Induction of z-conformation in ribosomal RNA by Z-DNA binding domain Z α of the human double-stranded RNA deaminase I (ADAR1)

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**INDUCTION OF Z-CONFORMATION IN RIBOSOMAL
RNA BY Z-DNA BINDING DOMAIN Z α OF THE HUMAN
DOUBLE-STRANDED RNA DEAMINASE I (ADAR1)**

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2012

**Induction of Z-conformation in ribosomal RNA by
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double-stranded RNA deaminase I (ADAR1)**

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ABBREVIATIONS

aa	amino acid
ADAR	adenosine deaminase acting on RNA
Bp	base pair
DNA	deoxyribonucleic acid
DSS	2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt
DTT	dithiothreitol
ds/ss	double strand/single strand
EDTA	ethylene glycol-bis (β -aminoethylether)-N, N' tetraacetic acid
FPLC	fast protein liquid chromatography
HSQC	heteronuclear single quantum coherence
IPTG	Isopropyl β -D-1-Thiogalactopyranoside
kDa	kilo Daltons
LB	Luria-Bertani
MS	mass spectrometry
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
nt	nucleotide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride

RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TROSY	transverse relaxation optimized spectroscopy
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

ABSTRACT

The Z-DNA/Z-RNA binding domain $Z\alpha$ of the human RNA editing enzyme double-stranded RNA deaminase I (ADAR1) is found to influence various biological functions, such as the DNA-mediated innate immune response, and transcriptional modulation of gene expression. Recently our collaborators have discovered that $Z\alpha_{ADAR1}$ binds stably to ribosomes inside *E. coli* and human cells leading to translational inhibition *in vitro* and *in vivo* (Feng, Li et al. 2011). We contributed to this fundamental discovery by demonstrating an involvement of the Z-RNA conformation in this process (Feng, Li et al. 2011). As a means to characterize the induction of Z-RNA we employed solution NMR spectroscopy. We determined 3D solution structures of $Z\alpha_{ADAR1}$ and $Z\alpha_{ADAR1}$ mutant (N43A, Y47A) and compared binding modes of these two proteins to Z-DNA, short (5-32 residues) dsRNA oligonucleotides derived from rRNA and *E.coli* ribosomes. We found that $Z\alpha_{ADAR1}$ has the ability to interact with Z-DNA, short dsRNA from rRNA and *E.coli* ribosomes with sufficiently high affinity and induce the B- to Z- or A- to Z- conformational changes in DNA and RNA, respectively. $Z\alpha_{ADAR1}$ mut also has the ability to interact with DNA and RNA, but with significantly lower affinity comparing to its wild type. $Z\alpha_{ADAR1}$ mut also lacks the ability to induce Z-conformation in DNA and RNA. We also mapped the binding interfaces of $Z\alpha_{ADAR1}$ to Z-DNA, Z-RNA and *E.coli* ribosomes, and found them to be identical. Based on this finding, we proposed that the role of $Z\alpha_{ADAR1}$ in binding to ribosomes and inhibiting translation is to recognize potentially Z-forming structures and/or induce localized structural transitions from A- to Z-conformations in ribosomal RNA.

In order to provide theoretical foundations for the analysis of NMR spectral responses of $Z\alpha_{\text{ADAR1}}$ to ribosomes we attempted to extend the conventional NMR relaxation theory to include a semi solid-state treatment of nuclear spin dynamics in large complexes. The main challenge to the classical NMR theory arises when one of the binding partners is so large that in the chemical shift time scale of solution NMR (milliseconds) it can be considered as practically static or solid-state-like. Therefore, our theoretical model shall predict whether the chemical shifts perturbations observed through the small binding partner can be used to delineate the involved binding interface, and whether changes of the positions of NMR resonances or only attenuation of the cross-peak intensities are to be expected upon binding to a solid partner.

I Introduction

Human beings are exposed to millions of potential pathogens every day, which induces lots of infectious diseases. Nowadays, infectious diseases cause about one-third of all human deaths in the world. The immune system functions to protect organisms from infections.

There are two major subdivisions of the human immune system, namely the innate immune system and the adaptive immune system. The innate immune system is a primary defense mechanism and is found in all classes of plants and animals. It acts nonspecifically by creating a barrier for pathogens and provides immediate but short-term protections.

In contrast the adaptive immune system, also known as the specific immune system, provides long-term effect, but requires longer time to be active against infections. It generates antibodies to specific kinds of pathogens to eliminate infection at the onset, and to protect organisms from re-exposure to the same pathogens (Bruce Alberts 2002).

I.1 The innate immune system

Generally, there are two working mechanisms of the innate immune system. The first one includes physical and chemical anatomical barriers, like human skin, mucus, and the acidic pH of the stomach, preventing pathogens from getting into a human body. The second mechanism includes recruitment of phagocytic cells and cellular intrinsic responses. The phagocytic cells such as

nertrophils, macrophages, and natural killer cells etc., recognize conserved features of pathogens and ingest microorganisms and foreign particles (Bruce Alberts 2002). The host cells have the ability to degrade single- and double-stranded RNA, which are common intermediates in viral replication. The ADAR family proteins play an important role in RNA modulation processes (Maas, Melcher et al. 1997).

I.2 The ADAR family

Double-stranded RNA deaminase (ADAR) is a protein family that acts on double-stranded regions of RNA to convert adenosine to inosine, which is then translated as guanosine (Maas, Melcher et al. 1997). This process leads to the site-specific substitution of one amino acid to another, thus altering the functional properties of a protein (Herbert, Schade et al. 1998). Adenosine to inosine (A- to -I) editing was shown to modulate the calcium permeability of neural glutamate receptors and reduce the G-protein coupling efficacy of serotonin 2C receptors (Sommer, Kohler et al. 1991; Burns, Chu et al. 1997). ADARs are involved in both non-specific and site-specific A- to -I editing. Extensive adenosine to inosine editing was reported for various virus infections and is one possible antiviral defense mechanism by disrupting viral open reading frames (ORF). The ADARs play important roles during innate immune response, exhibit antiviral activities, and are involved in apoptosis, embryogenesis, increasing neurotransmitter receptors diversity, as well as interfering with RNAi pathway (Toth, Zhang et al. 2006).

The ADAR family contains three members, ADAR1, ADAR2 and ADAR3.

ADAR1 is the first human family member isolated, and is ubiquitously expressed in all tissues that have been tested. ADAR1 maps to chromosome 1q21 and contains three dsRNA binding domains and a nuclear localization signal (Kim, Wang et al. 1994; Wang, Zeng et al. 1995). Subsequently, another mammalian enzyme, ADAR2, has been found to be 23% identical to ADAR1 amino acid sequence and contains a 54 amino acids N-terminal extension which includes an arginine-rich motif (O'Connell, Krause et al. 1995). ADAR2 is expressed in many tissues and is catalytically active (Melcher, Maas et al. 1996; O'Connell, Gerber et al. 1997). Human ADAR2 is encoded on chromosome 21q22.3 and has two dsRNA binding domains as well as the characteristic deaminase domain (Mittaz, Scott et al. 1997). ADAR3 localizes to chromosome 10p15 and contains an arginine- and lysine-rich domain that interacts with ssRNA and also has two dsRNA binding domains (Chen, Cho et al. 2000). It is expressed solely in the brain. Unlike ADAR1 and ADAR2, which are functional adenosine deaminases, human ADAR3, and its rat homologue RED2, lack deaminase activity (Chen, Cho et al. 2000).

Figure 1. The primary structures of ADARs from several species (Keegan, Leroy et al. 2004).

The Z-DNA binding domains (Z α and Z β), double-stranded RNA binding motif (DSRM) and adenosine deaminase motif (ADEAM) are shown. The hADAR1-S was translated starting at M296 in hADAR1-L. The lengths of ADARs are indicated at the right side. aa: amino acid.

ADARs are mostly conserved in their adenosine deaminase domains, but vary in dsRNA binding motifs and Z-DNA binding motifs (Figure 1) (Keegan, Leroy et al. 2004). According to Patterson and Samuel (Patterson and Samuel 1995), two different forms of ADAR1 proteins are expressed from the human ADAR1 gene: a 150 kDa interferon (IFN)-inducible form ADAE1-L (p150) and a smaller 110 kDa form ADAR1-S (p110). It was demonstrated that the constitutively expressed ADAR1-S was localized predominantly in or on the nucleus, whereas the IFN-inducible ADAR1-L was localized both in the cytoplasm and in the nucleus (Patterson and Samuel 1995). Both of the two proteins contain three dsRNA binding domains and one deaminase domain. Compared to ADAR1-S, ADAR1-L contains two additional Z-DNA binding domains. ADAR1-L has both functional nuclear localization signals (NLSs) and nuclear/nucleolus localization signal (NLS/NloS). Thus the full length ADAR1-L shows the characteristics of a shuttling protein (Eckmann, Neunteufl et al. 2001; Strehblow, Hallegger et al. 2002).

I.3 Z-DNA conformations

I.3.1 Structure of Z-DNA

DNA has been found to adopt three types of continuous major secondary structures, namely A- , B- and Z-DNA forms. A-DNA and B-DNA are both right-handed uniform double-helical structures, while Z-DNA is a left-handed double helix with a dinucleotide repeat and the backbone follows a zigzag pattern (Figure 2) (Ghosh and Bansal 2003). In living cells, B-DNA is the main conformation (Richmond and Davey 2003), although the first determined single-crystal X-ray structure of a DNA fragment was in Z-DNA form, as it was designated later (Wang, Quigley et al. 1979).

Z-DNA is a left-handed double helix with two anti-parallel strands held together by Watson-Crick base pairing. The sugar-phosphate backbone of Z-DNA shows a zigzag arrangement due to alternation of glycosidic bond linking bases and sugars between anti- and syn- conformations (Crawford, Kolpak et al. 1980). In contrast, all glycosidic bonds of B-DNA are in the anti-conformation, ensuring that the phosphate groups follow a smooth right-handed arc (Figure 2).

Figure 2. The structures of three distinct DNA conformations: A-, B-, and Z- conformation (Ghosh and Bansal 2003).

The nucleotides are colour-coded (cytosine in yellow, guanine in cyan, thymine in green and adenine in red).

Z-DNA conformations occur most readily in alternative purine/pyrimidine repeat sequences, as purines adopt the syn- conformation more readily than pyrimidines, especially with alternating $d(GC)_n$ repeats (Malfoy, Rousseau et al. 1986; Johnston 1992) and $d(GT)_n$ repeats. Z-DNA could also form in non-purine/pyrimidine repeat sequences if co-crystallized with Z-DNA binding domain (Ha, Choi et al. 2009).

Circular dichroism (CD) measurement and 1D ^{31}P NMR experiment were used to study Z-DNA structures (Pohl and Jovin 1972). In CD spectrum, the high value at around 260 nm and the low value around 290 nm indicate the Z-DNA conformation. The shift of the negative peak from 260 nm to around 290 nm was used to identify B- to Z- transitions (Figure 3) (Herbert, Alfken et al. 1997; Herbert, Schade et al. 1998; Kim, Hwang et al. 2009).

Figure 3. CD spectra of $d(CG)_n$ in B-conformation and change to Z-conformation in the presence of increasing amounts of Z α peptide.(Herbert, Alfken et al. 1997)

Numbers next to each spectra (solid lines) reflect the molar ratio of base pairs to peptide. The spectra obtained with DNA alone (B-conformation) and DNA in the presence of 10 mM $MgCl_2$ (Z-conformation) are also shown as reference. The conformations of $d(CG)_n$ base pairs gradually changed from B-conformation to Z-conformation with the decreasing molar ratio of base pairs to Z α peptide.

I.3.2 Existence of Z-DNA

Since Z-DNA conformation is energetically unfavored comparing with B-DNA conformation, high salt, high pressure or high temperature will be needed to promote Z-DNA formation *in vitro* (Pohl and Jovin 1972; Thamann, Lord et al. 1981; Irikura, Tidor et al. 1985). Z-DNA conformation could also be stabilized by DNA modifications such as methylation or bromination (Moller, Nordheim et al. 1984), or other agents that screen electrostatic repulsion between electronegative phosphate residues, which are located in close proximity in the Z-conformation (Rich, Nordheim et al. 1984).

The Z-DNA conformations exist *in vivo* in a dynamic state rather than a static state due to the requirement of the Gibbs free energy (Wittig, Dorbic et al. 1991). It has been found that Z-DNA conformations could be stabilized *in vivo* by negative supercoiling occurred during transcription (Liu and Wang 1987; Wittig, Dorbic et al. 1991). Supercoiling happened when additional twist was added to or subtracted from the DNA double helix. Negative supercoils can facilitate the energy required for B- to Z- DNA transition, thus play important role in stabilizing left-handed Z-DNA (Wittig, Dorbic et al. 1991). During transcription, the RNA polymerase creates positive supercoiled DNA ahead of it and negative supercoiled DNA behind it along the DNA template. Hence, Z-DNA is transiently induced and stabilized by this negative supercoiling behind the moving RNA polymerase. The negative supercoiled DNA is released quickly by topoisomerases, and reverts to double helix B-DNA (Figure 4) (Liu and Wang 1987; Wittig, Dorbic et al. 1991; Wittig, Wolfl et al. 1992; Wolfl, Martinez et al. 1996; Koster, Crut et al. 2010). This short-time existed

Z-DNA was also detected by Z-DNA-binding proteins (Nordheim, Tesser et al. 1982; Wittig, Dorbic et al. 1991).

Figure 4. A graphical illustration of DNA supercoils driven by transcription. (Koster, Crut et al. 2010)

The RNA polymerase is moving from left to right direction, uncoiling the double helix at the position where RNA polymerase interacts with DNA. This creates positive supercoils (+) in the template DNA ahead of it and leaves negative supercoils (-) behind it.

I.3.3 Biological function of Z-DNA

The biological role of Z-DNA is an area of active research. It has been shown that Z-DNA plays important roles in biological processes such as positive and negative transcriptional regulation (Nordheim and Rich 1983; Naylor and Clark 1990), general DNA recombination (Blaho and Wells 1987; Wahls, Wallace et al. 1990; Herbert and Rich 1999), as well as information propagation throughout the genomic DNA and genome integrity (Li, Xiao et al. 2009).

I.4 Z-RNA conformations

I.4.1 Structure of Z-RNA

A Z-DNA-like form could also be found in RNAs when Z-DNA forming sequences were transcribed into RNAs, which by analogy can be designated as Z-RNA. Although the low-energy forms of right-handed B-DNA and A-RNA are in very different structures, they adopt similar left-handed Z-conformations which can be seen in the crystal structures (Nakamura, Fujii et al. 1985; Teng, Liaw et al. 1989; Davis, Adamiak et al. 1990).

I.4.2 Existence of Z-RNA

The transition from the right-handed duplex of A-RNA to the left-handed Z-form is energetically less favorable than the B- to-Z DNA transition. Consequently, higher salt concentrations or lower dielectric solvents combined with higher temperatures are required to induce the transition *in vitro* than the equivalent B-to-Z transition of DNA (Klump and Jovin 1987). Experimental evidences showed that chemical bromination of poly[r(C-G)] could stabilize synthetic Z-RNA under physiological conditions (Hardin, Zarling et al. 1987). Z-RNA was

found to exist in both cytoplasm and nuclei in fixed cells, by using anti Z-RNA monoclonal antibody (Zarling, Calhoun et al. 1987). Cytoplasmic microinjection of Z-RNA antibodies could inhibit human cell growth (Zarling, Calhoun et al. 1990).

I.4.3 Biological function of Z-RNA

While the existence of Z-RNA conformations *in vivo* and *in vitro* is clearly proven via nuclear magnetic resonance (NMR), CD, absorption spectroscopy and X-ray crystallographic studies as mentioned above, the functional role of Z-RNA is less established (Rich and Zhang 2003). Structural studies showed that the conformation of Z-RNA was similar to that of Z-DNA. Z-RNA was also found to be immunogenic. Staining experiments with Z-RNA-specific antibodies showed that the antibody bound to fixed protozoan cells that were visualized by immunofluorescence microscopy. The antibodies were mostly found in the cytoplasm, which indicated that some cytoplasmic sequences existed as Z-RNA. Cytoplasmic microinjection of anti Z-RNA antibodies was found to inhibit cell multiplication. A possible physiological role for Z-RNA was suggested recently by (Brown, Lowenhaupt et al. 2000), who found that the Z-DNA-binding domain of ADAR1 could bind to Z-RNA and Z-DNA with similar affinity. Certain RNA viruses that replicate in the cytoplasm undergo considerable changes in sequence, which were probably the consequence of hyper-editing by ADAR1. Sequence analysis of the virus found in measles encephalitis showed that the RNA undergoes many edits: adenines are replaced by guanines, and uracils by cytosines. So, this virus has been extensively hyper-edited by the editing enzyme. Full length ADAR1 that

contains the Z-DNA-binding domain is up-regulated by the interferon response of the cell, which is triggered by the measles virus. Furthermore, ADAR1 accumulates in the cytoplasm where the measles virus replicates. So, it is possible that $Z\alpha$ ADAR1 binds to negatively torsionally strained double-stranded RNA, which might form during viral replication, targeting the editing enzyme to this site. The Z-DNA/Z-RNA-binding domain might have a role in the attempts of cells to inactivate the invading virus.

1.5 $Z\alpha_{ADAR1}$ domain and its interaction with Z-DNA and Z-RNA

Within ADAR family, only ADAR1-L contains two Z-DNA specific binding domains at its N terminus, named $Z\alpha$ and $Z\beta$. Z-DNA binding domains, which belong to the helix-turn-helix (HTH) family of proteins, have been shown to be conserved in human, rat, bovine, chicken and xenopus ADAR1 (Herbert, Alfken et al. 1997), suggesting its importance for the biological function of ADAR1. Figure 5 illustrated a possible model for the regulation of ADAR1 activity by Z-DNA. After Z-DNA was stabilized by the negative supercoiling generated by transcription behind a RNA polymerase, Z-DNA binding domain of ADAR1 recognized this short existed Z-DNA and bound to it with nanomolar affinity. The regulation function of ADAR1 was then activated. Transcription created a nascent RNA (pre-mRNA) and may form dsRNA when a pre-mRNA folds back on itself. The activated ADAR1 then initiated A-to-I modification on this dsRNA.

Figure 5. Model of the regulation function of ADAR1 activated by recognition of Z-DNA. (Herbert and Rich 1999)

Z-DNA formed behind a RNA polymerase, and stabilized by the negative supercoiling generated by transcription. ADAR1 recognized and bound to Z-DNA by its Z-DNA binding domain. Meantime, ADAR1 interacted with pre-mRNA created by RNA polymerase and regulated the A- to-I modification on this double stranded pre-mRNA

Z α _{ADAR1} alone is able to bind to Z conformation of DNA with very high affinity as shown by a band-shift assay (Herbert and Rich 1993). It strongly binds to Z-DNA even though there is a 10,000-fold mass excess of nonspecific B-DNA competitor around (Herbert, Spitzner et al. 1993; Herbert, Alfken et al. 1997). Z α _{ADAR1} can also form a complex with slightly different binding properties when interacting with Z β (Herbert, Schade et al. 1998; Schwartz, Lowenhaupt et al. 1999). By analytical ultracentrifugation and CD spectroscopy, it has been found that two Z α _{ADAR1} domains bind to one d(CGCGCG)₂ hairpin containing a stem of six base pairs in the Z-DNA conformation with two-fold symmetry with respect to the DNA helical axis (Schade, Behlke et al. 1999). Z α _{ADAR1} could induce and stabilize the transition of B- to Z-conformation at favorable sequences via an active B – Z transition mechanism in which one Z α _{ADAR1} first binds to B-DNA and then converts it to left-handed Z-DNA, the whole conformation is then stabilized by the binding of the second Z α _{ADAR1} molecule (Schade, Turner et al. 1999; Schwartz, Rould et al. 1999; Kang, Bang et al. 2009).

Recently, crystal structures of Z α _{ADAR1} with several DNA segments revealed that Z α _{ADAR1} binds to Z-formed DNAs of non-CG repeat DNA duplexes as well as d(CGCGCG)₂ in a well conserved binding mode, suggesting that Z-DNAs are recognized by Z α _{ADAR1} through common conformational features instead of the specific sequence and structural features (Herbert, Schade et al. 1998; Ha, Choi et al. 2009). Later on, a detailed analysis showed that Z α _{ADAR1} exhibits the sequence preference of d(CGCGCG)₂ > d(CACGTG)₂ > d(CGTACG)₂ through multiple sequence discrimination steps (Seo, Ahn et al. 2010).

Although both right-handed duplexes, A-RNA and B-DNA, adopt different conformational properties, the left-handed Z-form duplexes are similar (Nakamura, Fujii et al. 1985; Teng, Liaw et al. 1989; Davis, Adamiak et al. 1990). Mutations in Z α in ADAR1-L decreases the efficiency of editing of short dsRNA substrates (Herbert and Rich 2001), and the editing effect is greatly enhanced when a Z-RNA favored forming sequence is used as substrate (Koeris, Funke et al. 2005). The induction of ADAR1-L expression by interferon and inflammation, but not other ADARs, implies the special involvement of Z-DNA binding domains in anti-viral defense and inflammation.

Not only Z-DNA, Z-RNA could also be recognized and stabilized by Z α domain of ADAR1 *in vitro* (Brown, Lowenhaupt et al. 2000). Previous research found that Z α _{ADAR1} could induce a slow transition from right-handed A-form to the left-handed Z-form in duplex *r*(CG)₆ with a high activation energy of 38 kcal mol⁻¹, and the dsRNA immediately converts back to the A-form after dissociation, which indicates that Z α _{ADAR1} could not only recognize Z-RNA and convert the favorable dsRNA sequence from A-form to Z-form but also stabilize the dsRNA in the left-handed Z-conformation (Brown, Lowenhaupt et al. 2000). This conformational transition can be observed by shifts in the Raman spectrum and characteristic changes in the CD spectrum. The structure of Z α _{ADAR1} and a *dUr*(CG)₃ duplex RNA has been determined in a co-crystallized Z α _{ADAR1}-RNA complex (Placido, Brown et al. 2007). The structural analysis of Z α _{ADAR1}-RNA complex showed that Z α _{ADAR1} binds to the Z-RNA in the similar rule of the interaction with Z-DNA, in which two Z α _{ADAR1} monomers bind to one Z-RNA duplex and each Z α _{ADAR1} monomer interacts with a single RNA strand.

The overall conformational features and the interaction mode are well conserved between $Z\alpha_{\text{ADAR1}}$ -RNA and $Z\alpha_{\text{ADAR1}}$ -DNA complexes.

I.6 Structures of $Z\alpha_{\text{ADAR1}}$ domains and the complex with Z-DNA and Z-RNA

The binding of $Z\alpha_{\text{ADAR1}}$ to Z-DNA structure is very tight with a K_d of 4 nM. $Z\alpha_{\text{ADAR1}}$ could induce and stabilize the transition of B- to Z-conformation at favorable sequences (Schade, Turner et al. 1999; Schwartz, Rould et al. 1999). In the binding surface, extensive hydrogen bonds are formed between amino acid residues in $\alpha 3$ and $\beta 3$ and five consecutive phosphates in the DNA backbone with and without mediation of water, as shown in Figure 6. In addition to polar interactions, close van der Waals contacts are observed between the two perpendicular aromatic ring of Tyr177 and G4 base.

Figure 6. The binding surface between Z α_{ADAR1} and Z-DNA.(Schwartz, Rould et al. 1999)

A. Stereo-view from the axis orientation of helix $\alpha 3$. Extensive hydrogen bondings are indicated in the interface with or without mediation of water (green). The aromatic ring of Tyr177 is perpendicular with G4 base and involved in close van der Waals interaction. B. Schema of the binding surface. The residues critical to Z-DNA specific binding are listed and the interactions were showed. Dashed line stands for hydrogen bond and open circle for van der Waals contact.

G4 is in syn- conformation which is characteristic of Z-DNA. Pro192 and Pro193 in $\beta 3$ form yet another important van der Waals contact with the DNA backbone. In contrast to the predominant trans- peptide configuration, Pro192 forms a cis- peptide bond, rendering its uniqueness and importance in the binding. These residues interacting with DNA are highly conserved within known homologues of $Z\alpha_{\text{ADAR1}}$.

The crystal structures of $(Z\alpha_{\text{ADAR1}})_2$ -RNA complex show that $Z\alpha_{\text{ADAR1}}$ binding with Z-RNA shares many similarities with that of Z-DNA with respect to the 3-D structural features (Figure 7).

A

B

Figure 7. $Z\alpha_{ADAR1}$ binds to Z-DNA and Z-RNA.

$Z\alpha_{ADAR1}$ was labeled in light-green and showed as cartoon, while Z-DNA and Z-RNA were labeled in wheat and displayed as line. A. $Z\alpha_{ADAR1}$ binds to Z-DNA (PDB:1QBJ); B. $Z\alpha_{ADAR1}$ binds to Z-RNA (PDB: 2GXB)

Recently, it was found that $Z\alpha_{\text{ADAR1}}$ binds stably to ribosomes inside *E. coli* and human cells, and leads to translational inhibition *in vitro* and *in vivo* (Feng, Li et al. 2011). Comparing with a control probe, named $Z\alpha_{\text{ADAR1}} \text{ mut}$ (N43A, Y47A), which shares the very similar tertiary structures with $Z\alpha_{\text{ADAR1}}$ but fails to interact with RNA, $Z\alpha_{\text{ADAR1}}$ strongly binds to specific ribosomal RNA (rRNA) segments on *E.coli* ribosomes during a pull-down assay. After eluted, amplified and cloned for sequencing, a 17-mers RNA segment was found to present in high copy numbers in these RNA segments. NMR and CD measurements of $Z\alpha_{\text{ADAR1}}$ -rRNA complex suggest that $Z\alpha_{\text{ADAR1}}$ induces a transition of rRNA segment from A-form to Z-form during interaction.

I.7 Other Z-DNA binding domains

$Z\alpha_{\text{ADAR1}}$ belongs to a large helix-turn-helix family, and helix-turn-helix is a major motif capable of binding DNA. Since the discovery of $Z\alpha_{\text{ADAR1}}$, other Z-DNA binding proteins were discovered. These Z-DNA binding proteins have sequence similarity with $Z\alpha_{\text{ADAR1}}$ and interact with Z-DNA in a manner similar to $Z\alpha_{\text{ADAR1}}$ (Figure 8). DLM1 participates in the interferon-response pathway and is up-regulated in tissues bearing tumors (Fu, Comella et al. 1999). Co-crystallization of $Z\alpha_{\text{DLM1}}$ -Z-DNA complex showed that Z-DNA interacts with $Z\alpha_{\text{DLM1}}$ through the similar protein-DNA binding interface as $Z\alpha_{\text{ADAR1}}$ (Schwartz, Behlke et al. 2001). E3L, which is from vaccinia virus, acts as an inhibitor of interferon-response pathway, also contains Z-DNA binding domain at the N-terminal. NMR analysis showed the complex structure of $Z\alpha_{\text{E3L}}$ -Z-DNA, and revealed that the Z-DNA binding interface of $Z\alpha_{\text{E3}}$ is very similar to $Z\alpha_{\text{ADAR1}}$ (Kim, Muralinath et al. 2003; Kahmann, Wecking et al. 2004).

Figure 8. Sequence conservation among the Z-DNA binding domains from different proteins.(Schwartz, Behlke et al. 2001)

Z-DNA binding domains are aligned based on their amino acid sequences. Helices and strands are represented in the top by tubes and arrows, respectively. Residues conserved among these Z-DNA binding domains are highlighted in yellow. The sequences are as follows: DLM-1 from *Mus musculus* is mZa_{DLM} (GenBank accession number (acc: AF136520) and from *Homo sapiens*, hZa_{DLM} (acc: AJ300575); ADAR1 from *H. sapiens* is hZa_{ADAR} (acc: U18121); from *M. musculus*, mZa_{ADAR} (acc: AF052506); from *Rattus norvegicus*, rZa_{ADAR} (acc: U18942); from *Xenopus laevis*, xZa_{ADAR} (acc: U88065); from *Danio rerio*, dZa_{ADAR} (acc: AF124332); from *Fugu rubripes*, fZa_{ADAR} (acc: AAF69764); and E3L from *vaccinia virus* is vacZa_{E3L} (acc: S64006); from *Yaba-like disease virus*, yabZa_{E3L} (acc: AJ293568); and from *orf virus*, orfZa_{E3L} (acc: CAA10952).

II NMR methods to study Protein/RNA interactions

II.1 Kinetics of protein/RNA binding and associated conformational change dictates the choice of NMR methods.

In this thesis we limit the scope of the considered topics to the protein/double stranded RNA interactions as they manifest through chemical shifts perturbations. The review of the other NMR methods like direct or transferred NOEs is beyond the scope of this thesis. In particular, we focus on the two relevant cases: (i) a small protein (e.g. Z α) interacting with relatively short (5-32 nucleotides) dsRNA oligonucleotide and (ii) a small protein interacting with extremely large (relative to the protein) RNA complex such as functionally competent ribosome. Clearly, in both cases the choice of suitable NMR experiments and the corresponding analysis is dictated by the kinetic rates of binding and associated conformational changes. A typical goal of an NMR study is to determine full 3D configurations of the individual binding partners in the resulting complex when sufficiently high thermodynamic stability of that complex and slow complex dissociation rate are available or at least delineate binding interfaces of the interacting partners if the binding thermodynamics and kinetics are unfavorable. An impressive contemporary example of the structural investigation of the 3D structure of a complex between a protein and a double-stranded RNA is provided by studies of the recognition of the AGNN tetraloop RNA fold by the dsRNA-binding domain of Rnt1p RNase III (Wu, Henras et al. 2004; Gaudin, Ghazal et al. 2006). Multiple examples of the delineating binding interfaces are provided in this review (Schwalbe, Buck et al. 2007).

Solution NMR studies of interactions involving large molecules and assemblies is a very challenging area of research very frequently dwelling on the limits of applicability of the conventional relaxation theory of solution NMR (Foster, McElroy et al. 2007). In our opinion, the NMR spectroscopic manifestations of the binding of Z α to the functional ribosome deserves a separate analytical treatment, which is provided in the Results section of the thesis.

For the relatively small protein/ligand complexes a conventional chemical or conformational exchange treatment of NMR observables even in the cases of sparsely populated states is employed (Clore 2011). In the process of ligand binding to a small protein, the local environment around the binding sites changes, thus the chemical shift of the nucleus, which is extremely sensitive to the chemical environment, changes accordingly at the binding sites. The term exchange refers to the phenomenon that a nucleus exchanges between two or more environment in which its NMR parameters differ.

Conformational exchange, also named intramolecular exchange, is induced by motions of side-chains in proteins, unfolding of proteins, helix-coil transitions of nucleic acid or conformational equilibria. Proteins are dynamic systems with majority of the protein ensemble having the conformation at the low energy ground state (Frauenfelder, Sligar et al. 1991). However, they may undergo conformational transition to additional high energy sub-states which are important for some specific biological functions (Wand 2001; Kern and Zuiderweg 2003; Kraut, Carroll et al. 2003; Guo, Tolstoy et al. 2011). Figure 9 shows a conformational exchange between the active and inactive states of

the signaling protein NtrC (Kern and Zuiderweg 2003) .

Figure 9. An example of the conformational exchange among the active (orange) and inactive (blue) states of protein NtrC.(Kern and Zuiderweg 2003)

Much more complex exchange processes are theoretically expected and detected in RNA (Emani, Olsen et al. 2010; Bothe, Nikolova et al. 2011). Functional RNA molecules are conformationally dynamic and sample a multitude of dynamic modes over a wide range of frequencies. Thus, a comprehensive description of RNA dynamics requires the inclusion of a broad range of motions across multiple dynamic rates derived from multiple spectroscopies, as exemplified by NMR studies of non-rigid rotational motions in the HIV-1 transactivation response element (TAR) RNA in solution. The proposed theoretical models are shown to unify the experimental results generated by solution and solid-state NMR and provide a comprehensive view of the dynamics of HIV-1 TAR RNA, a well-known paradigm of an RNA where function requires extensive conformational rearrangements of this classical RNA-ligand interaction.

Chemical exchange is the process which involves the making and breaking of

chemical bonds, either by binding of small molecule to macromolecules, isotope exchange processes, or enzyme catalyzed reactions. Although no breakage of the chemical bonds is involved, we nevertheless group RNA binding to a protein under this category of exchange since the change in the local chemical environment of the involved spins is perturbed. When a ligand is added to a protein, the chemical shifts are perturbed with the increasing concentration of the ligand. These chemical shift perturbations are used to map the binding interface (Foster, Wuttke et al. 1998; Qin, Vinogradova et al. 2001; Zuiderweg 2002). Information in regards to interaction could be found out in a simple Heteronuclear Single-Quantum Correlation (HSQC) NMR experiment during titration process, like the chemical shift perturbations and line shape.

For illustrative purposes, we review a very basic, actually the simplest model of a chemical exchange process and discuss its implications for the NMR spectral features. To describe chemical exchange mechanism, we take the simplest two-site exchange system for consideration, in which a nucleus spin exchanges between A and B.

k_1 is the forward kinetic rate constant, and k_{-1} is the backward kinetic rate constant. The exchange rate in this system (k_{ex}) is defined by the sum of k_1 and k_{-1} . The nucleus spin has different local magnetization in each state, giving rise to different chemical shift frequencies, ω_A and ω_B , respectively. We have the chemical shift difference frequency between these two states ($\Delta\omega$), which

is determined by the absolute value of $(\omega_A - \omega_B)$. The magnetization in each state could be calculated through the approximate solutions of the Bloch-McConnell equations (Abergel and Palmer 2004).

To explain the kinetic properties in chemical exchange process, we consider not only the chemical shift perturbations ($\Delta\omega$), but also line shape. An important parameter of line shape is line width (full-width-at-half-high, λ), which is proportional to transverse relaxation rate (R_2). In chemical exchange process, the spin density operator corresponding to state A not only gives rise to a peak at resonance frequency of ω_A , but also undergoes excessive transverse relaxation due to chemical exchange with state B (R_{ex}). R_{ex} is proportional to the products of kinetic exchange rate and chemical shift perturbation.

Chemical exchange can be classified into three regimes based on the k_{ex} comparing with $\Delta\omega$: that is slow exchange when $k_{ex} \ll |\Delta\omega|$; intermediate exchange, $k_{ex} \sim |\Delta\omega|$; and fast exchange when $k_{ex} \gg |\Delta\omega|$ (Bain 1998). The chemical shift and line width of these chemical exchange regimes have different properties in spectra. In slow exchange, two peaks at frequency ω_A and ω_B appear according to the two states A and B, and the area of each peak is proportional to its concentration in the system. In fast exchange, a single peak comes out at the frequency between ω_A and ω_B , where the position is given by the mean of ω_A and ω_B , weighted by the concentration of the two states in the system. In intermediate exchange, a single peak appears at the same position as in fast exchange with the broadened line width. Figure 10

shows the effect of chemical exchange regimes on line shape.

Figure 10. Effect of chemical exchange on line shape.(Gordon Rule 2005)

The populations of both states were set to 1/2. The frequency difference of two states was set to 40 Hz. The exchange rates differ from 1 sec^{-1} to 10^4 sec^{-1} from A to E, respectively, which indicated the effects of chemical exchange on line shape from slow exchange to fast exchange.

The main challenge to those simplified NMR manifestations of the exchange processes arises when one of the binding partners is so large that in the chemical shift time scale of solution NMR (mili-seconds) it can be considered as practically static or solid state like. Thus, the real challenge is to develop a theory capable of predicting NMR observables in the case when a small ligand

(a protein $Z\alpha$ in our case) can be treated using the classical solution state Bloch-McConnell theory and Bloch-Wangsness-Redfield relaxation theory (http://dpwww.epfl.ch/cours/spin_dyn_final/bloch-wangsness-redfield.htm) and its binding partner is practically static or solid-state like thus being outside of the limits of the applicability of both theories. In particular, it will be critical to predict whether the chemical shifts perturbations observed through the small binding partner can be used to delineate binding interface, whether changes of the positions of NMR resonances or only attenuation of the cross-peak intensities are to be expected upon binding to a solid partner. Our attempt at constructing such a theory can be found in the Results section.

III Materials and Methods

III.1 Cloning and Expression of Z α ADAR1 and Z α ADAR1 mut

III.1.1 Cloning of Z α _{ADAR1} and Z α _{ADAR1} mut

Construction of expression vectors for Z α _{ADAR1} and Z α _{ADAR1} mut has been described in previous experiments (Li, Xiao et al. 2009). Briefly, the Z α _{ADAR1} domain, comprising residues 132-209 of human ADAR1, was cloned to the expression plasmid pET-17b with a FLAG-tag and Strep-tag II at its C-terminus. Mutations were introduced at N173A and Y177A (N43A, Y47A in our study) by assembly PCR and cloned into the same expression plasmid pET-17b as well as another expression plasmid pET-28a with a His-tag and a Thrombin cleavage site at its N-terminus.

III.1.2 Expression of Z α _{ADAR1} and Z α _{ADAR1} mut

For expression of Z α _{ADAR1} domain, the combined plasmid pET17b-Z α -FS was transformed into *E. coli* strain Rosetta (DE3) by electroporation, and cells were recovered for 30 mins at 37 °C. Bacteria were plated on LB agar with 34 ug/ml chloramphenicol and 100 ug/ml ampicillin and incubated overnight at 37 °C. A single colony was inoculated into 20 ml M9 minimal media contained 34 ug/ml chloramphenicol and 100 ug/ml ampicillin. After shaking overnight at 37 °C in a flask, the culture was diluted 50 times into M9 minimal media supplemented with 34 ug/ml chloramphenicol and 100 ug/ml ampicillin. For uniformly isotope labeling, the M9 minimal media contains 1 g/liter ¹⁵NH₄Cl and 2 g/liter ¹³C-glucose. Bacteria were grown at 37 °C shaking at 200rpm. Protein expression was induced at an optical density at 600 nm (OD₆₀₀) of 0.8 - 1.0 with 1 mM isopropyl thiogalactosidase (IPTG) at 20 °C. After overnight

incubation at 20 °C, cells were harvested by centrifugation at 4000 g for 15 min. The pellet was stored at -80 °C. Z α ADAR1mut containing FLAG-Strep-tag was expressed under the same conditions. Z α ADAR1mut containing His-tag was expressed in the same condition and procedure except using 30 ug/ml kanamycin instead of ampicillin.

III.1.3 Expression of deuterated Z α ADAR1

For expression of deuterated Z α ADAR1 domain, the host *E.coli* strain Rosetta (DE3) was freshly transformed. A single colony was inoculated into 5 ml unlabeled LB media containing 34 ug/ml chloramphenicol and 100 ug/ml ampicillin. After incubated overnight at 37 °C, cells were collected by centrifugation at 5,000 g and resuspended in 200 ml labeling M9 minimal media containing 100% D₂O supplemented with 1 g/liter ¹⁵NH₄Cl, and grown at 37 °C till OD₆₀₀ to 1.0. Subsequently, cells were diluted 5 times to total 1 liter deuterated labeling media. Protein overexpression was induced by 1 mM IPTG at OD₆₀₀ 0.8-1.0 at 20 °C. After overnight incubation at 20 °C, cells were harvested by centrifugation and stored at -80 °C.

III.2 Protein purification

III.2.1 Purification of Z α ADAR1 and Z α ADAR1 mut with FLAG-StreptII-tag

For Z α ADAR1 purification, cell pellet from 1 L M9 minimal media was resuspended in 20 ml HEPES resuspension buffer (50 mM HEPES, 500 mM NaCl, 0.125 mM PMSF, 1 mM DTT, pH 7.4), and sonicated (2 s on, 3 s off, power 24%) for 30 mins to perform cell lysis. Cell lysate was then centrifuged at 20,000 g for 30 mins at 4 °C. The supernatant containing the recombinant

proteins was collected and then affinity purified via 3 ml Strep-Tactin sepharose resins (IBA GmbH, Germany), and eluted with ST-elution buffer (50 mM HEPES, 50 mM NaCl, 2.5 mM desthiobiotin, pH7.4). After dialysis against the cation chromatography buffer (50 mM HEPES, 50 mM NaCl, 1 mM DTT, 0.125 mM PMSF, pH 7.4), the eluted protein was further purified through a cation exchange chromatography column (HiTrapTM SP HP Columns, GE Healthcare), and eluted with a 50-1000 mM NaCl gradient. Subsequently, protein fractions were combined and subjected to a size-exclusion column (HiLoadTM 16/60 Superdex 200 prep grade, Bio-Rad) with protein sample buffer (50 mM HEPES, 50mM NaCl, pH 6.8). Protein concentration was determined by spectrophotometer (NanoDrop ND-1000 Spectrophotometer). Deuterated Z α _{ADAR1} and Z α _{ADAR1} mut with FLAG-StrepII-tag proteins were expressed and purified as the same procedure described above.

III.2.2 Purification of Z α _{ADAR1} mut with His-tag

For purification of Z α _{ADAR1} mut with His-tag, cell pellet from 1 L M9 minimal media was resuspended in 20 ml Tris resuspension buffer (20 mM Tris-HCl, 150 mM NaCl, 0.125 mM PMSF, 10 mM β -mercaptoethanol, pH 8.0). After the same sonication and centrifugation procedure as Z α _{ADAR1}, the supernatant was then loaded onto a Ni-NTA column (HisTrapTM FF Columns, GE Healthcare), and eluted with the same buffer with 300 mM imidazole. After dialysis against the resuspension buffer to remove imidazole, the (His)₆-tag was removed by thrombin digestion at room temperature for 3 hours. For the second purification step, the protein was dialyzed into the same cation chromatography buffer (50 mM HEPES, 50 mM NaCl, 1 mM DTT, 0.125 mM PMSF, pH 7.4), and went

through the same cation exchange chromatography purification procedure as $Z\alpha_{\text{ADAR1}}$.

III.3 Expression and purification of *E.coli* ribosomes

The method of expressing *E.coli* ribosomes has been described Ederth's paper (Ederth, Mandava et al. 2009). Briefly, with the insertion of a (His)₆-tag at the C-terminus of the ribosomal protein L12, the *E. coli* strain JE28 was cultured in LB with 50 µg/ml kanamycin at 37 °C, and harvested by centrifugation when OD₆₀₀ reached 1.0. The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 10 mM MgCl₂, 150 mM KCl, 30 mM NH₄Cl, 200 µl/l PMSF, 0.5 mg/ml lysozyme, 10 µg/ml DNase I, pH 7.6), and lysed by sonication. After centrifuging at 18,000 rpm at 4 °C, the supernatant was affinity purified using a HisTrap column (HisTrapTM FF Columns, GE Healthcare) and eluted with 150 mM imidazole. Furthermore, the ribosomes were concentrated by ultracentrifugation, resuspended in polymix buffer (5 mM NH₄Cl, 95 mM KCl, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate and 1mM dithioerythritol) and shock-frozen in liquid nitrogen for storage. The purity of the expressed *E.coli* ribosomes was examined by agarose gel electrophoresis.

III.4 NMR Spectroscopy

III.4.1 Structure determination of $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1}}$ mut

NMR experiment were carried out on a 0.3 mM sample in buffer containing 50 mM HEPES, 50 mM NaCl, 5% D₂O, pH 6.8 at 25 °C on a 700 MHz Bruker

Avance II spectrometer, using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard. ^1H , ^{15}N and ^{13}C resonance assignments were obtained from the two-dimensional (2D) and three-dimensional (3D) heteronuclear correlation experiments, ^1H - ^{15}N transverse relaxation optimized spectroscopy (TROSY) and TROSY-HNCACB, respectively. Interproton distance restraints were derived from 3D ^{15}N -TROSY-nuclear Overhauser effect spectroscopy (NOESY). The chemical shifts were referenced to DSS directly for ^1H and indirectly for ^{13}C and ^{15}N spins. Spectra were processed using Topspin 2.1 and analyzed with CARA (www.nmr.ch). Peaks were manually picked from 3D ^{15}N -TROSY-NOESY spectrum. The peak list and the chemical shift assignments were used as the input for structure calculations by CYANA 2.0. .

III.4.2 Interaction Mapping with DNA fragment

Z forming DNA fragment $\text{d}(\text{CG})_3\text{T}_4(\text{CG})_3$ (referred as Z-DNA afterwards) was synthesized in Prof. Phan Ann Tuan's lab. For interaction mapping, 2D ^{15}N -TROSY spectra and 1D ^{31}P spectra were recorded on free $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1}}$ -DNA complex in HEPES buffer (50 mM HEPES, pH 6.8, 300mM NaCl, 5% D_2O) at 25 °C using Bruker Avance II spectrometers operating at 700 and 400 MHz respectively. 2D ^{15}N -TROSY spectra and 1D ^{31}P spectra of free $\text{Z}\alpha_{\text{ADAR1}}$ mut and $\text{Z}\alpha_{\text{ADAR1}}$ mut-DNA complex were recorded in the same condition.

III.4.3 Interaction Mapping with rRNA

17 mer rRNA fragment (5'-AUGGGGUGACUGCGUAC-3') and 30 mer rRNA

fragment (5'-CAGUGGGAGCCUCUAUGGGGUGACUGCGUA-3') were synthesized by external company. These rRNA fragments were derived from previous research. Briefly, recombinant plasmids pET17b - Z α _{ADAR1} and pET17b - Z α _{ADAR1} mut were transformed into *E.coli* strain BL21 (DE3) and cultured in LB media. Cell pellets were collected at OD₆₀₀ 0.9 and sonicated to make lysate. The protein-ribosomes complexes were purified using Strep-Tactin sepharose beads. The pulled-down material was then digested with RNase, and the RNA fragments which were still bound to Z α _{ADAR1} were eluted, PCR-amplified and cloned for sequencing. For interaction mapping, 2D ¹⁵N-TROSY spectra and 1D ³¹P spectra were recorded on free Z α _{ADAR1} and Z α _{ADAR1}-rRNA complex in buffer E (50 mM HEPES, pH 6.8, 50 – 300 mM NaCl, 5% D₂O) at 25 °C using Bruker Avance II spectrometers operating at 700 and 400 MHz respectively . 2D ¹⁵N-TROSY spectra and 1D ³¹P spectra of free Z α _{ADAR1} mut and Z α _{ADAR1} mut-rRNA complex were recorded in the same manner. Spectra were processed using Topspin 2.1 and analyzed using CARA.

III.4.4 Interaction Mapping with *E.coli* ribosomes

Several 2D TROSY spectra were recorded to follow the titration of 70S ribosomes binding with Z α _{ADAR1} and Z α _{ADAR1} mut at 25 °C using Bruker Avance II spectrometers operating at 700 MHz. These spectra were recorded in HEPES buffer (50 mM HEPES, 2 mM MgCl₂, 5% D₂O, pH 6.8) at different salt conditions from 50 mM NaCl to 300 mM NaCl.

IV Results and Discussion

IV.1 Expression and Purification of Z α _{ADAR1} and Z α _{ADAR1} mut

For protein expression, the fragment encoding Z α domain of human ADAR1 (amino acids 132-209) was inserted into a pET-17b expression vector which contains a FLAG-tag and a Strep-tag II at the C-terminus of the inserted fragment after a flexible linker GGSGG. The FLAG-tag is well known for its property of being recognized by an antibody, and proteins containing FLAG-tag were used for functional studies done by our collaborators. The Strep-tag II is used in future purification. The flexible linker serves to link the protein with the tag, and allows the protein to maintain flexibility and undergo conformational change during binding assay (Zaman and Kaazempur-Mofrad 2004; Li and Hazelbauer 2006). The fragment of Z α _{ADAR1} mut was inserted into the same plasmid as Z α _{ADAR1}, as well as a pET-28a vector which has a 6xHis-tag at the N-terminus of the inserted fragment. Both Strep-tag II and His-tag are necessary for the primary purification. The fragment encoding Z α _{ADAR1} mut also contains a Thrombin cleavage site at its N-terminus, which is useful in getting the pure protein without any tag attached on it.

These two recombinant vectors were expressed well in *E. coli* strain Rosetta (DE3) in M9 minimal media. High yield protein was produced after induction with IPTG. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed the protein fractions during expression and purification (Figure 11). It has been found that, when induced at 37 °C, both of the two proteins went partially into the insoluble protein fractions. However, when the induction temperature decreased to 20 °C, both of them appeared in the soluble part and no target

protein can be found in the pellet. So we expressed these two proteins at 20 °C and increased time to 16 h (overnight) to get high yield soluble proteins.

Both of the two proteins can be separated well from the cell supernatant in the first purification process, through Strep-Tactin sepharose resins and Ni-NTA column, respectively. After further cation exchange chromatography purification procedure, the target proteins were homogeneous and indicated by SDS-PAGE (Figure 11). Purification through size-exclusion column was applied if the proteins still contained impurities after cation exchange chromatography purification procedure.

In the Thrombin cleavage process, the amount of enzyme, the incubation time and temperature were optimized to be 1.5 u enzyme / mg protein for 3 hours at room temperature. The His-tag was partially cleaved when using lower amount of enzyme or at 4 °C, and the protein itself was also unspecifically cleaved by the enzyme when incubated longer than 3 hours at room temperature. The final yield of proteins is 2 mg ZαADAR1 / liter M9 media, 1 mg ZαADAR1 mut / liter M9 media.

Figure 11. Analysis of Z α_{ADAR1} and Z α_{ADAR1} mutant on SDS-PAGE.

Lane 1-6 are for Z α_{ADAR1} : lane 1, cell culture before induction; lane 2, cell culture after induction; lane 3, pellet after sonication; lane 4, supernatant after sonication; lane 5, collection after Strep-Tactin sepharose resins; lane 6, collection after cation exchange column. Lane 7-9 are for Z α_{ADAR1} mut: lane 7, collection after Ni-NTA purification; lane 8, protein product after thrombin cleavage; lane 9, collection after cation exchange column; MW: Molecular Weight.

IV.2 Expression and purification of *E.coli* ribosomes

To express *E.coli* ribosomes, we used recombinant *E. coli* strain JE28 containing a (His)₆-tag at the C-terminus of the ribosomal protein L12, and cultured in LB media. After centrifugation, the cell pellet was resuspended and purified through a Ni-NTA column and further by ultracentrifugation. The affinity-purified JE28 ribosomes were homogeneous 70S and indicated by agarose gel electrophoresis (Figure 12).

Figure 12. Agarose gel of purified *E.coli* ribosomes.

Clear bands indicated 30S and 50S ribosomal subunits, 700 bp and 1200 bp respectively, according to the DNA ladder.

IV.3 Structure Determination of Z α _{ADAR1} and Z α _{ADAR1} mut

The solution structure of Z α ADAR1 was resolved years ago, but no assignments were provided (Schade, Turner et al. 1999). In our study, we did NMR experiments on Z α ADAR1 (residues 132-209 of human ADAR1) and Z α ADAR1 mut (N43A, Y47A), and determined their solution structures. All the spectra were recorded at protein concentration of 0.3 mM at 25 °C. 1D ¹H NMR spectra of Z α ADAR1 and Z α ADAR1 mut showed well folded structures (Figure 13), and the narrow line width cross-peaks could be seen in 2D TROSY spectra (Figure 14). The data was analyzed by CARR. 3D TROSY-HNCACB was used for backbone assignment and HSQC-NOESY was for the side-chain assignment.

Figure 13. 1D ^1H NMR spectra of $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1 mut}}$

A. 1D ^1H NMR spectrum of $\text{Z}\alpha_{\text{ADAR1}}$; B. 1D ^1H NMR spectrum of $\text{Z}\alpha_{\text{ADAR1 mut}}$; C. 1D ^1H NMR overlapped amide proton region of $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1 mut}}$ (above: $\text{Z}\alpha_{\text{ADAR1 mut}}$; below: $\text{Z}\alpha_{\text{ADAR1}}$).

Figure 14. 2D ^{15}N -TROSY spectrum of $\text{Z}\alpha_{\text{ADAR1}}$

TROSY spectra of ^{15}N - ^{13}C -labeled $\text{Z}\alpha_{\text{ADAR1}}$ recorded on a 0.3 mM sample in buffer containing 50 mM HEPES, 50 mM NaCl, 10% D_2O , pH 6.8 at 25 °C on 700 MHz NMR spectrometer.

The pattern of hydrogen bonds is derived from the characteristic secondary chemical shifts of $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ spins locating the respective residues within α -helical or β -sheet regions, and verified against H-bonds found in the X-ray structures. We followed an established procedure described in detail here (Guntert 2004). The inclusion of H-bond related constraints resulted in improved regularity of the secondary structures in NMR ensemble. We admit that we used indirect way to establish H-bond pattern not rooted in the direct experimental data such as scalar coupling across H-bonds or temperature variation of the chemical shifts, since we expected very close similarity between the wild type and the mutant of $\text{Z}\alpha_{\text{ADAR1}}$, and the availability of NMR and X-ray structures for the wild type.

The structure of $\text{Z}\alpha_{\text{ADAR1}}$ was defined using 699 inter-residue and 162 intra-residue constraints, with a backbone Root Mean Square Deviation (RMSD) of 0.89 Å for the 20 conformers, and all of residues had backbone dihedral angles (ϕ and ψ) mapped to the most favored and additional allowed regions of the Ramachandran plot (table 1). The structure of $\text{Z}\alpha_{\text{ADAR1}}$ mut was defined using 641 inter-residue and 131 intra-residue constraints, with a backbone RMSD of 0.83 Å for the 20 conformers, and 98.2% of residues had backbone dihedral angles (ϕ and ψ) mapped to the most favored and additional allowed regions, and 1.8% in generously allowed regions of the Ramachandran plot (table 2. PDB: 2L54, RCSB: RCSB101968).

Table 1. NMR and refinement statistics for Z α _{ADAR1} structure

	Protein
NMR distance and dihedral constraints	
Distance constraints	
Total NOE	861
Intra-residue	162
Inter-residue	699
Sequential ($ i - j = 1$)	479
Medium-range ($ i - j < 4$)	167
Long-range ($ i - j > 5$)	53
Hydrogen bonds	27
Total dihedral angle restraints	
ϕ	55
ψ	55
Structure statistics	
Violations (mean and s.d.)	
Distance constraints (Å)	0.031±0.003
Dihedral angle constraints (°)	0.603±0.086
Max. dihedral angle violation (°)	0.7
Max. distance constraint violation (Å)	0.1
Average pairwise r.m.s. deviation** (Å)	
Heavy	1.48±0.37
Backbone	0.89±0.33
Ramachandran plot	
Most favored	89.4%
Additionally allowed	10.6%
Generously allowed	0.0%
Disallowed	0.0%

**Pairwise r.m.s. deviation was calculated among 20 refined structures.

Table 2. NMR and refinement statistics for Z α _{ADAR1} mut structure

	Protein
NMR distance and dihedral constraints	
Distance constraints	
Total NOE	772
Intra-residue	131
Inter-residue	641
Sequential ($ i - j = 1$)	369
Medium-range ($ i - j < 4$)	199
Long-range ($ i - j > 5$)	73
Hydrogen bonds	25
Total dihedral angle restraints	
ϕ	56
ψ	56
Structure statistics	
Violations (mean and s.d.)	
Distance constraints (Å)	0.038±0.002
Dihedral angle constraints (°)	0.943±0.062
Max. dihedral angle violation (°)	1.0
Max. distance constraint violation (Å)	0.1
Average pairwise r.m.s. deviation** (Å)	
Heavy	1.32±0.31
Backbone	0.83±0.33
Ramachandran plot	
Most favored	83.8%
Additionally allowed	14.4%
Generously allowed	1.8%
Disallowed	0.0%

**Pairwise r.m.s. deviation was calculated among 20 refined structures.

The structure of $Z\alpha_{\text{ADAR1}}$ consists of three α -helices (designated $\alpha 1$ (Q7 - E19), $\alpha 2$ (A28 - L35), and $\alpha 3$ (K39 - K52), respectively) and three β -strands (designated $\beta 1$ (G23 - T27), $\beta 2$ (K54 – G60) and $\beta 3$ (P63 – A68)). The $\alpha 2$ and $\alpha 3$ helices were jointed together by a short strand containing 3 amino acids indicating a helix-turn-helix (HTH) motif, which has the function of binding DNA and regulating gene expression in lots of proteins. $\alpha 1$ is oriented perpendicular to $\alpha 3$ and connects to the HTH motif through $\beta 1$. $\beta 2$ and $\beta 3$ locate at the C-terminal and are antiparallel to each other. $\beta 1$, which is oriented almost perpendicular to the C-terminal $\beta 2$ and $\beta 3$, contact $\beta 3$ through two hydrogen bonds between T26 and W65 (Figure 17).

IV.4 Structure comparison between Z α_{ADAR1} and Z α_{ADAR1} mut

Comparing with Z α_{ADAR1} , the backbone chemical shifts detected of Z α_{ADAR1} mut in TROSY only slightly deviate at N43, Y47 (N173, Y177 in ADAR1), which may indicate that the conformation of Z α_{ADAR1} mut is only slightly affected by the mutations (Figure 15). Since the FS tag of Z α_{ADAR1} is not cleaved, TROSY of Z α_{ADAR1} contains additional peaks which cannot be seen in spectrum of Z α_{ADAR1} mut. The small cross-peaks in the middle of Z α_{ADAR1} mut spectrum indicated that part of Z α_{ADAR1} mut got precipitate during the experiments. This situation was probably due to the His-tag cleavage process in protein purification, while a few Z α_{ADAR1} mut molecules in solution got unfolded after removing the tag. Since the cross-peaks corresponding to FS tag were separated well from cross-peaks corresponding to protein, we decided to use FS-Z α_{ADAR1} mut instead of His-tag-cleaved Z α_{ADAR1} mut in future titration experiments.

Figure 15. 2D ^{15}N -TROSY overlapped spectra of $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1}}$ mut.

Spectrum of $\text{Z}\alpha_{\text{ADAR1}}$ was indicated in red and spectrum of $\text{Z}\alpha_{\text{ADAR1}}$ mut was indicated in blue. The mutations were circled and showed as N43 and Y47. The spectrum of $\text{Z}\alpha_{\text{ADAR1}}$ mut was nearly identical to spectrum of $\text{Z}\alpha_{\text{ADAR1}}$, indicating the similar structures of these two proteins.

Direct comparison of $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts (Figure 16) reveal that $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1}}$ mut exhibit very similar secondary structures. The similar tertiary structures of the wild type and mutant are confirmed by reconstructed 3D structures (Figure 17). These indicate that the failure of recognizing Z-DNA by $\text{Z}\alpha_{\text{ADAR1}}$ mut is not due to the conformational change of the whole protein by mutation, but due to the substitutions of the two key residues in the Z-DNA-binding interface.

Figure 16. Comparison of chemical shifts between $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1 mut}}$.

A. Difference in ^1HN chemical shifts. The resonances corresponding to N43A and Y47A are indicated. B and C, secondary $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts detected in $\text{Z}\alpha_{\text{ADAR1}}$ (blue) and $\text{Z}\alpha_{\text{ADAR1 mut}}$ (red), respectively. Secondary shifts are the differences between experimentally observed values and the corresponding chemical shifts typically found in random coil proteins (Schwarzinger, Kroon et al. 2000).

Figure 17. Solution structure of Zα_{ADAR1} and Zα_{ADAR1} mut with mutation sites indicated.

A and B. The superposition of the NMR representative conformers of Zα_{ADAR1} and Zα_{ADAR1} mut respectively. C and D. Reconstructed NMR 3D structure of Zα_{ADAR1} and Zα_{ADAR1} mut respectively. The tertiary structure of Zα_{ADAR1} and Zα_{ADAR1} mut is very similar (left and right panel, respectively). The two key residues in the Z-DNA-binding interface in Zα_{ADAR1} (N43 and Y47) and the corresponding alanine substitutions in Zα_{ADAR1} mut are highlighted in red. E. The superposition of Zα_{ADAR1} (red), Zα_{ADAR1} mut (green) and crystal structure of Zα_{ADAR1} (yellow).

IV.5 Interaction with Z-DNA

To test the activity of our preparations of proteins, a complex with a Z-DNA forming oligonucleotide $d(\text{CG})_3\text{T}_4(\text{CG})_3$ was formed by titrating DNA into $\text{Z}\alpha_{\text{ADAR1}}$. Free $d(\text{CG})_3\text{T}_4(\text{CG})_3$ forms a hairpin with a 6-bp $d(\text{CG})_3$ stem in the B-DNA conformation in solution (Schade, Behlke et al. 1999). Figure 18 shows the change in the ^{31}P NMR spectrum of DNA fragment when adding $\text{Z}\alpha_{\text{ADAR1}}$. There is a specific displacement of ^{31}P resonances of DNA from positions at 1.5- 3 ppm to positions at 0.7, 1.3, 3.7, and 4.2 ppm during $\text{Z}\alpha_{\text{ADAR1}}$ binding. This is due to reversal in the direction of the phosphate backbone induced by Z-conformation. Yet no additional resonances appeared when the same DNA fragment was titrated with $\text{Z}\alpha_{\text{ADAR1}}$ mut, indicated that this DNA fragment was still in B-conformation upon addition of $\text{Z}\alpha_{\text{ADAR1}}$ mut.

Figure 18. 1D ^{31}P overlapped spectra of free DNA and protein-DNA complexes.

Free $d(\text{CG})_3\text{T}_4(\text{CG})_3$ forms B-conformation (blue). When adding $\text{Z}\alpha_{\text{ADAR1}}$ into DNA solution, the additional signals around 4 ppm and 1 ppm indicated Z-DNA formation (red). This DNA fragment was still in B-conformation in $\text{Z}\alpha_{\text{ADAR1}}$ mut-DNA complex (black).

The same results can be confirmed in 2D TROSY spectra. The superposition of the 2D TROSY spectra of free $Z\alpha_{\text{ADAR1}}$ and $(Z\alpha_{\text{ADAR1}})_2$ -DNA complex showed that several amide resonances move significantly, some vanish, and many show insignificant effects as a result of DNA binding and B-Z transition (Figure 19 A). The movements of resonances were observed during titration, and we found that the conformation changes significantly when the ratio of $Z\alpha_{\text{ADAR1}}$ and DNA is between 8:1 and 4:1. Significantly broadened cross-peaks in TROSY spectra indicated that the binding interface located mostly at the third helix including the two mutation sites (K40 – S48). Further broadened cross-peaks were observed at H29 at the N terminus of $\alpha 2$ with its side chain towards the DNA fragment, and K66 near the C terminus of $\beta 3$ indicating a long-range conformational change resulted from DNA binding (Figure 20). The cross-peaks shifted slightly at H29 and K40 when DNA fragment was titrated into $Z\alpha_{\text{ADAR1}}$ mut indicated that $Z\alpha_{\text{ADAR1}}$ mut may still bind to DNA fragment at low affinity, but lack the ability of inducing B- to Z- conformational change of DNA due to the two mutations at the binding sites (Figure 19 B). During these NMR experiments, $(Z\alpha_{\text{ADAR1}}$ mut) $_2$ -DNA complex precipitated after overnight measurement, while buffer containing $(Z\alpha_{\text{ADAR1}})_2$ -DNA complex was still clear after more than 2 days' experiments, indicating that $(Z\alpha_{\text{ADAR1}})_2$ -DNA complex was much more stable than $(Z\alpha_{\text{ADAR1}}$ mut) $_2$ -DNA complex at room temperature. The different binding characteristics of $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1}}$ mut with DNA fragment confirmed the previous results of N43, Y47 playing important roles in the interaction with DNA fragment as well as B-Z DNA conformational change.

Figure 19. Interaction mapping of Z α_{ADAR1} and Z α_{ADAR1} mut with Z-DNA

A. 2D ^{15}N -TROSY overlapped spectrum of Z α_{ADAR1} (red) and (Z α_{ADAR1}) $_2$ -DNA complex (blue). The labeled peaks show the amide chemical shift changes due to Z-DNA binding. B. 2D ^{15}N -TROSY overlapped spectrum of Z α_{ADAR1} mut (red) and (Z α_{ADAR1} mut) $_2$ -DNA complex (blue).

Figure 20. Perturbations of the backbone amide proton chemical shifts upon DNA binding to $\text{Z}\alpha_{\text{ADAR1}}$ and $\text{Z}\alpha_{\text{ADAR1}}^{\text{mut}}$, respectively.

We employed 5'- d(CG)3T4(CG)3-3' for DNA binding. The dashed horizontal lines indicate the maximum values of the chemical shift perturbations caused by oligonucleotide binding. The larger values of the perturbations resulted in severely broadened cross-peaks in 2D TROSY spectra and are designated as solid vertical lines connecting the dashed lines.

IV.6 Interaction with rRNA

In order to probe interactions with rRNA we used two synthetic RNA fragments, containing 17 and 30 oligonucleotides respectively. These fragments were derived from previous pull-down assay. In this assay, our collaborators prepared *E.coli* cells with expression vectors for $Z\alpha_{ADAR1}$ inside and used streptavidin-conjugated sepharose to pull-down probes from extracts of these *E.coli* cells (Feng, Li et al. 2011). It was found that only $Z\alpha_{ADAR1}$ remained stably associated with rRNA during pull-down and subsequent washing steps. The pulled-down material was then digested with RNase, and the RNA fragments which were still bound to $Z\alpha_{ADAR1}$ were eluted, PCR-amplified and cloned for sequencing. The 17-mer short RNA was the one presented in high copy numbers in these RNA segments. The 30-mer long RNA contained the core 17-mer RNA sequence and additional 13 nucleotides were predicted to form a hairpin structure according to the crystal structure of ribosome.

First we did NMR experiments of proteins binding to RNA fragments at low salt concentration (50 mM NaCl). 1D ^1H NMR spectra showed that the amide resonances changed significantly in both of $Z\alpha_{ADAR1}$ –17-mer rRNA complex and $Z\alpha_{ADAR1}$ mut – 17-mer rRNA complex when RNA concentration is only 1/10 of the protein concentration. During the titration process, the amide resonances did not change very much when the ratio between rRNA and $Z\alpha_{ADAR1}$ changed from 0.1 to 1 (Figure 21 A). This was confirmed in 2D-TROSY spectra. Around 40% of cross peaks were missing or shifted in 2D-TROSY spectrum at the time when initial aliquot of 17-mer rRNA was added into the protein solution, and no significant shifts could be detected

when the concentration of short RNA increased (Figure 21 B, C). 2D-TROSY spectrum of $Z\alpha_{\text{ADAR1}}$ – 30-mer rRNA complex showed similar characteristics (Figure 21 D). The 1D and 2D spectra were tested again with lower temperature, at 15 °C, with no difference noticed compare to the experiments done at 25 °C, indicating that the conformation change was not due to the high exchange rate between two conformations induced by specific binding. $Z\alpha_{\text{ADAR1}}$ mut – 17-mer rRNA complex showed similar 1D ^1H and 2D TROSY spectra (Figure 22). Combining with previous pull-down assay in which 17-mer rRNA did not bind to $Z\alpha_{\text{ADAR1}}$ mut, the conformation changes of $Z\alpha_{\text{ADAR1}}$ –17-mer rRNA complex and $Z\alpha_{\text{ADAR1}}$ mut – 17-mer rRNA complex were mostly due to the non-specific binding.

Figure 21. Interaction mapping of $Z\alpha_{ADAR1}$ with RNA at low salt concentration.

A. 1D ^1H NMR spectra of free $Z\alpha_{ADAR1}$, and $Z\alpha_{ADAR1}$ – 17-mer rRNA complex (red: free $Z\alpha_{ADAR1}$; blue: $Z\alpha_{ADAR1}$ titrated with 0.1x 17-mer rRNA; green: $Z\alpha_{ADAR1}$ titrated with 1x 17-mer rRNA). B. 2D ^{15}N TROSY overlapped spectra of free $Z\alpha_{ADAR1}$ (red), and $Z\alpha_{ADAR1}$ titrated with 0.1x 17-mer rRNA fragment (blue). C. 2D ^{15}N TROSY overlapped spectra of $Z\alpha_{ADAR1}$ titrated with 0.1x (red) and 1x (blue) 17-mer rRNA fragment. D. 2D ^{15}N TROSY overlapped spectra of $Z\alpha_{ADAR1}$ titrated with 1x 17-mer rRNA (red) and 1x 30-mer rRNA fragment (blue).

Figure 22. Interaction mapping of Z α _{ADAR1} mut with short RNA at low salt concentration.

1D ^1H NMR spectra of free Z α _{ADAR1} mut, and Z α _{ADAR1} mut – 17-mer rRNA complex (red: free Z α _{ADAR1} mut; blue: Z α _{ADAR1} mut titrated with 0.1x 17-mer rRNA; green: Z α _{ADAR1} mut titrated with 1x 17-mer rRNA).

Previous research found that either high salt concentrations or lower dielectric solvents combined with higher temperatures are required to induce the transition of RNA from A-form to Z-form (Hardin, Zarling et al. 1987; Klump and Jovin 1987). And P. Droge found that the pull-down assay did not work in the low salt condition as both Z α _{ADAR1} and Z α _{ADAR1} mut bind to rRNA segments, but work in the high salt condition resulting in specific rRNA segments binding with Z α _{ADAR1} and no rRNA segments binding with Z α _{ADAR1} mut (Feng, Li et al. 2011). To facilitate the conversion of potentially non-structured RNA into Z-RNA form and therefore to reduce the non-specific binding of Z α _{ADAR1} or Z α _{ADAR1} mut with rRNA, we decided to increase the salt concentration of the NMR buffer to 300 mM. The uniformly ^{15}N -labeled proteins were titrated with the unlabeled

17-mer rRNA segment, and the chemical shift perturbations of backbone residues and the intensities of cross-peaks were recorded using ^{15}N TROSY spectra.

After increasing salt concentration to 300 mM, the ^1H and ^{15}N resonances in $\text{Z}\alpha_{\text{ADAR1}}$ -rRNA complex shifted gradually with increasing rRNA-to- $\text{Z}\alpha_{\text{ADAR1}}$ ratio (Figure 23). The chemical shift perturbations (Figure 26, red lines) indicated that $\text{Z}\alpha_{\text{ADAR1}}$ binds to rRNA segment in the region corresponding to the third helix and the beginning of the second helix. The significantly broadened cross-peaks E10 and K65 indicate a long range conformational change during $\text{Z}\alpha_{\text{ADAR1}}$ binding. Most of the cross-peaks showed no shifting or attenuation in 2D TROSY spectrum of $\text{Z}\alpha_{\text{ADAR1}}$ mut-rRNA complex, while cross-peaks shifted slightly at E41 and E58, indicating that $\text{Z}\alpha_{\text{ADAR1}}$ mut may still bind to rRNA fragment with very low affinity (Figure 24).

Figure 23. Interaction mapping of $\text{Z}\alpha_{\text{ADAR1}}$ with 1x 17m rRNA in the HEPES buffer contains 300 mM NaCl.

2D ^{15}N TROSY overlapped spectra of $\text{Z}\alpha_{\text{ADAR1}}$ (red) and $\text{Z}\alpha_{\text{ADAR1}}$ – 17m rRNA complex in 300 mM NaCl (blue).

Figure 24. Interaction mapping of $Z\alpha_{ADAR1}$ mut with 1x 17m rRNA in the HEPES buffer contains 300 mM NaCl.

A. 1D ^1H NMR spectra of free $Z\alpha_{ADAR1}$ mut (red), $Z\alpha_{ADAR1}$ mut – 17m rRNA complex in 50 mM NaCl(blue), and $Z\alpha_{ADAR1}$ mut - 17m rRNA complex in 300 mM NaCl (green). B. 2D ^{15}N TROSY overlapped spectra of $Z\alpha_{ADAR1}$ mut (red) and $Z\alpha_{ADAR1}$ mut – 17m rRNA complex in 300 mM NaCl (blue).

In order to figure out the conformational change of rRNA fragment during protein binding, we also did 1D ^{31}P NMR experiments on free rRNA and protein-rRNA complex. In these ^{31}P spectra, we saw the displacement of ^{31}P resonances of rRNA from the bulk positions at 1.5-3 ppm to positions at 4.1, 4.5 and 6.7 ppm in ^{31}P spectra of $Z\alpha_{ADAR1}$ -rRNA complexes (Figure 25). This is due to reversal in the direction of the phosphate backbone induced by a Z-conformation. In 1:1 $Z\alpha_{ADAR1}$ -rRNA complexes of 30-mer rRNA, about 4 to 5 ^{31}P spins are found outside of the regular structure revealing quantitative and

specific interactions. In addition, comparison between ^{31}P spectra $\text{Z}\alpha_{\text{ADAR1}}$ -rRNA and $\text{Z}\alpha_{\text{ADAR1}}$ -DNA shows that 1 or 2 nucleotides were likely to be located at the Z junction, resulting in a signal at 6.7 ppm in rRNA spectra. No such shifting could be seen in ^{31}P spectrum of $\text{Z}\alpha_{\text{ADAR1}}$ mut – rRNA complex.

These results were consistent with the previous functional studies (Feng, Li et al. 2011) which $\text{Z}\alpha_{\text{ADAR1}}$ binds to rRNA fragment at high affinity while $\text{Z}\alpha_{\text{ADAR1}}$ mut dissociated with rRNA at high salt concentration, and indicated that rRNA may undergo A- to Z- conformational change as DNA did during $\text{Z}\alpha_{\text{ADAR1}}$ binding.

Figure 25. 1D ^{31}P overlapped spectra of protein-DNA and protein-RNA complexes.

A. free $\text{d}(\text{CG})_3\text{T}_4(\text{CG})_3$ DNA (blue) and DNA in complexes with $\text{Z}\alpha_{\text{ADAR1}}$ (red) and $\text{Z}\alpha_{\text{ADAR1}}$ mut (black). B. a vertically enhanced spectra of free 5'-AUGGGGUGACUGCGUAC-3' RNA (blue) and rRNA in complex with $\text{Z}\alpha_{\text{ADAR1}}$ (red) and $\text{Z}\alpha_{\text{ADAR1}}$ mut (black). The RNA oligo is heat-annealed.

IV.7 Comparison between protein-DNA and protein-rRNA complexes

Comparing the structures and binding interfaces of $Z\alpha_{\text{ADAR1}}$ -DNA and $Z\alpha_{\text{ADAR1}}$ -rRNA complexes, we found that DNA and rRNA fragments interact with $Z\alpha_{\text{ADAR1}}$ in the similar binding sites, which include the third helix and the N-terminal of the second helix. Both DNA and rRNA undergo B- to Z- or A- to Z- conformational change when adding $Z\alpha_{\text{ADAR1}}$. These results indicate that $Z\alpha_{\text{ADAR1}}$ specifically binds to DNA or RNA, and has the ability to induce and stabilize Z-conformation of nucleotides.

We also found that $Z\alpha_{\text{ADAR1}}$ mut was still capable of binding to DNA and rRNA, sharing the similar nucleotide binding interface mapped by chemical shift perturbations, but lack the function of stabilizing Z-conformations. It is clear that the presence of Y47A and N43A in $Z\alpha_{\text{ADAR1}}$ is crucial for stabilization of Z-conformations (Figure 26, 27).

Figure 26. Perturbations of the backbone amide proton chemical shifts upon DNA (blue) and RNA (red) binding to $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1 mut}}$, respectively.

We employed 5'- $d(\text{CG})_3\text{T}_4(\text{CG})_3$ -3' for DNA and 5'-AUGGGGUGACUGCGUAC-3' for RNA binding. The dashed horizontal lines indicate the maximum values of the chemical shift perturbations caused by oligonucleotide binding. The larger values of the perturbations resulted in severely broadened cross-peaks in 2D TROSY spectra and are designated as solid vertical lines connecting the dashed lines.

Figure 27. Mapping of $^1\text{H}_\text{N}$ chemical shift perturbations.

A. $\text{Z}\alpha_{\text{ADAR1}}$ -DNA complex. B. $\text{Z}\alpha_{\text{ADAR1}}$ -rRNA complex. C. $\text{Z}\alpha_{\text{ADAR1}}$ mut-DNA complex. D. $\text{Z}\alpha_{\text{ADAR1}}$ mut-rRNA complex. The color code used is: red, the cross-peaks in TROSY spectra of complexes are severely broadened; magenta, shifts are perturbed within 0.1-0.15 ppm range; yellow, 0.05-0.1 ppm; lightblue, 0.025-0.05 ppm; violet, 0.01-0.025 ppm; blue, below 0.01 ppm.

IV.8 Protein binding with ribosomes

To decide the molar ratio of proteins to 70S ribosomal subunits, a titration of 70S ribosomal subunits to both $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1}}^{\text{mut}}$ were recorded using a series of 2D TROSY spectra. We tested several molar ratios from $2.5 \times 10^3:1$ to 500:1 by gradually adding ribosomes into protein solution. At molar ratio of protein to 70S ribosomal subunits above $1.5 \times 10^3:1$, the cross-peaks in 2D TROSY spectrum of protein-ribosomes complex showed no chemical shifts perturbations nor slight attenuation when comparing with spectrum of free protein even in salt concentration as low as 50 mM. This suggests that the amount of ribosomes is too small to detect the conformational change upon interaction through NMR. When we decreased the molar ratio to 750:1 by adding more ribosomes into protein, the solution in NMR tube became opaque and lots of precipitates appeared. Finally the molar ratio of protein to 70S ribosomal subunits at $1 \times 10^3:1$ was chosen for further salt titration experiments.

At $1 \times 10^3:1$ molar ratio of protein to 70S ribosomal subunits, most of the signals disappeared at 50 mM salt concentration in both spectra of $Z\alpha_{\text{ADAR1}}$ – ribosomes and $Z\alpha_{\text{ADAR1}}^{\text{mut}}$ – ribosomes complexes. The cross-peaks in these spectra with high intensities corresponded to the C-terminal tag. These significantly broadened cross-peaks were probably due to none specific binding of both $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1}}^{\text{mut}}$ to ribosome. The same situation also happened in protein-rRNA complexes, in which most of the cross-peaks in TROSY spectra significantly broadened at low salt condition.

As salt concentration increase, the disappeared peaks became visible again.

Analyzing the intensities of all these cross-peaks during salt titration revealed that at salt concentration lower than 400 mM, some cross-peaks were more significantly broadened than others in $Z\alpha_{\text{ADAR1}}$ -ribosome complex; while no such intensity differences were seen in $Z\alpha_{\text{ADAR1}}$ mut-ribosome complex. These significantly broadened cross-peaks corresponded to the ribosomal binding site of $Z\alpha_{\text{ADAR1}}$. TROSY spectra of $Z\alpha_{\text{ADAR1}}$ mut-ribosome complex at 200 mM, 300 mM salt concentration displayed no differences comparing with spectrum of free $Z\alpha_{\text{ADAR1}}$ mut, indicating that the non-specific binding at low salt concentration dissociated when the salt concentration increased. At 400 mM salt concentration, TROSY spectra of both $Z\alpha_{\text{ADAR1}}$ -ribosome complex and $Z\alpha_{\text{ADAR1}}$ mut-ribosome complex showed the same spectra of free proteins, suggesting the abolishment of ribosomal binding with the proteins at high salt concentration (Figure 28). These findings agreed with the binding assay done in Prof. Droge's lab (Feng, Li et al. 2011). In their experiments, both $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1}}$ mut were found to bind to ribosome at low salt condition, but only $Z\alpha_{\text{ADAR1}}$ stably formed complex with ribosome at salt concentration between 100 mM and 300 mM. When salt concentration increased to 500 mM, both of them dissociated from ribosome. Later, we used salt concentration at 300 mM for interaction mapping in $Z\alpha_{\text{ADAR1}}$ -ribosome complex.

Figure 28. Intensities of selected cross-peak of protein-ribosome complexes in different salt concentration.

A. intensities of cross-peaks in Z α _{ADAR1}-ribosome complex; B. in Z α _{ADAR1mut}-ribosome complex. Color code: light blue, free protein; dark blue, complexes in 50 mM NaCl; red, in 100 mM NaCl; green, in 200 mM NaCl; purple, in 300 mM NaCl. Residue F16 located far away from the binding interface, while others are at or near the binding interface. Intensities were normalized to that of residue F16 in free protein condition.

Chemical shift and line shape are two important parameters for the determination of interaction surface. Comparing the chemical shift perturbations, we found that unlike $Z\alpha_{\text{ADAR1}}$ -rRNA complex, most of the cross-peaks of $Z\alpha_{\text{ADAR1}}$ shifted slightly during the binding of ribosome to $Z\alpha_{\text{ADAR1}}$ (Figure 29). This was probably due to the change in the chemical environment when bound with bulky ribosomes. To map the binding surface, we took cross-peak intensities for consideration.

Figure 29. Perturbations of the backbone amide proton chemical shifts upon RNA (blue) and ribosome (red) binding to $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1 mut}}$, respectively.

The dashed horizontal lines indicate the maximum values of the chemical shift perturbations caused by oligonucleotide binding. The larger values of the perturbations resulted in severely broadened cross-peaks in 2D TROSY spectra and are designated as solid vertical lines connecting the dashed lines.

Almost all cross-peak intensities in the $Z\alpha_{\text{ADAR1}}$ -ribosome complex showed more significant attenuation compared with that of $Z\alpha_{\text{ADAR1}}$ mut-ribosome complex, while some were broadened more than the others, indicating direct interaction sites with ribosomes. To map the binding interface of $Z\alpha_{\text{ADAR1}}$ to ribosomes, we carefully calculated the intensities of cross-peaks in 2D TROSY spectra, which were obtained in the buffer containing 300 mM NaCl. After modification of the intensities by multiplying a factor to average protein concentration and noise level in different spectra, the cross-peaks which had ratio of intensities of complex to free protein smaller than 5.0×10^{-2} were chosen and mapped to the solution structure of $Z\alpha_{\text{ADAR1}}$ (Figure 30). The choice of the cutoff is arbitrary. The reason that we set the cutoff at 0.05 is to identify a reasonably small number of residues on the protein surface interacting with ribosome, possibly delineating the actual binding site. The resulting number is comparable to the number of residues involved into the protein-RNA interface in protein-RNA complex. In addition, the strong cutoff serves as a filter for weaker and nonspecific binding. One can expect that since ribosome is a large molecule, and $Z\alpha_{\text{ADAR1}}$ may bind to ribosome through several binding sites. Through this cutoff we could eliminate the weakly binding sites and only consider the most affected amino acids by binding. We found that the binding sites located at the third helix (K40-V45, Y47-L49), and the second helix (A28, S32, K34). Long range conformational change also happened at the first helix (E10, Q11, I13) and the b-sheet C-terminal (L55, E58, K66). Nearly one third of the whole $Z\alpha_{\text{ADAR1}}$ protein was involved in the interaction with the bulky ribosomes.

Figure 30. Ratio of intensities of Z α _{ADAR1}-ribosome complex to free Z α _{ADAR1}.

Intensities are modified to average protein concentration and noise level in different spectra. Residues before 60 belong to protein, while residues from 80 to 100 belong to the C-terminal tag. The cross-peaks which have the ratio below 0.05 were chosen for interaction mapping. Residues from 60 to 80 corresponding to the link between protein and tag are not assigned.

IV.9 Comparison between $Z\alpha_{\text{ADAR1}}$ -rRNA and $Z\alpha_{\text{ADAR1}}$ -ribosomes complexes

Comparing the binding sites between $Z\alpha_{\text{ADAR1}}$ -ribosomes complex and $Z\alpha_{\text{ADAR1}}$ -rRNA complex, we found that these two complexes share the similar binding interface, which contains the second and third helix, and N-terminal of the first helix, as well as several amino acids from β -sheet C-terminal tail. These results indicated that $Z\alpha_{\text{ADAR1}}$ binds to ribosomes with the similar binding mode with $Z\alpha_{\text{ADAR1}}$ -rRNA complex.

We found that almost no cross-peak show significant perturbations during ribosomal binding as what they did during rRNA binding. Only cross-peaks corresponding to the binding interface attenuated more than others. Figure 31 shows that resonances of A28 and K39 backbone amides were shifted, the cross-peaks corresponding to residues H29, K66 and R44 broadened to disappear during the binding of $Z\alpha_{\text{ADAR1}}$ with rRNA, while all these cross-peaks were attenuated with no shifting in the TROSY spectrum of $Z\alpha_{\text{ADAR1}}$ -ribosome complex. Several cross-peaks which indicated the binding sites were selected (Figure 32). As shown in this figure, all cross-peaks of $Z\alpha_{\text{ADAR1}}$ shifted or disappeared during rRNA binding were attenuated without shifting during the binding process with ribosomes. No such shifting or disappearing of the binding site backbone amides peaks could be seen in the TROSY spectra of $Z\alpha_{\text{ADAR1mut}}$ -rRNA and $Z\alpha_{\text{ADAR1mut}}$ -ribosome complexes.

Figure 31. TROSY spectra of protein and protein-rRNA, protein-ribosome complexes.

A. $Z\alpha_{ADAR1}$; B. $Z\alpha_{ADAR1}$ -rRNA complex; C. $Z\alpha_{ADAR1}$ -ribosome complex; D. $Z\alpha_{ADAR1}$ mut; E. $Z\alpha_{ADAR1}$ mut-rRNA complex; F. $Z\alpha_{ADAR1}$ mut-ribosome complex. Residues locate at or near the binding interface are highlight.

Figure 32. Individual cross-peaks in TROSY spectra corresponding to residues around the binding site.

A, C, E for free $\text{Z}\alpha_{\text{ADAR1}}$ (black) and $\text{Z}\alpha_{\text{ADAR1}}$ -rRNA complex (red); B, D, F for free $\text{Z}\alpha_{\text{ADAR1}}$ (black) and $\text{Z}\alpha_{\text{ADAR1}}$ -ribosome complex (red)

IV.10 Multiple binding sites in $Z\alpha_{\text{ADAR1}}$ -ribosome complex

When we analyze the intensities of cross-peaks, we found that those cross-peaks far away from binding interface between $Z\alpha_{\text{ADAR1}}$ and ribosomes also got attenuated. This could be explained by the multiple interfaces between protein and ribosomes. Previous pull-down assay found not only the 17-mer rRNA (high confidence ribosome binding site) located at both small and large subunits, but also three additional rRNA segments bound to ribosome in less proximity (Feng, Li et al. 2011). Considering that the pull-down assay was done in a stringent manner, $Z\alpha_{\text{ADAR1}}$ may interact with ribosomes through all these possible rRNA segments in solution during our titration process.

The concentration of $Z\alpha_{\text{ADAR1}}$ to ribosomes in our titration experiments also needed to be taken into account. We used the molar ratio of $Z\alpha_{\text{ADAR1}}$ to ribosomes at $1 \times 10^3:1$, which means each ribosome was surrounded by lots of proteins. This gives proteins the possibility to interact with ribosomes at low affinity, non-favorable binding sites.

Taken together, it is possible that $Z\alpha_{\text{ADAR1}}$ interacts with ribosomes through different binding sites, and the most stable complex forms when the third helix of $Z\alpha_{\text{ADAR1}}$ faces the specific position of ribosomes containing the 17-mer rRNA, which was the similar binding interface as that of $Z\alpha_{\text{ADAR1}}$ -DNA and $Z\alpha_{\text{ADAR1}}$ -rRNA complexes.

IV.11 Comparing intensities of cross-peaks corresponding to protein and tag

Beside the points mentioned above, we also wondered if there are other mechanisms related to NMR causing the attenuation of nearly all cross-peaks. To facilitate this, we assigned the tag of the protein, and analyzed the intensities again. After cancelling out the effects of different protein concentration and noise level in each spectrum, we compared the intensities of cross-peaks belonging to the protein and the tag. The average intensities of cross-peaks from the tag got 7 times less than in free protein, while no such attenuation could be seen in the spectra of $Z\alpha_{\text{ADAR1}}$ -rRNA complexes. The intensities of cross-peaks from the tag in $Z\alpha_{\text{ADAR1}}$ -rRNA complexes have the comparable level with those in free $Z\alpha_{\text{ADAR1}}$, while only the cross-peaks at or near the binding site shifted and attenuated. This could be explained by conventional chemical exchange theory, in which only the spins undergone chemical exchange process have the performance of shifting or attenuation. However, chemical exchange mechanism cannot explain the attenuation of the tag in $Z\alpha_{\text{ADAR1}}$ -ribosome complexes.

We expressed and purified the deuterated $Z\alpha_{\text{ADAR1}}$ and did 2D TROSY NMR experiments on free D-protein and D-protein-ribosome complex. The ratio of intensities of D- $Z\alpha_{\text{ADAR1}}$ -ribosome complex to free D- $Z\alpha_{\text{ADAR1}}$ below 5.0×10^{-2} were chosen and mapped on the 3D structure of $Z\alpha_{\text{ADAR1}}$. It was found that deuteration did not affect the binding properties between $Z\alpha_{\text{ADAR1}}$ and ribosomes. The binding interface in D- $Z\alpha_{\text{ADAR1}}$ -ribosome complex located mainly at the third helix, as well as the N-terminal of the second helix.

Comparing between intensities of cross-peaks corresponding to protein residues and tag residues, we found that the cross-peaks from whole protein got less attenuated in D-protein-ribosome complex. The lines in Figure 33 B were more flattened from residues 60 to 80, while the intensities differences between binding sites and non-binding sites were much larger than figure of $Z\alpha_{\text{ADAR1}}$ -ribosome complex, making the cross-peaks in the binding interface significantly broadened than others.

Figure 33. Ratio of intensities of $Z\alpha_{ADAR1}$ -ribosome complex to free $Z\alpha_{ADAR1}$ (A) and D- $Z\alpha_{ADAR1}$ -ribosome complex to free D- $Z\alpha_{ADAR1}$ (B).

Intensities are modified to average protein concentration and noise level in different spectra. Residues before 60 belong to protein, while residues from 80 to 100 belong to the C-terminal tag. The cross-peaks which have the ratio below 5.0×10^{-2} were chosen for interaction mapping in both complexes.

IV.12 Dipole-dipole relaxation outside of Redfield limit

Consider the model described in figure 34:

Figure 34. Protein-ligand binding model.

Here B is a large aggregate (classified as NMR solid), A is a small molecule, and each of them carries a single proton spin (I and S , respectively). The angle θ is defined by the direction of the external polarizing magnetic field B_0 (conventionally assumed to be aligned vertically), and the direction defined by the interacting spins I and S .

Each of the small molecule A and the large aggregate B carries a single protein spin (I and S , respectively). Considering the complex A:B, we spell out the dipolar Hamiltonian for the spin pair (I , S), and only retain the secular part of dipolar interaction (equation 1.1 ~ 1.4). This approximation is valid if on-off exchange of the ligand A is not exceedingly fast. Specifically, the secular approximation is good when $\tau_{ex} \gg \omega_{0H}^{-1} \sim 200 \text{ ps}$ (in other words, focusing for a moment on Redfield limit, we assume that $J(\omega_H)$ can be neglected compared to $J(0)$). Normally, this would be a very safe assumption, provided that there is at least a minimal binding (as opposed to free diffusion of A relative to B).

$$H_{dip} = c_{dip} F_0 T_0^{dip} \quad (1.1)$$

$$c_{dip} = -\sqrt{6} \frac{\mu_0 \gamma_I \gamma_S \hbar}{4\pi r_{IS}^3} \quad (1.2)$$

$$F_0 = D_{0,0}^{(2)}(0, \theta, 0) = (3\cos^2\theta - 1)/2 \quad (1.3)$$

$$T_0^{dip} = \frac{1}{\sqrt{6}} (2I_Z S_Z - \frac{1}{2}(I_+ S_- + I_- S_+)) \quad (1.4)$$

Under certain numeric estimates, dipolar Hamiltonian can be rewritten as $2\pi D_{IS}(I_Z S_Z - \frac{1}{4}(I_+ S_- + I_- S_+))$ (similar to the scalar coupling $2\pi J_{IS} I_Z S_Z$ and chemical shift $2\pi \nu_{0I} I_Z$). Assuming for a moment that the effective proton-proton distance across the interface is 3 Å (probably reasonable given that in fact there are multiple protons on the side of B; more reliable estimates can be obtained using some kind of a model complex for which high-resolution coordinates are available), and that θ equals 90°, as shown in Figure 34. Then the interaction constant is estimated to be $D_{IS} \approx 4.45$ kHz.

As the on-off exchange occurs, I spin is exposed to the ‘dipolar field’ that alternates between 4.45 kHz (bound state) and 0 (free state). This is much stronger effect than modulation of the chemical shift, when I spin is exposed to the local field alternating between ν_{0I}^{bound} and ν_{0I}^{free} (typically, $\nu_{0I}^{bound} - \nu_{0I}^{free}$ is a fraction of 1 ppm on protons and, hence, on the order of hundreds of Hz). This is also much stronger effect than modulation of scalar couplings by exchange since any kind of scalar couplings in such a system are on the order of mere several Hz (e.g. scalar coupling across intermolecular hydrogen bond).

As for the angle θ , there are two options: (i) for a system such as Pf1 bacteriophage, which orients in the external magnetic field, θ can be set to a certain fixed value, for example $\theta = 90^\circ$ as shown in the plot, or alternatively, (ii) for a system such as crashed fibrils, the results ('relaxation' profiles) can be calculated first, and then the powder averaging can be performed. Since it is more general, the latter procedure is implemented here.

Let us investigate the transverse evolution of spin I from ligand A. When A is a part of the complex A:B and two spins are coupled through dipolar interaction the relevant Liouville space basis is $I_+, 2I_+S_Z, S_+, 2S_+I_Z$. This is a bit different from scalar coupling because of the presence of flip-flop terms in the Hamiltonian.

Now let us expand the Liouville space to include the blocks for free A (basis operator I_+^{free}) and free B (basis operator S_+^{free}). Let us further set up the complete superoperator matrix, $-iL + X$, where L is the Liouvillian (super-Hamiltonian) matrix, X is the exchange matrix.

$$\begin{bmatrix} I_+ \\ 2I_+S_Z \\ S_+ \\ 2S_+I_Z \\ I_+^{free} \\ S_+^{free} \end{bmatrix} L = \begin{bmatrix} \cdot & \pi D_{IS} & \cdot & 1/2 \pi D_{IS} & \cdot & \cdot \\ \pi D_{IS} & \cdot & 1/2 \pi D_{IS} & \cdot & \cdot & \cdot \\ \cdot & 1/2 \pi D_{IS} & \cdot & \pi D_{IS} & \cdot & \cdot \\ 1/2 \pi D_{IS} & \cdot & \pi D_{IS} & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \end{bmatrix} \quad (2.1)$$

$$\begin{bmatrix} I_+ \\ 2I_+S_Z \\ S_+ \\ 2S_+I_Z \\ I_+^{free} \\ S_+^{free} \end{bmatrix} X = \begin{bmatrix} -k_{off} & \cdot & \cdot & \cdot & k_1 & \cdot \\ \cdot & -k_{off} & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & -k_{off} & \cdot & \cdot & k_2 \\ \cdot & \cdot & \cdot & -k_{off} & \cdot & \cdot \\ k_{off} & \cdot & \cdot & \cdot & -k_{off} & \cdot \\ \cdot & \cdot & k_{off} & \cdot & \cdot & -k_{off} \end{bmatrix} \quad (2.2)$$

Here we assume that the amount of magnetization is proportional to the population of the corresponding species. For the exchange rates we have $k_1 = k_{on}[B]$ and $k_2 = k_{on}[A]$. Given that the amount of magnetization is proportional to the population of the respective species, we obtain the exchange matrix that satisfies the microscopic balance conditions, $k_{off}[AB] = k_{on}[A][B]$. To determine the populations of the species, we use trivial relationships involving analytical concentrations and presumed K_d value.¹

In principle, an empirical dissipation term could be added to $-iL + X$ (accounting for other spin interactions, such as for example spin-diffusion in solids). Such term could include effective auto-relaxation rates, $R_{2,solid}^{eff} \sim 1000 \text{ s}^{-1}$ (for protonated solid B) and $R_{2,liquid}^{eff} \sim 1 \text{ s}^{-1}$ (for small ligand A). However, we choose not to include these terms, since we intend to keep the model as simple as possible.

For the initial conditions we assume that I_+^{free} , $S_+^{free}I_+$, and S_+ are all excited with the same efficiency. This assumption is not unreasonable – hard proton

¹ In principle, we could also assume that all binding sites on the surface of B are saturated (excess ligand), so that there is no free B. In this case the size of the matrix Eq. (2) would be reduced to 5-by-5.

pulse can excite all spins across the spectrum, in solution as well as in solid. At the same time the assumption is crude – in reality, one should analyze the actual pulse sequence.

What quantity should be taken as an observable? If the exchange is slow, then I_+^{free} is the observable. If the exchange is fast, then $I_+^{free} + I_+$ is the observable. In general case, one should resort to numeric simulations to address this issue.

Our simulations of kinetics of binding between $Z\alpha_{ADAR1}$ (species [A]) and ribosomes (species [B]) assume the presence of many binding sites of A on the surface of B. This is not unreasonable assumption since we (and others) clearly demonstrated that $Z\alpha_{ADAR1}$ binds to any nucleotide sequences albeit with variable affinity. $Z\alpha_{ADAR1}$ may bind to many of the exposed nucleotides on the surface of ribosome, thus the potential binding sites per ribosome molecule could be expected to be in order of hundreds. The molar ratio of $Z\alpha_{ADAR1}$ to ribosome is 1000, but the ratio of [A] to [B], multiplied by the number of the expected binding sites is around 10. Thus, under [B] we understand the product of the ribosome concentration, [R], and the estimated number of the binding sites harbored on its surface, N, e.g. $[B]=[R]*N$. If ribosome harbors only one, highly selective, binding site, NMR observations of chemical shift changes in $Z\alpha_{ADAR1}$ at the experimentally used stoichiometry are clearly unfeasible. The latter was clearly demonstrated in our preliminary testing of the numerical model.

In the simulations illustrated below we found that the exchange is fast, i.e. $\tau_{ex} = 1/(k_{on}[B] + k_{off})$ is less than ca. 1 ms, which means that the two signals are past the coalescence point. Hence, we used $I_+^{free} + I_+$ as the observable (once again, numeric simulations are needed to rigorously mimic the experimental observable).

The results of the simulations are shown in Figure 35, and the parameters are listed in the figure caption. The trend in the results can be readily understood. If the exchange is too slow ($k_{on} = 10^7 s^{-1} M^{-1}, k_{off} = 10^3 s^{-1} M^{-1}, [B] = 1 mM, \tau_{ex} = 900 \mu s$) or too fast ($k_{on} = 10^9 s^{-1} M^{-1}, k_{off} = 10^5 s^{-1} M^{-1}, [B] = 1 mM, \tau_{ex} = 9 \mu s$) the decay of spin / magnetization is, in relative terms, slow. However, in the intermediate regime ($k_{on} = 10^8 s^{-1} M^{-1}, k_{off} = 10^4 s^{-1} M^{-1}, [B] = 1 mM, \tau_{ex} = 90 \mu s$) the decay is fast.

This kind of behavior is reminiscent of the familiar lineshape behavior – the signal is sharp when the exchange is slow or fast, but in the intermediate regime the signal experiences strong exchange broadening. Note that the regime where the decay is the fastest corresponds to $D_{IS}\tau_{ex} \approx 4.45 \cdot 10^3 Hz \times 90 \cdot 10^{-6} s = 0.4$ (i.e. this characteristic parameter is ~ 1 , as expected).

Figure 35. Decay of the spin I magnetization induced by exchange modulation of the (solid- state) dipolar coupling.

The affinity is assumed to be $K_d = 100\mu\text{M}$, the analytical concentration of the component B is 0.1 mM; the analytical concentration of the ligand A is 1 mM.

Note that in the above treatment we assumed that spins I and S are degenerate with respect to chemical shift. To test the effect of this approximation, we introduced the chemical shift difference between spins I and S . We ignored (generally much smaller) shifts between I in the free-state and I in the bound state, S in the free-state and S in the bound state. We only took into consideration the chemical shift offset between I and S (e.g. amide and methyl protons). The superoperator matrix, therefore, becomes $-iL - iZ + X$, where the Zeeman portion is:

$$\begin{bmatrix} I_+ \\ 2I_+S_Z \\ S_+ \\ 2S_+I_Z \\ I_+^{free} \\ S_+^{free} \end{bmatrix} \quad Z = \begin{bmatrix} \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \Delta\omega_{IS} & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \Delta\omega_{IS} & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \Delta\omega_{IS} \end{bmatrix} \quad (3)$$

The result remains qualitatively similar (as shown in Figure 36).

Figure 36. Decay of the spin I magnetization induced by exchange modulation of the (solid- state) dipolar coupling.

Same as Figure 35, but chemical shift offset $\Delta\omega_{IS}$ equal to 5 ppm (4 kHz) has been introduced.

Finally, it is interesting to derive the Redfield-limit behavior for this system. The results should be similar to scalar relaxation of the first kind (J-coupling modulated by exchange).

$$R_{ex} = (2\pi J)^2 \frac{1}{4} \left\{ \tau_{ex} + \frac{\tau_{ex}}{1 + (\omega_I - \omega_S)^2 \tau_{ex}^2} \right\} \quad (4)$$

In the above expression, Eq. (4), it is assumed that A is always in the bound state (i.e. concentration of the free A is zero).

Redfield-theory treatment of the problem at hand leads to the superoperator matrix $-iZ - \Gamma$, where the relaxation matrix Γ is given by:

$$\begin{bmatrix} I_+ \\ 2I_+S_Z \\ S_+ \\ 2S_+I_Z \\ I_+^{free} \\ S_+^{free} \end{bmatrix} \Gamma = \begin{bmatrix} R_a & \cdot & R_c & \cdot & \cdot & \cdot \\ \cdot & R_a & \cdot & R_c & \cdot & \cdot \\ R_c & \cdot & R_a & \cdot & \cdot & \cdot \\ \cdot & R_c & \cdot & R_a & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \end{bmatrix} \quad (5.1)$$

Where

$$R_a = P_{bound} (2\pi D_{IS})^2 \frac{1}{4} \left\{ \tau_{ex} + \frac{1}{4} \frac{\tau_{ex}}{1 + (\omega_I - \omega_S)^2 \tau_{ex}^2} \right\} \quad (5.2)$$

$$R_c = P_{bound} (2\pi D_{IS})^2 \frac{1}{8} \left\{ \tau_{ex} + \frac{\tau_{ex}}{1 + (\omega_I - \omega_S)^2 \tau_{ex}^2} \right\} \quad (5.3)$$

Here P_{bound} is a fraction of the bound state, $P_{bound} = [AB]/([A] + [AB])$; note also that the interaction constant D_{IS} still contains the spatial variable θ , so that the results still need to be powder-averaged.

We can further assume that the chemical shifts of spins I and S are not degenerate, such that the relaxation coupling R_c is inefficient (small term that couples two coherences processing at different frequencies can be neglected). Thus, what's left is the monoexponential relaxation R_a , Eq. (5.2),

that needs to be powder-averaged. The resulting profiles are shown in Figure 37.

Figure 37. Decay of the spin I magnetization induced by exchange modulation of the dipolar coupling.

Blue curves are from the extended simulation. Red curves are from the simplified Redfield-theory treatment (Eq. (5.2))

The results are as expected. For $k_{on} = 10^8 s^{-1} M^{-1}$ ($\tau_{ex} = 90 \mu s$) the characteristic parameter $D_{IS}\tau_{ex}$ is on the order of 0.4 (this estimate is discussed above). For Redfield treatment to be valid it is necessary to ensure that $D_{IS}\tau_{ex} \ll 1$. This is not the case here and, indeed, we observe that the Redfield-theory prediction (red curve) is significantly different from the numeric result (blue curve). On the other hand, for $k_{on} = 10^9 s^{-1} M^{-1}$ ($\tau_{ex} = 9 \mu s$) the characteristic parameter $D_{IS}\tau_{ex}$ is on the order of 0.04 and the condition $D_{IS}\tau_{ex} \ll 1$ is fulfilled. In this situation the Redfield-theory prediction matches

closely the numeric result.

The dephasing (relaxation) mechanism discussed above should, in principle, be operational for ligand spins close to the binding interface as well as ligand spins farther away from the binding interface. The key is that for a short period of time ligand becomes a 'solid', and hence each spin in the ligand experiences large dipolar fields. When ligand dissociates from the complex, these fields are effectively 'switched off'. Thus, a random modulation of dipolar fields by exchange.

Why is it that the spins closer to the binding interface experience stronger attenuation? We suggest that the proton density is higher near the interface compared to the portion of the ligand that extends into solvent. We verified that by using deuterated $Z\alpha_{\text{ADAR1}}$. It is important to check the initial conditions which are important. Indeed, in our simulations we assumed that all spins are excited uniformly. However, it might be that S magnetization will rapidly relax away, and the resulting 'saturation' will be transferred onto the ligand spin / proximal to the interface. These effects should be analyzed in relation to the specific pulse sequence.

Figure 38. Attenuation of the amplitude and the chemical shift perturbation of the NMR resonance line of the protein resonance in a free-state (higher spectrum) and in a transient complex with a large aggregate as predicted by the model of Eq. 1-3 (lower spectrum).

The kinetic parameters are as described above. The resonance offset is arbitrary set at -700 Hz.

In any event, this 'new relaxation mechanism' (due to modulation of the solid-state dipolar couplings by exchange) appears as a simple concept that's easy to visualize and model. It clearly predicts a pervasive attenuation of all spins in the small protein (not only those at the binding interface as predicted by the classical chemical exchange theory) with clear attenuation bias for the spins at the binding interface. The theory also predicts detectable displacements of the cross-peak positions of the protein in the dynamic complex with ribosome (Figure 38), the effect we indeed observed experimentally. It is clear that the new theory is still in development and requires some additional experimental verifications of its predictions.

As an alternative to our proposed fast exchange regime model modulating none-averaged dipolar interaction, a simple slow exchange model affecting only chemical shifts might be considered. In fact this slow exchange model constituted our initial hypothesis of the kinetics of $Z\alpha_{\text{ADAR1}}$ /ribosome interactions. However, we come up with three arguments against slow exchange regime hypothesis in $Z\alpha_{\text{ADAR1}}$ /ribosome complex in contrast to the fast exchange regime clearly established for $Z\alpha_{\text{ADAR1}}$ /RNA complex, which in fact called for the development of the new theory: 1) we observed reduction of the peak intensities also from the residues far from the interaction interface and the reduction of the intensities is spread through the protein. However, the residues situated immediately at the binding interface showed significantly stronger attenuation. This clearly contradicts to the slow exchange hypothesis predicting uniform decrease of intensities of all peaks, since the bound form of the protein is not detectable by NMR in solution; 2) Even in the slow exchange regime smaller but detectable shifts in the cross-peak positions are expected, which are not detected experimentally; 3) It is highly unlikely that ribosomal surface exposed RNA is drastically different to our free RNA thus considerably slowing down the exchange (across at least two orders of magnitude).

V Conclusion

The solution structures of $Z\alpha_{\text{ADAR1}}$ and $Z\alpha_{\text{ADAR1 mut}}$ were well defined using several constraints, with backbone RMSD around 0.8 Å for the 20 conformers, and more than 98.2% of residues had backbone dihedral angles (ϕ and ψ) mapped to the most favored and additional allowed regions of the Ramachandran plot. The similar tertiary structures of these two proteins indicate that the different binding affinity of proteins to nucleotides is not due to the conformational change of whole protein by mutation, but due to the substitutions of the two key residues in the Z-DNA-binding interface.

Comparing the different binding affinity of these two proteins to Z-DNA, rRNA and *E.coli* ribosomes, we found that $Z\alpha_{\text{ADAR1}}$ has the ability to interact with Z-DNA, rRNA and *E.coli* ribosomes in high affinity, and induces the B- to Z- or A- to Z- conformational change on Z-DNA and rRNA, respectively. $Z\alpha_{\text{ADAR1 mut}}$ also has the ability of interaction with Z-DNA and rRNA, but in very low affinity. $Z\alpha_{\text{ADAR1 mut}}$ also lack the ability to induce Z-conformation nucleotides.

We found that the three complexes, $Z\alpha_{\text{ADAR1}}$ - DNA, $Z\alpha_{\text{ADAR1}}$ - rRNA and $Z\alpha_{\text{ADAR1}}$ – ribosome share the similar interaction surfaces. This indicates that the functions of $Z\alpha_{\text{ADAR1}}$ binding ribosomes and inhibiting translation may relate to its function of recognizing the potential Z-forming structures and inducing A- to Z- transition on ribosomal RNA fragments.

The attenuation of all cross-peaks in TROSY spectra of $Z\alpha_{\text{ADAR1}}$ – ribosome complex indicates the potential multiple binding sites between $Z\alpha_{\text{ADAR1}}$ and

ribosomes. This phenomenon was explained by our new dipole-dipole relaxation theory outside of redfield limit.

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