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Article *in* Journal of biomolecular Structure & Dynamics · August 2015

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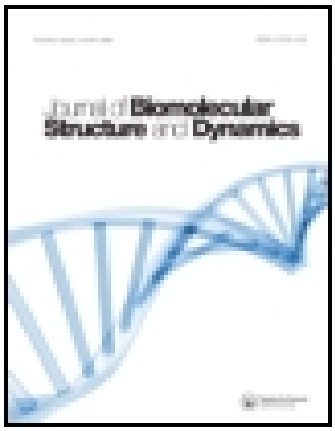

Journal of Biomolecular Structure and Dynamics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbsd20

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Accepted author version posted online: 21 Jul 2015.

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To cite this article: Mazen Y. Hamed & Gaurav Arya (2015): Zinc Finger Protein Binding to DNA: An Energy Perspective Using Molecular Dynamics Simulation and Free Energy Calculations on Mutants of both Zinc Finger Domains and their Specific DNA bases., Journal of Biomolecular Structure and Dynamics, DOI: 10.1080/07391102.2015.1068224

To link to this article: http://dx.doi.org/10.1080/07391102.2015.1068224

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Publisher: Taylor & Francis

Journal: *Journal of Biomolecular Structure and Dynamics*

DOI: http://dx.doi.org/10.1080/07391102.2015.1068224

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Zinc Finger Protein Binding to DNA: An Energy Perspective Using Molecular Dynamics Simulation and Free Energy Calculations on Mutants of both Zinc Finger Domains and their Specific DNA bases.

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Abstract

Energy calculations based on MM/GBSA were employed to study various zinc finger protein motifs binding to DNA. Mutants of both the DNA bound to their specific amino acids were studied. Calculated energies gave evidence for a relationship between binding energy and affinity of zinc finger motifs to their sites on DNA. ΔG values were -15.82(12), -3.66(12) and ¬12.14(11.6) Kcal/mol for finger one, finger two and finger three respectively. The mutations in the DNA bases reduced the value of the negative energies of binding (maximum value for ΔΔG = 42Kcal/mol for F1 when GCG mutated to GGG, and ΔΔG = 22 Kcal/mol for F2, the loss in total energy of binding originated in the loss in electrostatic energies upon mutation(r= 0.98). The mutations in key amino acids in the zinc finger motif in positions -1, 2, 3 and 6 showed reduced binding energies to DNA with correlation coefficients between total free energy and electrostatic was 0.99 and with Van der Waal was 0.93. Results agree with experimentally found selectivity which showed that Arginine in position -1 is specific to G, while Aspartic acid (D) in position 2 plays a complicated role in binding. There is a correlation between the MD calculated free energies of binding and those obtained experimentally for prepared zinc finger motifs bound to triplet bases in other reports (D J Segal, Dreier, Beerli, & Barbas, 1999) , our results may help in the design of zinc finger motifs based on the established recognition codes based on energies and contributing energies to the total energy.

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Introduction

Cys_2 His_2 Zinc finger proteins (ZF) are known to bind specifically to DNA sequences in transcription factor IIIA (Pabo, Peisach, & Grant, 2001; Eustermann et al., 2011; Carlson et al., 2010; Temiz & Camacho, 2009), they consist of two, three, or more fingers in the DNA binding protein. Each single finger domain consists of 30 amino acid residues with a ββα fold, the finger is stabilized by the structural zinc ion bound to two Histidine and two Cysteine residues in tetrahedral environment(Dreier, Segal, & Barbas, 2000; C O Pabo & Sauer, 1984). Naturally occurring ZF proteins in biological systems have been extensively studied and many x-ray crystal structures have been determined (Dreier et al., 2000; David J Segal, Crotty, Bhakta, Barbas, & Horton, 2006) . Progress in understanding protein-DNA recognition has made it possible to design many site specific regulatory ZF proteins that can alter gene expression in a specific way. Emergence of such systems has a wide range of important biomedical applications (Dreier et al., 2000; Ligands et al., 2004; Nakata et al., 2012; C O Pabo & Sauer, 1984; Carl O Pabo et al., 2001; Pavletich & Pabo, 1991; David J Segal et al., 2006). Also many other multi finger proteins were successfully engineered to bind specific DNA targets and this has a potential use in medicine (Dreier et al., 2000; Nakata et al., 2012; Carl O Pabo et al., 2001; D J Segal et al., 1999). Zinc finger proteins have potential as DNA binding domains of novel transcription factors (Carlson et al., 2010; Eustermann et al., 2011; A. N. Temiz & Camacho, 2009) which help in the treatment of diseases by employing gene therapy, the advantage of ZF proteins being their capability to bind larger binding sites(Nakata et al., 2012; Wolfe, Grant, Elrod-Erickson, & Pabo, 2001; Wolfe, Ramm, & Pabo, 2000) .

The issue of specificity of ZF proteins is still not fully understood, specifically the accuracy of the predicted modularity of ZF binding to DNA sequences , which means the ability

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to build segments from amino acids to form fingers and join the fingers together to fulfill certain function as engineered. The dissociation constants of many ZF-DNA sequences prepared by site directed mutagenesis were experimentally determined using gel shift mobility experiments (Dreier, Beerli, Segal, Flippin, & Barbas, 2001; Elrod-Erickson, Benson, & Pabo, 1998; Kang & Kim, 2000; Ligands et al., 2004; Carl O Pabo et al., 2001) with an error of $\pm$ 50% (Blancafort, Segal, & Barbas, 2004; Brayer, Kulshreshtha, & Segal, 2008; Cheng, Boyer, & Juliano, 1997; Dreier et al., 2000; Jamieson, Miller, & Pabo, 2003; Q. Liu, Xia, Zhong, & Case, 2002; Carl O Pabo et al., 2001; D J Segal et al., 1999; Uil, 2003; Wu, Yang, & Barbas, 1995). On the basis of these binding studies, amino acid residue-base specificities were assigned(Ligands et al., 2004; Carl O Pabo et al., 2001; D J Segal et al., 1999) supported by the known crystal structures. However, discrepancy between the predicted specificity and the experimentally found ones in determined x-ray structures still exists (David J Segal et al., 2006).

Statistically based specificities using the known ZF protein-DNA crystal structures derived by preparing databases and empirical rules there from were reported and compared with experimentally derived specificity with reasonable correlation(Desjarlais & Berg, 1992; Fu et al., 2009; Gromiha, Siebers, Selvaraj, Kono, & Sarai, 2005; J. Liu & Stormo, 2008; Paillard & Lavery, 2004; Rohs, West, Liu, & Honig, 2009) . Strategies for selection of specific ZF binding have also been developed(Greisman, 1997) .

Molecular dynamics has been employed as a powerful tool in solving complicated biological problems in protein folding, drug design, protein-protein interaction and DNA studies (Chang, McLaughlin, Baron, Wang, & McCammon, 2008; Fogolari, Brigo, & Molinari, 2003; Hou, Chen, McLaughlin, Lu, & Wang, 2006; Kollman et al., 2000; Naïm et al., 2007; Qiu, Shenkin, Hollinger, & Still, 1997; Rizzo, Aynechi, Case, & Kuntz, 2006; J. Wang, Morin, Wang,

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& Kollman, 2001; W. Wang et al., 2001) . Free energy calculations have been used as a powerful tool to study protein-ligand (Kollman et al., 2000) and protein-protein interactions (Chang et al., 2008; Hou et al., 2006). The MM-GBSA (Molecular Mechanics Surfaces area-generalized Born model) was introduced and used successfully in evaluating binding energies of proteins and their complexes with reasonable correlation to experimental values and giving a sound explanation of the contributing energies (Naïm et al., 2007; Rizzo et al., 2006; J. Wang et al., 2001; W. Wang et al., 2001). This method relies on the contributions of Van *der* Waals (VdW) and electrostatic energies as well as on nonpolar and relative solute entropy(Fogolari et al., 2003; Qiu et al., 1997). In this work, the VdW and electrostatic interactions between protein and ligand in the protein-DNA complex are calculated using molecular mechanics. The nonpolar contribution to solvation free energy is estimated employing the solvent accessible surface, and the electrostatic contribution to solvation is calculated by a continuum model (by solving the Generalized Born equation (Qiu et al., 1997). Entropy is usually calculated using normal mode calculation(Fogolari et al., 2003; Morozov, Havranek, Baker, & Siggia, 2005) . Snapshots are extracted from molecular dynamics trajectories and every snapshot is analyzed by MM/GBSA.

Here we employ MM/GBSA in calculating the free energy of binding for different sequences of zinc finger proteins to various targets of base combinations of DNA. Then the contributing energies to the total free energy of binding are analyzed to explore the capability of this method in estimating the specificity of ZF fingers binding to DNA bases. The outcome of this work can be used as a measurement of the predictive power of MD and MM-GBSA in designing Zinc fingers which bind specifically to certain base combinations of target DNA.

Attempts were made to identify the important features responsible for DNA binding using x-ray structures and bioinformatics in order to establish a recognition code and to predict

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specific binding. The extent to which these empirical rules can predict the capability of certain sequence to bind specifically to a designated DNA target so it can be used in zinc finger protein design was investigated. The indirect readout in the recognition process cannot be ignored and has been given attention (Rohs et al., 2009). In fact researchers, using selection strategies, have been able to develop new sequence specific DNA binding proteins which are capable of targeting unique sequences in the human genome with high affinity and modularity(Dreier et al., 2000; C O Pabo & Nekludova, 2000; C O Pabo & Sauer, 1984; Carl O Pabo et al., 2001; D J Segal et al., 1999) . However, until now, no simple recognition code could be established because of the problems of cross strand, inter-helical contacts and the flexibility of orientation of the recognition helix (Dreier et al., 2000; C O Pabo & Nekludova, 2000; Wolfe et al., 2001, 2000), along with the possibility of finger rotation(David J Segal et al., 2006) .

In this work the emphasis is on molecular aspects of recognition of amino acid residues on zinc fingers to DNA bases, the approach depends on mutating either the amino acid residues and keeping the DNA bases unchanged or mutating the DNA bases and keeping the amino acid sequence in the wild type unchanged. In another experiment both the zinc finger key residues and the DNA bases were mutated in a way to optimize the binding specificity to imitate the experimentally obtained data by random selection and mutagenesis (D J Segal et al., 1999).

The outcome of our calculations indicated a strong dependence on electrostatic energy in the binding process of zinc fingers to DNA and a high sensitivity of the calculated energy values to mutations, giving an indication of the predictive power of MD and MM-GBSA in this case. It also proved to have sufficient resolution, in this case, to predict residue dependent changes in free energy that correlate with the experimental results of Segal and Pabo (Miller & Pabo, 2001; Carl O Pabo et al., 2001; D J Segal et al., 1999). Based on these results we assign a functional

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dependence to each residue, which will help in the future design of zinc fingers capable of specific binding to DNA based on free energy of binding.

Methods :

Molecular dynamic simulations were performed using the Amber9.0 package (D. A. Case et al., 2006; D.A. Pearlman et al., 1995). Amber9 was used for MD simulations to optimize the zinc finger DNA complexes. A Zinc finger/DNA complex was downloaded from the PDB data bank (PDB code 1aay) with a resolution of 1.60*Å*. The PDB file was inspected, water and salt were removed and mutations were achieved using either pymol (DeLano, 2002) for the protein or using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen EF et al., 2004) for mutations in the DNA bases. Then Xleap (D. A. Case et al., 2006) was used to neutralize the system by adding sodium ions. The complex was solvated in aTIP3PBOX cubic water box with dimensions of 12.0 Å distance from solute. The structure was checked for errors and then converted to topology and coordinate files. Sander was used from the Amber9.0 package to minimize the structure. MD simulations were carried out under the NVT ensemble with heating, equilibration and production for 2 nanoseconds of simulation time. The output file was converted to PDB format using ambpdb from Amber and water was stripped out and the ions were removed, then the files were re-converted to topology and coordinate files in xleap and were used along with the Sander mdcrd output file in MM/GBSA to produce 100 to 150 trajectory files which were used to calculate the binding free energy, Normal mode calculation was used to calculate the entropy contribution.

Parameters used for MD calculations:

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A series of minimizations (maxcyc1000 ncycl 500 then 2500 ncycl 500 cycles of steepest descent at a time until RMSD is stable at a value less than 2 Å fluctuating around 1.4 Å) were performed to relax the system prior to MD simulations a 20000 step in total Particle Mesh Ewald calculation was used for calculating the long range electrostatic interactions. Shake was used to restrain bonds involving hydrogen atoms. The Time step for the MD simulations was 1.5 fs to 2 fs depending on the sensitivity of the system. Equilibration of the system employing MD followed these steps: first gradual heating from 0 to 300K in 50 ps (Langevin dynamics used for temperature control) followed by 50ps of density equilibration with weak restraints followed by 500ps of constant pressure equilibration at 300K. The production simulation was carried out under the same conditions as the final phase of equilibration for a total of 2ns with recording every 8 ps to obtain snapshots. Before using MM-GBSA the system equilibration was verified by considering temperature, density, total energy and root mean squared deviation of coordinates (RMSD). An RMSD value relative to the crystal structure of 1.5Å was deemed acceptable. The resulting trajectories were analyzed using the MM-GBSA modules in AMBER9.0.

Calculation of the Free Energy of Zinc finger interaction with DNA bases:

The Generalized Born model solvent area method in MM_GBSA in AMBER9.0 was employed for the calculations:

$$\Delta G_{binding(Solvated)} = \Delta G_{binding(vac)} + \Delta G_{Solvation(complex)} - (\Delta G_{solvation(Lig)} + \Delta G_{solvation(receptor)})$$

$$\Delta G_{solvation} = G_{electrostatic(\varepsilon=80)} - G_{electrostatic(\varepsilon=1)} + \Delta G_{hydrophobic},$$

The free energy of binding contains three major contributions: Van der Waals, electrostatic and solvation energies. The solvation energy contribution was calculated using both the polar

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contribution to solvation (GB) and the nonpolar contribution to solvation which was calculated based on accessible surface area (SA)(Qiu et al., 1997) .

Normal mode calculation was used to calculate entropy contributions, TΔS, from the same snapshots as were used for calculating $\Delta G_{binding}$. Then the absolute free energy of binding was calculated from

$$\Delta G = \Delta G_{binding} - T\Delta S$$

Results and Discussion

The recognition pattern of the zinc finger to DNA is shown in Figure 1, residues -1, 2, 3 and 6 are known to play the major role in the binding process. The aim of our calculations is to establish which part of the protein sequence is the possible recognition domain and which residue(s) play the crucial role in recognition and binding based on energy considerations. Non canonical interactions, which hamper the idea of simple recognition, have also to be considered. In general, amino acid residue A^1 on a protein interacts with its specific base B1 on the DNA and with other neighboring bases B2 and B3. On the other hand, base B1 from DNA may interact with more than one amino acid residue on the protein (A^1 and A^2) at the same time (C O Pabo & Sauer, 1984; Roy et al., 2012).

key positions in the zinc finger protein Figure 1 (namely -1, 3 and 6 in addition to Aspartate in position 2) which are known to bind specifically to DNA bases in each finger (C O Pabo & Sauer, 1984; Carl O Pabo et al., 2001; Pavletich & Pabo, 1991; Roy et al., 2012; D J Segal et al., 1999) were mutated in order to test the extent of modularity, specificity and any possible cooperativity. Aspartate in Position 2 (D^2) was reported to take part in binding to the bases C or A on the 5' strand opposite to a G base on the 3' strand, it also interacts with R^{-1} (see

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Figure 1 and Figure 5b). Mutants of residues 2 and 6 which are reported to play a role in binding either separately or jointly (Carl O Pabo et al., 2001; Roy et al., 2012) were studied. The role of other residues i.e. S^{-2}, S^{1} were also studied and the energy of binding was calculated in all cases.

1) Initial study of the binding of the zinc fingers to DNA

The free energy of binding of each of the zinc fingers from the x-ray structure, now termed F1, F2 and F3 were calculated (Table I). The total free energy (GBTOT) was -50(∓ 5) Kcal/mol for each finger, with varying contributions, the Van der Waal interactions were in the same range for all three fingers (- 35∓5 Kcal/mol). While the electrostatic interactions were maximum for F3 and lowest for F1, the free energy ΔG is the least negative for F2 namely – 3.5 Kcal/mol. ΔG was more negative for fingers one and three indicating stronger binding, the entropy factor did not show a considerable change within a margin of error for all three fingers. Thus, we can safely say that absolute free energies (ΔG) vary in the order F1 ≥ F3 >F2 with ΔH as the possible major contributor to the free energy, this may be an indication that F1 and F3 act as a hook to establish the binding of the protein to DNA.

Mutations of the DNA bases specifically bound to the Zinc fingers (each finger studied individually):

Figures 2a, 2b and 2c and inserts 2a and 2b show the results of an experiment where DNA bases were mutated leaving the protein sequence intact in its wild type form. Fingers one (F1), two (F2) and three (F3) showed reduced binding upon mutation of the DNA bases. The loss in binding energy upon mutating DNA bases confirms the natural selection of the binding sites as evident in the reduction of binding energy upon mutating the wild type bases(see insert to Figure 2a). The triplet **GCG** originally known to bind finger one (**F1**) (with the sequence

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SRSELTR) was mutated as shown in Figure 2a. The naturally occurring base triplet **TGG** which is specific to **F2** has the highest negative free energy compared to other base triplets. Mutation in the DNA triplet **TGG** specific for binding finger two (**F2**) (**SRSDHLTT**) in the wild type zinc finger protein is shown in Figure 2b. These mutations in the DNA bases resulted in lower free energy of binding. Changes in total binding energy, in this case, are paralleled by changes in both electrostatic and VdW energies. The weakest binding is observed for **ATA** for **F2**. The general trend is a loss in binding strength upon mutation of the wild type triplet **TGG** specific for finger two(**F2**). This loss in binding can be attributed mainly to loss in electrostatic energy upon mutation of bases. An increase in electrostatic energy values upon mutation of DNA bases in all three fingers was noted. It was more pronounced for **F2** than for **F1** and **F3**. Although electrostatic energy values increase considerably for **F3,** the values stay negative upon mutations. This is expected for the **F3** sequence due to it possessing an **R** residue at position 4 and a **K** residue at position 5. These both being polar basic residues, hence both are responsible for high electrostatic attraction. This observation, in addition to showing the effect of mutating the bases on reducing the free energy of binding, gives evidence of behavioral differences between **F1, F2** and **F3.** Thereby, supporting the individual role each finger plays in the DNA binding process, in agreement with (Eustermann et al., 2011) and (Roy et al., 2012) in which the authors highlight the different roles and importance of each finger. The total loss in free energy in all three fingers is about 30 Kcal/mol relative to the wild type binding energy. This loss varies according to the type of DNA base triplet combination (see inserts to Figure 2a and 2b). Table (II) columns 1, 2 and 3 give the correlations between individual energies contributing to the total free energy, it is clear in all fingers studied that the highest contribution is from electrostatic forces (H-bonds, water mediated H-bonds will be published later).

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Investigation of the binding of experimentally studied Zinc finger motifs to various DNA base combinations:

When each sequence is bound to its designated specific triplet as derived experimentally by random selection studied by Segal in (D J Segal et al., 1999), the free energy of binding is more negative than when that same sequence is bound to the wild type triplet (TGG), for example the sequence $SQ^{-1}SGD^{3}LRR^{6}$ binds GCA 1.6 times stronger than binding TGG (see Figure 5A and Tables II and III). This finding stands as a confirmation of the selectivity of sequences derived experimentally by Segal and co-workers. A good correlation is observed for F2 variants prepared by Segal when the individual energies (electrostatic, VDW, solvation) are correlated to the total free energy (GBTOT) as can be seen in Table (II) column 4. This observation confirms the naturally selected sequences bound to their triplets as prepared in (D J Segal et al., 1999).

Mutation of zinc finger protein amino acid sequence bound to wild type naturally occurring 11 base pairs

In order to study the effect of amino acid residue mutations on the free energy of binding, finger two was singled out due to its reported importance in the binding process. Each amino acid residue in finger two was mutated (finger two (F2) amino acid sequence from zif268 (pdb code 1aay) bound to its wild type 11bp (AGCGGGTGCGT) see Figure 1. The free energy of binding to the TGG region was calculated and the results are shown in Figure 3A and 3B. Figure 3 shows a great sensitivity of the binding free energy to the mutations of the residues in positions -

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1, 2, 3 and 6. It is evident from the mutations that the largest loss in binding energy took place upon mutation of the key residues responsible for specific binding (R^{-1}, H^3, T^6) see Figure 1 and 4. In this case, any loss in binding is attributed to the loss in net electrostatic energy. As shown in Figure 3b electrostatic energy changed noticeably (Correlation coefficient R= 0.99) and VdW energy (R=0.93) while desolvation energies did not show much change within the error margin upon mutation (R=0.21) (see Figure 3c and Table IV). The electrostatic part of the desolvation energy showed a good correlation unlike the hydrophobic part of the solvation energy (R=0.94). This is indicative of an electrostatic nature of the hydrated pocket involved in the process of binding.

The mutation of D^2 which is responsible for cooperative binding and also binding the opposite strand showed a significant loss in binding energy, implicating the importance of this residue for Zinc finger binding to DNA. The importance of the key residues responsible for DNA base binding in the zinc finger can be arranged according to the amount of lost binding energy upon mutating the residue, and they can be arranged as follows: D^2>R^{-1}>H^3 (T^6 was not reported to have such a strong binding with T of the DNA).

These mutations of the amino acid residues in the finger 2 sequence and the binding of the mutants to the WT 11bp of the DNA bound to ZF (AGCGTGGGCGG) revealed important information about the role of key residues -1,2,3,5 and 6, with special emphasis on residues-1,2,3 and 6. The total free energy of binding of finger 2 was -50.5$\pm$5 Kcal/mol. When residue **E** replaced residue **D** in position two, a noticeable loss in binding was observed. When Histidine in position 3 (known to bind the middle G in the TGG triplet) was mutated to **K,** the binding energy dropped by 8.0 ($\mp$ 4.4) Kcal/mol. The loss in binding energy was 18.0 $\pm$ 4.5 Kcal/mol when **R**

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in position -1 (which is known to bind G on the 3' side of TGG) was mutated to **K**. These results show the importance of R^{-1}, H^{3} and D^{2} in the DNA binding process.

It should be noted that some mutations, specifically the T^{5} to L and T^{6} to R mutants, showed enhanced binding (Figure 3A). This can be explained by the increased number of electrostatic interactions represented by hydrogen bonds to the phosphate backbone, which can be classified as nonspecific binding, thereby confirming the indirect effect of mutating an amino acid residue on directing side chains in the helix in order to make more nonspecific contacts. The increase in nonspecific binding is due to turning the helix into a different position upon mutation. No considerable change in entropy was recorded as indicated by the small change in TS upon mutation (Figure 3B). Indeed a mutagenesis study has shown (Chang et al., 2008) that a Leucine analogue exhibited an enhanced thermodynamic stability due to its side chain (a process dominated by solvent reorganization). This extra stability helps in exposing the polar surface of the helix to bind stronger to DNA bases. The L residue is known to enhance the α helical structure and consequently the electrostatic interactions (Tsui et al., 2000).

The hydrophobic contribution to solvation remained the same upon mutation of the F2 sequence. The drastic change occurred in the electrostatic interactions which changed the total free energy of binding. The free energy of binding upon mutation of residues in finger two has a high correlation with electrostatic interaction energy with an r value close to 1.0 indicating a large contribution from electrostatic energy.

Importance of R^{-1}

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Upon mutation of Arginine (R^{-1}) to other amino acid residues (Figure 4), namely lysine and Histidine (K and H), which have a resemblance to R (all three are polar basic amino acids and carry a positively charged Nitrogen) a loss in binding energy took place (up to 20Kcal/mol). The same behavior was observed upon mutating R to Q (a polar uncharged residue). The loss in binding became greater upon mutating R to the acidic residues Glutamine and Aspartic acid (E and D). It was reported that charged side chains and solvent to helix potential and field backbone contributions always come from the first two helical turns (Tsui et al., 2000). These results are in agreement of the extra binding of the N-terminal side of the helix, i.e S^{-2} R^{-1} S^{1} H^{3} and D^{2} fall in the range of these two turns. The binding distances of R^{-1} and H^{3} in F1 and F2 are demonstrated in the figures 5b and 5c.

Comparison of mutants of zinc finger domain sequences binding to other triplet base pairs

Finger two variants (J. Liu & Stormo, 2008; D J Segal et al., 1999) bound to 11 base pairs with the finger two target site mutated as shown in Figure4 were used to study the predictive power of the MD simulation in analyzing the binding of various amino acid sequences to specific base pair combinations. It can be seen that the stability of ZF-DNA complex, as indicated by the negative free energy, supports, in most of the cases, the experimentally obtained specificity of residues to their specific bases as obtained by K_d values from electrophoresis Gel shift assays (D J Segal et al., 1999) (Figure 5a) , for correlation between MD obtained values and experimental free energy values see Table III. The most noticeable free energy values are the specificity of residue

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R to the base G, residue Q to A and D to C, for example $R^{-1}K^{3}R^{6}$ showed enhanced stability for GGG over most ZF's which contain R in position 6 (see supplementary material). The amino acid combination also played a role in the stability, as can be seen in its extra stability over $R^{-1}H^{3}R^{6}$. The type of base combination also played a role in stability, for example RKR/GGG is more stable than RKR/GTG while in both cases R^{-1} and R^{6} are bound to G. The type of combination of residues also affected the free energy of binding, for example R-K-R showed stronger binding than R-H-R to GGG, although H was reported to be specific to G by Segel and co-workers (see supplementary Figures A and B). Position of the amino acid residue in the sequence has an effect on its strength of binding to its specific base, i.e whether the residue is in position -1, 3 or 6. For example Q-D-R binds specifically to GCA stronger than when D is in position -1 as the cases of D-H-R to GGC or DNR to GACshow. These findings show the importance of the positions of residues (-1, 3 or 6) in affecting the extent of binding.

The same argument holds true for other combinations, if we compare for example QDR/GCA, QNR/GAA, QSR/GTA, QSR/GTG and QDR/GCT. The outcome of this comparison is that Q^{-1} is specific to A, but Q results in more stable binding when present with D in position 3 than when present with T or S in position 3. These interactions affect the specificity of binding and support the hypothesis of cooperative binding. They also support the effect of interaction of residues with each other in the finger sequence (C O Pabo & Nekludova, 2000).

Breaking the total free energy of binding to its individual energies (Electrostatic, *Van der Waals* and surface area) shows which energy drives the binding process and which is most affected by mutation. It is obvious from the variation in VdW energy that it parallels the variation in total free energy. This makes it one of the major contributing energies to the free energy with the rest of the contribution from the electrostatic energy (loss in binding energy is

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mainly due to loss in electrostatic energy in all cases, see correlation diagrams). This contribution also depends on the type of residues and bases involved in binding; the electrostatic contribution from the point of view of base combination is maximum for GGG then GGA then GCA and GAA, so the order is GGG>GGA≥GCA=GAA.

Considering the amino acid combinations' effect on electrostatic energy, they can be arranged according to their electrostatic contribution: RK has the maximum contribution followed by QD, QH and QN. This can be verified further upon mutating the DNA bases.

This outcome demonstrates how complicated the recognition process can be (C O Pabo & Nekludova, 2000), and it also shows that co-operativity is a factor which cannot be ignored in the recognition process. A final outcome is that R and H are most specific to G, D and E are specific to C, T, S and E are specific to T and Q and N are specific to A.

The experimentally observed specificity of residues in positions -1,3 and 6 based on equilibrium binding studies showed that R and H were specific to G; Q and N and E bind specifically to A with Q the strongest binder; D and E bind C with D a stronger binder to C; T, S and E bind T. In order to study the effect of positions -2,1,2 and 5, finger two with the sequence SRSDHLTT was singled out for study, although experimental reports were mostly on –LVR in positions 5 and 6 instead of LTT (J. Liu & Stormo, 2008; Miller & Pabo, 2001; D J Segal et al., 1999). Our predicted values for a single finger 2 were (-50.0±4.5 Kcal/mol) and the absolute free energy was –3.5±11 Kcal/mol after considering the entropy TΔS. The details of calculated binding energies of several amino acid sequences and the experimentally calculates energies are shown in the supplementary figures.

Conclusion:

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F1 and F3 have higher negative hydrophobic energy than F2 (Table II). Desolvation of F3 is highest (expected because of the type of residues involved; ARSDERKR compared to both F1 and F2 which contain LT in positions 4 and 5 instead of the KR in F3). The solvation energy contribution is greatest for finger 3 then 2 then 1. The hydrophobic contribution to solvation is in the same range for all three fingers indicating that the solvation energy difference arises from polar contribution, this goes well with the more polar surface of finger three (see Table II).

Correlation for F1 and F2 (Table IV) is in the order Electrostatic>hydrophobic> Vdw while for F3 the correlation between total energy and hydrophobic energy is better than with electrostatic energy.

Total negative free energies GBTOT vary in the order: F3 ≥ F1> F2 (Table I). ΔG value is -3.5 Kcal/mol for all three fingers. Entropy contribution is close for all three fingers. Therefore, each finger has the same affinity to its specific base triplet and the process is mainly enthalpy driven. Van der Waal forces recorded similar values for all fingers, within the error margin (-35Kcal/mol). Electrostatic contribution is close for F1 and F2, but is slightly higher for F3.

Molecular dynamics simulations and MM/GBSA prove that the free energy of binding can be reasonably estimated for zinc finger proteins binding to DNA with correlation coefficients comparable to those reported in (J. Liu & Stormo, 2008) which employed context-dependent model for zinc finger-DNA interactions and compared it with other theoretically and experimentally obtained techniques , it was shown that the estimated free energy of binding gave acceptable correlation with values calculated experimentally from equilibrium studies (J. Liu & Stormo, 2008; D J Segal et al., 1999; Temiz & Camacho, 2009; Temiz et al., 2010). It was evident in all studies performed in this work that the driving force for binding of amino acid

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residues to DNA bases is electrostatic in nature, i.e. between amino acid side chains, DNA base side chains and the backbone phosphate (Roy et al., 2012; Temiz et al., 2010). These electrostatic attractions are expressed as hydrogen bonding between key residues on the N-terminus of the helix and the bases starting at the 3' end of the strand (Figure 5B and 5C). Non specific binding is part of the process which in some previous studies on DNA binding proteins was thought of as a transition state in the specific binding process.

Our studies showed that Arginine and Histidine are specific to G, while glutamine is specific to A and Aspartate is specific to C (see R—G and H—G distances in figures 5B and 5C). These findings are in agreement with experimental work by Segal (D J Segal et al., 1999). It was evident that position -1 in the helix plays a major role in recognition of the helix to its specific base triplet and in most cases Arginine recognized G and glutamine (Q) recognized A. In addition to that Aspartate in position 2 proved to be very important to the recognition and binding process as it forms four hydrogen bonds with R^{-1} and binds C or A on the opposite strand on the 5' side. This is in agreement with previous reports (Carl O Pabo et al., 2001; Temiz & Camacho, 2009; Temiz et al., 2010).

In all studies performed, mutations of the wild type zinc finger amino acid sequences resulted in lower energy of binding to the wild type eleven base pair DNA as can be seen in Figures 3 and 4. On the other hand, mutations of the specific DNA triplets (WT) resulted in lower binding energy of the zinc finger (Temiz & Camacho, 2009).

To optimize the selectivity, both the zinc finger helix and DNA triplets were mutated together, this resulted in maximum negative free energy of binding for the experimentally selected helices when compared to the binding energy of these helices to the original wild type

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triplet (TGG). It was evident in the study that finger 1 and 3 have a stronger binding than finger 2, which agrees with the proposed mechanisms that finger 1 plays the major role in recognition (Eustermann et al., 2011). It has been reported that due to the co-operatively of binding observed, it is difficult to design sequences with high specificity (Carlson et al., 2010). However, in the future, MM-GBSA and MD can help in designing new site-specific zinc finger proteins with optimum binding capacity to specific DNA bases by estimating the binding energy of a given sequence to a certain base pair combination. Indeed studies on ZF binding to DNA have already proved important in therapeutic studies in humans (Eustermann et al., 2011).

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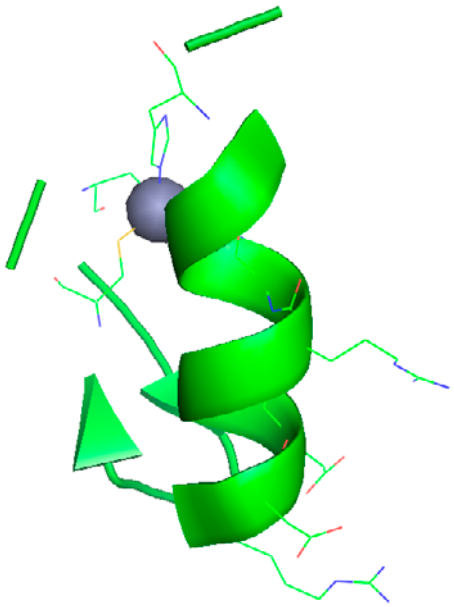

β 3

2

-1

-1 2 3 6

RPYACPVESCDRRFS**RSDEL**T**R**HIRIHTGQKPFQCRICMRNFS**RSDH**LT**T**HIRTHTGEKPFACDICGRKFA**RSDE**RK**R**HTK

IHLR F1 (α) F2 (α) F3 (α)

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Figure 1

Cys_2His_2 zinc finger motif and the sequence. Ribbon diagram (top) of finger 3 from the average structure: showing the two cysteine and two Histidine residues binding the zinc atom. Key

positions R^{-1}, D^{2}, E^{3} and R^{6} which bind the G C G on the major groove of the DNA. The amino acid sequence of zif268 with key positions which bind DNA bases are shown in boldface (bottom)

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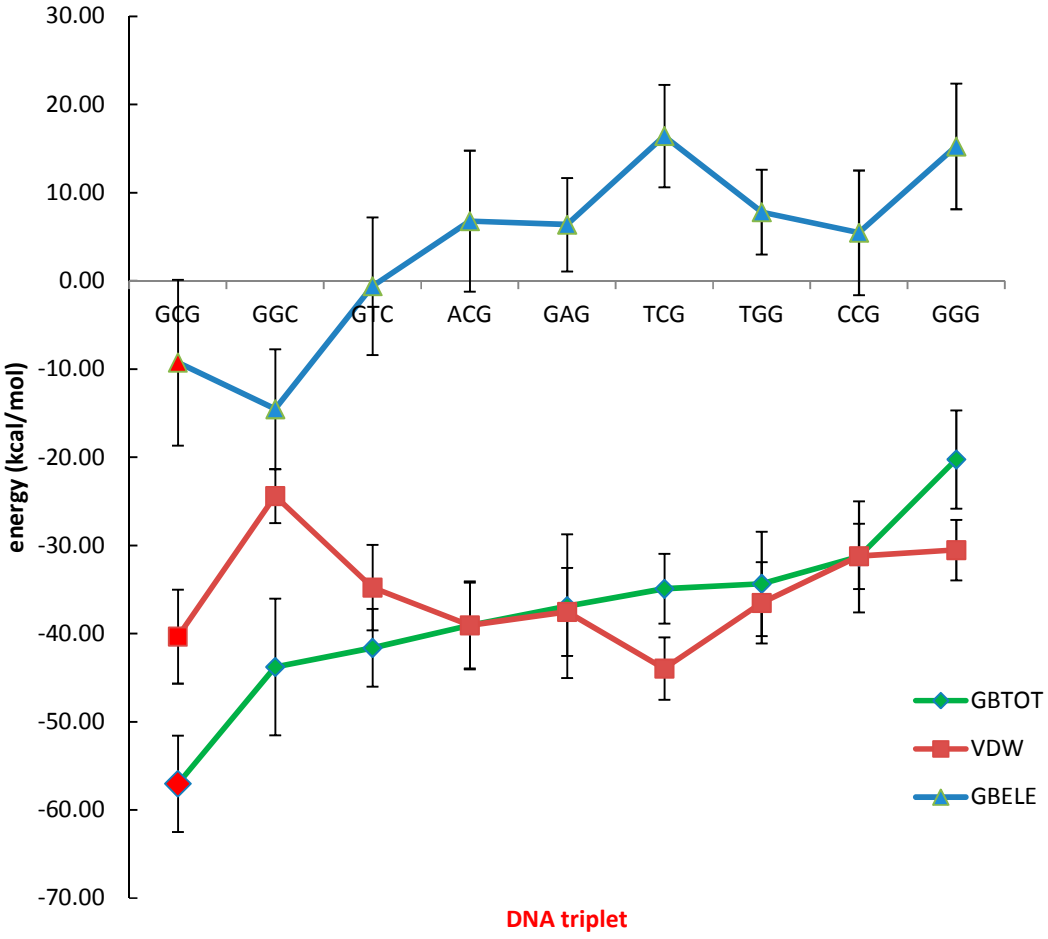

Figure 2(a):

The change in total energy (GBTOT) upon mutation of DNA bases specific for F1.Electrostatic contribution (GBELE) and *Van der Waals* contribution(VDW) to the total free energy (GBTOT) upon mutation of the specific bases (GCG) for finger one (F1) with the sequence SRSDELTR. Energy changes upon mutation of the DNA triplet (GCG) specific for finger one (F1) are labeled red. Total electrostatic energy GBELE is labeled Blue triangles. Van der Waals energy, Brick red (squares) total energy (GBTOT), green diamond.

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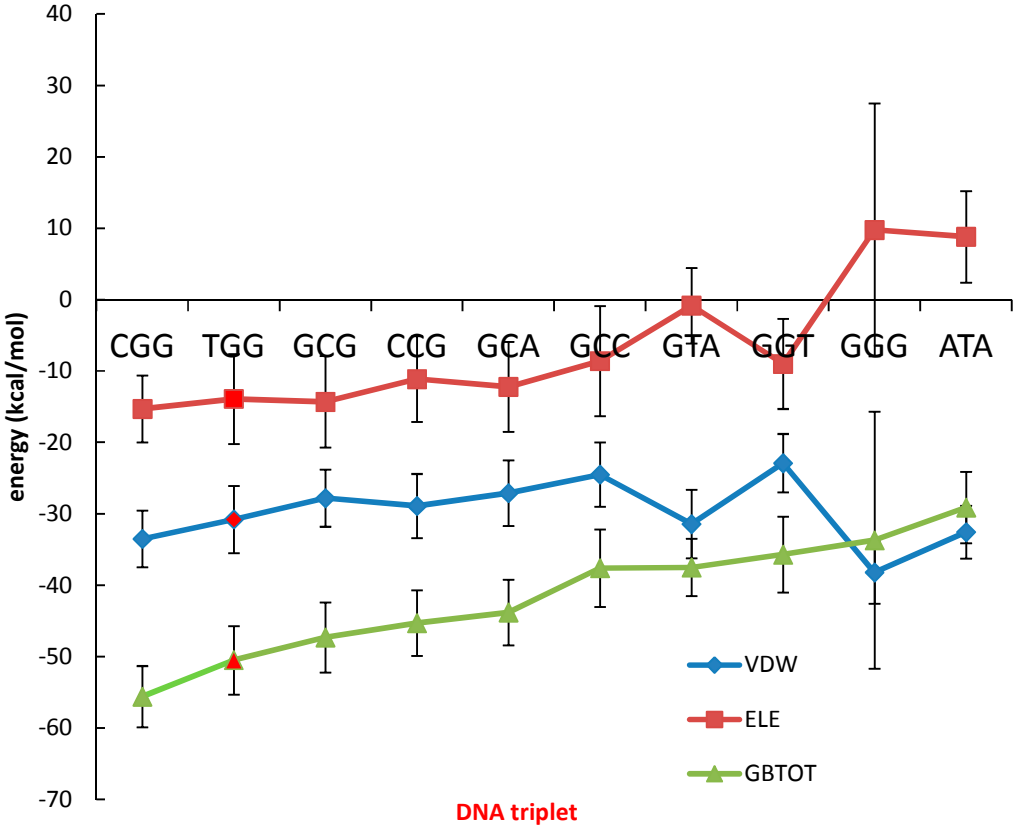

Figure2(b):

Loss in binding energy upon mutation of the specific base triplet (TGG) for finger two (F2) with the sequence SRSDHLTT. GBTOT represented as green triangles and green line, GBELE (Brick red squares and brick red line, VDW energy is labeled blue diamonds and blue line. The wild type naturally occurring triplet TGG is labeled red in all energies.

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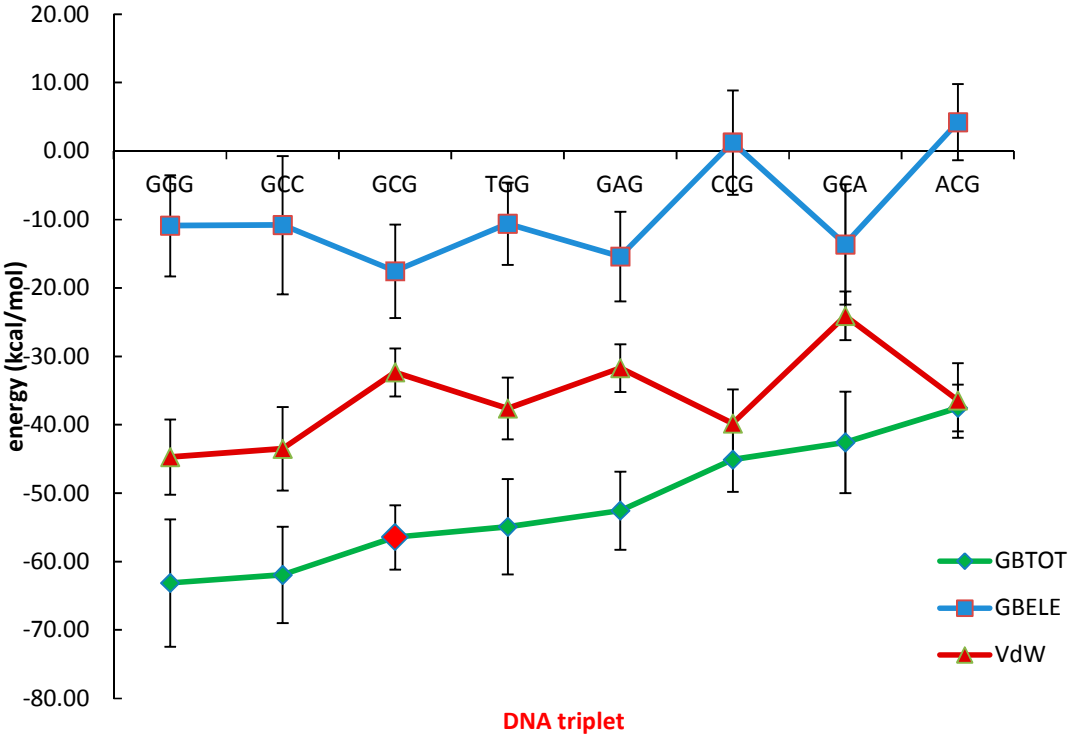

Figure2(c):

Loss in binding upon base mutation of the specific sequence (GCG) for F3. GBTOT is represented as green diamonds with green line, GBELE is represented as blue squares with blue line, VDW energy Brick red triangles and brick red line.

The label (red diamond) represent the wild type GCG triplet. The loss in binding energy (GBTOT) upon mutation of GCG is originated mainly in the loss in electrostatic forces (GBELE). For F3 energies are more negative than F1 and F2.

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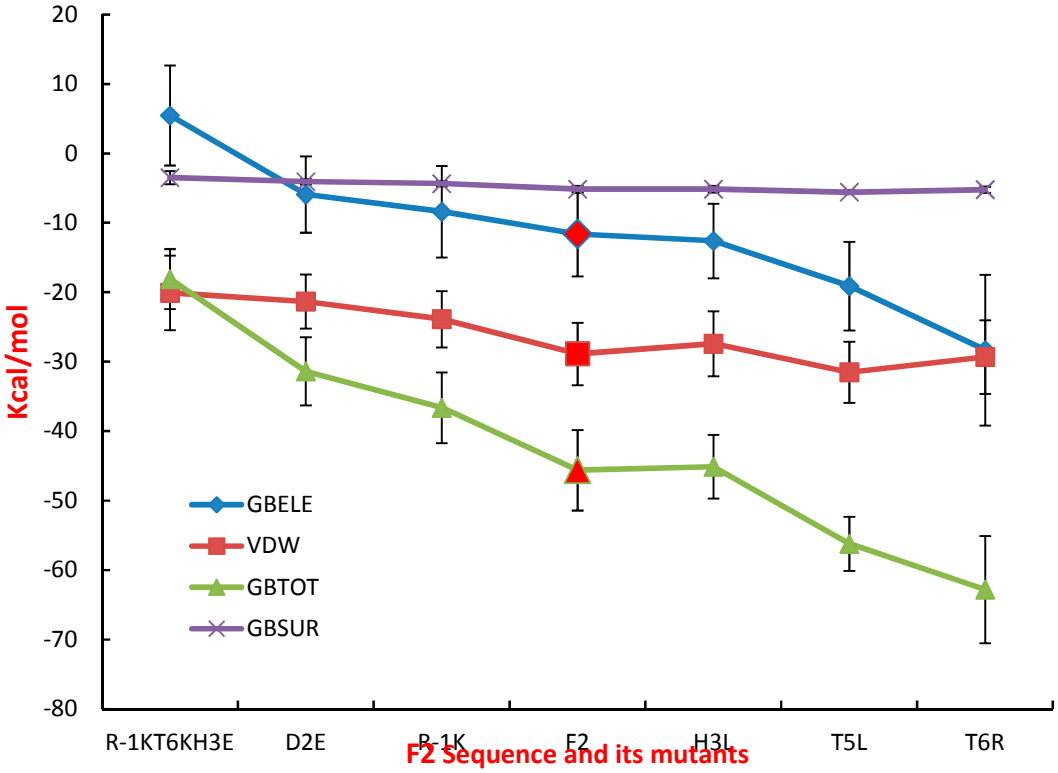

. **Figure3A:**

Plot of drop in energies of binding of finger two (F2) upon mutation of its amino acid sequence: studied with the wild type eleven base pairs specific for F2:

GBTOT (shown as green triangles and green line). GBELE (blue diamond and blue line. VdW (brick red squares and brick red line). GBSUR (purple(x) and purple line). The wild type finger is shown filled with red color.

D2E means D in position 2 is replaced by E, T5L means T in position 5 is replaced by L, T6R means T in position 6 is replaced by R. Its noticeable that GBSUR does not change considerably upon mutation of amino acid sequence.

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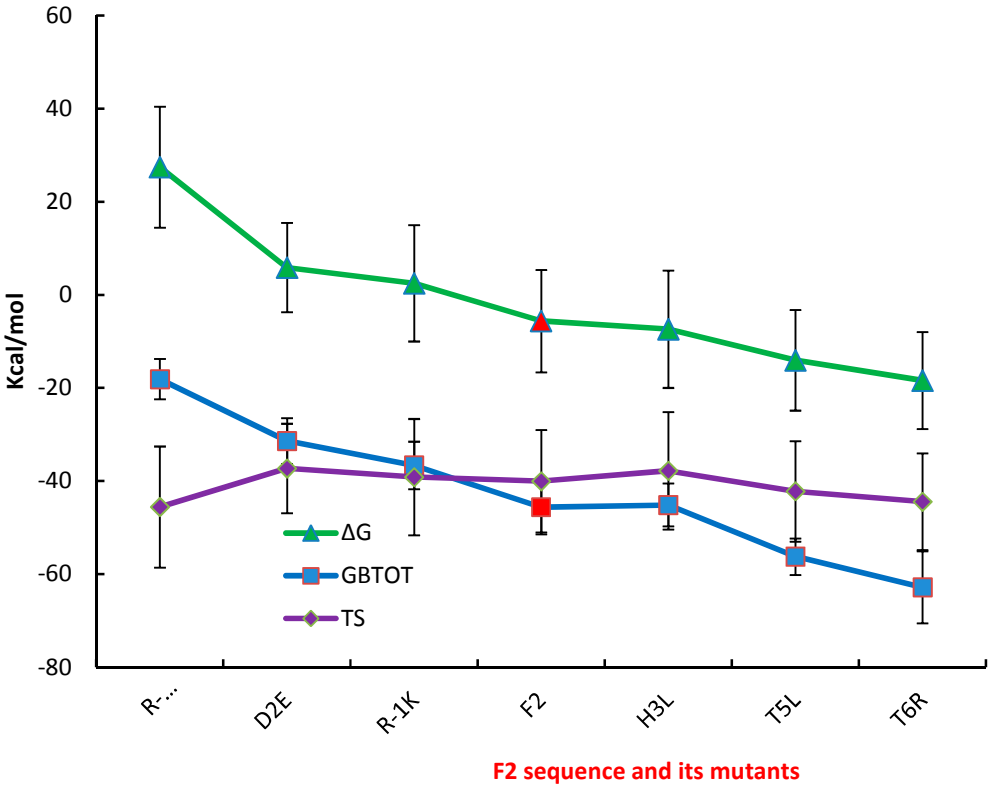

Figure3B: Calculation of ΔG from total energy and entropy factor

Energies of binding of finger 2 (F2) and its mutants to the 11bp WT DNA sequence: Absolute free energies. No considerable change in entropy upon mutations was observed. GBTOT (blue). TS (purple). ΔG = GBTOT –TS shown in green. GBTOT indicated by blue squares and blue line, TS is indicated by purple diamonds and purple line, ΔG = GBTOT –TS is indicated by the green triangles and green line

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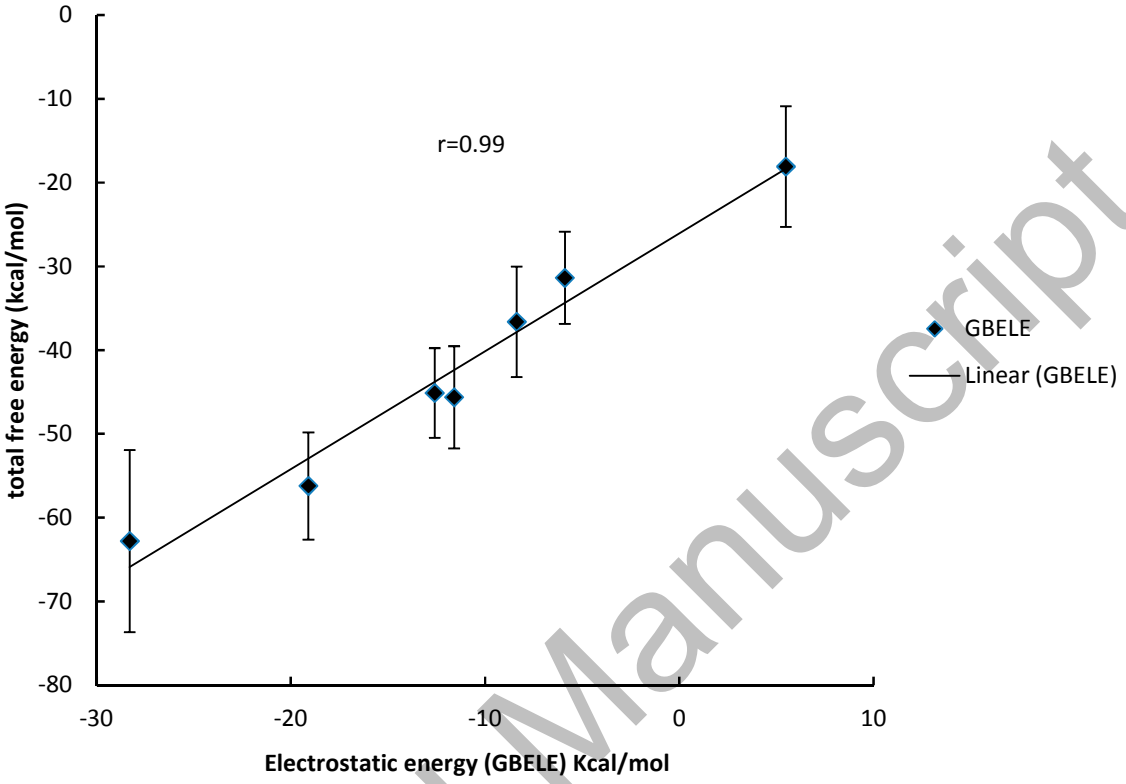

Figure 3C:

Correlation between total free energy of binding (GBTOT) and electrostatic energy (GBELE) of amino acid residues upon mutation of finger two (F2) sequence (Bound to the wild type 11bp). Values are taken from figure 3A. The r value of 0.99 indicates a strong correlation between the binding energy and electrostatic energy [see Table IV].

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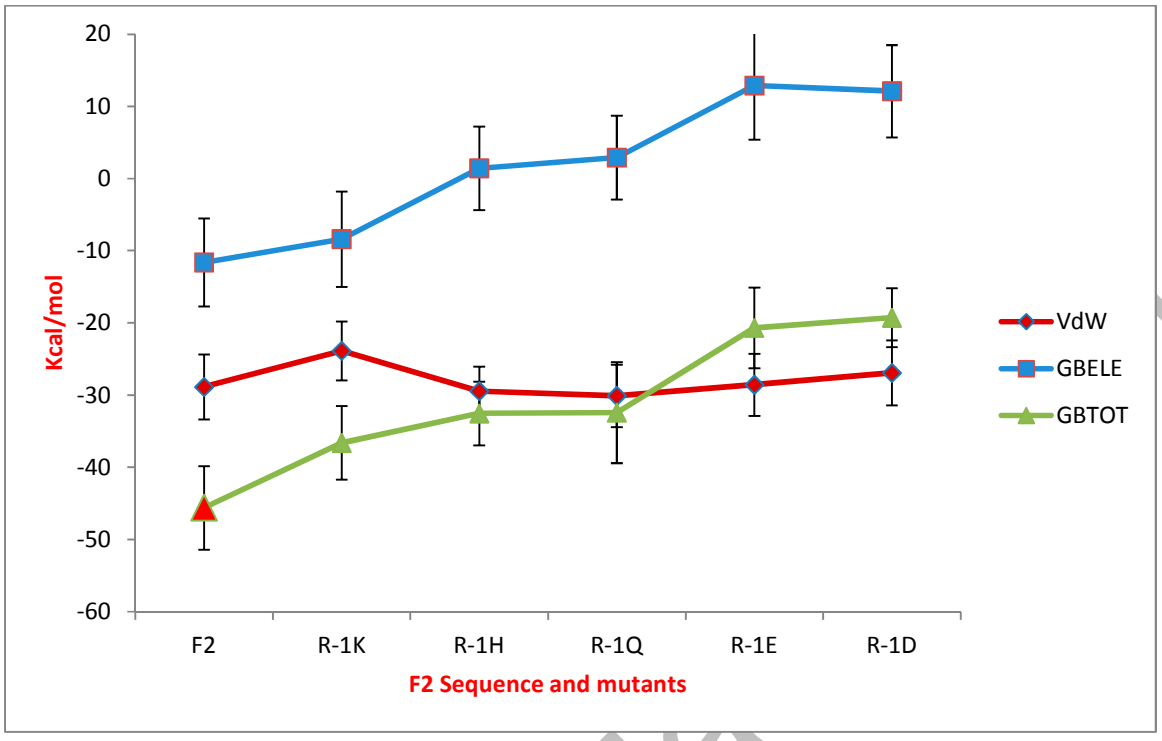

Figure 4: Effect of mutating R^{-1} on binding energies

The change in free energy of binding upon mutation of the Arginine residue (R^{-1}).Total free energy (green line and green diamonds). Electrostatic energy (blue line and squares). *Van der Waal* energy change (brick red line and diamonds).

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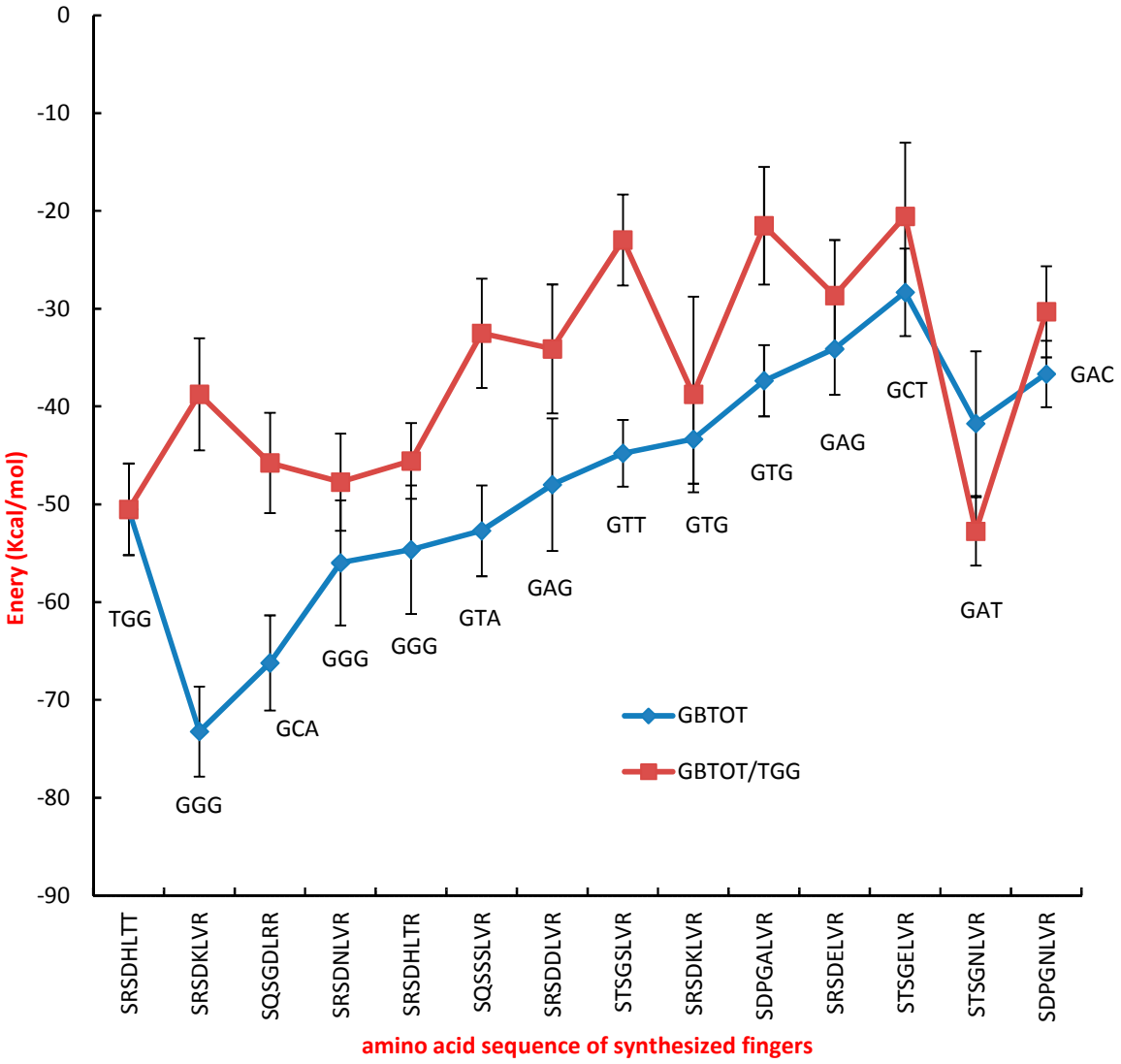

Figure 5A: Energies of binding certain designated sequences each to its specific triplet (blue) as compared to binding the same sequence to TGG triplet (brick red). Comparison between the free energy of binding for each sequence bound to TGG (shown in **Brick red** color line and squares) compared to the free energy of binding of that sequence to its specific DNA triplet found by experimental mutagenesis [11] (shown in blue Blue diamonds and blue line).

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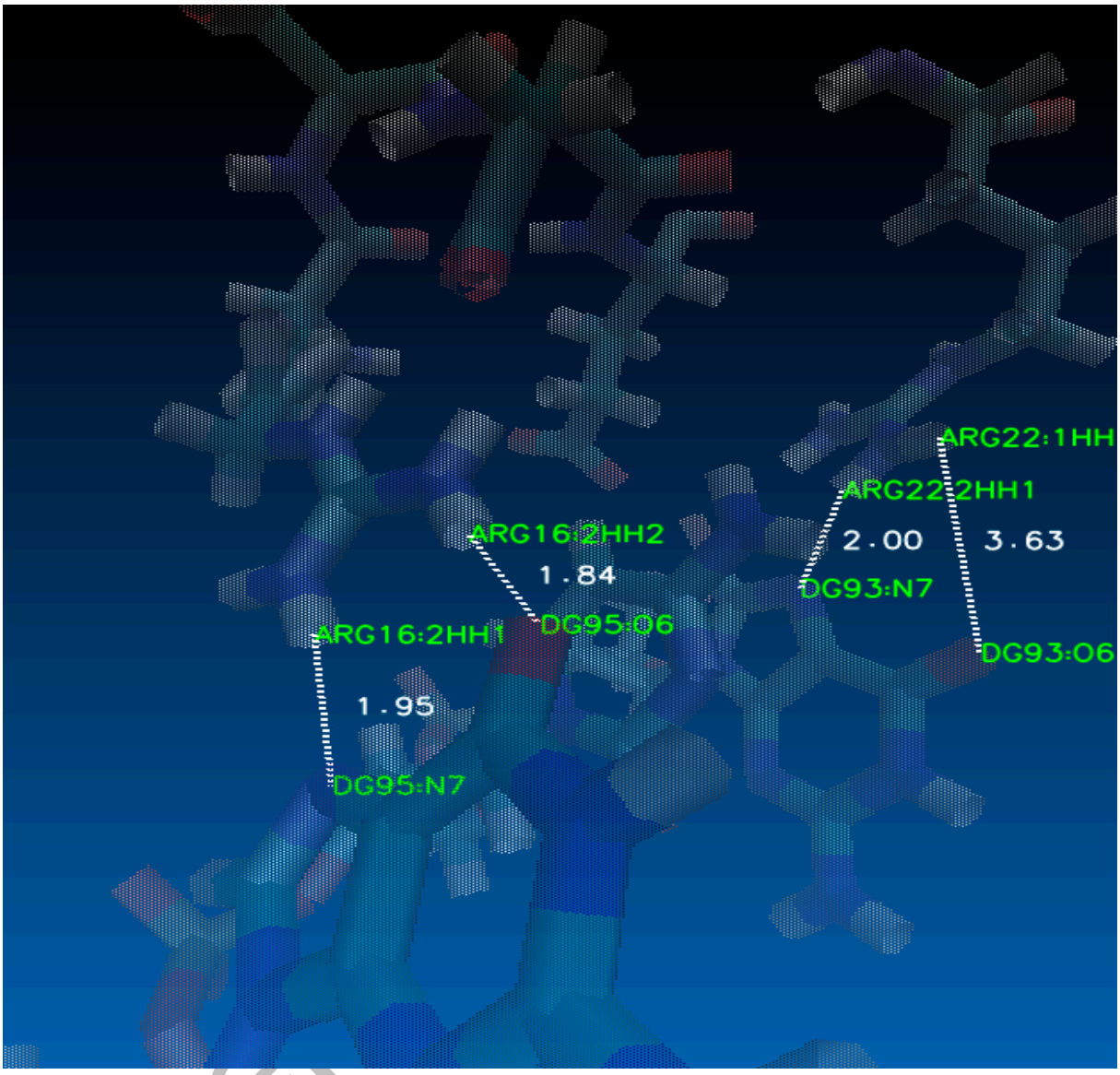

Figure 5B: Measured distances of amino acid residues on ZF1 from corresponding G bases taken from the average structure of 200 snapshots: R16 = R^{-1}, Arg22 = R^{6}. Distances show the binding of R^{-1} to N on G_2 (1.95Å) and R^{6} to N on G_1 (2.00 Å).

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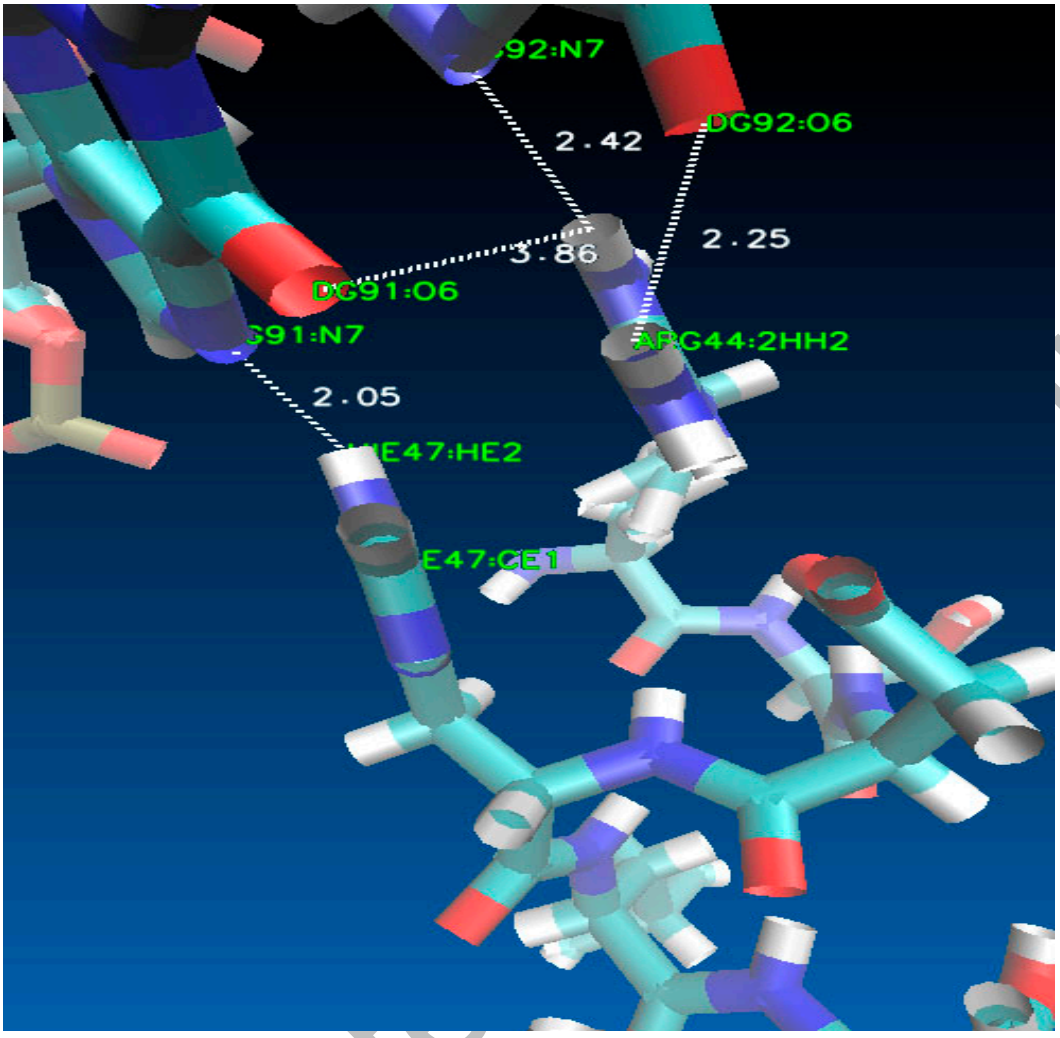

Figure 5C: ZF2 binding sites obtained from average structure: the figure shows the Histidine-G distances and Arginine-G distances in Angstroms

Binding of zinc finger F2 to DNA: R^{-1}-- G_1 (2.25 Å and 2.42Å) and H^3---- G_2 (2.05 Å)distances from their G respective binding sites (TG_2G_1) taken from average structure of 200 snapshots.

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Table I: Calculated energies for zinc fingers F1, F2, and F3: Total energy released (GBTOT) was calculated in a water box and using MM-GBSA, the net energy (**ΔG**) was calculated by subtracting TS from nmode calculations from the GBTOT. The contributing forces (VDW and electrostatic) to the total energy for each finger are shown.Electrostatic (GBELE) is greatest for F3.

	VDW	STD	GELE	STD	**GBTOT**	STD	TS	STD	**ΔG**	STD
F1	-39.13	4.15	-8.87	7.26	**-55.22**	5.98	-39.40	11.50	**-15.82**	12.96
F2	-30.79	4.65	-13.86	6.32	**-50.47**	4.83	-47.02	10.70	**-3.45**	11.74
F2*	-27.11	4.68	-13.03	6.70	**-45.42**	4.73	-41.76	12.00	**-3.66**	12.79
F3	-32.32	3.51	-17.55	6.82	**-56.44**	4.70	-44.30	10.61	**-12.14**	11.60

- Energy for Finger 2 was calculated in second experiment

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Table II: shows correlation (r values) of contributing forces (energies) to the total free energy of binding (GBTOT) upon mutation of DNA triplets. Column 5shows correlation for designed fingers bound to their designated DNA triplets (see Figure5A) as prepared experimentally by Segal et al [11]. The strong correlation between electrostatic forces (GBELE) and total energy released (GBTOT) is evident in all cases, also the strong correlation in column 5 between total energy and all individual contributing energies confirms the natural selection process in reference 11.

Correlation coefficient of individual energy with GBTOT (1)	F1/DNA (2)	F3/DNA(MUT) (3)	F2/DNA (4)	Mutations in F 2 sequence (mutant sequences based on Segal et al [11], each sequence bound to its specific base triplet) as in Figure 5A (5)
ELE	0.78	0.69	0.3	0.89
VdW	0.25	0.294	0.21	0.63
GAS	0.78	0.71	0.295	0.88
GBSUR	0.53	0.84	0.5	0.82
GB	0.74	0.67	0.25	0.87
GBSOL	0.74	0.67	0.25	0.87
GBELE	0.78	0.61	0.85	0.872

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Table III: Correlation Coefficients (r values) of individual energies to the absolute free energy **ΔG exp** for mutant sequences of finger two F2 in Figure 5A.Experimental values of **ΔG were calculated using the experimentally obtained K values in reference 11 and applying the equation ΔG= -RTlnK. The low correlation because the experimental values reported error was ± 50%**

Type of Energy	**Abbreviation**	**Correlation coefficient with ΔG exp[11]***
ΔG predicted from MD See reference [17]	ΔGMD	0.57
Total free energy of binding	GBTOT	0.483
Gas phase Electrostatic energy From MM	ELE	0.265
Total gas phase energy MM	GAS	0.265
Van der Waal	VDW	0.2
Sum of electrostatic energies from solvation and MM energies	GBELE	0.44
Sum of polar and nonpolar contributions to solvation	GBSOL	0.265
Electrostatic contribution to solvation	GB	0.265
Hydrophobic contribution to solvation	GBSUR	0.352
TΔS	TS	0.11

- ΔG exp calculated from K_d values obtained experimentally by Segal et al [11] using $\Delta G = RT\ln K_d$

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Table IV: Correlation coefficients (r) for different energy contributions to the total free energy of binding (GBTOT) of F2 mutant sequences bound to the specific naturally occurring 11bp DNA sequence (Figures 3a to 3c).

Energy component	**Correlation coefficient (r)**
Electrostatic	0.99
Van der Waal	0.93
Desolvation (electrostatic contribution) GB	0.46
Total desolvation (polar+ nonpolar)	0.46

The three major contributing forces in this case are electrostatic, VdW and hydrophobic forces.

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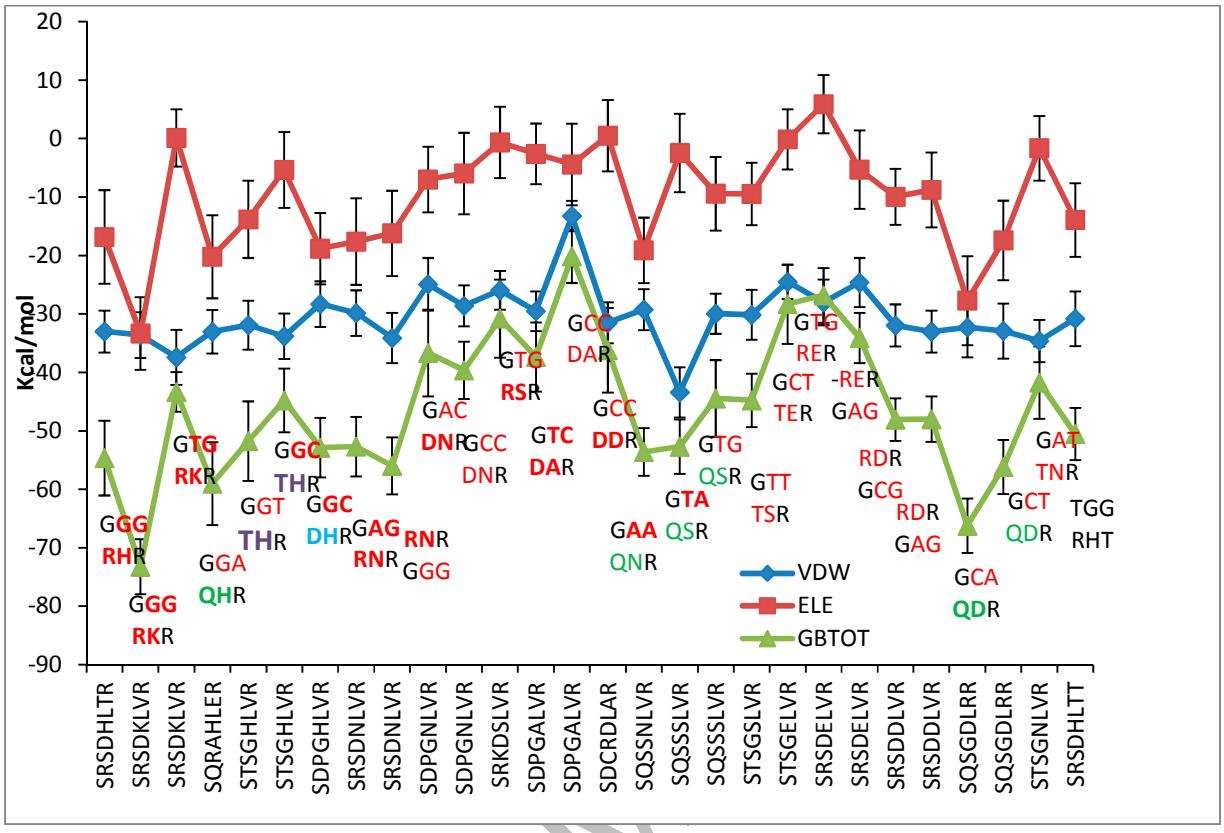

Figure A supplementary material: calculated energies of different finger sequences bound to their specific DNA triplets as prepared by Segel [11]. Brick red is electrostatic energy, blue diamonds and line is VDW forces. Green triangles and line is the total energy (GBTOT).

Every point is labeled by the most important amino acids in each sequence in Red bound to their specific DNA bases, for example the first point on the left R—H---R binds G—G—G and Q---D---R binds GCA, GAG and GCG with most negative energy for GCA, see conclusion part.

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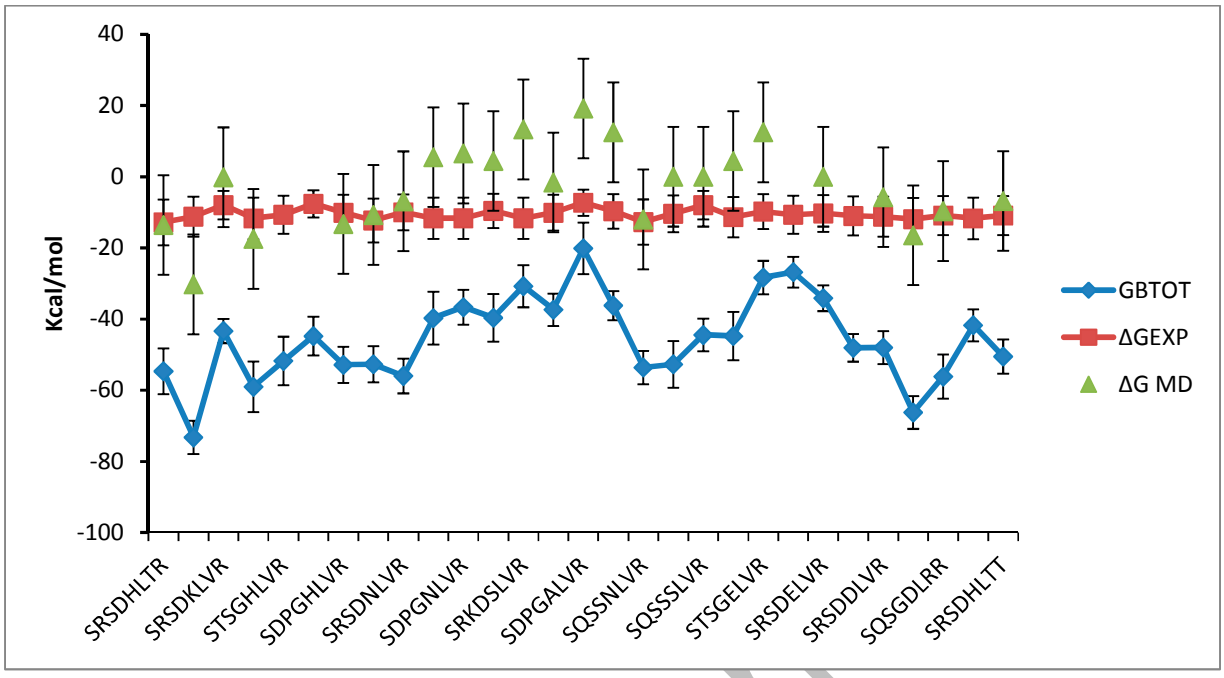

Figure B-supplementary: the energies GBTOT of synthesized sequences in Figure A supplementary were used to calculate ΔG from MD using TS and the equation ΔG_{MD}= GBTOT- TS scattered as green triangles around experimentally obtained values from gel-shift assays by Segel [11] labeled as ΔG_{exp} obtained from K values using ΔG= -RTlnK for different amino acid sequence arrangements

Red Bricks ΔG_{exp} , green triangles ΔG_{MD}, blue Diamond is GBTOT with added constant to fit the curve

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