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Engineering oxidoreductases: maquette proteins designed from scratch

Bruce R. Lichtenstein[*,1], **Tammer A. Farid**[*], **Goutham Kodali**[*], **Lee A. Solomon**[*], **J.L. Ross Anderson**[†], **Molly M. Sheehan**[*], **Nathan M. Ennist**[*], **Bryan A. Fry**[*], **Sarah E. Chobot**[‡], **Chris Bialas**[*], **Joshua A. Mancini**[*], **Craig T. Armstrong**[†], **Zhenyu Zhao**[*], **Tatiana V. Esipova**[*], **David Snell**[§], **Sergei A. Vinogradov**[*], **Bohdana M. Discher**[*], **Christopher C. Moser**[*], and **P. Leslie Dutton**[*]

[*]Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, 422 Curie Boulevard, Philadelphia, PA 19104, U.S.A.

[†]School of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

[‡]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, U.S.A.

[§]University of Chicago, 5747 S. Ellis Ave, Chicago, IL 60637, U.S.A.

Abstract

The study of natural enzymes is complicated by the fact that only the most recent evolutionary progression can be observed. In particular, natural oxidoreductases stand out as profoundly complex proteins in which the molecular roots of function, structure and biological integration are collectively intertwined and individually obscured. In the present paper, we describe our experimental approach that removes many of these often bewildering complexities to identify in simple terms the necessary and sufficient requirements for oxidoreductase function. Ours is a synthetic biology approach that focuses on from-scratch construction of protein maquettes designed principally to promote or suppress biologically relevant oxidations and reductions. The approach avoids mimicry and divorces the commonly made and almost certainly false ascription of atomistically detailed functionally unique roles to a particular protein primary sequence, to gain a new freedom to explore protein-based enzyme function. Maquette design and construction methods make use of iterative steps, retraceable when necessary, to successfully develop a protein family of sturdy and versatile single-chain three- and four-α-helical structural platforms readily expressible in bacteria. Internally, they prove malleable enough to incorporate in prescribed positions most natural redox cofactors and many more simplified synthetic analogues. External polarity, charge-patterning and chemical linkers direct maquettes to functional assembly in membranes, on nanostructured titania, and to organize on selected planar surfaces and materials. These protein maquettes engage in light harvesting and energy transfer, in photochemical charge separation and electron transfer, in stable dioxygen binding and in simple oxidative chemistry that is the basis of multi-electron oxidative and reductive catalysis.

Keywords

electron transfer; maquette; oxidoreductase; protein design; protein engineering; synthetic protein

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[1]To whom correspondence should be addressed (lichtenstein@gmail.com).

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Introduction

All too commonly, it is inferred that a specific biochemical function is necessarily linked with either a singular enzyme structure or a particular primary sequence. This ignores the apparent 'memory-less' random Markovian process of evolution that occurs down to the codon level [1]. An unknown and probably large variety of evolutionary pressures have contributed to the selection of the observed primary sequence and molecular structure, obfuscating and overlapping the multiplicity of biological roles that each amino acid plays [2,3]. These facts impair broad scientific goals directed at understanding and exploiting the fundamental principles and requirements for effective biological activity of natural enzymes. In some tantalizing cases, functionally identical enzymes from a variety of biological sources preserve 'active-site' residues; however, these residues may only afford the observed catalytic activity within their particular structural contexts. This makes it all but impossible to infer that those same residues placed appropriately will definitely yield the same function in some other protein structural family.

A practical understanding of the engineering of natural proteins relies on identifying the key structural and energetic terms naturally selected to carry out each specific enzymatic mechanism, as well as the quantification of the level of activity sufficient for adequate functional performance in an organism. In the absence of this information, it is understandable that researchers have assumed all things important and focused upon atomistic descriptions long thought to be essential for describing mechanistic detail. Unfortunately, leaps from this level of detail out to broader discussions of evolved activities take place without full knowledge of what, in Darwinian terms, natural selection acted upon, thereby limiting the scope of mechanistic conclusions.

Advances in rational protein design and engineering have allowed inferences gathered from bioinformatics studies of enzyme families to be tested directly [4–9]. This is particularly apparent in the growing literature of enzymes with substrate specificity rationally altered, usually by changing active-site loops [10,11]. Beyond substrate recognition and enzymatically competent binding, much recent progress has been made in wholescale modification or generation of catalytic activity in protein scaffolds [12–14]. The Baker group has led the way in producing enzymatic function built into natural scaffolds, including the creation of enzymes capable of catalysing reactions not known to occur naturally [15,16]. These experiments use a computational approach called Rosetta [17,18] and serve as a significant demonstration of our knowledge of both protein design and enzymatic mechanisms. Yet even with these remarkable successes, there remain open questions as to both the necessity of the computationally selected active-site residues and choice in protein scaffold, as well as the need for a computational approach at all in designing similar activity. The recapitulation of the novel Kempelimination enzymatic of a computationally redesigned protein [15] in a hand-redesigned variant of calmodulin [19] suggests that computational design is neither necessary for or uniquely capable of generating proteins with new non-natural activities.

Some of the major challenges in protein engineering are the creation of binding sites for small-molecule ligands and the tight stable binding of cofactors. Not surprisingly, there is less work on the rational design of novel oxidoreductase functions, which depend almost exclusively upon cofactor incorporation into protein scaffolds. Whereas some groups have succeeded in modifying natural oxidoreductases to accomplish additional desirable activities [20], insights into the fundamental requirements for the targeted function are obscured by the native biological function and role of the chosen scaffolds themselves. Our synthetic biology approach to creating and exploring oxidoreductase function divorces designed activities from naturally occurring scaffolds, and constructs protein maquettes from scratch.

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The maquette method

Defined as a non-complex functional protein scaffold, maquettes were born out of the need to cut through the frustrating complexity of natural oxidoreductases [21]. Study of natural oxidoreductases, especially those within the membranes of mitochondria and chloroplasts, is complicated by the many cryptic and overlapping spectral features of the various cofactors involved. Common biochemical techniques such as observing the effects of site-directed mutagenesis are blunted by this complexity and have left some of the salient features of their function undiscovered. Natural biological roles of these proteins add to their complexity, so studying oxidoreductase function specifically demands an approach that divests study from that biology.

Maquettes use established engineering parameters to generate simple non-natural four-α-helix bundles [22] (Figure 1), and build basic oxidoreductase activities into these scaffolds to allow us to understand and explore the engineering parameters of natural oxidoreductases [23]. The four-α-helix bundle was chosen as the scaffold form because its engineering principles are the most well defined of the self-contained tertiary structures. Functions were added by adjusting critical residues needed to bind to redox-active moieties. The combination of the established architecture and the relative ease at mutating these residues led to a rapid expansion in the nature of oxidoreductase function available for exploration in the maquettes. Indeed, iron and zinc tetrapyroles, iron–sulfur clusters, di-metal centres, flavins, quinones and nicotinamide have all been established in non-natural four-α-helix bundles ([24–31], and B.R. Lichtenstein and D. Snell, unpublished work) (Figure 2).

Although it is clear that cofactor binding of one form or another is a critical feature of the maquettes, our proteins are more than cofactor scaffolds and provide an excellent structural basis for more advanced oxidoreductase function such as charge separation and catalysis. Present maquette functions include redox-dependent conformational switching [32], molecular oxygen binding [33] and quinol–cytochrome *c* oxidoreductase activity [34]. Given the design versatility of the maquettes, we have come to view them as molecular laboratories and to establish each new design as one would an experimental set-up. We also believe that the approach is of a broader value to the scientific community and, in the present paper, seek to introduce our three-step process for maquette development.

Maquette step 1: choose function

As with other rational protein design approaches, an initial selection of function guides every design choice that follows. We define function as an activity, covering everything from adopting a desired secondary structure to cofactor binding to catalysis. Development of a maquette begins with a clear sense of a fundamental target function. These keystone functions will often serve as the basis for more advanced functions; for instance, oxidoreductase activity may require that a cofactor be bound, and this cofactor binding serves as a keystone function. Early maquettes focused almost exclusively on simple redox function imparted upon them by a bonded cofactor, yet additional functions such as native-like fold and molecular oxygen binding were also pursued. Each selected function serves as a constraint to design parameters that are guided by observations of Nature.

Maquette step 2: obtain engineering from observing Nature

The maquette scaffold, most commonly four-α-helix bundles, is the first engineering parameter considered. We have focused on incorporating oxidoreductase functions in four-α-helix bundles because this structure has well-established engineering requirements. Regan and DeGrado [22] demonstrated that peptides with polar and non-polar residues properly patterned around a heptad repeat would spontaneously assemble into soluble four-α-helical

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bundles. This so-called ‘binary patterning’ drives the bundle assemblies to form due to partitioning of hydrophobic residues into the protein core and polar residues on to the surface.

By starting with a well-defined scaffold with known engineering requirements, we are constrained in our placement of residues for keystone function and therefore guided in the selection of design criteria for additional functions. This constraint greatly simplifies matters and opens the way to a manual design process. In particular, residues essential for keystone functions are the first to be placed in the starting bundle sequence. Importantly to our work, cofactor number, placement, orientation, identity and anticipated redox potential are the most common design parameters that arise from chosen functions. The basic ligation residues and cofactor choices are usually the only design aspects taken directly from Nature and consist of a minimal part of the maquette sequence, as little as one residue.

The number and placement of cofactors is often guided by additional parameters obtained from our work defining empirical expressions describing electron transfer kinetics [35] (Figure 3). These expressions, derived from a semi-classical theory of electron transfer and parameterized by observation of events in natural oxidoreductases, serves to constrain the number and placement of cofactors and thus the residues necessary for their incorporation. The heptad repeating unit of the helix quantizes this selection parameter further, as not all cofactor distances are available in a four-α-helix bundle.

Subsequent sequence changes come about from additional constrains brought about by the choice of function. For instance, the iterative development of an oxygen-binding maquette involved the enhancement of the hydrophobicity of a haem-binding site followed by the introduction of buried charged residues to induce strain on the bis-histidine haem ligation [33,36]. Parameters such as these also arise from understanding the physical needs for function that are demonstrated in natural proteins. More than just mimicry, these considerations define a real set of engineering principles that can further guide design criteria for maquettes.

Before sequences are synthesized or expressed, some amount of manual sequence pruning is attempted to reduce steric clashes, poor geometries and incompatible charge–charge interactions; these steps are often aided by simple molecular modelling to assist in shape matching. Because the maquettes are not constructed from natural scaffolds, after the sequences have been finalized, we have the ability to reconcile the rationale for the selection of each amino acid throughout the design [21]. This greatly enhances our ability to properly assess and quantitatively ascribe to every amino acid its role in each observed maquette function, an understanding out of reach in complex natural proteins.

Maquette step 3: iterate

Unlike most other rational protein design methods, our approach makes explicit use of experimental characterization of each newly created maquette in the design process. Quantitative and qualitative details gathered from the results of experiments are used to guide further design choices meant to either establish the desired activity or test the necessity of our primary sequence selections. This repeated process, termed iterative protein design, has been used to introduce singular structure in either the apo [37] or holo [38] states of the maquettes from molten globular starting sequences and has been instrumental for rapid diversificiation of oxidoreductase activities accessible in the bundles.

The iterative process occurs at two levels. Point mutations, like those used to study natural proteins, are introduced to vary the observed properties of the maquettes, for instance, to convert a bis-histidine metal-porphyrin-binding site into a specific zinc-porphyrin-binding

site requires the mutation of a single histidine residue in our most recent maquette variants. A set of these iterations can result in profoundly different oxidoreductase activities, but does not result in a large-scale topological or sequence change (Figure 4, HT1 to HT3). At a higher level, what we term hyper-iteration results in generational changes, where fundamental properties of a prior maquette scaffold are maintained, but protein structural topology or sequence varies significantly (Figure 4, HT1 to HD3 to HM1). The distinction is made because, in all cases, hyper-iterative changes have granted us access to more advanced function often with greater control, which is not possible without a large number of point mutation iterative steps. Unlike evolutionary changes from natural and artificial selection, the iterative design processes are reversible, allowing us to counter the appearance of undesirable activities by continuing our design efforts from an 'earlier' iteration.

Progress

The course from the initial synthetic four-α-helix bundle maquette sequence, a homotetrameric protein, to our current work with expressed single chain monomers is an unbroken series of designed proteins (Figure 4). Representatives of almost all of the redox cofactors found in the oxidoreductases of respiration have been incorporated into this large family of maquettes, although, to date, only ferredoxin-like 4Fe–4S and not Rieske-like 2Fe–2S clusters have been incorporated.

Beyond simply binding cofactors and adopting native structures, advanced oxidoreductase functions have been developed in the maquettes through the iterative design process, including the aforementioned molecular oxygen binding, but also photoreduction and charge separation ([29], and G. Kodali and T.A. Farid, unpublished work). By using engineering principles gleaned from earlier soluble maquette work, we created amphiphilic proteins, which maintained many of the same functions [39]. These amphiphilic maquettes share very little primary sequence identity with the soluble ones and yet demonstrate complex oxidoreductase activities such as molecular oxygen binding and fully realized quinol–cytochrome *c* oxidoreductase activity reminiscent of the natural activity of the respiratory complex III [34]. Thus progress and understanding of oxidoreductase engineering parameters is not limited to use within only the continuous soluble maquette protein family tree. Indeed, recent efforts in our laboratory have been successful in incorporating haem-binding activity in new maquettes with no intentional sequence homology with previous maquettes or natural proteins for novel catalytic functions. Our ready ability to adjust the exterior residues of our maquettes without sabotaging the interior functions allows us to secure maquettes to each other [40] or to surfaces with electrode properties such as titania [41] and gold [42,43].

Conclusions

What is remarkable about the design process described in the preceding sections is that, despite a great deal of variation in the activities of the maquettes, the sequences of the α-helices have not varied extensively over iterations. This reveals that access to diverse oxidoreductase function in non-natural proteins is readily available using well-established engineering principles gained from observing Nature. Our approach differs from other rational design efforts in the use of non-natural protein scaffolds and the fundamentally iterative nature of our progression. By eliminating the biological complexity imperative with the use of natural protein scaffolds and selecting residue identity with well-established rationales, we are able to explore the fundamental engineering requirements of oxidoreductase activity in simple protein systems. Our continuing efforts are built around the idea that established sequences, which give desired keystone functions, can be combined to fully realize the complex oxidoreductase activities observed in Nature.

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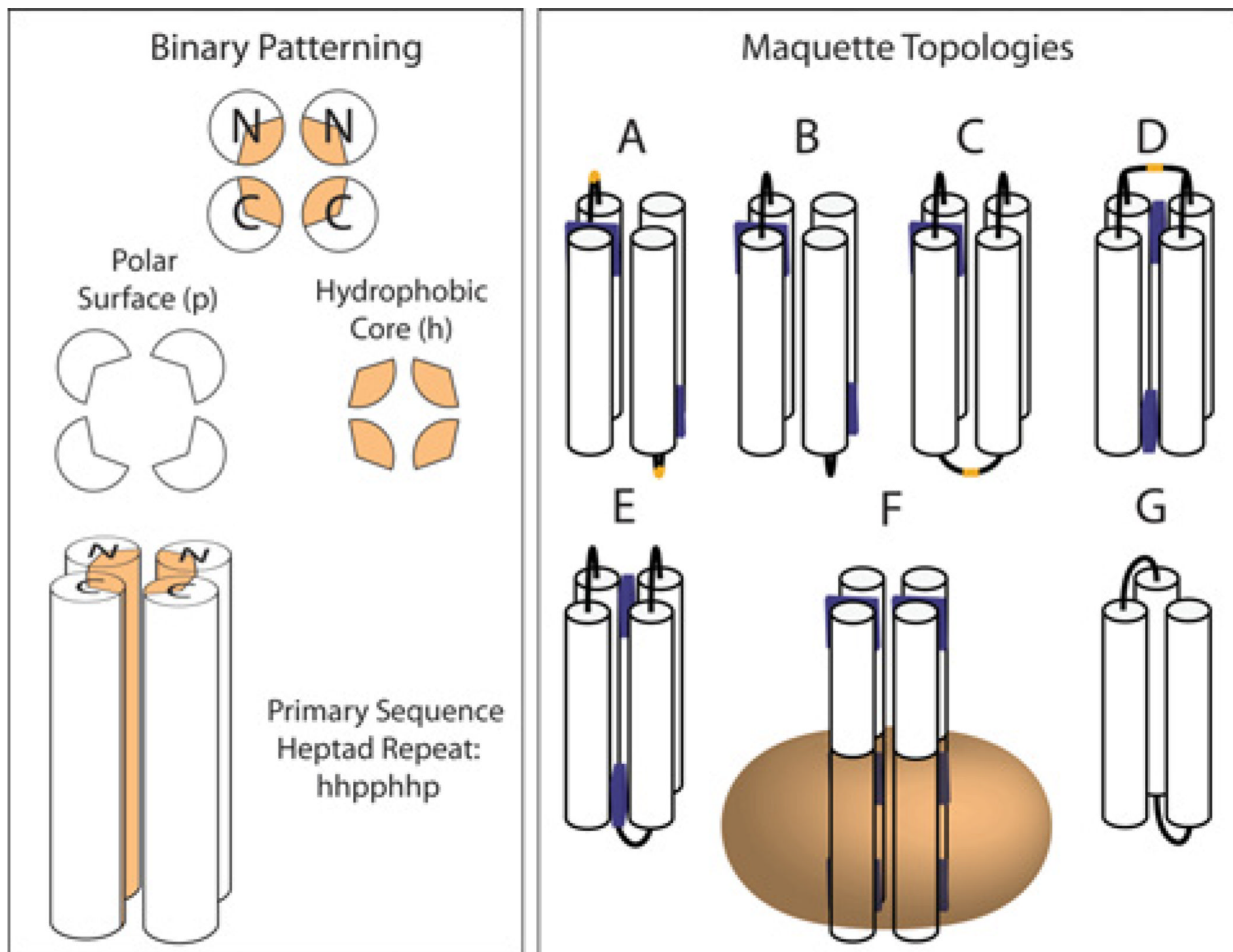

Figure 1. Maquette design, based on α-helical bundles, follows the principle of binary patterning
The specific topological assembly of the α-helices is controlled principally by connecting loops. Topological labels refer to sequences in Figure 4, and include both hydrophilic (A–E and G) and amphiphilic (F) maquettes. Cofactors such as tetrapyrroles (blue) are placed at selected positions along the bundle.

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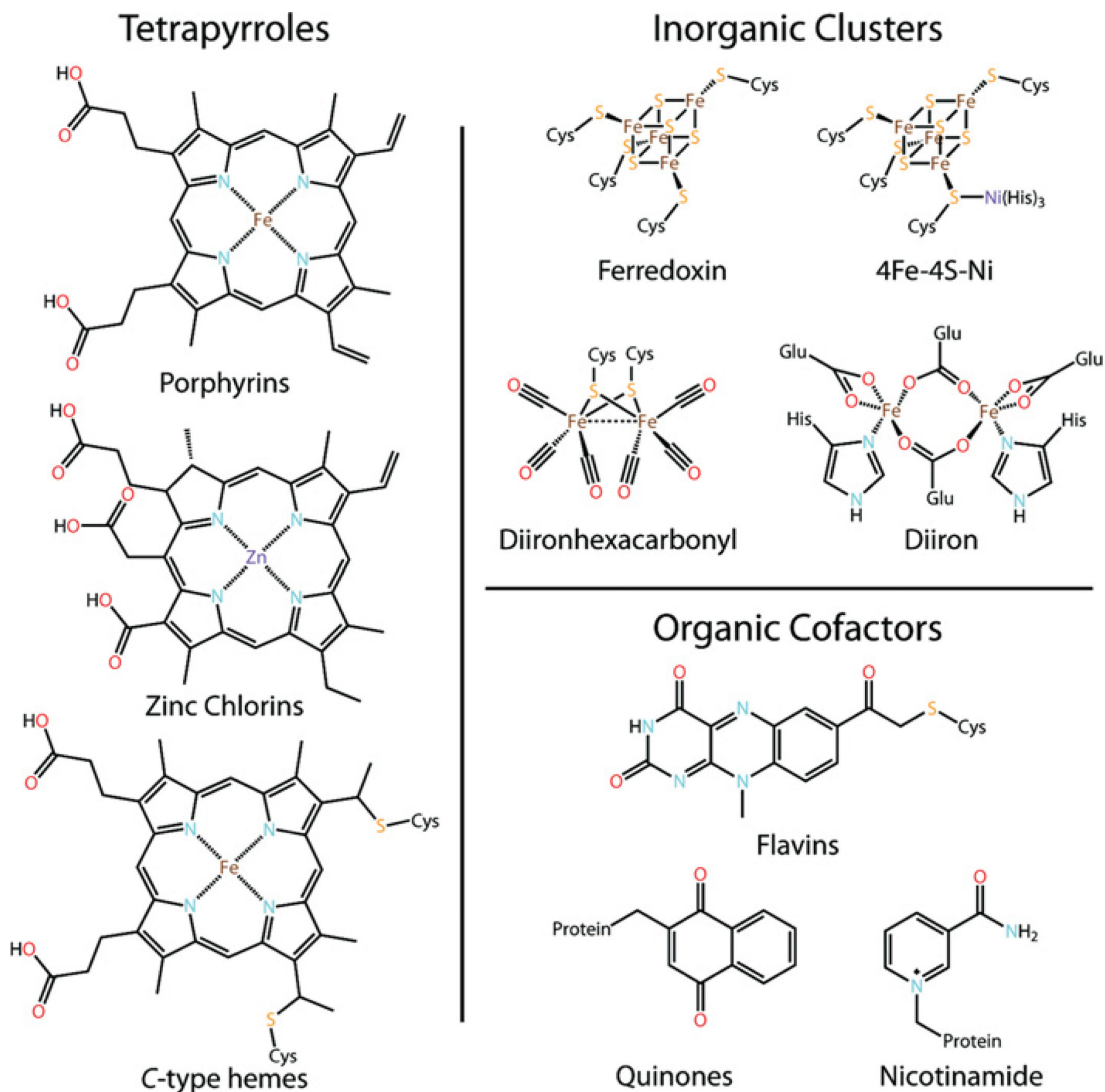

Figure 2.
Common biological redox cofactors and related novel cofactors incorporated into maquettes

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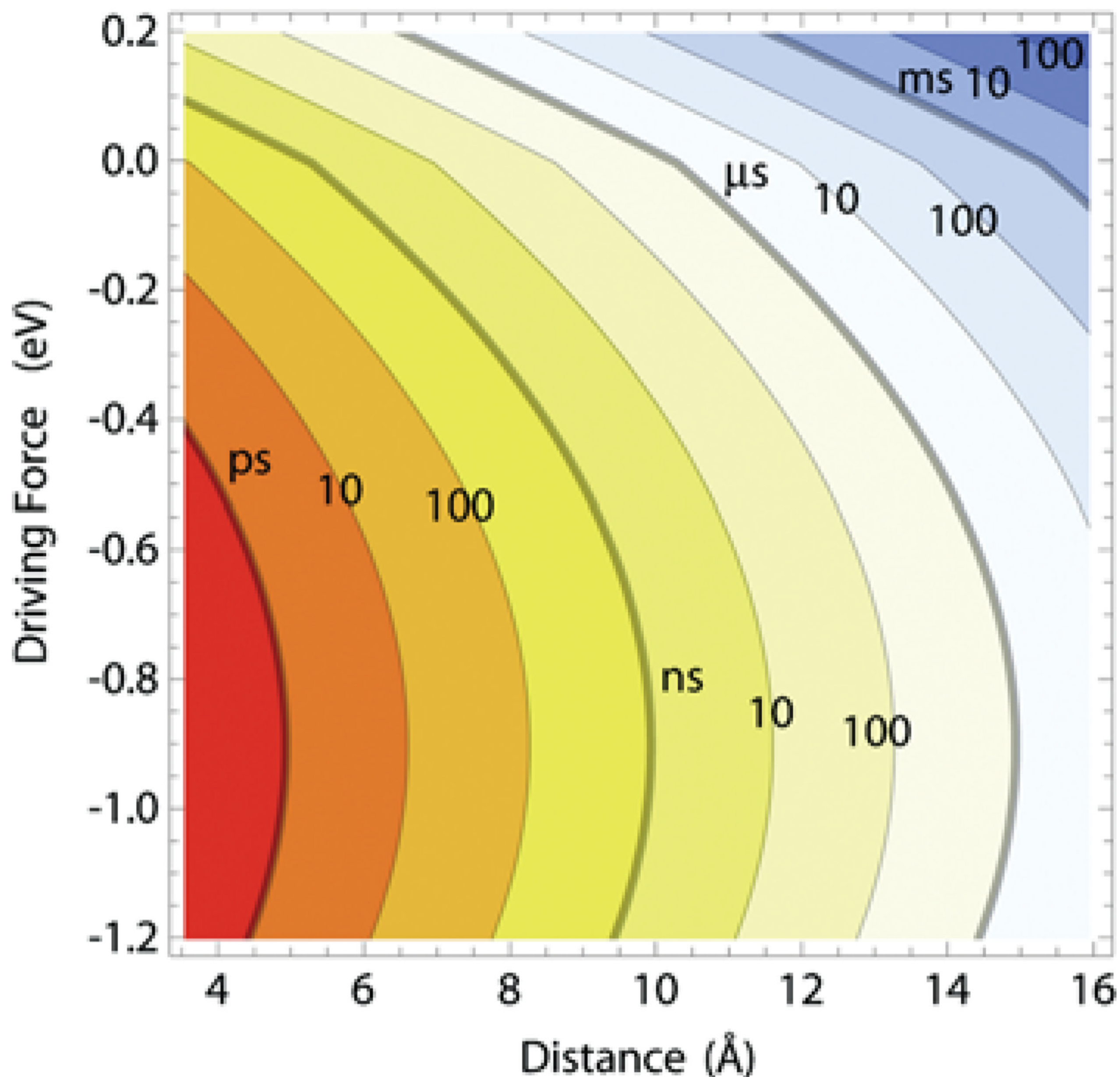

Figure 3. Electron-transfer reactions can be designed using parameters gleaned from natural engineering

Designing artificial electron-transfer proteins takes advantage of the observation that single-electron-transfer reactions in proteins occur by means of electron tunnelling with characteristic rates that depend on three adjustable parameters: distance, driving force and reorganization energy. This driving force and distance contour plot is illustrated with a typical reorganization energy of 0.9 eV.

Systematic Name	N-terminal Cap or Loop	Helix Sequence with relative Conservation of residue identity	C-terminal Cap or Loop	Topo-logy	Demonstrated Functions
HT1	CGGG	ELWKLHEELLKKFEELLKLHEERLKKL	CONH2	A	
HT2	CGGG	EIWKLHEEFLKKFEELLKLHEERLKKM	CONH2	A	
HT3	CGGG	EIWKQHEEALKKFEEALKQFEE-LKKL	CONH2	A	
HD1	Ac	LKKLREEALKLLEEFKKLLEEHLKWLE ELLKLHEELLKKCEELLKLAEERLKKL	GGGGGGGG- CONH2	B	
HD2	AcKKGCGG	KIWKLHEQFLQQFEQLLQLFEQQLQQL QIWQLHEQFLQQFEQLLQLFEQQLKKLKK	GGGGGG- CONH2	C	
HD3		EIWKQHEDALQKFEEALNQFED-LKQL EIWKQHEDALQKFEEALNQFED-LKQL	GGSGCGSGG- COOH	D	
HM1		EIWKQHEDALQKFEEALNQFED-LKQL EIWKQHEDALQKFEEALNQFED-LKQL EIWKQHEDALQKFEEALNQFED-LKQL EIWKQHEDALQKFEEALNQFED-LKQL	GGSGSGSGG- GGSGSGSGG- GGSGSGSGG- COOH	E	
HM2		KEAKEKHKKQLEEFFKKLWEKLQDNA KKAADEHQKQLKEFIEKLEKEFQDL EEAEKKHKEAQEKFEKLLKEFEELQKK KEKAEQEHKKAQEEFKKLKKQFEEELKKL	GGSGG- GGPKSENGG- GGSGG- COOH	E	
AT1	Ac	SSDPLVVAASIIGILHFILWILDRGGNGEIFKQHEEALKKFE	CONH2	F	
AT2	CGGG	EIWKQHEEALKKFFAFHFILPFIIMAIAMAHLLFLFGEG	CONH2	F	
AT3	CGGG	EIWKQHEEALKKFFLAHLLLALAFLALALAHLLL-AGEG	CONH2	F	
HZ1	Ac	RVKALEEKVKALEEKVKAL RIEELKKKWEELKKKIEEL EVKKVEEEVKKLEEEIKKL	GGGG- GGGG- CONH2	G	

Demonstrated Functions: Structure: Apo structured; Holo structured
Cofactor Binding: Metal tetrapyrroles; Ferredoxin (4Fe4S); Flavins; Quinones; Nicotinamide amino acid
Advanced Assembly: Protein semisynthesis; Protein monolayers
Advanced Oxidoreductase Activity: Binding-site-specific heme redox potentials; Charge separation/Photoreduction; Quinol-cyt c oxidoreductase; O_2 binding

Figure 4. Diverse functions have been demonstrated in maquettes with minor changes to primary sequence and topology

Examples of several generations of maquette design, with progression through **h**ydrophilic homo**t**etrameric (HT), **h**ydrophilic homo**d**imeric (HD) and **h**ydrophilic **m**onomeric (HM) four-α-helix bundle sequences, along with their topology (as shown in Figure 1) and various functions. HM2 represents one of the several recent maquettes that branch significantly away in primary sequence from the established soluble maquette family tree. Also included are three **a**mphiphilic homo**t**etrameric (AT) and three helix designs (HZ). AT1 places the hydrophilic helical sequence after an extended hydrophobic sequence, whereas AT2 and AT3 place it before.

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