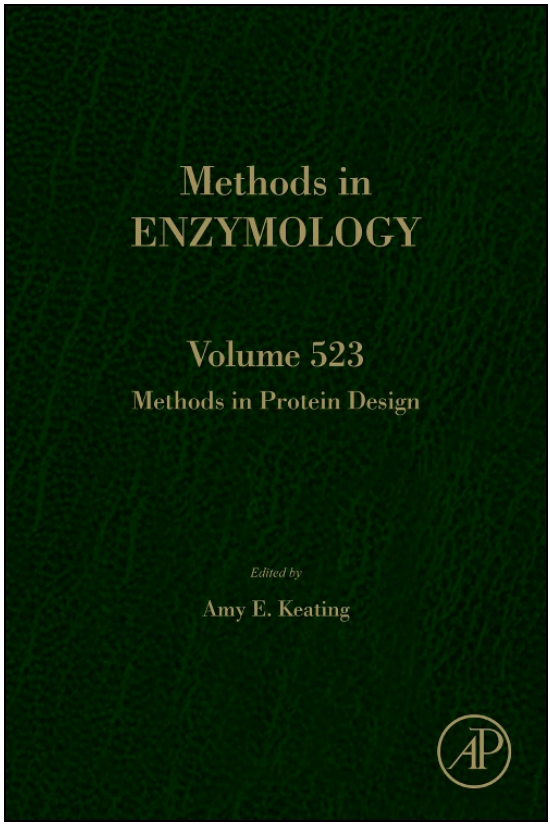

From: Timothy A. Whitehead, David Baker, Sarel J. Fleishman, Computational Design of Novel Protein Binders and Experimental Affinity Maturation.
In Amy E. Keating, editor: *Methods in Enzymology,* Vol. 523,
Burlington: Academic Press, 2013, pp. 1-19.
ISBN: 978-0-12-394292-0

CHAPTER ONE

Computational Design of Novel Protein Binders and Experimental Affinity Maturation

Timothy A. Whitehead[*,†,1], **David Baker**[‡], **Sarel J. Fleishman**[§,1]
[*]Department of Chemical Engineering and Materials Science, Michigan State University, East Lansing, Michigan, USA
[†]Department of Biosystems and Agricultural Engineering, Michigan State University, East Lansing, Michigan, USA
[‡]Department of Biochemistry, University of Washington and Howard Hughes Medical Institute, Seattle, Washington, USA
[§]Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel
[1]Corresponding authors: e-mail address: taw@msu.edu; sarel@weizmann.ac.il

Contents

Abstract

Computational design of novel protein binders has recently emerged as a useful technique to study biomolecular recognition and generate molecules for use in biotechnology, research, and biomedicine. Current limitations in computational design methodology have led to the adoption of high-throughput screening and affinity maturation techniques to diagnose modeling inaccuracies and generate high activity binders. Here, we scrutinize this combination of computational and experimental aspects and propose areas for future methodological improvements.

Methods in Enzymology, Volume 523
ISSN 0076-6879
http://dx.doi.org/10.1016/B978-0-12-394292-0.00001-1

1. INTRODUCTION

Molecular recognition underlies all of biological function including signaling, immune recognition, and catalysis. Molecular structures of thousands of naturally occurring protein interactions illuminate the physical basis for biomolecular recognition. These structures reveal very high shape complementarity between the interacting surfaces and energetically optimized interactions, including van der Waals, electrostatic, and hydrogen-bonding contacts. Computational modeling has been able to recapitulate some of these structural features to design novel protein–protein interactions (e.g., Huang, Love, & Mayo, 2007; Jha et al., 2010; Karanicolas et al., 2011; Liu et al., 2007), but until recently, the ability to design high-affinity and specific protein binders of naturally occurring biomolecules without recourse to existing cocrystal structures was not demonstrated, signifying gaps in our understanding of biomolecular recognition and frustrating attempts to program new molecular interactions that impact biological processes.

Recently, we described a new computational method for the design of protein binders, which focused on designing the surfaces of natural proteins of diverse folds to incorporate a region of high affinity for interaction with the target protein, and used this method to generate binders of the highly conserved stem region on influenza hemagglutinin (HA; Fleishman, Whitehead, Ekiert, et al., 2011; Fig. 1.1). The designs were found to interact specifically with the desired site, but initial binding affinities were low. We therefore combined computational design with *in vitro* affinity maturation to generate high-affinity binders of influenza HA that inhibited its cell-invasion function. The affinity maturation process also diagnosed inaccuracies in the energy function which underlies the computational design process, thereby suggesting a future route to improvements in our understanding of molecular recognition through the iterative application of *de novo* design and affinity maturation (Whitehead et al., 2012). Here, we provide an in-depth description of the computational and experimental techniques, focusing on what appear to us as the most fruitful ways to combine the computational and experimental aspects. We suggest areas where additional methodological developments are necessary for robust and reproducible design of protein binding to become routine.

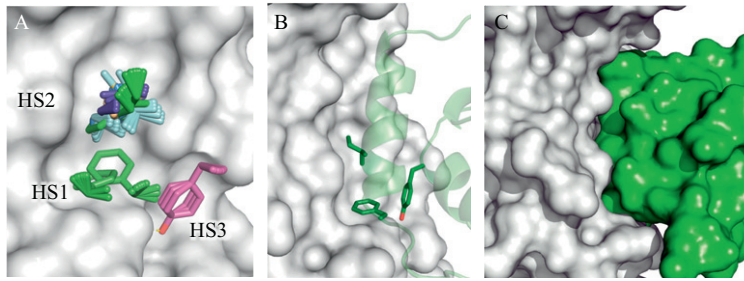

Figure 1.1 The computational design procedure realizes three features of natural protein–protein interactions: cores of high-affinity interactions with the target surface (A), favorable interactions among core residues (B), and high shape complementarity (C). (A) Three hotspot residue libraries (HS1, HS2, and HS3) were computed to form the idealized core of the interaction with the influenza hemagglutinin (HA) surface (gray). HS1 comprises two major configurations for a Phe aromatic ring and is supported by HS2, which contains the hydrophobic residues Phe, Leu, Ile, Met, and Val (green, purple, navy blue, cyan, and light brown, respectively), and HS3 comprises Tyr conformations. In the specific case of design of HA binders, the geometric constraint on HS2 is laxer than on the other hotspot positions and many different residues can be accommodated there. Residues from each hotspot-residue library interact favorably both with the target HA surface and the other hotspot-residue libraries, recapitulating two features common to many natural complexes: a core of highly optimized interactions with the target and internally stabilized contacts between key sidechains. (B) Cocrystal structure of HB80 and the HA surface. The hotspot residues are shown in dark green, realizing one of the energetically favorable combinations seen in panel a (comprising a Phe, Ile, and Tyr, for HS1, HS2, and HS3, respectively). (C) Common to many natural protein interactions, the surfaces of the designed and target proteins fit together snugly in a high shape complementary configuration. All molecular graphics were generated using PyMol (DeLano, 2002).

2. COMPUTATIONAL DESIGN OF BINDERS USING NOVEL SCAFFOLDS

Surveys of the molecular structures of protein–protein interactions have underscored the importance of high shape complementarity at the interface with many molecular structures showing interface-packing densities as high as those seen in protein cores (Lo Conte, Chothia, & Janin, 1999). Another feature of many protein–protein interfaces is energetically highly optimized interactions at the core of the interface, typically comprising long sidechains such as Tyr, Gln, and Leu. Such interaction hotspot regions contribute a large share of the binding energy (Bogan & Thorn,

1998; Clackson & Wells, 1995). In our preliminary design attempts (where the above features of optimized cores and high shape complementarity were the main selection criteria), we noted that the resulting designs still failed to embody another key feature of natural binders: when compared to natural binding surfaces, predicted hotspot residues on designed surfaces did not form appreciable stabilizing interactions with other structural elements in their host monomer (Fleishman, Khare, Koga, & Baker, 2011). The relative lack of stabilizing structural features in designed surfaces suggested that the designed surfaces were conformationally less rigid. We suggested that restricted sidechain plasticity was an important feature of binding surfaces, reducing entropy loss upon binding and precluding the reorganization of the binding surface into configurations that are incompatible with binding the target (Fleishman, Khare, Koga, et al., 2011). The organization of many natural hotspot regions into spatial clusters is potentially a negative design feature, disfavoring alternative conformations of the binding surface; due to spatial clustering, these alternative conformations are likely to introduce rotameric strain, voids, or clashes in the unbound protein. These three structural features—high shape complementarity, energetically optimized interactions between core residues on the binder with the target, and the clustering of these core residues—serve as the basis for the computational design method (Fleishman, Corn, Strauch, et al., 2011), and in the following, we discuss how each feature is realized in a computational design framework (Fig. 1.1).

The computational method was implemented as an extension of the Rosetta software suite for macromolecular modeling (Das & Baker, 2008). Rosetta provides implementations of many key functionalities in biomolecule structure prediction and design, providing a straightforward means to access sophisticated computational methods. Rosetta and the methods described here can be freely obtained by academic users through the RosettaCommons agreement.

3. TARGET SELECTION

In selecting a target for designing inhibitors, a number of structural and experimental considerations need to be taken into account. The combined computational–experimental approach that we describe requires that the protein that is targeted for binding is characterized with high-resolution molecular structures, is stable, and can be produced in good yield and purity for binding measurements. It is important to have a protein/small molecule

that binds at the target surface. Such preexisting binders provide valuable information on whether the target surface forms correctly in experiment, allowing confirmation that the designed binders target the intended site by serving as positive binding controls and as competitive inhibitors of the designed binders. Such preexisting binders might not be available for all desired applications. In such cases, amino acid substitutions at the target surface that disable binding to the designed proteins yet preserve the overall structure of the target can provide an alternative control for binding at the target site. Our computational strategy, which generates exposed hotspot sites of interaction, is best equipped to target concave protein surfaces. The conserved epitope of the soluble ectodomain of HA common to Group I influenza viruses exhibited all of these features: influenza HA is well characterized biochemically and structurally, it can be produced recombinantly in insect cells, and there exist multiple antibodies that bind at or near the epitope to serve as positive controls in experiments (Corti et al., 2011; Ekiert et al., 2009, 2011; Sui et al., 2009). It is also an important target for drug design, as binding in this region has been linked to preventing HA-mediated fusion of the viral and host endosomal membranes, thereby blocking viral entry into the cytoplasm of the host cell (Ekiert & Wilson, 2012).

4. GENERATING AN IDEALIZED CONCEPT OF THE HOTSPOT

A central element of the computational method is the construction of a spatial region in which high affinity, sidechain-mediated interactions are formed between the designed binder and the target protein; the designed sidechains should also be stabilized through intrachain interactions on the designed protein. As different surfaces on scaffold proteins for design present different ways in which to incorporate such key residues, we start the design process by precomputing a spatially clustered set of residue combinations (Fig. 1.1A). To date, we have generated binders with two to four hotspot positions, but the methods described below could be used to specify any number of hotspot positions. We previously provided examples based on natural protein–protein interactions for how to generate a hotspot conception when molecular structures of bound components are available (Fleishman, Corn, Strauch, et al., 2011). In the following, we describe in detail how to generate a hotspot region for a site that is known to serve in protein–protein interactions, but with minimal or no recourse to the natural binding mode; this approach was used to generate the hotspot region for

HA binding (Fleishman, Whitehead, Ekiert et al., 2011) and provides a way to generate protein binders with desired structural and biophysical properties without the limitations of naturally occurring binders (Fig. 1.1). To accomplish this, we docked aromatic residues against the hemagglutinin Trp21 on chain 2 of the HA (HA2) (HA residue numbering corresponds to the H3 subtype sequence-numbering convention) and isolated two major conformations of a Phe residue (other aromatic residue identities failed to produce energetically optimized configurations with respect to the target surface). For each Phe residue, we computed positions for backbone and Cβ atoms such that the aromatic ring moieties of the computed Phe residues intersected with the energetically optimized configurations computed in the previous step (inverse rotamers) (Fig. 1.1A); this step produces approximately a dozen different conformations for each of the two major configurations of the aromatic ring. All of these spatially clustered residues are saved in a hotspot-residue library for use in subsequent scaffold design steps. The HA target site is quite hydrophobic, and we extended this hotspot with hydrophobic residues (Phe, Leu, Ile, Met, Val) all of which formed favorable interactions both with the target HA surface and the previously computed hotspot Phe. A third Tyr hotspot position was extracted from antibody-bound structures of HA (Ekiert et al., 2009). This last hotspot residue was used in the design calculations that resulted in the binder codenamed HB80 (Fleishman, Whitehead, Ekiert, et al., 2011), but this design strategy, which included three hotspot residues, was found to be very restrictive (eliminating many potentially favorable designs), and the binder HB36 was computed without the Tyr hotspot residue. Once residue identities for the hotspot region were defined, the rigid-body conformations of these residues with respect to the target HA surface and to one another were computationally optimized by subjecting them to rigid-body docking and minimization simulations using Rosetta, and the lowest-energy conformations were isolated. Some of the hotspot-residue combinations, which appeared feasible and favorable at the initial hotspot construction phase, such as Val and Phe at HS2, failed to produce designed proteins with favorable energetic and structural characteristics, underscoring the importance of formulating a diverse hotspot concept. In summary, a hotspot region can be computed based on existing bound structures (Fleishman, Corn, Strauch, et al., 2011) or based solely on the molecular structure of the target protein (Fleishman, Whitehead, Ekiert et al., 2011). It is important to note that at this point of method development producing a hotspot concept requires human intervention in choice of target site and residue identities. Part of

the reason why this crucial step has not been automated stems from the fact that the energetics of residue binding to the target surface within the context of an entire protein are poorly modeled when these residues are dismembered from a protein as in the case of building a hotspot. We overcame this difficulty by testing diverse hotspot concepts as explained above. Recently, a method has succeeded in recapitulating natural hotspot residues and may serve in future design studies to automatically generate hotspot regions from scratch (Ben-Shimon & Eisenstein, 2010).

5. SELECTING SHAPE COMPLEMENTARY SCAFFOLD SURFACES FOR DESIGN

Shape complementarity is a key feature of biological protein–protein interactions (Lo Conte et al., 1999). Although certain protein families recur in biology as protein interaction modules (e.g., SH3, ankyrin, immunoglobulins; Pawson & Nash, 2003), we reasoned that using more scaffolds for design would increase the chances of designing proteins that exhibit high shape complementarity. We therefore used a set of more than 800 unique protein structures deposited in the Protein DataBank as scaffolds for design (Fleishman, Whitehead, Ekiert, et al., 2011). This library of protein structures was selected to improve the chances that the proteins would be easy to experimentally express and test; they contain no disulfide bridges, are relatively small (<250 amino acid residues), contain no small molecule ligands, and are predicted to be monomeric. This scaffold library could be periodically updated with newly deposited structures. Another potential extension may be to include structures of proteins containing disulfide bridges, as those may present stable scaffolds for design. A number of computational methods have been described that capitalize on the high shape complementarity of interacting molecular surfaces to predict the proteins' mode of interaction (e.g., Gabb, Jackson, & Sternberg, 1997; Katchalski-Katzir et al., 1992). In a step independent of the hotspot construction step above, we use an efficient docking method called PatchDock (Schneidman-Duhovny, Inbar, Nussinov, & Wolfson, 2005) to isolate hundreds of unique configurations for each scaffold protein that show high shape complementarity to the target. The PatchDock software is run externally to Rosetta as a precomputation step and the output files from PatchDock are read by the RosettaScripts (Fleishman, Leaver-Fay, Corn, et al., 2011) module of Rosetta to set up the configurations with which design simulations start.

6. INTERFACE DESIGN

At this point in the protocol, we have obtained hundreds of thousands of coarse-grained binding configurations of the scaffolds in the library docked against the target epitope. The next step is to computationally determine which scaffold surfaces can incorporate hotspot residues. We developed two approaches for placing the hotspot residues on the scaffold protein (Fleishman, Corn, Strauch, et al., 2011). The approaches either translate the scaffold protein such that residues in the vicinity of the precomputed hotspot residues align their backbone N—C and Cα–Cβ vectors perfectly with those of the hotspot residue (scaffold placement) or by starting from the PatchDock configuration and replacing a scaffold position of appropriate geometry with one of the hotspot residues in the library (hotspot placement). The scaffold placement approach reproduces with high fidelity the geometric relationships of the precomputed hotspot residue with the target surface, whereas the hotspot placement strategy allows for more slack in incorporating the hotspot residue. Choice of which algorithm to use in placement depends on the physicochemical nature of the hotspot positions: residues that form geometrically constrained interactions, such as hydrogen bonds should be positioned with scaffold placement, whereas sidechains that form hydrophobic interactions can be incorporated using hotspot placement. When applied sequentially, these two algorithms can be used to test the energetic compatibility of every combination of hotspot residues from all hotspot-residue libraries with the scaffold protein. This is an exhaustive approach, but one that is poorly scalable and we have found that for three or more hotspot positions a computationally less demanding strategy is needed. We developed an alternative simultaneous-placement procedure. This procedure focuses design calculations on a set of scaffold residues that provide the most optimal geometric compatibility with the hotspot-residue libraries and designs this set of residues simultaneously. Since this procedure does not iterate over each combination of hotspot residues defined in the hotspot-residue libraries, it is much more scalable and allows the design of, in principle, as many hotspot positions as needed. In practice, combinations of these three methods can be used for any design task. For instance, the accurate scaffold placement procedure would be applied to hotspot positions that are geometrically very constrained (e.g., HS3 in Fig. 1.1A), whereas all other hotspot positions would be designed with the simultaneous-placement strategy.

Every target surface has unique structural features that demand a tailored computational design protocol. We implemented all of the above algorithms

within the RosettaScripts framework allowing the protein designer to define specific constraints and filters in a versatile XML-style scripting language (Fleishman, Leaver-Fay, Corn, et al., 2011). For example, the HA target surface contains a recessed hydrophobic residue (Trp21) and an exposed backbone carbonyl. Following hotspot-residue placement, we found that many of the designs do not pack well against Trp21 or hydrogen bond with the backbone carbonyl. In the RosettaScripts framework, this problem was easily put right, without additional programming of the underlying C++ source code, by adding filters that prune modeling trajectories if these two constraints were not satisfied immediately following the hotspot placement steps. The RosettaScripts for running the HA design protocol and additional scripts for recapitulating a diverse set of natural complexes have been published providing computational design programs for a broad spectrum of molecular surfaces that could be used or modified to target other desired surfaces (Fleishman, Corn, Strauch, et al., 2011; Fleishman, Whitehead, Ekiert, et al., 2011).

Following the design of the core hotspot region, we use iterations of RosettaDesign and minimization (Kuhlman & Baker, 2000) to optimize the sequence of the scaffold protein for binding the target, while keeping the hotspot region fixed. The computational design strategy described here has been found to recapitulate natural binding interfaces with high sequence and conformational recovery rates (Fleishman, Corn, Strauch, et al., 2011). In the design of HA binders, we used small backbone motions to further optimize the binding interface, but in retrospect found that designed proteins that bound the target in experiment had very rigid backbones, where these motions had little effect (Fleishman, Whitehead, Ekiert, et al., 2011). In unpublished design work, we similarly found that all designs that bind their targets as intended contain a very high fraction of rigid secondary structural elements at the binding surface. The question of whether and what type of backbone motions are useful for interface design is the subject of ongoing research (Humphris & Kortemme, 2008; Zhang & Lai, 2012) and will impact our design capabilities as well as our understanding of the intimate connections between protein stability, conformational flexibility, and molecular function.

7. YEAST CELL-SURFACE DISPLAY AS A SCREENING METHOD FOR DESIGNED BINDERS

The advent of custom and affordable DNA synthesis allows the testing of scores of potential designs and greatly expands the diversity of designs that can be considered. This added capability necessitates a matching experimental method to screen for interactions. While every screening method to identify

putative binders has its advantages and pitfalls, the ideal features of a screening method would be the following: that the throughput of the experimental screening method be matched to that of the computational design process, that diverse proteins can be robustly expressed, that weak binders (dissociation constants in the micromolar range; designs with fast kinetic dissociation rates) be positively identified, and that false negatives and false positives be minimized. Screening methods previously used by design groups in published and unpublished work included pull-down approaches, ELISA with phage display (Gu et al., 1995), cell extracts, or purified proteins (Karanicolas et al., 2011), and purification followed by binding verification using surface plasmon resonance or fluorescence polarization. After critically evaluating these alternatives, we found yeast cell-surface display coupled to flow cytometry as best matched to our screening needs. Yeast cell-surface display is well documented methodologically (Chao et al., 2006). There is a demonstrated correspondence between dissociation constants (K_D) measured using yeast display and *in vitro* measurements on purified proteins up to $K_D = 100$ nM (Colby et al., 2004). It is possible to improve detection limits by increasing the effective affinity using avidity between the yeast surface and naturally oligomeric targets (like the trimeric influenza HA) or by creating oligomeric complexes of the target protein using a biotin-conjugated target protein bound to streptavidin (Chao et al., 2006). There are published protocols for screening as well as directed evolution approaches for affinity maturation. Finally, using the yeast display format described below, we have found that more than 80% of the designed proteins express robustly. In a side-by-side comparison, only 50% of these proteins could be solubly expressed in BL21 bacterial cells under standard expression protocols (Studier, 2005). Yeast display requires access to a flow cytometer, and affordable cytometers have been marketed in recent years, with many academic institutions having dedicated flow cytometry facilities. We recommend using flow cytometry for monitoring binding events. Although there is a literature on identifying weak interactions using yeast display coupled to magnetic bead screening (Ackerman et al., 2009), in our hands these systems were less robust for screening than flow cytometry. For reproducibility by other laboratories, we have made yeast display expression plasmids encoding 71 different designs available through AddGene (www.AddGene.com).

As *S. cerevisiae* surface-expressed proteins could be glycosylated by the cellular machinery, care must be taken to remove potential N-glycosylation consensus sequences near the designed surface. Unpaired surface exposed cysteines are also removed from the designs. We note that yeast are able

to express complicated multidomain and disulfide-linked proteins on their cell surface; thus the scaffold set used in the HA design effort could be expanded in future design efforts to include more diverse scaffolds.

One consideration of any screening method is the choice of a positive control to monitor binding events. Positive binding controls are important to ensure that the screen is run correctly; an ideal positive control would bind the targeted surface at the weak limits of detection. In our implementation, we chose the CR6261 antibody fragment (Fab) that was previously found to bind the targeted HA surface (Throsby et al., 2008). We created a CR6261 scFv using splice overlap extension PCR, displayed this construct on the cell surface, and verified binding against biotinylated HA. HA was biotinylated either through a genetically encoded Avi-tag or chemically using NHS-Ester chemistry (Pierce). We used alanine-scanning mutagenesis on multiple antibody residues directly contacting HA and combined several mutations to significantly reduce the affinity of the CR6261–HA interaction.

With this experimental setup in place, screening could be carried out at a high pace. Four days after DNA-encoding designs arrived, designs could be tested for binding. As the method is efficient, dozens of designs could be tested in a single afternoon by following the screening procedure in Chao et al. (2006). Once we isolated designs that bound the target, the yeast display system allowed rapid implementation of controls, including testing the nondesigned wild-type scaffold for binding and competitive binding experiments with the soluble CR6261 Fab. Combined, these controls were used to rapidly screen out those scaffolds that bound natively to the target and to cull designs that bound the target but not at the desired (and designed) location (Fig. 1.2).

8. AFFINITY MATURATION

Limitations in the energy function and design method resulted in designs that initially bound specifically but rather weakly to the target site. We wanted to identify amino acid substitutions that conferred tighter binding to the target. Identification of such substitutions is important for three reasons: by improving the affinity of our designs toward the target, we can improve biologically relevant function; consistency between the substitutions uncovered through affinity maturation and the designed model structures can affirm the designed binding mode, even in the absence of experimentally determined molecular structures of complexes, which are sometimes difficult to obtain; identifying substitutions that are clearly better

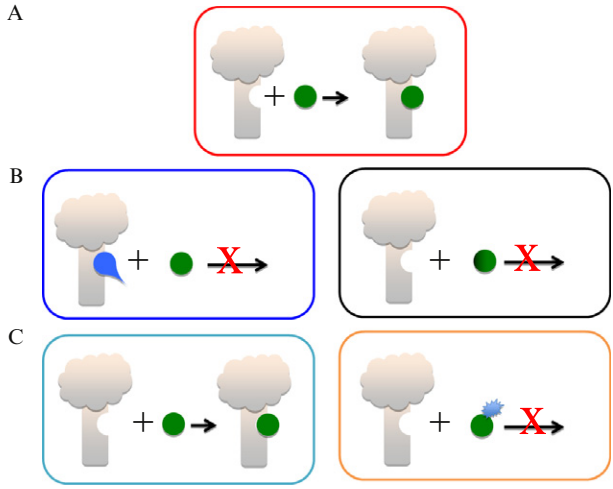

Figure 1.2 Yeast cell-surface display can be used to experimentally screen and test the precision of designed protein binders. (A) Screening for interactions is accomplished by incubation of a yeast-displayed designed protein (circle) with a purified, biotinylated target protein (gray stalk and head). After secondary labeling with a streptavidin-linked fluorophore, binding is measured by increased fluorescence of yeast cells as monitored by flow cytometry. (B) Precision of the designed binders can be tested using (left panel) competitive inhibition of the target surface with a small molecule/protein binder (teardrop) of the target surface and (right panel) coincubation of the target protein with a yeast-displayed scaffold from which the design was derived. The designed protein binds the targeted surface only if both experiments result in no increase in fluorescence. (C) Affinity maturation using yeast cell-surface display can further validate the accuracy of the design. Identification of mutations conferring affinity increases at the designed surface (left panel) suggests precision of the design if the improvements can be rationalized posteriori. Conversely, mutations conferring affinity increases distal to the designed surface (right panel) provide important clues that the protein is not binding the target as designed.

than the starting design helps, by retroactive rationalization, to improve the design process and identify inaccuracies in the energy function (Fig. 1.2).

We found yeast display coupled to flow cytometry to be well equipped for the affinity maturation process, as our binding screen could be readily reconfigured for cell sorting. In the affinity maturation protocol we implemented, single substitutions conferring large increases in affinity were isolated. Libraries encoding design variants could be generated either through site saturation mutagenesis (SSM) by the method of Kunkel (1985) or by error prone PCR (epPCR) (Genemorph II random mutagenesis kit, Stratagene) at low mutational loads (2–4 mutations/kb DNA),

followed by high efficiency transformation into *S. cerevisiae* EBY100 cells (Benatuil, Perez, Belk, & Hsieh, 2010). Constructs were combined with the yeast display backbone by homologous recombination, obviating a subcloning step. Retrospectively, the choice of mutagenesis procedure was not essential, as both SSM and epPCR approaches yielded the same consensus mutations. This may reflect the small size and high backbone rigidity of the designed scaffold proteins; with other scaffolds, the choice of library construction method may be significant. We recommend epPCR as it is considerably less laborious than SSM, and mutations are located throughout the protein sequence, not just at the designed epitope. This feature is particularly important for removing potential designs from consideration, as affinity-enhancing substitutions distal from the designed binding site provide clues that the design might not bind in the intended mode (Fig. 1.2). Care must be taken to sort the library under conditions where a single clone cannot dominate the library because in this case the mutation(s) responsible for increased affinity cannot be unequivocally determined. In our implementation, we used two (at most three) sorts of epPCR mutagenesis libraries at a sorting threshold of 5%. Sorting gates were set to collect cells with the tightest binding to the target protein. Following sorting, we ensured that the library was improved relative to the starting sequence by measuring dissociation constants using yeast cell-surface display against the soluble target protein. We then plated yeast cells and sequenced individual clones using yeast colony PCR to extract the DNA. Once affinity-enhancing substitutions were verified, they were directly incorporated into the original design framework, eliminating substitutions that might have arisen through genetic drift, for example, on protein surfaces away from the binding surface. The entire procedure of generating a library, selecting and identifying beneficial mutations, and testing them in a clean background took a little over 2 weeks.

9. WHAT WORKS, WHAT FAILS, AND WHAT IT MEANS

De novo design of protein binders is in its infancy. We have implemented a computational design strategy, which produced two different designed proteins that bound with atomic precision at the desired protein surface (Fleishman, Whitehead, Ekiert, et al., 2011). In unpublished work using the same methodology, we generated several binders of other protein targets, demonstrating the method's robustness and scope. By comparing the relatively small number of designed proteins which bind their targets with the much larger number of designs which fail to do so, we have learned important

lessons on biomolecular recognition (Fleishman, Whitehead, Strauch, et al., 2011). Similarly, by exhaustive characterization of working designs, we have been able to uncover weaknesses in the energy function used in design. We anticipate that the scrutiny of our design efforts by such computational and experimental methods will advance the ability to design new interactions in the future, and we have published the coordinates of all designed complexes that were experimentally tested (Fleishman, Whitehead, Ekiert, et al., 2011; Fleishman, Whitehead, Strauch, et al., 2011). In the following paragraphs, we describe two complementary approaches that we undertook in order to improve our understanding of molecular recognition and diagnose areas for future improvements (Fig. 1.3).

To get an unbiased view of structural features that distinguish computational designs from naturally occurring protein–protein interfaces, we compiled 88 designed proteins that expressed well and were tested for binding against three target proteins (HA, the human IgG1 Fc region, and an acyl carrier protein from *Mycobacterium tuberculosis*) (Fleishman, Whitehead, Strauch, et al., 2011). These designs did not bind their targets detectably. This set of protein structures was provided to 28 research groups that participated in the Critical Assessment of PRedicted Interactions experiment, and each group was asked to develop and disclose a computational metric for discriminating the nonbinding designs from naturally occurring interfaces. One of the surprising results came from an analysis by Haliloglu and coworkers revealing that many of the failed designs had binding surfaces that were predicted to be highly mobile and suggesting that these surfaces would not form as designed in experiment. This result underscores the importance of rigidity in functional surfaces: where such rigidity is not provided by the protein's secondary structure, the design effort was largely in vain. This insight has been translated to a simple computational filter, which tests whether the scaffold surface for design is embedded within the scaffold protein to stabilize it, demonstrating how *de novo* design, experimental characterization, and posteriori analysis can be used to diagnose and improve our understanding and ability to design binders. A recent cocrystal structure from another *de novo* protein binding study showed that plasticity either at the level of the sidechains or the backbone can lead to proteins which recognize their targets through quite different binding modes than those that were planned (Karanicolas et al., 2011) and might also have a role in the reduced effectiveness of *de novo* designed enzymes (Fleishman & Baker, 2012). Two directions for future research are consequently how to stabilize backbones that are poorly embedded in the scaffold protein, such as

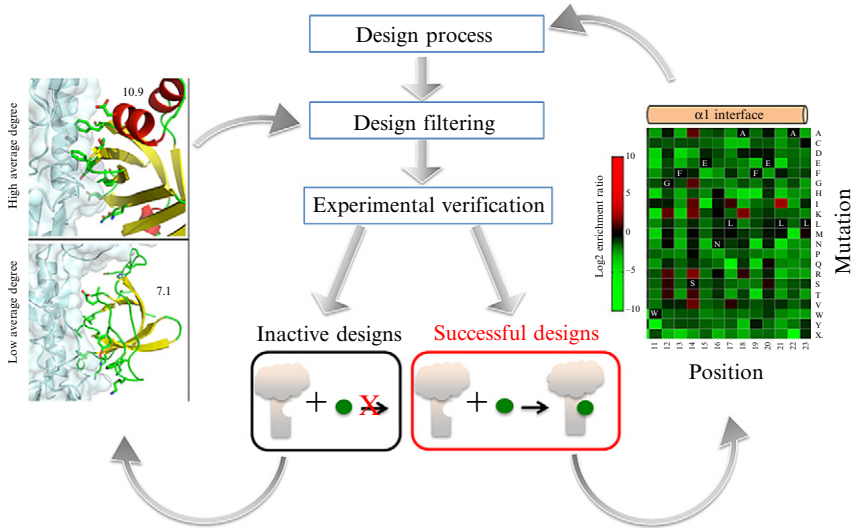

Figure 1.3 A schematic for how computation and experiment have been integrated to probe the physical basis for molecular recognition and to generate novel proteins of biomedical potential. Experimental screening of designs using yeast cell-surface display leads to their classification as active or inactive. Inactive designs can be compared to native protein interfaces (arrow pointing left): metrics that discriminate inactive designs from natural proteins can be used to formulate automated computational filters to prune unpromising designs in future design efforts and highlight areas for future methodological improvements in design. By contrast, successful designs (arrow pointing right) can be experimentally probed for detailed structure–function relationships using newly developed next generation sequencing technologies. Here, a plot showing enrichment ratio (a proxy for affinity) as a function of point mutation is shown for the interface stretch of one of the designs. This wealth of information can be used to identify limiting approximations in the energetic potential underlying the design calculations. By identifying mutations that clearly improve binding, this dataset can also be used to program high affinity and specificity binders from these initial designs that could subsequently be used as therapeutics. *The left-hand side of panel was reproduced with permission from Fleishman, Whitehead, Strauch, et al. (2011), and right-hand side with permission from Whitehead et al. (2012).*

unstructured regions, and how to predict the stability of designed surfaces. This is particularly relevant for efforts to design antibodies or antibody-like scaffolds for therapeutically relevant targets.

In a second approach, we sought to fully characterize the effects on binding affinity and specificity of substitutions on the designed binders. Using the affinity maturation approach mentioned above, we had identified a handful of substitutions that increased the affinity of our designs to HA (Fleishman,

Whitehead, Ekiert, et al., 2011). The substitutions that increased affinity delineated potential shortcomings in the energy model that undergirds the design calculations, yet the data were too sparse to use our findings to improve the computational design process. To understand more completely the shortcomings of our energy model, we extended a recently described approach for experimentally mapping the affinity contributions of residues at binding interfaces using high-throughput sequencing to encompass much larger sets of positions (Araya & Fowler, 2011; Fowler et al., 2010). Very briefly, we transformed SSM libraries encoding all possible single point mutants of our designs into yeast and used fluorescence-activated cell sorting to select variants that bound the target protein HA (Whitehead et al., 2012) We then used Illumina DNA sequencing to sequence the entire population of design variants before and after selection. In so doing, a comprehensive sequence–function map for nearly every possible single point substitution in HB36 and HB80 was generated. As these maps were generated using selections for binding of H1 and H5 HA subtypes and at differing selection stringencies, for both designs we were able to determine the sequence determinants for affinity and subtype specificity.

For both designs, the sequence–function map identified many affinity-enhancing substitutions, and computational modeling indicated that a large fraction of these improved the long-range electrostatic complementarity of the designed binders with the HA surface. Long-range electrostatics effects are notoriously difficult to model (Fleishman & Baker, 2012), and the unprecedented amount of experimental data generated by this experimental approach provided an opportunity to test a variety of different electrostatic models, ultimately leading us to develop a rapidly computable static Poisson–Boltzmann electrostatics model that can be used as a final step in the design process to ensure that the designed proteins exhibit high charge complementarity with the target molecular surface. These maps enabled us to improve dissociation constants of both designs against HA to picomolar levels through the combination of many of these beneficial substitutions. The best design variant neutralized two different H1 influenza strains at doses comparable to the CR6261 antibody and is currently being tested for influenza abatement in live animal models. Thus, the combination of *de novo* protein design with comprehensive sequence–function mapping by deep sequencing can be used to generate proteins of potential therapeutic relevance.

Computational design of interactions holds great promise for extending our understanding of biomolecular recognition and ability to design novel proteins with useful molecular functions (Fleishman & Baker, 2012).

Although the field is young, computational design has already generated proteins with antiviral potential. Pressing areas for future development include a more general method for modeling of a hotspot region, the ability to design conformationally stable loop regions in functional sites, and improvements in the accuracy of the energy function. These abilities will open the way to routine and robust generation of novel biomolecules for biomedicine, biotechnology, and research.

ACKNOWLEDGMENTS

The authors thank past and present members of the Baker lab for many contributions to the methods described here, and Eva-Maria Strauch, Jim Stapleton, and Ravit Netzer for comments. Research in the Whitehead lab is supported by the National Science Foundation. Research in the Baker lab is supported by the National Institutes of Health, the Defense Advanced Research Projects Agency, the Defense Threat Reduction Agency, and the Howard Hughes Medical Institute. Research in the Fleishman lab is supported by the Geffen Fund, the Yeda-Sela Center, a donation from Sam Switzer and Family, a Marie Curie Integration Grant, an Alon Fellowship, the Israel Science Foundation, and the Human Frontier Science Program. SJF is the incumbent of the Martha S Sagon Career Development Chair.

REFERENCES

Ackerman, M., Levary, D., Tobon, G., Hackel, B., Orcutt, K. D., & Wittrup, K. D. (2009). Highly avid magnetic bead capture: An efficient selection method for de novo protein engineering utilizing yeast surface display. *Biotechnology Progress*, *25*, 774–783.

Araya, C. L., & Fowler, D. M. (2011). Deep mutational scanning: Assessing protein function on a massive scale. *Trends in Biotechnology*, *29*, 435–442.

Benatuil, L., Perez, J. M., Belk, J., & Hsieh, C. M. (2010). An improved yeast transformation method for the generation of very large human antibody libraries. *Protein Engineering, Design and Selection*, *23*, 155–159.

Ben-Shimon, A., & Eisenstein, M. (2010). Computational mapping of anchoring spots on protein surfaces. *Journal of Molecular Biology*, *402*, 259–277.

Bogan, A. A., & Thorn, K. S. (1998). Anatomy of hot spots in protein interfaces. *Journal of Molecular Biology*, *280*, 1–9.

Chao, G., Lau, W. L., Hackel, B. J., Sazinsky, S. L., Lippow, S. M., & Wittrup, K. D. (2006). Isolating and engineering human antibodies using yeast surface display. *Nature Protocols*, *1*, 755–768.

Clackson, T., & Wells, J. A. (1995). A hot spot of binding energy in a hormone-receptor interface. *Science*, *267*, 383–386.

Colby, D. W., Kellogg, B. A., Graff, C. P., Yeung, Y. A., Swers, J. S., & Wittrup, K. D. (2004). Engineering antibody affinity by yeast surface display. *Methods in Enzymology*, *388*, 348–358.

Corti, D., Voss, J., Gamblin, S. J., Codoni, G., Macagno, A., Jarrossay, D., et al. (2011). A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*, *333*, 850–856.

Das, R., & Baker, D. (2008). Macromolecular modeling with rosetta. *Annual Review of Biochemistry*, *77*, 363–382.

DeLano, W. L. (2002). *The PyMol molecular graphics systems*. Palo Alto, CA: DeLano Scientific.

Ekiert, D. C., Bhabha, G., Elsliger, M. A., Friesen, R. H., Jongeneelen, M., Throsby, M., et al. (2009). Antibody recognition of a highly conserved influenza virus epitope. *Science*, *324*, 246–251.

Ekiert, D. C., Friesen, R. H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science*, *333*, 843–850.

Ekiert, D. C., & Wilson, I. A. (2012). Broadly neutralizing antibodies against influenza virus and prospects for universal therapies. *Current Opinion in Virology*, *2*, 134–141.

Fleishman, S. J., & Baker, D. (2012). Role of the biomolecular energy gap in protein design, structure, and evolution. *Cell*, *149*, 262–273.

Fleishman, S. J., Corn, J. E., Strauch, E. M., Whitehead, T. A., Karanicolas, J., & Baker, D. (2011). Hotspot-centric de novo design of protein binders. *Journal of Molecular Biology*, *413*, 1047–1062.

Fleishman, S. J., Khare, S. D., Koga, N., & Baker, D. (2011). Restricted sidechain plasticity in the structures of native proteins and complexes. *Protein Science*, *20*, 753–757.

Fleishman, S. J., Leaver-Fay, A., Corn, J. E., Strauch, E. M., Khare, S. D., Koga, N., et al. (2011). RosettaScripts: A scripting language interface to the Rosetta macromolecular modeling suite. *PLoS One*, *6*, e20161.

Fleishman, S. J., Whitehead, T. A., Ekiert, D. C., Dreyfus, C., Corn, J. E., Strauch, E.-M., et al. (2011). Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. *Science*, *332*, 816–821.

Fleishman, S. J., Whitehead, T. A., Strauch, E. M., Corn, J. E., Qin, S., Zhou, H. X., et al. (2011). Community-wide assessment of protein-interface modeling suggests improvements to design methodology. *Journal of Molecular Biology*, *414*, 289–302.

Fowler, D. M., Araya, C. L., Fleishman, S. J., Kellogg, E. H., Stephany, J. J., Baker, D., et al. (2010). High-resolution mapping of protein sequence-function relationships. *Nature Methods*, *7*, 741–746.

Gabb, H. A., Jackson, R. M., & Sternberg, M. J. (1997). Modelling protein docking using shape complementarity, electrostatics and biochemical information. *Journal of Molecular Biology*, *272*, 106–120.

Gu, H., Yi, Q., Bray, S. T., Riddle, D. S., Shiau, A. K., & Baker, D. (1995). A phage display system for studying the sequence determinants of protein folding. *Protein Science*, *4*, 1108–1117.

Huang, P. S., Love, J. J., & Mayo, S. L. (2007). A de novo designed protein protein interface. *Protein Science*, *16*, 2770–2774.

Humphris, E. L., & Kortemme, T. (2008). Prediction of protein-protein interface sequence diversity using flexible backbone computational protein design. *Structure*, *16*, 1777–1788.

Jha, R. K., Leaver-Fay, A., Yin, S., Wu, Y., Butterfoss, G. L., Szyperski, T., et al. (2010). Computational design of a PAK1 binding protein. *Journal of Molecular Biology*, *400*, 257–270.

Karanicolas, J., Corn, J. E., Chen, I., Joachimiak, L. A., Dym, O., Peck, S. H., et al. (2011). A de novo protein binding pair by computational design and directed evolution. *Molecular Cell*, *42*, 250–260.

Katchalski-Katzir, E., Shariv, I., Eisenstein, M., Friesem, A. A., Aflalo, C., & Vakser, I. A. (1992). Molecular surface recognition: Determination of geometric fit between proteins and their ligands by correlation techniques. *Proceedings of the National Academy of Sciences of the United States of America*, *89*, 2195–2199.

Kuhlman, B., & Baker, D. (2000). Native protein sequences are close to optimal for their structures. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 10383–10388.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proceedings of the National Academy of Sciences of the United States of America*, *82*, 488–492.

Liu, S., Zhu, X., Liang, H., Cao, A., Chang, Z., & Lai, L. (2007). Nonnatural protein-protein interaction-pair design by key residues grafting. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 5330–5335.

Lo Conte, L., Chothia, C., & Janin, J. (1999). The atomic structure of protein-protein recognition sites. *Journal of Molecular Biology*, *285*, 2177–2198.

Pawson, T., & Nash, P. (2003). Assembly of cell regulatory systems through protein interaction domains. *Science*, *300*, 445–452.

Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., & Wolfson, H. J. (2005). PatchDock and SymmDock: Servers for rigid and symmetric docking. *Nucleic Acids Research*, *33*, W363–W367.

Studier, F. W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expression and Purification*, *41*, 207–234.

Sui, J., Hwang, W. C., Perez, S., Wei, G., Aird, D., Chen, L. M., et al. (2009). Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nature Structural and Molecular Biology*, *16*, 265–273.

Throsby, M., van den Brink, E., Jongeneelen, M., Poon, L. L., Alard, P., Cornelissen, L., et al. (2008). Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS One*, *3*, e3942.

Whitehead, T. A., Chevalier, A., Song, Y., Dreyfus, C., Fleishman, S. J., De Mattos, C., et al. (2012). Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing. *Nature Biotechnology*, *30*, 543–548.

Zhang, C., & Lai, L. (2012). Automatch: Target-binding protein design and enzyme design by automatic pinpointing potential active sites in available protein scaffolds. *Proteins*, *80*, 1078–1094.