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Thermodynamic profiling of HIV RREIIB RNA – zinc finger interactions

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Abstract

The interactions between the HIV Rev responsive element (RRE) RNA and the HIV regulatory protein Rev, is crucial for the HIV life cycle. We have previously shown that single C₂H₂ zinc fingers, have the same binding site as the Rev peptide and exhibit nanomolar affinities. In this study, the specific role of amino acid side chains and molecular processes involved with complex formation were investigated by perturbation to the binding energetics via changes in temperature, pH, buffers, salt concentrations as well as zinc finger (znf) and RNA mutations, by isothermal titration calorimetry (ITC). Interestingly, despite the large cationic charge on the znfs, the number of interactions with the RNA phosphate backbone was lower than intuitively expected. The presence of binding induced protonation was established by ITC and localized to a histidine on the znf β -sheet, by NMR. The ΔC_p of znf-RNA binding was observed to be substantially negative and could not be accounted for by conventional solvent accessible surface area models. An alternative model (50), based on the extent of hydrogen bond changes as a result of differences in ligand induced water displacement at the binding site, provided reasonable explanation of the trends in ΔC_p , as well as ΔH and ΔS . Our studies show that incorporation of favorable interactions at the solvent excluded binding interface can be used to alleviate the unfavorable enthalpic penalties of displacing water molecules from the hydrated RNA surface.

Keywords

zinc finger; thermodynamics; solvent; specific heat; RNA protein interactions

Introduction

In the early phases of the human immunodeficiency virus type 1 (HIV-1) life cycle, the viral pre-mRNA undergoes complete splicing resulting in only 2 kilobase (kb) mRNA transcripts that are translated to the regulatory proteins: Tat, Nef and Rev (1,2). The production of structural, enzymatic and accessory viral proteins for the assembly of the progeny virus, requires the viral pre-mRNA to be diverted in sufficient quantities from the host cell nuclear splicing machinery and translocated to the cytoplasm as unspliced (~9kb) and singly spliced

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(~ 4kb) transcripts (3–5). This shift from the production and cytoplasmic appearance of fully spliced to unspliced and singly-spliced mRNA transcripts marks the transition from viral latency to the late phase of the HIV-1 replication cycle (3,6–8). The nuclear export of the viral pre-mRNA in unspliced and singly spliced forms is facilitated by the interaction between the regulatory protein Rev and the Rev responsive element (RRE), a 234-nucleotide-long untranslated RNA structure located within the env gene of the viral RNA genome (3,9,13, 14). Rev initially binds RRE at a high affinity-binding site, localized to a relatively small stem loop structure, RREIIB (10,11), followed by subsequent oligomerisation of up to a total of 8 Rev molecules through RNA-protein contacts (12). This forms the nuclear export signal, which utilizes the cellular nuclear export machinery to translocate to the cytoplasm. In the cytoplasm the dissociation of the complex releases the unspliced mRNA for translation while Rev shuttles itself back to the nucleus for the next round of HIV mRNA export (6,8). Thus the initial Rev-RREIIB interaction is vital for the subsequent production of viral assembly components; efficient prevention of this interaction could effectively abolish the production of infectious virions.

The interaction between Rev and RREIIB has been characterized using biochemical, mutational and structural methods (15,16,17). NMR structural studies have utilized a RREIIB analog RREIIBTR and the Rev peptide, a 17 amino acid arginine rich motif (Rev_{34–50}), to demonstrate that binding occurs in the major groove of the RNA (18). Rev peptide–RREIIBTR interaction induces formation of 2 new purine-purine base pairs (G-G and G-A) (19). These base pairs are formed in the bulge in the stem loop IIB and are critical to Rev binding.

An extensive list of strategies for inhibiting the pathogenesis of the virus has been documented (20). To interfere with the essential RRE–Rev interaction, these approaches use RNA based strategies such as anti-sense RNA, RNA decoys, RNA aptamers, ribozymes siRNA, protein based strategies which involve transdominant negative proteins (TNP), chimeric nucleases, intracellular antibodies, peptides (21,22) and small organic compounds (23,24). We have previously demonstrated that C₂H₂ zinc finger proteins (znfs) (ZNF29 and ZNF29G29R), designed by phage display (25), bind the same RNA bulge that Rev utilizes with nanomolar affinities (26). In a regular RNA helix the major groove is narrow while for RREIIB the major groove is opened up at the bulge containing the purine purine mismatches. This facilitates access of alpha helical recognition elements of Rev as well as znfs proteins. Our current studies focus on understanding the energetics of the underlying molecular processes that facilitate RNA – znf recognition. We have studied the energetic perturbations to the znf-RNA system resulting from mutations on ZNF29 and the RNA target, RREIIBTR, by isothermal titration calorimetry (ITC). Effects of salt, pH, temperature and the role of solvent on the binding were also investigated. Our findings suggest strategies for enhancing binding affinities of zinc finger proteins to the target RNA.

Results and Discussion

Design of zinc finger and RNA mutants

The sequence of the zinc finger was been previously optimized by phage display, which produced a number of high affinity RREIIB binding proteins (25). Subsequently we determined the NMR structures of ZNF29 and a mutant ZNF29G29R (26) which exhibits a higher affinity for the RNA. Using this phage display generated high affinity scaffold we now seek to optimize RNA binding by changing additional key residues to assess their binding contributions. The mutant znfs are named based on the position of the mutation relative to ZNF29 (Figure 1A); the rationales involved in the choice of the position and type of mutation are as follows:

(I) β sheet mutations: Histidine at position 6 could potentially be involved in stacking with an RNA base. The ZNF29H6A and ZNF29H6K mutants test this possibility. If the interaction

involves aromatic stacking, reduced affinities are expected for both mutations. The ZNF29R12A mutant queries the role of a charged polar side chain on the β -sheet, to RNA binding. (II) Mutations at the tip of the zinc finger: The ZNF29N15A and ZNF29D16A mutants examine the involvement of the amino acids at the tip of the zinc finger to RNA binding. Zinc fingers are known to utilize their tips in DNA as well as RNA binding (27,28). (III) Mutations on the α helix: The ZNF29N21A mutant examines if the asparagine at this position serves a similar role as the analogous asparagine in the Rev peptide (18), which bridges the G47 – A73 base pair in the Rev-RREIBTR complex. The importance of the side chain length was investigated using ZNF29N21Q. In addition, a double mutant, ZNF29N21Q29R, was designed based on the analysis of the binding affinities of the single mutants.

None of the mutations involve zinc coordinating residues or residues involved in the hydrophobic packing of the zinc finger core. Nevertheless, we have established that all mutants fold into the characteristic $\beta\beta\alpha$ fold of zinc fingers from the presence of signature chemical shifts (Phe14 ζ H and His27 δ 2H) (26,29) in 1D 1 H NMR spectra in D₂O (data not shown). The constant structure of the scaffold allows us to evaluate the effect of individual side chains to the binding interaction.

The “wild type” RNA in our binding study is RREIBTR. RREIBTR is a modified version of the RREIB RNA and has been shown to retain all elements necessary for specific binding of Rev and Rev peptides (19). For ease of reference, regions of the RNA have been referred to as upper stem, middle stem, bulge and lower stem, as shown in Figure 1B. The RNA mutants probe the effects of base pair disruption on znf binding. Four of these mutants involve substitution of the guanosine in the middle stem (G50 and G70) and the bulge (G48-G71 and G47) with a 2-aminopurine while the other two involve substitution of the cytosine in the middle stem (C49, C79) with uracils.

To ascertain similar interaction of the mutant znfs with RREIBTR RNA, binding was monitored by NMR (Figure 2). While we observe new and shifted imino proton resonances from the bulge regions/middle stem for all mutants, other RNA regions are not affected. Specifically, the base pairs in the upper (G53–G64), near the lower stem (G41–C46) or the hairpin loop (G55) in the all RREIBTR complexes, retain essentially the same chemical shift, as the free RNA. The middle stem/bulge region was also implicated in the binding site from steady state fluorescence data (Supplementary data S10). Thus, all the new znf mutants studied in this work have the same binding site on the RNA, the bulge region, as determined previously for ZNF29 and ZNF29G29R (26), enabling the comparison of their thermodynamic binding parameters.

Binding affinities of znf mutants – RREIBTR

Thermodynamic parameters (ΔH , ΔS , K_d) for the binding of zinc finger proteins to RREIBTR were determined by ITC (Table 1). All binding isotherms could be fit to a single binding site model with a 1:1 stoichiometry. The free energy of binding at 25°C for all znf – RNA interactions had favorable enthalpy and entropy contributions. Changes in binding affinity for the znf mutants are based on the control, RREIBTR-ZNF29 interaction.

(I) β sheet mutations—While a mutation to alanine H6A lowered the affinity ~ 1.4 fold, the lysine mutation H6K resulted in increased affinity ~ 1.6 fold. There are differences in the imino spectra of ZNF29H6K vs ZNF29 RNA binding while the spectra of ZNF29H6A is similar to ZNF29 (Figure 2). Hence, the trend in the affinity of the H6 mutants cannot categorically rule out aromatic interactions, as mutation to lysine may have compensated for the absence of an aromatic interaction. Nevertheless, these mutations implicate the involvement of H6 in RNA binding. The possibility of binding linked protonation/deprotonation of this histidine was also investigated. The R12A mutation on the second β -strand of ZNF29 caused a ~ 1.8 fold reduction

in the binding affinity. This was surprising since zinc fingers generally do not utilize the β -sheet in their interactions with RNA (27,28). The imino spectra of the ZNF29R12A – RREIBTR complex is essentially superimposable to that of the control, suggesting that the interaction could be an electrostatic contact between the R12 side chain and the RNA phosphodiester backbone.

(II) Mutations at the tip of the zinc finger—The mutations at the tip of znf, ZNF29N15A and ZNF29D16A, resulted in the lowest binding affinities (~2.2 and ~3 fold lower for the N15A and D16A mutations respectively). In addition, the imino proton peaks that appeared on complex formation of these 2 mutants with RREIBTR, displayed distinct differences in their chemical shifts and intensities, compared to the control (Figure 2). The reduced affinities, along with the differences in the imino spectra of the complexes with RREIBTR indicate that these residues (N15 and D16) are directly involved in the formation of new base pairs in the complex.

(III) Mutations on the α helix—Mutations at position 21, N21A and N21Q, had opposite effects on the RNA binding affinity. While the mutation to alanine reduced binding affinity ~1.6 fold, there was a ~1.9 fold increase for N21Q. The imino proton spectra of ZNF29N21A - RNA was similar to that of the control but there were noticeable differences in the new imino proton peaks for ZNF29N21Q – RREIBTR. These comparisons suggest that the side chain of N21 does not contact a base pair as N40 in the Rev peptide (18). The longer sidechain of the N21Q mutant could result in altered protein-protein contacts. *In silico* mutation (N21Q) of the free ZNF29 predicts a hydrogen bond between Q21 and R17 (data not shown) (Swiss PDB Viewer, (30)). Thus the increased affinity of ZNF29N21Q could possibly arise from different positioning of the R17 side chain.

Binding affinities of RREIBTR mutants – ZNF29G29R

The thermodynamic binding parameters of ZNF29G29R to the RNA mutants detailed in Figure 1B are also presented in the Table 1. All RNA mutants bound ZNF29G29R with 1:1 stoichiometry. The free energy of binding had favorable enthalpy and entropy contributions for all RNA mutants – ZNF29G29R interactions, at 25 °C, except for the RNA mutant with a 2-aminopurine substitution at position G70 (RREIBTRG70_2AP) which has favorable enthalpy but unfavorable entropy contribution. While all the RNA mutants displayed lower binding affinities to ZNF29G29R than the wild type RNA, there is a trend depending on the position of the mutation. Any mutation disrupting the potential G70 – C49 base pair (middle stem) in the complex caused a drastic reduction in znf binding affinity (~11.8 fold lower for RREIBTRG70_2ap and ~8.9 fold lower for RREIBTRC49U). The 2-aminopurine substitutions at the bulge (RREIBTRG48G71_2ap, RREIBTRG47_2ap) reduced znf binding affinity ~5 – 6 fold while alteration of the G50 – C69 base pair had the least deleterious effect on znf binding (~3.7 and ~4.5 fold lower for RREIBTRG50_2ap and RREIBTRC69U respectively). The trend in the enthalpy and entropy components of the free energy of interaction for both protein and RNA mutants are discussed in further detail in later sections.

Binding linked protonation and pH effects

The potential for binding induced protonation was examined by determining the binding enthalpies at pH 6.2 and 8.0 using buffers with different heats of ionization. The observed binding enthalpy for ZNF29–RREIBTR is expressed as (31,32,39):

$$\Delta H_{\text{obs}}^i = \Delta H_{\text{int}} + \Delta n_{\text{H}} (\Delta H_{\text{Lp}} + \Delta H_{\text{b}}^i) \quad (1)$$

where, ΔH_{obs}^i is the enthalpy observed in the ITC experiment at the respective pH and buffer, ΔH_{int} is the enthalpy of fully protonated znf binding RNA, Δn_{H} is the proton uptake per mole of complex formation, $\Delta H_{\text{L,p}}$ is the enthalpy of binding linked ligand protonation (all potential protonation sites) and ΔH_{b}^1 is the enthalpy of buffer ionization. For complex formation in buffers with different heats of ionization at the same pH and temperature:

$$\Delta n_{\text{H}} = (\Delta H_{\text{obs}}^2 - \Delta H_{\text{obs}}^1) / (\Delta H_{\text{b}}^2 - \Delta H_{\text{b}}^1) \quad (2)$$

Δn_{H} was determined to be 0.24 and 0.61 at pH's 6.2 and 8.0 respectively (Figure 3A) and signifies a net uptake of protons. These values indicate the presence of a single protonation site whose pK_{a} is increased due to binding. The pH range suggests the involvement of a histidine and was determined by NMR to be H6, since complex formation resulted in substantial chemical shifts changes for H6 while no changes are observed for the zinc coordinating histidines (Figure 3C). In the free ZNF29, the pK_{a} of H6 is 6.7, determined from the change in the δ^2 ^{13}C and ^1H chemical shifts (Figure 3B). Deprotonation of H6 in the free ZNF29 is characterized by increasing ^{13}C and decreasing ^1H chemical shifts (supplementary data, S2). In the ZNF29-RNA complex the pK_{a} of H6 is expected to be higher; unfortunately precipitation at higher pH's precluded a direct measurement. However, the ^{13}C chemical shift decrease and the ^1H chemical shift increase (H6 δ^2 complex) supports a higher level of H6 protonation in the complex as compared to the free znf at pH 7 (Figure 3C). Using the proton uptake determined previously, a simulation places the estimated pK_{a} of H6 in the complex around 8.3 (Figure 3B)^R, corresponding to an increase of 1.6 pK_{a} units.

If binding induced protonation of the H6 side chain is the only event contributing to the enthalpy, then the enthalpies of ZNF29H6A – RREIBTR complex formation should remain constant in the pH range 6.2 – 8.0. However, this was not the case (Figure 3D); there is a linear increase in the binding enthalpy with increasing pH for ZNF29H6A. Considering potential groups on znf and RNA that may be protonated in this pH range, the most likely candidate is the N-terminal NH_3^+ for which pK_{a} of 8.0 is predicted (PROPKA (33)). We note that the enthalpy of H6A–RREIBTR complex formation increases linearly with lowered fractional protonation of this NH_3^+ group (Figure 3D). The N-terminal is located near the tip of the zinc finger, which is known to be involved in binding, since mutations (D16A, N15A) drastically affect the binding. We therefore hypothesize that interactions between the RNA and the tip of the znf is hampered by an intra- protein interaction involving the N-terminal NH_3^+ , possibly by an ion pair with D16. The znf tip–RNA interactions could involve disruption of this ion pair resulting in an enthalpic penalty. In such a scenario, the increasing pH will result in lowered levels of NH_3^+ protonation and consequently less energy is expended in breaking this ion-pair on binding. As a result, such an effect will manifest itself in more exothermic enthalpies with increasing pH, as observed. The distinct chemical shift changes ($^{13}\text{C}_{\alpha}$ - $^1\text{H}_{\alpha}$) of the N-terminal methionine (data not shown) upon binding RNA supports a change in the environment of M1. The ZNF29 binding enthalpy in Figure 3D is non-linear since it involves 2 processes, namely a linear contribution from ion-pair disruption and a non-linear contribution from binding induced protonation. The correlation between Δn_{H} and pH is non-linear (Figure 3B, equation 1). The highest contribution to ZNF29 binding enthalpy due to pH-influenced processes is predicted to be at around pH 7.5, in agreement with the maximal proton uptake (Δn_{H}) at this pH (Figure 3B, D).

^RThe H6 δ^2 chemical shifts in the complex could only be determined up to pH 8.8 beyond which precipitation prohibited measurements. Consequently, the end point of the pH titration of the complex could not be established. The pK_{a} of H6 in the complex was estimated by iterative use of pK_{a} values in the Henderson-Hasselbalch equation that reproduced proton uptake numbers determined experimentally (n_{H} values from Figure 3A).

We note that elevated pKa's for the RNA bases adenosine and cytidine have been reported for locally crowded phosphodiester backbones in ribozymes (34). However, pKa shifts for the RREIBTR RNA were not considered since the interaction site does not contain such structural features and the CD spectra does not support major rearrangement upon binding (Figure 7A).

Effects of salt

The extent of electrostatic contributions to the binding free energies was assessed by evaluating the affinities of ZNF29, ZNF29G29R and ZNF29R12A to RREIBTR under varying salt concentrations (NaCl) (Figure 4A). The absence of significant conformational changes in the complex was confirmed from the observation that the imino spectra of ZNF29 bound RREIBTR are salt independent (supplementary data, S3).

The salt dependence was analyzed using (35):

$$-\delta \log (K_{\text{obs}}) / \delta \log [\text{NaCl}] = m'\psi + k \quad (3)$$

where m' is the number of ion pairs formed resulting in cation release from the RNA, ψ is the fractional neutralization of the RNA backbone phosphates by thermodynamically bound cations, and k is the fraction of ions released by the protein on binding. Binding of ZNF29G29R exhibited the highest salt dependence followed by ZNF29, while binding of ZNF29R12A was salt independent. For ZNF29 and ZNF29G29R, a linear decrease of the binding affinities was observed with increasing salt concentration, in the range $100 \text{ mM} \leq [\text{NaCl}] \leq 175 \text{ mM}$, with $\delta \log (K_{\text{obs}}) / \delta \log [\text{NaCl}]$ of 2.19 and 2.94 respectively, indicating net release of ions (Na^+ and Cl^-), while at lower salt concentrations the binding affinities did not increase linearly (below 100 mM). Similar departures from linearity have also been observed in previous studies of protein-DNA binding studies at lower salt concentrations (36). This trend was attributed to change in the occupancy of ion binding sites on the protein resulting from transfer of the protein ion-binding surface from the bulk solution to a different ionic environment in the vicinity of the nucleic acid upon binding (high cation concentration, low anion concentration). The presence of weak anion binding by the znf and subsequent release on RNA binding was indicated from the decreasing overall ΔH_{obs} (less exothermic) and increasing overall ΔS_{obs} , when the anion was changed from a strongly hydrated (F^-) to a weakly hydrated anion (Br^-) (Figure 4B). Hence, changes in the occupancy of the anion binding sites on the znf would explain the curvature of $\log (K_{\text{obs}})$ vs $\log [\text{NaCl}]$, at low salt concentrations.

From the difference in $\delta \log (K_{\text{obs}}) / \delta \log [\text{NaCl}]$ of ZNF29 and ZNF29G29R and recognizing that ZNF29G29R has one additional charge, we estimate ψ to be 0.75. This value of ψ is reasonable for an oligomer like RREIBTR, which is expected to have a lower axial charge density than a long helix (0.88) due to end effects as well as irregular charge density at the bulge.

RNA binding by ZNF29G29R, ZNF29, and ZNF29R12A results in net release of 2.94, 2.19 and 0 ion pairs respectively indicating that R12 in ZNF29 and R29 in ZNF29G29R are involved in ionic interactions with the RNA phosphate backbone. The linear extrapolation, of the integrated form of equation 3, to 1 M NaCl can be used to calculate the free energy of binding in the absence of ion release (ΔG_0^0) (supplementary data, S4) (35). For ZNF29 and ZNF29G29R - RNA binding, ΔG_0^0 values are -6.34 and -5.91 kcal/mol at 298 K and consequently the free energy contribution of ion release (cations and anions, at 0.1 M salt, 298 K) are -2.96 and -3.96 kcal/mol respectively.

Temperature dependence of znf – RNA binding enthalpy

The temperature dependence of ΔH_{obs} for ZNF29 and ZNF29G29R RNA binding has been evaluated from 20 to 35 °C (Figure 5). For ZNF29G29R, the free energy of binding is enthalpy and entropy-driven in this temperature range. For ZNF29, the free energy of binding is enthalpy and entropy driven below 302 K while being enthalpy-driven and entropy-opposed above that. For both znfs, ΔG_{obs} is nearly constant in this temperature range. In cases when one or both biomolecules undergo thermally induced unfolding the relationship between ΔH_{obs} and temperature is non-linear (38). Both ZNF29 and ZNF29G29R RNA binding show a linear temperature dependence confirming that the components do not unfold in the studied range. Moreover, the znf – RNA complex, the free znfs, and the free RNA have been independently confirmed to be stable at these temperatures, which simplifies the analysis (data not shown).

The changes in the heat capacity of binding (ΔC_p) are -403 ± 40 and -274 ± 34 cal mol⁻¹ K⁻¹ for ZNF29 and ZNF29G29R respectively. These substantial changes in binding heat capacity may arise from several contributing factors as discussed below where the buffer ionizations only can account for a small portion of ΔC_p (-28 cal/mol for sodium phosphate buffer at pH 7) (39).

Major structural changes of either biomolecule or both on complex formation can have significant contributions to ΔH_{obs} and consequently affect ΔC_p (38). However, the CD spectra of free and complexed RNA show only minor differences which suggests minimal RNA structural changes upon binding (Figure 7A). In addition, chemical shifts of the residues in the hydrophobic core have similar chemical shifts for the free and bound znf (Figure 7B). Since the znf hydrophobic core is composed of side chains from the β -sheet as well as the α -helix, this indicates that on binding the znfs do not undergo any major conformational changes either. Thus, large structural perturbations are not the cause for the significant ΔC_p . While RNA binding will result in znf side chain rearrangements, this is not expected to contribute substantially to ΔC_p . The imino spectra of ZNF29 and ZNF29G29R RNA complexes are essentially superimposable indicating that the binding of both znfs result in the same changes. Therefore the formation of new base pairs can not account for the sizeable difference in their ΔC_p values ($|\Delta \Delta C_p| = 129$ cal mol⁻¹ K⁻¹) and are also not expected to be major contributors to the over all heat capacity changes.

A negative ΔC_p in biomolecular interactions has been linked to the extensive dehydration of non-polar (ΔA_{np}) compared to polar surfaces (ΔA_{p}) (either by the conformational rearrangement of the interacting molecules or by burial of nonpolar interfacial surfaces) (40, 41). The surface dehydration contribution to ΔC_p can be described by the empirical correlation:

$$\Delta C_p = \alpha \Delta A_{\text{np}} - \beta \Delta A_{\text{p}} \quad (6)$$

where α and β are positive coefficients (40–44) while ΔA_{np} and ΔA_{p} are negative quantities since they represent surface area burial. We have estimated the values of net change in solvent accessible surface area (SASA)^N, ΔA_{np} and ΔA_{p} (protein and RNA) (supplementary data, S6, S7) using a 1.5 Å solvent probe (45). The estimated net ΔA_{np} and ΔA_{p} , for ZNF29 – RREIBTR complex formation, are -751 and -1888 Å² respectively, while for ZNF29G29R – RREIBTR, the net ΔA_{np} and ΔA_{p} values are -743 and -1983 Å². Based on these values complex formation

^NSASA calculations were performed as described in the methods section. Since the structures of the free and znf complexed RNA are not available, the values presented here serve only as guidelines to underscore the extensive burial of polar over non-polar SASA. Even when the SASA calculations were performed by unstacking the bases in the middle stem and the bulge, for the free RREIB structure, ΔC_p calculated from semi-empirical models (Supplementary data S8) were not in the range of experimentally determined values. Intuitively too, the surface of the interacting elements that would be buried on complex formation, the α -helix of the znfs and RNA major groove at the bulge, involve more polar than non-polar SASA.

with either znf is expected to bury more polar than non-polar SASA. Even using different values for α and β (supplementary data, S8), surface area burial is still not able to explain the substantial negative values of the change in observed heat capacity and the $\Delta\Delta C_p$ for ZNF29 versus ZNF29G29R – RREIBTR binding. Failure to account for negative ΔC_p by conventional surface area models focusing on nonpolar surface dehydration alone have also been reported for other biomolecular interactions (46,47).

It has been suggested that ΔC_p contribution from protonation effects are compensated by opposing changes in the buffer (49) and therefore maybe disregarded. However, recent studies have shown that this assumption might be too simplistic (32,48). While we have not evaluated ΔC_p contribution of protonation effects, we recognize that $\Delta\Delta C_p$ for ZNF29 versus ZNF29G29R – RREIBTR binding cannot be explained by the temperature dependence of the induced protonation effect. Furthermore, trends in ΔH_{obs} for znf and RNA mutational studies prompted us to evaluate alternative models.

Cooper Models explain ΔC_p and trends in binding enthalpy and entropy of mutant znfs and mutant RNAs

Large negative values of ΔC_p for biomolecular interactions have been rationalized by the involvement of water (50,51). In this model, trends in ΔH and ΔC_p are evaluated on the basis of differences in the extent of hydrogen bond changes as a result of differences in ligand induced water displacement at the binding site. Water is treated as 1) solvating the macromolecular cavity and the ligand 2) bulk water and 3) non displaced water on ligand binding, where the occupancy of 1 and 2 are temperature dependent resulting in the following predictions (50):

- a. The binding enthalpy of a ligand that displaces more water molecules from the macromolecular cavity is less exothermic.
- b. Water involvement can result in negative ΔC_p . Specifically, a ligand displacing more water is predicted to have a less negative ΔC_p . Each additional water molecule displaced increases ΔC_p by $+18 \text{ cal mol}^{-1} \text{K}^{-1}$.

Despite its simplicity this model has proven useful to rationalize ΔH and ΔC_p values for biomolecular interactions in water (51).

The salt dependent behavior of ZNF29G29R implicates that the terminal arginine makes an electrostatic contact with the RNA phosphate backbone. Arginine interactions with the phosphate backbone are enthalpically favorable (52). Thus ZNF29G29R binding is expected to result in a more exothermic binding compared to ZNF29. Yet, the opposite is observed. We note that ZNF29G29R binding would displace more water than ZNF29 and the observed binding enthalpies are in agreement with the Cooper model. Similarly, as predicted, ΔC_p of ZNF29 binding is more negative than that of ZNF29G29R. This model predicts (from $|\Delta\Delta C_p|$) ZNF29G29R displaces ~ 7 more water molecules than ZNF29, in good agreement with the number estimated (~ 7 to 8) from the difference in their solvent excluded volumes ($110 \text{ \AA}^3$).

We also observe similar enthalpic trends for most of the other ZNF29 mutants, signifying solvent role in a similar fashion (Figure 6A). Although N15 and D16 are a part of the znf – RNA interactions, as evidenced from imino proton data, the binding enthalpies of N15A and D16A mutants are slightly more exothermic as compared to ZNF29, instead of being lower. Similarly, ZNF29R12A binding is more exothermic than ZNF29G29R despite the missing electrostatic interactions (salt studies). The mutants H6A and H6K cannot have binding induced protonation effects because they lack the histidine and yet mutation to an alanine is more exothermic than to lysine. All the above comparisons involve mutations from bulkier to smaller amino acids, which result in fewer displaced water molecules on binding.

Consequently, such lowered displacement of water molecules results in more exothermic binding enthalpies as predicted by the Cooper model. In comparison to the ZNF29 binding enthalpy, a mutation to smaller non polar amino acid thus has 2 opposing effects: an enthalpic loss due to the absence of the potential favorable interaction, compensated by an enthalpic gain due to lower displacement of water. The binding enthalpies of mutations that are secluded from the znf- RNA - solvent interface (Figure 6C), thus reflect the potential interaction, as we observe in the case of the mutants N21A and N21Q.

The enthalpic gain from lowered displacement of water molecules to the bulk solvent is also observed for RNA mutations that affect the geometry of the RREIBTR bulge. The change in the binding site geometry thus results in fewer displaced water molecules and consequently, is manifested as higher binding enthalpies accompanied by lower entropic contributions to the binding free energy (Figure 6B). This effect is more pronounced for modifications at the middle stem, which alter the compact geometry of the binding site by prevention of possible base pair formation (2-aminopurine substitutions). Modifications at the bulge G48G71-2ap, G47_2ap resulted in less exothermic binding, indicating their involvement at the RNA-protein interface devoid of solvent.

While displacement of water molecules from the hydrated RNA surface contributes to the binding free energy via the entropic gain, it is accompanied by unfavorable enthalpic contributions, which mitigates the net favorable impact on the overall binding energetics (Supplementary data S9).

Conclusion

In this study we have performed a thermodynamic investigation of the znf – RNA interactions utilizing isothermal titration calorimetry and NMR spectroscopy. We have previously shown that znf – RNA binding is dependent on the znf $\beta\beta\alpha$ architecture (26). Our current studies focus on enumerating the role of amino acid sidechains on the RNA - znf interactions. We observe that although coupling of several molecular processes hamper specific allocation of contributions to the binding free energy; insight into the nature of interactions can still be obtained.

The zinc finger motif is ubiquitous amongst nucleic acid binding proteins. Specifically, C₂H₂ znf motifs belonging to TFIIIA have been shown to interact with their RNA targets through multiple modes of recognition (53). Structural and biochemical studies have determined that the residues at the tip of the helix are critical to binding (53). Our studies also show the importance of the residues at these positions, N15 and D16, to RNA binding. It was surprising to observe the involvement of the β -sheet residues (H6 and R12) in znf - RNA interactions, since zinc fingers do not generally use any component of their β -sheet in RNA binding (27, 28,53). Both H6 and R12 residues flank the β sheet (Figure 6C), and binding induced protonation of the H6 residue as well as salt studies on the ZNF29R12A mutant binding suggests their role in contacts to the phosphodiester backbone. These residues may serve as anchoring the znf to the RNA in coordination with other interactions in the widened RNA major groove. The removal of any of these anchors may introduce conformational flexibility of the RNA phosphate backbone at these sites, as evidenced by the increased binding entropy of these mutants (Figure 6A), as compared to ZNF29.

The free energy of znf – RNA association contains contributions from several processes. Amongst them, the favorable contribution from ion release on binding to the overall free energy accounts for ~ 32 % and ~ 40% of the observed total binding free energy for ZNF29 and ZNF29G29R respectively (100 mM NaCl, 298 K). Consequently, this indicates that molecular recognition between the znf and the RNA is driven by fewer electrostatic contacts on the RNA

phosphate backbone than one might expect. Therefore znf – RNA association is largely driven by favorable contributions arising mainly from interactions in the RNA major groove. These favorable interactions counteract the unfavorable contributions from loss of translational and rotational degrees of freedom, which can be substantial (54). Unlike protein-DNA complexes, protein- RNA complexes have a lower level of participation of the phosphate backbone in interactions. Statistical analyses of protein RNA complexes (55–57) have shown that H-bonds from arginine/lysine side chains to the RNA 2' OH account for a significant portion of the molecular contacts. We note that the screening of the znf phage display library against the RREIIB target was performed in the presence of excess tRNA, which might have efficiently eliminated znf sequences where recognition was predominantly driven by less specific RNA phosphate backbone interactions.

The perturbations to the ZNF29 – RREIIBTR binding free energy, through znf or RNA mutations, is limited to a window of ± 1 kcal/mol, although substantial differences are observed in their enthalpic and entropic components. Specifically, for ZNF29 and ZNF29G29R binding, the ΔC_p as well as the $\Delta\Delta C_p$ (ZNF29 vs ZNF29G29R) could not be explained by conventional solvent accessible surface area models. The use of Cooper models with respect to dehydration at the binding interface and their subsequent effect on the respective enthalpies, entropies as well as the nature of ΔC_p , provided a satisfactory explanation. Additionally, analyses of the enthalpy using buffers with different heats of ionization clearly demonstrated the presence of binding induced protonation.

A more detailed understanding of the molecular processes in this znf – RNA system enabled the design of a znf with higher RREIIBTR binding affinity. The interaction of the terminal arginine (ZNF29G29R) with the RNA phosphate backbone, though favorable, is alleviated by a substantial enthalpic penalty due to displacement of solvent. The binding of the mutant ZNF29N21Q however shows how such enthalpic penalties can be mitigated with favorable interactions at the binding interface that is secluded from the solvent. This information has been successfully used to design the double mutant ZNF29N21QG29R (Table 1), where both the enthalpic and entropic contributions to free energy are favorably increased, compared to ZNF29. Such an approach may be instrumental in improving binding affinity of drugs guided by thermodynamic studies.

MATERIALS AND METHODS

Proteins and RNA

Site directed mutants of the ZF29 plasmid were obtained using Stratagene's QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) and transformed into BL21DE3 pLys S cells (Novagen). Expression, purification and characterization were done following previous protocols (26). RNA – Gel purified RREIIB-TR RNA and the 2-aminopurine modified RREIIB-TR RNA sequences were obtained from Dharmacon Research, Inc. with 2' protection groups and treated as described previously (26).

Isothermal Titration Calorimetry

All ITC experiments were recorded on a VP-ITC micro-calorimeter (MicroCal, LLC, Northampton, MA) with 10 μ L aliquots of the protein into RREIIB-Tr RNA every 400 seconds. Unless otherwise stated, ITC experiments were done at 25 °C in a standard ITC buffer (10 mM sodium phosphate, 100 mM NaCl, and 200 μ M β -mercaptoethanol) with ZNF and RNA concentrations of 65 – 85 μ M and 4.5 – 5 μ M respectively. ITC samples were degassed, thermostatted and then adjusted to pH 7.0. In all experiments, the pH difference between the titrant and analyte were less than 0.02. Equilibrium constants (K_a), ΔH , and number of binding sites (n) were obtained from a one-site model curve fit using VPViewer2000 and Origin 7

(OriginLab Corp, Northampton, MA). The heat of dilution was subtracted by applying a linear fit to the saturation portion of titration curve. ΔG and ΔS were calculated from: $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$ (where R is the gas constant ($1.987 \text{ cal mol}^{-1} \text{ K}^{-1}$) and T is the temperature in K). The specific heats (ΔC_p) were obtained from: $\Delta C_p = \partial \Delta H / \partial T$. The contribution from buffer ionizations was calculated from $\Delta n_H \Delta C_{p \text{ buffer}}$ (32), where Δn_H is the number of protons uptake per mole of complex and $\Delta C_{p \text{ buffer}}$ is the heat capacity change from the deprotonation of the buffer.

Buffer protonation measurements were recorded at pH 6.2 in MES buffer (2-(N-morpholino) ethanesulfonic acid), phosphate, and sodium cacodylate buffer and at pH 8.0 in tricine buffer, triethanol amine, EPPS buffer (3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid), and phosphate buffer. All buffers contained 10 mM buffer, 100 mM NaCl and 200 μM β -mercaptoethanol. Published values for the heats of protonation for the buffers are as follows: MES, 3.71 kcal/mol; phosphate, 1.22 kcal/mol; sodium cacodylate, -0.47 kcal/mol; tricine, 7.63 kcal/mol; triethanolamine, 8.02 kcal/mol; and EPPS, 5.15 kcal/mol (39).

NMR spectroscopy

NMR samples were prepared in the ITC buffer containing 10% D_2O . Imino proton spectra were collected over a range of 50 – 200 mM NaCl. All NMR experiments were acquired at 25°C (unless otherwise state) on a Bruker Avance 600 using a 5 mm QXI triple resonance Z-gradient probehead (Bruker). Data was processed with XWINNMR 3.5. The 1D imino proton spectra for both RREIBTR RNA and RREIBTR-ZNF complexes were collected using a 1-1 jump and return pulse sequence (58). Heteronuclear single quantum correlation (HSQC) spectra using echo-antiecho-TPPI and shaped pulses for the ^{13}C inversion were recorded using z gradients ($2\text{K} \times 512$, 48 scans, 1.5-s presaturation). (59). The 2D data were processed with a shifted sinebell (SSB) window function and transformed with the following processing parameters: $4\text{K} \times 4\text{K}$, SSB of 2 in f2 and f1. The ^{13}C chemical shifts in the HSQC spectrum at pH of 7 and 25 °C was referenced indirectly to the ^1H internal standard (DSS). This spectral reference was used to reference all the other HSQC spectra (different pH's).

Circular Dichroism

All CD spectra were recorded on a Jasco J720 spectrophotometer from 200 to 330 nm at 200 nm/min with a 1 nm bandwidth using a 0.2 cm cuvette. 20 repetitive scans were averaged and smoothed by Savitzky–Golay smoothing filter in the CD software package Jasco Spectra Manager v1.5.

Surface Area Calculations

Solvent accessible surface area (SASA) was calculated using a 1.5 solvent probe using the radii set of Richards et al. (61) for both Tables S6 and S7 (supplementary data), using the program Surface Racer (45). The changes in area were calculated as: $\Delta (\text{SASA}) = \text{SASA}_{\text{complex}} - (\text{SASA}_{\text{free RNA}} + \text{SASA}_{\text{free protein}})$ For the free proteins, ZNF29 and ZNF29G29R, the pdb files 2AB3 and 2AB7 were used. The free RNA structure of RREIBTR is not available. Therefore the pdb for free RNA structure was created from the Rev bound RREIBTR (18) (pdb id 1ETF) by manually deleting the Rev peptide. Docking the free protein helix in the major groove of the free RNA created the pdb used for the respective complex. The A_{OH} for ZNF29G29R (free and bound) is assumed to be the same as for ZNF29.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

ZNF29 and RREIBTR mutations. (A) ZNF29 (26) NMR solution structure with the position of the mutations displayed (side chains). The tip of the znf is the turn connecting the β -sheet to the α -helix. (B) RREIBTR sequence with the same numbering as Battiste et al. (19). RNA mutants with 2-aminopurine substitutions are indicated by ●, while mutations from a cytosine to a uracil are indicated by ■. All mutations are single changes except G48 – G71 where both guanosines are replaced by 2 – aminopurines. The RNA mutants are named based on the position and the type of mutation. Mutations involving 2-aminopurine substitutions are: RREIBTRG50_2ap, RREIBTRG70_2ap, RREIBTRG48G71_2ap, RREIBTRG47_2ap. Mutations involving replacement of the cytosine by a uracil are: RREIBTRC49U, RREIBTRC69U.

Figure 2.

Imino proton spectra of free and znf bound RREIIBTR RNA. Znf:RNA molar ratios of 1:1 were achieved by titrating protein into RNA at 298 K in ITC buffer. The assignment of the free RNA is based on previously published data (19), except for G55, which was identified from the imino spectrum of a smaller RNA oligonucleotide representing the upper stem (C51-G67) (data not shown). The dotted lines indicate imino resonances that show minor shifts on znf addition or do not shift at all. These resonances represent base pairing of the upper stem including the GCAA tetraloop (G53, G55, G64) and the lower stem (G42, G76, G77) thus precluding them from being a part of the binding region and consequently localizing the bound znfs to the region including the bulge and the middle stem. The resonances at around 13.97

and 13.72 were identified as U66 and U45 respectively from NOESY cross-peaks for the ZNF29-RREIIBTR complex (data not shown). However, absence of NOE's from the new peaks (12.45–12.75, 11.75) to previously identified resonances prevented their identification.

Figure 3.

Binding linked protonation and pH effects. (3A) The experimentally determined enthalpies (ΔH_{obs}) of ZNF29 – RREIBTR binding at the pH's, 6.2 and 8.0, are plotted against the enthalpies of buffer ionization (ΔH_b). The ΔH_b values are taken from Fukada and Takahashi (39). The ΔH_{obs} (kcal/mol) values at pH 6.2 are: -3.94 ± 0.11 (MES, $\blacktriangle$), -4.96 ± 0.08 (cacodylate, $\blacktriangledown$) and -4.47 ± 0.07 (sodium phosphate, $\circ$) while at pH 8 are: -5.06 ± 0.04 (Tricine, ∇), -4.45 ± 0.08 (triethanolamine, $\blacklozenge$), -6.38 ± 0.10 (EPPS, $\blacksquare$) and -8.76 ± 0.08 (sodium phosphate, $\circ$). The Δn_H (equation 2) values are determined to be 0.24 and 0.61 at pH's 6.2 and 8 respectively and indicate proton uptake on binding.

(3B) The fractional change in H6 protonation for free ZNF29 was determined from the ^1H ($\delta 2$) ($\bullet$) and ^{13}C ($\delta 2$) ($\blacktriangle$) fractional chemical shift changes (chemical shifts at the respective pH are listed in the supplementary data, S2). The fractional change in protonation is displayed as fully protonated (0) to fully deprotonated (1). The dashed line represents the fit to the equation, fractional change = $(10^{\text{pH}-\text{pK}_a})/(1+10^{\text{pH}-\text{pK}_a})$. The solid black line represents the fractional protonation change calculated from the above equation for $\text{pK}_a = 8.3$. The solid red line is the theoretically calculated Δn_H for a pK_a shift from 6.7 (free) to 8.3 (bound). The

experimentally determined Δn_H , from Figure 3A are marked by $\oplus$. (3C) Overlay of the ^{13}C - ^1H HSQC spectra for the free (blue) and RREIBTR bound ZNF29 (red) shows distinct changes in chemical shift ($^1\text{H}\delta 2$ & $^{13}\text{C}\delta 2$) for H6 while the zinc coordinated histidines (H23 & H27) do not shift.

(3D) Binding enthalpies (ΔH_{obs}) and errors for ZNF29 (■) and H6A (●) are plotted against the fractional deprotonation (f_p) of the N-terminal NH_3^+ group (pK_a 8.0). The pH's for the corresponding fractional deprotonation are also displayed. The solid line represents a linear regression fit ($\Delta H_{\text{obs}} = \Delta H_{\text{int}} + f_p \cdot \Delta H_{\text{ipd}}$) for H6A binding enthalpies (ΔH_{obs}) with increasing pH and has a slope of $-5.90 \pm 0.6 \text{ kcal mol}^{-1}$ (ΔH_{ipd} , enthalpy of ion pair disruption). The fractional deprotonation was calculated by the Henderson–Hasselbalch equation. Additional thermodynamic parameters for Figures 3A and 3D are listed in supplementary data, S11.

Figure 4.

Salt dependence of znf - RNA binding. (A) RREIBTR binding affinity (K_a) for ZNF29, ZNF29G29R and ZNF29R12A are plotted against salt (NaCl) concentrations. The linear correlation between salt dependence and binding affinity have been analyzed by equation 3. All the above experiments were conducted at 298 K with the same buffer conditions (10 mM sodium phosphate, 200 μ M β -mercaptoethanol, pH 7.0 $\pm$ 0.02) under varying NaCl concentrations. (B) Thermodynamic parameters of ZNF29G29R – RREIBTR binding determined at 100 mM NaF, NaCl and NaBr. Entropic contributions are expressed as $-T\Delta S_{obs}$ (298 K). Previous studies have shown that anions interacting weakly with a protein when released on DNA binding make the overall ΔH_{obs} less exothermic (37).

Figure 5.

Enthalpy - entropy compensation for the binding of ZNF29 and ZNF29G29R to RREIBTR. Thermodynamic parameters for ZNF29G29R binding are displayed as: ΔH ■, $T\Delta S$ ●, ΔG ▲ and for ZNF29 as: ΔH □, $T\Delta S$ ○, ΔG ▼. The free energy was calculated by $\Delta G = -RT \ln (K_a)$ and entropic contributions by $T\Delta S = \Delta H - \Delta G$. The linear regression fit of the enthalpic dependence on temperature yielded ΔC_p for ZNF29 and ZNF29G29R binding to be -403 ± 40 and $-274 \pm 34 \text{ cal mol}^{-1} \text{ K}^{-1}$ respectively. A non-zero heat capacity is also indicated by a non linear correlation between $\ln (K_a)$ and $1/T$ (Supplementary data S5). Symbols encompass the error associated. All the above data is also presented in the supplementary data, S11.

Figure 6.

Enthalpic and entropic contributions to free binding energy of binding at 298 K. The binding enthalpies (black bars) of ZNF29 and mutants to RREIBTR (A) and RNA mutants to ZNF29G29R (B) are displayed in the order of increasing exothermicity. Entropic contributions (white bars) are expressed as $-\Delta S$. Errors in ΔH are from curve fitting of the binding isotherm in Origin. (C) The molecular surface of the free ZNF29G29R (pdb id: 2AB7) (VMD) (60) is shown with the positions of the mutations rendered opaque. The protrusions from the surface for mutational positions at the protein – RNA – solvent interface affecting water displacement on binding are shown in red. The surface of the helical residues (residue #'s 17–19, 22, 24–26, 28) surrounding N21 (yellow) is shown in transparent blue. The backbone of the znf is displayed as a cartoon trace.

Figure 7.

Absence of major structural rearrangements for the RNA and ZNF29 on binding. (A) CD spectra of free RREIBTR RNA (dashed) and ZNF29 complexed RNA (1:1 complex) (solid) at a concentration of 10 μM . Spectra (20 scans) were acquired in a 0.2 cm cuvette using a scanning speed of 200 nm/min. The spectrum of free ZNF29 was subtracted from that of the complexed RNA. (B) Overlay of the aromatic region of the ^{13}C - ^1H HSQC spectra for free (blue) and RREIBTR bound ZNF29 (red). Assignments are indicated for the free protein. All spectra (CD & NMR) were acquired at pH 7.0, 298 K in ITC buffer.

Energetics of ZNF – RNA binding at 298 K, pH 7.0. Binding affinities are expressed as dissociation constants (K_d) where $K_d = 1/K_a$, the association constant obtained by curve-fitting the ITC binding isotherm in Origin 7.0 software. The errors reported are also from curve fitting to a 1:1 site binding model. The binding free energies were calculated from $\Delta G = -R \ln(K_a)$, and the entropies from $\Delta S = (\Delta H - \Delta G) / T$. ZNF concentrations were in the range of 65 – 85 μ M, while RNA was 4.5 – 5 μ M. All experiments were performed in ITC buffer (10 mM sodium phosphate, 100 mM NaCl, 200 μ M β -mercaptoethanol). Representative ITC traces are included in supplementary data S1. The heats of dilution (kcal/mol) were: ZNF29: –0.4, 29R: –0.4, H6K: –0.4, R12A: –0.4, N15A: –0.4, D16A: –0.4, H6A: +3.5, N21A: +2, N21Q: +2.

Table 1

Protein	RNA	n	K_d nM	ΔG kcal mol ⁻¹	ΔH kcal mol ⁻¹	ΔS cal mol ⁻¹
ZNF29	RREIBTR	1.02	149 ± 21	-9.3 ± 0.1	-7.2 ± 0.1	7.2
ZNF29H6A	RREIBTR	0.94	207 ± 25	-9.1 ± 0.1	-5.3 ± 0.1	13
ZNF29H6K	RREIBTR	1.01	95 ± 29	-9.6 ± 0.2	-4.3 ± 0.1	17.7
ZNF29R12A	RREIBTR	0.98	266 ± 10	-9.0 ± 0.0 ^a	-5.7 ± 0.0 ^a	11
ZNF29N15A	RREIBTR	1.09	322 ± 60	-8.9 ± 0.1	-8.2 ± 0.2	2.3
ZNF29D16A	RREIBTR	1.00	450 ± 45	-8.7 ± 0.1	-8.0 ± 0.2	2.4
ZNF29N21A	RREIBTR	0.94	232 ± 40	-9.0 ± 0.1	-6.7 ± 0.2	8.1
ZNF29N21Q	RREIBTR	0.94	80 ± 31	-9.7 ± 0.2	-7.8 ± 0.2	6.3
ZNF29G29R	RREIBTR	0.99	57 ± 6	-9.9 ± 0.1	-5.5 ± 0.1	14.6
ZNF29N21Q29R	RREIBTR	1.06	35 ± 10	-10.2 ± 0.2	-7.6 ± 0.2	8.6
ZNF29G29R	RREIBTRG70_2ap	1.00	671 ± 147	-8.41 ± 0.1	-10.1 ± 0.5	-5.8
ZNF29G29R	RREIBTRC49U	1.07	510 ± 64	-8.57 ± 0.1	-6.1 ± 0.1	8.4
ZNF29G29R	RREIBTRG48G71_2ap	1.04	324 ± 46	-8.84 ± 0.1	-4.7 ± 0.1	14
ZNF29G29R	RREIBTRG47_2ap	1.02	303 ± 57	-8.88 ± 0.1	-4.3 ± 0.1	15.3
ZNF29G29R	RREIBTRG50_2ap	0.93	210 ± 46	-9.10 ± 0.1	-7.8 ± 0.2	4.5
ZNF29G29R	RREIBTRC69U	0.98	254 ± 82	-8.99 ± 0.1	-8.6 ± 0.3	1.5

^aErrors in ΔG and ΔH are 0.02 and 0.04 kcal mol⁻¹ respectively.