Abstract

Proteins perform an amazingly diverse set of functions in all aspects of life. Critical to the function of many proteins are the highly specific three-dimensional structures they adopt. For this reason, there is strong interest in learning how to rationally design proteins that adopt user-defined structures. Over the last 25-years there has been significant progress in the field of computational protein design as rotamer-based sequence optimization protocols have enabled accurate design of protein tertiary and quaternary structure. In this award article I will summarize how the molecular modeling program Rosetta is used to design new protein structures and describe how we have taken advantage of this capability to create proteins that have important applications in research and medicine. I will highlight three protein design stories: the use of protein interface design to create therapeutic bispecific antibodies, the engineering of light-inducible proteins that can be used to recruit proteins to specific locations in the cell, and the de novo design of new protein structures from pieces of naturally occurring proteins.

Essential to the function of all proteins is their capacity to specifically bind other molecules. Regardless of whether the binding partner is a single atom or a chromosome, binding specificity is derived from the ability of proteins to fold into unique three-dimensional structures with well-defined binding pockets and surfaces. Because of the strong link between protein structure, binding, and function, there is longstanding interest in learning how to design proteins that adopt predetermined structures and complexes (1). In addition to providing an approach for creating molecules with important applications in medicine, protein design tests our understanding of the critical determinants of protein structure and function (2).

For the last 20 years my research has focused on the development and application of computational methods for protein design. This work began while I was a postdoctoral fellow in David Baker’s laboratory at the University of Washington and has continued in the context of the RosettaCommons consortium, a large set of research groups (now over 60 groups) that collaborate to develop and extend the capabilities of the molecular modeling program Rosetta. While Rosetta was first developed for predicting protein structure (3), it is now widely used for a variety of protein design problems including stabilizing naturally occurring proteins (4), the de novo design of new protein structures (2, 5), and the design of protein complexes (6).

Protein design with Rosetta.
All computational methods that have been developed for protein design include protocols for optimizing amino acid sequences for a specified protein structure (figure 1) (7). These protocols aim to identify sequences that form tightly packed hydrophobic cores, satisfy the hydrogen bond capacity of all backbone and side chain polar groups, and minimize torsional strain. The sequence optimization protocol in Rosetta contains two primary components: an energy function for ranking the fitness of alternative sequences for a particular protein structure and a search protocol to find lower energy sequences.

The energy function in Rosetta is a linear combination of a Lennard-Jones potential that models van der Waals forces and steric repulsion, an implicit solvation model that penalizes the burial of polar groups, an orientation-dependent hydrogen bonding term, a short range electrostatics potential and knowledge-based potentials for scoring side chain and backbone torsion energies (5, 8, 9). We use an implicit solvation model as it is computationally too expensive to model bulk water during a design simulation. Since first creating the energy function, we have placed a strong emphasis on parameterizing it to produce protein models that reproduce structural and sequence features observed in naturally occurring proteins (10). For instance, the hydrogen bond potential and electrostatics potential in Rosetta were parameterized simultaneously so that the combined model favors hydrogen bond distances and orientations that closely resemble those in high-resolution crystal structures (11). To explicitly tune the energy function for protein design we make frequent use of sequence recovery tests. In these benchmarks, Rosetta is used to redesign the sequences of a large set of naturally occurring proteins and complexes. Although it is not expected that the naturally occurring sequence for a protein will necessarily be the most stable, we have found that parameterizing the energy function to produce sequences that are similar to native sequences generates designs with high stabilities and good solubilities. Recently, Frank DiMaio and Hahnbeom Park have further improved the Rosetta energy function by parameterizing it on features from small molecules as well as macromolecules (9).

With an energy function in place it is possible to search for alternative sequences with lower energy. Because the size of sequence space even for a small protein is astronomical, the search protocol cannot explicitly enumerate all possible sequences and conformations of the amino acid side chains. Instead, researchers have developed a variety of deterministic (converges to the same answer each time) and stochastic (can give different answers each time it is run) algorithms for identifying low energy sequences (7). Deterministic methods like dead-end elimination are guaranteed to find the optimal solution if they converge but are computationally expensive and usually can only be applied to a region of a protein. Rosetta uses a stochastic approach based on Monte Carlo sampling to optimize the sequence of a protein (12). Single amino acid substitutions are automatically accepted if they lower the energy of the protein and accepted with some probability if the energy is raised. The probability for accepting an unfavorable change is governed by the Metropolis Criterion which lowers the probability of acceptance when the energy changes are large or the system is at a lower temperature. Simulations are started with a high temperature to allow energy barriers to be crossed and then the temperature is slowly cooled to let the system settle into an energy minimum. Despite the simplicity of this optimization scheme independent trajectories starting from different random sequences but with the same protein backbone converge on sequences that are very similar to each other, suggesting that the system is not being trapped in false minima far from the global minima.

Some advantages of the Monte Carlo-based sequence optimization scheme in Rosetta is that it can be applied to large systems (hundreds to thousands of residues) and that it runs quickly; a sequence can be designed for a 100-residue protein in a few minutes on a single computer processor. It is important that sequence optimization be rapid, as for many projects in protein design it is necessary to search through both backbone conformational space and sequence space to find low energy sequence/structure pairs (5). For instance, when
designing a protein de novo there is no guarantee that the initial model of the protein backbone will be designable, i.e. that there is any combination of the 20 naturally occurring amino acids that can stabilize the structure. We have found that it is usually necessary to make small adjustments to the protein backbone to find a structure where the side chains can pack tightly and buried polar groups can form hydrogen bonds. One of the strengths of Rosetta is that it has a variety of protocols for sampling alternative backbone conformations (13-15). This is a consequence of all the structure prediction capabilities that have been introduced into the software, and the collaborative nature of the team developing Rosetta.

With effective protocols for sequence optimization and backbone sampling it is possible to address a wide range of problems in protein design. The sequence optimization protocols in Rosetta can be used to closely pack protein cores (2, 16), create favorable interactions at a protein-protein interface (17) and form tight interactions with ligands (18). Here, to convey the broad range of problems that can be solved with computational protein design, I will summarize three recent design projects from our laboratory at the University of North Carolina.

Creating bispecific antibodies with interface design.

Bispecific antibodies are engineered antibodies that can bind to two antigens simultaneously. There is strong interest in bispecific antibodies because they can be used to gain higher specificity binding to particular cell types (i.e. cells that display both antigens recognized by the bispecific) and they can be used to co-localize different cell types (19). Bispecific antibodies that simultaneously bind receptors on the surface of T cells and receptors on the surface of tumor cells are currently used in the clinic to treat cancer (20). Because of their utility, a number of strategies have been developed for creating bispecific antibodies. Most approaches involve fusing fragments of one antibody with fragments from another antibody. This can be effective, but these antibodies typically loose some of the features that make antibodies effective therapeutics. The antibody fragments often have poor stability and short serum half-lives. To circumvent these problems, we collaborated with Steve Demarest’s group at Eli Elly to develop a method for creating bispecific antibodies that more closely resemble naturally occurring IgG antibodies (21-23). Given two monoclonal antibodies, call them A and B, we wanted to create antibodies where one arm of the antibody had the light chain of A paired with the heavy chain of A, and the second arm of the antibody had the light chain of B paired with the heavy chain of B. The challenge is that if one simultaneously expresses two light chains and two heavy chains in a single cell there is no strong reason why the light chain from the first antibody should not assemble with the heavy chain from the second antibody and vice versa. In fact, there are ten different ways that the four chains can assemble, with only one of them being the desired assembly (figure 2).

A solution to the assembly problem was proposed by a research team at Genentech over 20 years ago (24). They realized that by redesigning the interfaces between antibody chains that it would be possible to change how they assemble. In their case they focused on breaking symmetry between the two antibody heavy chains to allow the formation of heavy chain heterodimers (in naturally occurring antibodies the heavy chains homodimerize). This is a necessary step in creating bispecific IgG antibodies, but it still does not solve the pairing problem between the light and heavy chains. Inspired by Genentech’s approach, we used Rosetta to create altered specificity interfaces between antibody heavy and light chains so that we could direct the proper assembly of bispecific IgG antibodies.

In an IgG antibody the constant domain of the light chain (C\textsubscript{L}) makes a tight interface with the first constant domain (C\textsubscript{H1}) of the heavy chain. Our goal was to make mutations to the two domains, the C\textsubscript{L} and the C\textsubscript{H1}, so that the redesigned variants would interact with each other but would no longer interact with the wildtype domains. To identify such mutations, we used a protocol for multistate protein design that we have created in Rosetta (25). During multistate design, an amino acid sequence is optimized for more than one feature. In this case, we wanted to simultaneously favor the interaction
between the redesigned C_L and C_H1 domains, while disfavoring interactions between the redesigns and the wild type sequences. Designing against a particular outcome is often referred to as “negative design” in the protein engineering field.

Twenty redesigned C_L-C_H1 interfaces predicted to be orthogonal to the wild type interface were chosen for experimental characterization (23). Approximately half of the designs failed to express at levels similar to the wild type protein. Most of these failures were cases where a large number of mutations were made to the interface (>10 mutations). Of the designs that expressed well, two of them (CRD1 and CRD2) displayed strong specificity for the redesign over the wild type partners. Crystal structures of the designs showed that the Rosetta modeling correctly predicted how the redesigned side chains would interact. CRD1 relies on both redesigned hydrophobic packing as well as redesigned electrostatic interactions to create specificity, while CRD2 relies primarily on perturbed packing between hydrophobic amino acids.

Interestingly, creating an altered specificity interaction between the C_L and C_H1 domains was not fully sufficient for forcing the desired light chain/heavy chain pairing when assembling bispecific antibodies. Design simulations identified additional mutations in the framework regions of the variable domains (V_H and V_L) that further enhanced specificity and when combined with the mutations in the constant domains promoted the proper assembly of bispecific IgG antibodies. Particularly exciting was the finding that the mutations worked for a variety of antibody pairs that we tested and that the in vivo pharmacokinetic parameters and the thermodynamic stability of the bispecific IgGs were similar to their parental monoclonal antibodies. We have demonstrated that the bispecific IgG antibodies can be used to bind to two cell surface receptors simultaneously and that they can be used to redirect T cells to kill tumor cells by engineering one arm of the antibody to bind the CD3 receptor on the surface of T cells and the other arm to recognize the EGFR receptor which is overexpressed on some cancers. The designed mutations are now being used by Eli Lilly and the start-up company Dualogics to create novel bispecific antibodies with therapeutic potential.

**Designing light sensitive proteins for Optogenetics.**

Optogenetics is a field of research based on using light to control biological processes in living systems. The advantage of using light to control cell signaling is that it affords precise spatial and temporal control. With a laser it is possible to activate a protein within a specific region of a cell or within a specific set of cells in an organism, and with some light sensitive systems it is possible to rapidly and reversibly turn the system on and off by simply turning the laser on and off. Optogenetics first gained traction with the use of naturally occurring light-sensitive proteins such as channelrhodopsin (26), but recently scientists have also engineered a variety of proteins that allow control over biological processes (27).

In our laboratory we have focused on redesigning the light-sensitive LOV2 domain from the *Avena sativa* protein phototropin to be a general tool for controlling cell signaling pathways. The LOV2 domain binds a flavin mononucleotide cofactor that when activated with blue light forms a metastable covalent bond with a cysteine in the core of the protein. This new covalent bond leads to structural perturbations throughout the protein and destabilizes interactions with a long α-helix (Jα-helix) at the C-terminus of the protein (figure 3) (28). Once the interactions are disrupted the Jα-helix rapidly undocks from the protein and unfolds. We and others have shown that this undocking event can be used to control the accessibility of proteins that are fused near the end of the Jα-helix (29-31). In particular, partially embedding a peptide in the last few residues of the Jα-helix has proven to be a robust strategy for regulating the binding of the peptide to other proteins. For instance, by placing a nuclear localization signal in the end of the Jα-helix we created a switch that would only localize to the nucleus after activation with blue light (32-34).

One challenge we have faced with some peptides we have attempted to cage with the LOV2 domain is that even in the dark they have
exhibited appreciable affinity for their binding partner. This was true of our first attempt to control binding between the ssrA peptide and its binding partner SspB (29). Since the ssrA motif and the SspB protein are bacterial and do not interact with eukaryotic proteins we hypothesized that placing this interaction under the control of light would provide a powerful system for controlling protein-protein interactions in eukaryotic systems. By fusing the LOV2-ssrA construct to protein X and SspB to protein Y it should be possible to regulate the proximity of X and Y with light. However, a simple fusion of the ssrA peptide near the end of the Jα-helix only results in a two-fold change in affinity for SspB with light stimulation. The change was so small because in the dark LOV2-ssrA bound to SspB with an affinity that was only 2-fold less than affinity of the uncaged peptide.

We used Rosetta in a couple ways to improve the dynamic range of the LOV2-ssrA switch (35). In both cases, the goal was to increase how tightly the ssrA peptide was held against the LOV2 domain in the dark. First, we modeled extensions off the C-terminus of LOV2-ssrA to see if any residues added to the end of the peptide could help hold it against the LOV2 domain. We observed that adding a single phenylalanine to the C-terminus created favorable hydrophobic contacts with a small hydrophobic pocket on the surface of the LOV2 domain. This interaction was later confirmed with a crystal structure (figure 3) of the final engineered protein.

Our second approach to stabilize the dark state of LOV2-ssrA was to combine computational protein design with high-throughput selection experiments. We performed computational site saturation mutagenesis with Rosetta to identify point mutations likely to stabilize interactions between the core region of LOV2 domain and the Jα-helix. We then constructed a combinatorial library encompassing these mutations and used phage display to select library members that bind more tightly to SspB in the dark than in the light. The final selected sequence included several mutations on the surface of a β-sheet on the LOV2 domain that pack against the Jα-helix as well as mutations in a hinge-like loop that connects the Jα-helix to the rest of the domain. Our final designed sequence, named iLID for improved light-inducible dimer, shows a 58-fold change in affinity between the dark state (47 μM) and the lit state (800 nM).

In the last few years iLID has been used to control a diverse set of biological processes (36). It has been used to induce protrusions at specific regions on a cell surface by recruiting guanine exchange factors to that region of the cell (37). The Kiyomitsu laboratory has used the switch for spatial control of spindle-pulling forces in a dividing cell by inducing an interaction between NuMA and dynein (38), and the Brangwynne lab has used iLID to control localized liquid-liquid phase separation in living cells by recruiting intrinsically disordered macromolecules to each other (39).

### Requirement-driven protein design with SEWING.

The photoswitch and antibody projects that I have described are examples of template-based design, that is where the sequence and structure of a naturally evolved protein is modified to create a new function. Another type of protein design is de novo protein design (2). In de novo design, novel protein backbones and sequences are generated from scratch, guided by physiochemical constraints. Most projects in de novo protein design begin with defining the target fold/topology that one would like to create, and then molecular modeling is used to create a protein backbone (or an ensemble of backbones) that adopt that fold(5). This is followed by rotamer-based sequence optimization to find a sequence that will stabilize the desired structure. This process is excellent for testing our understanding of protein folding and stability, but if the end goal of a project is to create a protein with a particular function, there may be many alternative protein folds that can provide the desired functionality. For this reason, we have been working on an approach to de novo protein design that does not require the user to predefine the target protein fold. Instead, the design process begins with a set of requirements that one wishes to satisfy. These requirements can be quite general, for instance creating proteins that have a particular size groove on their surface, or they can
be more specific such as the inclusion of a particular functional motif. One problem that we are currently working on is the design of metal binding proteins, in this case the design requirement is that the protein include a metal-binding motif.

To enable requirement-driven design we have created a design approach called SEWING that allows for the rapid generation of alternative protein structures that satisfy the design requirements (40). SEWING works by piecing together portions of naturally occurring protein structures. To date, we have focused on using supersecondary structures – two secondary structures connected by a loop – as the building blocks for SEWING. We refer to these pieces of naturally occurring proteins as substructures. Starting from a single substructure, new proteins are assembled by sequentially adding substructures off the N- or C-terminus. Substructures are candidates for being pieced together if the C-terminal portion of one substructure structurally aligns with the N-terminal portion of another substructure (or vice versa), and if they do not clash when superimposed. A Monte Carlo assembly process is used to add and remove substructures from the design model, favoring models that optimize a low resolution score function that rewards placing $\alpha$-helices near each other at distances similar to those seen in naturally occurring proteins. The assembly process is rapid and tens of thousands of alternative backbone models can be created in a few hours on a single computer processor.

As a first experimental test for SEWING we aimed to create diverse helical bundle proteins with five or 6 helices. Eleven proteins were selected for experimental study. Four of the proteins were monomeric, helical as evidenced by circular dichroism, and unfolded cooperatively upon thermal denaturation. One of the designs was exceptionally stable and did not unfold even at 100°C. A crystal structure of the design showed sub-angstrom agreement with the design model. The protein incorporates several interesting features including a kinked helix and a small cavity in the protein core.

To perform requirement-driven protein design with SEWING we have added several features to the protocol (41). SEWING can now be used to build proteins around small functional motifs. To design metal binding proteins we are starting with a single helix that contains two amino acid side chains coordinating a metal, and then during backbone assembly with SEWING tertiary structures are searched for that bring in additional residues that can coordinate metal. Similarly, we are building proteins around peptide motifs that are known to interact with other proteins. The design process results in an expanded protein interface with the binding partner providing an opportunity to increase binding affinity and specificity.

Learning from past design successes and failures.

I have summarized results from three design projects that were a success and produced well-folded and functional proteins. However, in these projects many design sequences did not express or perform as intended and in some design projects we have pursued in the laboratory none of the designed proteins behaved as desired. From a single design project it is difficult to pinpoint the key factors that give rise to success or failure, but as our laboratory and others continue to work on a diversity of protein design problems it is possible to gain some insight into the existing challenges in the field (42). A few years ago our laboratory analyzed structural features in design models from several projects aimed at designing de novo protein-protein interactions (43). We observed that almost all of the design successes relied on protein-protein interfaces that were dominated by hydrophobic interactions. Attempts to design more polar interfaces (despite their prevalence in nature) generally resulted in proteins that did not interact. This observation spurred us and others to improve how we evaluate the energy of hydrogen bonding interactions and develop methods for effectively sampling buried hydrogen bond networks (11, 44, 45). The design of buried polar interactions continues to be a significant challenge for the field, but there have been some impressive successes designing coiled-coils with buried hydrogen bond networks (44).

One interesting result that has been obtained in many de novo and template-based design projects is the generation of proteins with
exceptionally high thermostabilities (5, 16, 40, 46). This indicates that naturally occurring proteins are generally not optimized for high stability. This could be for a variety of reasons. The function of many proteins requires them to adopt alternative conformations, overstabilization of one state could prevent switching to the alternative state. Once the thermostability of a protein reaches a certain level above the ambient temperature that an organism experiences, there may be little benefit or even a cost to raising stability further. For instance, placing hydrophobic amino acids at residues that are only partially buried often leads to a boost in protein stability (47), but is likely to have deleterious effects on the solubility of the protein.

**Conclusion**

It is an exciting time for the field of structure-based protein design as the focus begins to shift from developing design methods towards applying these methods to important problems in biology and medicine (48). For instance, I have described our use of the modeling program Rosetta to create bispecific antibodies with therapeutic potential and our engineering of light sensitive switches that control signaling pathways in living cells and animals. Our lab and others are exploring a variety of additional problems in protein design including the creation of more effective vaccines (49), the generation of self-assembling protein materials(6), and the design of protein inhibitors and activators for disease relevant signaling pathways (50). Future challenges for the field are numerous including the rational design of enzymes with activities comparable to naturally occurring enzymes, the *de novo* design of proteins that incorporate allostery as an additional layer of regulation and the design of functional loops such as those found in the antigen binding sites of antibodies.

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**Conflict of Interest**

Brian Kuhlman has financial interest in a company, Dualogics, that is commercializing the technology for making bispecific antibodies.
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Footnotes

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Figure 1. Computational Protein Design with Rosetta. Given a protein structure (for instance the one shown to the left), the sequence optimization protocol in Rosetta searches for amino acid side chains that will maximize the stability of the protein. Protein stability is evaluated using an energy function that (1) favors tight van der Waals contacts, (2) penalizes the burial of polar groups, (3) rewards hydrogen bonds with good geometries, (4) favors interactions between unlike charges, (5) and prefers side chain conformations (“rotamers”) that are frequently observed in naturally occurring proteins.
Figure 2. **Design of altered specificity protein-protein interfaces promotes assembly of bispecific antibodies.** (A) There are ten different ways that two antibody light chains and two antibody heavy chains can assemble if co-expressed in a single cell. Only one of these possibilities matches the desired bispecific IgG where one arm of the antibody binds to one antigen and the other arm binds to a different antigen. (B) To promote assembly of properly formed bispecific antibodies we used Rosetta to redesign interactions between the antibody constant domains (shown as bumps and holes in the figure) so that the heavy chains would heterodimerize and each light chain would partner with the correct heavy chain. (C) Structures of a redesigned interface between the antibody light and heavy chains. The left panel shows that the redesign (cyan) introduces a bump in the light chain with leucine 135 mutated to phenylalanine and that this is accommodated with a hole in the heavy chain by mutating phenylalanine 174 to a threonine. An additional valine to phenyalanine at position 190 in the heavy chain further stabilizes the interface by making pi stacking interaction with phenyalanine 135. The right panel shows a close match between the design model and a crystal structure of the redesign.
Figure 3. *Design of a light inducible protein dimer.* (A) The peptide, ssrA, was embedded in the Jα-helix of the LOV domain so that in the dark it is sterically blocked from binding SspB, but when the Jα-helix undocks from the LOV domain upon stimulation with blue light the ssrA peptide can bind SspB. (B) A crystal structure of the engineered switch (called iLID for improved Light Inducible Dimer) with the flavin cofactor shown in stick format. (C) The crystal structure of iLID shows that a phenylalanine introduced at the C-terminus of the ssrA peptide packs against the surface of the LOV domain and stabilizes the closed state, (D) and that redesigned residues in the hinge connecting the Jα-helix to the LOV domain also form stabilizing interactions. (E) The designed switch can be used along with a laser (illumination of the yellow box of the middle panel) to recruit protein to a specific region of a cell. In this experiment, iLID was fused to a protein that anchors it in the plasma membrane and SspB was fused to a red fluorescent protein. The three panels show before illumination (top), during illumination (middle) and after illumination (after). Recruitment of SspB to the illuminated spot is readily apparent.
Figure 4. *De novo protein design with SEWING.* (A) During SEWING pieces of naturally occurring proteins (shown in the circles) are sequentially chimerized to create a larger structure. The design CA01, was created from pieces of four proteins (1-4). The final assembly (green) is then input into the sequence optimization protocol of Rosetta to design a sequence for the protein. (B) Superposition of the design model and crystal structure of CA01. (C) Denaturation experiments followed by circular dichroism show that CA01 is exceptionally stable and only unfolds at high temperatures if chemical denaturant (guanidine hydrochloride) is also present. (D) We are now using SEWING to perform requirement-driven design. In this example, SEWING is being used to build a protein around a naturally occurring peptide (red) and form additional contacts with the binding partner (gray).
Designing protein structures and complexes with the molecular modeling program
Rosetta
Brian Kuhlman

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