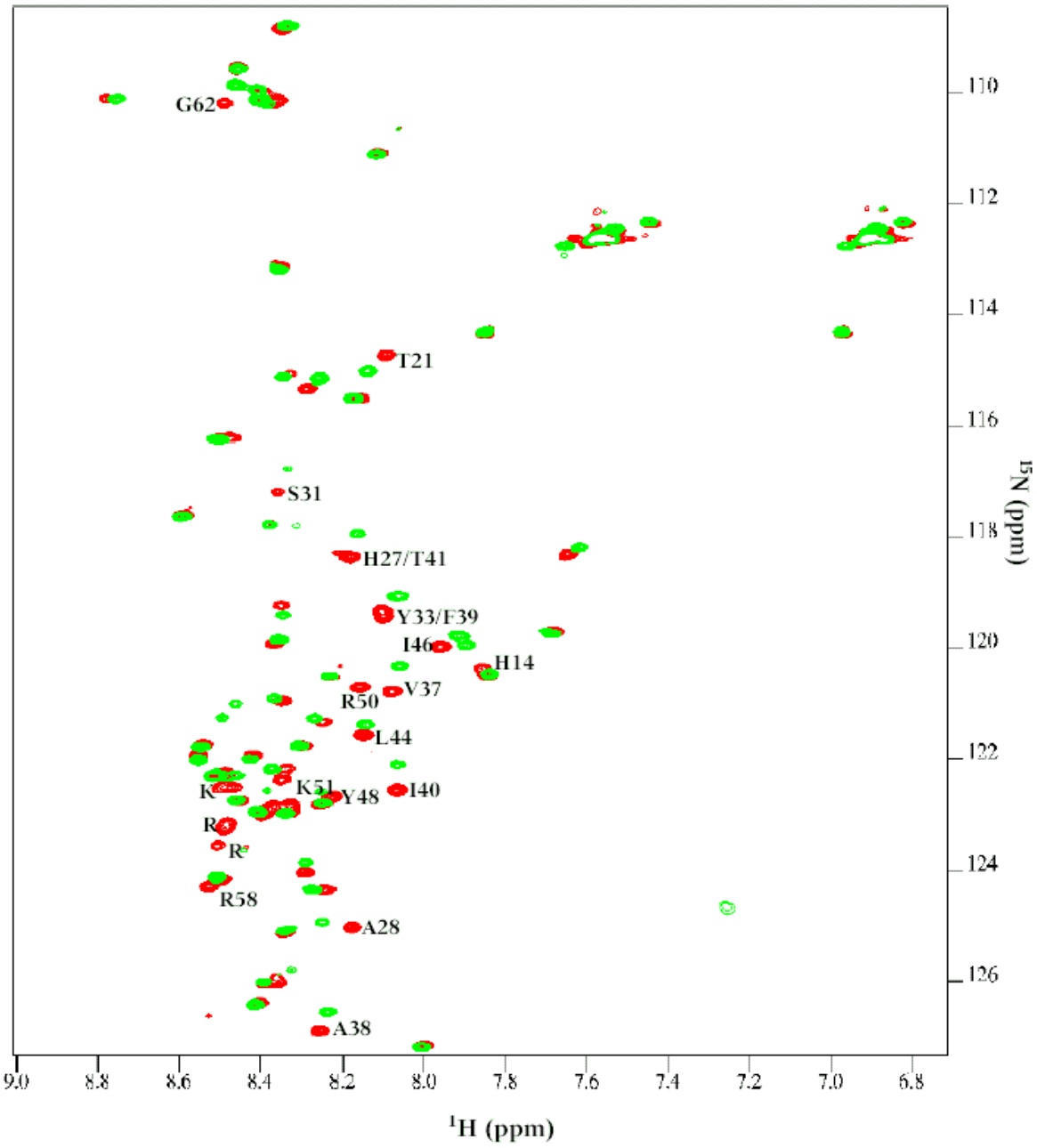


Fig. 4.6 ^{1}H-^{15}N HSQC spectra of Tat-Cys^{-}. Superposition of ^{1}H-^{15}N HSQC spectra of ^{15}N labeled Tat-Cys^{-} in the absence (red) and presence (green) of TAR RNA. The concentrations of free ^{15}N labeled Tat-Cys^{-} is 0.3 mM and the complex contains 0.3 mM of ^{15}N labeled Tat-Cys^{-} and 0.3 mM of unlabeled TAR RNA. Only those resonances that showed significant changes in chemical shifts after the addition of TAR RNA are indicated. The spectra were recorded at 25 °C in a buffer containing 10 mM potassium phosphate pH 6.4, 150 mM NaCl and 90/10% H_2O/D_2O.

4.1.6 Homonuclear TOCSY of TAR RNA

The biochemical analysis identified that Tat binds to TAR RNA around the U-rich bulge region and the two base pairs above and below the bulge significantly contribute to Tat binding (Churcher et al., 1993; Delling et al., 1992; Weeks et al., 1991). In order to verify whether Tat-Cys$^-$ binds TAR RNA at the UUU bulge, ^{1}H-^{1}H TOCSY of TAR RNA was measured, both in the absence and presence of Tat-Cys$^-$. In the ^{1}H-^{1}H TOCSY spectrum, H5-H6 cross peaks of all the pyrimidine bases can be observed. Thus, one can unambiguously identify the binding region. The cross peaks from nucleotides located in the bulge region and immediately neighboring bases-pairs to bulge showed significant chemical shift changes upon the addition of Tat-Cys$^-$ (Fig. 4.7).

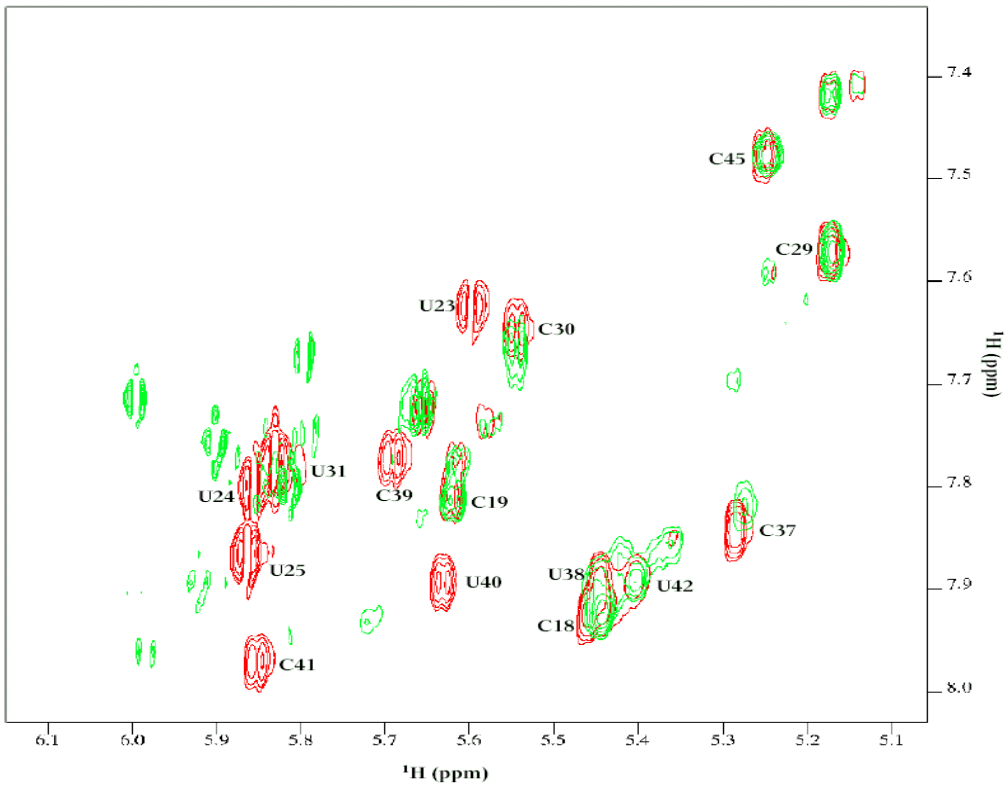


Fig. 4.7 ^{1}H-^{1}H TOCSY of TAR RNA. TOCSY spectrum of TAR RNA recorded at 25 °C with a mixing time of 100 ms. Free TAR RNA (red) and 1:1 molar ratio of TAR:Tat-Cys$^-$ (green) in a buffer containing 10 mM potassium phosphate, pH 6.4, 150 mM NaCl, 90/10 % H_2O/D_2O. H5-H6 cross peaks are labeled for the free TAR RNA.

The cross peak of U23 showed a dramatic chemical shift change in the presence of Tat-Cys$^-$ which was identified to be critical for the recognition of Tat (Berkhout and Jeang, 1989). The cross peaks from nucleotides located in the loop and lower stem region are unaffected by the addition of Tat-Cys$^-$ indicating that Tat-Cys$^-$ binds to TAR RNA around the bulge region.

4.1.7 Backbone dynamics

Backbone motions on the ps to ns timescale can be detected with $\{^1H\}$-^{15}N steady state NOE. Backbone dynamics of Tat-Cys$^-$ in the presence and absence of TAR RNA were analysed by recording $\{^1H\}$-^{15}N steady-state NOE. For free Tat-Cys$^-$, $\{^1H\}$-^{15}N steady-state NOE measurements at 14.1 T resulted in either negative signal intensities or missing signals in the saturated subspectrum compared to the unsaturated subspectrum. Thus, the intensity ratio of the saturated versus the nonsaturated subspectrum is either '0' or less for all residues, except for very few residues in the amino terminus (Fig. 4.8), clearly pointing to a high flexibility of Tat-Cys$^-$. The acidic region of Tat-Cys$^-$ may be interpreted in terms of decreased flexibility of this region as compared to the rest of the protein. In the presence of TAR RNA, $\{^1H\}$-^{15}N steady-state NOE for a few residues in the core region of Tat-Cys$^-$ resulted in positive values in between 0.2 to 0.4 (Fig.4.7). However, the values did not reach the typical range (>0.65) found in folded and rigid polypeptide chains. $\{^1H\}$-^{15}N steady-state NOE values could not estimated for the basic region since they were missing in both the saturated and unsaturated subspectra due to intermediate exchange on NMR time.

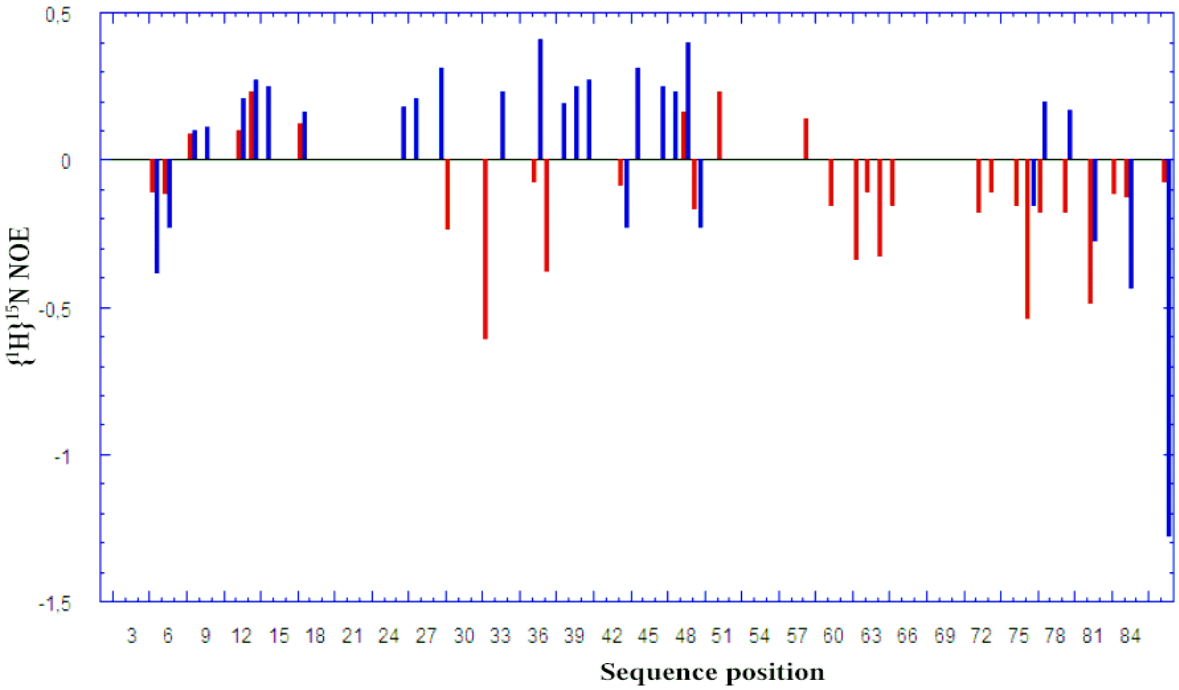


Fig.4.8 $\{^1H\}^{15}N$ steady state NOE. Plot of $\{^1H\}^{15}N$ steady state NOE values for the free Tat-Cys$^-$ (red) and in the presence of TAR RNA (blue), 1:1 molar ratio of Tat-Cys$^-$:TAR. Missing bars correspond to either missing signals in the saturated subspectrum or indicate that no reliable data were obtained.

4.2 Structure determination of the NELF-E RRM

The elongation of transcription of HIV-1 RNA at TAR is highly regulated by positive and negative factors, including NELF. The NELF-E, subunit of the heterotetrameric NELF harbors an RNA recognition motif (RRM) at the C-terminus and its RNA binding activity is critical for the function of NELF. Since NELF-E RRM plays critical role in regulation of HIV-1 transcription, this work is mainly focussed on the structure determination of NELF-E RRM in solution and binding studies of NELF-E RRM with HIV-1 TAR RNA by NMR spectroscopy.

4.2.1 Assignment of backbone chemical shifts

In order to obtain sequential backbone resonance assignments of NELF-E RRM standard triple resonance experiments were recorded as shown in Tables 3.1 and 3.2 to correlate the resonances of the peptide backbone [H^N(i), N^H(i), C^α(i), H^α(i), C^α(i-1), H^α(i-1), C (i) and C (i-1)].

An ^{1}H-^{15}N HSQC experiment contains the ^{15}N- and H^N-resonances, therefore allowing the use of this pair of spins as reference and starting point for further assignment of other resonances. The HNCA experiment, for example, correlates the H^N and ^{15}N chemical shifts of residue (i) with the C^α shifts of residue (i) (via $^1J_{NC\alpha}$ » 7-12 Hz) and residue (i-1) (via $^2J_{NC\alpha}$ < 8 Hz), thereby providing sequential connectivity information. A complementary experiment to the HNCA, the HN(CO)CA correlates, in contrast, the amide proton and nitrogen resonances of an amino acid only with the C^α chemical shift of its preceding residue. This is due to the fact that this technique uses a relay mechanism, transferring magnetisation from ^{15}N to C^α via the intermediate carbonyl nucleus. Generally, the intensity of the C^α (i) and C^α (i-1) of the HNCA cross peaks can be differentiated on the basis of their relative intensity. Thus, the HN(CO)CA proposes only an unambiguous assignment in case of accidental overlap of intra- and inter-residue H^N-N-C^α correlations in the HNCA. An HNHA experiment completes the assignment of the α-resonances. The spins H^α(i) are correlated to the H^N via a $^3J_{HNH\alpha}$(i) coupling that connects only the resonances of the same residue.

To circumvent the overlap of the α-resonances, further experiments were required for the chemical shifts of side-chain carbon and proton spins (especially C^α and H^β) to achieve the sequential assignment. It follows then the recommended set of experiments HNCO, HNCA,

HN(CO)CA, HNCACB, CBCA(CO)NH, HNHA, and HBHA(CO)NH. C^{α} and C^{β} are of prime importance, because their chemical shifts show a large spectral dispersion (C^{α} » 25 ppm; C^{β} » 60 ppm), and these shifts are characteristic for the identification of the amino acids (Fig. 2.6).

The most important experiment for the assignment of the backbone resonances is the HNCACB. This experiment yields the C^{β} shifts [in position (i) and (i-1)] in addition to those coming from the HNCA. The C^{α} and C^{β} correlations have opposite signs and can thus be distinguished. The resonances in the (i)-position can be discriminated from those in the (i-1)-position on their different intensity as explained for the HNCA experiment.

The "domino pattern" that obtained for NELF-E RRM from amino acid L42 to Y45 during the sequential assignments with the triple resonance spectra is shown in Fig. 4.9. It shows the superposition of CBCA(CO)NH and HNCACB spectrum of the corresponding amino acid. The side chain resonances of glutamine and asparagine is assigned using the HNCA spectrum, and by observing the characteristic intra-residual NOEs in ^{15}N NOESY-HSQC. Almost the complete assignment of the ^{1}H-^{15}N HSQC spectrum of NELF-E RRM is shown in Fig. 4.10. and a very good dispersion of amide resonances between 6.6 to 9.9 ppm in the ^{1}H-^{15}N HSQC spectrum is an indication of folded protein.

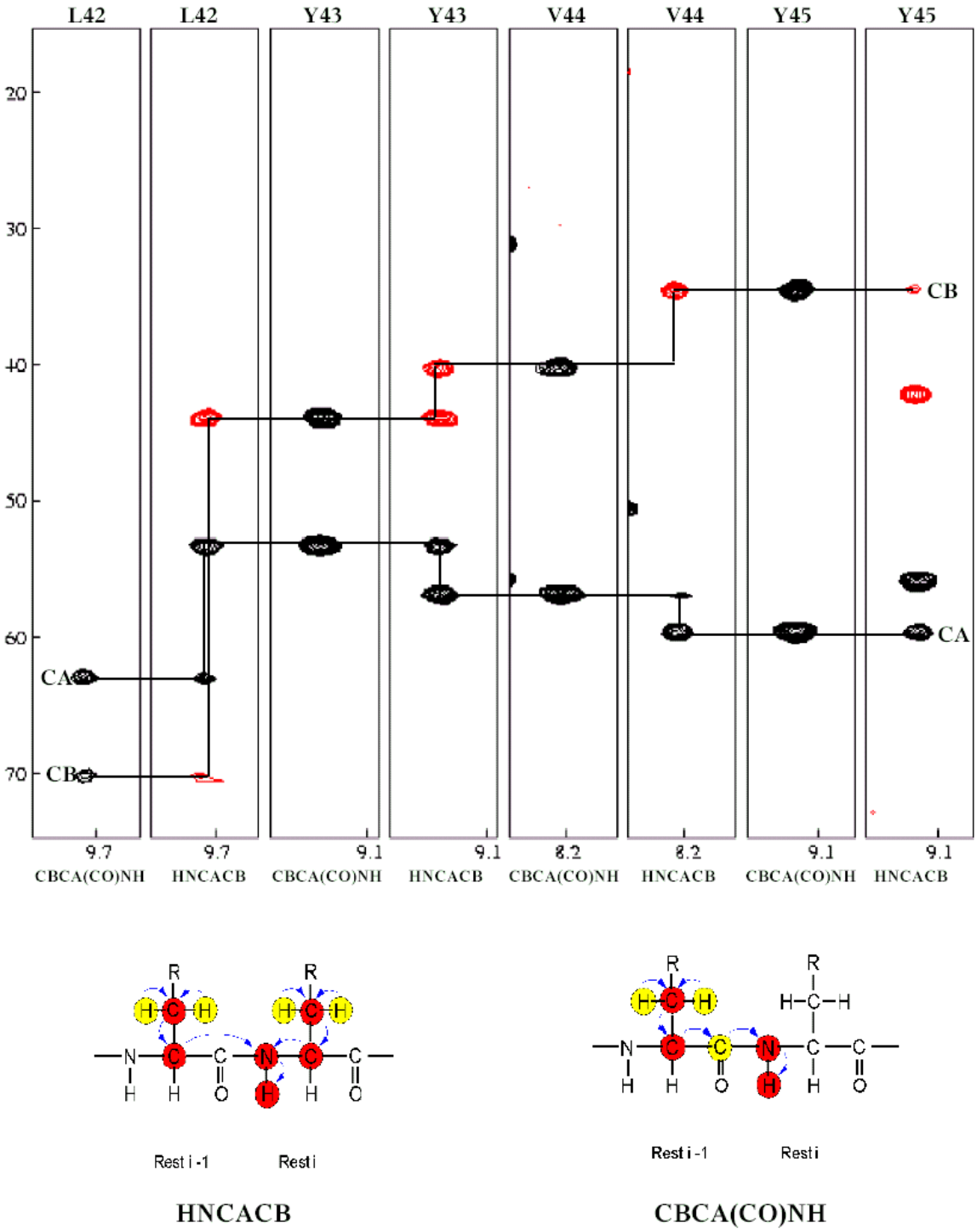


Fig. 4.9 Backbone assignment of NELF-E RRM. Strips from two spectra are shown, corresponding to a single amino acid. Several of these strips are placed in a row to show the sequential connectivities from each amino acid to the preceding one. The flow of magnetisation in these two experiments are indicated by arrows.

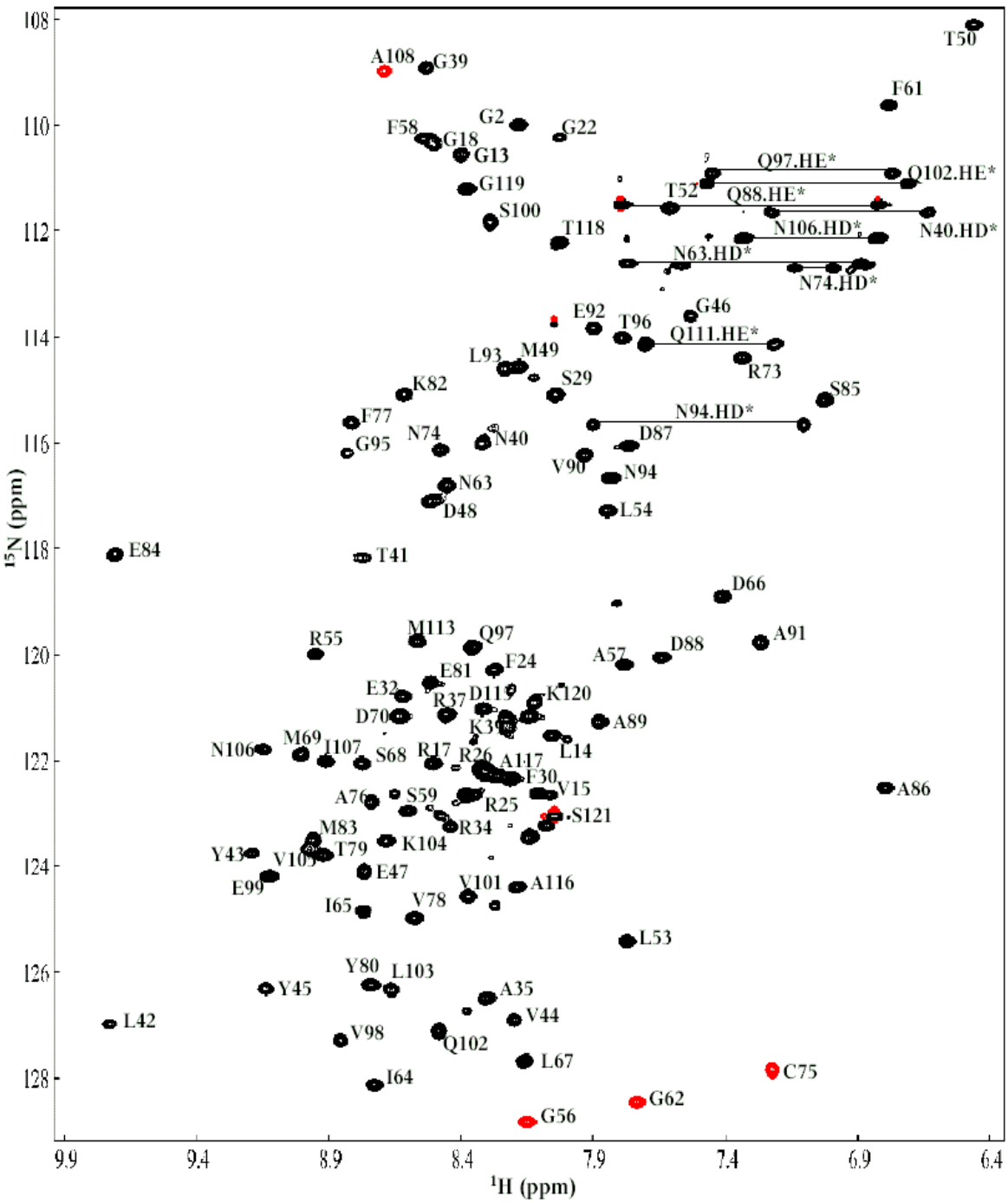


Fig. 4.10 ^{1}H-^{15}N HSQC of NELF-E RRM. ^{1}H-^{15}N HSQC spectrum of uniformly ^{15}N labeled NELF-E RRM (positive signals in black and negative signals in red). The negative signals are aliased in ^{15}N dimension. All the amide proton resonances are labeled.

4.2.2 Assignment of side chain chemical shifts

Assignment of the side-chain resonances and especially of the proton chemical shifts is a prerequisite for analysing NOE interactions which yield important distance restraints. For example, valine and leucine side-chains are often located in the hydrophobic core of the protein thus giving a lot of distance constraints resulting in structural information. The success of this assignment is then directly related to the structure quality. With the known H^{α} and C^{α} chemical shifts, assignment of the side chain resonances was completed by recording 3D HCCH-TOCSY. In a HCCH-TOCSY spectrum, the trace of a side chain is followed by observing the whole spin system via the proton chemical shifts. A small number of side-chain protons, generally the amino groups of arginine and lysine residues remain unassigned due to weak signals or ambiguity. The alpha proton/carbon region of NELF-E RRM in *constant time* evolution ^{1}H-^{13}C HSQC spectrum is shown in Fig. 4.11. The up field shifts and low field shifts of alpha proton resonances from 2.6 to 5.8 ppm indicates NELF-E RRM possesses an α-helical and β-sheet secondary structure. Resonance of the methyl groups of alanine, valine, leucine, Isoleucine, and threonine are often found in hydrophobic interactions in the core of protein and provide long range distance restrains for the tertiary structure calculation. A good dispersion of these proton and carbon resonances is necessary, in order to be able to identify inter residual NOEs in NOESY experiments. Most of the methyl groups showed an isolated signal in the ^{1}H-^{13}C HSQC spectrum (Fig. 4.12) which is a further indication of compact structure. Methyl groups from V78 and L93 showed a strong up field shift of -0.05 ppm, which is a typical chemical shifts for those resonances close to the aromatic ring in the core of the protein. The aromatic resonances were assigned by observing the characteristic inter-residual NOEs in ^{1}H-^{13}C aromatic NOESY-HSQC, ^{1}H-^{13}C NOESY-HSQC and ^{1}H-^{15}N NOESY-HSQC. The aromatic region of ^{1}H-^{13}C HSQC is shown in Fig. 4.13, and ζ proton resonances of F58, F61 and F77 were not assigned due to severe overlap.

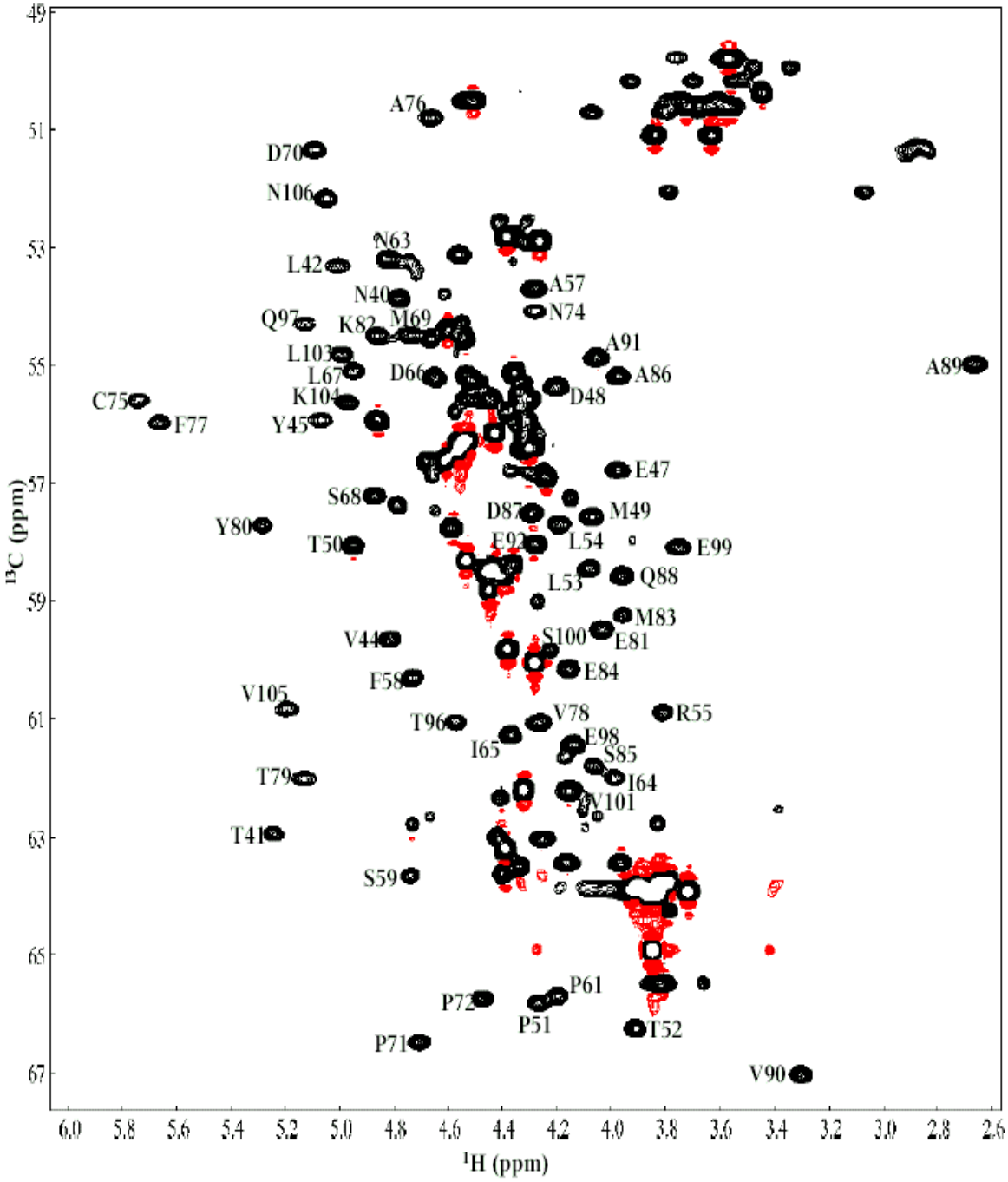


Fig. 4.11. Alpha proton/carbon region of NELF-E RRM. Alpha proton/carbon region of NELF-E RRM in *ct*-^{1}H^{13}C-HSQC spectra. Some alpha proton resonances located in the random coil region (4.4 ppm) are not assigned due to severe overlap.

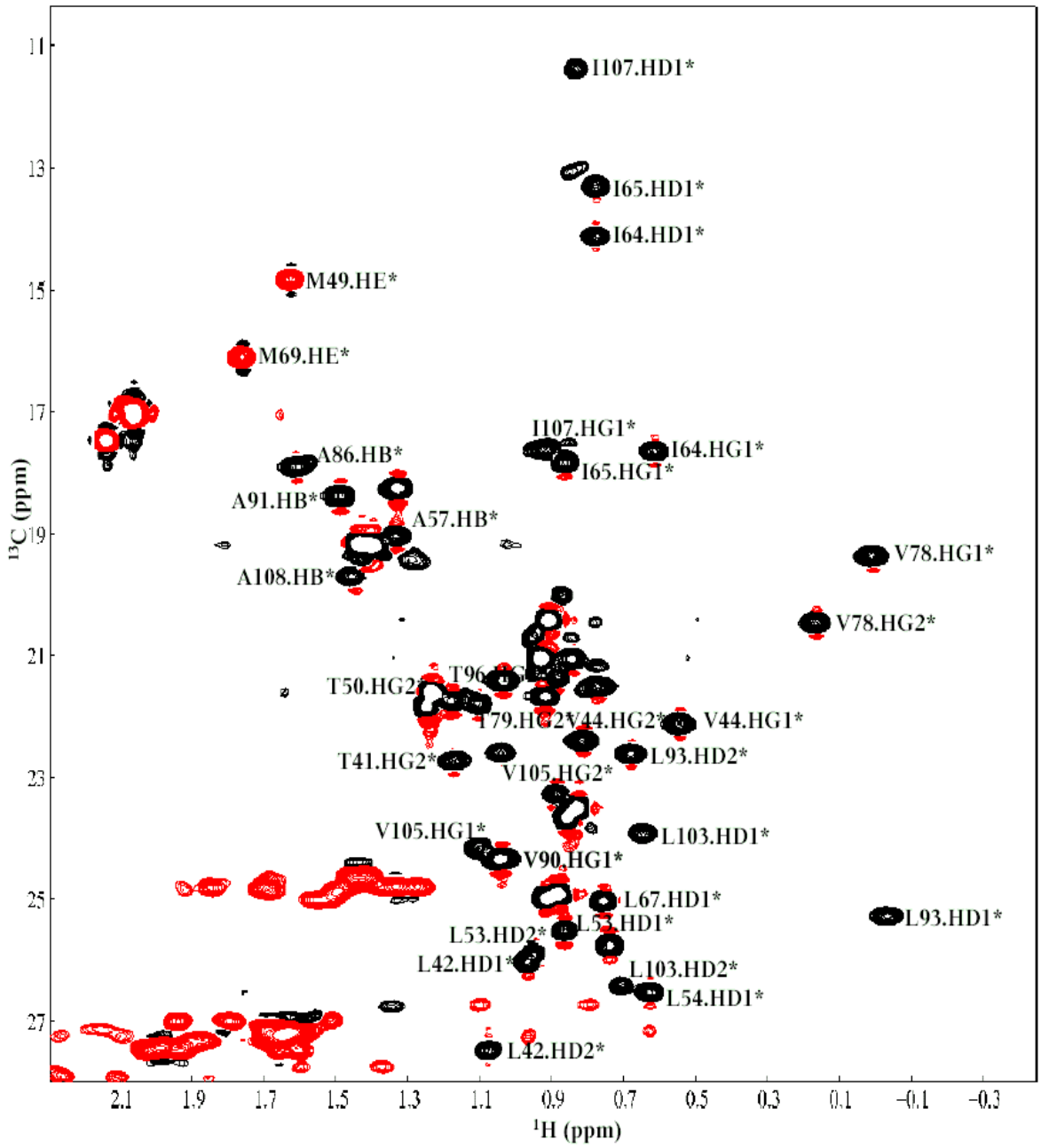


Fig. 4.12 Methyl proton/carbon region of NELF-E RRM. Methyl proton/carbon region of NELF-E RRM in *ct*-^{1}H^{13}C-HSQC spectra. A good dispersion of methyl proton/carbon resonances is observed. Almost complete assignment of the methyl groups located in the structured region is shown.

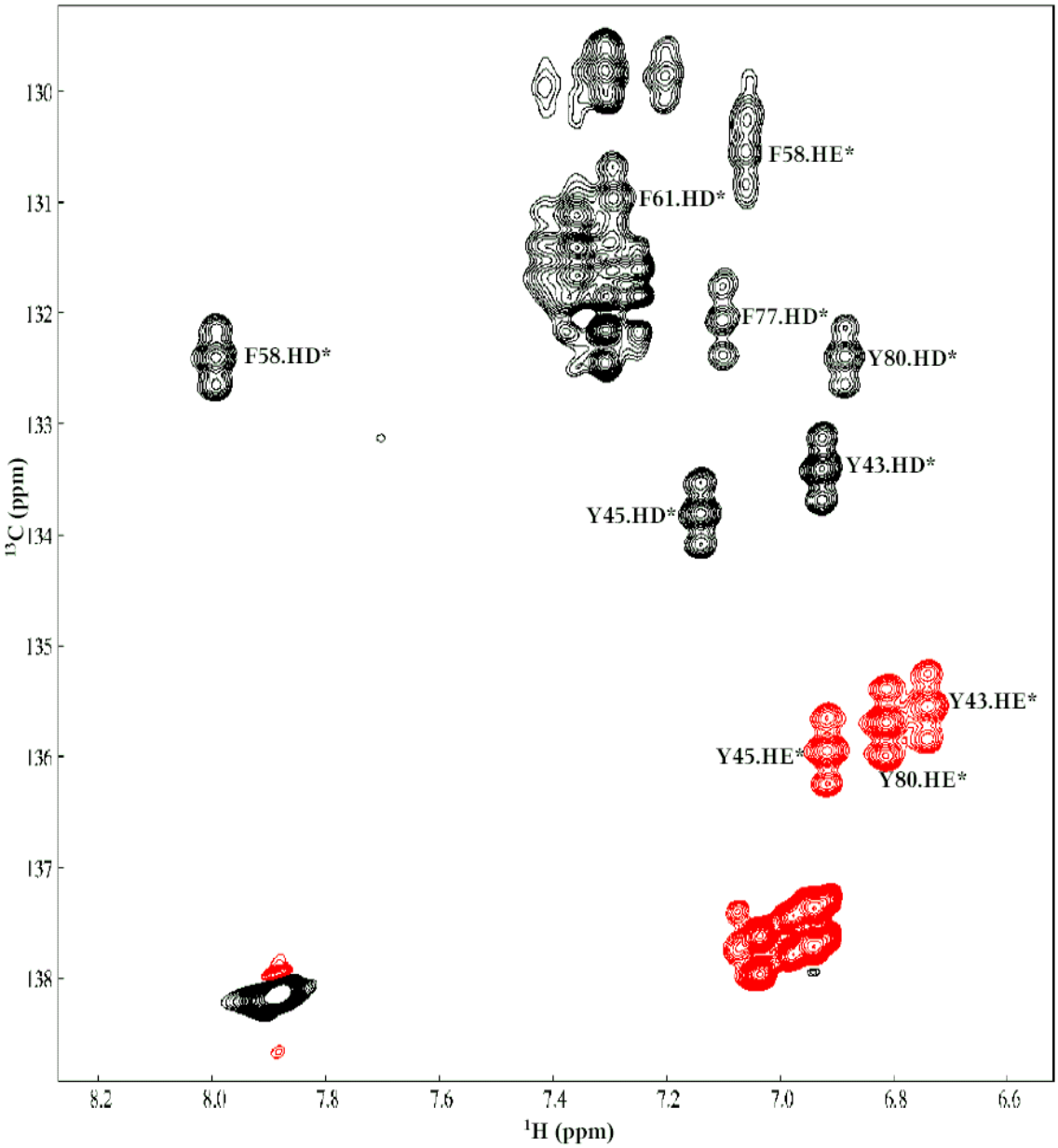


Fig. 4.13 Aromatic proton/carbon region of NELF-E RRM. Aromatic proton/carbon region of NELF-E RRM in *ct*-^{1}H^{13}C-HSQC spectra. The assignment of the aromatic residues is indicated.

4.2.3 Secondary structure

The secondary structure of NELF-E RRM was determined using H^{α}, C^{α}, and C' chemical shift indices (Wishart et al., 1994). Sequence plots of secondary chemical shifts in NELF-E RRM are shown in Fig.5.6 for H^{α}, C^{α} and C'. If this secondary chemical shift value exceeds or falls below a certain interval around random coil chemical shifts, a chemical shift index (CSI) (+1) or (-1) is assigned to that particular nucleus, and helices/strands are identified from opposite CSI being constant over at least three to four residues (Zhang and Formann-Kay 1997). From CSI data four β-sheets (β1: T41-Y45, β2: I64-D70, β3: C75-T79, and β4: L103-I107) and two α-helices (α1: P51-F61, and α2: M83-E92) are clearly predicted in NELF-E RRM (Fig. 4.14).

4.2.4. Hydrogen bonds

Amide protons, whose exchange rate is slowed down relatively to the other, are particularly found in secondary structure elements, in which they are involved in hydrogen bonding. Amide proton exchange rates were obtained by NEWMEXICO experiment for NELF-E RRM (Chapter 3.7.8). For the structure calculation of NELF-E RRM, 24 of slowly exchanging amide protons (N40, L42, Y43, V44, Y45, G56, A57, F58, S59, S68, C75, A76, F77, V78, Y80, D87, Q88, A89, V90, A91, E92, L103, K104, and N106) were used in the form of additional distance restrains. Hydrogen bonds were included in the final structure calculation as described in Chapter 3.7.8.

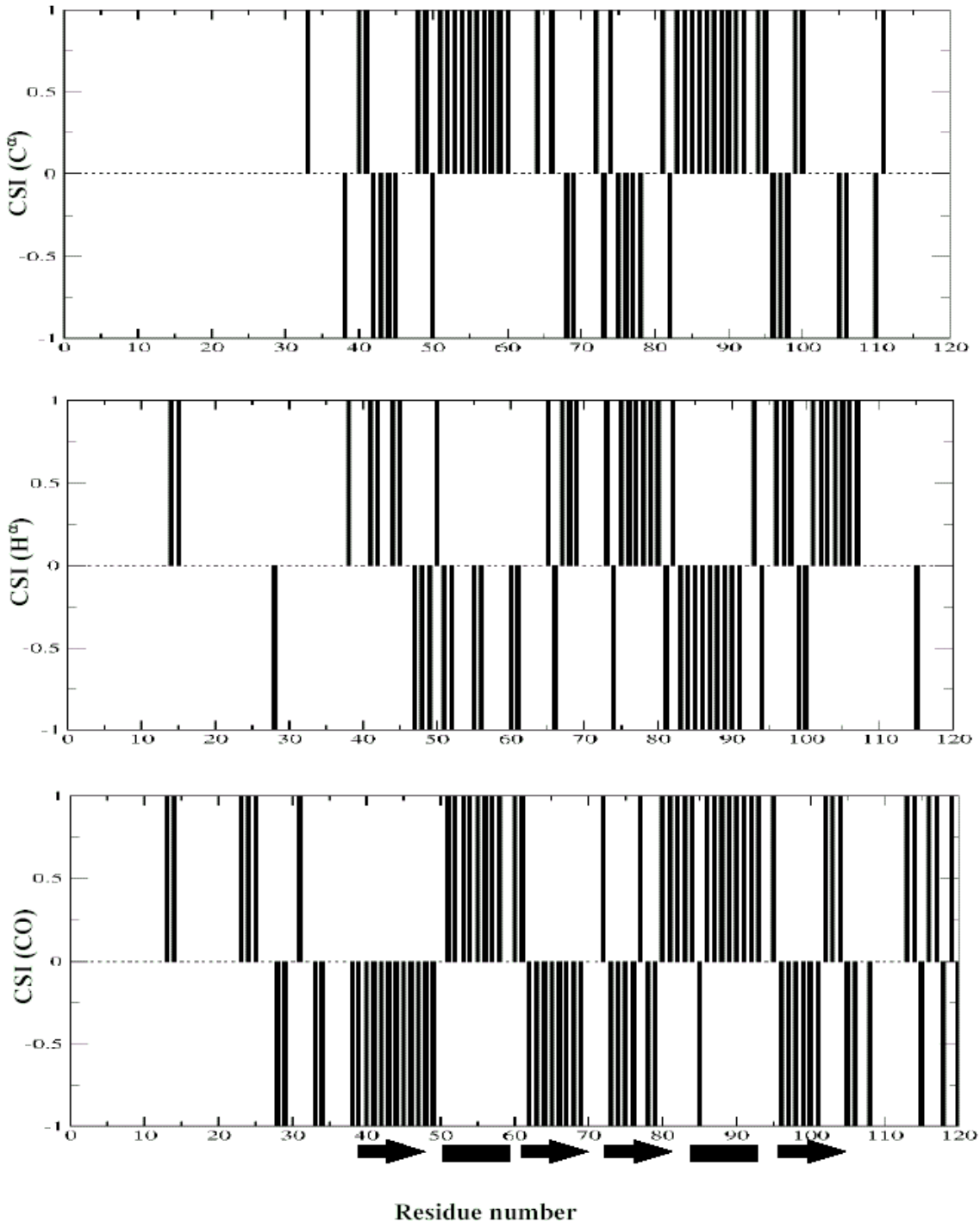


Fig. 4.14 Prediction of secondary structure using chemical shift index

4.2.5. Dihedral angle restraints

The dihedral angle restraints for the NELF-E RRM was determined indirectly via the ratios of the cross peak to diagonal signal in the HNHA spectrum as described in Chapter 3.7.8. The coupling constants for NELF-E RRM were scaled with another factor 1.1 to account for the different relaxation behavior of H^N and H^α nuclei. The typical $^3J(H^NH^\alpha)$ coupling constants found for an α-helix and antiparallel β-sheets are <6 and >8 Hz, respectively. For the final structure calculation of NELF-E RRM, in total 32 dihedral angle restraints, derived from $^3J(H^NH^\alpha)$ coupling constants were included. Residues with scalar coupling constants below 6 Hz were restrained to dihedral angles between - 80° and - 40°, residues showing coupling constants above 8 Hz were restricted to dihedral angles of - 160° to - 80° (Schweimer et al., 2002). Glycines were omitted, since they are not stereospecifically assigned and the coupling constants are likely to be affected by cross relaxation (Vuister and Bax 1993). The typical $^3J(H^NH^\alpha)$ coupling constants were observed for the secondary structural elements predicted from CSI (Fig. 4.15).

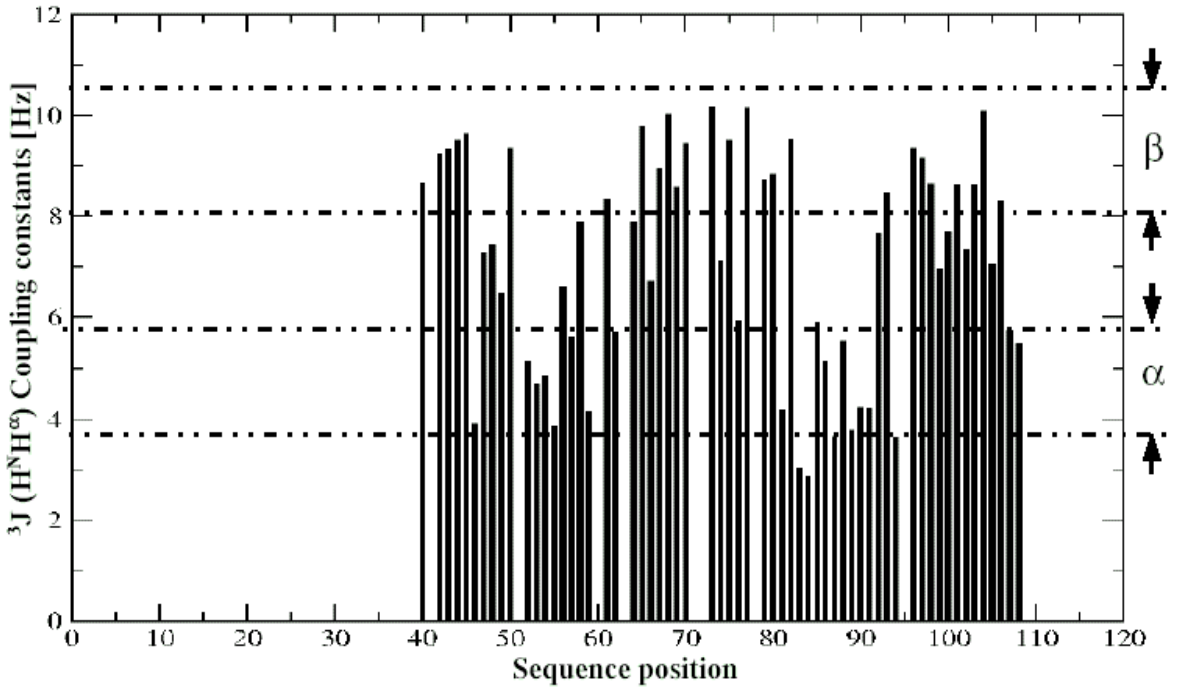


Fig. 4.15 $^3J(H^NH^\alpha)$ coupling constants. The regions which have been assigned to be α-helical or β-strand dominated are enclosed by dashed lines. Missing bars indicate residues which were not assigned or where no reliable coupling constants could be estimated

4.2.6 {^{1}H}-^{15}N steady state NOE

The internal flexibility of the protein backbone can be well understood by measuring the {^{1}H}-^{15}N steady state NOE. To investigate the dynamics of NELF-E RRM, {^{1}H}-^{15}N steady state NOE was recorded at 14.1 T on a uniformly ^{15}N labeled NELF-E RRM and {^{1}H}-^{15}N steady state NOE values were estimated as described in Chapter 3.7.10. The {^{1}H}-^{15}N steady state NOE values cluster around 0.7, indicating the absence of pronounced motions on the ps to ns time scales for nearly all the residues in between N40 and A108 (Fig. 4.16). Residues located in the amino and carboxy terminal regions showed either negative {^{1}H}-^{15}N steady state NOE values or missing signals in the saturated sub-spectrum, which is a clear indication of a high degree of flexibility. This is further supported by observing the chemical shifts of 20 residues from the amino terminus and 12 residues from carboxy terminus in the random coil region and a lack of medium/long range NOEs in the NOESY spectrum.

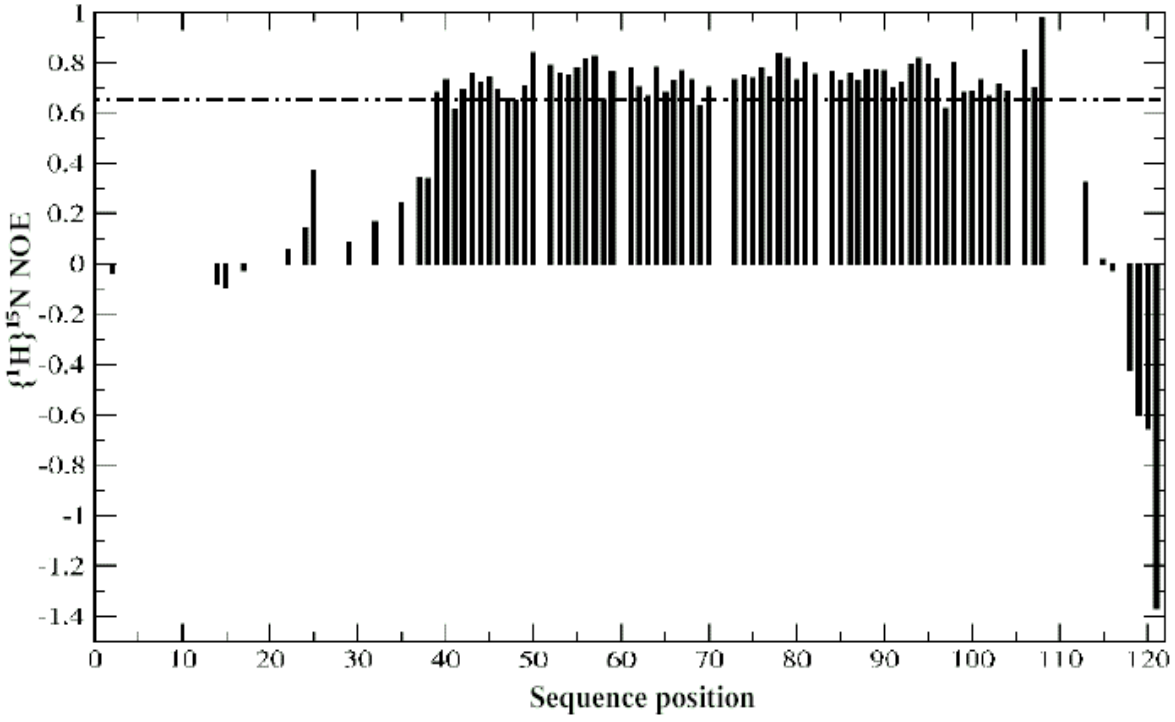


Fig. 4.16 {^{1}H}-^{15}N steady state NOE. {^{1}H}-^{15}N steady state NOE values as a function of the amino acid sequence of NELF-E RRM. Typical ranges (>0.65) found in rigid-folded polypetide chains are shown by the dashed line.

4.2.7 Analysis of NOESY spectra

The finding of a tertiary structure results in the success of all the steps discussed in the preceding chapters. The last stage in the long procedure of protein structure determination by NMR spectroscopy consists in the collection of tertiary structural information. To date, several techniques with different levels of development offer the possibility to gain this information. The classical way to determine the tertiary structure of biomolecules is based on inter-proton distances derived from NOESY cross peaks.

Following the assignment of NOE connectivities were accomplished using a combination of complementary HSQC based 3D-NOESY experiments as described in Chapter 3.7.5. As an optimal NOE mixing time a value of τ_{mix} = 120 ms was employed, a time span where the relaxation of the 14 kDa protein was still in tenable limits and spin diffusion did not pose a problem. First of all, only cross peaks connecting the β-strands were sought, as they provide the information about the topology of the protein. The H^N-H^α contacts across the β-strands serves as a confirmation. For an antiparallel β-sheet, as is the case for NELF-E RRM, an inter-strand H^N-H^N NOE cross peaks, for example V78 to L42, necessarily has to be accompanied by a H^N-H^α cross peak from V78 to Y43 as shown in Fig. 4.17. Typical for antiparallel β-sheets are extremely strong H^α-H^α cross peaks, because they are directly facing each other and their distance is below 2.2 Å in an ideal antiparallel β-sheet. Whereas in α-helices $d_{NN}(i,i+1)$ = 2.8 Å is short and several medium range NOEs up to $d_{\alpha N}(i,i+4)$ and $d_{NN}(i,i+4)$ were observed. NOESY cross-peaks were divided into four classes, strong, medium, weak and very weak, which resulted in restraints on upper distances of 3.0, 4.0, 5.0 and 6.0 Å, respectively. In total 1926 NOE distance restraints were evaluated, which makes approximately 27 restraints per structured residue. 468 of these NOE restraints were intra-residual contacts, 406 were derived from sequential inter-residue contacts, 30 % of all restraints (623) were long range NOEs and 15 % (281) medium range (Table 4.1). The high number of long and medium range restraints forms the basis for the well defined structure of NELF-E RRM.

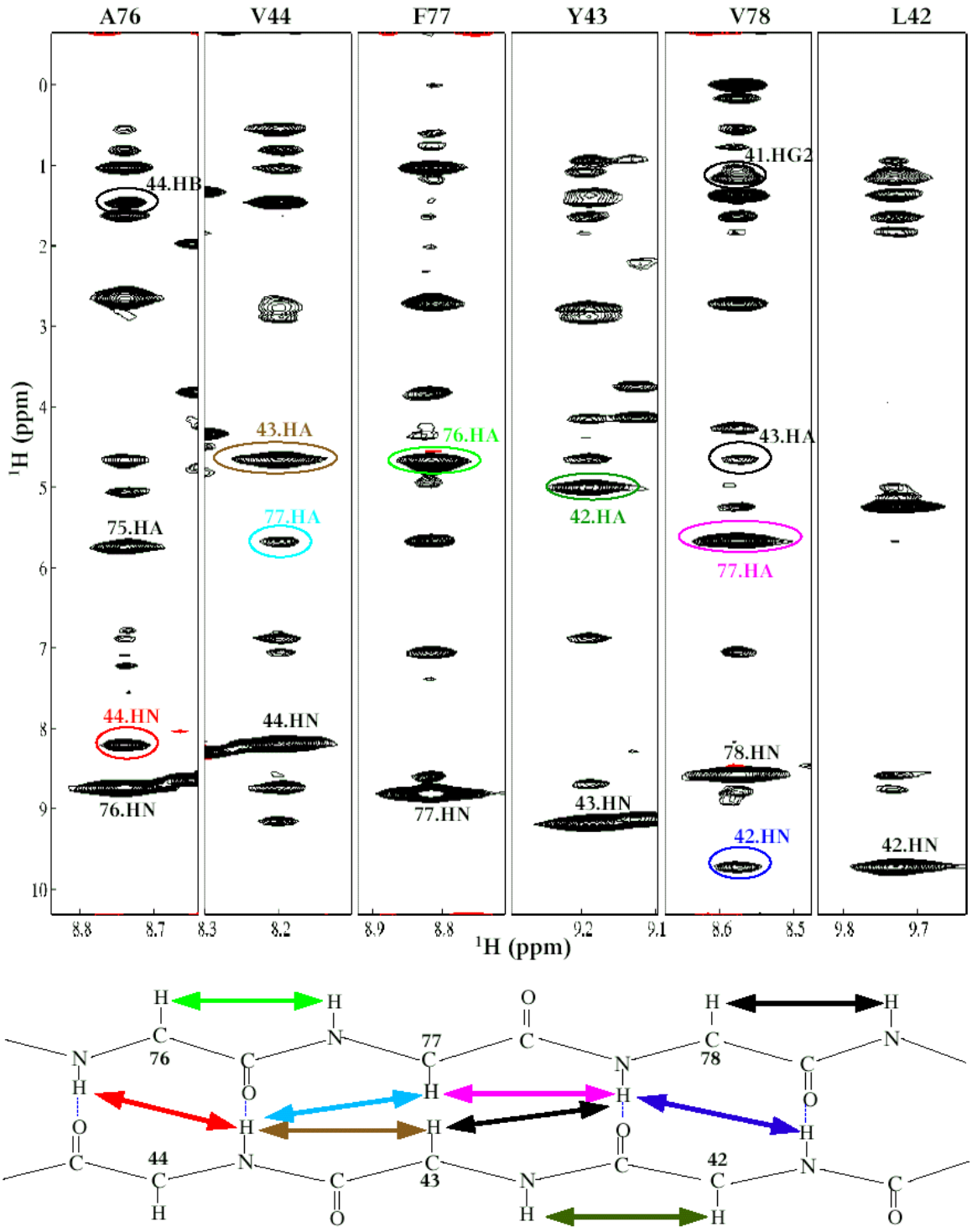


Fig. 4.17 NOESY spectra of NELF-E RRM. Slices from ^{15}N NOESY-HSQC spectrum of the few residues located in the central β-strands are placed in a row. Characteristic NOEs observed in the antiparallel β-strands are shown with double headed arrows and the corresponding NOEs are represented with the respective colour in the spectra.

4.2.8 Structure calculation

The structure calculations of NELF-E RRM were performed with the program XPLOR 3.8.5.1 using a three-step simulated annealing protocol with floating assignments of prochiral groups as described in Chapter 3.7.9.2. In a first step, 200 structures were calculated using 1926 distance, 24 hydrogen bond and 32 dihedral angle restraints. The 40 structures with the lowest energy were then refined using 55 $^{1}D(^{1}H^{N}, ^{15}N)$ RDCs with harmonic potential (Tjandra et al., 1997). Dipolar couplings of flexible residues showing a $\{^{1}H\}$-^{15}N NOE below 0.65 at 14.1 T were excluded from the calculations. The tensor components of the alignment were optimised with a grid search by varying the axial components D_a and the rhombicity R in steps of 0.5 and 0.1, respectively. The initial values of D_a and R were estimated from the distribution of the $^{1}D(^{1}H^{N}, ^{15}N)$ (Clore et al., 1998), and a molecular dynamics run was performed for each pair of D_a and R, yielding an axial component of 9.0 Hz, and a rhombicity of 0.4 for the energetically most favorable combination of D_a and R.

The 20 structures showing the lowest values of the target function excluding the database potential were further analysed with X-PLOR (Bruenger 1993), MolMol (Koradi et al., 1996) and PROCHECK 3.5.4 (Morris et al., 1992; Laskowski et al., 1996). The resulting ensemble of 20 structures shows a high coordinate precision of 0.28 Å for the heavy backbone atoms and 0.64 Å for all heavy atoms for residues G39-A108, corresponding to the structurally defined domain, as well as good stereochemical properties reflected by the fact that 91 % of residues are located in the most favored regions of the Ramachandran plot (Table 4.1).

The solution structure of NELF E RRM exhibits a compact βαββαβ fold with a four stranded antiparallel β sheet (β1 = N40-Y45, β2 = I64-A70, β3 = C75-Y80, β4 = Q102-I107) that packs against two helices (α1 = P51-F61 and α2 = M83-L93) which are oriented approximately perpendicular to each other (interhelix angle = 114.3° ± 1.8°). One side of the β- sheet is packed with two α-helices to form a hydrophobic core of the protein and the other side is solvent exposed. Residues T96 and E97 form an additional short β-strand aligned antiparallel to β4, thus extending the β-sheet (Fig. 4.18). This is similar to other RRMs where a β-hairpin is found in the sequence region between α2 and β4 (Volpon et al., 2005).

experimental restraints		
distance restraints	total	1926
	intraresidual sequential medium-range long-range	468 406 281 623
dihedral angles		32
dipolar couplings		55
hydrogen bonds (two restraints each)		24
molecular dynamics statistics		
energies (kcal/mol)		
	E_{pot}	14.1 ± 1.4
	E_{bond}	0.58 ± 0.06
	E_{angle}	6.7 ± 0.6
	E_{impr}	2.3 ± 0.2
	E_{repel}	2.4 ± 0.3
	E_{NOE}	1.3 ± 0.5
	E_{cdih}	0.03 ± 0.03
	E_{sani}	0.7 ± 0.2
RMSDs from ideal distances (Å)	bond lengths distance restraints	0.00068 ± 0.00004 0.0036 ± 0.0007
RMSDs from ideal angles (deg)	bond angles dihedral angle restraints	0.14 ± 0.06 0.15 ± 0.15
RMSDs from dipolar couplings (Hz)		0.11 ± 0.02
atomic coordinate precision (RMSD) (Å)		
backbone heavy atoms		0.28 (Gly39-Ala108)
heavy atoms		0.64 (Gly39-Ala108)
Ramachandran plot statistics		
residues in		
	most favored regions	91.0%
	allowed regions	9.0%

Table 4.1: Structural statistics and atomic R.M.S. Deviations of NELF-E RRM.

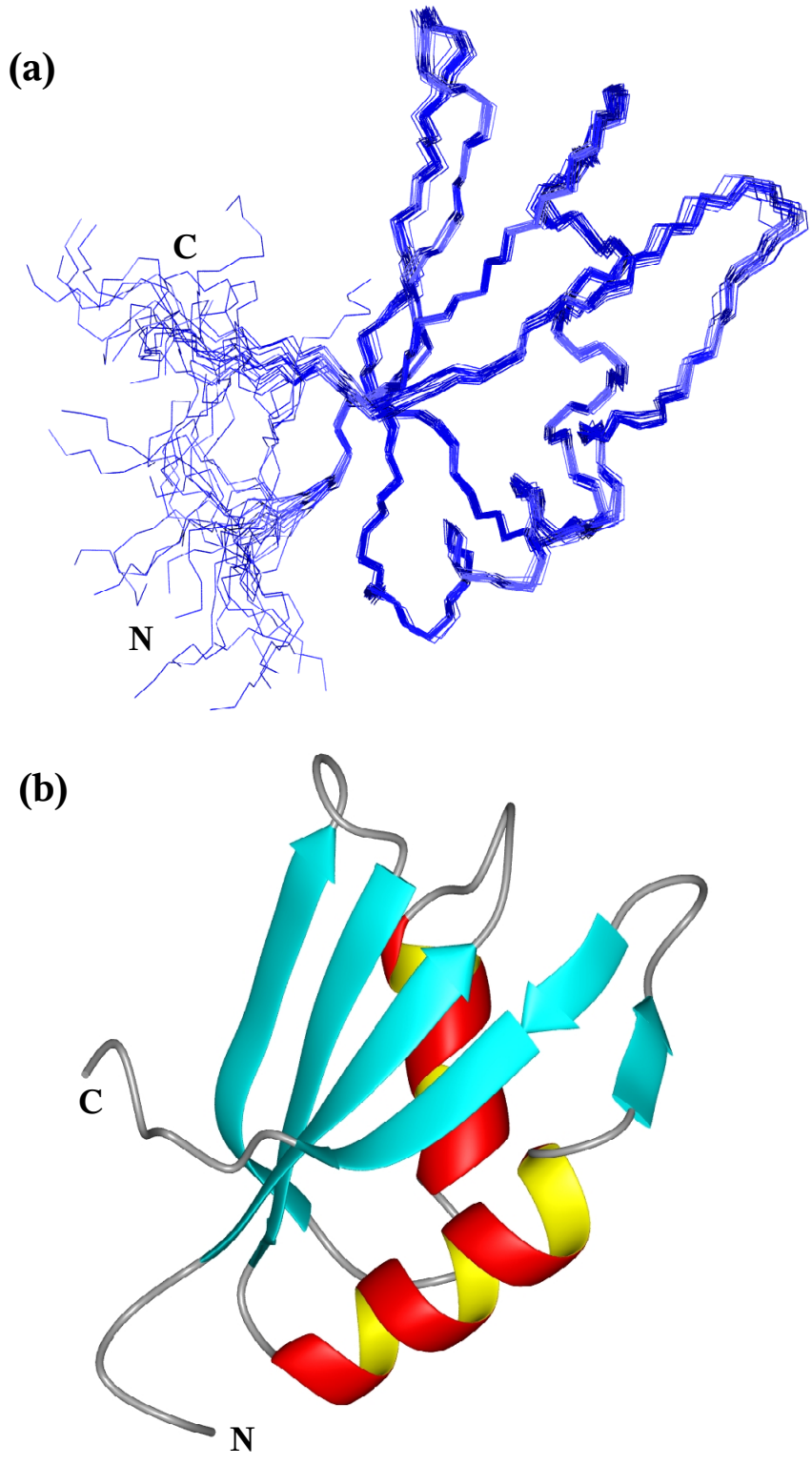


Fig. 4.18 Solution structure of NELF-E RRM. (a) Overlay of the 20 structures (residues A35-M113) showing the lowest values of the target function excluding the database potential. **(b)** Schematic presentation of the NELF-E RRM structure. The figure is generated using MOLMOL (Koradi et al., 1996).

4.3 RNA binding studies on NELF-E RRM

4.3.1 Interaction of NELF-E RRM with HIV-1 TAR RNA

It has been shown that NELF-E binds to HIV-1 TAR RNA *via* its RRM to arrest the elongation complex of HIV-1 transcription (Fujinaga et al., 2004). In order to obtain further details into the NELF-E RRM:TAR complex at the atomic level, NMR titration experiments were employed. Amide ($^{1}H^{N}$, ^{15}N) chemical shifts are very sensitive to local structural changes. Therefore, observation of chemical shift changes on titration of a binding partner to a ^{15}N labeled protein provides a powerful method for the identification the of binding interface. Addition of TAR RNA to uniformly ^{15}N labeled NELF-E RRM showed significant chemical shift changes in the ^{1}H-^{15}N HSQC spectrum (Fig. 4.19). Residues located in the two central β-stands (T41, L42, V44, Y45, C75, F77, and V78) showed remarkable chemical shift changes upon addition of TAR RNA and the resonances located in the amino terminal region of α_2 helix (K82, M83, E84, and D87) were missing in the spectra due to intermediate exchange on NMR time scale. Amide resonances located in β_4 strand (K104, N106, and I107) also showed significant chemical shift changes after the addition of TAR RNA, suggesting these residues are involved in binding. The chemical shift changes for residues located in strands β_1 and β_3 indicate the typical binding of RNA to the RRM by stacking of bases onto the two conserved aromatic residues Y43 and F77. The large chemical shift changes seen in the M113 and A116 resonances on TAR titration could be an indication of a conformational change in the region which is highly flexible in free NELF E RRM. The RNA binding region is mainly located on the β-sheet surface. The carboxy terminus of NELF-E RRM and animo terminus of α_2 helix are contributing to the RNA binding. Fig. 4.20 shows the normalised chemical shift changes and the surface representation of NELF-E RRM highlighting the binding interface and the residues that exhibit chemical shift changes upon binding to TAR RNA.

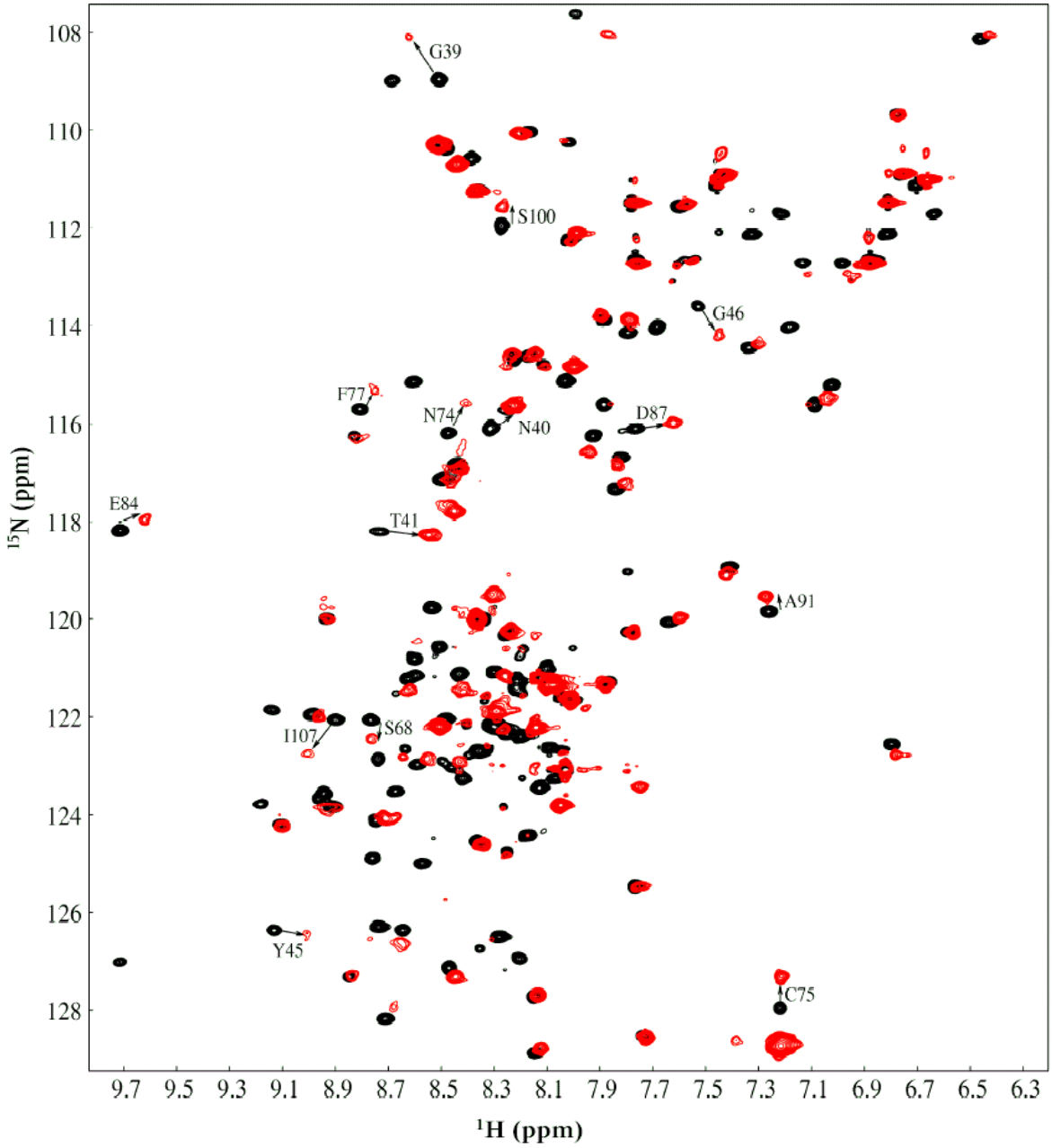


Fig. 4.19 Overlay of the ^{1}H-^{15}N HSQC spectra of free NELF-E RRM (black) and the NELF-E:TAR RNA complex (red). The amino acid resonances showing significant chemical shift changes upon addition of an equimolar amount of TAR RNA are indicated by arrows.

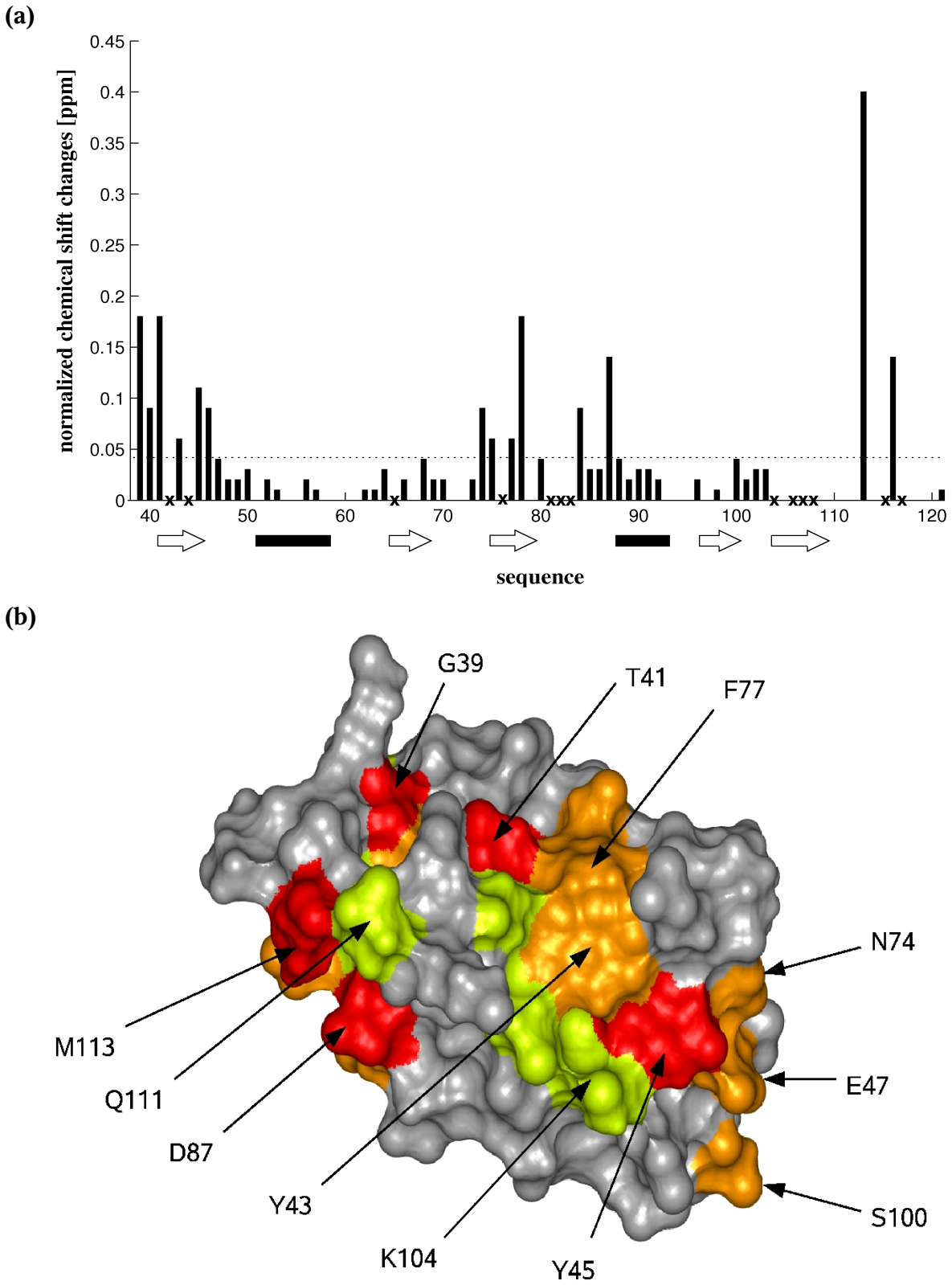


Fig. 4.20 Identification of the binding interface. (a) Normalised chemical shift changes of NELF E RRM upon TAR RNA binding. The normalised chemical shift changes (weighted geometric average of $^{1}H^{N}$ and ^{15}N chemical shift changes) are shown as a function of the primary sequence. Changes larger than 0.04 ppm (dotted line) were considered as significant. Resonances disappearing after TAR binding

due to extreme line broadening by chemical exchange are marked with an x. **(b)** Surface representation of NELF-E RRM highlighting the binding interface. Residues with resonances showing significant chemical shift changes upon TAR binding ($0.04 < \Delta\delta \leq 0.1$), including the highly conserved Y43 and F77 are shown in yellow. Those resonances showing chemical shift changes with $\Delta\delta > 0.1$ are shown in light red, and residues whose resonances could not be detected due to extreme line broadening are shown in light green.

4.3.2. Interaction of NELF-E RRM with RNA oligonucleotides

Fujinaga and coworkers (Fujinaga et al., 2004) showed that NELF-E binds to the lower stem region of HIV-1 TAR to arrest the elongation complex. RNA binding studies were preformed with six RNA oligonucleotides derived from the lower stem of HIV-1 TAR RNA (Fig. 4.21.b), to identify the high affinity binding sequence and to understand RNA recognition of NELF-E RRM. In order to identify the interaction between NELF-E RRM and RNA, ^{15}N labeled protein was titrated with the unlabeled RNA oligonucleotides. An overlay of ^{1}H-^{15}N HSQC spectra at each NMR titration step is shown in Fig. 4.21, 4.22, 4.23, 4.24, 4.25, and 4.26. NELF-E RRM binds to the various RNA oligonucleotides and they showed a different exchange regime on NMR time scale indicating that NELF-E RRM binds to RNA oligonucleotides with different affinities (Since all RNA oligonucleotides possess almost the same length it can be assumed that they have similar diffusion rates). For example titration of NELF-E RRM with TAR1-10 is in the fast exchange regime on NMR time scale (Fig. 4.21). Thus, the resonances of the nuclei affected by RNA binding gradually shift their position from the resonance for the free state towards the resonance of the bound state. In most cases this facilitates an unambiguous assignment of the amide proton and nitrogen resonances as they can be traced along their way. Interestingly, in all titration experiments almost the same set of amide resonances showed chemical shift changes upon the addition of RNA which implies NELF-E RRM binds to all RNA oligonucleotides in a very similar manner. Amide resonances located in the loop between β_3 and α_2 (E81, K82, and M83) and carboxy terminal region (M113, D115, A116, and A117) of NELF-E RRM showed remarkable chemical shift changes along with the amide resonances located in the β-strands. Thus, the RNA binding interface is mainly located on β-sheet surface, the β_3-α_2 loop region and carboxy terminus of NEFL-E RRM could be making substantial contributions to RNA binding.

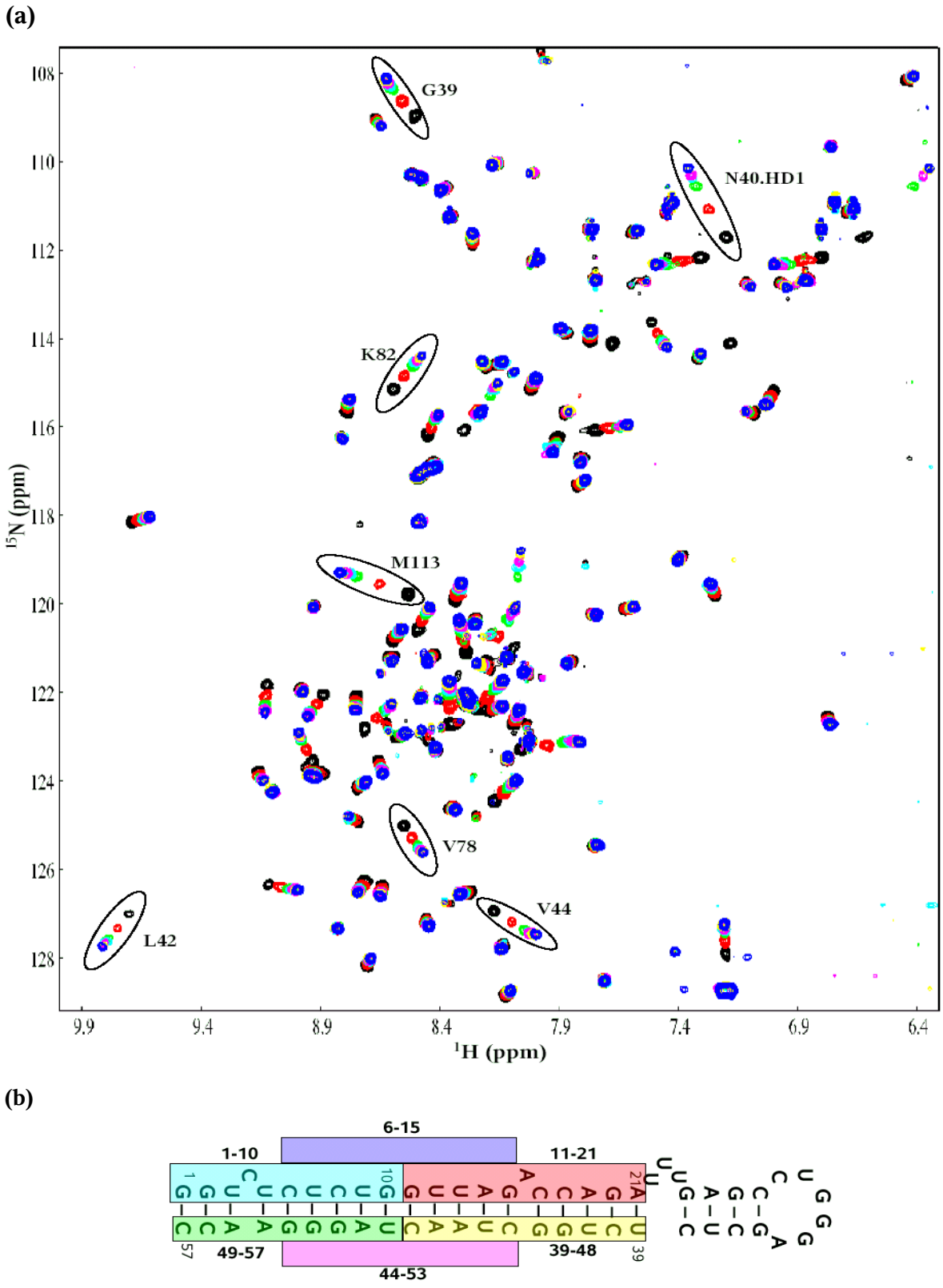


Fig. 4.21 Titration of NELF-E RRM with TAR1-10. (a) Overlay of the ^{1}H-^{15}N HSQC spectra recorded during the titration with different RNA/protein ratios. Black: 0.0, Red: 0.25, Green: 0.5, Cyan: 0.75, Magenta:1.0, yellow: 2.0, and Blue: 3.0. The amide resonances which showed strong chemical

shift changes are indicated. **(b)** RNAs used for the binding studies are shown in different colours.

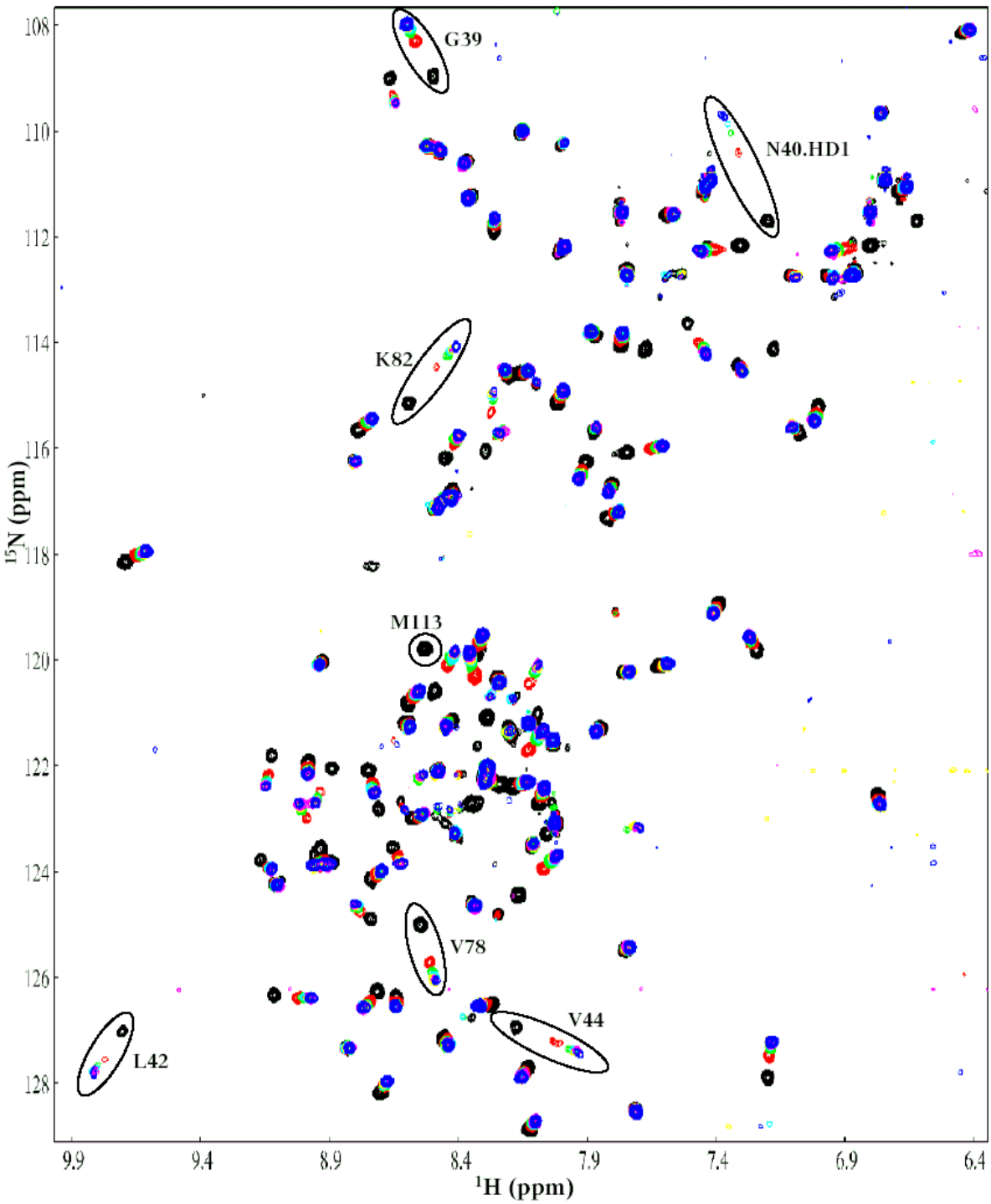


Fig. 4.22 Titration of NELF-E RRM with TAR6-15. Overlay of the ^{1}H-^{15}N HSQC spectra recorded during the titration with different RNA/protein ratios. Black: 0.0, Red: 0.2, Green: 0.4, Cyan: 0.6, yellow: 0.8, Magenta:1.0, and Blue: 1.5.

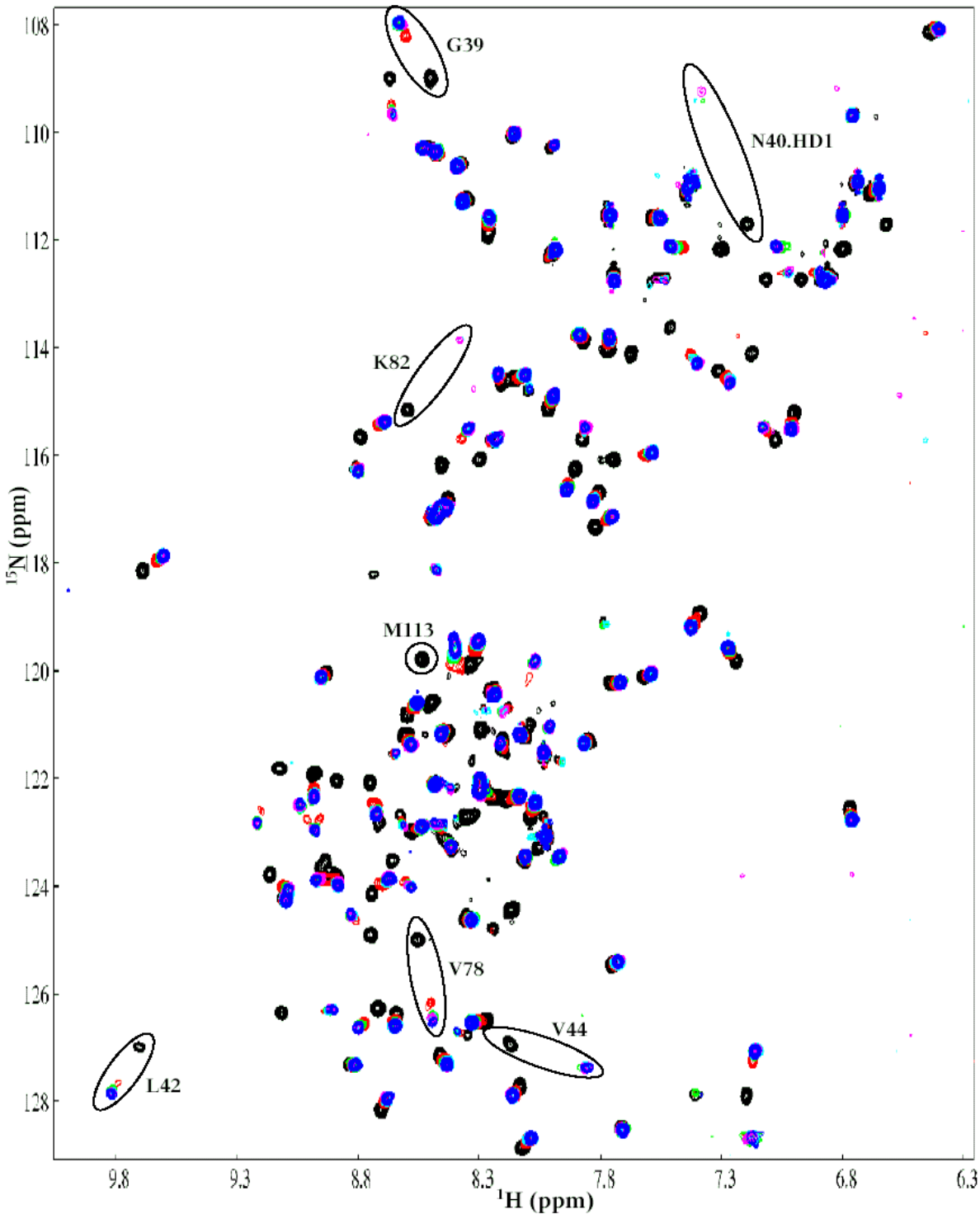


Fig. 4.23 Titration of NELF-E RRM with TAR11-21. Overlay of the ^{1}H-^{15}N HSQC spectra recorded during the titration with different RNA/protein ratios. Black: 0.0, Red: 0.25, Green: 0.5, Cyan: 0.75, Magenta:1.0, and Blue: 1.5.

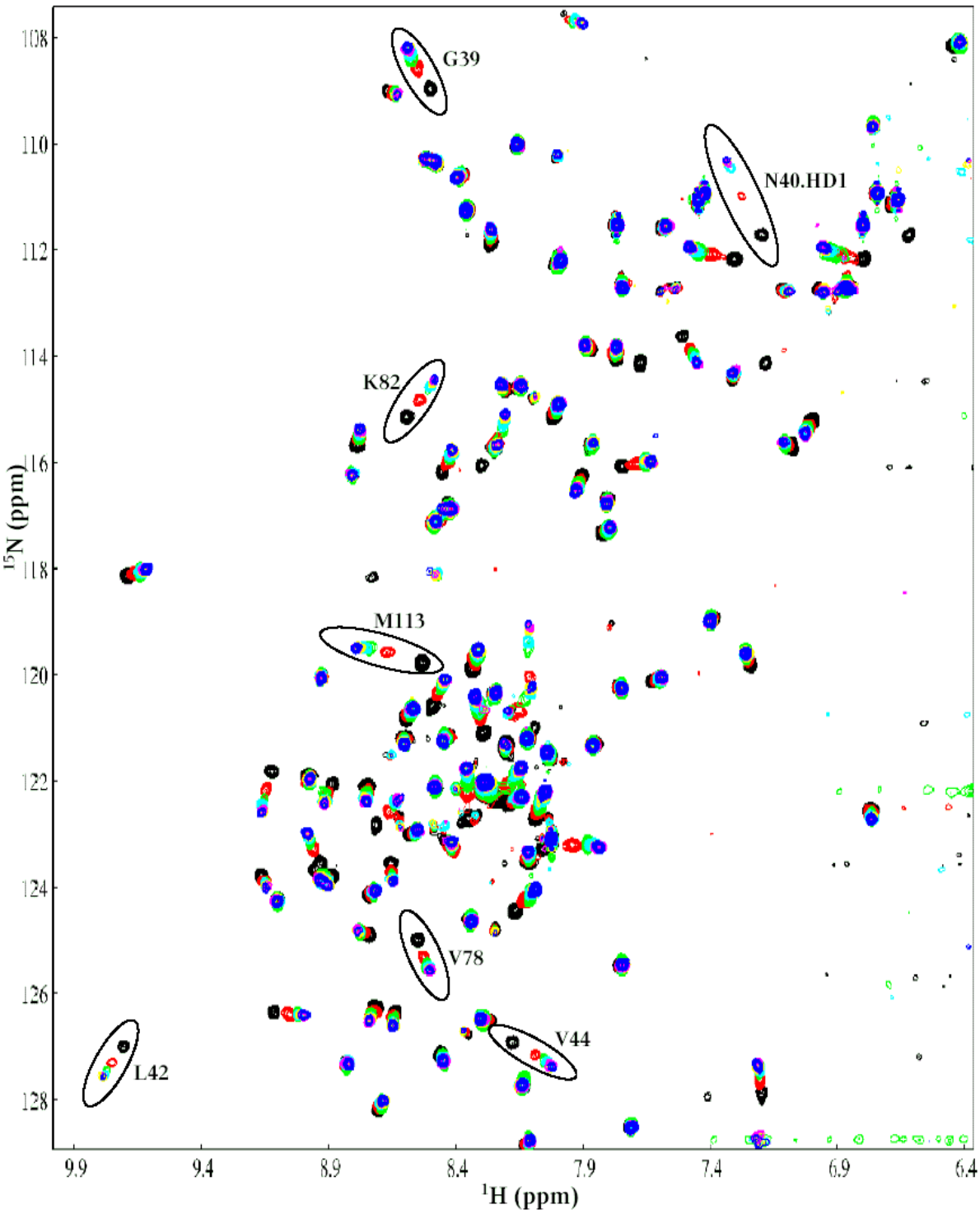


Fig. 4.24 Titration of NELF-E RRM with TAR39-48. Overlay of the ^{1}H-^{15}N HSQC spectra recorded during the titration with different RNA/protein ratios. Black: 0.0, Red: 0.25, Green: 0.5, Cyan: 0.75, yellow: 1.0, Magenta: 2.0, and Blue: 2.5.

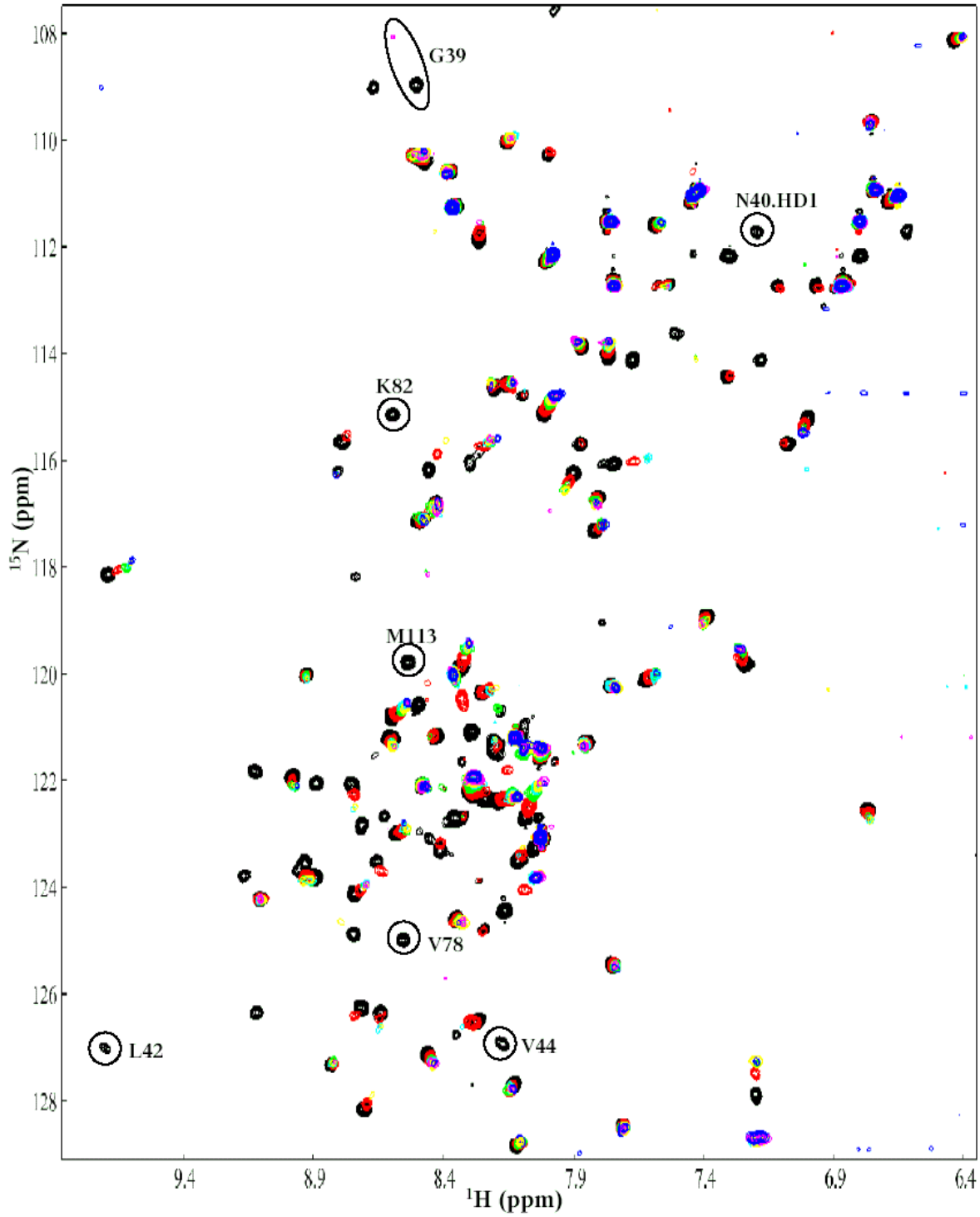


Fig. 4.25 Titration of NELF-E RRM with TAR44-53. Overlay of the ^{1}H-^{15}N HSQC spectra recorded during the titration with different RNA/protein ratios. Black: 0.0, Red: 0.25, Green: 0.5, Cyan: 0.75, yellow: 1.0, Magenta:1.5, and Blue: 2.0.

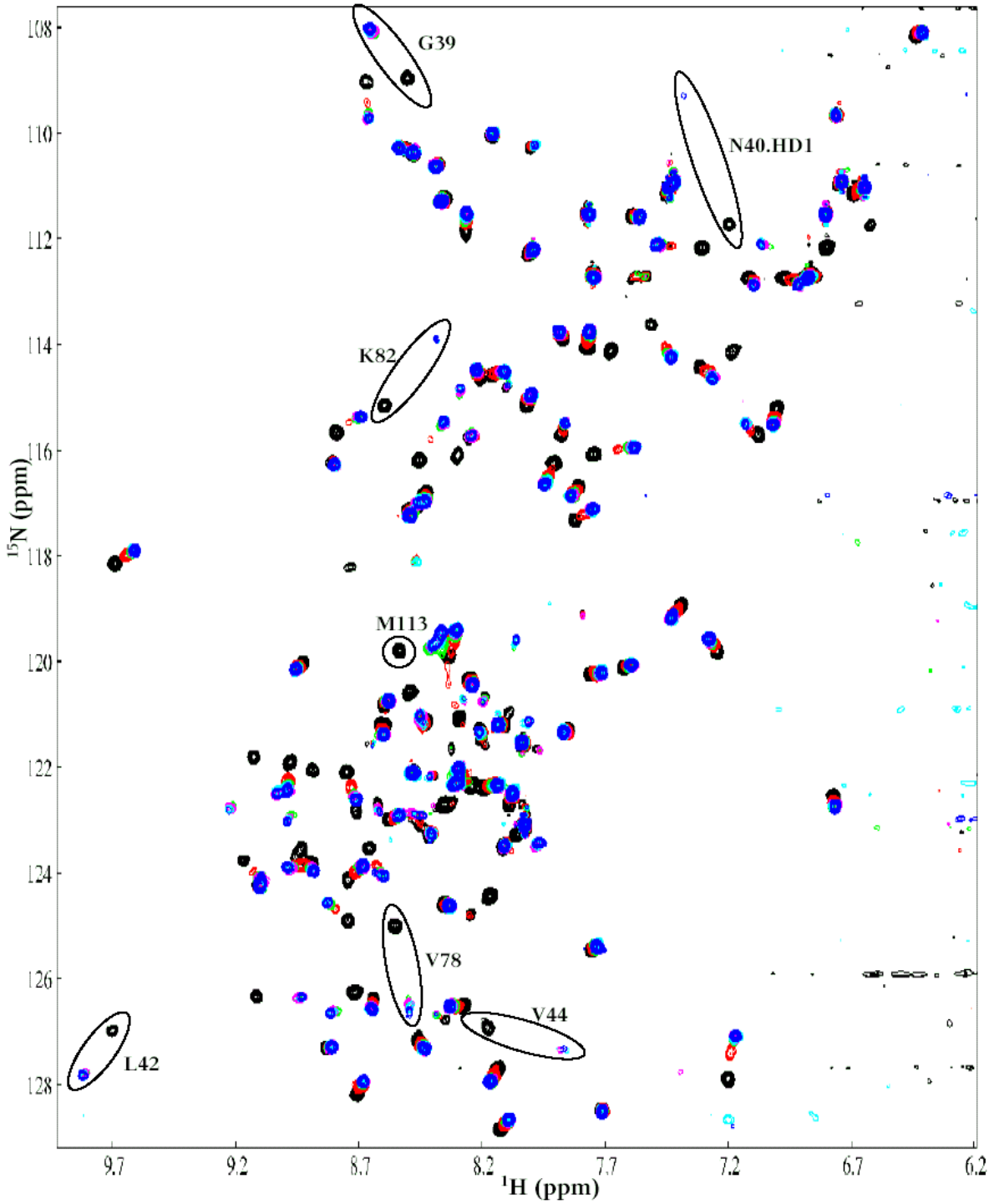


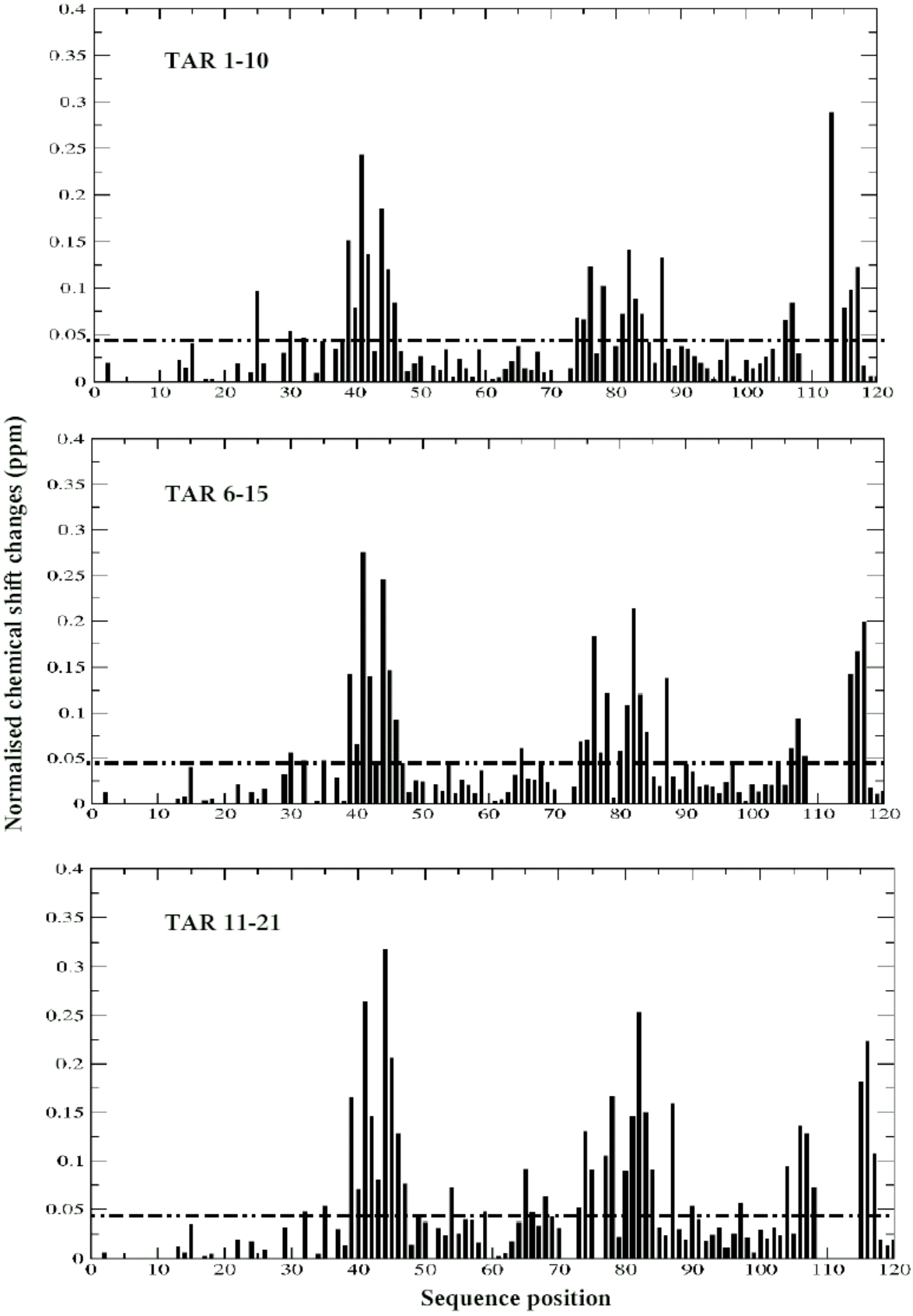
Fig. 4.26 Titration of NELF-E RRM with TAR49-57. Overlay of the ^{1}H-^{15}N HSQC spectra recorded during the titration with different RNA/protein ratios. Black: 0.0, Red: 0.2, Green: 0.4, Cyan: 0.6, Magenta: 0.8, and Blue: 1.0.

4.3.3 Normalised chemical shift changes

Normalised chemical shift changes are expressed as the weighted geometric average of $^{1}H^{N}$ and ^{15}N chemical shift changes for each residue.

$$\Delta\delta_{norm} = \sqrt{(\Delta\delta_{1H})^2 + 0.1(\Delta\delta_{15N})^2}$$

Normalised chemical shift changes larger than 0.04 ppm are considered significant (Hajduk et al., 1997) as indicated by dashed line in Fig 4.27. Normalised chemical shift changes for NELF-E RRM:TAR44-53 complex was not obtained since the amide resonances disappeared in the final spectra of the titraion due to intermediate chemical exchange on NMR time scale. Fig. 6.10 shows the normalised chemical shift changes as a function of the primary sequence upon the addition of RNA. The backbone amide protons and nitrogens located in the β-sheets, loop between β_3 and α_2, and the carboxy terminus are strongly influenced. Among the NELF-E RRM:TAR complexes, NELF-E RRM:TAR49-57 and NELF-E RRM:TAR11-21 showed the strongest chemical shift changes. For example, the normalised chemical shift change for V44 in NELF-E RRM:TAR1-10 and NELF-E RRM:TAR49-57 complexes are 0.185 and 0.308 ppm, respectively.


TAR 1-10
TAR 6-15
TAR 11-21
Normalised chemical shift changes (ppm)
Sequence position
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 10 20 30 40 50 60 70 80 90 100 110 120

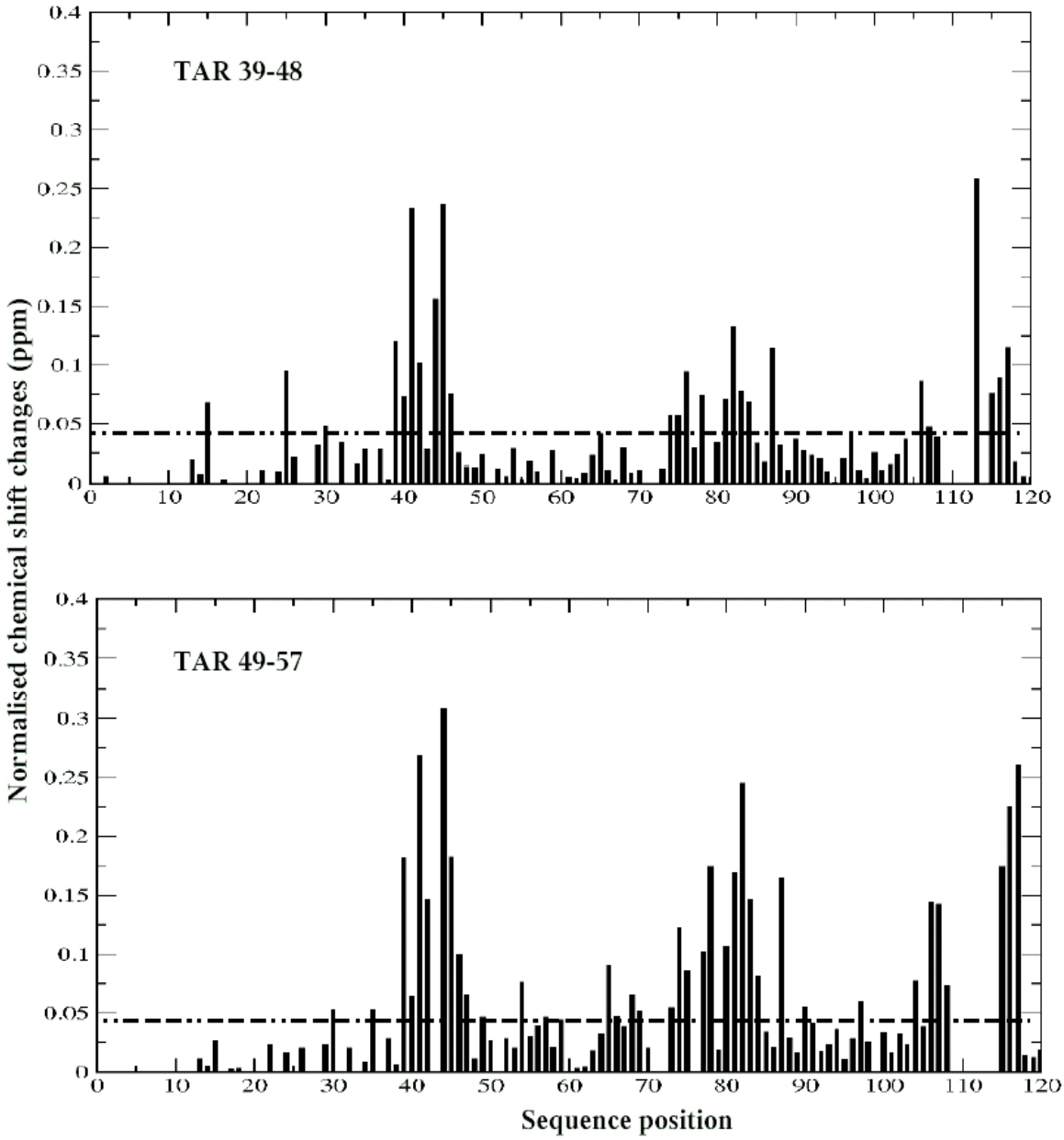


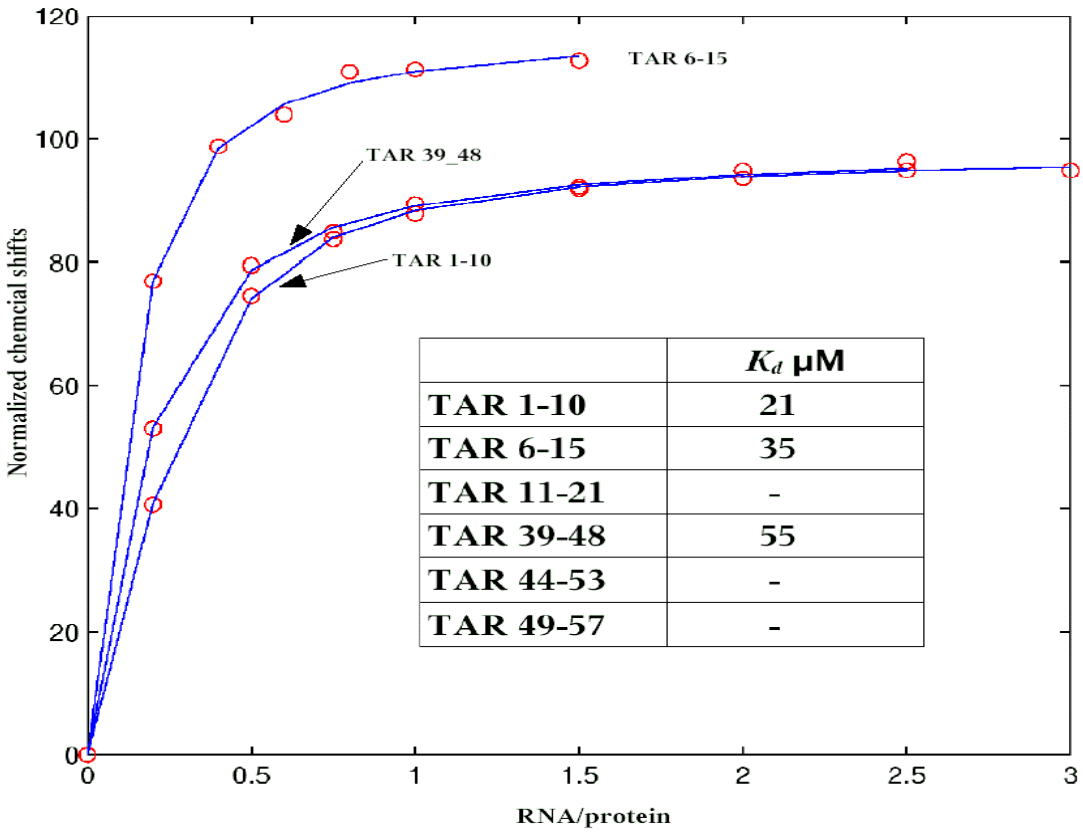
Fig. 4.27 Normalised weighted chemical shift changes for NELF-E RRM upon binding to RNA oligonucleotides. Normalised chemical shift changes larger than 0.04 ppm were considered to be significant and are indicated by a dashed line.

4.3.4 Dissociation constants for NELF-E RRM-TAR complexes

The dissociation constant K_d is determined from the changes of chemical shifts of ^{15}N-labeled NELF-E RRM observed in an ^{1}H-^{15}N HSQC after gradual addition of the corresponding unlabeled binding partner. Changes of chemical shifts of signals in the fast-exchange limit were fitted to the following equation for a two-state model.

$$\delta_{obs} = \delta_P + (\delta_{PL} - \delta_P)\left[\frac{\{K_D + (1+r)[P]_0\}}{2[P]_0} - \frac{\sqrt{(K_D + (1+r)[P]_0)^2 - 4[P]_0^2 r}}{2[P]_0}\right]$$

δ_{obs}, δ_P, and δ_{PL} are the chemical shifts for the actual mixture, the free protein, and the completely bound protein, respectively. $[P]_0$ is the total concentration of NELF-E RRM, and r describes the NELF-E RRM/RNA ratio.



	K_d µM
TAR 1-10	21
TAR 6-15	35
TAR 11-21	-
TAR 39-48	55
TAR 44-53	-
TAR 49-57	-

Fig. 4.28 Dissociation constants for NEFL-E RRM-TAR complexes. Fitting of the curves yielded the calculated K_d values shown in the inset . Normalised chemical shift changes of G39 were used.

Titrations of ^{15}N labeled NELF-E RRM with TAR1-10, TAR 6-15, and TAR 39-48 allowed the observation of chemical shift changes with fast exchange behavior for resonances located in the RNA binding region of NELF-E RRM. Thus, a K_d-values of 21, 35, and 55 μM could be determined (Fig. 4.28).

4.3.5 Structure determination of RNA bound NELF-E RRM

Among all the NELF-E RRM-TAR titration experiments, titration of NELF-E RRM with TAR49-57 showed the strongest chemical shift changes and slow exchange behavior on NMR time scale allowing further structural characterisation of this complex. Structural characterisation of this complex would give further insights into the large chemical shift changes observed in the carboxy terminus and β_3-α_2 loop region of NELF-E RRM during the titration experiments.

4.3.5.1 Resonance assignments of NELF-E RRM in the complex

Backbone resonances of NELF-E RRM in the complex with TAR49-57 were mainly assigned by following the titration steps. Except for the resonances involved in RNA binding, all other resonances could be assigned straightforwardly since they did not show any chemical shift perturbations. Resonances showing a large chemical shift change were assigned by observing the characteristic NOEs in ^{15}N NOESY-HSQC spectra. Almost completely assigned ^{1}H-^{15}N HSQC of NELF-E RRM in the complex with TAR49-57 is shown in Fig. 4.29. Many protein resonances in the complex gave chemical shifts and patterns of NOE cross peaks similar to those observed for free protein. Thus, most of the side chain resonances were assigned by comparing the *constant time* ^{1}H-^{13}C HSQC spectra of free and RNA bound NELF-E RRM. Side chain resonance assignments for those residues involved in RNA binding were achieved by following the trace of side chain protons in 3D HCCH-TOCSY and identifying characteristic NOEs in ^{13}C NOESY-HSQC spectrum.

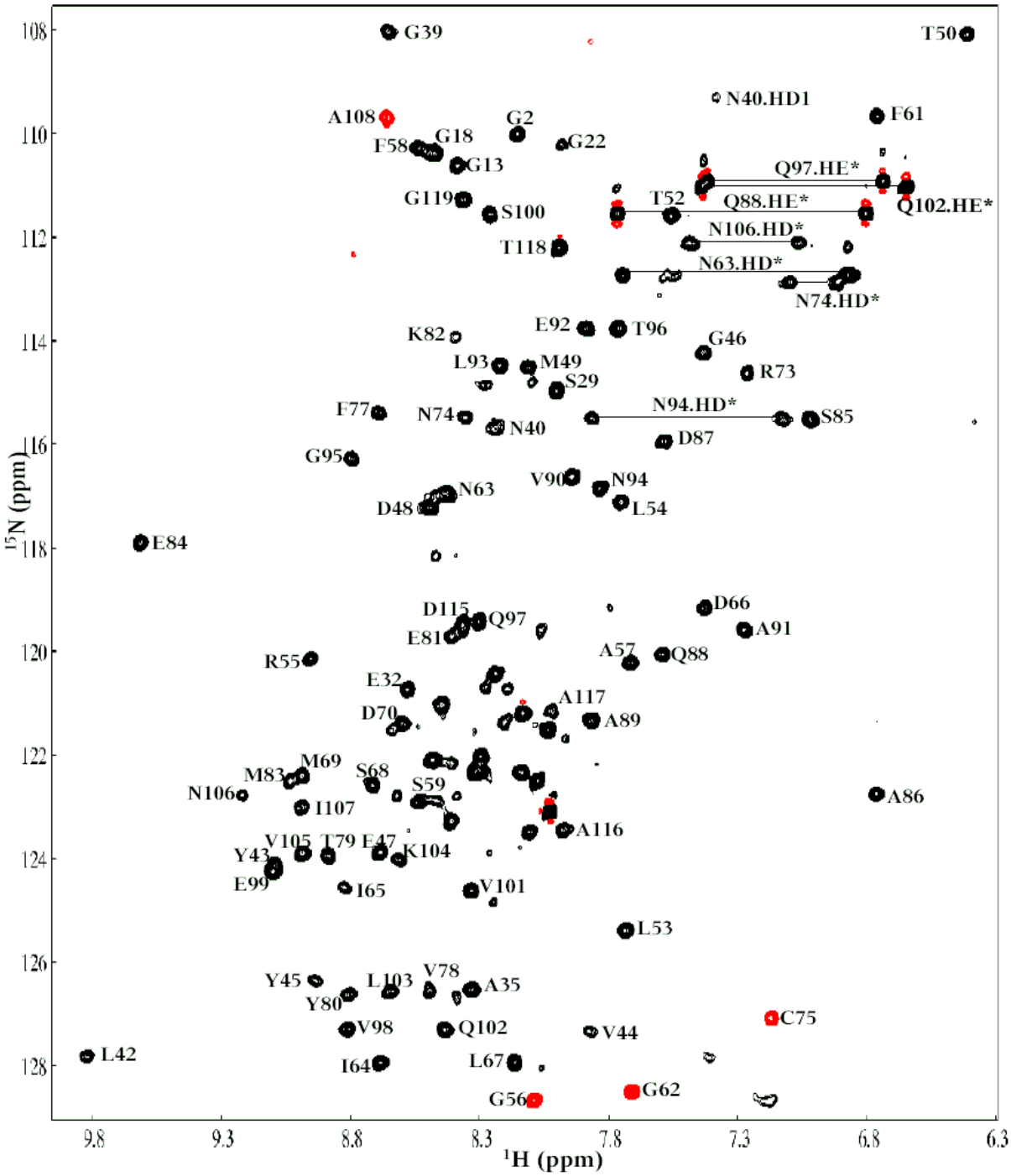


Fig. 4.29 ^{1}H-^{15}N HSQC of NELF-E RRM in complex with RNA. ^{1}H-^{15}N HSQC spectrum of uniformly ^{15}N labeled NELF-E RRM in the presence of one molar equivalent of TAR49-57 (positive signals in black and negative signals in red). The negative signals are aliased along the ^{15}N dimension. All the amide proton resonances are labeled.

4.3.5.2 Analysis of NOESY spectra of NELF-E RRM in the complex with RNA

The NOE derived distance restraints for the RNA bound NELF-E RRM were obtained using the restraints for the free NELF-E RRM as a starting point. The 1926 distance restraints observed for free NELF-E RRM are also found in the complex with TAR49-57. In addition to these distance restraints, another 40 inter-residual NOEs were observed for RNA bound NELF-E RRM, which were mainly derived from the carboxy terminal and β_3-α_2 loop regions. The chemical shifts and NOE connectivities for residues located in the core of the protein showed no significant changes in RNA bound NELF-E RRM, indicating that the local conformation remains unchanged in this region. Fig. 4.30 shows strips from the ^{13}C NOESY-HSQC spectrum of the NOE cross peak pattern of L93, M83, and Q111, for the free NELF-E RRM and NELF-E RRM:TAR49-57 complex. Inter-residual NOEs observed between L93 and F58 showed no significant changes in free and RNA bound NELF-E RRM which is clearly indicating that the overall global fold of the NELF-E RRM did not change. When compared with free NELF-E RRM significant changes were observed in the NOE pattern of M83 and Q111 for the RNA bound NELF-E RRM. For NELF-E RRM in the complex with RNA, strong NOEs were observed between the gamma protons of M83 and Q111, and methyl groups of A117 and T79, respectively (Fig. 4.30). The presence of these strong inter-residual NOEs in the RNA bound NELF-E RRM indicating that the carboxy terminus of NELF-E RRM is undergoing a conformational change upon binding to TAR49-57.

4.3.5.3 Structure of RNA bound NELF-E RRM

In total 1980 distant restraints, 32 dihedral angles, and 24 hydrogen bonds were used for the structure calculation. The structure calculations of RNA bound NELF-E RRM were performed with the program XPLOR 3.8.5.1 using a three-step simulated annealing protocol as described in Chapter 4.2.8. Overlay of the resulting ensemble of 20 structures and the ribbon representation of the lowest energy structure are shown in Fig 4.31. The overall global fold of NELF-E RRM in the complex with RNA did not change. However, the carboxy terminus of NELF-E RRM is undergoing a confromational change by adopting a small 3_{10} helix with residues M113, L114, and D115 around the loop between β_3 and α_2. The large chemical shift changes observed in carboxy terminus and β_3-α_2 loop regions are attributed to the confromational change upon binding to RNA.

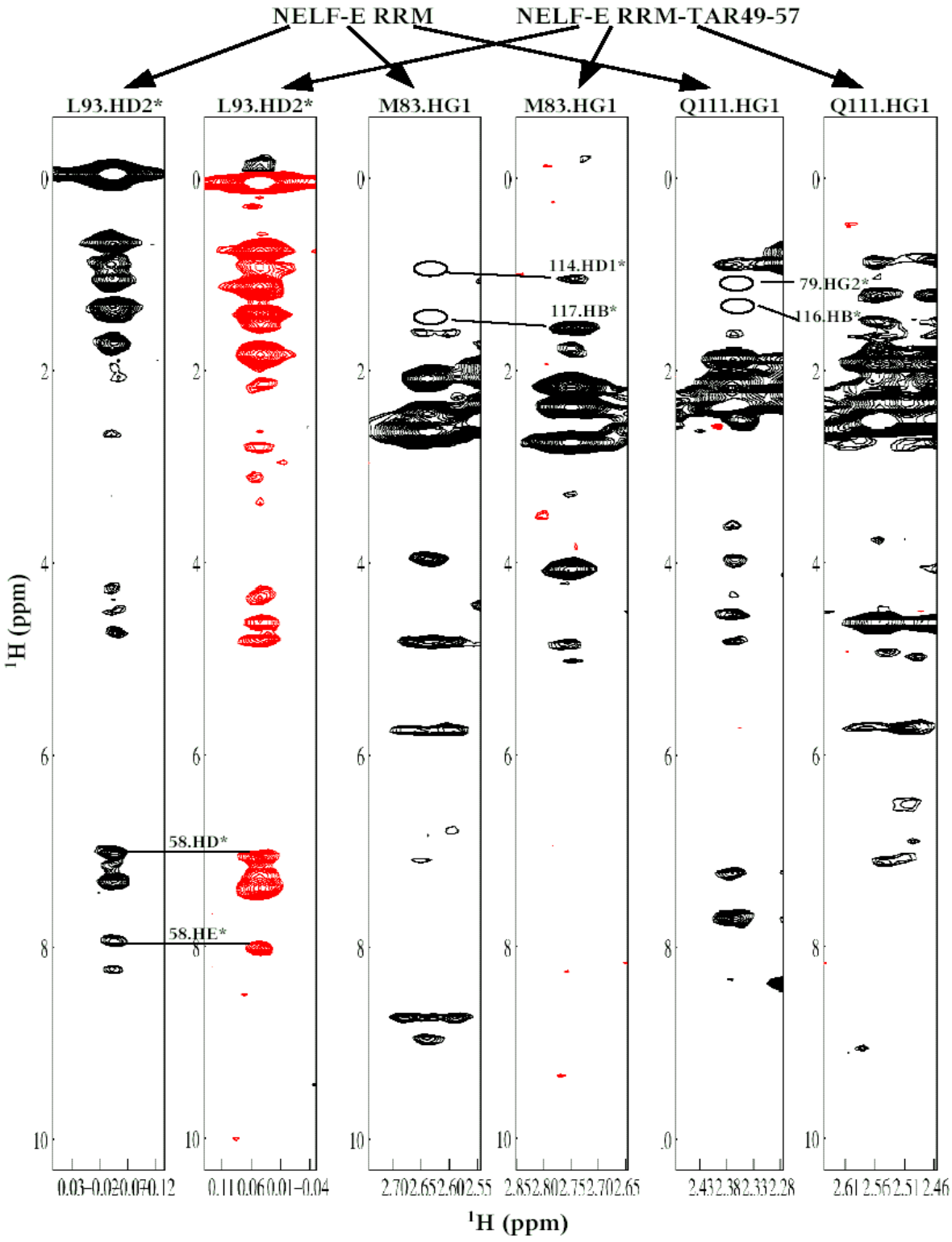


Fig. 4.30 Comparison of ^{13}C NOESY-HSQC spectra of free and RNA bound NELF-E RRM. Inter-residual NOEs observed between the carboxy terminus and around the loop β_3-α_2 region are labeled. Corresponding NOEs not present in the free NELF-E RRM are indicated with open circles.

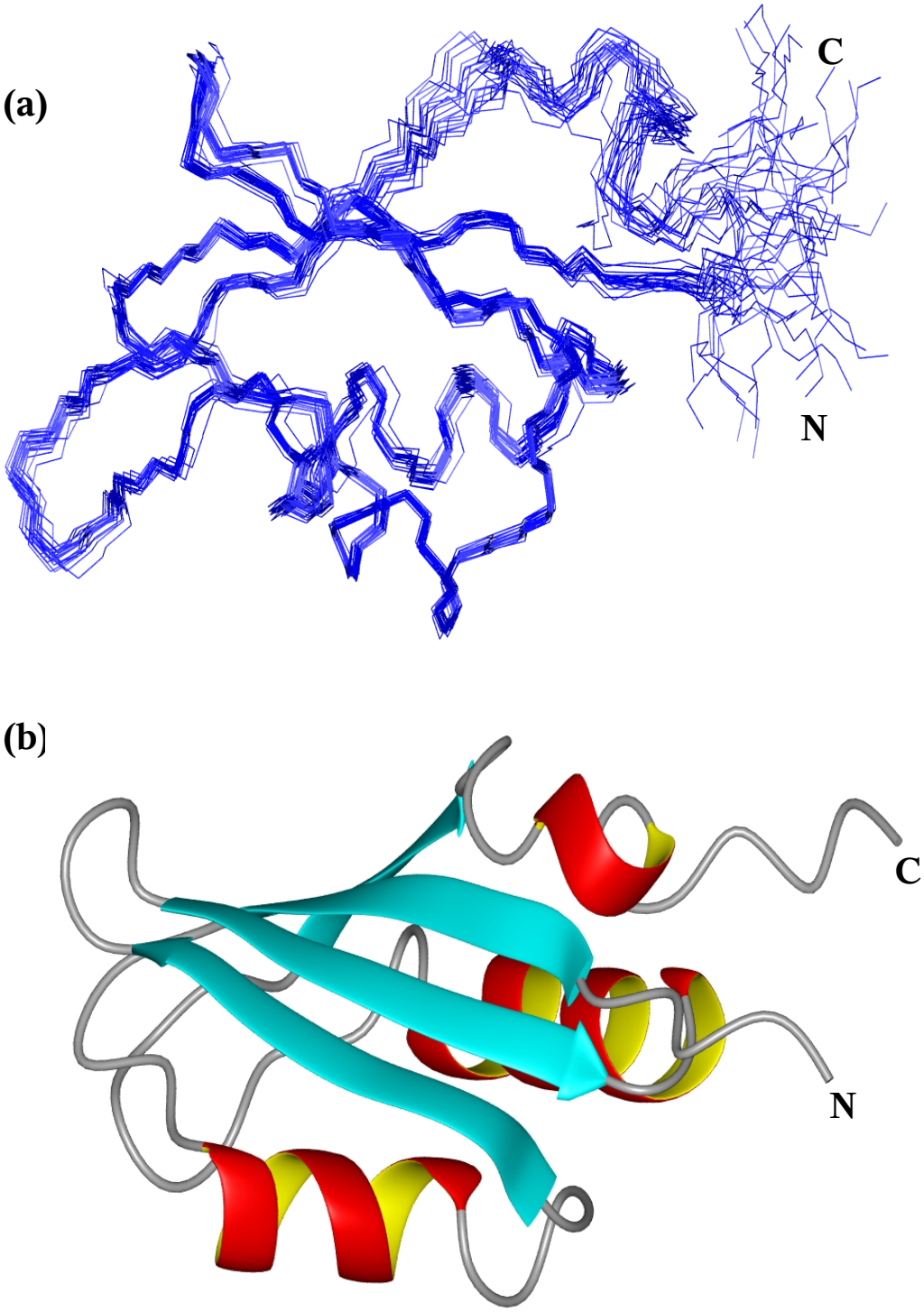


Fig. 4.31 Structure of NELF-E RRM in the complex with TAR49-57. (a) Overlay of the 20 structures (residues A35-S121) showing the lowest values of the target function excluding the database potential. **(b)** Ribbon diagram of the lowest energy structure. The figure is generated using MOLMOL (Koradi et al., 1996).

5 Discussion

5.1 HIV-1 Tat-Cys$^-$-TAR complex.

The HIV-1 Tat protein is required for virus production. Tat stimulates the elongation efficiency of Rpol II by hyperphosphorylation of its C-terminal domain through interaction with the p-TEFb and TAR RNA (Chapter 1.2.6). It has been suggested that compounds are able to bind TAR with high affinity and specificity thereby disrupting the Tat/TAR interaction could serve as potential drugs against HIV. Thus, structural details of the HIV-1 Tat/TAR complex are indispensable for drug discovery. Up to now the structural details of this important complex are not well defined at the atomic level. Biochemical identification reveals that the core and basic regions of Tat are required for high affinity and specificity to TAR RNA.

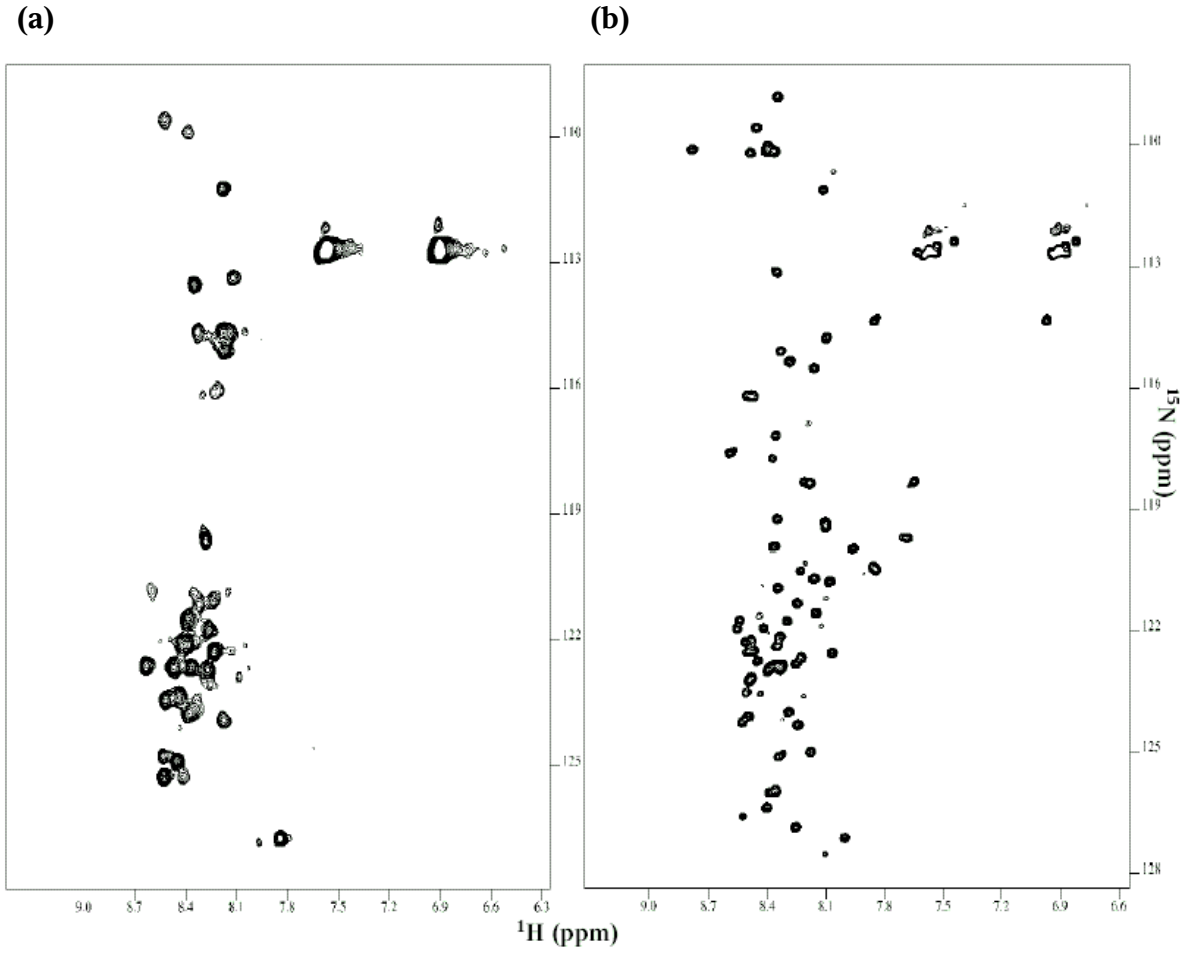


Fig. 5.1 Comparison of ^{1}H-^{15}N HSQC of wild type Tat and Tat-Cys$^-$. (a) ^{1}H-^{15}N HSQC of wild type Tat protein. **(b)**^{1}H-^{15}N HSQC of Tat-Cys$^-$ protein.

The wild type Tat protein has 7 cysteines in a span of 16 amino acids (cysteine rich region). These cysteines readily oxidise which leads to protein aggregation thus hampering the effort of protein purification on a large scale (Bayer et al., 1995). It is almost impossible to get sufficient concentrations of HIV-1 wild type Tat for structural studies. These cysteines are required for recruiting the p-TEFb onto elongation complex, but not needed for the binding of Tat to TAR RNA *in vitro* (Churcher et al., 1993; Fujinaga et al., 2002). Thus, mutation of all cysteines to serines or alanines leads to a more soluble mutant Tat protein, Tat-Cys$^-$, which is far easier to handle. ^{1}H-^{15}N HSQC spectra of wild type HIV-1 Tat protein and Tat-Cys$^-$ are shown in Fig. 5.1. Only few amide cross peaks were observed in ^{1}H-^{15}N HSQC spectra of wild type Tat protein. The extremely line broadening of peaks in the wild type Tat spectra is a clear indication of protein aggregation. Whereas in the ^{1}H-^{15}N HSQC spectra of Tat-Cys$^-$ almost all the amide cross peaks were present and showing sharp and intense signals indicating that Tat-Cys$^-$ exists as a monomer. Thus, Tat-Cys$^-$ was used for the binding studies with TAR RNA.

CD spectra of TAR RNA in the presence of Tat-Cys$^-$ showed a significant change at 265 nm (Fig. 4.4) indicating that Tat-Cys$^-$ induced a conformational change in TAR RNA upon binding. Varani and coworkers (Aboul-ela et al., 1995) showed that within free TAR RNA, bulge nucleotides appear to be at least partially stacked between two A-form helices. When a TAR RNA-Tat complex is formed with a peptidomimetic Tat, the bases in the bulge become unstacked and the two double stranded helices become coaxially stacked. Thus, the apparent net loss of stacking interactions is consistent with the observed decrease in the CD signal at 265 nm. Whereas the difference CD spectra of Tat-Cys$^-$ did not show any characteristics of secondary structure formation. However, some residual structure is observed in Tat-Cys$^-$ upon binding to TAR RNA (Fig. 4.4).

The amide cross peaks of the core and the basic region of Tat-Cys$^-$ showed either significant chemical shift changes or disappeared in ^{1}H-^{15}N HSQC spectra (Fig. 4.6) upon the addition of TAR RNA is indicating that these regions of Tat-Cys$^-$ are involved in TAR RNA binding. Upon the addition of Tat-Cys$^-$ to TAR RNA, the chemical shift changes were observed for the bases located in the bulge region of TAR RNA in ^{1}H-^{1}H TOCSY spectra (Fig. 4.7), which is a further indication of Tat-Cys$^-$ is binding to TAR RNA and able to induce a conformational change. The two base pairs above and below the bulge region of TAR RNA are significantly contributing to the recognition of Tat-Cys$^-$, which is in good agreement with biochemical data (Churcher et al., 1993). $\{^1$H$\}$-^{15}N steady state NOE of Tat-Cys$^-$ both in the presence and

absence of TAR RNA showed in the Fig. 4.8 is indicating that there is a change in the internal dynamics of Tat-Cys$^-$ upon binding to TAR RNA. Increased steady state NOE values of the core region of Tat-Cys$^-$ in the presence of TAR RNA indicate that backbone motions of the core region in the complex with TAR RNA are restricted up to certain extent. However, steady state NOE values of the core region of Tat-Cys$^-$, even in the complex with TAR RNA are lower than those of the structured proteins (Fig. 4.8). Biochemical analysis reveals that the core region of Tat is important for binding of TAR RNA specifically (Churcher et al., 1993), however, the core region remains unstructured even in the complex with TAR RNA. Probably the Tat protein adapts its conformation to the TAR RNA structure and the RNA folds around the Tat basic and core regions. The precise three dimensional structure of TAR RNA could be a major determinant of Tat/TAR recognition. The mutant of Tat basic region, KKKRKKKKK, showed the same affinity and specificity to TAR RNA as wild type Tat 49-58 peptide (Calnan et al., 1991a; Calnan et al., 1991b; Tan and Frankel, 1992). Only R52 is making a sequence specific contact to TAR RNA, but the over all charge needs to be maintained to provide the nonspecific electrostatic contacts and to increase the RNA binding affinity (Calnan et al., 1991b). Unlike HIV-1 Tat, mutation of single amino acid in the basic region of bovine immunodeficiency virus (BIV) Tat protein has a dramatic affect on binding to BIV TAR (Chen and Frankel, 1995; Carolina et al., 2001; Xie et al., 2004). Puglisi and coworkers (Puglisi et al., 1995) showed that in contrast to HIV-1 Tat, BIV Tat adopts a β- hairpin structure when it binds to BIV TAR. Thus, mutations in the BIV Tat basic region could affect the conformation required for the binding of BIV Tat to its target RNA leading to a decrease in affinity, whereas HIV-1 Tat allowed the mutations in basic region except for R52. This reveals that, HIV-1 Tat does not require a fixed structure to bind to TAR RNA. Therefore, it is tempting to speculate that HIV-1 Tat protein remains unstructured upon binding to its target RNA.

5.2 Analysis of sequence and structure of NELF-E RRM

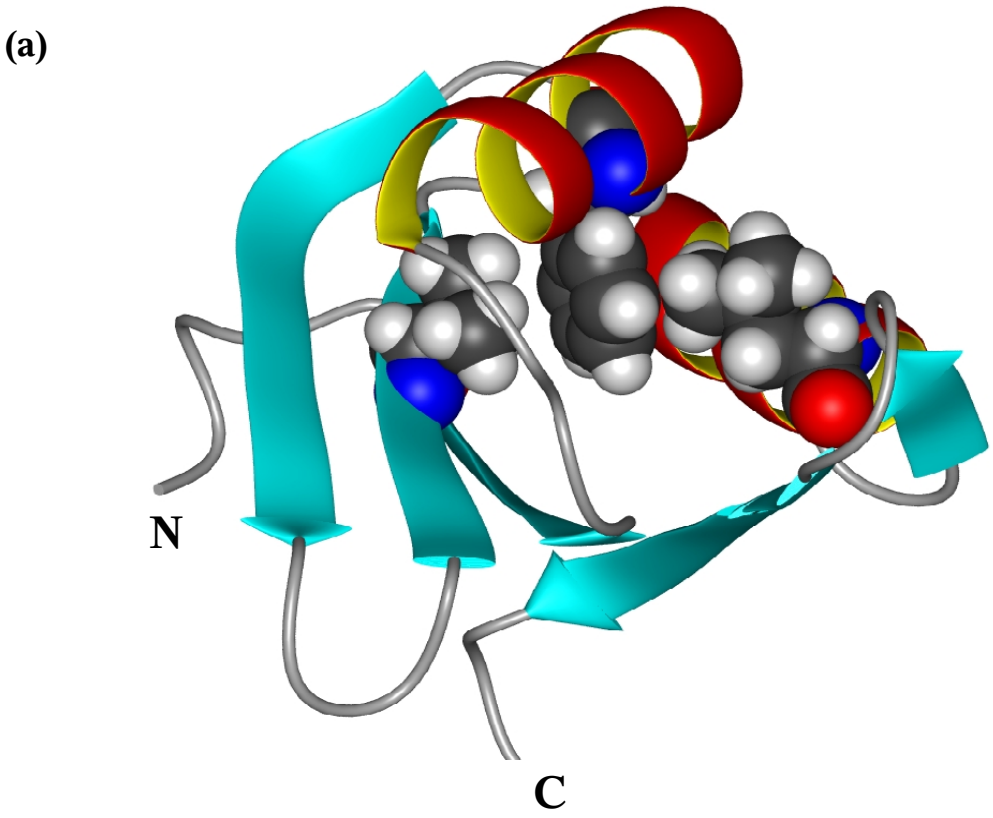
The elongation of transcription of HIV-1 RNA by cellular Rpol II is highly regulated by positive and negative factors. The detailed understanding of negative and positive transcription regulation of HIV-1 would help to design a new drug targets against HIV-1.

Thus, the structure of NELF-E RRM was determined using NMR spectroscopy. The $\{^1H\}$-^{15}N heteronuclear steady state NOE experiment at 14.1 T (Fig. 4.16) shows values around 0.6-0.8 for the region K38-R109, while outside of this region the heteronuclear NOE decreases towards the termini. Additionally, the assigned residues from the terminal regions show chemical shifts typically found for highly flexible polypeptides. This is characteristic for a compactly folded domain within the region K38-R109 and unstructured termini. Missing assignments of amide resonances can therefore be explained by conformational or solvent exchange. Due to the flexible character of the termini, residues M1-R34 and L114 -S121 were excluded from structure determination. The structure of the NELF-E RRM exhibits a βαββαβ topology with a four antiparallal β- sheet surface is packed against two α- helices. The two α-helices are oriented almost perpendicularly to each other. The loop between α_2 and β_4 forms a small β- hairpin structure

Sequence alignment of NELF-E RRM with other RRMs (Fig. 5.2), whose structures in complex with RNA are known showed that NELF-E RRM possesses a considerable structural similarity with other RRMs in spite of the low sequence homology (approximately 25%). However, the loops between the secondary structure elements of RRMs are usually disordered and of various lengths. It is assumed that the different specificities and binding affinities of RRMs are, among other factors, modulated by variations in these loop regions (Maris et al., 2005). In most of the cases loop between α_2 and β_4 adopts a small β hairpin structure. The ribonucleo protein (RNP) motifs are conserved among the RRMs (Fig. 5.2). The highly conserved aromatic amino acids in the RNP motifs are thought to be involved in stacking interaction with RNA bases .

Fig 5.2 Sequence alignment of RRMs from different proteins. Sequence alignment of NELF-E RRM with the RRMs from U1A spliceosomal protein (U1A, Allain et al., 1996), sex lethal protein (SxL, Handa et al., 1999), poly adenine binding protein (PABP, Deo et al., 1999), heteronuclear ribonucleo protein (hnRNP, Ding et al., 1999) and human homologs of *Drosophila* proteins (HuD, Wang et al., 2001) is performed using T-COFFEE programme (www.expasy.org). The conserved RNP motifs, hydrophobic and aromatic amino acids are shown in gray, blue and red colours, respectively.

The hydrophobic core of the protein is stabilised by the residues L42, V44, M49, L54, F58, F61, I64, L67, M69, A76, V78, Y80, L93, L103, and V105 which is evidenced by the inter-residual NOEs between the hydrophobic groups of these amino acids. Among the numerous hydrophobic contacts, F58 from the helix1 is involved in hydrophobic interaction with V78 from the central β strand (β_3) and L93 from helix2, thus F58 is sand-witched between V78 and L93 (Fig.7.2.a). Interestingly, these amino acids are conserved in all other known RRMs as well and adopt a very similar conformation (Fig. 7.2.b). It appears that these three amino acid side chains could play a crucial role in stabilising the RRM structure.


(a)
N
C

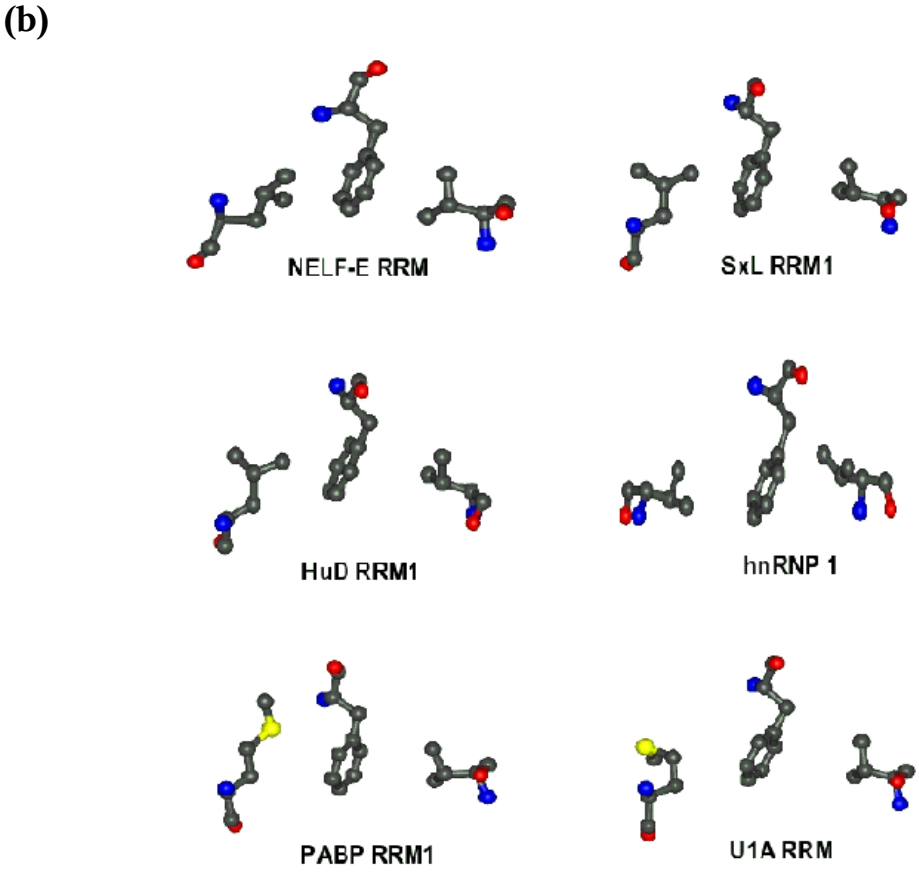

(b)
NELF-E RRM
SxL RRM1
HuD RRM1
hnRNP 1
PABP RRM1
U1A RRM

Fig 5.3 Hydrophobic interaction of the conserved amino acids in the RRM core. (a) Packing of F58, V78 and L93 amino acids are displayed as space-filled atoms in the ribbon representation of the NELF-E RRM structure. **(b)** These highly conserved hydrophobic amino acids from different RRMs are shown in ball & stick representation.

5.3 RNA binding studies on NELF-E RRM

It has been shown recently that NELF-E binds to a wide variety of RNAs, amongst them the HIV-1 TAR RNA (Yamaguchi et al., 2002). To obtain further details into the RNA recognition of NELF-E RRM, RNA binding studies were performed with various RNAs. ^{1}H-^{15}N HSQC experiments were used to monitor the interaction between NELF-E RRM and RNA. NELF-E RRM interacts with all RNAs used in this study. The binding NELF-E RRM to various RNAs by using always the same set of amide resonances (Fig. 4.21, 4.22, 4.23, 4.24, 4.25, and 4.26) indicating that it binds to RNAs in a very similar manner. However, NELF-E RRM binds to various RNAs with different affinities as indicated by different exchange behavior on NMR time scale (Fig.7.3). The ability of NELF-E RRM to bind different single stranded RNAs with high affinity (low μM range) is not surprising, since NELF is involved in the regulation of several genes other than the HIV-1 (Yamaguchi et al., 2002; Wu et al., 2003). Thus, NELF-E RRM showed a broad range of specificity to RNA. Among the RNA oligonucleotides used for the binding studies TAR49-57 showed the strongest chemical shift perturbations with slow exchange behavior on NMR time scale, which is amenable to further structural characterisation of NELF-E RRM in the presence of RNA.

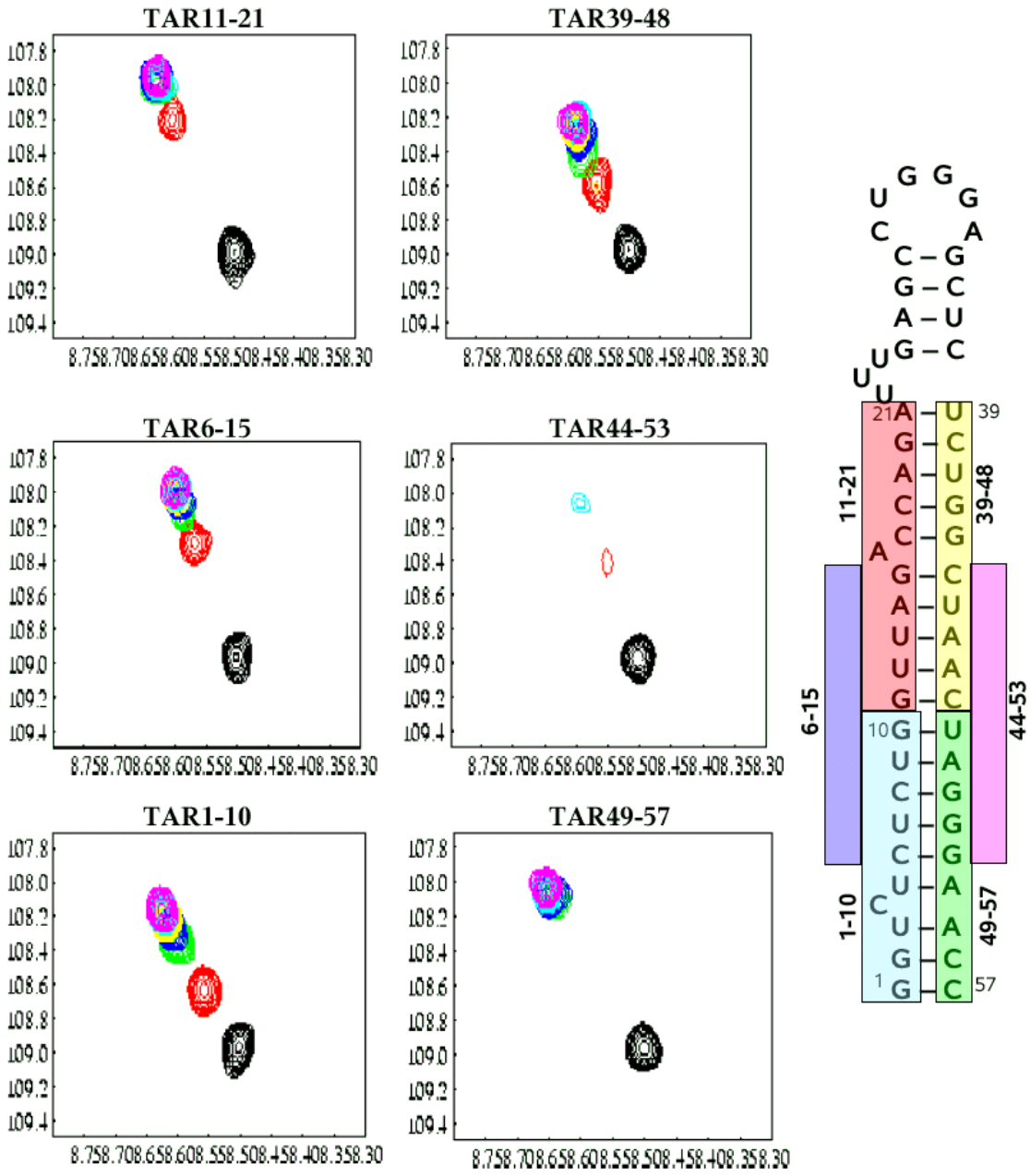


Fig. 5.4 Titration of NELF-E RRM with single stranded TAR RNA oligonucleotides. (a) Overlay of G39 amide cross peak in ^{1}H-^{15}N HSQC spectra at each titration step. **(b)** Sequence of the corresponding TAR RNA oligonucleotides are shown in different colours.

5.4 Mapping of RNA binding interface.

Mapping of the RNA binding interface on the ribbon diagram of NELF-E RRM and the comparing with other RNA binding interfaces of the known RRM-RNA complex structures reveals an intriguing differences (Fig. 7.4). Addition of RNA results in remarkable chemical shift changes for resonances located in the β strands indicating the typical binding of RNA to the RRM by stacking of bases onto the two conserved aromatic residues Y43 and F77. The large chemical shift changes observed upon the addition of various RNAs for the resonances located in the C-terminus of NELF-E RRM imply a structural change of this region which is highly flexible in free state. Structural changes of the carboxyl terminus are observed, e.g., in hnRNP1 RRM1, where the corresponding region is also unstructured in the free state and adopts a 3_{10} helix upon RNA binding (Maris et al., 2005). The additional β-strand of the NELF E RRM (T96-E97) does not exhibit significant chemical shift changes, indicating that this region is not involved in RNA recognition. This is in contrast to the RRM from TcUBP1, where large chemical shift changes of the resonances of the additional β-hairpin indicate interaction with RNA (Volpon et al., 2005). Unusual chemical shift changes are found for several residues in the loop preceding helix 2 (E81-E84, D87). This region is not involved in RNA binding in complexes with known structures (Allian, et al., 1996; Deo et al., 1999; Handa et al., 1999; Wang et al., 2001), and the distance between the RNA contact sites and helix 2 is probably too large for the induction of secondary chemical shift changes. A contribution of these large chemical shift changes observed in the loop preceding helix 2 and C-terminal region were revealed by the further structural characterisation of the NELF-E RRM:TAR49-57 complex.

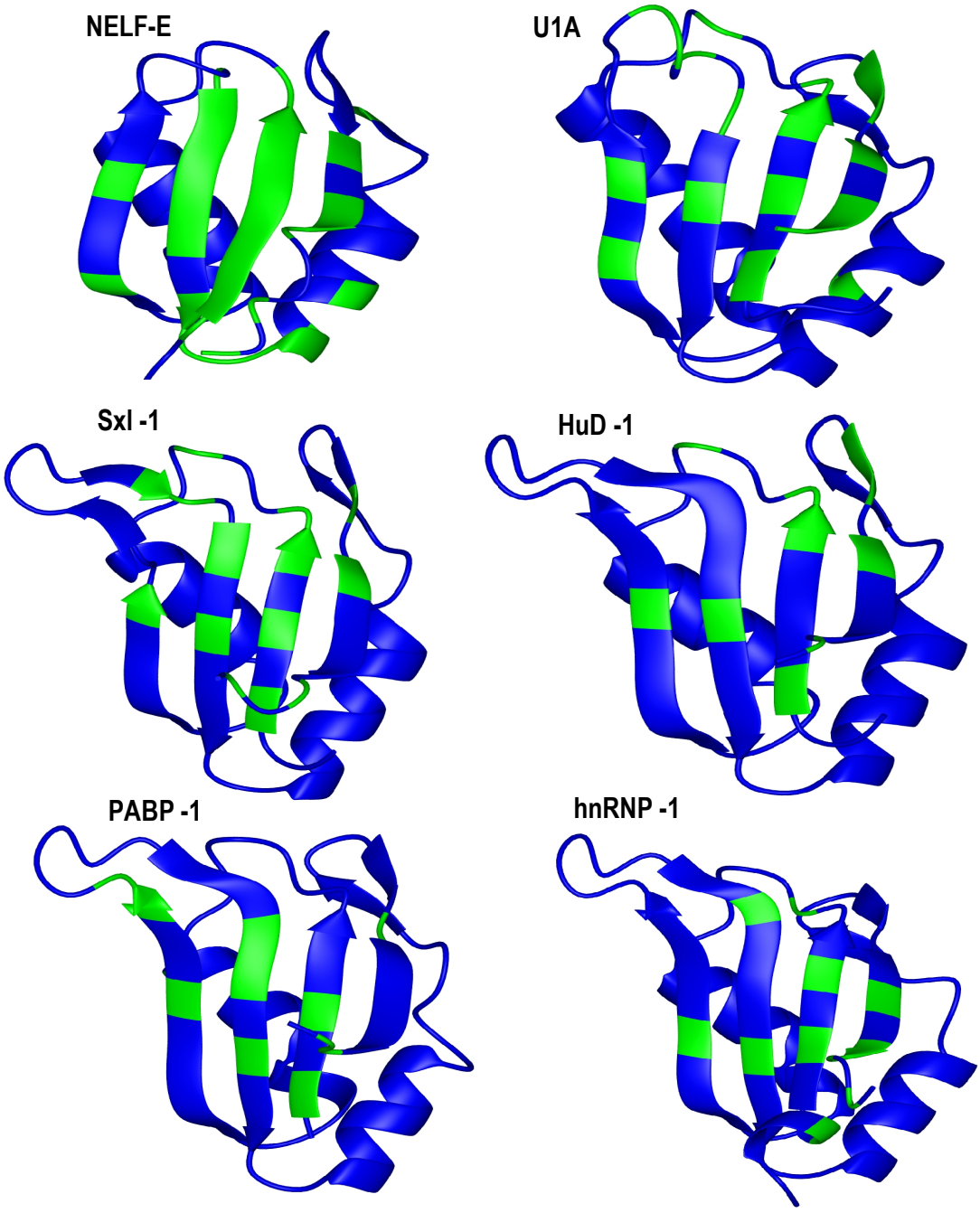


Fig. 5.5 Mapping of the RNA binding interface. Ribbon representation of RRMs whose structures in the complex with RNA are known. Residues involved in RNA binding are highlighted in green. For NELF-E RRM the amide chemical shift perturbation is used to map the RNA binding interface..

5.5 Characterisation of the RNA bound conformation of NELF-E RRM

In the RNA bound NELF-E RRM, 40 inter-residual NOEs were observed in between the C-terminal region of NELF-E RRM and the residues located around the loop between β_3 and α_2. The C-terminal region of NELF-E RRM in the RNA bound conformation adopts a small 3_{10} helix around the loop between β_3 and α_2 (Fig. 7.5) which is highly flexible in the free state.

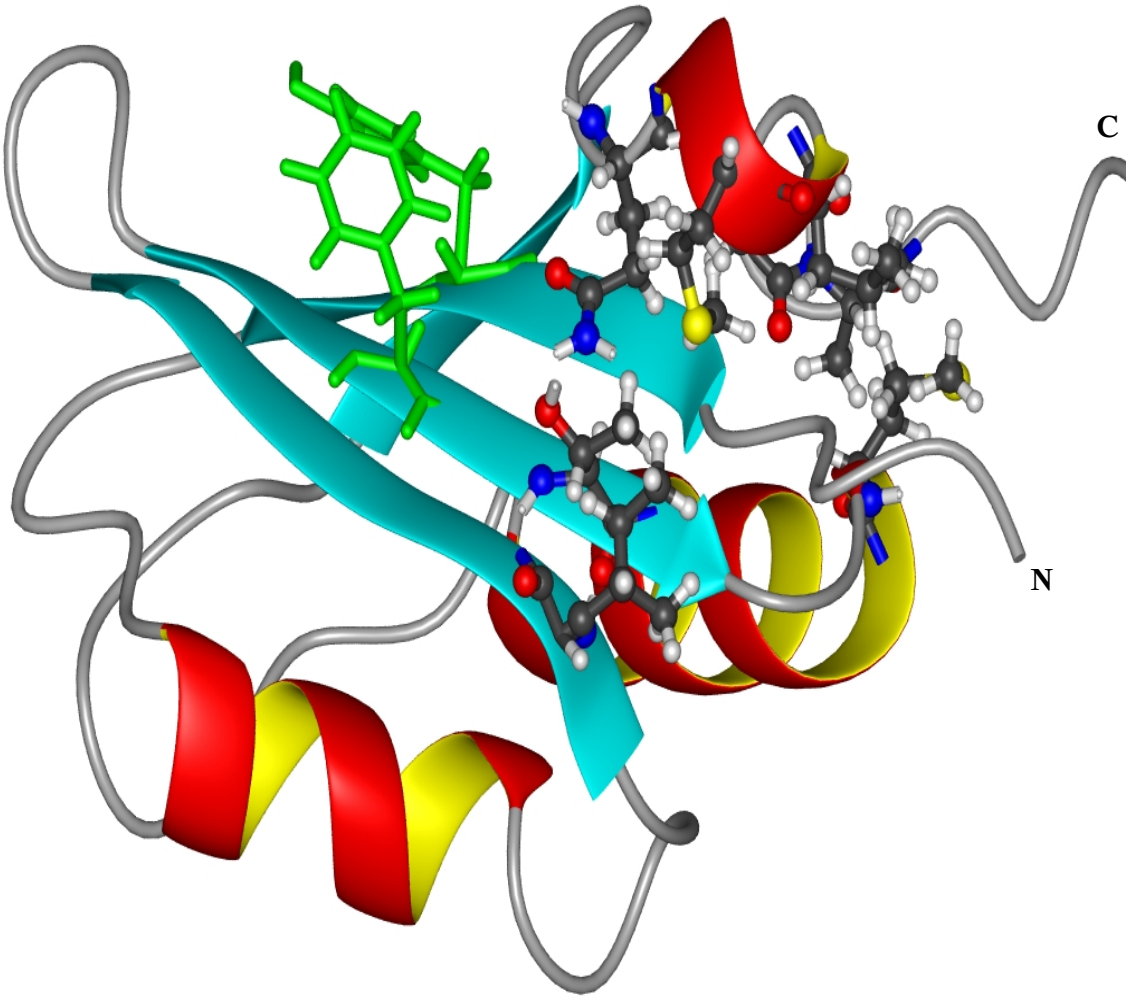


Fig. 5.6 Structure of the RNA bound conformation of NELF-E RRM. The residues involved in hydrophobic interactions to stabilise the C-terminal region of NELF-E RRM are shown in ball & stick representation. The highly conserved aromatic amino acids located in RNP1 and RNP2 are represented by sticks (green).

The C-terminus of NELF-E RRM is stabilised by the numerous hydrophobic contacts (I65, T79, M83, M113, L114, A116, A117, and T118) in the RNA bound conformation. The large

chemical shift changes observed in the loop preceding helix 2 and C-terminal regions are attributed to the conformational changes in these regions upon binding to RNA. Unfortunately, no intermolecular NOEs were observed for the 1:1 complex of NELF-E RRM and TAR49-57 which are crucial for the structure determination of the complex. The basic residues located C-terminal to β_4 (R109, K110) could be involved in electrostatic interaction with the phosphate backbone of the RNA to achieve high affinity. In the RNA bound conformation, the C-terminal region of NELF-E RRM is placed close to the conserved aromatic amino acids and is probably involved in RNA binding (Fig. 5.6). This structural rearrangements reinforces the concept of binding by induced fit. The C-terminal region of RRM from the U1A spliceosomal protein adopts an α-helix in both the free and RNA bound conformation (Allain et al., 1996). This region is involved in the RNA binding and contributing to the specific recognition of an RNA hairpin (Allain et al., 1997). However, Further structural studies on NELF-E RRM and its complex structure with RNA will contribute to understand how NELF-E RRM can bind a wide variety of RNAs with high affinity.

6 Summary

After HIV enters the host cell the viral RNA is reverse transcribed into double stranded DNA and then integrated into the host genome. At this step, transcription of HIV RNA by Rpol II is tightly regulated by several cellular factors including NELF, DSIF, and pTEFb, and the virus encoded Tat protein. The work presented in this thesis is mainly focussed on understanding the mechanism of termination and antitermination of HIV-1 transcription.

Some of the cysteines in the wild type HIV-1 Tat are required for the Zn^{2+} dependent interaction with pTEFb, but not needed for the binding of TAR RNA *in vitro*. These cysteines are readily oxidised and leading to the protein aggregation. Thus, a mutant, Tat-Cys$^-$, lacking all cysteines was used for binding studies with TAR RNA. ^{1}H-^{15}N HSQC and ^{1}H-^{1}H TOCSY spectra were measured to monitor the interaction between Tat-Cys$^-$ and TAR RNA. The results presented indicate Tat-Cys$^-$ is binding to TAR RNA around the bulge region and is able to induce a conformational change in TAR RNA. This is further supported by observing a change in the CD signal at 265 nm upon addition of Tat-Cys$^-$ to TAR RNA. $\{^1$H$\}$-^{15}N steady state NOE experiments showed that the core region of Tat remains unstructured even in the complex with TAR RNA. Based on the NMR and CD spectroscopic data presented in this thesis, and data published by others it is tempting to speculate that the core and basic regions of Tat-Cys$^-$ remain unstructured upon binding to TAR RNA.

Furthermore, the solution structure of the RNA binding domain of NELF-E, NELF-E RRM, was determined using multidimensional NMR spectroscopy. The data reveal that the structure of NELF-E RRM exhibits a βαββαβ topology. The atomic coordinates were deposited in the protein data bank (pdb) under the accession code 2BZ2. RNA binding studies were performed with TAR RNA and various oligoribonucleotides. NMR studies with NELF-E RRM:TAR complexes indicate NELF-E RRM binds to various RNAs in a very similar manner but with different affinities. Mapping of chemical shift perturbations on the structure of NELF-E RRM indicate that the RNA binding interface is mainly located on the β-sheet surface. Large chemical shift perturbations are observed for the residues located in the flexible C-terminal region of NELF-E RRM and in the loop between β_3 and α_2. Among the various RNAs tested, TAR49-57 displayed the highest affinity to NELF-E RRM. Further structural characterisation of the NELF-E RRM:TAR49-57 complex revealed that the C-terminal region of NEFL-E RRM adopts a small 3_{10} helix around the β_3-α_2 loop and is stabilised by several hydrophic

interactions. The C-terminal region of NELF-E RRM in the RNA bound conformation is very close to the RNA binding interface and could be involved in the RNA binding. However, further structural characterisation of NELF-E RRM in the complex with RNA will be needed to fully understand the mechanism of RNA recognition.

7 Zusammenfassung

Nachdem HIV in die Wirtszelle eingetreten ist, wird die virale RNA durch Reverse Transkription in doppelsträngige DNA umgewandelt und anschließend ins Wirtsgenom integriert. Ab diesem Zeitpunkt wird die Transkription der HIV RNA durch die Rpol II streng durch verschiedene zelluläre Faktoren, wie NELF, DSIF und pTEFb, sowie durch das virale Tat Protein reguliert. Die Ergebnisse, die in dieser Arbeit präsentiert werden, konzentrieren sich hauptsächlich auf das Verständnis des Mechanismus der Termination und Antitermination der HIV-1 Transkription.

Einige der Cysteine des Wildtyp Tat Proteins sind für die Zn^{2+}-abhängige Wechselwirkung mit pTEFb erforderlich. Allerdings werden sie für die Bindung von Tat an die TAR RNA *in vitro* nicht benötigt. Deswegen wurde eine Mutante, Tat-Cys^-, der alle Cysteine fehlen, für Bindungsstudien mit TAR RNA verwendet. Um die Wechselwirkung von Tat-Cys^- mit TAR zu analysieren, wurden ^1H-^{15}N HSQC und ^1H-^1H TOCSY Spektren gemessen. Die dargestellten Ergebnisse deuten darauf hin, dass Tat-Cys^- an die Bulge Region (Ausbuchtung) der TAR RNA bindet, und dass das Protein in der Lage ist, Konformationsänderungen in der TAR RNA herbeizuführen. Dieses Ergebnis wird durch den Befund einer Änderung des CD-Signals bei 265 nm nach Zugabe von Tat-Cys^- zur TAR RNA weiter unterstützt. $\{^1H\}$-^{15}N *steady state* NOE Experimente zeigen, dass die Kernregion (core) von Tat auch im Komplex mit der TAR RNA unstrukturiert bleibt. Auf der Grundlage der in dieser Arbeit gezeigten Ergebnisse und der Ergebnisse anderer Gruppen kann man annehmen, dass die Kernregion und die basische Region von Tat-Cys^- nach der Bindung an TAR RNA unstrukturiert bleiben.

Darüber hinaus wurde die Struktur der RNA-bindenden Domäne von NELF-E, NELF-E RRM, mit Hilfe multidimensionaler NMR Spektroskopie in Lösung bestimmt. Die Ergebnisse zeigen, dass die Struktur von NELF-E RRM eine βαββαβ Topologie aufweist. Die Atom-Koordinaten wurden in der Proteindatenbank (pdb) unter der Eintragsnummer 2BZ2 hinterlegt. Es wurden RNA Bindungsstudien mit der TAR RNA und verschiedenen Oligoribonukleotiden durchgeführt. NMR Studien mit NELF-E RRM:TAR Komplexen zeigen, dass NELF-E RRM verschiedene RNAs in ähnlicher Weise, jedoch mit unterschiedlichen Affinitäten, bindet. Die Kartierung der Änderung der chemischen Verschiebungen auf der Struktur von NELF-E RRM

zeigen, dass sich die RNA Bindungsfläche hauptsächlich auf der Oberfläche des β-Faltblatts befindet. Große Änderungen der chemischen Verschiebungen können für die Reste in der flexiblen C-terminalen Region von NELF-E RRM und in der Schleife zwischen β_3 und α_2 beobachtet werden. Unter den verschiedenen getesteten RNAs besaß TAR49-57 die höchste Affinität für NELF-E RRM. Zusätzliche strukturelle Charakterisierungen zeigten, dass die C-terminale Region von NELF-E RRM eine kleine 3_{10} Helix in der Region der β_3-α_2-Schleife ausbildet und durch verschiedene hydrophobe Wechselwirkungen stabilisiert wird. Die C-terminale Region von NELF-E RRM befindet sich in der RNA-gebundenen Form sehr nahe an der RNA Bindungsfläche und könnte in der RNA Bindung beteiligt sein. Allerdings ist eine weitere Charakterisierung von NELF-E RRM im Komplex mit RNA notwendig um den Mechanismus der RNA Erkennung vollständig aufzuklären.

8 Abbreviations

ε	molar extinction coefficient
1D	one dimensional
2D	two dimensional
3D	three dimensional
AIDS	acquired immunodeficiency syndrome
APS	ammonium peroxy disulfate
BIV	bovine immunodeficiency virus
CD	circular dichroism
CDK9	cyclin dependent kinase 9
CTD	carboxy terminal domain
DRB	5,6-dichloro-1-β-D-ribifuranosylbenzimidazole
DSIF	DRB sensitivity inducing factor
DSS	2,2-dimethyl-2-silapentanesulfonate
E. coli	Escherichia coli
FID	free induction decay
FPLC	fast perfomance liquid chromatography
HDAg	hepatitis delta antigen
HIV	human immunodeficiency virus
hnRNP	heteronuclear ribonucleo protein
HSQC	heteronulcear single quantum coherence
HuD	human homologs of *Drosophila* proteins
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo dalton
LTR	long terminal repeat
MEXICO	measurement of exchange rates in isotopically labeled compounds
MRW	mean residue ellipticity
NELF	negative elongation factor
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser enhancement spectroscopy
OD	optical density

PABP	poly adenine binding protein
PDB	protein data bank
PMSF	phenylmethylsulfonylflouride
p-TEFb	positive transcription elongation factor b
RDC	residual dipolar coupling
RMSD	root mean square deviation
RNP	ribonucleo protein
Rpol II	RNA polymerase II
RRE	Rev response element
RRM	RNA recognition motif
RT	reverse transcriptase
SA	simulated annealing
SAR	structure activity relationship
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sxl	sex lethal protein
TAR	transactivation response
Tat	transactivator of transcription
TD	time domain
TOCSY	total correlated spectroscopy
TPPI	time proportional phase incrimination
TS2	trace element solution 2

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10 Appendex

10.1 Nucleotide sequence of Tat-Cys$^-$

```
gtatacgagctgggccagctgggcttgtagcttggcaccttggtggggcca

H  M  L  D  P  V  D  P  N  I  E  P  W  N  H  P  G

agagtcggcttttggcgaaggttggcacgagtgcgattctttaggcgaatg
S  Q  P  K  T  A  S  N  R  A  H  A  K  K  S  A  Y

gtgagggtccaacgaaagtagtggtttccagacccataaagcatgccagca
H  S  Q  V  A  F  I  T  K  G  L  G  I  S  Y  G  R

ttctttgcagcagtcgcagcagcaggcagagtcccaccagtttgagtggtt
K  K  R  R  Q  R  R  R  P  S  Q  G  G  Q  T  H  Q

ctgggctagggctttgttggcagaagagttggggcgccactgggctgacca
D  P  I  P  K  Q  P  S  S  Q  P  R  G  D  P  T  G

ggctttcttattattcgactcg
P  K  E  *  *  A  E  -
```

10.2 Nucleotide sequence of NELF-E RRM

```
atgggcagcagccatcatcatcatcacagcagcggcctggtgccgcgcggcagc
  M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S

catatgggtccgttccgccgttctgattccttccctgaacgtcgcgccccgcgtaaa
  H  M  G  P  F  R  R  S  D  S  F  P  E  R  R  A  P  R  K

gggaacacattatatgtttatggcgaagacatgaccccgactcttttgcgcggtgcc
  G  N  T  L  Y  V  Y  G  E  D  M  T  P  T  L  L  R  G  A

ttctccccccttcggcaacattatcgatctgtctatggaccctccgcgtaattgcgcg
  F  S  P  F  G  N  I  I  D  L  S  M  D  P  P  R  N  C  A

tttgtcacctacgaaaaaatggaaagcgcagatcaagctgtggccgaactgaatgga
  F  V  T  Y  E  K  M  E  S  A  D  Q  A  V  A  E  L  N  G

acgcaggttgagtcggtccagctcaaagtgaacattgcgcgcaagcagccgatgctg
  T  Q  V  E  S  V  Q  L  K  V  N  I  A  R  K  Q  P  M  L

gatgccgctactggcaagtcttag
  D  A  A  T  G  K  S  -
```

10.3 Chemical shifts of NELF-E RRM at pH 6.9 and 25 °C

1	MET	HA	C	-100.000	-100.000
1	MET	HB1	C	-100.000	-100.000
1	MET	HB2	C	-100.000	-100.000
1	MET	HG1	C	-100.000	-100.000
1	MET	HG2	C	-100.000	-100.000
1	MET	HE*	C	-100.000	-100.000
2	GLY	HN	N	8.181	109.994
2	GLY	HA1	C	-100.000	-100.000
2	GLY	HA2	C	-100.000	-100.000
3	SER	HN	N	-100.000	-100.000
3	SER	HA	C	-100.000	-100.000
3	SER	HB1	C	-100.000	-100.000
3	SER	HB2	C	-100.000	-100.000
4	SER	HN	N	-100.000	-100.000
4	SER	HA	C	-100.000	-100.000
4	SER	HB1	C	-100.000	-100.000
4	SER	HB2	C	-100.000	-100.000
5	HIS	HN	N	-100.000	-100.000
5	HIS	HA	C	-100.000	-100.000
5	HIS	HB1	C	-100.000	-100.000
5	HIS	HB2	C	-100.000	-100.000
5	HIS	HD2	C	-100.000	-100.000
5	HIS	HE1	C	-100.000	-100.000
6	HIS	HN	N	-100.000	-100.000
6	HIS	HA	C	-100.000	-100.000
6	HIS	HB1	C	-100.000	-100.000
6	HIS	HB2	C	-100.000	-100.000
6	HIS	HD2	C	-100.000	-100.000
6	HIS	HE1	C	-100.000	-100.000
7	HIS	HN	N	-100.000	-100.000
7	HIS	HA	C	-100.000	-100.000
7	HIS	HB1	C	-100.000	-100.000
7	HIS	HB2	C	-100.000	-100.000
7	HIS	HD2	C	-100.000	-100.000
7	HIS	HE1	C	-100.000	-100.000
8	HIS	HN	N	-100.000	-100.000
8	HIS	HA	C	-100.000	-100.000
8	HIS	HB1	C	-100.000	-100.000
8	HIS	HB2	C	-100.000	-100.000
8	HIS	HD2	C	-100.000	-100.000
8	HIS	HE1	C	-100.000	-100.000
9	HIS	HN	N	-100.000	-100.000
9	HIS	HA	C	-100.000	-100.000
9	HIS	HB1	C	-100.000	-100.000
9	HIS	HB2	C	-100.000	-100.000
9	HIS	HD2	C	-100.000	-100.000
9	HIS	HE1	C	-100.000	-100.000
10	HIS	HN	N	-100.000	-100.000
10	HIS	HA	C	-100.000	-100.000
10	HIS	HB1	C	-100.000	-100.000
10	HIS	HB2	C	-100.000	-100.000
10	HIS	HD2	C	-100.000	-100.000
10	HIS	HE1	C	-100.000	-100.000
11	SER	HN	N	-100.000	-100.000
11	SER	HA	C	-100.000	-100.000
11	SER	HB1	C	-100.000	-100.000
11	SER	HB2	C	-100.000	-100.000
12	SER	HN	N	-100.000	-100.000
12	SER	HA	C	-100.000	-100.000
12	SER	HB1	C	-100.000	-100.000

12	SER	HB2	C	-100.000	-100.000
13	GLY	HN	N	8.398	110.560
13	GLY	HA*	C	4.403	45.420
14	LEU	HN	N	8.055	121.538
14	LEU	HA	C	4.315	55.348
14	LEU	HB*	C	1.605	42.563
14	LEU	HG	C	1.624	27.022
14	LEU	HD1*	C	0.925	24.854
14	LEU	HD2*	C	0.870	23.710
15	VAL	HN	N	8.111	122.628
15	VAL	HA	C	4.415	59.842
15	VAL	HB	C	2.062	32.798
15	VAL	HG1*	C	0.947	21.215
15	VAL	HG2*	C	0.947	20.539
16	PRO	HA	C	4.412	63.135
16	PRO	HB1	C	2.300	32.306
16	PRO	HB2	C	1.909	32.306
16	PRO	HG1	C	2.072	27.375
16	PRO	HG2	C	1.977	27.306
16	PRO	HD1	C	3.904	51.205
16	PRO	HD2	C	3.698	51.205
17	ARG	HN	N	8.505	122.047
17	ARG	HA	C	4.315	56.417
17	ARG	HB1	C	1.809	30.860
17	ARG	HB2	C	1.883	30.906
17	ARG	HG*	C	1.659	27.111
17	ARG	HD*	C	3.213	43.350
17	ARG	HE1	N	-100.000	-100.000
18	GLY	HN	N	8.500	110.371
18	GLY	HA1	C	-100.000	43.338
18	GLY	HA2	C	-100.000	-100.000
19	SER	HN	N	-100.000	-100.000
19	SER	HA	C	-100.000	-100.000
19	SER	HB1	C	-100.000	-100.000
19	SER	HB2	C	-100.000	-100.000
20	HIS	HN	N	-100.000	-100.000
20	HIS	HA	C	-100.000	-100.000
20	HIS	HB1	C	-100.000	-100.000
20	HIS	HB2	C	-100.000	-100.000
20	HIS	HD2	C	-100.000	-100.000
20	HIS	HE1	C	-100.000	-100.000
21	MET	HN	N	-100.000	-100.000
21	MET	HA	C	-100.000	55.265
21	MET	HB1	C	-100.000	31.965
21	MET	HB2	C	-100.000	-100.000
21	MET	HG1	C	-100.000	33.200
21	MET	HG2	C	-100.000	-100.000
21	MET	HE*	C	-100.000	-100.000
22	GLY	HN	N	8.025	110.223
22	GLY	HA1	C	-100.000	-100.000
22	GLY	HA2	C	-100.000	-100.000
23	PRO	HA	C	-100.000	63.318
23	PRO	HB1	C	-100.000	32.072
23	PRO	HB2	C	-100.000	-100.000
23	PRO	HG1	C	-100.000	27.022
23	PRO	HG2	C	-100.000	-100.000
23	PRO	HD1	C	-100.000	49.881
23	PRO	HD2	C	-100.000	-100.000
24	PHE	HN	N	8.271	120.294
24	PHE	HA	C	4.598	57.810
24	PHE	HB*	C	3.082	39.288
24	PHE	HD*	C	-100.000	-100.000
24	PHE	HE*	C	-100.000	-100.000
24	PHE	HZ	C	-100.000	-100.000
25	ARG	HN	N	8.358	122.638
25	ARG	HA	C	4.373	56.418

25	ARG	HB*	C	1.967	30.778
25	ARG	HG1	C	-100.000	-100.000
25	ARG	HG2	C	-100.000	-100.000
25	ARG	HD1	C	-100.000	-100.000
25	ARG	HD2	C	-100.000	-100.000
25	ARG	HE1	N	-100.000	-100.000
26	ARG	HN	N	8.301	122.139
26	ARG	HA	C	4.324	56.714
26	ARG	HB*	C	2.016	30.461
26	ARG	HG1	C	-100.000	-100.000
26	ARG	HG2	C	-100.000	-100.000
26	ARG	HD1	C	-100.000	-100.000
26	ARG	HD2	C	-100.000	-100.000
26	ARG	HE1	N	-100.000	-100.000
27	SER	HN	N	8.365	117.370
27	SER	HA	C	-100.000	58.525
27	SER	HB1	C	-100.000	63.794
27	SER	HB2	C	-100.000	-100.000
28	ASP	HN	N	-100.000	-100.000
28	ASP	HA	C	4.422	54.472
28	ASP	HB1	C	2.684	41.139
29	SER	HN	N	8.040	115.096
29	SER	HA	C	4.479	58.431
29	SER	HB*	C	3.878	64.107
30	PHE	HN	N	8.201	122.335
30	PHE	HA	C	-100.000	56.068
30	PHE	HB1	C	-100.000	38.961
30	PHE	HB2	C	-100.000	-100.000
30	PHE	HD*	C	-100.000	-100.000
30	PHE	HE*	C	-100.000	-100.000
30	PHE	HZ	C	-100.000	-100.000
31	PRO	HA	C	-100.000	63.700
31	PRO	HB1	C	-100.000	32.148
31	PRO	HB2	C	-100.000	-100.000
31	PRO	HG1	C	-100.000	27.464
31	PRO	HG2	C	-100.000	-100.000
31	PRO	HD1	C	-100.000	50.764
31	PRO	HD2	C	-100.000	-100.000
32	GLU	HN	N	8.619	120.796
32	GLU	HA	C	-100.000	56.920
32	GLU	HB1	C	-100.000	30.050
32	GLU	HB2	C	-100.000	-100.000
32	GLU	HG1	C	-100.000	-100.000
32	GLU	HG2	C	-100.000	-100.000
33	ARG	HN	N	-100.000	-100.000
33	ARG	HA	C	-100.000	57.105
33	ARG	HB1	C	-100.000	30.754
33	ARG	HB2	C	-100.000	-100.000
33	ARG	HG1	C	-100.000	-100.000
33	ARG	HG2	C	-100.000	-100.000
33	ARG	HD1	C	-100.000	-100.000
33	ARG	HD2	C	-100.000	-100.000
33	ARG	HE1	N	-100.000	-100.000
34	ARG	HN	N	8.439	123.268
34	ARG	HA	C	-100.000	55.666
34	ARG	HB1	C	-100.000	31.052
34	ARG	HB2	C	-100.000	-100.000
34	ARG	HG1	C	-100.000	-100.000
34	ARG	HG2	C	-100.000	-100.000
34	ARG	HD1	C	-100.000	-100.000
34	ARG	HD2	C	-100.000	-100.000
34	ARG	HE1	N	-100.000	-100.000
35	ALA	HN	N	8.304	126.499
35	ALA	HA	C	-100.000	50.486
35	ALA	HB*	C	-100.000	18.435
36	PRO	HA	C	4.392	62.943

36	PRO	HB1	C	2.304	32.318
36	PRO	HB2	C	1.899	32.318
36	PRO	HG*	C	2.027	27.464
36	PRO	HD1	C	3.821	50.675
36	PRO	HD2	C	3.625	50.675
37	ARG	HN	N	8.455	121.140
37	ARG	HA	C	4.335	56.190
37	ARG	HB*	C	1.822	30.906
37	ARG	HG*	C	1.645	27.375
37	ARG	HD*	C	3.222	43.527
37	ARG	HE1	N	-100.000	-100.000
38	LYS	HN	N	8.214	121.235
38	LYS	HA	C	4.540	55.491
38	LYS	HB1	C	1.918	34.348
38	LYS	HB2	C	1.747	34.348
38	LYS	HG1	C	1.703	29.140
38	LYS	HG2	C	1.767	29.140
38	LYS	HD*	C	1.449	24.728
38	LYS	HE1	C	3.022	42.237
38	LYS	HE2	C	2.990	42.237
39	GLY	HN	N	8.531	108.917
39	GLY	HA*	C	4.052	45.679
40	ASN	HN	N	8.316	116.038
40	ASN	HA	C	4.793	53.950
40	ASN	HB1	C	3.022	38.384
40	ASN	HB2	C	2.817	38.384
40	ASN	HD21	N	7.234	111.650
40	ASN	HD22	N	6.633	111.637
41	THR	HN	N	8.770	118.175
41	THR	HA	C	5.244	62.890
41	THR	HB	C	3.932	70.430
41	THR	HG2*	C	1.186	22.774
42	LEU	HN	N	9.736	126.985
42	LEU	HA	C	5.019	53.338
42	LEU	HB1	C	1.634	43.937
42	LEU	HB2	C	1.362	43.937
42	LEU	HG	C	1.833	27.152
42	LEU	HD1*	C	1.089	27.568
42	LEU	HD2*	C	0.968	25.852
43	TYR	HN	N	9.196	123.755
43	TYR	HA	C	4.637	56.878
43	TYR	HB1	C	2.896	40.205
43	TYR	HB2	C	2.795	40.205
43	TYR	HD*	C	6.880	133.330
43	TYR	HE*	C	6.770	135.706
44	VAL	HN	N	8.205	126.894
44	VAL	HA	C	4.833	59.568
44	VAL	HB	C	1.488	34.514
44	VAL	HG1*	C	0.837	22.462
44	VAL	HG2*	C	0.564	22.098
45	TYR	HN	N	9.137	126.298
45	TYR	HA	C	5.078	55.960
45	TYR	HB*	C	2.843	42.177
45	TYR	HD*	C	7.097	133.770
45	TYR	HE*	C	6.880	135.960
46	GLY	HN	N	7.538	113.616
46	GLY	HA*	C	3.835	45.665
47	GLU	HN	N	8.764	124.104
47	GLU	HA	C	4.002	56.801
47	GLU	HB1	C	1.854	30.164
47	GLU	HB2	C	1.931	30.325
47	GLU	HG1	C	2.259	36.822
47	GLU	HG2	C	2.183	36.822
48	ASP	HN	N	8.512	117.116
48	ASP	HA	C	4.207	55.378
48	ASP	HB1	C	2.861	39.288

48	ASP	HB2	C	2.675	39.214
49	MET	HN	N	8.173	114.545
49	MET	HA	C	4.080	57.575
49	MET	HB1	C	2.035	34.695
49	MET	HB2	C	1.448	34.695
49	MET	HG1	C	2.248	30.820
49	MET	HG2	C	1.778	30.820
49	MET	HE*	C	1.624	14.836
50	THR	HN	N	6.458	108.096
50	THR	HA	C	4.964	57.911
50	THR	HB	C	4.655	70.292
50	THR	HG2*	C	1.236	21.631
51	PRO	HA	C	4.226	65.845
51	PRO	HB1	C	2.427	31.744
51	PRO	HB2	C	2.072	31.788
51	PRO	HG1	C	2.273	28.081
51	PRO	HG2	C	2.125	28.081
51	PRO	HD1	C	4.087	50.765
51	PRO	HD2	C	3.834	50.765
52	THR	HN	N	7.607	111.569
52	THR	HA	C	3.884	66.362
52	THR	HB	C	4.305	68.570
52	THR	HG2*	C	1.253	21.784
53	LEU	HN	N	7.774	125.426
53	LEU	HA	C	4.080	58.428
53	LEU	HB1	C	1.909	43.048
53	LEU	HB2	C	1.703	43.113
53	LEU	HG	C	1.625	27.516
53	LEU	HD1*	C	0.979	26.088
53	LEU	HD2*	C	0.881	25.593
54	LEU	HN	N	7.850	117.277
54	LEU	HA	C	4.197	57.858
54	LEU	HB1	C	1.931	42.489
54	LEU	HB2	C	1.176	42.593
54	LEU	HG	C	1.745	27.308
54	LEU	HD1*	C	0.903	23.294
54	LEU	HD2*	C	0.651	26.528
55	ARG	HN	N	8.952	119.998
55	ARG	HA	C	3.816	60.927
55	ARG	HB1	C	1.889	29.504
55	ARG	HB2	C	1.931	29.596
55	ARG	HG1	C	1.756	28.972
55	ARG	HG2	C	1.581	28.920
55	ARG	HD1	C	3.254	42.997
55	ARG	HD2	C	3.156	42.997
55	ARG	HE1	N	-100.000	-100.000
56	GLY	HN	N	8.147	128.829
56	GLY	HA1	C	4.061	47.349
56	GLY	HA2	C	3.861	47.349
57	ALA	HN	N	7.793	120.204
57	ALA	HA	C	4.276	53.720
57	ALA	HB*	C	1.341	19.095
58	PHE	HN	N	8.539	110.237
58	PHE	HA	C	4.755	60.390
58	PHE	HB1	C	3.285	38.470
58	PHE	HB2	C	3.033	38.470
58	PHE	HD*	C	7.954	132.340
58	PHE	HE*	C	7.054	-100.000
58	PHE	HZ	C	-100.000	-100.000
59	SER	HN	N	8.601	122.951
59	SER	HA	C	4.751	63.564
59	SER	HB*	C	4.266	62.863
60	PRO	HA	C	4.197	65.683
60	PRO	HB1	C	2.026	31.457
60	PRO	HB2	C	0.519	31.457
60	PRO	HG*	C	1.767	28.140

60	PRO	HD1	C	3.801	52.055
60	PRO	HD2	C	3.090	52.055
61	PHE	HN	N	6.785	109.617
61	PHE	HA	C	4.403	58.379
61	PHE	HB1	C	3.395	39.015
61	PHE	HB2	C	2.842	39.015
61	PHE	HD*	C	7.250	130.900
61	PHE	HE*	C	-100.000	-100.000
61	PHE	HZ	C	-100.000	-100.000
62	GLY	HN	N	7.737	128.459
62	GLY	HA1	C	4.221	45.269
62	GLY	HA2	C	4.007	45.269
63	ASN	HN	N	8.455	116.806
63	ASN	HA	C	4.823	53.204
63	ASN	HB*	C	2.867	38.880
63	ASN	HD21	N	7.774	112.606
63	ASN	HD22	N	6.880	112.606
64	ILE	HN	N	8.733	128.129
64	ILE	HA	C	3.982	62.016
64	ILE	HB	C	1.703	38.768
64	ILE	HG11	C	1.865	27.984
64	ILE	HG12	C	0.247	27.984
64	ILE	HG2*	C	0.629	17.679
64	ILE	HD1*	C	0.794	14.196
65	ILE	HN	N	8.772	124.854
65	ILE	HA	C	4.373	61.599
65	ILE	HB	C	1.869	38.505
65	ILE	HG11	C	1.122	26.736
65	ILE	HG12	C	0.814	26.736
65	ILE	HG2*	C	0.881	17.835
65	ILE	HD1*	C	0.794	13.208
66	ASP	HN	N	7.412	118.877
66	ASP	HA	C	4.657	55.240
66	ASP	HB1	C	2.622	44.848
66	ASP	HB2	C	2.302	44.848
67	LEU	HN	N	8.163	127.700
67	LEU	HA	C	4.950	55.067
67	LEU	HB1	C	1.703	44.791
67	LEU	HB2	C	1.275	44.725
67	LEU	HG	C	-100.000	-100.000
67	LEU	HD1*	C	0.773	25.270
67	LEU	HD2*	C	1.408	28.452
68	SER	HN	N	8.772	122.050
68	SER	HA	C	4.862	57.209
68	SER	HB*	C	3.826	65.529
69	MET	HN	N	9.005	121.878
69	MET	HA	C	4.745	54.492
69	MET	HB*	C	2.035	34.429
69	MET	HG1	C	2.543	31.935
69	MET	HG2	C	2.358	31.935
69	MET	HE*	C	1.760	16.108
70	ASP	HN	N	8.632	121.167
70	ASP	HA	C	5.100	51.423
70	ASP	HB1	C	3.429	41.093
70	ASP	HB2	C	2.292	41.093
71	PRO	HA	C	4.706	66.494
71	PRO	HB1	C	2.532	-100.000
71	PRO	HB2	C	2.142	-100.000
71	PRO	HG1	C	2.304	-100.000
71	PRO	HG2	C	2.000	-100.000
71	PRO	HD1	C	3.779	-100.000
71	PRO	HD2	C	3.598	-100.000
72	PRO	HA	C	4.471	65.853
72	PRO	HB1	C	2.446	31.542
72	PRO	HB2	C	1.723	31.467
72	PRO	HG1	C	2.095	28.348

72	PRO	HG2	C	2.040	28.348
72	PRO	HD1	C	3.932	50.098
72	PRO	HD2	C	3.714	50.098
73	ARG	HN	N	7.336	114.410
73	ARG	HA	C	4.501	55.321
73	ARG	HB*	C	2.290	31.457
73	ARG	HG1	C	-100.000	-100.000
73	ARG	HG2	C	-100.000	-100.000
73	ARG	HD1	C	-100.000	-100.000
73	ARG	HD2	C	-100.000	-100.000
73	ARG	HE1	N	-100.000	-100.000
74	ASN	HN	N	8.474	116.121
74	ASN	HA	C	4.293	54.071
74	ASN	HB1	C	2.817	37.450
74	ASN	HB2	C	2.522	37.446
74	ASN	HD21	N	7.139	112.714
74	ASN	HD22	N	6.985	112.664
75	CYS	HN	N	7.220	127.876
75	CYS	HA	C	5.753	55.480
75	CYS	HB1	C	2.686	32.843
75	CYS	HB2	C	2.622	32.843
76	ALA	HN	N	8.739	122.779
76	ALA	HA	C	4.667	50.729
76	ALA	HB*	C	1.047	24.412
77	PHE	HN	N	8.816	115.621
77	PHE	HA	C	5.655	55.986
77	PHE	HB*	C	2.701	41.715
77	PHE	HD*	C	7.054	132.040
77	PHE	HE*	C	7.380	131.345
77	PHE	HZ	C	-100.000	-100.000
78	VAL	HN	N	8.577	124.982
78	VAL	HA	C	4.266	61.079
78	VAL	HB	C	1.380	34.784
78	VAL	HG1*	C	0.181	20.487
78	VAL	HG2*	C	0.028	19.395
79	THR	HN	N	8.921	123.801
79	THR	HA	C	5.107	62.098
79	THR	HB	C	3.786	70.238
79	THR	HG2*	C	1.122	21.725
80	TYR	HN	N	8.737	126.239
80	TYR	HA	C	5.303	57.698
80	TYR	HB1	C	3.610	42.426
80	TYR	HB2	C	2.664	42.426
80	TYR	HD*	C	6.826	132.340
80	TYR	HE*	C	6.705	135.524
80	TYR	OH	O	9.735	-100.000
81	GLU	HN	N	8.517	120.534
81	GLU	HA	C	4.041	59.542
81	GLU	HB1	C	2.193	31.113
81	GLU	HB2	C	2.253	31.113
81	GLU	HG1	C	2.445	36.198
81	GLU	HG2	C	2.270	36.198
82	LYS	HN	N	8.611	115.096
82	LYS	HA	C	4.853	54.599
82	LYS	HB1	C	1.953	34.429
82	LYS	HB2	C	2.193	34.431
82	LYS	HG1	C	1.526	25.114
82	LYS	HG2	C	1.570	25.114
82	LYS	HD1	C	1.764	-100.000
82	LYS	HD2	C	-100.000	-100.000
82	LYS	HE1	C	3.047	42.281
82	LYS	HE2	C	3.003	42.281
83	MET	HN	N	8.958	123.503
83	MET	HA	C	3.972	59.149
83	MET	HB1	C	2.074	32.370
83	MET	HB2	C	2.139	32.370

83	MET	HG*	C	2.653	32.143
83	MET	HE*	C	-100.000	-100.000
84	GLU	HN	N	9.715	118.126
84	GLU	HA	C	4.168	60.262
84	GLU	HB*	C	2.055	28.654
84	GLU	HG1	C	2.478	36.822
84	GLU	HG2	C	2.368	36.822
85	SER	HN	N	7.021	115.177
85	SER	HA	C	4.075	61.731
85	SER	HB1	C	3.397	62.685
85	SER	HB2	C	2.500	62.620
86	ALA	HN	N	6.795	122.522
86	ALA	HA	C	3.990	55.239
86	ALA	HB*	C	1.620	17.890
87	ASP	HN	N	7.766	116.039
87	ASP	HA	C	4.295	57.550
87	ASP	HB1	C	2.632	40.790
87	ASP	HB2	C	2.686	40.790
88	GLN	HN	N	7.642	120.061
88	GLN	HA	C	3.982	58.630
88	GLN	HB1	C	2.150	28.400
88	GLN	HB2	C	2.062	28.400
88	GLN	HG1	C	2.554	33.859
88	GLN	HG2	C	2.368	33.859
88	GLN	HE21	N	7.796	111.496
88	GLN	HE22	N	6.823	111.496
89	ALA	HN	N	7.876	121.269
89	ALA	HA	C	2.652	55.090
89	ALA	HB*	C	1.419	19.323
90	VAL	HN	N	7.934	116.227
90	VAL	HA	C	3.327	67.030
90	VAL	HB	C	2.114	32.012
90	VAL	HG1*	C	1.056	21.423
90	VAL	HG2*	C	1.056	24.438
91	ALA	HN	N	7.264	119.780
91	ALA	HA	C	4.041	54.889
91	ALA	HB*	C	1.488	18.523
92	GLU	HN	N	7.896	113.818
92	GLU	HA	C	4.285	58.064
92	GLU	HB1	C	2.094	30.963
92	GLU	HB2	C	1.690	30.963
92	GLU	HG1	C	2.390	36.666
92	GLU	HG2	C	2.281	36.661
93	LEU	HN	N	8.226	114.596
93	LEU	HA	C	4.540	55.107
93	LEU	HB1	C	1.742	44.025
93	LEU	HB2	C	1.057	44.025
93	LEU	HG	C	1.362	26.788
93	LEU	HD1*	C	0.695	22.670
93	LEU	HD2*	C	-0.016	25.166
94	ASN	HN	N	7.835	116.672
94	ASN	HA	C	4.373	56.883
94	ASN	HB1	C	3.268	39.015
94	ASN	HB2	C	3.082	39.015
94	ASN	HD21	N	7.899	115.662
94	ASN	HD22	N	7.101	115.648
95	GLY	HN	N	8.830	116.192
95	GLY	HA1	C	4.227	46.330
95	GLY	HA2	C	3.883	46.330
96	THR	HN	N	7.793	114.006
96	THR	HA	C	4.579	61.119
96	THR	HB	C	4.236	71.174
96	THR	HG2*	C	1.195	21.725
97	GLN	HN	N	8.356	119.871
97	GLN	HA	C	5.136	54.273
97	GLN	HB1	C	1.850	30.377

97	GLN	HB2	C	1.898	30.377
97	GLN	HG*	C	2.073	33.911
97	GLN	HE21	N	7.451	110.910
97	GLN	HE22	N	6.774	110.910
98	VAL	HN	N	8.854	127.300
98	VAL	HA	C	4.148	61.540
98	VAL	HB	C	1.859	33.665
98	VAL	HG1*	C	0.925	21.631
98	VAL	HG2*	C	0.870	21.319
99	GLU	HN	N	9.127	124.206
99	GLU	HA	C	3.728	58.039
99	GLU	HB*	C	2.172	27.211
99	GLU	HG1	C	2.314	36.402
99	GLU	HG2	C	2.237	36.402
100	SER	HN	N	8.287	111.825
100	SER	HA	C	4.217	59.857
100	SER	HB*	C	3.972	63.485
101	VAL	HN	N	8.368	124.567
101	VAL	HA	C	4.158	62.268
101	VAL	HB	C	2.259	33.286
101	VAL	HG1*	C	0.903	21.379
101	VAL	HG2*	C	0.783	21.529
102	GLN	HN	N	8.480	127.128
102	GLN	HA	C	4.540	55.579
102	GLN	HB1	C	2.093	28.830
102	GLN	HB2	C	1.977	28.830
102	GLN	HG*	C	2.241	33.730
102	GLN	HE21	N	7.474	111.110
102	GLN	HE22	N	6.713	111.110
103	LEU	HN	N	8.665	126.341
103	LEU	HA	C	4.980	54.810
103	LEU	HB1	C	2.040	44.641
103	LEU	HB2	C	1.122	44.829
103	LEU	HG	C	1.765	26.580
103	LEU	HD1*	C	0.728	26.372
103	LEU	HD2*	C	0.662	23.918
104	LYS	HN	N	8.684	123.509
104	LYS	HA	C	4.970	55.701
104	LYS	HB1	C	2.083	34.429
104	LYS	HB2	C	1.999	34.429
104	LYS	HG1	C	1.707	24.992
104	LYS	HG2	C	1.521	24.992
104	LYS	HD*	C	1.738	29.405
104	LYS	HE*	C	3.011	42.237
105	VAL	HN	N	8.974	123.671
105	VAL	HA	C	5.215	60.730
105	VAL	HB	C	1.898	35.703
105	VAL	HG1*	C	1.122	24.178
105	VAL	HG2*	C	1.056	22.566
106	ASN	HN	N	9.143	121.793
106	ASN	HA	C	5.048	52.075
106	ASN	HB1	C	2.903	43.048
106	ASN	HB2	C	2.773	43.048
106	ASN	HD21	N	7.329	112.135
106	ASN	HD22	N	6.823	112.135
107	ILE	HN	N	8.908	122.021
107	ILE	HA	C	4.184	61.690
107	ILE	HB	C	1.898	37.183
107	ILE	HG11	C	1.625	27.776
107	ILE	HG12	C	1.384	27.776
107	ILE	HG2*	C	0.947	17.523
107	ILE	HD1*	C	0.834	11.380
108	ALA	HN	N	8.691	108.985
108	ALA	HA	C	4.400	52.573
108	ALA	HB*	C	1.440	19.792
109	ARG	HN	N	8.300	119.780

109	ARG	HA	C	-100.000	56.702
109	ARG	HB1	C	-100.000	30.308
109	ARG	HB2	C	-100.000	-100.000
109	ARG	HG1	C	-100.000	-100.000
109	ARG	HG2	C	-100.000	-100.000
109	ARG	HD1	C	-100.000	-100.000
109	ARG	HD2	C	-100.000	-100.000
109	ARG	HE1	N	-100.000	-100.000
110	LYS	HN	N	-100.000	-100.000
110	LYS	HA	C	-100.000	54.387
110	LYS	HB1	C	-100.000	34.599
110	LYS	HB2	C	-100.000	-100.000
110	LYS	HG1	C	-100.000	-100.000
110	LYS	HG2	C	-100.000	-100.000
110	LYS	HD1	C	-100.000	-100.000
110	LYS	HD2	C	-100.000	-100.000
110	LYS	HE1	C	-100.000	-100.000
110	LYS	HE2	C	-100.000	-100.000
111	GLN	HN	N	8.939	123.560
111	GLN	HA	C	-100.000	59.286
111	GLN	HB1	C	-100.000	32.829
111	GLN	HB2	C	-100.000	-100.000
111	GLN	HG1	C	-100.000	-100.000
111	GLN	HG2	C	-100.000	-100.000
111	GLN	HE21	N	7.705	114.127
111	GLN	HE22	N	7.211	114.141
112	PRO	HA	C	4.348	63.561
112	PRO	HB1	C	2.209	32.141
112	PRO	HB2	C	1.828	32.141
112	PRO	HG1	C	1.975	27.287
112	PRO	HG2	C	1.819	27.287
112	PRO	HD*	C	3.625	50.764
113	MET	HN	N	8.565	119.749
113	MET	HA	C	4.477	55.701
113	MET	HB1	C	-100.000	32.671
113	MET	HB2	C	-100.000	-100.000
113	MET	HG1	C	-100.000	-100.000
113	MET	HG2	C	-100.000	-100.000
113	MET	HE*	C	-100.000	-100.000
114	LEU	HN	N	8.264	123.760
114	LEU	HA	C	-100.000	55.661
114	LEU	HB1	C	-100.000	42.781
114	LEU	HB2	C	-100.000	-100.000
114	LEU	HG	C	-100.000	27.287
114	LEU	HD1*	C	-100.000	24.992
114	LEU	HD2*	C	-100.000	23.845
115	ASP	HN	N	8.317	121.040
115	ASP	HA	C	4.569	54.397
115	ASP	HB1	C	2.718	41.093
115	ASP	HB2	C	2.620	41.093
116	ALA	HN	N	8.187	124.382
116	ALA	HA	C	4.263	52.848
116	ALA	HB*	C	1.394	19.255
117	ALA	HN	N	8.253	122.286
117	ALA	HA	C	4.364	52.906
117	ALA	HB*	C	1.448	19.275
118	THR	HN	N	8.029	112.216
118	THR	HA	C	4.315	62.220
118	THR	HB	C	4.226	70.060
118	THR	HG2*	C	1.237	21.639
119	GLY	HN	N	8.375	111.219
119	GLY	HA*	C	4.002	45.516
120	LYS	HN	N	8.139	121.182
120	LYS	HA	C	4.442	56.171
120	LYS	HB1	C	1.914	33.325
120	LYS	HB2	C	1.767	33.325

120	LYS	HG*	C	1.449	24.551	121	SER	HA	C	-100.000	60.142
120	LYS	HD*	C	1.707	28.876	121	SER	HB1	C	-100.000	64.818
120	LYS	HE*	C	3.022	42.203	121	SER	HB2	C	-100.000	-100.000
121	SER	HN	N	8.045	123.059						

10.4 Distance restraints used for the structure determination of NELF-E RRM

(resid	83	name	HN	)	(resid	84	name	HN	)	4.00	(resid	68	name	HN	)	(resid	78	name	HN	)	6.00
(resid	80	name	HN	)	(resid	42	name	HN	)	6.00	(resid	44	name	HN	)	(resid	78	name	HN	)	5.00
(resid	59	name	HN	)	(resid	80	name	HH	)	6.00	(resid	56	name	HN	)	(resid	58	name	HN	)	5.00
(resid	58	name	HN	)	(resid	80	name	HH	)	5.00	(resid	57	name	HN	)	(resid	58	name	HN	)	3.00
(resid	62	name	HN	)	(resid	80	name	HH	)	5.00	(resid	65	name	HN	)	(resid	81	name	HN	)	5.00
(resid	85	name	HN	)	(resid	84	name	HN	)	3.00	(resid	80	name	HN	)	(resid	81	name	HN	)	5.00
(resid	86	name	HN	)	(resid	84	name	HN	)	5.00	(resid	101	name	HN	)	(resid	102	name	HN	)	5.00
(resid	42	name	HN	)	(resid	43	name	HN	)	5.00	(resid	49	name	HN	)	(resid	48	name	HN	)	4.00
(resid	108	name	HN	)	(resid	43	name	HN	)	4.00	(resid	64	name	HN	)	(resid	63	name	HN	)	5.00
(resid	78	name	HN	)	(resid	43	name	HN	)	6.00	(resid	38	name	HN	)	(resid	37	name	HN	)	5.00
(resid	107	name	HN	)	(resid	106	name	HN	)	5.00	(resid	62	name	HN	)	(resid	63	name	HN	)	5.00
(resid	98	name	HN	)	(resid	99	name	HN	)	5.00	(resid	73	name	HN	)	(resid	74	name	HN	)	3.00
(resid	104	name	HN	)	(resid	45	name	HN	)	4.00	(resid	75	name	HN	)	(resid	74	name	HN	)	3.00
(resid	46	name	HN	)	(resid	45	name	HN	)	5.00	(resid	41	name	HN	)	(resid	40	name	HN	)	5.00
(resid	76	name	HN	)	(resid	45	name	HN	)	6.00	(resid	39	name	HN	)	(resid	40	name	HN	)	5.00
(resid	101	name	HN	)	(resid	99	name	HN	)	5.00	(resid	90	name	HN	)	(resid	93	name	HN	)	5.00
(resid	100	name	HN	)	(resid	99	name	HN	)	4.00	(resid	92	name	HN	)	(resid	93	name	HN	)	3.00
(resid	68	name	HN	)	(resid	69	name	HN	)	5.00	(resid	89	name	HN	)	(resid	93	name	HN	)	6.00
(resid	70	name	HN	)	(resid	69	name	HN	)	5.00	(resid	94	name	HN	)	(resid	93	name	HN	)	3.00
(resid	95	name	HN	)	(resid	105	name	HN	)	5.00	(resid	91	name	HN	)	(resid	93	name	HN	)	5.00
(resid	104	name	HN	)	(resid	105	name	HN	)	5.00	(resid	45	name	HN	)	(resid	44	name	HN	)	5.00
(resid	53	name	HN	)	(resid	55	name	HN	)	5.00	(resid	68	name	HN	)	(resid	67	name	HN	)	5.00
(resid	65	name	HN	)	(resid	79	name	HN	)	5.00	(resid	59	name	HN	)	(resid	56	name	HN	)	6.00
(resid	80	name	HN	)	(resid	79	name	HN	)	5.00	(resid	57	name	HN	)	(resid	56	name	HN	)	3.00
(resid	82	name	HN	)	(resid	83	name	HN	)	5.00	(resid	53	name	HN	)	(resid	56	name	HN	)	5.00
(resid	56	name	HN	)	(resid	55	name	HN	)	3.00	(resid	59	name	HN	)	(resid	58	name	HD*	)	5.00
(resid	57	name	HN	)	(resid	55	name	HN	)	5.00	(resid	57	name	HN	)	(resid	58	name	HD*	)	4.00
(resid	66	name	HN	)	(resid	79	name	HN	)	4.00	(resid	61	name	HN	)	(resid	58	name	HD*	)	6.00
(resid	106	name	HD21)		(resid	107	name	HN	)	5.00	(resid	87	name	HN	)	(resid	90	name	HN	)	5.00
(resid	85	name	HN	)	(resid	83	name	HN	)	5.00	(resid	105	name	HN	)	(resid	94	name	HN	)	6.00
(resid	108	name	HN	)	(resid	107	name	HN	)	5.00	(resid	55	name	HN	)	(resid	54	name	HN	)	3.00
(resid	67	name	HN	)	(resid	79	name	HN	)	6.00	(resid	95	name	HN	)	(resid	96	name	HN	)	3.00
(resid	70	name	HN	)	(resid	77	name	HN	)	5.00	(resid	59	name	HN	)	(resid	57	name	HN	)	5.00
(resid	101	name	HN	)	(resid	98	name	HN	)	4.00	(resid	97	name	HN	)	(resid	96	name	HN	)	5.00
(resid	97	name	HN	)	(resid	98	name	HN	)	5.00	(resid	52	name	HN	)	(resid	53	name	HN	)	4.00
(resid	100	name	HN	)	(resid	98	name	HN	)	5.00	(resid	85	name	HN	)	(resid	87	name	HN	)	5.00
(resid	94	name	HN	)	(resid	95	name	HN	)	5.00	(resid	86	name	HN	)	(resid	87	name	HN	)	3.00
(resid	97	name	HE21)		(resid	98	name	HN	)	5.00	(resid	61	name	HN	)	(resid	62	name	HN	)	3.00
(resid	78	name	HN	)	(resid	77	name	HN	)	5.00	(resid	90	name	HN	)	(resid	88	name	HN	)	5.00
(resid	102	name	HN	)	(resid	98	name	HN	)	6.00	(resid	89	name	HN	)	(resid	88	name	HN	)	3.00
(resid	48	name	HN	)	(resid	47	name	HN	)	5.00	(resid	91	name	HN	)	(resid	88	name	HN	)	6.00
(resid	49	name	HN	)	(resid	47	name	HN	)	6.00	(resid	54	name	HN	)	(resid	52	name	HN	)	6.00
(resid	66	name	HN	)	(resid	65	name	HN	)	3.00	(resid	76	name	HN	)	(resid	46	name	HN	)	6.00
(resid	44	name	HN	)	(resid	76	name	HN	)	4.00	(resid	47	name	HN	)	(resid	46	name	HN	)	5.00
(resid	63	name	HD21)		(resid	64	name	HN	)	6.00	(resid	101	name	HN	)	(resid	97	name	HE21)		5.00
(resid	75	name	HN	)	(resid	76	name	HN	)	5.00	(resid	67	name	HN	)	(resid	66	name	HN	)	5.00
(resid	98	name	HN	)	(resid	103	name	HN	)	5.00	(resid	64	name	HN	)	(resid	66	name	HN	)	6.00
(resid	102	name	HN	)	(resid	103	name	HN	)	5.00	(resid	44	name	HN	)	(resid	77	name	HE*	)	6.00
(resid	96	name	HN	)	(resid	103	name	HN	)	5.00	(resid	88	name	HE21)		(resid	61	name	HE*	)	5.00
(resid	74	name	HN	)	(resid	70	name	HN	)	6.00	(resid	62	name	HN	)	(resid	61	name	HD*	)	5.00
(resid	75	name	HN	)	(resid	70	name	HN	)	4.00	(resid	41	name	HN	)	(resid	40	name	HD21)		6.00
(resid	85	name	HN	)	(resid	82	name	HN	)	5.00	(resid	80	name	HN	)	(resid	40	name	HD21)		6.00
(resid	86	name	HN	)	(resid	82	name	HN	)	6.00	(resid	59	name	HN	)	(resid	61	name	HD*	)	6.00
(resid	42	name	HN	)	(resid	78	name	HN	)	3.00	(resid	58	name	HN	)	(resid	61	name	HD*	)	6.00
(resid	84	name	HN	)	(resid	82	name	HN	)	5.00	(resid	92	name	HN	)	(resid	61	name	HZ	)	5.00
(resid	40	name	HN	)	(resid	82	name	HN	)	6.00	(resid	47	name	HN	)	(resid	45	name	HD*	)	6.00

(resid 76 name HN) (resid 45 name HD*) 6.00
(resid 104 name HN) (resid 45 name HD*) 5.00
(resid 46 name HN) (resid 45 name HD*) 4.00
(resid 68 name HN) (resid 77 name HD*) 6.00
(resid 86 name HN) (resid 85 name HN) 3.00
(resid 57 name HN) (resid 58 name HE*) 6.00
(resid 106 name HN) (resid 43 name HD*) 5.00
(resid 45 name HN) (resid 43 name HD*) 5.00
(resid 47 name HN) (resid 45 name HE*) 6.00
(resid 76 name HN) (resid 43 name HD*) 6.00
(resid 44 name HN) (resid 43 name HD*) 4.00
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(resid 78 name HN) (resid 43 name HA) 5.00
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(resid 53 name HN) (resid 50 name HB) 4.00
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(resid 92 name HN) (resid 93 name HA) 6.00
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(resid 103 name HN) (resid 102 name HA) 3.00
(resid 39 name HN) (resid 38 name HA) 4.00
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(resid 37 name HN) (resid 36 name HA) 4.00
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(resid 92 name HN) (resid 91 name HA) 4.00
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(resid 61 name HN) (resid 62 name HA2) 6.00
(resid 40 name HD22) (resid 86 name HA) 6.00
(resid 42 name HN) (resid 41 name HB) 5.00
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(resid 48 name HN) (resid 47 name HA) 3.00
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(resid 97 name HE22) (resid 100 name HB*) 6.00
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(resid 53 name HN) (resid 52 name HA) 4.00
(resid 73 name HN) (resid 72 name HD1) 4.00
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(resid 77 name HN) (resid 68 name HB*) 5.00
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(resid 49 name HN) (resid 46 name HA*) 6.00

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(resid 59 name HN) (resid 58 name HB1) 5.00
(resid 93 name HN) (resid 94 name HB1) 6.00
(resid 56 name HN) (resid 55 name HD1) 6.00
(resid 95 name HN) (resid 94 name HB2) 5.00
(resid 93 name HN) (resid 94 name HB2) 5.00
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(resid 83 name HN) (resid 40 name HB1) 5.00
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(resid 57 name HN) (resid 58 name HB2) 5.00
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(resid 86 name HN) (resid 40 name HB1) 6.00
(resid 64 name HN) (resid 63 name HB*) 4.00
(resid 82 name HN) (resid 63 name HB*) 6.00
(resid 44 name HN) (resid 43 name HB1) 5.00
(resid 104 name HN) (resid 45 name HB*) 4.00
(resid 49 name HN) (resid 48 name HB1) 5.00
(resid 89 name HN) (resid 61 name HB2) 5.00
(resid 62 name HN) (resid 61 name HB2) 4.00
(resid 83 name HN) (resid 40 name HB2) 6.00
(resid 44 name HN) (resid 43 name HB2) 5.00
(resid 107 name HN) (resid 106 name HB2) 4.00
(resid 108 name HN) (resid 106 name HB2) 6.00
(resid 68 name HN) (resid 77 name HB*) 4.00
(resid 78 name HN) (resid 77 name HB*) 4.00
(resid 47 name HN) (resid 48 name HB2) 6.00
(resid 76 name HN) (resid 75 name HB1) 4.00
(resid 93 name HN) (resid 89 name HA) 4.00
(resid 49 name HN) (resid 48 name HB2) 5.00
(resid 92 name HN) (resid 89 name HA) 4.00
(resid 46 name HN) (resid 75 name HB2) 5.00
(resid 61 name HN) (resid 89 name HA) 6.00
(resid 84 name HN) (resid 83 name HG*) 5.00
(resid 65 name HN) (resid 66 name HB1) 5.00
(resid 67 name HN) (resid 66 name HB1) 5.00
(resid 77 name HN) (resid 69 name HG1) 5.00
(resid 70 name HN) (resid 69 name HG1) 6.00
(resid 92 name HN) (resid 88 name HG1) 6.00
(resid 87 name HN) (resid 85 name HB2) 6.00
(resid 62 name HN) (resid 85 name HB2) 5.00
(resid 46 name HN) (resid 74 name HB2) 4.00
(resid 86 name HN) (resid 85 name HB2) 4.00
(resid 83 name HN) (resid 84 name HG1) 6.00
(resid 82 name HN) (resid 81 name HG1) 6.00
(resid 54 name HN) (resid 51 name HB1) 6.00
(resid 52 name HN) (resid 51 name HB1) 4.00
(resid 89 name HN) (resid 88 name HG2) 5.00
(resid 79 name HN) (resid 66 name HB2) 5.00
(resid 70 name HN) (resid 73 name HB*) 6.00
(resid 74 name HN) (resid 73 name HB*) 5.00
(resid 93 name HN) (resid 92 name HG2) 5.00
(resid 67 name HN) (resid 66 name HB2) 5.00
(resid 53 name HN) (resid 51 name HG1) 6.00
(resid 52 name HN) (resid 51 name HG1) 4.00
(resid 75 name HN) (resid 73 name HB*) 4.00
(resid 48 name HN) (resid 49 name HG1) 6.00
(resid 63 name HN) (resid 81 name HB2) 5.00
(resid 63 name HD21) (resid 81 name HB2) 6.00
(resid 97 name HE22) (resid 102 name HG*) 6.00
(resid 100 name HN) (resid 99 name HB*) 4.00
(resid 74 name HD21) (resid 47 name HG2) 6.00
(resid 85 name HN) (resid 82 name HB2) 4.00
(resid 74 name HD22) (resid 47 name HG2) 6.00
(resid 88 name HN) (resid 90 name HB) 6.00
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(resid 55 name HN) (resid 51 name HB2) 6.00
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(resid 52 name HN) (resid 51 name HB2) 4.00
(resid 73 name HN) (resid 72 name HG2) 5.00
(resid 102 name HE22) (resid 97 name HG*) 6.00
(resid 77 name HN) (resid 69 name HB*) 5.00
(resid 70 name HN) (resid 69 name HB*) 4.00
(resid 37 name HN) (resid 36 name HG*) 5.00
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(resid 81 name HN) (resid 82 name HG2) 6.00
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(resid 43 name HN) (resid 108 name HB*) 5.00
(resid 45 name HN) (resid 44 name HB) 5.00
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(resid 90 name HN) (resid 89 name HB*) 3.00
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(resid 68 name HN ) (resid 67 name HD1* ) 4.00
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(resid 94 name HN ) (resid 93 name HD1* ) 5.00
(resid 104 name HN ) (resid 103 name HD2* ) 5.00
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(resid 67 name HN ) (resid 64 name HG2* ) 5.00
(resid 66 name HN ) (resid 64 name HG2* ) 3.00
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(resid 93 name HN) (resid 93 name HD1*) 4.00
(resid 54 name HN) (resid 54 name HD2*) 4.00
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(resid 47 name HG1 ) (resid 45 name HE* ) 6.00
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(resid 78 name HG1*) (resid 67 name HD1*) 5.00
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(resid 51 name HB2) (resid 67 name HD1*) 4.00
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(resid 107 name HD1*) (resid 107 name HN) 5.00
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(resid 98 name HB) (resid 98 name HN) 4.00
(resid 95 name HA1) (resid 95 name HN) 3.00
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(resid 101 name HB) (resid 101 name HN) 3.00
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(resid 93 name HB2) (resid 93 name HN) 4.00
(resid 93 name HA) (resid 93 name HN) 3.00
(resid 93 name HG) (resid 93 name HN) 3.00
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(resid 58 name HB2) (resid 58 name HD*) 3.00
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(resid 43 name HB2) (resid 43 name HD*) 3.00

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(resid 82 name HG1) (resid 82 name HA) 3.00
(resid 63 name HB*) (resid 63 name HA) 3.00
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(resid 69 name HG2) (resid 69 name HA) 4.00
(resid 69 name HB*) (resid 69 name HA) 3.00
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(resid 102 name HB2) (resid 102 name HA) 4.00

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(resid 106 name HA) (resid 106 name HB2) 4.00

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(resid 51 name HB2) (resid 51 name HG1) 3.00
(resid 92 name HB2) (resid 92 name HG2) 3.00
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(resid 43 name HD*) (resid 104 name HB2) 6.00
(resid 45 name HE*) (resid 104 name HB2) 6.00
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(resid 77 name HD*) (resid 42 name HB1) 6.00
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(resid 43 name HD*) (resid 105 name HG2*) 6.00
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(resid 43 name HD*) (resid 44 name HG1*) 6.00
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(resid 45 name HD*) (resid 103 name HD2*) 6.00
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(resid 58 name HD*) (resid 93 name HD2*) 4.00
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(resid 51 name HA) (resid 54 name HB2) 5.00
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(resid 69 name HE*) (resid 51 name HD2) 4.00
(resid 69 name HE*) (resid 51 name HD1) 5.00
(resid 69 name HE*) (resid 54 name HD2*) 4.00
(resid 69 name HE*) (resid 76 name HB*) 4.00
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(resid 69 name HE*) (resid 70 name HN) 5.00
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(resid 44 name HB) (resid 58 name HE*) 5.00
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(resid 54 name HN) (resid 54 name HG) 3.00
(resid 54 name HN) (resid 54 name HB1) 3.00
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(resid 50 name HN) (resid 49 name HG1) 5.00
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(resid 74 name HB2) (resid 45 name HE*) 5.00
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(resid 54 name HN) (resid 56 name HN) 5.00
(resid 55 name HN) (resid 55 name HB2) 3.00
(resid 58 name HN) (resid 60 name HD2) 4.00
(resid 61 name HN) (resid 58 name HA) 4.00
(resid 63 name HN) (resid 62 name HA2) 3.00
(resid 66 name HN) (resid 65 name HG12) 5.00
(resid 70 name HN) (resid 76 name HA) 3.00
(resid 73 name HN) (resid 72 name HG1) 5.00
(resid 76 name HN) (resid 44 name HB) 4.00
(resid 83 name HN) (resid 82 name HB1) 5.00
(resid 85 name HN) (resid 84 name HB*) 4.00
(resid 86 name HN) (resid 80 name HB2) 5.00
(resid 86 name HN) (resid 85 name HA) 4.00
(resid 87 name HN) (resid 87 name HB2) 3.00
(resid 87 name HN) (resid 88 name HN) 3.00
(resid 87 name HN) (resid 89 name HN) 5.00
(resid 88 name HN) (resid 88 name HB1) 3.00
(resid 88 name HN) (resid 88 name HB2) 3.00
(resid 88 name HN) (resid 86 name HN) 5.00
(resid 89 name HN) (resid 61 name HB1) 5.00
(resid 89 name HN) (resid 61 name HD*) 5.00
(resid 90 name HN) (resid 89 name HA) 5.00
(resid 90 name HN) (resid 88 name HA) 5.00
(resid 91 name HN) (resid 88 name HA) 5.00
(resid 92 name HN) (resid 88 name HA) 5.00
(resid 94 name HN) (resid 92 name HN) 5.00
(resid 98 name HN) (resid 97 name HB1) 5.00
(resid 98 name HN) (resid 100 name HA) 6.00
(resid 99 name HN) (resid 98 name HG1*) 4.00
(resid 100 name HN) (resid 98 name HG2*) 5.00
(resid 101 name HN) (resid 98 name HG2*) 4.00
(resid 101 name HN) (resid 98 name HG1*) 5.00
(resid 101 name HN) (resid 98 name HB) 5.00
(resid 101 name HN) (resid 100 name HA) 4.00
(resid 102 name HN) (resid 101 name HG1*) 5.00
(resid 103 name HN) (resid 98 name HG2*) 5.00
(resid 103 name HN) (resid 102 name HB1) 4.00
(resid 104 name HN) (resid 44 name HG1*) 5.00
(resid 104 name HN) (resid 103 name HG) 5.00
(resid 106 name HN) (resid 105 name HG1*) 4.00
(resid 106 name HN) (resid 43 name HB1) 5.00
(resid 108 name HN) (resid 109 name HN) 5.00
(resid 42 name HD1*) (resid 90 name HN) 4.00
(resid 104 name HB1) (resid 45 name HD*) 5.00
(resid 104 name HD*) (resid 45 name HD*) 5.00
(resid 47 name HA) (resid 74 name HB2) 5.00
(resid 49 name HB1) (resid 54 name HD1*) 5.00
(resid 49 name HB1) (resid 54 name HD2*) 5.00
(resid 49 name HB1) (resid 69 name HE*) 5.00
(resid 49 name HB2) (resid 69 name HE*) 5.00
(resid 49 name HE*) (resid 69 name HE*) 5.00
(resid 51 name HB1) (resid 67 name HD1*) 5.00
(resid 53 name HD2*) (resid 103 name HD2*) 5.00
(resid 54 name HB1) (resid 67 name HD1*) 5.00
(resid 54 name HD1*) (resid 78 name HG2*) 5.00
(resid 54 name HD1*) (resid 103 name HD2*) 4.00

(resid 54 name HD2*) (resid 67 name HD2*) 5.00
(resid 54 name HD2*) (resid 69 name HG1) 5.00
(resid 54 name HD2*) (resid 76 name HB*) 4.00
(resid 57 name HB*) (resid 93 name HD1*) 4.00
(resid 61 name HB1) (resid 80 name HH) 5.00
(resid 61 name HB1) (resid 89 name HB*) 5.00
(resid 61 name HB1) (resid 85 name HA) 5.00
(resid 88 name HB1) (resid 61 name HD*) 5.00
(resid 88 name HB2) (resid 61 name HD*) 5.00
(resid 88 name HG1) (resid 61 name HD*) 5.00
(resid 88 name HG2) (resid 61 name HD*) 4.00
(resid 89 name HA) (resid 61 name HD*) 5.00
(resid 62 name HA1) (resid 85 name HB2) 5.00
(resid 64 name HG11) (resid 80 name HE*) 5.00
(resid 65 name HN) (resid 79 name HB) 5.00
(resid 65 name HG11) (resid 79 name HB) 5.00
(resid 65 name HD1*) (resid 79 name HB) 5.00
(resid 65 name HD1*) (resid 81 name HA) 4.00
(resid 67 name HD2*) (resid 78 name HG1*) 5.00
(resid 69 name HB*) (resid 76 name HB*) 5.00
(resid 90 name HG1*) (resid 106 name HA) 5.00
(resid 61 name HE*) (resid 93 name HD2*) 4.00
(resid 61 name HE*) (resid 92 name HB1) 4.00
(resid 77 name HD*) (resid 108 name HB*) 5.00
(resid 80 name HD*) (resid 58 name HD*) 5.00
(resid 80 name HD*) (resid 85 name HB1) 5.00
(resid 80 name HD*) (resid 86 name HB*) 4.00
(resid 61 name HZ) (resid 93 name HN) 5.00
(resid 61 name HZ) (resid 93 name HG) 5.00
(resid 61 name HZ) (resid 92 name HG2) 5.00
(resid 58 name HD*) (resid 86 name HA) 5.00
(resid 58 name HD*) (resid 54 name HG) 6.00
(resid 58 name HD*) (resid 89 name HB*) 5.00
(resid 43 name HE*) (resid 45 name HD*) 4.00
(resid 43 name HE*) (resid 77 name HE*) 4.00
(resid 43 name HE*) (resid 45 name HB*) 3.00
(resid 45 name HE*) (resid 104 name HD*) 5.00
(resid 45 name HE*) (resid 47 name HG*) 5.00
(resid 80 name HE*) (resid 93 name HD2*) 5.00
(resid 80 name HE*) (resid 85 name HB2) 5.00
(resid 80 name HE*) (resid 61 name HD*) 5.00
(resid 43 name HD*) (resid 108 name HB*) 5.00
(resid 43 name HD*) (resid 106 name HA) 5.00
(resid 61 name HD*) (resid 93 name HD2*) 4.00
(resid 61 name HD*) (resid 93 name HD1*) 5.00
(resid 61 name HD*) (resid 80 name HH) 5.00
(resid 61 name HD*) (resid 58 name HA) 5.00
(resid 61 name HE*) (resid 58 name HA) 5.00

(resid 61 name HZ) (resid 89 name HA) 5.00
(resid 96 name HN) (resid 105 name HG2*) 6.00
(resid 57 name HN) (resid 93 name HD1*) 6.00
(resid 106 name HN) (resid 44 name HG1*) 6.00
(resid 68 name HN) (resid 67 name HD2*) 6.00
(resid 78 name HN) (resid 67 name HD1*) 6.00
(resid 53 name HD2*) (resid 49 name HG1) 6.00
(resid 103 name HD1*) (resid 54 name HA) 6.00
(resid 88 name HB1) (resid 85 name HB2) 6.00
(resid 88 name HB1) (resid 89 name HN) 6.00
(resid 102 name HB2) (resid 103 name HN) 6.00
(resid 55 name HG2) (resid 67 name HD1*) 6.00
(resid 52 name HA) (resid 53 name HB2) 6.00
(resid 49 name HB2) (resid 47 name HN) 6.00
(resid 106 name HA) (resid 107 name HG12) 6.00
(resid 86 name HB*) (resid 85 name HB1) 6.00
(resid 86 name HB*) (resid 90 name HB) 6.00
(resid 101 name HG2*) (resid 103 name HB1) 6.00
(resid 49 name HA) (resid 53 name HD2*) 6.00
(resid 93 name HD1*) (resid 96 name HB) 6.00
(resid 54 name HD1*) (resid 105 name HG1*) 6.00
(resid 44 name HA) (resid 104 name HA) 6.00
(resid 103 name HD1*) (resid 103 name HB1) 6.00
(resid 81 name HB1) (resid 81 name HA) 6.00
(resid 38 name HB2) (resid 38 name HA) 6.00
(resid 72 name HD1) (resid 72 name HG2) 6.00
(resid 103 name HA) (resid 103 name HB1) 6.00
(resid 81 name HA) (resid 81 name HN) 6.00
(resid 104 name HG1) (resid 104 name HE*) 6.00
(resid 80 name HD*) (resid 40 name HB1) 6.00
(resid 108 name HN) (resid 90 name HG2*) 6.00
(resid 61 name HN) (resid 64 name HD1*) 6.00
(resid 96 name HN) (resid 93 name HD1*) 6.00
(resid 49 name HN) (resid 54 name HD2*) 6.00
(resid 74 name HN) (resid 72 name HG2) 6.00
(resid 70 name HN) (resid 77 name HB*) 6.00
(resid 106 name HN) (resid 42 name HB1) 6.00
(resid 59 name HN) (resid 60 name HB2) 6.00
(resid 83 name HN) (resid 40 name HD21) 5.00
(resid 103 name HD1*) (resid 57 name HB*) 6.00
(resid 104 name HA) (resid 94 name HD21) 6.00
(resid 96 name HG2*) (resid 98 name HG2*) 6.00
(resid 50 name HG2*) (resid 51 name HB2) 6.00
(resid 83 name HA) (resid 84 name HG1) 6.00
(resid 81 name HA) (resid 65 name HB) 6.00
(resid 98 name HA) (resid 98 name HG2*) 3.00
(resid 88 name HB1) (resid 88 name HG2) 3.00
(resid 80 name HD*) (resid 81 name HB1) 5.00

10.5 Dihedral angle restraints

(resid 41 and name C) (resid 42 and name N)
(resid 42 and name CA) (resid 42 and name C) 1.0 -120 40 2

(resid 42 and name C) (resid 43 and name N)
(resid 43 and name CA) (resid 43 and name C) 1.0 -120 40 2

(resid 43 and name C) (resid 44 and name N)
(resid 44 and name CA) (resid 44 and name C) 1.0 -120 40 2

(resid 44 and name C) (resid 45 and name N)
(resid 45 and name CA) (resid 45 and name C) 1.0 -120 40 2

(resid 51 and name C) (resid 52 and name N)
(resid 52 and name CA) (resid 52 and name C) 1.0 -60 20 2

(resid 52 and name C) (resid 53 and name N)
(resid 53 and name CA) (resid 53 and name C) 1.0 -60 20 2

(resid 53 and name C) (resid 54 and name N)
(resid 54 and name CA) (resid 54 and name C) 1.0 -60 20 2

(resid 54 and name C) (resid 55 and name N)
(resid 55 and name CA) (resid 55 and name C) 1.0 -60 20 2

(resid 56 and name C) (resid 57 and name N)
(resid 57 and name CA) (resid 57 and name C) 1.0 -60 20 2

(resid 58 and name C) (resid 59 and name N)
(resid 59 and name CA) (resid 59 and name C) 1.0 -60 20 2

(resid 67 and name C) (resid 68 and name N)

(resid 68 and name CA) (resid 68 and name C) 1.0 -120 40 2

(resid 68 and name C) (resid 69 and name N)
(resid 69 and name CA) (resid 69 and name C) 1.0 -120 40 2

(resid 69 and name C) (resid 70 and name N)
(resid 70 and name CA) (resid 70 and name C) 1.0 -120 40 2

(resid 74 and name C) (resid 75 and name N)
(resid 75 and name CA) (resid 75 and name C) 1.0 -120 40 2

(resid 76 and name C) (resid 77 and name N)
(resid 77 and name CA) (resid 77 and name C) 1.0 -120 40 2

(resid 78 and name C) (resid 79 and name N)
(resid 79 and name CA) (resid 79 and name C) 1.0 -120 40 2

(resid 79 and name C) (resid 80 and name N)
(resid 80 and name CA) (resid 80 and name C) 1.0 -120 40 2

(resid 82 and name C) (resid 83 and name N)
(resid 83 and name CA) (resid 83 and name C) 1.0 -60 20 2

(resid 83 and name C) (resid 84 and name N)
(resid 84 and name CA) (resid 84 and name C) 1.0 -60 20 2

(resid 84 and name C) (resid 85 and name N)
(resid 85 and name CA) (resid 85 and name C) 1.0 -60 20 2

(resid 85 and name C) (resid 86 and name N)
(resid 86 and name CA) (resid 86 and name C) 1.0 -60 20 2

(resid 86 and name C) (resid 87 and name N)
(resid 87 and name CA) (resid 87 and name C) 1.0 -60 20 2

(resid 87 and name C) (resid 88 and name N)
(resid 88 and name CA) (resid 88 and name C) 1.0 -60 20 2

(resid 88 and name C) (resid 89 and name N)
(resid 89 and name CA) (resid 89 and name C) 1.0 -60 20 2

(resid 89 and name C) (resid 90 and name N)
(resid 90 and name CA) (resid 90 and name C) 1.0 -60 20 2

(resid 90 and name C) (resid 91 and name N)
(resid 91 and name CA) (resid 91 and name C) 1.0 -60 20 2

(resid 95 and name C) (resid 96 and name N)
(resid 96 and name CA) (resid 96 and name C) 1.0 -120 40 2

(resid 96 and name C) (resid 97 and name N)
(resid 97 and name CA) (resid 97 and name C) 1.0 -120 40 2

(resid 97 and name C) (resid 98 and name N)
(resid 98 and name CA) (resid 98 and name C) 1.0 -120 40 2

(resid 100 and name C) (resid 101 and name N)
(resid 101 and name CA) (resid 101 and name C) 1.0 -120 40 2

(resid 102 and name C) (resid 103 and name N)
(resid 103 and name CA) (resid 103 and name C) 1.0 -120 40 2

(resid 103 and name C) (resid 104 and name N)
(resid 104 and name CA) (resid 104 and name C) 1.0 -120 40 2

10.6 Hydrogen bonds

(resid 56 and name HN) (resid 52 and name O) 2.3
(resid 56 and name N) (resid 52 and name O) 3.3

(resid 57 and name HN) (resid 53 and name O) 2.3
(resid 57 and name N) (resid 53 and name O) 3.3

(resid 58 and name HN) (resid 54 and name O) 2.3
(resid 58 and name N) (resid 54 and name O) 3.3

(resid 59 and name HN) (resid 55 and name O) 2.3
(resid 59 and name N) (resid 55 and name O) 3.3

(resid 87 and name HN) (resid 83 and name O) 2.3
(resid 87 and name N) (resid 83 and name O) 3.3

(resid 88 and name HN) (resid 84 and name O) 2.3
(resid 88 and name N) (resid 84 and name O) 3.3

(resid 89 and name HN) (resid 85 and name O) 2.3
(resid 89 and name N) (resid 85 and name O) 3.3

(resid 90 and name HN) (resid 86 and name O) 2.3
(resid 90 and name N) (resid 86 and name O) 3.3

(resid 91 and name HN) (resid 87 and name O) 2.3
(resid 91 and name N) (resid 87 and name O) 3.3

(resid 92 and name HN) (resid 88 and name O) 2.3
(resid 92 and name N) (resid 88 and name O) 3.3

(resid 44 and name HN) (resid 76 and name O) 2.3
(resid 44 and name N) (resid 76 and name O) 3.3

(resid 76 and name HN) (resid 44 and name O) 2.3
(resid 76 and name N) (resid 44 and name O) 3.3

(resid 42 and name HN) (resid 78 and name O) 2.3
(resid 42 and name N) (resid 78 and name O) 3.3

(resid 78 and name HN) (resid 42 and name O) 2.3
(resid 78 and name N) (resid 42 and name O) 3.3

(resid 40 and name HN) (resid 80 and name O) 2.3
(resid 40 and name N) (resid 80 and name O) 3.3

(resid 80 and name HN) (resid 40 and name O) 2.3
(resid 80 and name N) (resid 40 and name O) 3.3

(resid 103 and name HN) (resid 96 and name O) 2.3
(resid 103 and name N) (resid 96 and name O) 3.3

(resid 68 and name HN) (resid 77 and name O) 2.3
(resid 68 and name N) (resid 77 and name O) 3.3

(resid 77 and name HN) (resid 68 and name O) 2.3
(resid 77 and name N) (resid 68 and name O) 3.3

(resid 75 and name HN) (resid 70 and name O) 2.3
(resid 75 and name N) (resid 70 and name O) 3.3

(resid 43 and name HN) (resid 106 and name O) 2.3
(resid 43 and name N) (resid 106 and name O) 3.3

(resid 106 and name HN) (resid 43 and name O) 2.3
(resid 106 and name N) (resid 43 and name O) 3.3

(resid 45 and name HN) (resid 104 and name O) 2.3
(resid 45 and name N) (resid 104 and name O) 3.3

(resid 104 and name HN) (resid 45 and name O) 2.3
(resid 104 and name N) (resid 45 and name O) 3.3

10.7 Residual dipolar couplings

(resid 39 and name N)
(resid 39 and name HN) -0.08

(resid 40 and name N)
(resid 40 and name HN) 0.08

(resid 43 and name N)
(resid 43 and name HN) -7.48

(resid 44 and name N)
(resid 44 and name HN) -4.63

(resid 45 and name N)
(resid 45 and name HN) -4.73

(resid 46 and name N)
(resid 46 and name HN) -0.05

(resid 47 and name N)
(resid 47 and name HN) -4.16

(resid 48 and name N)
(resid 48 and name HN) -0.03

(resid 49 and name N)
(resid 49 and name HN) 0.84

(resid 52 and name N)
(resid 52 and name HN) 0.81

(resid 53 and name N)
(resid 53 and name HN) -2.3

(resid 54 and name N)
(resid 54 and name HN) -3.35

(resid 55 and name N)
(resid 55 and name HN) -0.75

(resid 56 and name N)
(resid 56 and name HN) -0.32

(resid 57 and name N)
(resid 57 and name HN) -2.21

(resid 58 and name N)
(resid 58 and name HN) -3.17

(resid 59 and name N)
(resid 59 and name HN) 6.56

(resid 61 and name N)
(resid 61 and name HN) -0.95

(resid 63 and name N)
(resid 63 and name HN) 5.32

(resid 64 and name N)
(resid 64 and name HN) 3.97

(resid 65 and name N)
(resid 65 and name HN) -9.05

(resid 66 and name N)
(resid 66 and name HN) -4.84

(resid 67 and name N)
(resid 67 and name HN) 7.45

(resid 68 and name N)
(resid 68 and name HN) -6.51

(resid 70 and name N)
(resid 70 and name HN) -2.65

(resid 73 and name N)
(resid 73 and name HN) -5.54

(resid 74 and name N)
(resid 74 and name HN) -4.49

(resid 75 and name N)
(resid 75 and name HN) 4.54

(resid 76 and name N)
(resid 76 and name HN) -6.97

(resid 77 and name N)
(resid 77 and name HN) -6.84

(resid 78 and name N)
(resid 78 and name HN) -8.94

(resid 79 and name N)
(resid 79 and name HN) -5.54

(resid 80 and name N)
(resid 80 and name HN) -6.51

(resid 81 and name N)
(resid 81 and name HN) 7.19

(resid 82 and name N)
(resid 82 and name HN) 12.08

(resid 84 and name N)
(resid 84 and name HN) 2.46

(resid 85 and name N)
(resid 85 and name HN) 4.27

(resid 86 and name N)
(resid 86 and name HN) 10.62

(resid 87 and name N)
(resid 87 and name HN) 4.84

(resid 88 and name N)
(resid 88 and name HN) -0.89

(resid 89 and name N)
(resid 89 and name HN) 5.73

(resid 90 and name N)
(resid 90 and name HN) 9.68

(resid 91 and name N)
(resid 91 and name HN) 3.27

(resid 92 and name N)
(resid 92 and name HN) 1.7

(resid 93 and name N)
(resid 93 and name HN) 12.84

(resid 94 and name N)
(resid 94 and name HN) 5.41

(resid 98 and name N)
(resid 98 and name HN) -0.19

(resid 99 and name N)
(resid 99 and name HN) -2.22

(resid 100 and name N)
(resid 100 and name HN) -4.84

(resid 101 and name N)
(resid 101 and name HN) -6.03

(resid 102 and name N)
(resid 102 and name HN) -1.22

(resid 103 and name N)
(resid 103 and name HN) -2.27

(resid 104 and name N)
(resid 104 and name HN) -5.33

(resid 106 and name N)
(resid 106 and name HN) -6.21

(resid 107 and name N)
(resid 107 and name HN) -6.89

10.8 Chemical shifts of NELF-E RRM in the complex with TAR49-57 at pH 6.9 and 30 °C

35	ALA	HN	N	8.245	126.420
35	ALA	HA	C	-100.000	-100.000
35	ALA	HB*	C	-100.000	-100.000
36	PRO	HA	C	-100.000	-100.000
36	PRO	HB1	C	-100.000	-100.000
36	PRO	HB2	C	-100.000	-100.000
36	PRO	HG1	C	-100.000	-100.000
36	PRO	HG2	C	-100.000	-100.000
36	PRO	HD1	C	-100.000	-100.000
36	PRO	HD2	C	-100.000	-100.000
37	ARG	HN	N	8.374	121.030
37	ARG	HA	C	-100.000	-100.000
37	ARG	HB1	C	-100.000	-100.000
37	ARG	HB2	C	-100.000	-100.000
37	ARG	HG1	C	-100.000	-100.000
37	ARG	HG2	C	-100.000	-100.000
37	ARG	HD1	C	-100.000	-100.000
37	ARG	HD2	C	-100.000	-100.000
37	ARG	HE1	N	-100.000	-100.000
38	LYS	HN	N	8.149	119.860
38	LYS	HA	C	4.754	54.189
38	LYS	HB1	C	2.010	35.044
38	LYS	HB2	C	1.802	35.044
38	LYS	HG1	C	1.515	-100.000
38	LYS	HG2	C	-100.000	-100.000
38	LYS	HD1	C	-100.000	-100.000
38	LYS	HD2	C	-100.000	-100.000
38	LYS	HE1	C	3.089	-100.000
38	LYS	HE2	C	-100.000	-100.000
39	GLY	HN	N	8.712	107.990
39	GLY	HA1	C	4.046	45.460
39	GLY	HA2	C	-100.000	-100.000
40	ASN	HN	N	8.301	115.650
40	ASN	HA	C	4.851	54.110
40	ASN	HB1	C	3.307	37.840
40	ASN	HB2	C	2.741	37.840
40	ASN	HD21	N	7.472	109.330
40	ASN	HD22	N	6.358	109.340
41	THR	HN	N	-100.000	-100.000
41	THR	HA	C	5.399	63.408
41	THR	HB	C	4.146	69.932
41	THR	HG2*	C	1.512	23.095
42	LEU	HN	N	9.901	127.870
42	LEU	HA	C	5.093	53.264
42	LEU	HB1	C	1.719	43.593
42	LEU	HB2	C	1.402	43.593
42	LEU	HG	C	1.911	27.040
42	LEU	HD1*	C	1.166	27.719
42	LEU	HD2*	C	1.034	25.857
43	TYR	HN	N	9.191	124.140
43	TYR	HA	C	4.421	-100.000
43	TYR	HB1	C	2.860	40.305
43	TYR	HB2	C	2.582	40.305
43	TYR	HD*	C	-100.000	-100.000
43	TYR	HE*	C	-100.000	-100.000
44	VAL	HN	N	7.976	127.360
44	VAL	HA	C	4.808	59.322
44	VAL	HB	C	1.421	34.360

44	VAL	HG1*	C	0.815	22.400
44	VAL	HG2*	C	0.544	22.057
45	TYR	HN	N	9.024	126.390
45	TYR	HA	C	4.979	55.676
45	TYR	HB1	C	2.809	42.210
45	TYR	HB2	C	-100.000	-100.000
45	TYR	HD*	C	7.104	-100.000
45	TYR	HE*	C	6.891	-100.000
46	GLY	HN	N	7.510	114.220
46	GLY	HA1	C	3.829	45.362
46	GLY	HA2	C	3.829	45.362
47	GLU	HN	N	8.747	123.880
47	GLU	HA	C	3.992	56.715
47	GLU	HB1	C	1.883	30.463
47	GLU	HB2	C	1.957	30.463
47	GLU	HG1	C	2.286	36.795
47	GLU	HG2	C	2.191	36.795
48	ASP	HN	N	8.536	117.200
48	ASP	HA	C	4.253	55.212
48	ASP	HB1	C	2.904	39.150
48	ASP	HB2	C	2.703	39.150
49	MET	HN	N	8.189	114.560
49	MET	HA	C	4.123	57.508
49	MET	HB1	C	2.040	34.618
49	MET	HB2	C	1.457	34.618
49	MET	HG1	C	-100.000	-100.000
49	MET	HG2	C	-100.000	-100.000
49	MET	HE*	C	1.613	14.682
50	THR	HN	N	6.505	108.060
50	THR	HA	C	5.017	57.962
50	THR	HB	C	4.706	70.148
50	THR	HG2*	C	1.332	21.551
51	PRO	HA	C	4.344	65.795
51	PRO	HB1	C	2.465	31.784
51	PRO	HB2	C	2.122	31.784
51	PRO	HG1	C	2.191	27.891
51	PRO	HG2	C	2.336	27.891
51	PRO	HD1	C	4.146	50.691
51	PRO	HD2	C	-100.000	50.691
52	THR	HN	N	7.635	111.520
52	THR	HA	C	3.979	66.158
52	THR	HB	C	4.100	68.434
52	THR	HG2*	C	1.314	21.773
53	LEU	HN	N	7.808	125.410
53	LEU	HA	C	4.131	58.357
53	LEU	HB1	C	1.946	42.975
53	LEU	HB2	C	1.772	42.975
53	LEU	HG	C	1.661	27.468
53	LEU	HD1*	C	1.028	25.954
53	LEU	HD2*	C	0.922	25.602
54	LEU	HN	N	7.845	117.150
54	LEU	HA	C	4.262	57.578
54	LEU	HB1	C	1.977	42.328
54	LEU	HB2	C	1.200	42.328
54	LEU	HG	C	1.790	27.147
54	LEU	HD1*	C	0.933	23.145
54	LEU	HD2*	C	0.655	26.634
55	ARG	HN	N	9.020	120.050
55	ARG	HA	C	3.876	60.888
55	ARG	HB1	C	1.953	29.577
55	ARG	HB2	C	1.953	29.577
55	ARG	HG1	C	1.820	28.960
55	ARG	HG2	C	1.626	28.960
55	ARG	HD1	C	3.303	42.907
55	ARG	HD2	C	3.202	42.907
55	ARG	HE1	N	-100.000	-100.000

56	GLY	HN	N	8.183	128.700
56	GLY	HA1	C	4.102	47.210
56	GLY	HA2	C	3.945	47.210
57	ALA	HN	N	7.833	120.270
57	ALA	HA	C	4.359	53.589
57	ALA	HB*	C	1.407	19.048
58	PHE	HN	N	8.601	110.250
58	PHE	HA	C	4.792	60.209
58	PHE	HB1	C	3.327	38.390
58	PHE	HB2	C	3.110	38.390
58	PHE	HD*	C	-100.000	-100.000
58	PHE	HE*	C	8.017	-100.000
58	PHE	HZ	C	7.084	-100.000
59	SER	HN	N	8.614	122.860
59	SER	HA	C	4.808	63.538
59	SER	HB1	C	4.329	62.913
59	SER	HB2	C	4.329	62.913
60	PRO	HA	C	4.282	65.683
60	PRO	HB1	C	0.581	31.418
60	PRO	HB2	C	2.101	31.418
60	PRO	HG1	C	1.815	28.131
60	PRO	HG2	C	1.815	28.131
60	PRO	HD1	C	3.861	52.059
60	PRO	HD2	C	3.140	52.059
61	PHE	HN	N	6.850	109.700
61	PHE	HA	C	4.507	58.310
61	PHE	HB1	C	3.455	38.916
61	PHE	HB2	C	2.921	38.916
61	PHE	HD*	C	7.337	-100.000
61	PHE	HE*	C	7.412	-100.000
61	PHE	HZ	C	7.236	-100.000
62	GLY	HN	N	7.803	128.520
62	GLY	HA1	C	4.289	45.291
62	GLY	HA2	C	4.085	45.269
63	ASN	HN	N	8.519	117.020
63	ASN	HA	C	4.922	52.932
63	ASN	HB1	C	2.936	38.628
63	ASN	HB2	C	2.936	38.628
63	ASN	HD21	N	7.833	112.710
63	ASN	HD22	N	6.951	112.710
64	ILE	HN	N	8.746	127.930
64	ILE	HA	C	4.098	61.936
64	ILE	HB	C	1.769	38.775
64	ILE	HG11	C	1.923	28.059
64	ILE	HG12	C	0.295	28.059
64	ILE	HG2*	C	0.697	17.548
64	ILE	HD1*	C	0.848	14.059
65	ILE	HN	N	8.906	124.530
65	ILE	HA	C	4.492	61.310
65	ILE	HB	C	2.000	38.474
65	ILE	HG11	C	1.161	26.702
65	ILE	HG12	C	0.847	26.702
65	ILE	HG2*	C	0.949	17.930
65	ILE	HD1*	C	0.859	13.730
66	ASP	HN	N	7.516	119.140
66	ASP	HA	C	4.704	55.521
66	ASP	HB1	C	2.733	45.294
66	ASP	HB2	C	2.352	45.294
67	LEU	HN	N	8.229	127.890
67	LEU	HA	C	4.984	54.981
67	LEU	HB1	C	1.820	44.444
67	LEU	HB2	C	1.284	44.444
67	LEU	HG	C	-100.000	-100.000
67	LEU	HD1*	C	0.822	25.094
67	LEU	HD2*	C	1.445	28.587
68	SER	HN	N	8.806	122.560

68	SER	HA	C	4.967	57.167
68	SER	HB1	C	4.048	65.319
68	SER	HB2	C	3.958	65.319
69	MET	HN	N	9.049	122.420
69	MET	HA	C	4.735	54.522
69	MET	HB1	C	-100.000	-100.000
69	MET	HB2	C	-100.000	-100.000
69	MET	HG1	C	2.634	31.907
69	MET	HG2	C	2.429	31.907
69	MET	HE*	C	1.793	15.868
70	ASP	HN	N	8.619	120.620
70	ASP	HA	C	5.139	51.406
70	ASP	HB1	C	3.590	40.931
70	ASP	HB2	C	2.283	40.931
71	PRO	HA	C	4.763	66.382
71	PRO	HB1	C	-100.000	-100.000
71	PRO	HB2	C	-100.000	-100.000
71	PRO	HG1	C	-100.000	-100.000
71	PRO	HG2	C	-100.000	-100.000
71	PRO	HD1	C	3.852	49.731
71	PRO	HD2	C	3.610	49.731
72	PRO	HA	C	4.523	65.753
72	PRO	HB1	C	2.510	31.570
72	PRO	HB2	C	1.772	31.570
72	PRO	HG1	C	2.168	-100.000
72	PRO	HG2	C	2.168	-100.000
72	PRO	HD1	C	4.024	50.219
72	PRO	HD2	C	3.750	50.219
73	ARG	HN	N	7.365	114.560
73	ARG	HA	C	4.570	55.160
73	ARG	HB1	C	1.835	29.000
73	ARG	HB2	C	1.660	29.000
73	ARG	HG1	C	2.398	31.253
73	ARG	HG2	C	-100.000	-100.000
73	ARG	HD1	C	3.420	43.104
73	ARG	HD2	C	3.358	43.104
73	ARG	HE1	N	-100.000	-100.000
74	ASN	HN	N	8.445	115.460
74	ASN	HA	C	4.266	53.834
74	ASN	HB1	C	2.785	37.502
74	ASN	HB2	C	2.442	37.502
74	ASN	HD21	N	7.177	112.830
74	ASN	HD22	N	6.991	112.830
75	CYS	HN	N	7.269	127.180
75	CYS	HA	C	5.726	55.415
75	CYS	HB1	C	2.522	32.643
75	CYS	HB2	C	-100.000	-100.000
76	ALA	HN	N	8.590	122.120
76	ALA	HA	C	-100.000	-100.000
76	ALA	HB*	C	1.012	24.402
77	PHE	HN	N	8.782	115.390
77	PHE	HA	C	5.622	55.855
77	PHE	HB1	C	-100.000	-100.000
77	PHE	HB2	C	-100.000	-100.000
77	PHE	HD*	C	-100.000	-100.000
77	PHE	HE*	C	-100.000	-100.000
77	PHE	HZ	C	-100.000	-100.000
78	VAL	HN	N	8.581	126.440
78	VAL	HA	C	4.301	60.958
78	VAL	HB	C	1.445	34.362
78	VAL	HG1*	C	0.240	20.421
78	VAL	HG2*	C	0.059	19.332
79	THR	HN	N	8.960	123.990
79	THR	HA	C	5.326	61.941
79	THR	HB	C	3.901	69.907
79	THR	HG2*	C	1.219	22.184

80	TYR	HN	N	8.883	126.670
80	TYR	HA	C	5.399	57.664
80	TYR	HB1	C	3.703	42.219
80	TYR	HB2	C	2.793	42.219
80	TYR	HD*	C	-100.000	-100.000
80	TYR	HE*	C	-100.000	-100.000
81	GLU	HN	N	8.488	119.690
81	GLU	HA	C	4.111	59.290
81	GLU	HB1	C	2.333	31.214
81	GLU	HB2	C	2.271	31.214
81	GLU	HG1	C	2.523	36.413
81	GLU	HG2	C	2.365	36.413
82	LYS	HN	N	8.461	113.920
82	LYS	HA	C	5.016	54.140
82	LYS	HB1	C	2.260	34.425
82	LYS	HB2	C	2.260	34.425
82	LYS	HG1	C	1.647	24.995
82	LYS	HG2	C	1.612	24.995
82	LYS	HD1	C	1.859	29.057
82	LYS	HD2	C	-100.000	-100.000
82	LYS	HE1	C	3.116	41.951
82	LYS	HE2	C	-100.000	-100.000
83	MET	HN	N	9.102	122.530
83	MET	HA	C	4.069	59.568
83	MET	HB1	C	2.166	32.424
83	MET	HB2	C	-100.000	-100.000
83	MET	HG1	C	2.748	32.845
83	MET	HG2	C	2.378	32.845
83	MET	HE*	C	-100.000	-100.000
84	GLU	HN	N	9.695	117.940
84	GLU	HA	C	4.236	60.041
84	GLU	HB1	C	2.143	28.643
84	GLU	HB2	C	-100.000	-100.000
84	GLU	HG1	C	2.547	36.782
84	GLU	HG2	C	2.432	36.782
85	SER	HN	N	7.110	115.500
85	SER	HA	C	4.130	61.838
85	SER	HB1	C	3.496	62.400
85	SER	HB2	C	2.541	62.400
86	ALA	HN	N	6.863	122.760
86	ALA	HA	C	4.084	55.210
86	ALA	HB*	C	1.766	18.071
87	ASP	HN	N	7.682	115.980
87	ASP	HA	C	4.389	57.467
87	ASP	HB1	C	2.813	40.852
87	ASP	HB2	C	2.743	40.852
88	GLN	HN	N	7.674	119.920
88	GLN	HA	C	4.039	58.497
88	GLN	HB1	C	2.221	28.275
88	GLN	HB2	C	2.143	28.275
88	GLN	HG1	C	2.618	33.778
88	GLN	HG2	C	2.435	33.778
88	GLN	HE21	N	7.824	111.450
88	GLN	HE22	N	6.882	111.450
89	ALA	HN	N	7.949	121.330
89	ALA	HA	C	2.796	54.933
89	ALA	HB*	C	1.537	19.343
90	VAL	HN	N	8.034	116.630
90	VAL	HA	C	3.365	67.091
90	VAL	HB	C	2.203	32.177
90	VAL	HG1*	C	1.130	21.315
90	VAL	HG2*	C	1.130	24.375
91	ALA	HN	N	7.354	119.620
91	ALA	HA	C	4.127	54.812
91	ALA	HB*	C	1.572	18.327
92	GLU	HN	N	7.962	113.770

92	GLU	HA	C	4.356	57.926
92	GLU	HB1	C	2.162	30.977
92	GLU	HB2	C	1.747	30.977
92	GLU	HG1	C	2.440	36.613
92	GLU	HG2	C	2.345	36.613
93	LEU	HN	N	8.311	114.550
93	LEU	HA	C	4.625	55.810
93	LEU	HB1	C	1.814	44.135
93	LEU	HB2	C	1.132	44.135
93	LEU	HG	C	1.413	26.769
93	LEU	HD1*	C	0.748	22.626
93	LEU	HD2*	C	0.046	25.333
94	ASN	HN	N	7.911	116.840
94	ASN	HA	C	4.434	56.689
94	ASN	HB1	C	-100.000	-100.000
94	ASN	HB2	C	-100.000	-100.000
94	ASN	HD21	N	7.926	115.400
94	ASN	HD22	N	7.208	115.400
95	GLY	HN	N	8.890	116.350
95	GLY	HA1	C	4.287	46.087
95	GLY	HA2	C	3.958	46.087
96	THR	HN	N	7.862	113.920
96	THR	HA	C	4.660	60.879
96	THR	HB	C	4.273	71.094
96	THR	HG2*	C	1.246	21.650
97	GLN	HN	N	8.365	119.550
97	GLN	HA	C	5.209	54.168
97	GLN	HB1	C	1.952	30.466
97	GLN	HB2	C	1.884	30.466
97	GLN	HG1	C	2.122	33.846
97	GLN	HG2	C	-100.000	-100.000
97	GLN	HE21	N	7.494	110.850
97	GLN	HE22	N	6.816	110.850
98	VAL	HN	N	8.898	127.260
98	VAL	HA	C	4.200	61.348
98	VAL	HB	C	1.910	33.604
98	VAL	HG1*	C	0.982	21.700
98	VAL	HG2*	C	0.911	21.100
99	GLU	HN	N	9.158	124.200
99	GLU	HA	C	3.817	57.954
99	GLU	HB1	C	2.224	27.111
99	GLU	HB2	C	-100.000	-100.000
99	GLU	HG1	C	2.325	36.410
99	GLU	HG2	C	-100.000	-100.000
100	SER	HN	N	8.330	111.660
100	SER	HA	C	4.278	59.775
100	SER	HB1	C	4.231	63.302
100	SER	HB2	C	4.032	63.302
101	VAL	HN	N	8.413	124.550
101	VAL	HA	C	4.212	62.077
101	VAL	HB	C	2.307	33.199
101	VAL	HG1*	C	0.944	21.305
101	VAL	HG2*	C	0.821	21.508
102	GLN	HN	N	8.505	127.320
102	GLN	HA	C	4.594	55.087
102	GLN	HB1	C	2.129	28.624
102	GLN	HB2	C	2.036	28.624
102	GLN	HG1	C	2.292	33.540
102	GLN	HG2	C	2.254	33.540
102	GLN	HE21	N	7.515	110.980
102	GLN	HE22	N	6.733	110.980
103	LEU	HN	N	8.714	126.610
103	LEU	HA	C	5.045	54.736
103	LEU	HB1	C	2.074	44.820
103	LEU	HB2	C	1.161	44.820
103	LEU	HG	C	1.829	26.500

103	LEU	HD1*	C	0.755	26.421
103	LEU	HD2*	C	0.683	23.965
104	LYS	HN	N	8.695	124.080
104	LYS	HA	C	5.029	55.484
104	LYS	HB1	C	2.042	34.523
104	LYS	HB2	C	2.12 9	34.523
104	LYS	HG1	C	1.520	24.900
104	LYS	HG2	C	1.770	24.900
104	LYS	HD1	C	1.802	29.445
104	LYS	HD2	C	-100.000	-100.000
104	LYS	HE1	C	3.075	41.940
104	LYS	HE2	C	-100.000	-100.000
105	VAL	HN	N	9.054	123.920
105	VAL	HA	C	5.191	60.841
105	VAL	HB	C	1.939	35.656
105	VAL	HG1*	C	1.148	24.107
105	VAL	HG2*	C	1.095	22.610
106	ASN	HN	N	9.302	122.780
106	ASN	HA	C	5.215	51.626
106	ASN	HB1	C	3.048	43.842
106	ASN	HB2	C	2.756	43.842
106	ASN	HD21	N	7.580	112.010
106	ASN	HD22	N	7.142	112.010
107	ILE	HN	N	9.053	122.980
107	ILE	HA	C	4.160	61.669
107	ILE	HB	C	2.002	36.141
107	ILE	HG11	C	1.652	-100.000
107	ILE	HG12	C	1.558	-100.000
107	ILE	HG2*	C	0.919	17.745
107	ILE	HD1*	C	0.876	10.217
108	ALA	HN	N	8.739	109.610
108	ALA	HA	C	4.776	52.621
108	ALA	HB*	C	1.848	19.568
109	ARG	HN	N	-100.000	-100.000
109	ARG	HA	C	4.393	56.117
109	ARG	HB1	C	1.921	31.007
109	ARG	HB2	C	1.830	31.007
109	ARG	HG1	C	-100.000	-100.000
109	ARG	HG2	C	-100.000	-100.000
109	ARG	HD1	C	3.242	43.268
109	ARG	HD2	C	-100.000	-100.000
109	ARG	HE1	N	-100.000	-100.000
110	LYS	HN	N	-100.000	-100.000
110	LYS	HA	C	-100.000	-100.000
110	LYS	HB1	C	-100.000	-100.000
110	LYS	HB2	C	-100.000	-100.000
110	LYS	HG1	C	-100.000	-100.000
110	LYS	HG2	C	-100.000	-100.000
110	LYS	HD1	C	-100.000	-100.000
110	LYS	HD2	C	-100.000	-100.000
110	LYS	HE1	C	-100.000	-100.000
110	LYS	HE2	C	-100.000	-100.000
111	GLN	HN	N	-100.000	-100.000
111	GLN	HA	C	4.629	55.160
111	GLN	HB1	C	2.312	32.900
111	GLN	HB2	C	1.938	32.900
111	GLN	HG1	C	2.550	33.053
111	GLN	HG2	C	2.450	33.053
111	GLN	HE21	N	-100.000	-100.000
111	GLN	HE22	N	-100.000	-100.000
112	PRO	HA	C	4.374	64.087
112	PRO	HB1	C	1.840	32.648
112	PRO	HB2	C	-100.000	-100.000
112	PRO	HG1	C	2.122	-100.000
112	PRO	HG2	C	-100.000	-100.000
112	PRO	HD1	C	3.792	-100.000

112	PRO	HD2	C	-100.000	-100.000
113	MET	HN	N	-100.000	-100.000
113	MET	HA	C	-100.000	-100.000
113	MET	HB1	C	-100.000	-100.000
113	MET	HB2	C	-100.000	-100.000
113	MET	HG1	C	-100.000	-100.000
113	MET	HG2	C	-100.000	-100.000
113	MET	HE*	C	2.087	17.484
114	LEU	HN	N	7.707	123.050
114	LEU	HA	C	4.440	-100.000
114	LEU	HB1	C	1.837	42.643
114	LEU	HB2	C	1.768	42.643
114	LEU	HG	C	-100.000	-100.000
114	LEU	HD1*	C	1.078	24.873
114	LEU	HD2*	C	1.000	24.983
115	ASP	HN	N	8.440	119.520
115	ASP	HA	C	4.605	54.621
115	ASP	HB1	C	2.770	40.719
115	ASP	HB2	C	2.674	40.719
116	ALA	HN	N	8.055	123.460
116	ALA	HA	C	4.323	53.075
116	ALA	HB*	C	1.492	54.000
117	ALA	HN	N	8.109	121.130
117	ALA	HA	C	4.645	52.597
117	ALA	HB*	C	1.553	19.359
118	THR	HN	N	8.064	112.100
118	THR	HA	C	4.434	62.134
118	THR	HB	C	4.369	69.841
118	THR	HG2*	C	1.325	21.620
119	GLY	HN	N	8.432	111.230
119	GLY	HA1	C	-100.000	-100.000
119	GLY	HA2	C	-100.000	-100.000
120	LYS	HN	N	8.203	121.180
120	LYS	HA	C	4.510	56.113
120	LYS	HB1	C	1.980	33.268
120	LYS	HB2	C	1.840	33.268
120	LYS	HG1	C	1.531	-100.000
120	LYS	HG2	C	-100.000	-100.000
120	LYS	HD1	C	-100.000	-100.000
120	LYS	HD2	C	-100.000	-100.000
120	LYS	HE1	C	-100.000	-100.000
120	LYS	HE2	C	-100.000	-100.000
121	SER	HN	N	8.094	123.030
121	SER	HA	C	-100.000	-100.000
121	SER	HB1	C	-100.000	-100.000
121	SER	HB2	C	-100.000	-100.000

10.9 An additional distance restraints used for structure determination of RNA bound NELF-E RRM

(resid	113	name	HE*)	(resid	65	name	HD1*)	3.00	(resid	113	name	HE*)	(resid	112	name	HD1)	5.00
(resid	113	name	HE*)	(resid	79	name	HG2*)	3.00	(resid	116	name	HA)	(resid	115	name	HA)	5.00
(resid	113	name	HE*)	(resid	116	name	HB*)	5.00	(resid	116	name	HA)	(resid	65	name	HD1*)	5.00
(resid	83	name	HE*)	(resid	118	name	HG2*)	4.00	(resid	118	name	HN)	(resid	117	name	HA)	4.00
(resid	83	name	HE*)	(resid	117	name	HB*)	3.00	(resid	118	name	HN)	(resid	117	name	HB*)	5.00
(resid	82	name	HA)	(resid	117	name	HB*)	3.00	(resid	116	name	HN)	(resid	115	name	HN)	4.00
(resid	83	name	HG1)	(resid	117	name	HB*)	3.00	(resid	116	name	HN)	(resid	40	name	HA)	5.00
(resid	111	name	HG1)	(resid	116	name	HB*)	5.00	(resid	116	name	HB*)	(resid	40	name	HA)	6.00
(resid	111	name	HG2)	(resid	116	name	HB*)	5.00	(resid	116	name	HN)	(resid	115	name	HA)	3.00
(resid	111	name	HG2)	(resid	79	name	HG2*)	4.00	(resid	115	name	HN)	(resid	114	name	HA)	3.00
(resid	111	name	HG1)	(resid	79	name	HG2*)	4.00	(resid	115	name	HN)	(resid	114	name	HB1)	4.00
(resid	111	name	HG1)	(resid	65	name	HD1*)	6.00	(resid	115	name	HN)	(resid	114	name	HN)	4.00
(resid	114	name	HD1*)	(resid	117	name	HB*)	5.00	(resid	117	name	HN)	(resid	115	name	HA)	5.00
(resid	114	name	HD1*)	(resid	83	name	HE*)	4.00	(resid	116	name	HB*)	(resid	117	name	HA)	4.00
(resid	118	name	HB)	(resid	117	name	HB*)	6.00	(resid	117	name	HB*)	(resid	116	name	HA)	4.00
(resid	118	name	HA)	(resid	117	name	HB*)	4.00	(resid	111	name	HB1)	(resid	79	name	HG2*)	4.00
(resid	111	name	HG1)	(resid	114	name	HB1)	5.00	(resid	111	name	HB2)	(resid	79	name	HG2*)	4.00
(resid	111	name	HG1)	(resid	114	name	HB2)	5.00	(resid	39	name	HN)	(resid	38	name	HA)	3.00
(resid	115	name	HA)	(resid	112	name	HA)	6.00	(resid	39	name	HN)	(resid	38	name	HB1)	3.00
(resid	113	name	HE*)	(resid	112	name	HA)	5.00	(resid	39	name	HN)	(resid	38	name	HB2)	3.00

10.10 Xplor protocols.

10.10.1 Generate_structure.inp

```
! generiert structure-output fuer coordinaten-generation

topology

                @../PARAMETER/topallhdg.pn

end

parameter

                @../PARAMETER/parallhdg_min.pn

end

segment
name=""
chain
@./toph19.pep
link pept head - * tail + * end
first prop tail + pro end          ! special n-ter for PRO
first nter              tail + * end
last cter head - *                      end
sequence @../PSF/nelf.seq end

end

end

delete
```

```
selection=(((resid 1:34) or (resid 114:121)) and name *)
end

write structure output=../PSF/nelf_short.psf end

stop
```

10.10.2 Generation of template coordinates

```
remarks  file  nmr/generate_template.inp
remarks  generates a "template" coordinate set.  This produces
remarks  an arbitrary extended conformation with ideal geometry.
remarks  author: Axel T. Brunger

{====>}
structure @../PSF/nelf_short.psf end              {* read structure file *}

parameter
{====>}
@../PARAMETER/parallhdg_min.pn                    {* read parameters *}
end

topology
presidue NDIS
delete bond 1SG 2SG
delete angle 1CB 1SG 2SG
delete angle 1SG 2SG 2CB
end

end

{====>}
{* if your protein contains S-S bridges appropriately modify and   *}
{* then uncomment the following lines.                             *}



vector ident (x) ( all )
vector do (x=x/10.) ( all )
vector do (y=random(0.5) ) ( all )
vector do (z=random(0.5) ) ( all )

vector do (fbeta=50) (all)          {* friction coefficient, in 1/ps *}
vector do (mass=100) (all)           {* heavy masses, in amu          *}

parameter
nbonds
cutnb=5.5 rcon=20. nbxmod=-2 repel=0.9  wmin=0.1 tolerance=1.
rexp=2 irexp=2 inhibit=0.25
end

end

flags exclude * include bond angle vdw end

minimize powell nstep=50  nprint=10 end
```

```
flags include impr end

minimize powell nstep=50 nprint=10 end

dynamics  verlet
nstep=50  timestep=0.001 iasvel=maxwell  firsttemp= 300.
tcoupling = true  tbath = 300.   nprint=50  iprfrq=0
end

parameter
nbonds
rcon=2. nbxmod=-3 repel=0.75
end

end

minimize powell nstep=100 nprint=25 end

dynamics  verlet
nstep=500  timestep=0.005 iasvel=maxwell  firsttemp= 300.
tcoupling = true  tbath = 300.   nprint=100  iprfrq=0
end

flags exclude vdw elec end
vector do (mass=1.) ( name h* )
hbuild selection=( name h* ) phistep=360 end
flags include vdw elec end

minimize powell nstep=1000 nprint=50 end

                                {* write coordinates *}

remarks produced by nmr/generate_template.inp
write coordinates output=../PSF/nelf_short.pdb end

stop
```

10.10.3 Simulated annealing protocol for structure determination

```
remarks sa_l.inp
remarks Author: Michael Nilges

evaluate ($ini_count = $StartStructure)
evaluate ($end_count = $MaxStructure)

evaluate ($iniseed = 100046)
evaluate ($iniseed2 = 554321)  !changed HS
evaluate ($iniseed3 = 204875)  !changed HS
evaluate ($iniseed4 = 395164)  !changed HS

evaluate ($init_t = 2000 )  {* initial simulated annealing temperature *}
evaluate ($high_steps = 20000)  !20000
evaluate ($cool1_steps = 30000) !30000
evaluate ($cool2_steps = 15000) !15000

evaluate ($fileroot = $WorkingDirectory + $PDB_Name)
```

```
evaluate ($template = "../PSF/nelf_short.pdb")

structure @@../PSF/nelf_short.psf end

parameter @@../PARAMETER/parallhdg_min.pn end

!parameter  nbfix S  S  462  13.6  462  13.6 end

!parameter

!   bond (name sg) (name sg) 0.0 TOKEN

!   angle (all) (name sg) (name sg)  0.0 TOKEN

!end

evaluate ($DistanceRestraint = $WorkingDirectory + $FileRestraints)

noe
reset
nrestraints = 5000                ! allocate space for NOEs
ceiling 100

class
dist @$DistanceRestraint

set echo on message on end

averaging  * sum
potential  * soft
scale      * 1.0
sqconstant * 1.0
sqexponent * 2
soexponent * 1
rswitch    * 1.0
sqoffset   * 0.0
asymptote  * 2.0
end

restraints dihedral
nassign=1000
                  @@../INPUT/phi.list
end

couplings
potential harmonic
class phi
force 1.0
nres 300

  set echo on message on end

!  @../input/coup1.list

  !set echo off message off end

end

!inserted HS
```

```
evaluate ($krama = 1.0)
evaluate ($ramacoff = 10.0)
rama
nres=10000

!set message off echo off end

!@../PARAMETER/gaussians/shortrange_gaussians.tbl
!@../PARAMETER/gaussians/new_shortrange_force.tbl
end

set message on echo on end

!@../PARAMETER/gaussians/newshortrange_setup.tbl

flags exclude * include bonds angle impr vdw noe cdih  end

set echo on message on end

@setup_swap_orig.hs

vector ident (store2) (store1)

parameter
improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
end

set echo false message false end

vector do (fbeta=10) (all)    {* friction coefficient for MD heatbath, in 1/ps *}
vector do (mass=100) (all)

evaluate ($kcdih = 5)
restraints dihedral
scale=$kcdih
end

evaluate ($count = $ini_count)
evaluate ($max_count = $end_count + $ini_count)
while ($count < $max_count ) loop main

evaluate ($count=$count+1)
evaluate ($nreassign = 0)

coor @@$template

evaluate ($seed =$count*$iniseed)

if ($count > 60) then
evaluate ($seed =($count-60)*$iniseed2)
end if

if ($count > 120) then
evaluate ($seed =($count-120)*$iniseed3)
end if

if ($count > 180) then
evaluate ($seed =($count-180)*$iniseed4)
```

```
end if

set seed $seed end
@sa_l_randomchain.xplor

evaluate ($cpu1 = $cpu)

evaluate ($final1_t = 1000)      { K }
evaluate ($final2_t = 100)      { K }
evaluate ($tempstep = 50)      { K }

evaluate ($ncycle = ($init_t-$final1_t)/$tempstep)
evaluate ($nstep = int($cool1_steps/$ncycle))

@@sa_l_initial_values.xplor

parameter
angle (store2) (all) (store2) $ini_flt TOKEN
angle (all)    (all) (store2) $ini_flt TOKEN
end

parameter nbonds
atom cutnb 12 tolerance 3.5 repel=1.2 wmin 0.5
rexp=2 irexp=2 rcon=1. Nbxmod 4
end end

@@sa_l_reduced.xplor  { defines store1 }

constraints
interaction (all) (not store1) weights * 1 angl 1.0 impr 1.0 vdw 0.0 elec 0 end
interaction (store1) (store1)  weights * 1 angl 1.0 impr 1.0 vdw 0.1 elec 0 end
end

{* 1 ================================================= initial minimization  *}
restraints dihedral   scale=5.   end
noe potential * soft scale * 1.0 asymptote * 2.0  end
minimize powell nstep=50 drop=10.  nprint=25 end

{* 2 ================================================ high temperature dynamics *}

flags include bond angl impr vdw noe cdih end

@sa_cyto_hightemp.xplor

flags include bond angl impr vdw noe cdih rama end

{* 3 ================================================ cooling 1 *}

@sa_cyto_cool1.xplor

{* 4 ================================================ cooling 2 *}

@sa_cyto_cool2.xplor

{* 5 ================================================= final minimization *}

evaluate ($swap = 1.001)
flags exclude * include noe end
```

```
@swap15v.xplor
flags include bond angl impr vdw noe cdih rama  end

minimize powell nstep=500 drop=10.0 nprint=25 end

flags exclude * include bond angl impr vdw noe cdih end

minimize powell nstep=500 drop=10.0 nprint=25 end

flags include bond angl impr vdw noe cdih rama  end

{* 6 ========================================= write out the final structure(s) *}

evaluate ($filename=$fileroot+ encode($count)+ ".pdb")
evaluate ($fname=$fileroot+ encode($count)+".prt")

print threshold=0.3 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$violations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=0.5 angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks initial random number seed: $seed
remarks ============================================================
remarks           overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ============================================================
remarks           bonds,angles,impropers,noe,cdih
remarks rms-dev.: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ============================================================
remarks             noe,  cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ============================================================
write coordinates sele= (not (resid 500)) output =$filename end

set print = $fname end
noe print thresh = 0.1 end
close $fname end

end loop main

stop
```

10.10.4 Simulated annealing protocol for the structure refinement

```
remarks sa_l.inp
remarks Author: Michael Nilges

evaluate ($ini_count =  $StartStructure)
evaluate ($end_count = $EndStructure)
evaluate ($ini_zeit = 0)
evaluate ($end_zeit = 5)

evaluate ($iniseed = 100046)
evaluate ($iniseed2 = 554321)  !changed HS
evaluate ($iniseed3 = 204875)  !changed HS
evaluate ($iniseed4 = 395164)  !changed HS
evaluate ($init_t = 1000 )  {* initial simulated annealing temperature *}
evaluate ($high_steps = 10000) !50000
evaluate ($cool_steps = 100000) !50000

evaluate ($fileroot = $WorkingDirectory + $PDB_Name)

structure @@$FilePSF end
structure @../PSF/axis_hs.psf  end

parameter @@../PARAMETER/parallhdg_min.pn end
param @../PARAMETER/para_axis_3.pro  end

evaluate ($DistanceRestraint = $WorkingDirectory + $FileRestraints)
evaluate ($DihedralRestraint = $WorkingDirectory + $FileDIH)
evaluate ($RDCRestraint = $WorkingDirectory + $FileRDC)

noe
reset
nrestraints = 6500                ! allocate space for NOEs
class      dist @$DistanceRestraint
set echo on message on end

ceiling=1000
averaging  * sum
potential  * square
scale      * 50.
sqoffset   * 0.0
sqconstant * 1.0
sqexponent * 2
rswitch    * 0.5
end

restraints dihedral
nassign=1000
@@$DihedralRestraint
end

restraints dihedral
nassign=1000
@@$DihedralRestraint
end

evaluate ($ksani = 0.01)

sani
```

```
nres=400
class JNH
force $ksani
potential harmonic
coeff 0.0 $Dab $Rhom
@$RDCRestraint
end

sani class JNH force 0 end

parameter                    {*Parameters for the repulsive energy term.*}
nbonds
repel=0.75                {*Initial value for repel--modified later.*}
rexp=2 irexp=2 rcon=1.
nbxmod=3
wmin=0.01
cutnb=4.5 ctonnb=2.99 ctofnb=3.
tolerance=0.5
end
end

restraints dihedral
scale=5.
end

set echo off message off end

evaluate ($krama = 1.0)
evaluate ($ramacoff = 10.0)
rama
nres=10000
set message off echo off end
!@../PARAMETER/gaussians/shortrange_gaussians.tbl
!@../PARAMETER/gaussians/new_shortrange_force.tbl
end

set message on echo on end

!@../PARAMETER/gaussians/newshortrange_setup.tbl

flags exclude * include bonds angle impr vdw noe cdih coup sani harm rama end

set echo on message on end

@setup_swap_orig.hs

vector ident (store2) (store1)

parameter
improper (store2) (store2) (all) (all) 0.0 TOKEN TOKEN
improper (all) (all) (store2) (store2) 0.0 TOKEN TOKEN
end

evaluate ($count = $ini_count)
while ($count < $end_count ) loop main
evaluate ($count=$count+1)
evaluate ($nreassign = 0)
evaluate ($e_prev = 10000)
evaluate ($e_act = 10000)
```

```
evaluate ($template = $FileTemplate+ encode($count)+ ".pdb")
evaluate ($zeit = $ini_zeit)
while ($zeit < $end_zeit ) loop intra
evaluate ($zeit=$zeit+1)
evaluate ($nreassign = 0)

coor @@$template
coor @../PSF/axis.pdb

evaluate ($seed =($count+$zeit)*$iniseed)

if ($count > 60) then
evaluate ($seed =($count-60)*$iniseed2)
end if

if ($count > 120) then
evaluate ($seed =($count-120)*$iniseed3)
end if

if ($count > 180) then
evaluate ($seed =($count-180)*$iniseed4)
end if

set seed $seed end

{*Friction coefficient for MD heatbath, in 1/ps.   *}
vector do (fbeta=10) (all)

{*Uniform heavy masses to speed molecular dynamics.*}
vector do (mass=100) (not (resid 500 or resid 600))
vector do (mass  = 30.0) (resid 500 or resid 600)

! Fixing the axis using harmonic restraint
! leave out, let both rotate

vector do (refx=x) (all)
vector do (refy=y) (all)
vector do (refz=z) (all)

constraints fix ((resid 500 or resid 600) and name OO) end

!Original part from RNA-refinement protocol

vector do (vx=maxwell($init_t)) (all)
vector do (vy=maxwell($init_t)) (all)
vector do (vz=maxwell($init_t)) (all)

!swap adopted from Nilges Protocol

evaluate ($swap = 1.001)
flags exclude * include noe end
@swap15v.xplor
flags exclude * include bond angl impr vdw noe cdih coup sani harm rama end

restraints dihedral   scale=5.   end
noe asymptote * 1.0  end

constraints interaction
(all) (all) weights * 1 end end
```

```
!   minimize powell nstep=500 drop=10.  nprint=25 end

{* ========================================== High-temperature dynamics.*}

minimize powell nstep=500 drop=10.  nprint=25 end

constraints interaction (all) (all)
weights * 1  end end

eval ($init_kcdih=50)
eval ($end_kcdih=55)
eval ($cdihstep=1)
eval ($kcdih=49)
eval ($ncycle=($end_kcdih-$kcdih)/$cdihstep)
eval ($nstep= int($high_steps/$ncycle))
eval ($i_cdih=0)

while ($i_cdih < $ncycle) loop cdih1
eval ($i_cdih = $i_cdih+1)
eval ($kcdih = $kcdih+$cdihstep)

restraints dihedral scale=$kcdih end

dynamics  verlet
nstep=$nstep    timestep=0.0005   iasvel=current   firstt=$init_t
tcoupling=true  tbath=$init_t  nprint=50  iprfrq=0
end

end loop cdih1

constraints interaction (all) (all) weights * 1 end end

restraints dihedral   scale=50.   end

evaluate ($final_t = 300)      { K }
evaluate ($tempstep = 25)      { K }

evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
evaluate ($nstep = int($cool_steps/$ncycle))

evaluate ($ini_rad  = 0.9)        evaluate ($fin_rad  = 0.75)
evaluate ($ini_con=  0.003)       evaluate ($fin_con=  4.0)

evaluate ($ini_sani = 0.01)         evaluate ($fin_sani = 1.0)
evaluate ($sani_fac = ($fin_sani/$ini_sani)^(1/$ncycle))
evaluate ($ksani = $ini_sani)
sani class JNH force $ksani end

evaluate ($bath  = $init_t)
evaluate ($k_vdw = $ini_con)
evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
evaluate ($radius=    $ini_rad)
evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

evaluate ($i_cool = 0)
while ($i_cool < $ncycle) loop cool
evaluate ($i_cool=$i_cool+1)

evaluate ($bath  = $bath  - $tempstep)
```

```
evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
evaluate ($radius=max($fin_rad,$radius*$radfact))
evaluate ($ksani  = $ksani*$sani_fac)

sani class JNH force $ksani end

parameter  nbonds repel=$radius   end end
constraints interaction (not name SG) (all)
weights * 1. vdw $k_vdw end end

dynamics  verlet
nstep=$nstep time=0.0005 iasvel=current firstt=$bath
tcoup=true tbath=$bath nprint=$nstep iprfrq=0
end

evaluate ($critical=$temp/$bath)

if ($critical >  10. ) then
display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
stop

end if  end loop cool

{* ================================================== Final minimization.*}

constraints interaction (all) (all) weights * 1. vdw 1. end end

parameter
nbonds
repel=0.75              !changed HS, original value 0.80
rexp=2 irexp=2 rcon=1.
nbxmod=3
wmin=0.01
cutnb=6.0 ctonnb=2.99 ctofnb=3.
tolerance=1.5
end
end

minimize powell nstep=200 drop=10.0 nprint=25 end

flags exclude * include bond angl impr vdw noe cdih coup sani harm rama end

minimize powell nstep=1000 drop=10.0 nprint=25 end

flags exclude * include bond angl impr vdw noe cdih coup sani harm end

sani class JNH force 0 end

{* 6 ======================================= write out the final structure(s) *}

evaluate ($filename=$fileroot+ encode($count)+ ".pdb")
evaluate ($outname=$fileroot+ encode($count)+ ".axis")
evaluate ($fname=$fileroot+ encode($count)+".prt")
evaluate ($sname=$fileroot+ encode($count)+".sani")
evaluate ($dname=$fileroot+ encode($count)+".dih")
evaluate ($angname=$fileroot+ encode($count)+".ang")

print threshold=0.3 noe
evaluate ($rms_noe=$result)
```

```
evaluate ($violations_noe=$violations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=0.5 angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks initial random number seed: $seed
remarks =============================================================
remarks          overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih, $sani
remarks =============================================================
remarks          bonds,angles,impropers,noe,cdih
remarks rms-dev.: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks =============================================================
remarks            noe,  cdih
remarks violations.: $violations_noe, $violations_cdih
remarks =============================================================

evaluate ($e_act = $ener)

if ($e_act < $e_prev) then

evaluate ($e_prev = $e_act)
write coordinates sele= (resid 500) output =$outname end
write coordinates sele= (not (resid 500)) output =$filename end
set print = $fname end
noe print thresh = 0.1 end
close $fname end

set print = $sname end
sani print thresh = 0.0 end
close $sname end

set print = $dname end
print thresh = 0.001 cdih end
close $dname end

set print = $angname end
print thresh = 0.8 angles end
close $angname end

end if

end loop intra

end loop main

stop
```

11 Acknowledgements

The work presented in this thesis was done under the dynamic guidance of Prof. Dr. Paul Rösch at the Department of Structure and Chemistry of Biopolymers of the University of Bayreuth, Germany, in the period between Sep 2003 and Aug 2006. I would like to express heartfelt thanks to my supervisor Prof. Dr. Paul Rösch for excellent research facilities, his confidence and the freedom to pursue the projects with own ideas, helpful discussions, creation of friendly working environment and unrestricted support in all concerns.

I would like to express my deepest sense of gratitude to Prof. Dr. Birgitta Wöhrl for her help and valuable discussions during my entire thesis work. I owe her thanks for being the first one to introduce me the practical aspects of molecular biology.

I am very thankful to Dr. Kristian Schweimer for introducing me to the fascinating world of protein NMR spectroscopy and subsequently helping me in the NMR data acquisition, analysis and valuable discussions.

I would like to acknowledge Liane Neumann for the cloning, expression and purification of NELF-E RRM, Dr. Stephan Schwarzinger for his timely help, scientific and general discussions, Rainer Hofmann for helping me in the computational work and friendly discussions, Andrea Hager for ordering chemicals, and lab technicians Ulrike Herzing, Nadine Herz and Ramona Heißmann for vitro transcription of RNA and Katrin Weiß for the protein expression and purification.

I would like to thank Gudrun Wagner, Angela Rösler, and Violaine Zigan for their help in administration work during my entire stay here

My thanks to all present and former members of the Department for being very helpful to me. Particularly, Dr. Finn Baur, Dr. Anke Eisenmann, and Dr. Klaus Vitzithum for giving me valuable advises.

Finally I thank my parents for ensuring that I have a meaningful education, and whose love and sacrifices can never be repaid.

My apologies to the others who I have not mentioned by name, I am indebted to them for the many ways they helped me.

12 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Bayreuth, den

Nageswara Rao Jampani