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Boosting protein stability with the computational design of β-sheet surfaces

Doo Nam Kim,[1] Timothy M. Jacobs,[2] and Brian Kuhlman[1,3]*

[1]Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina
[2]Program in Bioinformatics and Computational Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina
[3]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Received 20 October 2015; Revised 18 December 2015; Accepted 21 December 2015
DOI: 10.1002/pro.2869
Published online 23 December 2015 proteinscience.org

Abstract: β-sheets often have one face packed against the core of the protein and the other facing solvent. Mutational studies have indicated that the solvent-facing residues can contribute significantly to protein stability, and that the preferred amino acid at each sequence position is dependent on the precise structure of the protein backbone and the identity of the neighboring amino acids. This suggests that the most advantageous methods for designing β-sheet surfaces will be approaches that take into account the multiple energetic factors at play including side chain rotamer preferences, van der Waals forces, electrostatics, and desolvation effects. Here, we show that the protein design software Rosetta, which models these energetic factors, can be used to dramatically increase protein stability by optimizing interactions on the surfaces of small β-sheet proteins. Two design variants of the β-sandwich protein from tenascin were made with 7 and 14 mutations respectively on its β-sheet surfaces. These changes raised the thermal midpoint for unfolding from 45°C to 64°C and 74°C. Additionally, we tested an empirical approach based on increasing the number of potential salt bridges on the surfaces of the β-sheets. This was not a robust strategy for increasing stability, as three of the four variants tested were unfolded.

Keywords: protein stability; computational protein design; Rosetta molecular modeling program; β-sheets; electrostatic interactions; charge zipper proteins

Introduction

Approximately one quarter of all known protein structures are comprised almost exclusively of β-strands and connecting loops.[1] These proteins often adopt β-sandwich or β-barrel folds in which it is common for one face of a β-sheet to point towards the hydrophobic core of the protein while the other face points towards solvent. As would be expected, the core facing residues play a critical role in determining protein stability as they form tight van der Waals and hydrogen bonding interactions with other residues in the protein. However, the solvent-facing residues can also play a strong role in dictating protein stability, as they frequently form specific interactions with residues from neighboring β-strands as well as nearby residues on the same β-strand. For this reason, there has been considerable effort aimed at understanding the sequence and structure features that contribute to β-sheet stability.[2–5]

Mutagenesis studies and statistical analyses of naturally occurring β-sheets have shown that some amino acids have a greater intrinsic propensity to adopt β-strands. The β-branched amino acids (Ile, Val, and Thr) and aromatic residues are overrepresented in β-strands, while the charged amino acids (Arg, Lys, Glu, and Asp) and turn residues (Gly and Pro) are underrepresented. Similar studies have also examined the preferences for various amino acids to be placed near each other on adjacent

Additional Supporting Information may be found in the online version of this article.

Conflict of interest: The authors have no conflict of interest with this work.

Grant sponsor: NIH; Grant number: GM073960.

*Correspondence to: Brian Kuhlman, Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC. E-mail: bkuhlman@email.unc.edu

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β-strands.[6,7] Two of most favored pairings are aromatic pairs and the formation of salt bridges using aspartate or glutamate paired with arginine or lysine. These preferences have been used widely to design and stabilize model β-hairpins and β-sheets constructed from synthetic peptides,[8,9] but there have been relatively few studies that have focused on using these principles for the large-scale redesign of β-sheets that are incorporated in folded proteins.

An important feature of β-sheets in well-folded proteins is that they are fairly rigid, and each residue in the sheet has a unique set of phi and psi angles as well as a unique set of neighbors, each with distinct geometries that dictate which direction side chains will be projected. An important consequence of this variability is that although there are general preferences for particular amino acids and amino acid pairs to stabilize β-sheets, the preferred amino acid at a specific residue position depends strongly on the precise structure surrounding that residue.[7] This complexity and diversity suggests that the most advantageous methods for designing β-sheets will be approaches that take into account the multiple factors that contribute to stability including: side chain rotamer preferences, van der Waals interactions, hydrogen bonding, desolvation effects, and electrostatics.[10]

Over the last 20 years, methods for computational protein design have emerged as a powerful approach for optimizing sequences based on multicomponent energy functions. These protocols have been used to stabilize proteins, design new protein structures and interactions, and more recently create large macromolecular assemblies.[11–13] In these studies, β-sheet surfaces have been designed in the context of larger goals, but there have been few studies that have specifically probed how effective these approaches are at designing β-sheet surfaces. For instance, is it possible to dramatically stabilize naturally occurring proteins by just redesigning their β-sheet surfaces? Mayo and coworkers optimized an energy function for the design of β-sheet surfaces and tested the protocol on the redesign of β-sheets from two proteins, in one case there was a modest decrease in protein stability and in the other case the melting temperature increased by 8°C.[14] In this study, we used the molecular modeling program Rosetta to redesign β-sheet surfaces of the fibronectin type III domain of the protein tenascin (TNfn3).

TNfn3 forms a Greek key fold with three β-strands in one sheet and four β-strands in a second sheet. It has been studied extensively as a model system for protein folding and stability,[15,16] and previous studies have demonstrated that its stability can be improved via mutation. In most cases, the stabilizing mutations have been located in the protein core, or the redesigns included a mixture of mutations from various regions of the protein.[17–19]

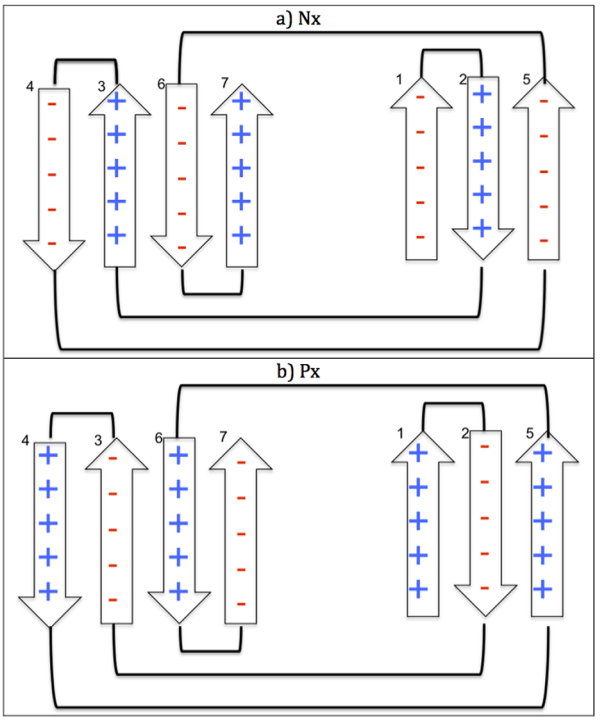


Figure 1. Conceptual overview of the charge zipper scheme for the TNfn3 β-sandwich fold. By mutating residues on the surface exposed faces of the two β-sheets it is possible to create a scenario where every strand is paired with a strand of opposite sign in three-dimensional space, but strands that are close in primary sequence, but are not paired, have the same charge. (a) A charge zipper that starts with a negatively charged β-strand. (b) A charge zipper that starts with a positively charged β-strand.

Unlike the Mayo study, we did not employ an energy function and modeling protocol specifically created for β-sheet surfaces, but rather used the all-atom energy function in Rosetta, which has been parameterized with a diverse set of sequence design and structure prediction tests.[20,21] The primary components of the energy function are a damped Lennard-Jones potential that models dispersion forces and steric repulsion, an implicit solvation model that penalizes the burial of polar groups, an orientation-dependent hydrogen bonding term that has been parameterized to be used with damped Coulomb electrostatics, and knowledge-based terms that score dihedral preferences and the intrinsic preferences of the amino acids to be in alternative secondary structures. The Coulomb electrostatics term is a more recent addition to the Rosetta force field that has been benchmarked computationally,[21] but few experimental tests have been performed with it.

In addition to designing β-sheet surfaces with Rosetta, we also tested an empirical approach based on increasing the number of salt bridges (glutamate or aspartate paired with lysine or arginine) between strands on the surface of the β-sheets. This

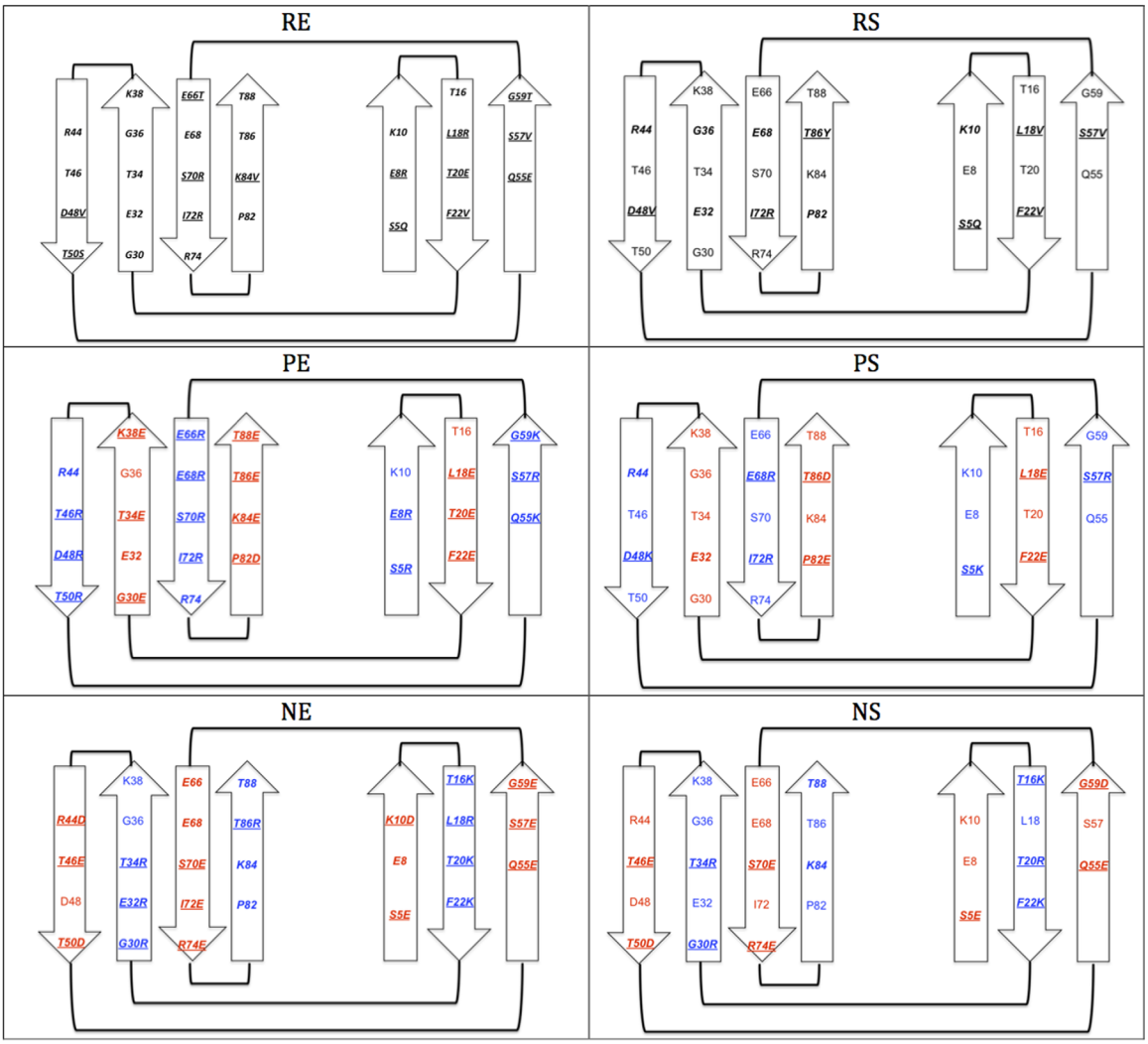


Figure 2. Surface exposed β-sheet residues for the various designs. Mutated residues are underlined, and the residues that were allowed to vary in the simulations were shown in bold italic. RE, RS design, (PE) exhaustively designed charge zipper starting with positively charged β-strand, (PS) sparsely designed charge zipper starting with positively charged β-strand, (NE) exhaustively designed charge zipper starting with negatively charged β-strand, (NS) sparsely designed charge zipper starting with negatively charged β-strand.

approach was inspired by previous studies that demonstrated that arrays of salt bridges could be used to favor the formation of heterodimeric over homodimeric coiled-coils.[22] Charge repulsion between like-charged groups disfavored homodimers while charge attraction favored the heterodimers. A significant challenge in the design of β-sheet proteins is how to specify which β-strands will pair with each other. This is especially problematic for tertiary folds in which strands distant in primary sequence are paired in the final folded structure. Kinetically, it is more straightforward for strands close in primary sequence to pair, and many structure prediction algorithms suffer from predicting too many local contacts when performing *ab initio* structure prediction on β-sheet proteins.[23] TNfn3 is an excellent example of a protein with a topology that is difficult for design and prediction and contains β-strand contacts distant in primary sequence; it includes strand pairing between the third and sixth β-strands as well as the second and fifth β-strands. Interestingly, we observed that through mutation it is possible to place charged residues on TNfn3 in such a way that every β-strand has the opposite charge of the β-strands that are paired with it, and that β-strands that are close in primary sequence, but are not paired in the final structure, end up with the same charge (Fig. 1). We reasoned that this arrangement of charges should favor the folding and stability of the protein by creating favorable electrostatic interactions in the folded state, while simultaneously disfavoring kinetically accessible misfolded states.

Both mutational studies and statistical analyses of β-sheet sequences indicate that there is a strong energetic bonus for placing lysines and arginines

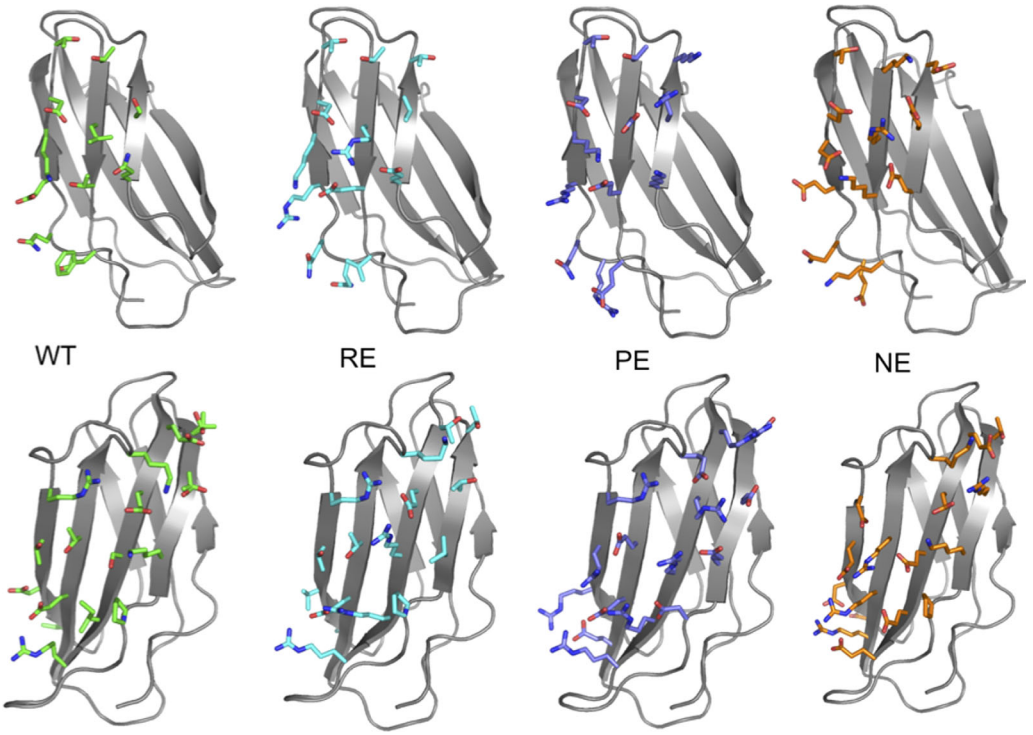


Figure 3. Wild type TNfn3 (WT) and redesigns (RE, PE, NE) with surface exposed residues displayed in sticks. The top row shows the 3-stranded β-sheet and the bottom row shows the 4-stranded β-sheet. The structures are oriented in the same fashion as the illustrations shown in Figure 2, in that on the 3-stranded sheet G59 is at the top right, and in the 4-stranded sheet residue 88 is at the top right. T86 and T88 in the WT protein are shown with two alternative conformations as observed in the crystal structure.

across from glutamates or aspartates in β-sheets, while there is an energetic penalty for placing like-charged amino acids near each other.[6,24] However, charged residues also have lower intrinsic preferences for adopting β-strands.[7,25,26] This suggests that although charge patterning may stabilize the desired pair interactions, the new charged residues may also disfavor β-strand formation.

Results

To test the Rosetta design protocol and energy function on β-sheet surfaces we designed and characterized two variants of TNfn3. In the exhaustive simulation, all surface positions on both β-sheets of TNfn3 were allowed to vary. This included 18 positions on the four-stranded sheet and 10 positions on the three-stranded sheet (Fig. 2). All amino acids except for cysteine and proline were allowed at each position. Interestingly, Rosetta only mutated 5 residues on the four-stranded sheet and mutated 8 residues on the three-stranded sheet (Fig. 3). All but one residue on strands 3 and 4, which are in the four-stranded sheet, were kept as the wild-type amino acid. We refer to this design as RE, for Rosetta exhaustive. The total calculated energy for RE is −195 REUs (Rosetta Energy Units, negative values are more favorable) relative to −180 REUs for the wild type protein. The hydrogen bond score is more favorable for RE compared to the WT protein (−14 vs. −10 REUs), as well the electrostatics term (−67 vs. −62; Table I). New interactions predicted to occur in RE include hydrogen bonds between T66 and E68, E68 and R70, and D11 with R18.

We also performed a design run in which only 8 residues were allowed to vary on the 4-stranded sheet and 5 residues on the 3-stranded sheet (Fig. 2). These residues were picked to emphasize the formation of new pair contacts between strands. Residues 44, 36, 68, and 86 were all varied and form a line across the 4-stranded β-sheet, similarly with residues 48, 32, 72, and 82. This design simulation produced a sequence with 3 mutations on the 4-stranded sheet and 4 mutations on the 3-stranded sheet. We refer to this design as RS, for Rosetta sparse. The total calculated energy for RS was −190 REU. As with the exhaustive design, there were improved hydrogen bonding and electrostatics energies compared to the wild type sequence with scores of −67 and −11 REUs respectively. New interactions included a hydrogen bond between E32 and R72, and a tight valine-valine interaction formed between V18 and V57.

To test the empirical approach of explicitly adding more salt bridges to β-sheet surfaces we constructed four variants of TNfn3. In two of the

Table I. *Computed Stabilities for Proteins*

Type	Name	Salt bridges[a]	Total energy[b]	Coulomb term[c]	hbond_sc[d]	Solvation[e]	vdw[f]
Reference	WT[g]	2	−180	−62	−10	226	−359
Rosetta designed	RE	6	−195	−67	−14	232	−368
	RS	4	−190	−67	−11	229	−365
Charge Zipper	NE	14	−188	−77	−15	241	−372
	NS	10	−183	−69	−12	234	−370
	PE	18	−192	−89	−20	248	−373
	PS	6	−182	−71	−13	242	−370

[a] Number of salt bridges on the β-sheet surfaces. Explanation of a salt bridge is in Supporting Information materials.
[b] Total energy for the protein as computed with Rosetta (unit is REU).[27]
[c] Coulombic electrostatic potential with a distance-dependent dielectric (unit is REU).[21]
[d] Sidechain-sidechain hydrogen bond energy (unit is REU).
[e] Lazaridis-Karplus solvation energy (unit is REU).
[f] van der waals (= "Lennard-Jones attractive between atoms in different residues" + "Lennard-Jones repulsive between atoms in different residues"; unit is REU).
[g] Fibronectin type III domain from tenascin (PDB code: 1ten).

variants we varied most of the residues that were varied in the RE (exhaustive) simulation. In one of these cases, we started the charge patterning with the first β-strand forced to be negatively charged, while in the second case we started with the first β-strand positively charged. We refer to these designs as NE, for negative exhaustive, and PE, positive exhaustive. In PE, the first, fourth, fifth and sixth β-strands are positively charged, while the other strands are negatively charged. The reverse is true for NE. To pick which charged residues were placed at each residue position, we performed a constrained design simulation with Rosetta where residues on the positive strands were constrained to lysine or arginine, and residues on the negative strands were constrained to be aspartate or glutamate. The final PE and NE designs have 22 and 19 mutations, respectively, and were predicted to include 18 and 14 surface salt bridges, respectively. Interestingly, the total score for the PE design is more favorable than the score for the NE design, −192 versus −188 REUs. One contribution to this difference is that the NE design results in a higher net charge for the protein (−14) compared with PE (−5) and wild type (−9; Table II).

In addition to the charge patterned exhaustive designs, we also created a PS (positive sparse) and a NS (negative sparse) design. These simulations used the same charge patterning rules that were used for the PE and NE designs. The PS design has 9 mutations relative to wild type and the NS design has 12 mutations.

All six of the designs (RE, RS, PE, PS, NE, and NS) along with the wild type protein were expressed in *E. coli* and purified with metal affinity chromatography followed by gel filtration. Circular dichroism was used to determine if the proteins were folded. At low concentrations of salt, RE, RS, and PE all exhibited a CD spectrum consistent with a folded β-protein, while PS, NE, and NS have CD spectra indicative of random coil (Fig. 4). The thermal stabilities of the folded proteins were measured by monitoring the CD signal at 220 nm as a function of temperature. Both of the Rosetta designed sequences were dramatically stabilized relative to the WT protein with thermal unfolding temperatures of 74.1°C (RE) and 64.1°C (RS) compared with 45.4°C for the wild type protein. Like the WT protein, the designs also refolded when returning to room temperature (Supporting Information Fig. S5). These experiments were performed with 0*M* NaCl. At a concentration of 1 M sodium chloride, the designs were also more stable than the wild type protein, 58.2°C (WT), 82.2°C (RE), and 77.7°C (RS). Similar increases in stability were observed for RE and RS in chemical denaturation experiments with guanidine hydrochloride (Table II, Supporting Information Fig. S6).

Table II. *Experimentally Measured Stabilities for Proteins*

Type	Name	Net charge	Mutations	T_m (0*M* NaCl)	T_m (1*M* NaCl)	*m* (Kcal/mol * M)	ΔGu (0*M* GdnHCl) (Kcal/mol)
Reference	WT	−9	0	45.4°C	58.2°C	2.42	3.82
Rosetta designs	RE	−5	14	74.1°C	82.2°C	2.73	6.86
	RS	−7	7	64.1°C	77.7°C	2.56	5.27
Charge Zipper	NE	−14	19	Not folded	Not folded	Not folded	Not folded
	NS	−12	12	Not folded	Not folded	Not folded	Not folded
	PE	−5	22	49.9°C	47.6°C	N/A	N/A
	PS	−6	9	Not folded	48.3°C	N/A	N/A

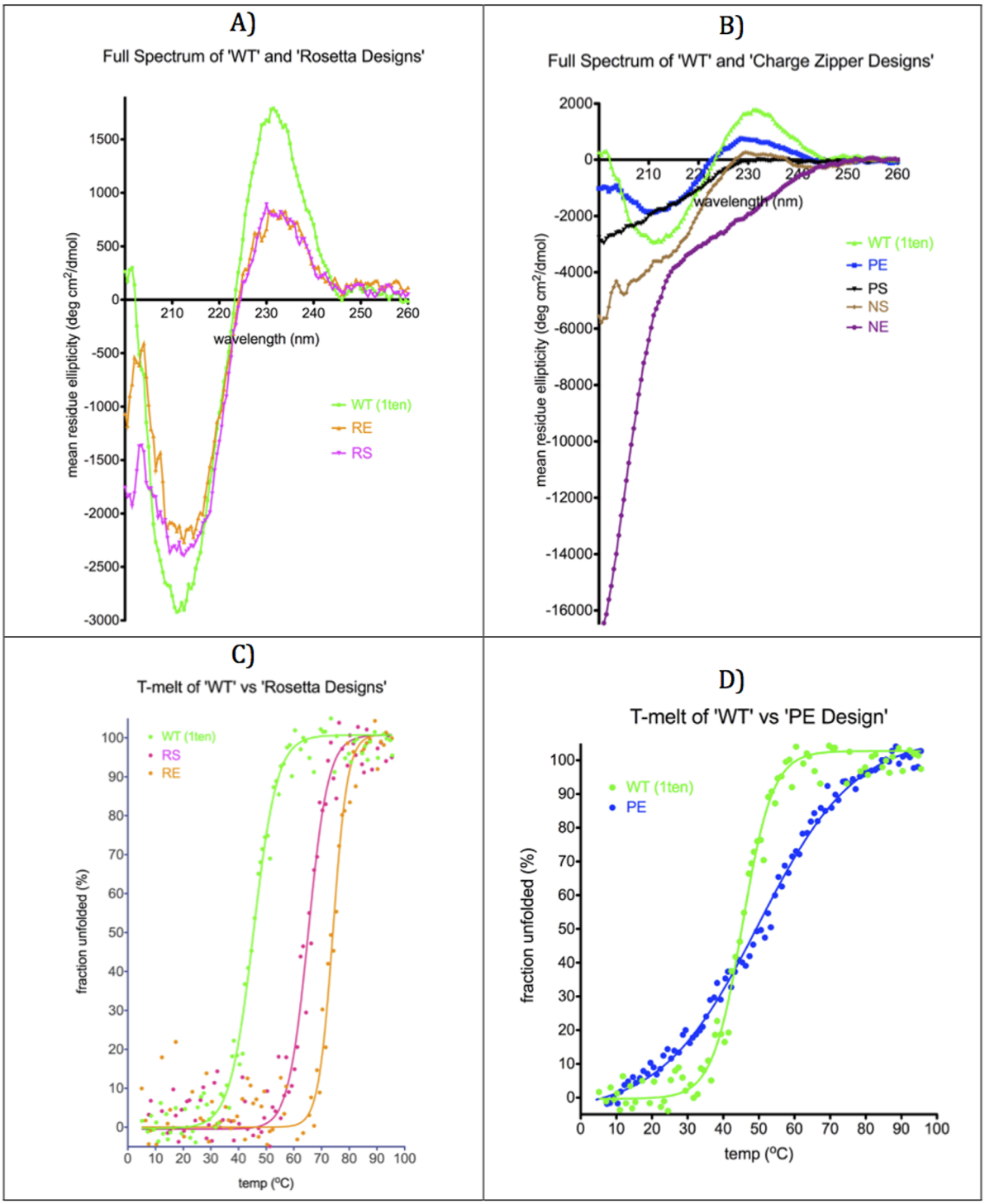
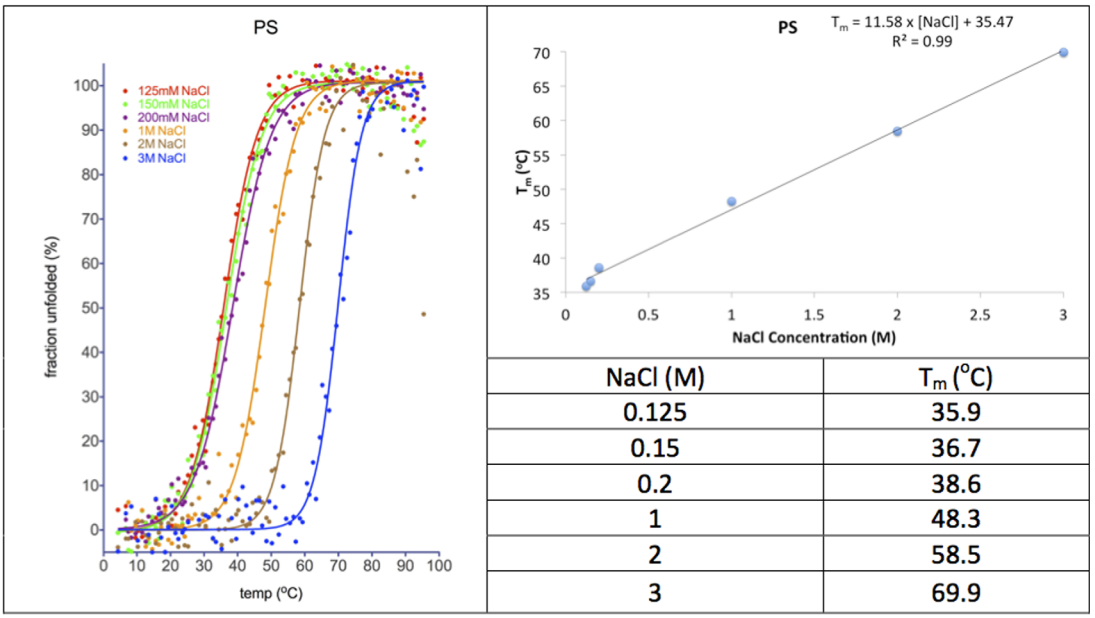


Figure 4. Circular dichroism spectra and thermal denaturation experiments of the Rosetta designs (panels A and C) and the charge zipper designs (panels B and D). All experiments were performed at pH 7.0 in 20 m*M* sodium phosphate.

Of the charge zipper designs, only PE is folded at low concentrations of salt and has a thermal unfolding temperature that is 5°C greater than the wild type protein. Interestingly however, PE is not stabilized by salt like the wild type protein, and at 1*M* NaCl has a thermal stability that is 11°C lower than the wild type protein. Intrigued by the dramatic changes in stability with changes in salt concentration, we examined NE, NS, and PS to determine if they could be induced to fold by adding salt. NS and NE did not fold, but PS was dramatically stabilized with the addition of NaCl (Supporting Information Fig. S5). The thermal unfolding temperature of PS varied linearly with salt and the protein reached a thermal unfolding temperature of 48°C in 1*M* NaCl and 70°C at 3*M* NaCl (Fig. 5).



NaCl (M)	T_m (°C)
0.125	35.9
0.15	36.7
0.2	38.6
1	48.3
2	58.5
3	69.9

Figure 5. T_m measurements for the charge zipper design PS as a function of NaCl concentration in 20 m*M* sodium phosphate, pH 7.

Discussion

Our results demonstrate that protein stability can be dramatically increased by redesigning only the solvent exposed face of small β-sheet proteins. Since the Rosetta design protocol aims to optimize several energetic features, including van der Waals contacts, intrinsic secondary structure preferences and electrostatic interactions, it is not straightforward to assign the increase in stability to any single feature. However, it is interesting that like WT TNfn3 both of the Rosetta designs, RE and RS, are stabilized by high salt concentrations. This suggests that the stability of these variants is not entirely dependent on the formation of salt-bridges between oppositely charged amino acids, as these interactions are predicted to become weaker at higher salt concentrations. Consistent with this conclusion, explicitly placing oppositely charged amino acids on the surface was not a simple recipe for boosting the stability of TNfn3. Three of the four charge zipper designs failed to fold at low salt concentrations. The charge zipper design that does fold, PE, is unlike the other TNfn3 variants, in that it is destabilized by high salt concentrations. This suggests that the redesign did have the intended effect of making protein stability more dependent on surface electrostatic interactions. In contrast to our results with β-sheets, surface salt bridges have been shown to have a more dominant role in stabilizing helical proteins.[22,28,29] This is likely to be in part because the charged amino acids, Arg, Lys, Glu, and Asp have a higher intrinsic propensity to be in helices compared with β-strands.[26]

One of our goals in testing charge patterning on TNfn3 was our hope that it would provide a way to dictate, which β-strands would pair with each other, and in particular destabilize pairing between strands that are close in primary sequence but are not intended to be paired. We thought that this would be a simple approach to incorporate in the *de novo* design of β-sandwich proteins, a problem that is still unsolved. The results suggest that charge patterning does not provide a simple solution, and indicate that the correct strand pairing will need to be specified by the many different structural features that go into determining β-sheet stability.

It is striking that in the design simulation where all residues on the surfaces of the β-sheets were allowed to vary, Rosetta only mutated 14 out of 28 residues. This is despite the fact that the design simulation starts from a completely random sequence, and uses a stochastic sampling protocol to find a low energy sequence. This suggests that most native residues on the β-sheet surfaces of TNfn3 are already optimized for stability, and highlights the fact that every residue in a β-sheet is in a unique environment, where the most favorable residue depends on the precise positioning of neighboring backbone atoms.[30]

Materials and Methods

Computational design and analysis of proteins

We redesigned the β-sheet surfaces on the WT fibronectin type III β-sandwich from tenascin (PDB code: 1ten) using the molecular modeling program Rosetta to perform rotamer-based sequence optimization in combination with backbone refinement. The protocol iterated five times between the PackRotamersMover (rotamer optimization) and the FastRelax protocol

(backbone refinement).[31] The script used to perform these simulations is provided in the Supporting Information. Residues not allowed to change their amino acid identities were allowed to adopt different rotamers ("NATAA"); 1,000–10,000 independent simulations were performed for each set of design parameters (80–800 cpu hours spent, number of design trajectories did not affect greatly the final design selection), and the lowest energy sequence for each set was selected for experimental characterization.

Protein expression and purification

All proteins were expressed using a 6-Histidine tagged PQE-80L vector in the BL21* strain of *E. coli*. Isopropyl β-D−1-thiogalactopyranoside (IPTG) was used at 0.4∼0.8 OD_{600} to induce and the proteins were expressed overnight at 18°C. Cell pellets were sonicated, and after additional centrifugation, supernatant was applied to a Ni-NTA column (GE healthcare). The purified solutions were further purified by size exclusion chromatography (GE healthcare HiLoad 16/60 Superdex 75 pg or HiLoad 16/600 Superdex 200 pg).

Circular dichroism

Secondary structure identification and melting temperature measurement were performed using circular dichroism with JASCO J-815 CD spectrometer. All measurements were done with 20 μ*M* protein concentration. All mean residue ellipticity values shown in this article are CD values of protein sample after extracting CD values of buffer only. Data Integration Time (D.I.T) for ellipticity measurements was increased to 8 s from 4 s especially when high concentration of NaCl was used as buffers. When high concentration of NaCl was used as buffers, analysis of full spectrum of the ellipticity was not meaningful when wavelength is less than 205 nm. Nonlinear regression (sigmoidal dose-response) was used to fit all melting temperatures by Prism software ver. 5.0a.[32] Similar thermal unfolding temperatures were obtained by fitting the data to the Gibbs Helmholtz equation with nonlinear regression by Mathematica 10.[33]

Fluorescence

All chemical denaturations were evaluated by measuring fluorescence emission spectra (310–400 nm) with a Fluoromax 3 spectrofluorometer. Similar as in Gilbreth *et al*.,[17] we plotted fluorescence intensity vs. [GdnHCl] at wavelength 365 nm after excited at 295 nm. All measurements were performed with 5 μ*M* protein concentration at 20 m*M* sodium phosphate pH 7.0 except PS where the measurement was done in 20 m*M* sodium phosphate pH 7.0 and 100 m*M* NaCl.

Acknowledgment

The authors thank Dr. Bryan Der, Dr. Ryan Hallet, Dr. Hayretin Yumerefendi, and Dr. Joseph Harrison for guidance in cloning and protein purification, and Dr. Ashutosh Tripathy for helpful discussions.

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