High-resolution design of a protein loop

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Despite having irregular structure, protein loops often adopt specific conformations that are critical to protein function. Most studies in *de novo* protein design have focused on creating proteins with regular elements of secondary structure connected by very short loops or turns. To design longer protein loops that adopt specific conformations, we have developed a protocol within the Rosetta molecular modeling program that iterates between optimizing the sequence and conformation of a loop in search of low-energy sequence–structure pairs. We have tested the procedure by designing 10-residue loops for the connection between the second and third strand in the β -sandwich protein tenascin. Three low-energy designs from 7,200 flexible backbone trajectories were selected for experimental characterization. All three designs, called LoopA, LoopB, and LoopC, adopt stable folded structures. High-resolution crystal structures of LoopA and LoopB have been solved. LoopB adopts a structure very similar to the design model (0.46 Å rmsd), and all but one of the side chains are modeled in the correct rotamers. LoopA crystallized at low pH in a structure that differs dramatically from our design model. It forms a strand-swapped dimer mediated by hydrogen bonds to protonated glutamic acids. Gel filtration indicates that the protein is not a dimer at neutral pH. These results suggest that the high-resolution design of protein loops is possible; however, they also highlight how small changes in protein energetics can dramatically perturb the low free energy structure of a protein.

computational protein design | loop modeling | tenascin

Protein loops often adopt specific conformations that are critical to protein function. Many protein active sites contain residues that are located in loops, and protein–protein interactions are frequently mediated by loops. Despite the clear importance of loops, most studies in *de novo* protein design have not involved the creation of longer loops that adopt specific conformations but rather have focused on proteins that consist almost entirely of α -helices or β -strands connected by short turns or loops (1–8).

There are several reasons why designing ordered loops may be especially challenging. Unlike α -helices and β -sheets, the backbone hydrogen bonding potential of a loop is not automatically satisfied. For a loop conformation to have low free energy, it is important that each polar group in the loop is hydrogen bonding with another group in the protein or is accessible to water. However, if too much of the loop is exposed to water, then it is less likely that it will adopt a unique conformation. Additionally, unlike α -helices and β -strands, there are not well determined amino acid preferences for forming a well ordered loop. Protein engineers have shown that helices can be built by favoring sequences rich in alanine, leucine, lysine, and glutamate, whereas the β -branched amino acids, threonine, valine, and isoleucine, prefer to form β -strands (9–11). Loops can be designed by favoring sequences enriched in glycine and polar amino acids, but sequences of this type are unlikely to form a unique conformation (12). Well ordered loops typically have diverse sequences that form specific tight packing interactions within the loop and with the rest of the protein.

Designing a new loop requires specification of the new backbone coordinates and the amino acid sequence. This is a difficult problem, because most arbitrarily chosen protein backbones are unlikely to be designable, i.e., there will be no amino acid sequences that pack on the structure with energies that are comparable with

what are observed for naturally occurring proteins (13). Designable backbones can be created by using heuristics derived from naturally occurring protein structures, or structure prediction protocols can be used in tandem with sequence optimization protocols to search for low-energy sequence–structure pairs (2, 6, 14–18). Here, we examine whether the second approach, combining structure prediction with automated sequence optimization, can be used to design loop sequences that adopt unique conformations. Schliebs and coworkers (19) have used a similar strategy to introduce four mutations into an eight-residue loop in triosephosphate isomerase. In their study, Monte Carlo sampling of loop conformations was interspersed with hand-picked mutations.

A variety of methods have been developed to predict the structures of protein loops (20). In general, loop prediction protocols have two primary components, a procedure for searching through the various conformations a loop might adopt and an energy function for evaluating the relative favorability of these conformations. Often, conformational sampling is aided by using loop conformations from other proteins as starting points for structure optimization. We use the molecular modeling program Rosetta to perform sequence design and loop modeling. Rosetta was first developed for *ab initio* structure prediction but has since been expanded to contain protocols for high-resolution structure refinement, loop modeling, molecular docking, and protein design (6, 21–24). The Rosetta energy function used for high-resolution refinement and design emphasizes short range interactions: steric repulsion, van der Waals interactions within 5.5 Å, torsion energies, hydrogen bonding, and a desolvation penalty for bringing atoms close to other polar atoms (25, 26). Sequence and conformational space are searched with a Monte Carlo optimization procedure. Single amino acid substitutions or backbone torsion angle perturbations are evaluated with the Metropolis criterion. In the last stage of refinement, the Monte Carlo moves are followed by gradient-based minimization of torsion angles before comparing the energy of the structure to the most recently accepted structure. To design protein loops, we have combined Rosetta's loop modeling protocols with sequence optimization. The protocol iterates between refining the structure of a loop and designing a sequence for the loop. We generally perform thousands of independent trajectories, because each individual simulation eventually gets trapped in a local energy minimum.

To experimentally test the protocol, we have examined whether we can design new backbone conformations and sequences for the 10-residue loop that connects the second and third β -strands from the third fibronectin type III domain from tenascin-C (27). In these studies, the WT loop is removed from the protein, and the new loop

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Abbreviation: SASA, solvent accessible surface area.

Data deposition: X-ray coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 2RB8 (LoopB) and 2RBL (LoopA)].

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Fig. 2. Models and sequences of the redesigned proteins. (A) Designed loops (blue, LoopA; cyan, LoopB; fuchsia, LoopC) compared with the WT loop structure (red, WT). (B–E) Models of the WT and the designed loops.

LoopC backbones the scores are -137 , -140 , and -143 kcal/mol respectively.

Structure Prediction with the Designed Loop Sequences. During the loop design protocol, small perturbations in backbone motion are used to look for local minima in structure space, but the protocol does not include any explicit tests that would probe whether the design sequence prefers significantly different alternative conformations. To computationally test whether our designed sequences prefer the designed target conformations, we performed structure prediction with the loop sequences. In these simulations, the only input into Rosetta is the sequence of the loop and the structure of the scaffold. Structure prediction is performed by using fragment-based insertion with a round of low-resolution scoring followed by high-resolution scoring (32). A cyclic coordinate descent algorithm is used to close loops after insertions (33). As is the usual strategy with Rosetta, thousands of independent structure prediction simulations were run with each sequence. For all three sequences, the lowest-energy structure predictions resembled the design models (backbone rmsd < 0.8 Å), and there was an increase in energy as the rmsd values for the models increased by > 1 Å (Fig. 3). Pairwise backbone rmsd values were calculated between each of the designed loops and the lowest-energy structure prediction for each loop (SI Table 4). There was a closer agreement between matched pairs, i.e., LoopA structure prediction compared with the LoopA design, than there was between unmatched pairs, i.e., LoopA structure prediction compared with the LoopB design model.

The lowest-energy structure predicted for LoopB is similar to the design model (backbone rmsd = 0.77 Å), but there are noticeable differences in the backbone positions of residues 24–26. In the predicted structure, the carbon alpha positions of these residues are shifted ≈ 1 Å toward the N-terminal tail of tenascin, and proline 24 is better packed in the structure prediction model. The SASAprob score (see *Materials and Methods*) of proline 24 is 0.29 in the structure prediction model and 0.09 in the original design model. During structure prediction with the LoopB sequence, there were models created that more closely matched the LoopB design model (backbone rmsd < 0.3 Å), but these models scored worse (Fig. 3).

Table 2. Sequences and scores of the redesigns

		SASAprob			
		Residue			
Protein	Total Score	24	28	31	Sequence
WT	−148	0.42	0.05	0.05	22-FKPLAEIDGI-31
LoopA	−152	0.12	0.52	0.52	SMQLSQLEGI
LoopB	−149	0.09	0.09	0.09	MPPSQVDGF
LoopC	−156	0.002	0.02	0.02	ALPSRPLDGF

The SASAprob scores, which reflect the quality of packing for individual residues, are shown for residues buried in the center of the loop. A SASAprob score of 0.20 for a leucine indicates that 80% of the leucines in a similar environment in the PDB are better packed (see *Methods*).

Our design procedure did not find the lower-energy conformations for the LoopB sequence, indicating that in the future that it will be advantageous to perform more aggressive sampling of conformational space, including *de novo* structure prediction, when iterating between sequence design and structure optimization.

Experimental Characterization. All three designed proteins were expressed in *Escherichia coli*. One-dimensional ^1H NMR spectra of each protein indicate that they are well folded and adopt β -sheet structures (SI Fig. 8). Circular dichroism was used to probe the thermal stability of the proteins (Fig. 4). LoopB and LoopC have midpoints of thermal unfolding (T_m) that are similar to the WT protein, whereas the T_m for LoopA is 15°C lower than the that of WT protein. All three proteins return to the folded state when the temperature returns to 25°C .

Crystal trays were set up for all three designs. Crystals that diffract at high resolution were obtained for LoopA and LoopB. The crystal structure for LoopB was solved to a resolution of 1.45 Å with an R factor of 17% and an R_{free} of 19%. There is good agreement between the design model and the crystal structure. The design model was superimposed on the crystal structure by aligning the loop residues and residues that make contact with the loop (residues 4–8, 20–31, 48–55, and 72–74) (Fig. 5). Based on this superposition, the rmsd between the backbone atoms in the designed loop and the crystal structure was 0.46 Å. The lowest-energy *de novo* structure prediction for LoopB had a slightly better match to the crystal structure, the rmsd was 0.42 Å. In the crystal structure the backbone atoms of residues 24–26 were located between the design model prediction and the *de novo* prediction. The B values for the atoms in the redesigned loop are comparable with the other loops in the protein, and are all < 30 Å 2 .

All of the side-chain rotamers in the design model were predicted correctly except for Gln-26. In the crystal structure, the side chain of Gln-26 forms hydrogen bonds with the backbone nitrogen and side-chain oxygen of Asp-2 (Fig. 5) and has low B values (< 25). We were curious whether this side chain was not predicted correctly because of changes in the backbone coordinates of the residues or because of the Rosetta energy function. To test between these two options, the backbone coordinates of the crystal structure were used as the template for a Rosetta side-chain repacking simulation with the designed sequence. Like the design model, the repacked structure did not have hydrogen bonds between Asp-2 and Gln-26. The Rosetta energy function disfavors the crystal structure rotamer for several reasons. First, the rotamer adopted in the crystal structure is especially rare in the Dunbrack rotamer library (34, 35), and Rosetta assigns an internal energy of 3.0 kcal/mol to this rotamer. The Rosetta-preferred rotamer has a rotamer score of 0.8 kcal/mol. Second, Rosetta only assigns weak scores to the two putative hydrogen bonds. Both have scores weaker than 0.5 kcal/mol because the distances and angles between the groups are suboptimal. Favorable hydrogen bonds in Rosetta score near 2 kcal/mol. Third, there is a desolvation penalty (> 3 kcal/mol) for removing the glutamine side chain from water. It is difficult to determine which of these scores is most misrepresenting the true

Fig. 3. Structure prediction with the designed sequences. Ten thousand independent prediction trajectories were run for each design, and the Rosetta energies of the models were plotted against the backbone rmsd of the predicted structures compared with the design models.

energetics of Gln-26, and this result demonstrates the challenge of balancing nearly equal and opposite energy terms.

LoopA crystallized at low pH (3.0). The resolution of the structure was 2.1 Å with an R_{free} value of 30%. Under these conditions, the structure of the protein does not look similar to the design model. In the crystal, the protein adopts a 50/50 mixture of domain-swapped dimers and monomers (Fig. 6). In the domain-swapped structure, the designed loop opens up to allow strands 1 and 2 to insert into the partner molecule. The loop is stabilized by a set of hydrogen bonds involving acidic side chains that appear to be protonated. Unfortunately, there is not clear electron density for the loop residues in the monomeric chain. This suggests that, at low pH, the loop is not adopting a specific conformation in the monomer. To further characterize the monomer-dimer equilibrium, gel filtration experiments were performed at a variety of pHs. At neutral pH, the protein has an apparent molecular mass that is close to the predicted mass for a monomer, whereas at low pH, the apparent molecular mass is 18 kDa, halfway between a monomer and dimer. In both cases, the concentration of the protein sample loaded on to the gel filtration column was 2 mM. These results, combined with the decreased stability of LoopA at pH 7, suggest that the designed loop may not have a strong preference for adopting the target conformation.

Fig. 4. Thermal unfolding of the designed sequences as monitored with circular dichroism.

None of the scores that were used to evaluate the design models suggested that LoopA would be more prone than the other loop designs to forming a domain swap interaction. Domain swapping has been observed in many proteins, including *de novo* designed proteins (36, 37). From these studies, it is clear that subtle changes in environment or sequence can promote swapping. Unlike the sequences of LoopB, LoopC, and the WT protein, LoopA does not have any prolines in the redesigned loop. In the domain-swapped crystal structure of LoopA, residues 23, 27, 29, and 30 have ϕ angles that are incompatible with a proline. This suggests that the prolines in the other designed loops could play a role in preventing domain-swapping by disallowing conformational changes required for opening up the loop. However, it should be noted that, in a previous

Fig. 5. Structural alignment between the crystal structure and the design model. (A) The crystal structure of LoopB (green) aligned with the design model of LoopB (mauve). The backbone atoms of residues 4–8, 20–31, 48–55, and 72–74 were used for the alignment. (B) Close-up of glutamine 26.

Fig. 6. The crystal structure of LoopA at low pH. (A) The repeating unit contains a domain-swapped dimer (cyan, chain 1; green, chain 2) and a monomer (purple). Electron density is not present for the redesigned loop in the monomer. In the dimer, the loop opens up, and strands 1 and 2 insert into the partner molecule. (B) The designed loop appears to be stabilized by protonated glutamic acid residues.

study, we used a designed proline to favor domain swapping (38). In summary, the LoopA results highlight the diversity of structures that a designed sequence can adopt and shows that explicit negative design would be useful if the competing states could be identified *a priori*.

Conclusion

Our results indicate that, with the current Rosetta energy function and sampling techniques, it is possible to design a 10-residue loop with high accuracy. LoopB is stabilized by tight packing interactions between hydrophobic side chains in the center of the loop. In the future, it will be interesting and important to test whether novel protein loops can be designed by forming new hydrogen bonding interactions. In this study, we used *de novo* structure prediction simulations to test whether our designed sequences prefer the target conformations. In all three cases, the lowest-energy structure prediction resembled the design models, but, in the case of LoopB, there were a few residues that were shifted by ≈ 1 Å from the design model. In the crystal structure of LoopB, these residues adopted a position that was between the design model and the structure prediction. In future designs with more complicated target structures, it may be even more useful to evaluate designed sequences with *de novo* structure prediction simulations. These simulations can provide templates for creating even lower-energy sequence–structure pairs, and they can be used to determine whether negative design will be needed to disfavor competing states.

Materials and Methods

Iterative Backbone and Sequence Optimization. Rosetta's standard full atom energy function was used for structure prediction and sequence design (6, 21). Rotamer-based sequence optimization was performed as described in ref. 39. Dunbrack's backbone-dependent rotamer library was used with extra subrotamers created by varying all χ_1 angles and the χ_2 angles on aromatic residues plus or minus one standard deviation from the most preferred χ angles (34). Rosetta's backbone torsion angle energies are based on the prob-

ability of observing a particular ϕ and ψ angle for a given amino acid in the protein database (21). During sequence design, all 10 residues in the designed loop were allowed to vary to any amino acid except for cysteine, and the neighboring residues were allowed to adopt alternative side-chain conformations. Nine rounds of iterative sequence design and backbone optimization were used to search for low-energy sequence–structure pairs.

Backbone flexibility was restricted to the loop. Backbone optimization was performed by using Monte Carlo optimization. Only torsion angles are explicitly varied during the procedure. A single Monte Carlo move consisted of (i) a small change to the ϕ and ψ angles of the loop residues (up to five residues are varied simultaneously), (ii) a quick optimization of side-chain rotamers, and (iii) gradient-based optimization of backbone and side-chain torsion angles. After performing these three steps, the energy of the new structure is compared with the energy of the protein before the move, and the Metropolis criterion is used to decide whether the move should be accepted. Two types of moves were used to create the initial perturbation to the backbone: small random changes ($\approx 1^\circ$ perturbations) and shear moves. A shear move consists of a small change to a ϕ angle compensated by a change in the opposite direction to the ψ angle. Fast rotamer optimization was performed by cycling over each side chain once (in random order) and choosing the lowest-energy rotamer given the current environment. Gradient-based minimization was performed with a conjugate gradient protocol that calculates the first derivative of the energy function for each torsion angle that is being varied (21, 22). A score that favors a low rmsd between the first and last residue of the loop and the protein scaffold was used to keep the loop closed during backbone optimization.

De Novo Structure Prediction of Loop Sequences. The structures of the designed sequences were predicted by using a recently developed Monte Carlo-based loop modeling protocol in Rosetta (40). The sequence of the loop is used to pick overlapping three-residue fragments from the PDB with similar sequences. These fragments are then randomly combined to create a starting structure for optimization. The first round of optimization is performed with a low-resolution model of the protein that favors good backbone torsion angles and backbone hydrogen bonding. The second round is performed in high-resolution full atom mode and combines small and shear moves (see above) with gradient-based minimization and Dunbrack's cyclic coordinate descent algorithm for loop closure (33). Rotamer repacking is also performed after every 20 backbone trials. Thousands of Monte Carlo moves are considered. For each sequence, 10,000 independent trajectories were performed.

SASApack and SASAprob Scores. A score based on accessible surface areas (SASApack score) was used to evaluate the quality of packing of the design models. The SASA of each residue in the protein was calculated with two different probes (radii equal to 0.5 Å and 1.4 Å), and the difference was compared with the average difference seen in the PDB for a particular amino acid in a similar buried environment (31). A larger-than-average difference between SASA with a 0.5 Å probe and SASA with a 1.4 Å probe (SASApack score > 0 Å²) indicates poor packing and the presence of small voids that cannot be filled with water. By construction, 50% of proteins in the protein database have average per-residue SASApack scores < 0.0 Å². The SASAprob score is the probability of observing an amino acid in the PDB in a similar environment with a higher SASApack score. A SASAprob score of 0.95 indicates that a residue is more tightly packed than 95% of similar residues in the PDB.

Protein Expression and Purification. Genes for the redesigned proteins were constructed with cassette mutagenesis in the pET21b expression vector. The proteins were expressed in the *E. coli* BL21 strain at 37°C with 0.5 mM isopropyl β -D-thiogalactoside used for

induction. The proteins were purified with a Ni⁺ affinity column followed by size-exclusion chromatography (Superdex 75).

NMR. The redesigned proteins (≈ 1 mM) were equilibrated in a 20 mM sodium phosphate/0.5 M NaCl (pH 7.4) buffer, and one-dimensional ¹H NMR spectra were recorded at 25°C on a 700-MHz Varian (Palo Alto, CA) spectrometer.

Circular Dichroism. CD data were collected on a Jasco (Tokyo, Japan) J-810/815 CD spectrometer with a 1-mm path-length cuvette. Protein concentrations were 50 μ M in a solution of 10 mM potassium phosphate, pH 7.0. The CD signal was monitored at 200 nm as a function of temperature (10–90°C). The fraction of unfolded was calculated assuming that the CD signal of the unfolded and folded protein varies linearly with temperature.

Crystallization, X-Ray Diffraction, and Structure Determination. The hanging-drop vapor diffusion method was used for crystallization trials of the three designed proteins at room temperature. LoopA (≈ 20 mg/ml in 100 mM NaCl/20 mM Tris (pH 7.4) was mixed with an equal volume of well buffer of 2.0 M ammonium sulfate, pH 3.0/10% additive 0.1 M cupric chloride (Hampton Research, Aliso Viejo, CA) was added to the drop. Ethylene glycol (20%) was used

as the cryoprotectant. Crystals of LoopB (≈ 35 mg/ml in 100 mM NaCl/20 mM Tris, pH 7.4) were grown against a well buffer of 3.8 M sodium formate/5% glycerol, pH 7.5. Glycerol (20%) was used as the cryoprotectant. Diffraction data of LoopA were collected at the Advanced Photon Source at Argonne National Laboratory, Beamline 22-ID (Southeast Regional Collaborative Access Team). Diffraction data of LoopB were collected at the Beamline X29A at Brookhaven National Laboratory.

The data were indexed and processed with the program HKL2000 (41). The structures of both LoopA and LoopB were solved by molecular replacement, using the programs AmoRe (42) and Phaser (43, 44). WT tenascin (PDB code 1TEN) was used as the initial search model. The models were then refined against the synchrotron data to resolutions of 1.45 and 2.1 Å, respectively (SI Table 5). Alternating cycles of model building with the program O (45) and refinement with the programs CNS (46) and Refmac (47) were used to determine the final structure. The geometry of the final model was assessed with Procheck software (48).

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