Progress in Computational Protein Design

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Abstract

Current progress in computational structure-based protein design is reviewed in the areas of methodology and applications. Foundational advances include new potential functions, more efficient ways of computing energetics, flexible treatments of solvent, and useful energy function approximations, as well as ensemble-based approaches to scoring designs for inclusion of entropic effects, improvements to guaranteed and to stochastic search techniques, and development of methods to design combinatorial libraries for screening and selection. Applications include new approaches and successes in the design of specificity for protein folding, binding, and catalysis, in the redesign of proteins for enhanced binding affinity, and in the application of design technology to study and alter enzyme catalysis. Computational protein design continues to mature and its future is bright.

INTRODUCTION

Computational protein design is continually developing as a practical option for solving protein engineering problems. Much progress has been made from early proof-of-concept redesigns of protein cores and full proteins, with current research addressing a wide diversification of problems. Investigations pursue both scientific and engineering goals in tandem, using design to test and advance our understanding of underlying biophysical interactions.

Developments have been made toward both grand challenges, and toward more immediate practical applications. Grand challenges tend to be *de novo* design problems, such as the creation of novel protein folds, binding interfaces, or enzymatic activities. More immediate practical applications involve the redesign of existing proteins, for increased thermostability, altered binding specificity, improved binding affinity, enhanced enzymatic activity, or altered substrate specificity.

An increasingly common limitation in design is the choice of objective function. Few problems can be adequately addressed by the straightforward energy-minimization of a single protein state. Instead, multi-objective searches are ideal for designing specificity (to

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stabilize one or more states relative to others), improving binding affinity (to increase interaction while maintaining folding stability), and designing *de novo* proteins (to avoid alternate structures and aggregation). Furthermore, enzyme design may benefit from more detailed objectives than simply binding the transition state and coordination of key active-site functional groups.

In this review we address progress in structure-based computational protein design in the past two years (since 2005). Other recent reviews provide additional background and viewpoints [1–8]. Here we highlight progress in design methodology and in applications, and discuss some emerging themes.

PROGRESS IN METHODOLOGY

Energy functions

Protein design technology relies on pairing energy functions to evaluate candidates with search algorithms to examine large combinatorial collections of candidates. These interdependent foundational methodologies continue to be improved in ways that promise to increase the accuracy, efficiency, and scope of computational protein design applications. Work on energy functions includes understanding and validating their applicability to design studies, developing new potentials and target functions where appropriate, and improving efficiency through both better algorithms and approximations.

An energy function for nucleic acids and their interactions with proteins has been developed by Siggia, Baker, and their colleagues and validated for protein–DNA binding specificity [9]. Effects are attributed to direct readout through intermolecular electrostatics and packing terms and to indirect readout through intramolecular terms describing the DNA conformation. In other work, a classical mechanical description of interactions for dimanganese centers has been developed by Spiegel, DeGrado, and Klein (including implicit treatment of charge transfer and polarization effects) that accounts for the reduced and oxidized form [10]; successful enzyme design requires the surrounding protein to stably bind multiple states of the metal center, as a prerequisite for carrying out catalysis. This important step, as well as additional work for other metal centers, will be beneficial to metalloenzyme design.

Progress has been made in the inclusion of solvent and solvent-mediated effects into protein engineering computations. For compatibility with discrete search approaches, Mayo and colleagues have developed a pairwise approximation to continuum electrostatics and implemented it with the finite-difference Poisson–Boltzmann model [11]. The true problem is not pairwise because the desolvation of one side chain or the interaction of a pair of side chains depends on the shape of the protein and solvent regions, which is defined by the placement of all other side chains. The approximation involves a reduced representation of the protein structure built from the backbone and a single or a pair of side chains. There is a sense that continuum models appear to be an efficient approach for treating the important effects of the solvent environment, both directly in accounting for important desolvation effects, and indirectly through screening of charge, polar, and hydrogen bonding interactions. Wodak and colleagues have raised questions about the applicability of some implicit solvent models to design, where they found insufficient penalty for the burial of unsatisfied polar groups [12]. Clearly these questions need to be addressed in future studies.

The placement of individual water molecules, particularly bridging protein complexes, is important in natural and designed proteins. Baker and colleagues have introduced a new energetic description of water-mediated hydrogen bonds and combined it with a "solvated rotamer" approach to place interfacial water molecules using conventional rotamer search

techniques [13**]. In our own protein redesign work, we treat buried, crystallographic water molecules with rotameric conformational freedom and the option to be replaced by new side-chain growth (SM Lippow *et al.*, unpublished).

Significant computational expense is required to assemble individual and pair energy contributions for combinatorial search; efficiencies achieved in this area are valuable. A trie data structure was used by Leaver-Fay, Kuhlman, and Snoeyink to eliminate redundant atom–atom calculations in the assembly of pair energies, which led to a four-fold speedup in this portion of the calculation [14].

The approximation of physical potentials with cluster expansion techniques was undertaken by Keating and colleagues [15,16**]. Energies for a subset of the search space were computed and used to train a set of expansion coefficients, which expressed the design energy in terms of sequence as opposed to structure, effectively integrating over rotamers for each residue. The resulting reduced search was reasonably accurate and extremely fast.

Potential energy functions describe the underlying interactions in protein systems, but folding and binding free energies, as well as kinetic binding and catalytic rates, result from an analysis of ensembles and include energetic and entropic contributions. New studies have expanded design considerations from single structures to explicit consideration of ensembles and their associated entropy. Donald and colleagues have formulated the protein design problem using a target function constructed from conformational ensembles rather than a single conformation and validated its use in redesigning substrate specificity [17**]. Kuhlman and colleagues used a different procedure based on Monte Carlo (MC) search to include side-chain conformational entropy in the design of 110 native protein backbones [18**]. They found very little difference in the resulting sequence designs whether entropy was included or not, with the largest differences involving long, flexible side chains. Even if conformational entropy contributions are not dominant in protein design calculations, the use of ensembles is likely to have other benefits in protein design engineering.

Schreiber and colleagues have developed and validated an approach to design mutations leading to faster and tighter binding complexes through enhancement of the electrostatic contribution to the association rate [19 and references therein], using a computational treatment of the electrostatic interaction energy. Our group has developed an approach for identifying opportunities to introduce noncontacting residues near a binding interface that enhance affinity by virtue of paying very little desolvation penalty yet making larger "action-at-a-distance" intermolecular interactions [20]. Based on different mechanistic principles, there is some but not very much overlap between designs made by this and the Schreiber approach. What is interesting about both approaches is that they are computationally very rapid because they don't require full repacking calculations.

An important question remains concerning which properties need to be explicitly accounted for in design, and which others "come along for the ride". For example, focusing on protein stability appears to lead to designed proteins with appropriate kinetic pathways to the folded state with perhaps no need for explicit consideration of folding kinetics in the design. However, the same is not true for aggregation properties. Varani, Baker, and their colleagues redesigned, synthesized, and studied a variant of human U1A protein with 65 substitutions in 95 residues [21]. NMR showed that not only the backbone structure but also its dynamics were reproduced in the redesign. As the authors point out, the computation aims to reproduce the existence of a minimum in the free energy surface corresponding to the native backbone structure; in doing so, the shape of the surface appears also to have been reproduced. While more work needs to be done to assess the generality of this result, it

suggests a certain insensitivity of backbone dynamics to at least some details of side chain packing.

Search and optimization procedures

The tremendous advances in protein design studies over the past ten years result from the maturation of a number of component technologies, including combinatorial discrete search and optimization methodology. This was led by the adoption of guaranteed discrete approaches such as dead-end elimination, A*, and integer programming, as well as faster, non-guaranteed methods including MC and self-consistent mean field theory. New improvements to foundational search methodology continue to drive innovation.

Donald and colleagues have made progress bridging guarantees in discrete search space with the complexities introduced when energy minimization in continuous space is considered for all members of the discrete space [22**]. In other work, Xie and Sahinidis recast the hierarchy used in guaranteed discrete search by explicitly considering residue elimination in addition to rotamer elimination, which speeds calculations by one to two orders of magnitude [23]. Allen and Mayo report two enhancements to the stochastic optimizer FASTER that result in up to two orders of magnitude speedup [24**]. The work points out benefits of selection of appropriate initial configurations and positions for relaxation following a perturbation. Hom and May present a new MC and FASTER implementation for carrying out fixed composition sequence design, which may have benefits due to uncertainties in modeling the unfolded state [25].

Saven and colleagues present improved sampling techniques based on MC and biased MC with replica exchange for use in extracting residue-specific probability distributions for protein design [26]. The probability distribution formulation is particularly relevant for the design of protein libraries for analysis by selection or high-throughput screening approaches. Saven, Boder, and their colleagues have fully connected probabilistic design and library construction through the development of altered machine protocols for automated DNA sythesizers to produce a pool of DNA corresponding to the desired protein sequence distribution [27]. Maranas and colleagues present an iterative procedure to design combinatorial libraries using mixed-integer linear programming and demonstrate it to explore multiple mutations of a starting sequence to improve the properties of the resulting protein [28]. Taken together, new algorithmic approaches provide greater efficiencies for exploring larger spaces and phrasing different optimization problems.

PROGRESS IN APPLICATIONS

Specificity

The term specificity describes several protein phenomena: selective binding to certain ligands, enzymatic activity for particular substrates, and the overall protein fold that a particular sequence adopts. In some cases, the design challenge is in creating new recognition, but other times the difficulty is avoiding undesired recognition. Designing specificity often requires a combination of positive and negative design, but positive alone may be sufficient in some cases. Negative design poses a more difficult search problem, as one needs to directly address the structure prediction problem.

The design of specificity would be simplified if one only needed to consider positive design of a desired state, with specificity for that state a convenient by-product of optimization. Sauer and colleagues made a head-to-head comparison of a pure positive-design protocol and an explicit specificity approach for the redesign of a homodimer into a heterodimer [29**]. Their specificity design protocol yielded heterodimer specificity, but at the cost of protein stability. Baker and colleagues also explored two strategies for the design of a

specificity switch at a protein–protein interface [30**]. Their direct affinity design protocol led to the creation after two design rounds of a 300-fold specificity switch over one of the non-cognate interactions. Sampling known variation in rigid-body binding orientation likely contributed to the specificity, and despite the creation of a novel hydrogen-bond network, the majority of the specificity switch was due to a single hydrophobic mutation. In addition, explicit negative design was used by Baker and colleagues to successfully redesign the specificity of an endonuclease [31**], by Keating and colleagues to convert a homotetramer into a heterotetramer [32], and by Jasanoff, Tidor, and their colleagues to alter calmodulin specificity [33]. These data together support the need for elements of negative design for creating protein–protein interaction specificity.

De novo protein design often requires an amino-acid sequence to fold to a specific, single structure, yet protein conformational change often mediates function. Ambroggio and Kuhlman designed a single sequence to adopt two distinct folds, using direct stability design simultaneously for both states [34**]. The new protein switches from a zinc finger-like fold to a trimeric coiled-coil fold depending on pH or transition metals. The protein is aggregation prone from hydrophobic residues on the surface, reflecting the greater need for negative design to avoid undesired interactions.

Affinity

For the redesign of improved protein binding affinity, energy function accuracy is critical. Binding affinity is usually modified within a few orders of magnitude, making calculations of single kcal/mol changes important. Redesign from nanomolar to picomolar affinities is a particular challenge for a variety of maturation technologies.

Several groups have made progress toward protein–protein or protein–peptide binding affinity redesign. Springer, Baker, Desjarlais, and their colleagues redesigned the low-affinity ICAM-I/LFA-I interaction using a variety of structure-based techniques, achieving 20-fold improvement to 12 nM by combining designed single mutations [35]. However, predictions from visualization-based expert design included the majority of the affinity-enhancing mutations, and exhibited a higher success rate than the computational methods. Sood and Baker designed N- or C-terminal extensions to increase protein–peptide interaction using a novel technique that combines backbone and side-chain sequence and conformational search [36]. The results were modest, though, with 1.3- and 2.3-fold improvement in their two test cases. Van Vlijmen, Clark, and their colleagues achieved 8-fold improvement to 850 pM by combining four single mutations designed from a variety of available computational techniques [37]. Their success rate was 12% across 83 constructed mutants, but would have been 26% with a retroactive analysis. Dahiyat, Lazar, and their colleagues redesigned the Fc/FcγR interaction, yielding an Fc variant with over 100-fold improvement to 2 nM, after greater than 200 Fc variants were tested [38]. These results illustrate a need for reliable redesign methods, and indicate an absence of redesign to high-affinity picomolar interactions.

In our own lab, in collaboration with K. Dane Wittrup, we redesigned multiple antibody–antigen interactions (SM Lippow *et al.*, unpublished). Using novel selection criteria based on calculations of improved binding electrostatics, we achieved a success rate for single mutations of over 60%. We combined single or double mutations to improve the lysozyme-binding antibody D44.1 by 140-fold to 30 pM, and the anti-EGFR therapeutic antibody cetuximab (Erbitux) by 10-fold to 52 pM. Our methods also identified known affinity-enhancing mutations in the anti-fluorescein antibody 4-4-20 and the anti-VEGF therapeutic antibody bevacizumab (Avastin).

The design of novel protein–protein interactions or small-molecule binding sites presents additional challenges for conformational search. DeGrado and colleagues developed a procedure to create a new protein framework for binding a specified cofactor and designed a four-helix bundle that binds a metalloporphyrin cofactor [39**]. Yang, Hellinga, and their colleagues designed a calcium binding site into the cell adhesion protein CD2 using an approach that evaluates potential binding sites for compatibility with ideal geometry [40**].

Enzymes

It is not well understood how natural enzymes function, and thus the optimization objectives for computational enzyme design are unclear. Factors that may be important include binding to transition state, accommodation of substrate, release of product, protein flexibility and dynamics, and active site catalytic residues. Chakrabarti, Klibanov, and Friesner found that they could recover the majority of wild-type enzyme sequences by optimizing enzyme–substrate binding affinity while imposing geometric constraints on catalytic side-chain conformations [41**]; however, it is unclear if this is sufficient for design. Current work has enforced known key active site contacts [41**,42] or a known description for transition state and function group geometry [43,44].

Mayo and colleagues explored enzyme redesign by stabilizing transition-state binding and only mutating second-shell positions. For the redesign of *E. coli* chorismate mutase, one of five single mutations predicted to maintain activity increased efficiency by 60% [42]. Separately, redesign of the imipenemase IMP-1 predicted a mutation that removes a hydroxyl group, and a double mutation that transfers the hydroxyl group [45]. Hydroxyl transfer altered substrate specificity, whereas the presence of both hydroxyl groups turned out to increase catalytic efficiency. Methods remain to be developed for the computational improvement of catalytic activity.

For the *de novo* design of enzymes, conformational search of both active site residue placement and small molecule rigid body placement complicates calculations. Baker and colleagues developed methods for the placement of a predefined active site, and developed an *in silico* benchmark for 10 chemical reaction types [43]. Their procedure searches a protein for candidate active site locations, and then designs the surrounding protein for binding to the transition state. Mayo and colleagues developed methods for small-molecule placement in enzyme design [44]. They incorporated small-molecule rotational and translational search into protein design, and added energy biasing to favor side-chain–ligand contacts deemed necessary for catalysis or binding. Work by Chakrabarti, Klibanov, and Friesner may also be useful for guiding the search for protein scaffolds suitable for introduction of *de novo* activity [46]. Computational enzyme design remains a significant challenge, with a rare success reported by Hellinga and colleagues [47**].

Though computational design has been used to stabilize proteins, enzyme stabilization is complicated by the need to maintain catalytic activity. Stoddard, Baker, and their colleagues thermostabilized the enzyme yeast cytosine deaminase by 10 °C through combination of three synergistic mutations [48**]. They optimized for protein stability while fixing the active site and contacting side chains. It remains unclear to what degree distal residues play a role in catalysis; this work demonstrates that the enzyme core can be modified independent of the active site functionality.

DISCUSSION

Electrostatics

A common difficulty reported in computational design efforts is the accurate evaluation of electrostatic solvation and interaction terms. Electrostatics in protein design has been

previously reviewed [7], and here we highlight continued challenges as exemplified by recent design work.

Electrostatics has affected design methods in various ways. On one hand, designed structures have been subsequently discarded due to unsatisfied or sub-optimal hydrogen bonding for altered protein–protein specificity [30**] or protein–peptide binding [36]. On the other hand, protein–DNA designs have been selected based on the hydrogen-bonding energy contribution, which was "more highly predictive of the specificity of the native enzyme than the total energy of the complex", though the relative binding affinity prediction for the redesigned endonuclease was inaccurate [31**]. In addition, we found in our redesign of interactions that the electrostatic term of the binding energy was a better predictor than the total energy for affinity improvements (SM Lippow *et al.*, unpublished).

Results from computation design indicate additional progress needed in the treatment of electrostatics. In affinity redesign, successful mutations were "almost exclusively nonpolar or aromatic, suggesting that packing interactions are predicted more accurately than electrostatic or polar interactions" [35]. For wild-type side-chain placement, "structural accuracy is somewhat lower for polar and charged side chains compared with nonpolar side chains", though the authors attribute overall success in part to the use of a continuum electrostatic model [46]. In enzyme thermostabilization, "redesigns involving incorporation or alteration of polar or charged residues in the core … were less successful than mutations involving substitution of one hydrophobic side chain for another.… Furthermore, modeling of interactions involving buried polar and charged side chains in the enzyme core is an area for future development" [48**]. Additionally, authors have pointed out the challenge of predicting polar residues engaged in hydrogen bonds with ligand [41**], the implied need for an improved treatment of electrostatics [42], the need for an energy-biasing step to make up for many shortcomings including electrostatics and solvation modeling [44], and the challenge that protein design presents implicit solvation models [12].

Human intervention

Most design methods are not free of human intervention. The use of hand curation is common for selecting or refining designs, as opposed to a fully automated methodology. For instance, designs have been removed that had unsatisfied hydrogen bonding [30**], 15% of structures with best binding energy were discarded due to visual inspection of sub-optimal packing or hydrogen bonding [36], predictions were "inspected visually, and if we observed an improvement in packing, additional intermolecular hydrogen bonds, or an increase of intermolecular hydrophobic contacts, we decided to make and express the mutants" [37], "the designed Ca^{2+}-binding site in CD2 (Ca-CD2) was finally selected after careful evaluation" [40**], and N- and C-terminal helix-capping residues were added to a four-helix bundle design [39**]. Hand curation of designs can be critical for success, but limit the transferability of methods for use in new systems or by other researchers. Furthermore, these methods limit the ability to investigate and improve our understanding of the underlying biophysical interactions.

Bound water molecules

Structure determination of computationally-designed proteins has revealed deficiencies in the modeling of explicit, bound water molecules found at interfaces and binding sites. A water molecule was not predicted yet found crystallographically at a protein–protein interface [30**] and a protein–DNA interface [31**]. Redesign has failed to displace a bound water molecule [30**], and wild-type redesign has failed to recapitulate a water molecule [36]. In our own work, we designed high-affinity improvements including a double mutation predicted to displace a bound water molecule; a structure of our mutant

complex has yet to be determined (SM Lippow *et al.*, unpublished). We expect that improved handling of the conformational freedom of explicit, bound water molecules will continue to play an important role in design.

Independent designs & subsequent combination

Several successful design efforts have used an iterative procedure. In a first step, many small, independent designs are carried out, and predictions are experimentally validated. In a second step, successful mutations are combined for greater improvement. In redesigning binding affinity, Springer, Baker, Desjarlais, and their colleagues were unsuccessful in their simultaneously-designed mutations, yet successful in combining single mutations [35]. The same was true for enzyme thermostabilization [48**]. The combination of separate, smaller designs was also used by our own group (SM Lippow *et al.*, unpublished), and others [37,38,42]. This divide, conquer, and recombine technique is powerful in that it reduces combinatorial complexity and isolates potentially destabilizing or unbeneficial mutations; however, the capability of computational design to search vast sequence spaces is not taken advantage of fully.

OUTLOOK

Computational protein design is thriving, with more ambitious challenges being achieved together with the development of improved methodology that is on its way to becoming robust. Design using physics-based energy functions provides a more direct test of our understanding of biophysical interactions and might be applicable to a broader class of problems, yet knowledge-based functions are more commonly used. Future development of the field will be advanced more by an understanding of failures than successes, and the widespread adoption of fully automated design (removing human intervention) will lead to better estimation of the inherent robustness and transferability of design technology. Current approaches are efficient and enable many practical protein engineering applications already; future advances will expand the realm of possibilities and increase reliability.

Acknowledgments

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