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Novel proteins: from fold to function

Betsy A Smith and Michael H Hecht

The field of *de novo* protein design, though only two decades old, has already reached the point where designing and selecting novel proteins that are functionally active has been achieved several times. Here we review recently reported *de novo* functional proteins that were developed using various approaches, including rational design, computational optimization, and selection from combinatorial libraries. The functions displayed by these proteins range from metal binding to enzymatic catalysis. Some were designed for specific applications in engineering and medicine, and others provide life-sustaining functions *in vivo*.

Address
Departments of Chemistry and Molecular Biology, Princeton University, Princeton, NJ 08544, United States

Corresponding author: Hecht, Michael H (hecht@princeton.edu)

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Introduction

The sequences and structures of natural proteins are the results of eons of evolutionary selection. Some features of these proteins are crucial for their functions, while others are merely 'evolutionary baggage' that came along for the ride. Designing proteins *de novo* provides an opportunity to separate the crucial from the coincidental. Design also allows scientists and engineers to explore beyond what has already appeared in nature, and to devise structures and functions that are possible, but have not yet been sampled by nature. In just over 20 years, since the first *de novo* designed proteins were reported [1,2], many different structures have been described [3]. Some are recapitulations of three-dimensional structures that occur frequently in nature, while others were designed to fold into topologies that had not been seen previously [4–6]. Although the design and optimization of stable structures continues as an active research area [7], the next step — incorporating functional activity into *de novo* proteins — is becoming a major focus of the field.

This review will focus on proteins that are not based on natural sequences. We emphasize recent achievements; readers are advised to consult other reviews for discussions of earlier work on the binding activities of *de novo* proteins and peptides [6,8,9].

Proteins designed to bind metals

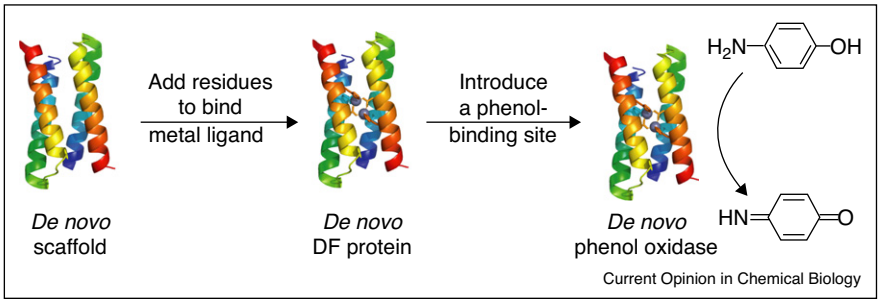
One of the simplest protein functions is binding, and the simplest ligand bound by native proteins is a metal ion. Indeed, nearly a third of natural proteins contain a metal-binding site [8]. Thus, it is not surprising that some of the first functional *de novo* proteins were designed to bind metals such as zinc or mercury [10,11]. One class of these metal-binding proteins was based on a helix-loop-helix dimer and known as the duo-ferri (DF) proteins because the earliest versions bound two irons [12]. Members of this family are water-soluble and can form complexes with several different metals [13]. Some possess ferroxidase activity [14], and a DF protein was shown to react with O_2 to form an oxidized biferric species [15]. A recent variant was designed with a binding site for phenol in close proximity to the iron-binding sites. This protein uses O_2 to oxidize Fe^{II} to Fe^{III}, and then oxidizes the bound phenol while reducing the iron back to Fe^{II}. Like natural enzymes, the *de novo* protein releases product and repeats the catalytic process (Figure 1) [16•].

DF proteins are not the only *de novo* proteins designed to bind metals. Another was designed based on a simple amphipathic repeating peptide that trimerizes into a three-helix bundle. Incorporation of one cysteine per helix led to the co-ordination of various metals, including Cd (II) in a rare trigonal geometry [17]. Recently, a four-helix bundle protein was designed to bind Fe_4S_4 in its hydrophobic core. This is particularly noteworthy given that natural Fe_4S_4-binding proteins are not α-helical and generally bind the ligand in flexible loops [18••].

Proteins designed to bind targets ranging from small cofactors to large receptors

Four-helix bundles are relatively easy to design, and numerous functions have been designed onto this structural scaffold. In most cases, the structure was designed first, and function was added in a subsequent stage. A function that has been explored extensively in four-helix bundles is the ability to bind heme and related porphyrins [19–21]. One *de novo* four-helix bundle protein was altered to bind heme simply by adding four histidine residues at appropriate positions [22]. A variant was further engineered by the addition of a lipophilic maquette to insert into lipid membranes, and the bound heme was shown to be active in redox coupled proton exchange across the membrane [23]. Another approach used a library of four-helix bundle proteins containing a

Figure 1



Steps in creating a *de novo* phenol oxidase. The DF protein was created by adding metal-binding side chains to a helix-loop-helix dimer. Residues capable of binding phenols were rationally designed into this protein to create the phenol-binding site. The final protein catalyzes the oxidation of amino phenol and is active through multiple rounds of catalysis. Structure drawn in pymol [50] from 1EC5 [12].

single histidine that bound heme and catalyzed its oxidative degradation [24].

Sequences need not be complex to bind heme. Starting with a four-helix bundle scaffold containing only three types of amino acids, heme-binding was designed by the addition of histidines [25••]. After further refinement to improve stability and structure, this protein was eventually developed into the first *de novo* heme protein that co-ordinates O_2 and maintains it in an oxy-ferrous state rather than immediately reducing it. This capability, similar to natural globins, was achieved by designing the structure to exclude water from the core. Further modifications will allow systematic variations to evaluate the factors that affect O_2 stability [25••].

Quinones have also been targeted for the design of novel binding proteins. In one example, a three-helix bundle was designed to bind 2,6-dimethylbenzoquinone (DMBQ) via a cysteine side chain. The properties of DMBQ bound to the protein were compared to its properties bound to a free cysteine. The pK_A of the quinone was similar when bound to the protein or the free cysteine, suggesting that this interaction alone dominates the pK_A. Conversely, the reduction potential was significantly different, implying that the rest of the protein plays a role in this case [26]. This example demonstrates how *de novo* proteins can be used to assess the minimal requirements for function in the absence of the evolutionary baggage that complicates the sequences and structures of naturally evolved proteins.

Targets for binding need not be limited to small molecules. For example, a four-helix bundle sequence initially designed using only seven different amino acids [27] was later redesigned by incorporating several key residues that favored interaction with the interleukin 4 receptor. This *de novo* mimic of IL-4 bound the receptor with an IC_{50} of 27 μM — lower than that measured for native IL-4 [28].

Beyond binding: novel proteins for catalytic and biological functions

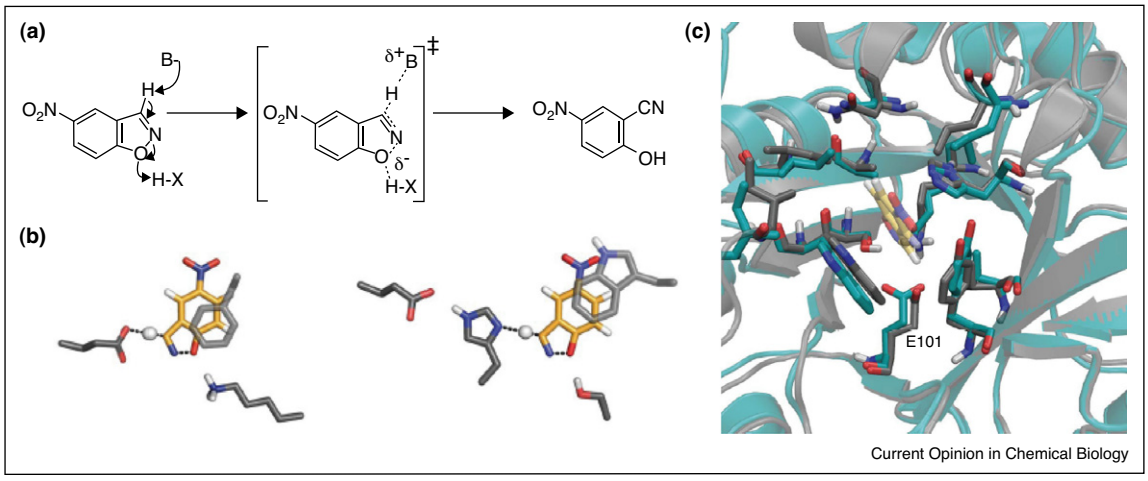
Proteins can be designed to mimic functions that occur in very specific tertiary structures. For example, a *de novo* protein designed to mimic the rubredoxin β-sheet structure was shown to bind iron and remain stable for 16 cycles of oxidation–reduction [29]. In another example, a library of proteins was designed to fold into the secondary and tertiary structure of the helical bundle protein chorismate mutase, using a limited library of possible amino acids. Using a selection in chorismate mutase-deficient *E. coli*, a number of proteins were found that were able to rescue the cells [30]. More recently, it was shown that the protein could be limited to nine amino acids while still rescuing the mutant cells [31].

Protein minimization can be accomplished by grafting the functional features that occur naturally on a large scaffold onto a small scaffold that was designed *de novo*. For example, Ghirlanda and coworkers grafted key residues from a large protein important in phagocytic macrophage activity onto a *de novo* three-helix bundle, producing a novel protein that showed native-like activity in macrophages [32]. As this protein has two threonines that can be glycosylated, it has also been used to investigate the energetic effects of glycosylation [33].

Not all biological functions involve ligand binding or enzymatic catalysis. Protein design has also been used to devise pores in lipid bilayers. For example, the novel protein, SGP, not only formed pores; it also showed antitumor properties in animal models [34]. *De novo* proteins are also finding use in fields outside of biology; designed helical dimers can be adsorbed onto gold surfaces, and the electronic properties of the gold are modulated depending on whether the dimers are parallel or antiparallel [35].

Design can also be used to evaluate different structural folds and to determine which are best suited for particular

Figure 2



Creating a *de novo* protein to perform the Kemp elimination. **(a)** Kemp elimination. **(b)** Two examples of active-site motifs that were used as input for modeling. **(c)** Comparison of computed structure (gray) bound to transition state (yellow) with solved crystal structure of a *de novo* enzyme in the unbound state (cyan). Figure adapted with permission from [38••], © 2008 Nature Publishing Group.

functions. For example, Baker and coworkers used computational methods to search over 10^{18} possibilities in 71 different scaffolds for a protein that would catalyze a retro-aldol reaction. They constructed and characterized the 72 most likely contenders, and of these, 32 had detectable activity [36•]. A similar approach was used to design proteins to facilitate the Kemp elimination, a reaction not catalyzed by any known enzyme. In this reaction, a base catalyzes the ring-opening of a benzisoxazole to produce an α-cyanophenol [37] (Figure 2a). To design a protein that catalyzed this reaction, computational design was first used to graft various basic residues onto a variety of protein scaffolds (Figure 2b and c). After computational optimization, 59 proteins were expressed and tested experimentally, and eight of these showed catalytic activity [38••]. Using *in vitro* evolution by random mutation and shuffling, an enzyme was eventually developed that had a rate enhancement 1.18×10^6 fold above the uncatalyzed reaction.

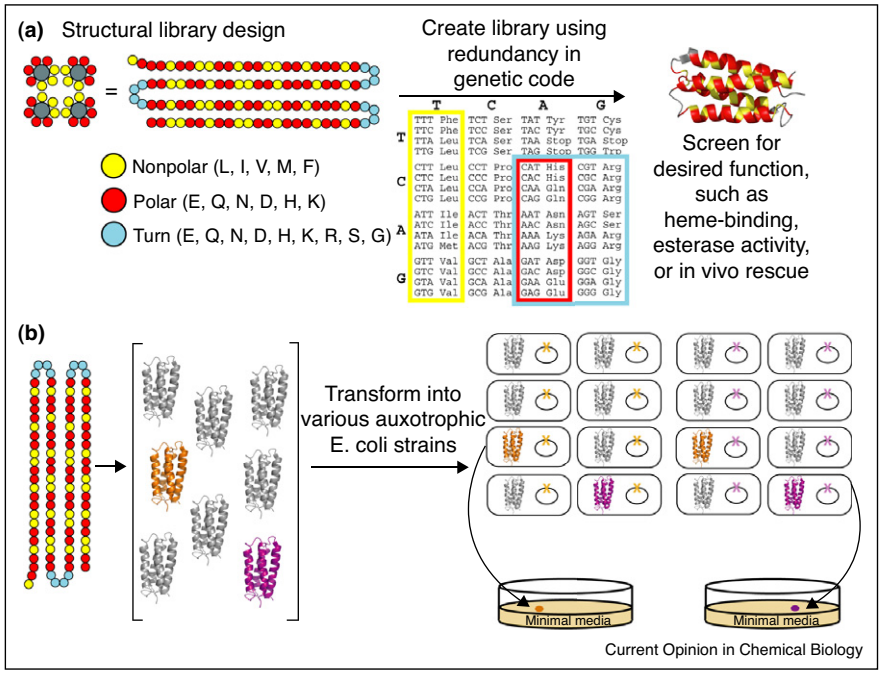
Functional proteins from combinatorial libraries of novel sequences

An alternative approach to residue-by-residue rational design is to construct large libraries of novel sequences and then screen for function. If the libraries are constructed randomly, then the vast majority of sequences will not be functional, and finding rare functional sequences will require screening through enormous libraries. Nonetheless, a pioneering study by Keefe and Szostak selected four ATP-binding proteins from a random library containing 6×10^{12} sequences 80 amino acids in length [39]. One of these proteins was subjected to directed evolution and characterized crystallographically. During this process, it was discovered that the protein not only bound ATP, but also hydrolyzed it to ADP [40].

Since well-defined protein functions typically depend on well-ordered structures, collections that favor folded structures are likely to contain a much higher fraction of functionally active proteins. One way to bias a library in favor of folded structures is to design a library of sequences to fold into one particular topology, such as a four-helix bundle, while still allowing combinatorial diversity in sequence, thereby facilitating the possibility of many different functions. This can be achieved by using a binary code for protein design, in which each position in the sequence is specified as polar or nonpolar, but the identity of each side chain is allowed to vary (Figure 3a) [41].

Our laboratory has used the binary code strategy to design several libraries, including both α-helical and β-sheet topologies. The α-helical collections have yielded functionally active protein with a surprisingly high frequency. For example, heme-binding was observed for approximately half of the members of a binary-patterned library of *de novo* four-helix bundles [42]. These heme-binding proteins were assayed for peroxidase activity. A large proportion showed activity, and one promoted rapid catalytic turnover [43]. When exposed to immobilized heme on a solid surface, this protein catalyzed peroxidase activity about half as well as horseradish peroxidase, a protein that evolved over millions of years to perform this reaction [44]. The proteins in our libraries of four-helix bundles were subsequently evaluated for activities that do not require cofactors, such as esterase and lipase functions. About 30% of the library showed esterase activity, and 20% lipase activity [45••]. Interestingly, a

Figure 3



(a) Creation of a *de novo* four-helix bundle library using binary patterning. **(b)** Screening the binary-patterned library for proteins that rescue auxotrophic *E. coli* strains on minimal media. Structures drawn in pymol [50] from 1P68 [51].

significant proportion was active in all three assays, indicating a high level of promiscuity. This is consistent with hypotheses about the early evolution of natural proteins, which suggest that 'primitive enzymes possessed a very broad specificity, permitting them to react with a wide range of related substrates.' [46] Such broad specificity would have facilitated life at the early stages of evolution because it would have 'maximized the catalytic versatility of an ancestral cell that functioned with limited enzyme resources.' [46]

Another approach to library design is to base the library on the sequence and structure of a natural scaffold and randomize selected parts to introduce side chains that might support catalytic activity. For example, a library based on a nonenzymatic zinc finger scaffold was designed by completely randomizing side chains in two loop regions. The collection of 4×10^{12} sequences was screened for ATP-binding ability [47]. The same library was also screened for RNA ligase activity. Sequences that catalyzed the reaction were subjected to mutagenesis and optimization. Ultimately a protein was obtained that increased the rate of ligation 2×10^6-fold and was active for multiple turnovers [48].

Novel proteins that function *in vivo*

Although the field of protein design has focused primarily on devising novel proteins that function *in vitro*, a long-term goal is to produce novel macromolecules that provide essential cellular functions in living systems. A major advantage of working with activity *in vivo* is that one does not have to rely on engineered screens. Instead, one can use more powerful life-or-death genetic selections. Our laboratory has used selections *in vivo* to probe a library of 1.5×10^6 novel four-helix bundles for proteins capable of rescuing strains of *E. coli* that were deleted for natural genes essential for growth on minimal media. Since the library was not designed for any specific function, a variety of auxotrophic strains were screened. Not surprisingly, most of the auxotrophic strains were not rescued. However, several deletions of conditionally essential genes were rescued by proteins from our library (Figure 3b). These include deletions of the following genes and proteins: SerB, which encodes phosphoserine phosphatase; GltA, which encodes citrate synthase; IlvA, which encodes threonine deaminase; and Fes, which encodes enterobactin esterase [49••]. Although the binary-patterned library was designed solely for folding into a particular structure, several proteins from this library can substitute for various different natural proteins, none of which have structures that resemble a four-helix bundle. These results demonstrate that novel proteins that are unrelated to natural sequences can provide functions that sustain the growth of living organisms.

Conclusion

De novo proteins offer promise in many areas of research, from basic biology to applications in engineering and

medicine. Design can be used to increase activity, enhance protein stability and shelf life, decrease protein size, and uncover information about the mechanisms of reactions. Moreover, compared to standard organic chemistry procedures, protein catalysts are environmentally more benign. Increased computational power and better modeling allow more of the work to be done rapidly before entering a laboratory, and enhance the likelihood that laboratory experiments will succeed. In just over two decades since the first *de novo* proteins were designed, the field has reached a stage where it is now possible to design structures never seen before in nature, to catalyze reactions for which no natural enzyme exists, and to isolate sequences that have no biological ancestors but nonetheless enable the growth of living cells.

Acknowledgement

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