

Predicting the conformational preferences of proteins using a physics-based free energy method

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Abstract

Calculation of free energy differences is of central importance in the simulation of biochemical systems. It is particularly difficult to calculate between pairs of macromolecular conformations as well as a computationally expensive task with existing methods. In this work, confinement approach is used to calculate absolute free energies of biomolecular systems. This method provides two main advantages: it does not require a reaction coordinate or transition path and it is fast to compute. Free energy calculated can be decomposed into a per residue contribution in an approximate way. Per residue free energy allows us to identify the reason behind conformational preferences in biomolecules. Through out the article we show its use in different challenging modeling problems. In particular, we show its use in predicting the conformational preference of chameleon sequences (sequences with high sequence identity and different folds). This sequence dependent conformational preferences and per residue free energy decomposition set the stage for the use of this method in protein design.

[Guys - I recommend we get a better name than confinement. It's totally uninformative. In my view, better would be 'confine-and-configure' or something. Please give it some thought.]

1 Introduction

In some problems of protein science, you want to know the relative stability of a protein's conformation *A* compared to its conformation *B*. We call this the *difference free energy* (DFE). For example, in allosteric mechanisms, a protein often adopts one conformation when a ligand is bound and another conformation when no ligand is bound¹. To understand the mechanism requires knowledge of the relative free energies of the protein conformations. Another example is in protein structure prediction, for example as practiced in the CASP competition². If you are using some computational model and have predicted two putative native structures, *A* and *B*, you want to compute which has the lower free energy, in order to know which is the more native-like. In a third example, you may want to know whether mutating a few amino acids in a protein could cause the protein to switch from one stable conformation to another, because that could have important consequences for biological mechanisms and disease. And fourth, sometimes binding a ligand induces a protein from one conformation to another. A quantitative understanding of such *induced-fit* situations requires knowledge of the difference free energy of the protein in the two states. In all these cases, it would be useful to have a computational method that can be used efficiently with atomically detailed physical forcefield models to compute the free energy differences between different given protein conformations.

Methods like thermodynamic integration^{11,12} which are successful in alchemical free energy calculation may not be as useful for calculation of conformational free energy. One widely explored strategy is to use molecular dynamics simulations along some putative reaction coordinate pathway from conformation *A* to *B*^{1,3-8}. **we need many refs here: cite Elber's milestones, Jhih-Wei Chu, and the many other path-sampling approaches.]** The free energy along this reaction coordinate can then be determined using methods such as umbrella sampling¹⁰. Such approaches have several limitations. First, it is necessary to know a reaction pathway from *A* to *B*.

If conformations A and B are quite different, then it can be challenging to find such paths. Second, these methods are computationally slow. To get an accurate estimate of the total free energy difference $\Delta G = G_B - G_A$ requires accurate determinations of the many small free energy differences $A \rightarrow 1 \rightarrow 2 \dots \rightarrow B$, so each microstep requires substantial amounts of sampling. And third, these methods are prone to large errors, because the pathway error is a sum of many errors among the many microsteps.

Will it be useful to add methods like PMF or replica exchange based free energy methods ?

[Are all these methods below ones that don't involve paths? Also, are there other key methods we should cite? We need a good scholarly list of all the relevant works.] The calculation of protein conformational free energy has been successfully attempted by a number of groups²⁴⁻³². Some of these methods like reference system method²⁴, deactivated morphing²⁵, confinement method²⁸⁻³⁰ take an alternative strategy for computing difference free energies that does not require knowing a pathway from A to B . [please add the several new Karplus papers from 2012-2013.]

Here, we adopt the confinement method of Tyka et al²⁸ and Cecchini et al.²⁹. This method also resembles the ‘confine-and-release’ method^{13, 14, 15}. [cite mobley’s jctc 07 (ref 216 on our labsite), and mobley’s recent j chem phys review, and mobley (ref 199) j chem phys, and others that are relevant.]. The method is based on the thermodynamic cycle showns in Figure 1. To start, conformation A is an ensemble. We reduce ensemble A to one particular microstate A^* in that ensemble by applying restraints in an MD simulation. In the same way, we reduce conformational ensemble B to one of its microstates, B^* . Then, we simply compute the free energy difference between the microstates as $\Delta G_{A^*,B^*} = \Delta H_{A^*,B^*}$ (which is just an enthalpy difference since there is no conformational entropy difference between the microstates). $\Delta H_{A^*,B^*}$ is readily calculated using normal mode analysis. In this way, we can calculate the free energy difference between the two end states without needing to define a physical path or reaction coordinate connecting the two states.

[Figure 1 about here.]

For this fig, let's make A and A^* green and B and B^* red or something, to

distinguish them visually. Let's also blur the ensemble more relative to the microstate, so it's clearer the distinction.]

Previously, the confinement method has been validated on small model peptides^{28;29}. Here, we show that the confinement method can also be applied successfully to larger systems, such as the series of chameleon proteins designed by Orban and co-workers^{34–36;38}, which can switch between two completely different folds with only few-amino-acid changes in sequence. We also show that our computed difference free energies are useful in evaluating the quality of CASP target protein predictions. This could be of significant value for ultimately improving the energetics in protein-structure models, not just the structures. And, finally, we show that we can approximately decompose difference free energies into individual amino acid components. This offers the opportunity for diagnosing the structural basis for difference free energies, which can be useful for interpreting biological mechanisms.

2 Results and Discussion

2.1 The confinement method succeeds at some basic consistency checks

First, we validated that our implementation of the confinement method produces results compatible with previous calculations reported in the literature. The method has previously been applied to a 16 amino acid residue β -hairpin from protein G, known as BHP²⁹. We calculated the free energy difference between the native conformation (called bhp1), which has a two-stranded β -sheet and a three-stranded β -sheet called bhp3. Our confinement calculation shows that bhp1 is more stable by 1.7 kcal/mol, consistent with 4 μ s equilibrium simulations showing that bhp1 is more favorable configuration by 1.8 kcal/mol, and in agreement with previous calculations²⁹.

Second, we looked at 6 target proteins from the CASP9 experiment. For each target, we examined up to 5 submitted models. We computed the difference free energy between the true native and the best model. Figure 2 and Table 1 shows that, in 5 out of 6 cases, the confinement method assigns

a lower free energy to the experimentally determined structure than to any of the decoys. Other well-known discriminators can also successfully make this recognition⁴⁰; it is just an independent useful validation that the confinement calculations make sense.

[Figure 2 about here.]

[Table 1 about here.]

[I think a better notation is $\Delta\Delta G = G_{best\ decoy} - G_{native}$, rather than having the arrow.]

2.2 The confinement method correctly predicts the structures of chameleon sequences

We also tested the confinement method predictions for difference free energies on the chameleon sequences of Orban et al.^{34–39}; these are instances in which two highly similar sequences fold into remarkably different structures. Orban and co-workers have designed a protein-G-like sequence of 56-residues that is marginally stable in one of two possible folds. By mutating key residues in this sequence they are able to stabilize one fold or the other (see Figure 4). We call conformation “ β ” that fold into the protein-G-like that fold into a $4\beta + \alpha$ fold, and we call conformation “ α ” that fold into 3α helical folds. One pair of sequences (GA88/GB88) is 88 percent identical in sequence, differing in seven positions. Another pair (GA95/GB95) is 95 percent identical, differing in three positions. Accurately predicting the structural preferences of these structures presents a serious challenge for computational methods^{34–39}.

[Figure 3 about here.]

We initially approached this problem by making a model of each sequence with the same backbone structure as its partner chameleon sequence. For example, we took the sequence of GA88 and built a model with the same overall structure as GB88. We then used the confinement method to assess

the free energy difference between the experimentally determined structure of GA88 and the model (with the GA88 sequence and the GB88 structure). The confinement method was able to predict the conformational preferences correctly for all four sequences (data not shown). This is, however, not surprising. It is well known^{19, 40} that it is easy to distinguish computational models from native structures. To avoid this potential problem, we instead computed relative free energy of two different computer generated models for each sequence. One model is based on the α structure and the other on the β structure (see Supporting Information for details on the modeling procedure). This is a much more realistic test of the confinement method's ability to accurately calculate relative free energies.

[It's not clear what this test is and why it's better, and why there are only 5 sequences. Please clarify.] Now there are 4 sequences as they have the experimentally available structure. The fifth sequence is discussed at the end of the paragraph.

Figure 4 shows that the confinement method identifies the correct structure for all four sequences. One hypothesis for fold switching of chameleon sequences is that the structural transitions require states with diminished stability³⁷. It is believed that if the free energy of the native state and the alternative state are within a range of 5 kcal/mol, then it is possible for the native state to be destabilized relative to the alternative state with only small changes in sequence. The stability of the native state can decrease for a number reason ranging from chemical modification, breaking of disulphide bonds or mutations—as is the case here. The calculated free energy differences range from around 3.5 to 5.0 kcal/mol, which is consistent with the above hypothesis^{35–37}. In a more recent study³⁸, the amino acid residue at position 45 (Tyr for β and Leu for α) was found to be important for switching between α and β conformation. This inspired us to introduce another mutation at 45 position. For our future reference this Tyr-45-Ala mutation of GB95 sequence will be known as GB98. Interestingly, with this mutation, the equilibrium shifts to α conformation as it gains 3.84 Kcal/Mol stability compare to β conformation and indicate the importance of Tyr-45 residue. It will be interesting to know the actual experimental structure which can validate our result.

2.3 Per-residue free energy calculation can identify the mechanistic detail behind conformational preferences

To understand the mechanism behind the conformational preferences of the different protein sequences, we decomposed the calculated free energy into per-residue contributions in an approximate way²⁸. For this purpose, the confinement free energy, $\Delta G_{A,A^*}$ and $\Delta G_{B,B^*}$ of each residue is calculated in the usual numerical way as described the method section. We call this method approximate as we ignore the contribution from the normal mode or quasiharmonic analysis. Instead, decomp module of amber is used to calculate the internal energy of each residue from the final restrained trajectory.

[Arijit – I didn't find this methods calculation. We should at least put in a sentence or two here summarizing it.] This helps us to identify important residues that stabilize a particular conformation. The per residue free energy, $\Delta\Delta G((\beta) - (\alpha))$ is shown in Figure 4. In this plot, residues colored in blue favor the α structure, residues in red favor the β structure, and white residues have no preference.

[Figure 4 about here.]

Although the overall free energy difference between the two structures is small (within 3-5 Kcal/Mol), the individual residues can show marked preferences for being in either α or β conformation. Such differences can be understood by looking in detail at the local environment for those residues. For example, the region around residue 7 forms a random coil in the α structure, whereas it is forming a beta sheet structure in the β structure. These residues strongly favour the locally well packed and hydrogen bonded environment found in the β sheet. Overall, favoring α or β structure is a delicate balance, where the relative global free energy difference is small and the contributions of different per residue tendencies balance out. It is therefore very likely that by changing key residue preferences the global fold preferences can be changed.

Three residues are mutated between GA95 and GB95, at positions 20, 30, and 45. In GA95, these residues (L20, I30 and L45) stabilize the α structure. In GB95, two of these residues (F30 and Y45) favor the β structure, because

they have large solvent exposed surface areas in the α structure, but are more buried in the β structure. Additionally Y45 forms a hydrogen bond with D47 in the β structure. On the other hand, residue A20 from GB95 still favors the α structure, although not as strongly as L20 in GA95.

The α and β sequences are nearly identical, so there are some common features observed for all sequences. Roles of some important residues in stabilizing either the α or β conformation and possible reasons for such conformational preferences are summarized in supporting information Figure S1. The experimental observations classified the protein into two parts: Amino acids 9-51 are fully structured in both folds, whereas residues 1-8 and 52-56 are unstructured in the α fold, but form a β -strand in the β fold. Most of the amino acid residues in the region 1-9 have negative per-residue free energies, which means that these residues favor the β structure.

In addition to the direct effects of the mutation, there are also indirect effects due to small perturbations due to the mutations. For example, the L20A mutation causes a slight repacking around residue 20. This causes large changes in the per-residue free energies of nearby residues T25 and A26. However, these differences largely cancel and the overall effect of the L20A mutation is weaker than either the I30F or L45Y mutations.

Such per-residue free energy decompositions allow us to understand the driving forces behind protein folding and conformational change and may be useful for designing proteins with specific structures and functions.

2.4 Application of confinement method for structure prediction

We applied the confinement method as a “meta-predictor” for structure prediction. Here, the task is to correctly identify the most accurate models out of a set of “decoys” generated by different methods during the Critical Assessment of Structure Prediction (CASP) experiment. CASP is a blind test in which different groups apply methods to predict the 3-dimensional structure of proteins from their sequences. Each group is allowed to submit five possible structures, which they are supposed to rank from best to worst. We have performed two experiments centered on CASP.

In the first experiment, we examined several targets and tried to rank-order the predictions made by the same group (same methodology used to generate the models), in others the structures were drawn from several different groups (different methodology used). The goal of this experiment is to determine whether the physics-based confinement method can correctly identify more native-like structures as having low free energies.

Our second experiment was to see if the confinement method can identify structures that are missed by other meta-prediction servers. Most successful meta-prediction servers are based on the idea of consensus: if many different prediction methods produce similar results, then that is probably a correct prediction. This is often a powerful heuristic, but it can miss cases where there is a very good result that is only predicted by one method. We chose several cases where such structures were missed and assessed if the confinement method can correctly identify these accurate models.

As is common in the CASP experiment, we assess our results in terms of Global Distance Test Total Score (GDT-TS)⁴⁵, which is a C α based measure of structural accuracy. It can be understood roughly as the percentage of residues that are correctly positioned in the model (range 0 to 100, higher is better). The initial models for the test was chosen from different server groups those have traditionally done well in past CASP events.

2.4.1 The confinement method can correctly rank different models generated by the same methodology and distinguish the native structure

Here, our task is to rank different submitted models of CASP9 targets that have been generated with the same methodology. In absence of any other reliable alternative, we compare all our free energy based ranking with the GDT-TS based ranking which are available in CASP website.

The first test case is a protein BVU3908 from *Bacteroides vulgatus* whose PDB id and CASP target code are 2L01 and T0559 respectively. The best predictor group for this 69 amino acid long target was "BAKER-ROSETTASERVER". We initially checked similarity between the models submitted by them and discarded two from the analysis on the basis of being too similar to some of the other models. The GDT_TS score and rmsd values as shown in Figure 5 indicate that model 1 was predicted correctly,

whereas the order of model 3 and 5 was wrong. The main difference between model 3 and rest of the models was the orientation of the first alpha helix. On the other hand, as shown in Figure 5 the confinement method not only can differentiate the native structure from the submitted models, the ranking also correlates perfectly with the GDT_TS score.

Arijit- we could probably make a better visualization of this figure above by putting it onto a toy landscape: x-axis is rmsd, y-axis is DDG, and above each of the 4 points on this graph will be the ribbon diagram and the GDT-TS. I suggest we do that with the fig below too. Let's don't use 4 different colors for the fig below. Let's just show differences in structures in some different color, or show them all as green.]

[Figure 5 about here.]

To further test the method we continued with the example of protein BT2368 from *Bacteroides thetaiotaomicron*. The PDB id of this 74 amino acid residue protein is 2L02 and CASP target code is T0560. We compared the free energy difference between the native structure and the two of the five submitted models from the group "Splicer". The remaining three models were discarded as they were too similar to the rest of the models. As shown in Figure 6, we can identify the native state correctly and our calculated free energy based ranking matches well with the GDT_TS score.

[Figure 6 about here.]

2.4.2 The confinement method can rank models generated by different methods

Our next test was between models that are produced with different methodologies. Here, our model system was a fas apoptosis inhibitory protein molecule whose pdb id and CASP target codes are 3MX7 and T0540 respectively. This protein contain 8 beta strands and 90 amino acid residues. The top models from groups "LTB" and "Mufold" was chosen for analysis. We will call this as Model 1 and Model 2 for future references. As summerized in Figure 7, the free energy based ranking once again correlates well with the GDT_TS based score.

[Figure 7 about here.]

2.4.3 Per residue free energy calculation identifies the residues that are responsible for differences between two conformations

[Arijit - This and the following sections need to be shortened and made more concise.]

In section 2.3 we discussed how per residue free energy calculation can identify the mechanistic detail behind conformational preference of a chameleon sequence. In this section, our aim is to apply the same method and try to understand whether per residue free energy can help us identify residues which stabilizes/destabilizes a particular region of a protein. The difference here is that the protein has similar fold with only mismatch in some region. To investigate this, we choose a domain of adhesion exoprotein from *Pediococcus pentosaceus* (pdb id=2KYW) from protein data bank and its best predicted model of CASP9 (casp id=T0569, submitted by 'Mufold' group which had a GDT_TS of 78). We first calculated the free energy of the whole protein and found that the native structure is stabilized by 20 Kcal/Mol (see supporting information Figure S2).

Next we proceed with the per residue free energy calculation. Our effort clearly identifies two hydrophobic residues Val-59 and Ile-61, which destabilizes the submitted model with respect to the crystallographic structure. The sidechains of these hydrophobic residues are oriented towards the protein hydrophobic core in the native NMR structure but oriented outside protein and solvent exposed in the model. These residues are the primary reason why two of the beta sheets in the native structure are disordered in the model structure (see Figure 8 and supporting information Figure S3). This method also identifies some other residues which stabilize/destabilize either of the conformation. For example, Lys-76 stabilizes the model as it has a H-bond with Asp-11 which is missing in the native structure (Figure 8 and supporting information figure S3).

[Figure 8 about here.]

2.4.4 Failures in the confinement method

Despite the great success in most of the studied systems, there are few failures, specially when the GDT scores of the compared structures are very close. We observed this for a 54 amino acid residue long engineered protein from Asr4154 protein (pdb id: 2L09 and CASP code T0538). Like previous cases, we kept the native structure and include models from the group "PconsR" (GDT_TS=96.23, model1), "Shell" (GDT_TS = 90.09, model2) and "FOLDIT" (GDT_TS = 86.32, model3) for analysis. Contrary to our expectation, model1 was found to be more stable than the crystal structure (see Figure 9). In order to rationalize such obseravtion we calculates per residue free energy between crystal structure and model1. The results show us that despite the small variation at the backbone level (as shown by high GDT_TS scores and low RMSDs), the sidechains are oriented in very different ways, giving rise to large differences in the stabilization of certain residues. In particular, some of the differences arise from different salt bridge patterns (Arg-32 with Glu-35, Glu-28 with Lys-24 and Arg-26 with Glu-50 in crystal versus Arg-32 with Glu-28 in model 1) and certain flexible polar residues exposed to the surface (Lys-24 in model 1, which has an entropic gain from absence of salt bridge and is stabilized by interactions with the solvent) (See supporting information Figure S4). All in all, this unexpected result just shows us that this method is sensitive to local interactions such as those happening from side chain reorientation and also indicate the limitations of GDT_TS based ranking.

[Figure 9 about here.]

2.4.5 What can we say about low resolution models?

So far we have seen that the method is good at predicting preferences when the structures are not very far from the native. But the question remains how far from native can we go and still see that the method produces correct result. In this section we explored this question with models from extracellular domain of the jumping translocation breakpoint protein (pdb id: 2KJX). Most of the group could only generate low resolution models for this CASP9 target (id: T0531) . In our comparison, we choose five models by the group MUFOULD-MD, which was the best performing group for

this target with their best model had a GDT_TS value of 44. The result presented in Figure 10 shows: 1.) native is correctly identified as expected and 2.) Surprisingly there is a high level of correlation between the GDT ranking and the free energy ranking for model 1 and model 3, the rest three structures with GDT_TS score less than 35 are ordered incorrectly. It is worth to note that models 2 and 3 have the same GDT and very different free energies, meaning that the actual ordering could change a lot⁴⁶. It is encouraging that at least the method can pick out the best model even though it is got a low GDT score: 44.

[Figure 10 about here.]

2.4.6 Can the confinement method perform better quality assessment in protein structure prediction?

[Arijit - let's make the following section more concise and focused.]

A part of the CASP experiment is dedicated to the quality assessment (QA) of predicted models⁴⁴. Here, predictors were asked to score each model on a scale (known as qmode) from 0 to 1, with higher values corresponding to better models⁴⁴. It will be interesting to know how confinement method perform in quality assessment compare to the other groups in CASP9. We investigated this using couple of CASP Targets. Here, we present a case, where confinement method perform well than the top performing group MUFOLD-WQA⁴⁷ of CASP9. We choose two models from CASP target T0538. They were model 3 submitted by PconsR (GDT_TS = 96, qmode 1 = 0.5434) and model 5 from the MULTICOM-NOVEL (GDT_TS = 83, qmode 1 = 0.5865). Both are server predicted models and the qmode value presented are from MUFOLD-WQA⁴⁷. We choose these two models as the model by PconsR was the most accurate predicted model for this target, whereas the other model was predicted best by QA test of MUFOLD-WQA. The calculated free energy using confinement method indicate that the model by PconsR is more stable by 3.9Kcal/Mol which support the GDT_TS trend. There is no doubt that the consensus approach can predict the model quality in a faster manner. But we expect confinement method can predict the model quality in a relatively expensive but much more accurate way.

3 Conclusion

We have described a computational method called confinement for computing the difference free energy from a protein conformation *A* to *B*. We show that it can give accurate values, even for relatively large conformational changes. We have demonstrated that it can discriminate which Orban chameleon peptides fold into a 3-helix bundle vs. a $4\beta+1$ helix structure. We show that it can discriminate between good and bad CASP models. Perhaps most importantly, we have shown that it can be used to give residue-level insights into what are the dominant structural factors in a protein that are responsible for the difference free energies. A key advantage is that this method does not require any reaction coordinate, or sampling a pathway from conformation *A* to *B*. We have tested the method for structures of proteins having up to 100 amino acids. The computational cost for a 56-residue protein is about 4 hours [on 1 gpu] for 20 ns of confinement [Is 20ns all you need?].

I need 21 (confinement run) x 20 ns for single confinement

[If this is a relevant limitation, let's say something about it.] I think we need to write the normal mode issue and number of residues that we can handle. If one of you contribute here that will be great.

4 Method

The confinement method has been described in details in ref. by Tyka et al.²⁸ and Cecchini et al.²⁹. The basic approach of the confinement approach is the same in both these papers. A thermodynamic cycle is used to compute the free energy between conformations *A* and *B*. However, there are some small technical differences between the two approaches. Here we briefly describe the procedure that we used.

1. In the first step, a minimization of *A* and *B* conformations are performed. These minimized conformations (A^* and B^*) are the reference conformation of that state.
2. The free energy of confining the ensemble (*A* or *B*) to a microstate (A^* or B^*) is calculated. This is done by gradually applying larger and

larger harmonic restraints on all the atoms of the biomolecule. This is done by running 21 molecular dynamics simulation (each 20 ns long) for each leg of the thermodynamic cycle, where the harmonic restraint force constant was scaled from 0.00005 Kcal/Mol (mostly free) to 81.92 Kcal/Mol (frozen in one microstate). In this final restrained state, the rotational contribution to the free energy is frozen out and the only remaining contribution is the vibrational part. The free energy for this step is estimated from the fluctuations around the reference structure using a numerical approach developed by Tyka et. al.²⁸. The confinement free energy calculated in this way is recorded as $\Delta G_{A,A^*}$ and $\Delta G_{B,B^*}$ as shown in Figure 1.

3. Finally the thermodynamic cycle is closed by calculating the free energy between the final restrained state A* and B* using normal mode analysis or quasiharmonic analysis. The free energy calculated in this way is shown as $\Delta G_{A^*,B^*}$ in Figure 1.
4. The full free energy, $\Delta G_{A,B}$ between the two state A and B is calculated using the equation $\Delta G_{A,B} = \Delta G_{A,A^*} - \Delta G_{B,B^*} + \Delta G_{A^*,B^*}$

All calculations were performed with the amber 11 suit of programs^{41, 42} in combination with ff99SB forcefield⁴⁹ and generalized born implicit solvent⁵⁰. Interestingly, we extend the method for calculation of per residue free energy in an approximate way. For this purpose, the confinement energy, $\Delta G_{A,A^*}$ and $\Delta G_{B,B^*}$ of each residue is calculated in the usual numerical way as described in ref. by Tyka et al.²⁸. The internal energy of each residue is calculated using the decomp module of amber from the final restrained trajectory. We call this method approximate as we ignore the entropic contribution from the normal mode or quasiharmonic analysis. However, this contribution to the total free energy is much smaller, which allow us to study the mechanistic details of conformational preference of each residue.

References

- [1] Elber, R. A Milestoning Study of the Kinetics of an Allosteric Transition: Atomically Detailed Simulations of Deoxy Scapharca Hemoglobin. Biophysical J., 2007, 92, 85-87.

- [2] Moult, J.; Fidelis, K.; Kryshtafovych, A.; Tramontano, A. Critical assessment of methods of protein structure prediction (CASP)-round IX. *Proteins*, 2011, 79, 1-5.
- [3] West, A.M.; Elber, R.; Shalloway, D. Extending molecular dynamics time scales with milestoning: example of complex kinetics in a solvated peptide. *J Chem Phys*. 2007, 126, 145104-145104.
- [4] Haas, K.; Chu, J.W. Decomposition of energy and free energy changes by following the flow of work along reaction path. *J. Chem. Phys.* 2009, 131, 144105-144111.
- [5] Jnsson, H.; Mills, G.; Jacobsen, K.W. Nudged Elastic Band Method for Finding Minimum Energy Paths of Transitions, in Classical and Quantum Dynamics in Condensed Phase Simulations, Ed. B. J. Berne, G. Ciccotti and D. F. Coker, 385 (World Scientific, 1998).
- [6] E, W.; Ren, W.; Vanden-Eijnden, E. Simplified and improved string method for computing the minimum energy paths in barrier-crossing events. *J. Chem. Phys.* 2007, 126, 164103.
- [7] Dellago, C.; Bolhuis, P.G.; Geissler, P.L. Transition Path Sampling, *Adv. Chem. Phys.* 2002, 123, 1-84.
- [8] Cheng, X.; Wang, H.; Grant, B.; Sine, S.M.; McCammon, J.A. Targeted Molecular Dynamics Study of C-Loop Closure and Channel Gating in Nicotinic Receptors. 2006, 9, 134.
- [9] Elber, R. Long-timescale simulation methods. *Cur. Opin. in Str. Biol.* 2005, 15, 151-156.
- [10] Torrie, G. M.; Valleau, J. P. Nonphysical sampling distributions in Monte Carlo free-energy estimation: Umbrella sampling (1977) *J. Comput. Phys.* 23, 187
- [11] Tironi, I.G.; van Gunsteren, W.F. A molecular-dynamics simulation study of chloroform. *Mol. Phys.* 1994, 83, 381-403.
- [12] Meirovitch, H. Recent developments in methodologies for calculating the entropy and free energy of biological systems by computer simulation. *Current Opinion in Structural Biology*, 2007, 17, 181-186.

- [13] Mobley, D.L.; Chodera, J.D.; Dill, K.A. The combining and release method: obtaining correct binding free energies in the presence of protein conformational change. *Journal of Chemical Theory and Computation* 2007, 3, 1231-1235.
- [14] Mobley, D.L.; Klimovich, P.V. Perspective: Alchemical free energy calculations for drug discovery. *J. Chem. Phys.* 2012, 137, 230901-12.
- [15] Mobley, D.L.; Chodera, J.D.; Dill, K.A. On the use of orientational restraints and symmetry corrections in alchemical free energy calculations. *J. Chem. Phy.* 2006, 125, 084902.
- [16] Chipot, C.; Shell, M.S.; Pohorille, A. Introduction, in Chipot, C., Pohorille, A., editors. *Free Energy Calculations: Theory and Applications in Chemistry and Biology*. Springer Series in Chemical Physics, vol. 86. Berlin and Heidelberg: Springer; 2007, p. 132.
- [17] Jorgensen, W.L. The many roles of computation in drug discovery, *Science* 2004, 303, 18138.
- [18] Gilson, M.K.; Zhou, H.X. Calculation of protein-ligand binding affinities. *Annu Rev Biophys Biomol Struct.* (2007) 36, 21-42.
- [19] Handl, J.; Knowles, J.; Lovel, S.C. Artefacts and biases affecting the evaluation of scoring functions on decoy sets for protein structure prediction. *Bioinformatics*, 2009, 25, 1271-1279.
- [20] Dill, K.A.; H.S. Chan. From Levinthal to Pathways to Funnels: The "New View" of Protein Folding Kinetics. *Nature Structural Biology* 4, 10-19 (1997)
- [21] Dill, K.A.; Ozkan, S.B.; Shell, M.S.; Weikl, T.R. The protein folding problem. *Annual Review of Biophysics* (2008), 37, 289-316.
- [22] Anfinsen. C.B. Principles that Govern the Folding of Protein Chains. *Science* (1973) 181, 223-230.
- [23] Christ, C.D.; van Gunsteren, W.F. Enveloping distribution sampling: A method to calculate free energy differences from a single simulation, *J. Chem. Phys.* (2007), 126, 184110.

- [24] Ytreberg, F.; Zuckerman, D. Simple estimation of absolute free energies for biomolecules. *J. Chem. Phys.* 2006, 124, 104105.
- [25] Park, S.; Lau, A.; Roux, B. Computing conformational free energy by deactivated morphing. *J. Chem. Phys.* 2008, 129, 134102
- [26] Zheng, L.; Chen, M.; Yang, W. Random walk in orthogonal space to achieve efficient free-energy simulation of complex systems, *Proc. Natl. Acad. Sci.* 2008, 105 (51), 20227.
- [27] Shell, S.M. A replica-exchange approach to computing peptide conformational free energies. *Mol. Sim.* 2010, 7, 505-515.
- [28] Tyka, M.; Clarke, A.; Sessions, R. An Efficient, Path-Independent Method for Free-Energy Calculations. *J.Phys.Chem. B* 2006, 110, 17212-17220.
- [29] Cecchini, M., Krivov, S.V., Spicthy, M., Karplus, M. Calculation of free-energy differences by confinement simulations. Application to peptide conformers. *J. Phys. Chem. B.* 2009, 113, 9728-9740.
- [30] Ovchinnikov, V.; Cecchini, M.; Karplus, M. A Simplified Confinement Method for Calculating Absolute Free Energies and Free Energy and Entropy Differences. *J. Phys. Chem. B.* 2013, 117, 750-762.
- [31] Spicthy, M.; Cecchini, M.; Karplus, M. Conformational Free-Energy Difference of a Miniprotein from Nonequilibrium Simulations. *J. Phys. Chem. Lett.*, 2010, 1, 1922-1926.
- [32] Strajbl, M.; Sham, Y.Y.; Vill, J.; Chu, Z.-T.; Warshel, A. Calculations of Activation Entropies of Chemical Reactions in Solution. (2000) 104, 4578-4584.
- [33] Krivov, S.; Karplus, M. Hidden complexity of free energy surfaces for peptide (protein) folding *Proc. Natl. Acad. Sci. U.S.A.* 2004, 101, (41), 14766.
- [34] Alexander, P.A.; He, Y.; Chen, Y.; Orban, J. Bryan, P. The design and characterization of two proteins with 88% sequence identity but different structure and function. *Proc. Natl. Acad. Sci.* 2007, 104 (29), 11963-11968.

- [35] He, Y.; Chen, Y.; Alexander, P.A.; Orban, J. NMR structures of two designed proteins with high sequence identity but different fold and function. *Proc. Natl. Acad. Sci.* 2008, 105 (38), 14412-14417.
- [36] Alexander, P.A.; He, Y.; Chen, Y.; Orban, J. Bryan, P. A minimal sequence code for switching protein structure and function. *Proc. Natl. Acad. Sci.* 2009 , 106(50), 21149-21154.
- [37] Bryan, P.N.; Orban, J. Proteins that switch folds. *Curr Opin Struct Biol.* 2010, 20(4), 482-488.
- [38] He, Y.; Chen, Y.; Alexander, P.A.; Bryan, P.N.; Orban, J. Mutational tipping points for switching protein folds and functions. *Structure.* 2012, 20(2), 2 83-91.
- [39] Shortle, D. One sequence plus one mutation equals two folds. *Proc. Natl. Acad. Sci.* 2009, 106(50), 21011-21012.
- [40] Sheffler, W.; Baker, D. RosettaHoles: Rapid assessment of protein core packing for structure prediction, refinement, design, and validation. *Protein Sci ence.* 2009, 18(1), 229-239.
- [41] D.A. Case, T.A. Darden, T.E. Cheatham, III, C.L. Simmerling, J. Wang, R.E. Duke, R. Luo, R.C. Walker, W. Zhang, K.M. Merz, B. Roberts, S. Hayik, A. Roitberg, G. Seabra, J. Swails, A.W. Goetz, I. Kolossvry, K.F. Wong, F. Paesani, J. Vanicek, R.M. Wolf, J. Liu, X. Wu, S.R. Brozell, T. Steinbrecher, H. Gohlke, Q. Cai, X. Ye, J. Wang, M.-J. Hsieh, G. Cui, D.R. Roe, D.H. Mathews, M.G. Seetin, R. Salomon-Ferrer, C. Sagui, V. Babin, T. Luchko, S. Gusarov, A. Kovalenko, and P.A. Kollman (2012), AMBER 12, University of California, San Francisco.
- [42] Goetz, A.W.; Williamson, M.J.; Xu, D.; Poole, D.; Le Grand, S.; Walker, R.C. Routine microsecond molecular dynamics simulations with AMBER - Part I: Generalized Born. *J. Chem. Theory Comput.* 2012, 8(5) 1542.
- [43] MacCallum, J.; Perez, A.; Schnieders, MJ.; Hua, L.; Jacobson, M.P.; Dill, K.A. Assessment of protein structure refinement in CASP9. *Proteins*, 2011, 79, 74-90.

- [44] Kryshtafovych, A.; Fidelis, K; and Tramontano, A. Evaluation of model quality predictions in CASP9. *Proteins*, 2011, 79, 91106
- [45] Zemla, A. LGA: a method for finding 3D similarities in protein structures. *Nucleic Acids Res* 2003, 31, 33703374.
- [46] Perez, A.; Yang, Z.; Bahar, I.; Dill, K.A.; MacCallum, J.L.; FlexE: Using Elastic Network Models to Compare Models of Protein Structure. *J. Chem. Theory Comput.*, 2012, 8, 3985-3991.
- [47] Wang, Q.; Vantasin, K.; Xu, D.; Shang, Y. MUFOLD-WQA: A new selective consensus method for quality assessment in protein structure prediction. *Proteins*, 2011, 79: 185-195.
- [48] Case, D.A.; Cheatham, III, T.E.; Darden, T.; Gohlke, Luo, H.R.; Merz, Jr., K.M.; Onufriev, A; Simmerling, C.; Wang, B.; R. Woods, R. The Amber biomolecular simulation programs. *J. Computat. Chem.* (20005) 26, 1668-1688.
- [49] Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins.*, 2006, 65, 712-725.
- [50] Mongan, J.; Simmerling, C.; A. McCammon, J.; A. Case, D.; Onufriev, A. Generalized Born with a simple, robust molecular volume correction. *J. Chem. Theory Comput.*, 2006, 3, 156-169.

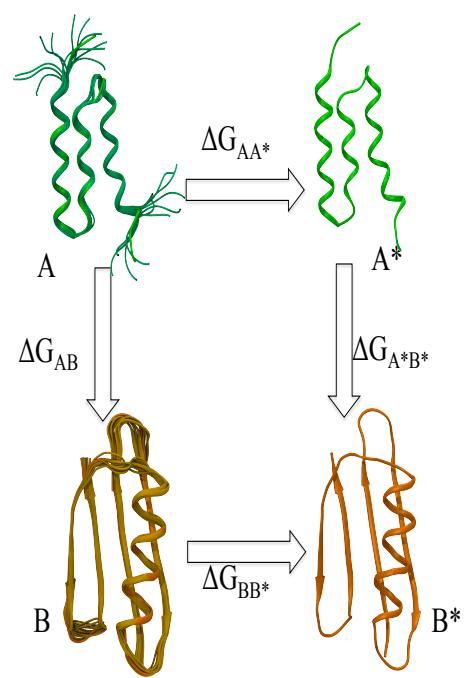


Figure 1: Graphical representation of the thermodynamic cycle involving confinement method.

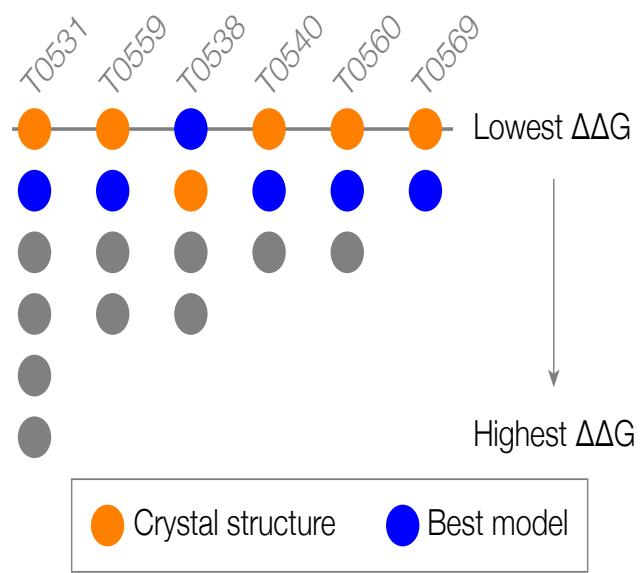
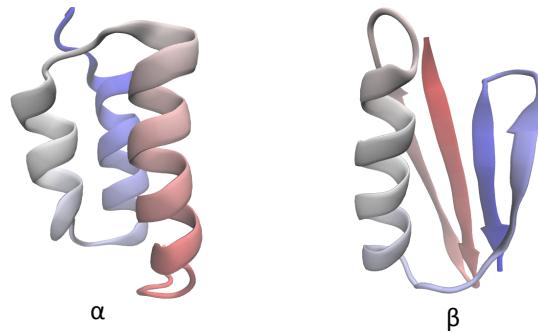


Figure 2: In 5 out of 6 CASP Target, the confinement method assigns a lower free energy to the experimentally determined native structure.

12345678901234567890123456789012345678901234567890123456
 2jws: TTYKLILNLKQAKEEAIKELVDA**GIAEKYIKL****I**ANAKTVEGVWT**L**KDE**I**LTFVTVE **GA88**
 2jwu: TTYKLILNLKQAKEEAIKELVDA**ATAEKYFKLY**ANAKTVEGVWT**Y**KDE**T**KTFVTVE **GB88**
 2kdl: TTYKLILNLKQAKEEAIKE**L**VDAGTAEKY**I**KLIANAKTVEGVWT**L**KDE**I**KTFVTVE **GA95**
 2kdm: TTYKLILNLKQAKEEAIKE**A**VDAGTAEKY**F**KLIANAKTVEGVWT**Y**KDE**I**KTFVTVE **GB95**



Sequence	Experimental Fold		Calculated Fold		Calculated $\Delta G_{\beta} - \Delta G_{\alpha}$ (Kcal/Mol)
	α	β	α	β	
GA88	✓		✓		+3.94± 0.51
GB88		✓		✓	-4.36±0.46
GA95	✓		✓		+3.48±0.47
GB95		✓		✓	-5.01±0.49

Figure 3: Confinement method correctly predicts the structural preferences of four chameleon sequences. The top part of the figure represent four sequences used in this study along with the protein data bank id. Each sequence adopts either a three-helix bundle fold (denoted α) or $4\beta + \alpha$ fold (denoted β). The relative free energies of the two folds are reported for each sequence.

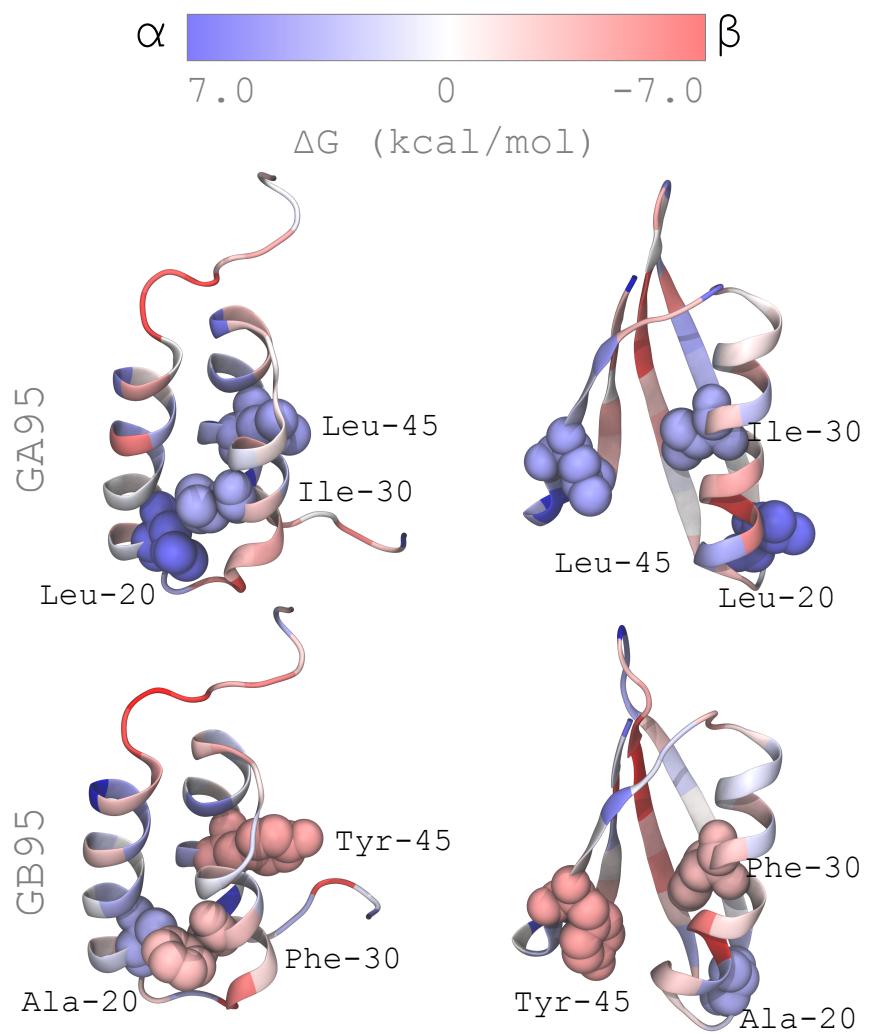


Figure 4: The α and β conformation associated with GA95 and GB95 sequences are colored according to the relative per residue free energy value. In this figure, residues colored in blue favor the α structure, residues in red favor the β structure, and white residues have no preference. Some of the mutated residues are also labelled.

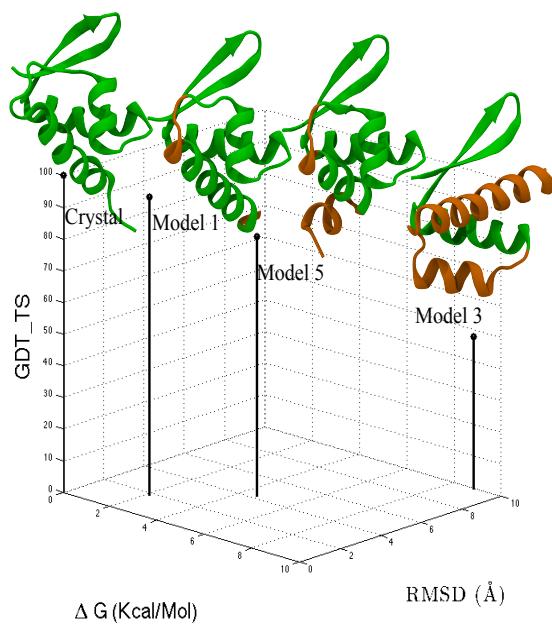


Figure 5: The native and three submitted model structure, along with their GDT_TS, RMSD and relative Free energy values of protein BVU3908 from *Bacteroides vulgatus* (PDB id: 2L01 and CASP code: T0559).

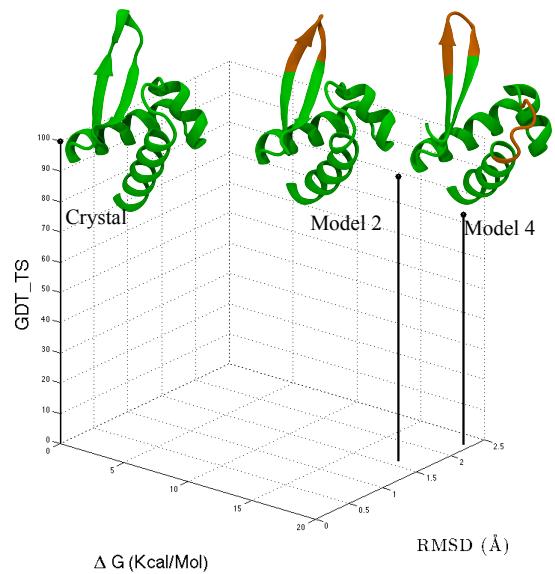


Figure 6: Native and two model structure of protein BT2368 from *Bacteroides thetaiotaomicron* (pdb id: 2L02 and CASP code: T0560). The two models were from the group "Splicer".

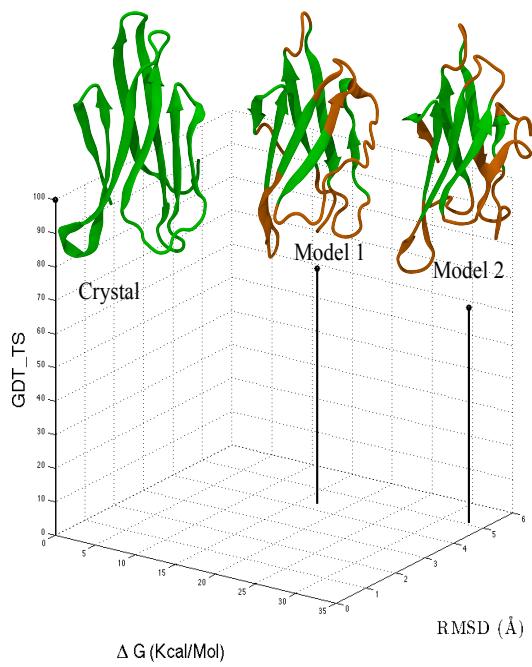


Figure 7: X-ray crystallographic structure and two submitted models of fas apoptosis inhibitory protein (pdb id: 3MX7 and CASP code: T0540). Model 1 and Model 2 in this analysis were submitted by the group LTB and MUFOLD respectively.

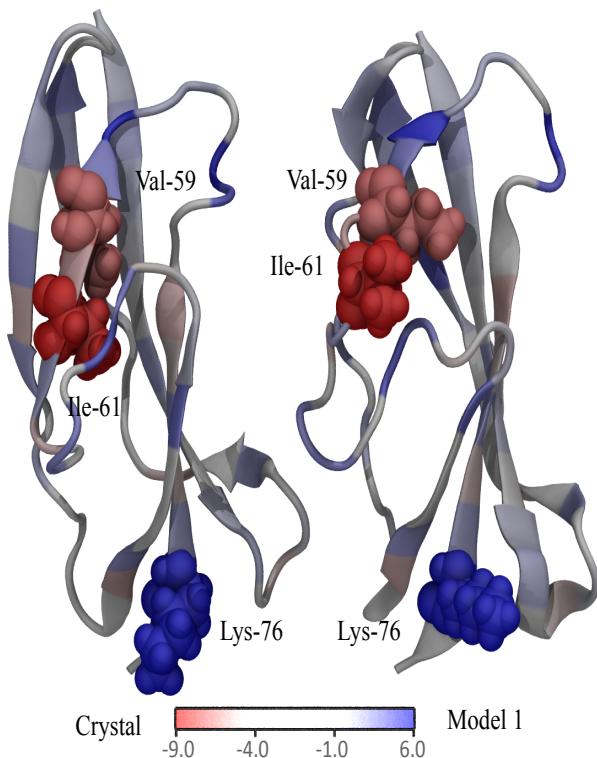


Figure 8: To better understand the reason behind difference in conformation, structure of CASP Target 569 (pdb id: 2KWy) and a generated model was colored according to the per residue free energy value. The amino acid residues that are colored in deep red and deep blue stabilizes the crystal and the model respectively, while the residues with light blue color does not have a preference. Some of the important residues are also labelled.

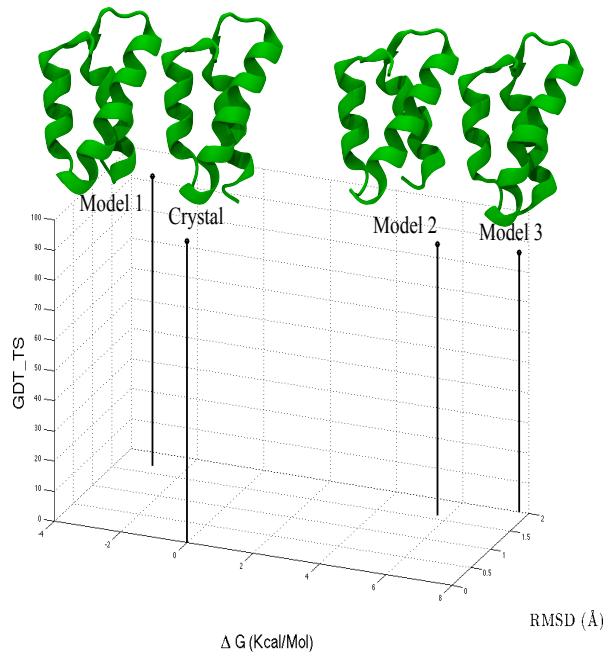


Figure 9: The native and three model structure of engineered protein from Asr4154 protein (PDB ID: 2L09 and CASP code:T0538). The model 1,2 and 3 are from the group PconsR, Shell and FOLDIT respectively.

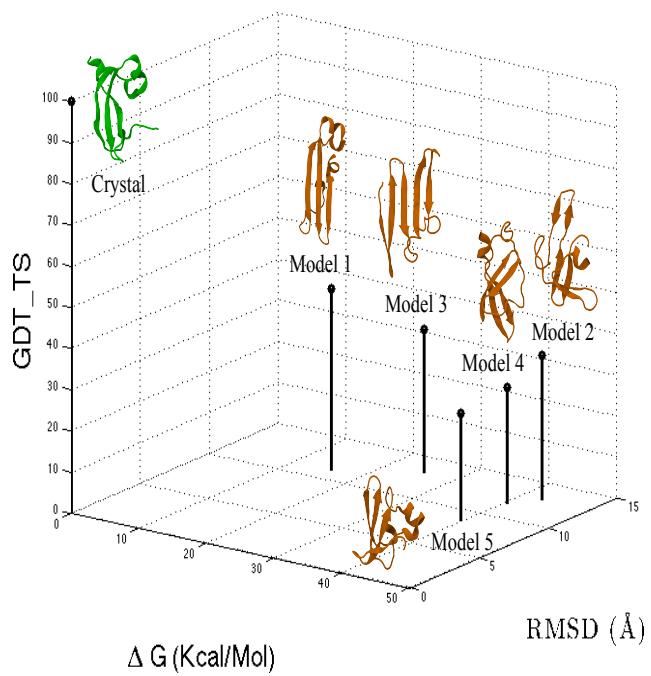


Figure 10: The native structure and 5 models of extracellular domain of the jumping translocation breakpoint protein (pdb id: 2KJX and the CASP code: T0531).

Table 1: The confinement method assigns a more favorable free energy to the experimentally determined structure than to computer-generated predictions. For each target, we examined as many as five predictions submitted by CASP participants. We report the free energy difference between the most favorable decoy and the experimentally determined structure. Positive $\Delta\Delta G$ values indicate that the experimental structure is predicted to be more favorable than any of the decoys.

CASP Target	PDB Identifier	$\Delta\Delta G = G_{best\ decoy} - G_{native}$ (kcal/mol)
T0531	2KJX	11.15 ± 0.70
T0538	2L09	-3.00 ± 0.47
T0540	3MX7	16.94 ± 0.49
T0559	2L01	2.10 ± 0.24
T0560	2L02	18.00 ± 0.49
T0569	2KWy	20.01 ± 0.69