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De Novo Construction of Redox Active Proteins

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Abstract

Relatively simple principles can be used to plan and construct de novo proteins that bind redox cofactors and participate in a range of electron-transfer reactions analogous to those seen in natural oxidoreductase proteins. These designed redox proteins are called maquettes. Hydrophobic/hydrophilic binary patterning of heptad repeats of amino acids linked together in a single-chain self-assemble into 4- α -helix bundles. These bundles form a robust and adaptable frame for uncovering the default properties of protein embedded cofactors independent of the complexities introduced by generations of natural selection and allow us to better understand what factors can be exploited by man or nature to manipulate the physical chemical properties of these cofactors. Anchoring of redox cofactors such as hemes, light active tetrapyrroles, FeS clusters, and flavins by His and Cys residues allow cofactors to be placed at positions in which electron-tunneling rates between cofactors within or between proteins can be predicted in advance. The modularity of heptad repeat designs facilitates the construction of electron-transfer chains and novel combinations of redox cofactors and new redox cofactor assisted functions. Developing de novo designs that can support cofactor incorporation upon expression in a cell is needed to support a synthetic biology advance that integrates with natural bioenergetic pathways.

1. INTRODUCTION

While the natural amino acids Trp, Tyr, and Cys are important participants in radical electron transfer under appropriate conditions (Stubbe & van der Donk, 1998), the vast majority of natural oxidoreductases exploit redox active cofactors to guide electron tunneling between redox centers and manage the electron-transfer reactions at sites of catalysis (Page, Moser, Chen, & Dutton, 1999). By constructing cofactor binding de novo proteins (maquettes) analogous to natural redox proteins, we can better understand the default properties of these cofactors in a protein matrix independent of the complexities introduced by repeated cycles of natural selection and gain insight into how redox proteins may have operated early in the evolution of life. Such designs also help us to isolate and understand the means by which protein can be engineered to manipulate cofactor properties. De novo design also supports the combination of protein cofactors in novel ways to explore functions unseen in natural systems.

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A wide range of redox active centers have been successfully incorporated into de novo designed proteins (for review, see Prabhulkar, Tian, Wang, Zhu, & Li, 2012; Zastrow & Pecoraro, 2013). With a view of potential synthetic biology engineering (Akhtar & Jones, 2014), this work will concentrate on the design and construction of de novo proteins that bind natural redox cofactors and offer the potential for being exploited for intracellular expression and redox cofactor assembly to interface with electron-transfer pathways within the cell.

To be most useful, these de novo protein frames need to be large enough to surround the redox cofactors within a stable protein fold that is tolerant to multiple, diverse changes in amino acid sequence. Furthermore, to make interpretation of the effects of the protein environment on cofactor function as clear as possible, the folding and amino acid sequence should be relatively elementary, selected according to basic principles of design with minimal reference to complex natural protein sequences. In our experience, de novo designed 4-helix bundles based on a binary patterning of hydrophobic and hydrophilic amino acids fulfills these requirements for a large number of redox cofactors, ranging from redox active iron tetrapyrroles such as heme, light active zinc tetrapyrroles, iron sulfur clusters, flavins, and quinones (Farid et al., 2013; Gibney, Mulholland, Rabanal, & Dutton, 1996; Hay, Westerlund, & Tommos, 2007; Lichtenstein et al., 2015; Robertson et al., 1994; Sharp, Moser, Rabanal, & Dutton, 1998). By linking all helices into a single chain via short, flexible loops, bundle assembly under in vivo conditions is simplified.

2. SEQUENCE SELECTION: BINARY PATTERNING

First-principles protein folding design exploits the hydrogen-bonding pattern of the peptide backbone in an alpha helix that completes two full helical turns for every seven residues. Imposing a binary hydrophobic/polar pattern on each heptad of amino acids in a de novo sequence enables helices to fold and stabilize by self-associating with other helices to bury the nonpolar residues in the bundle core (Fig. 1). Traditionally, based on the sequence of the early structurally resolved fibrous 2-helix coiled-coil protein tropomyosin (McLachlan & Stewart, 1975), the heptad positions are lettered sequentially from *a* through *g*, with the *a* and *d* positions associated with core hydrophobic residues.

When the heptads include a third hydrophobic residue, in either the *e* or *g* position, either HPPHPP or HPPPHPP where H are hydrophobic residues and P polar residues, 4-helix bundles are stabilized with either positions *ade* or *adg* in the core (Liu et al., 2007). The extremely elementary sequence of the three amino acids glutamate (E), leucine (L), and lysine (K) in a 25 amino acid LEELLKK heptad repeat, has a seven turn, 40Å helix length appropriate for core burial of multiple redox cofactors extending the sequence of a relatively simple earlier de novo bundle forming helix sequence (Ho & DeGrado, 1987). The binary patterned helices are linked together into a single chain with glycine (G) and serine (S) rich loops such as GGSGSGSGG. Single-chain designs have strong advantages over previous bundle designs of symmetric tetramers and dimers, not only because they favor exclusive folding into 4-helix assemblies, but also because they afford greater design freedom in asymmetric sequence changes while engineering the environment of incorporated redox cofactors. Simple sequences such as these can provide extremely robust helical bundle

frames that resist unfolding even at near boiling temperatures. For spectroscopic convenience of monitoring maquette concentration, a Trp residue with absorbance at 280 nm is usually included in the sequence, for example replacing the middle S within the second loop.

In the assembly of a single-chain 4-helix bundle, we employ two distinct topologies of helical threading that pack nonpolar residues in the core. Loops connecting helices can proceed circumferentially around the 4-helix cylinder (Fig. 1, *left*) such that helices in contact have an antiparallel arrangement and residues at position *d* in the heptad form potential packing interactions with corresponding *d* residues in adjacent helices offset along the bundle long axis nearly a full helical turn. Similarly, *e* position residues interact with adjacent *e* residues. A binary patterned heptad repeat that obeys this pattern is LQQLQX where Q is Gln and X is E for one sequential pair of helices and K for the other pair. This fully antiparallel arrangement is common in natural helical coiled-coil proteins. Knobs and holes or ridges and grooves formed by large and small protruding core residues in adjacent helices help to stabilize the bundle structure as helices twist around one another in a left-handed super-helical pitch down the central axis of the bundle. This left-handed twist supports a helical pitch of 3.5 residues per alpha helical turn.

In our alternative single-chain topology, there is a mix of parallel and antiparallel helical neighbors (Fig. 1, *right*). The second loop does not proceed along the perimeter of the bundle, but crosses the central axis with a zigzag threading. This topology tends to be favored with the binary patterned heptad repeat LEELLKK. Helical interfaces are more heterogeneous. Instead of the two types of helical interfaces, as in the all antiparallel bundle, there are now three types of helical interfaces. Knob and hole packing in the parallel helices 1 and 3 as well as 2 and 4 have core residues in the *d* position interacting with *e* position residues in the adjacent helix (Huang et al., 2003). Abundant Glu and Lys residues can form stabilizing, charge neutralizing intrahelical salt bridges. However, this topology also supports stabilizing interhelical salt bridges between oppositely charged *b* and *g* position residues. Between antiparallel helices 1 and 2 the *d* residues interact with each other. Between helices 3 and 4 the *e* residues are close enough to interact. Helices in this bundle topology are observed to be straighter with less coiling than a traditional coiled coil (Huang et al., 2003; Skaliky, Gibney, Rabanal, Bieber Urbauer, & Dutton, 1999). With less coiling, the alpha helical pitch increases from 3.5 to about 3.6 residues per turn. At this pitch, if enough turns are added to each helix, the bundle core heptad residues will gradually shift from *adg* to *abe*.

For any maquette sequence that obeys binary patterning, negative design can make the difference between folding into one or the other helical threading (Betz & DeGrado, 1996; Marsh & DeGrado, 2002; Summa, Rosenblatt, Hong, Lear, & DeGrado, 2002), for example, by arranging for favorable E–K salt bridges between adjacent helices in one threading and/or electrostatic repulsion between similarly charged residue in another threading or by including core residues of different sizes that will pack differentially in parallel and antiparallel orientations. Fig. 2 illustrates the conflicting electrostatic interactions between helices with swapped helical threading of the heptad repeats of Fig. 1. Negative design can also suppress multiple interchangeable conformations on the residue level and support

folding into singular structures that simplify experimental interpretation of the manipulation of cofactor properties.

Maquette helical bundle structures are generally highly tolerant to changes in amino acid sequence. For example, exterior polar residues can be changed to adjust the net charge from highly negative (−14) to near neutral to positive (+11), which can be exploited to electrostatically manipulate the redox midpoint potential of bound cofactors or to diversify the polar amino acids to simplify NMR structure analysis. Maquette bundles are also generally forgiving to changes in core residues, tolerating departures from strict binary patterning while still displaying robust folding and self-assembly. Most importantly, maquettes are readily adapted by introducing residues to covalently attach redox cofactors and have been shown to be highly malleable to the introduction of cofactors of a wide range of shapes and sizes.

3. SEQUENCE SELECTION: COFACTOR SELF-ASSEMBLY

Cells often employ ligases and accessory maturation proteins to insert cofactors into specific locations with a specific geometry in natural proteins. Although we have observed accessory proteins recognizing and interfacing with unnatural maquette partners (for example, Anderson et al., 2014), in general maquettes are intentionally designed without sequence similarity to any natural protein, and productive interaction with accessory proteins inside the cell cannot be taken for granted. Instead *de novo* redox proteins are designed with an underlying physical chemistry that supports cofactor self-assembly, both *in vivo* and *in vitro*.

For sufficiently nonpolar cofactors, such as heme, simple hydrophobic partitioning is adequate to insert cofactors into a helical bundle core (Solomon, 2013; Solomon, Kodali, Moser, & Dutton, 2014). However, in practice, redox maquettes exploit the chemistry of nitrogen and sulfur of His and Cys to covalently secure the cofactors into specific designed positions.

4. BINDING HEME REDOX COFACTORS

Natural proteins frequently use His to anchor the central metal redox active iron in heme tetrapyrroles, either in a bis-His ligation, as in cytochromes *b*, or a single His ligation, as in hemoglobin (Reedy & Gibney, 2004). Indeed, during *in vivo* expression in *Escherichia coli*, maquettes can bind cellular heme, especially if heme synthesis is stimulated by addition of heme precursor aminolevulinic acid to the grown medium. When relatively nonpolar tetrapyrrole cofactors such as heme are added to maquettes *in vitro*, cofactor aggregation can compete with cofactor binding. To minimize aggregation, tetrapyrrole cofactors are added in successive 0.2 equivalent aliquots from an ~1 mM stock solution in DMSO at intervals of 10 min with stirring. Maquette concentrations are typically tens of μM. After an excess of heme is added, unbound heme cofactor and DMSO are then removed by passing through a PD-10 G25 desalting column (GE). Bis-His heme binding is confirmed by conspicuous absorption shifts of the Soret and alpha bands, greater than the subtle bandshifts seen on heme hydrophobic partitioning. For binding affinities measurements, stock heme concentration is confirmed by the hemochrome assay (Berry & Trumpower, 1987). For tetrapyrrole binding

titrations in general, bandshift spectra on binding, even if subtle, can be analyzed via singular value decomposition analysis fit to a binding model for accurate K_D values.

Many histidine heme binding de novo helical bundle designs have been explored, from symmetric tetramers (Robertson et al., 1994) to dimers (Ghirlanda et al., 2004) to combinatorial libraries (Moffet et al., 2000). To help minimize sequence complexity, we apply two fundamental principles to single-chain helical bundles based on the elementary LEELLKK and LQQLQX motifs to secure heme cofactor binding. The first is to simply replace the first interior *a* position leucine with His (H) on each helix to provide the thermodynamically favorable iron ligation by histidine nitrogen. In the elementary maquette sequence based solely on the LEELLKK heptad with four identical helices joined by loops, this in itself is sufficient to secure μ M heme binding affinity with no attempt to provide a preformed cavity. The second principle is to provide a primitive accommodation for bound cofactor bulk in the core by shortening Leu to Ala in adjacent helices near the cofactor ligating residues while bulking up core Leu to Phe in remote positions. This second step increases heme affinity by more than a thousand.

In contrast to the fully antiparallel helical topology, bis-His heme ligation between helices 1 and 3 and between 2 and 4 in the mixed antiparallel/parallel helix threading requires helical rotation to appropriately orient *a* position His residues toward heme iron (Fig. 3). With this rotation, some binary patterned residues are partly buried while others are partly exposed, putting strain on the iron ligating histidines. While such strain may be useful for designs that encourage ligand exchange, such as His displacing O₂ binding in oxygen transport (Farid et al., 2013; Koder et al., 2009), we normally avoid excessive hydrophobic residue exposure by replacing *D* position Leu near the end of helices 1 and 2 with a polar residue such as Glu.

In these designs, packing of the maquette core adjusts around bis-His ligated hemes with a diversity of peripheral groups in addition to heme B protoporphyrin IX. These include mesoporphyrin IX, deuteroporphyrin IX, etioporphyrin, isohematoporphyrin, 2,6-diacetyl deuteroporphyrin IX, 2,6 dinitrile porphyrin, and even bulkier groups such as in tetracarboxyphenyl porphyrin or heme A with a farnesyl polyisoprene tail (Gibney et al., 2000; Solomon et al., 2014). By placing a periplasm export tag on the N-terminus of mixed parallel/antiparallel maquettes and using a CXXCH sequence motif for one of the two His groups at a heme binding site, during *E. coli* expression this maquette will interact with the natural cytochrome *c* maturation system and covalently link the Cys groups to heme vinyls creating a heme C (Anderson et al., 2014).

Maquettes also provide a robust frame for creating alternative six-coordinate heme-metal ligation patterns in otherwise similar protein environments by changing just one or two amino acids. Table 1 and Fig. 4 show a variety of coordinate ligations of heme between diagonal helices 1 and 3 in the antiparallel maquette of Fig. 1. These include His-Cys, His-Met, His-Tyr, His-Lys, and His-Ala (Table 1 and Fig. 3, *left*). In general, sites designed to be single His and five coordinate ligation bind heme only weakly. This discrimination can be exploited for site specificity when creating mixed Fe and Zn tetrapyrrole designs as the two tetrapyrroles, respectively, prefer bis-His and single His ligation patterns. On the other hand, in the absence of His, placing a single Cys in maquette cores opposed by a non-coordinating

group, akin to the Cys-Ala pattern seen in natural P-450 enzymes, leads to secure heme binding (Fig. 4, *right*).

Unlike the mixed parallel/antiparallel design, the diagonal ligation of bis-His sites in the antiparallel bundle does not lead to helical strain on heme binding and is relatively resistant to displacement of His by added diatomic ligands such as CO and O₂. Both bis-His heme ligation in a mixed parallel/antiparallel bundle with helical strain (Farid et al., 2013), and Cys-Ala in either the mixed parallel/antiparallel bundle or the antiparallel helical bundle without strain are open to binding diatomic ligands to the Fe sixth coordination site (Fig. 4, *right*).

5. ELECTRON-TRANSFER REACTIONS OF HEME MAQUETTES

At rates that vary considerably according to details of maquette design and the redox potential of the heme, reduced heme will bind and eventually reduce O₂ (Farid et al., 2013). Indeed, when a continuous supply of reducing equivalents is supplied to the bis-His heme site of the mixed parallel/antiparallel bundle by anchoring the maquette to a gold electrode surface and performing cyclic voltammetry, a clear catalytic wave of multiple turnover O₂ reduction can be seen (Fig. 5). Peak catalytic reduction current occurs as expected around the redox midpoint potential of the heme with at least 50 catalytic turnovers per maquette.

Interprotein electron transfer takes place when heme maquettes are mixed with natural redox proteins such as cytochrome *c* (Fry, Solomon, Dutton, & Moser, 2016) or with other heme maquettes (Solomon, 2013). Electron transfer upon stopped-flow mixing is easily monitored when the donor and acceptor hemes have conspicuously different visible spectra, such as when a reduced lower redox potential heme B maquette is mixed with a higher potential heme C containing cytochrome *c*, or when a reduced heme B maquette is mixed with a red shifted, high potential 2,6-diacetyl deuteroporphyrin IX maquette.

In the absence of obligatory and rate limiting proton-transfer reactions, intraprotein electron transfer between fixed cofactors placed within a single maquette is often controlled by the rate of electron tunneling. Because this rate has an exponential dependence on the edge-to-edge distance between cofactors (Moser, Keske, Warncke, Farid, & Dutton, 1992), the dynamics of an electron-transfer system can be engineered by selecting the position of the cofactor anchoring amino acid along the bundle helices (Fig. 6). For porphyrin tetrapyrrole cofactors anchored with *a* position residues four helical turns apart, this is about 16 Å edge-to-edge and leads to ~1 ms electron-tunneling times at low driving force, analogous to transmembrane heme-to-heme electron transfer in cytochrome *bc*₁. Larger driving force electron transfer at this distance can be as fast as 10 μs. At three helical turns, this distance shrinks to around 9 Å and rates can be expected to be four orders of magnitude faster. To position tetrapyrrole cofactors closer together for more rapid electron tunneling, cofactors need to be anchored to different helices to avoid steric clash.

Both intraprotein and interprotein heme maquette electron-transfer rates are resolved by light activation. For example, photolysis of CO bound to reduced maquette heme frees the heme for electron transfer with redox partners (Fry et al., 2016). With somewhat less control

over electron-transfer distances, externally attached, light activatable redox centers such as bipyridyl ruthenium have been attached to external His or Cys. Best control is offered by light activation of redox cofactors within the maquette core, such as a flavin (Sharp, Moser, et al., 1998) or a Zn tetrapyrrole (Farid et al., 2013).

6. BINDING LIGHT-ACTIVATED ZN TETRAPYRROLE COFACTORS

Light activatable Zn tetrapyrroles, including Zn porphyrins, chlorins, and bacteriochlorins, need only one amino acid ligand for ligation to the maquette frame. In natural proteins, most light activatable chlorins and bacteriochlorins are either a noncovalently bound free base, or ligate a Mg metal, which is more abundant than Zn under nonacidic conditions, using His or Lys. Zn bacteriochlorins are found naturally in the acidophilic photosynthetic bacterium *Acidiphilium rubrum* and various Zn tetrapyrroles can be found in other organisms with altered or absent Mg-chelatases (Jaschke, Hardjasa, Digby, Hunter, & Beatty, 2011). Indeed, there is speculation that heme containing cytochromes *b* may have an evolutionary link with early photosynthesis (Xiong & Bauer, 2002) and that in the absence of a Mg-chelatase, Zn replacement of iron may have supported a light-activated role in early life (Jaschke et al., 2011).

Zn tetrapyrroles are bound to maquettes in vitro from stock DMSO solutions much the same as the procedure reported above with Fe tetrapyrroles. Bis-His sites can bind one or two Zn tetrapyrroles as well (Cohen-Ofri et al., 2011; Sharp, Diers, Bocian, & Dutton, 1998). For this reason, in mixed iron/zinc tetrapyrrole maquette designs, a reasonably tight binding heme should be added to fill a bis-His site to a near 1:1 stoichiometry before binding Zn tetrapyrrole. Similarly, maquette interiors accommodate a wide range of peripheral substituents and ring sizes for Zn tetrapyrroles. As a practical matter, the general pattern for rapid and strong binding of Zn tetrapyrroles to maquettes while avoiding counterproductive cofactor aggregation is to have a mix of hydrophobic and hydrophilic peripheral substituents, preferably arranged in an opposing, amphiphilic pattern.

In redox cofactor dyad maquettes with a bis-His site first bound with heme and a second His site four turns away bound with a Zn porphyrin, light activation results in photoreduction of the heme on the 1 ms timescale (Farid et al., 2013). Light-activated electron transfer from Zn chlorin is faster, ~50 μ s, presumably because of the change in driving force and shorter electron-tunneling distance with the larger chlorin conjugated ring system (Farid et al., 2013). In these dyads, charge separation has nearly the same energy as charge recombination, which leads to comparable rates and transient populations of the charge-separated state of about 30%. Like natural systems, we introduce a third redox cofactor for increased distance of charge separation to further stabilize light-activated charge-separated states.

7. IRON-SULFUR CLUSTER COFACTORS

Another common and apparently evolutionary ancient type of redox center are iron-sulfur clusters (Blöchl, Keller, Wachtershäuser, & Stetter, 1992). In our experience, Fe₄S₄ cubane clusters readily insert into Cys rich maquette loops containing a ferredoxin consensus motif

CIGCGAC. Indeed, a short and flexible 16 amino acid sequence KLCEGGCIACGACGGW will spontaneously incorporate Fe₄S₄ clusters (Gibney et al., 1996) as well as other metals such as Co (Kennedy, Petros, & Gibney, 2004). This sequence was inspired by short hexadecapeptide (Frausto da Silva & Williams, 2001) akin to sequences found in natural proteins.

Fe₄S₄ cluster insertion into apo-maquette proteins is carried out under anaerobic, reducing conditions (added 2-mercaptoethanol) such that all Cys residues are reduced. FeCl₃ and Na₂S are slowly added to sixfold stoichiometric excess. Excess reagents are then removed by dialysis or PD-10 G25 buffer exchange column. Redox potentials of these clusters are pH dependent and in the -225 to -375 mV range (Kennedy & Gibney, 2002). Loop insertion leaves the helices free to bind other cofactors such as heme (Gibney et al., 1996).

In nature, an FeS cluster ligating Cys sulfur is sometimes used to ligate additional metals to form extended clusters, manipulating redox potentials, and facilitating catalysis. This strategy was used to modify the maquette frame just described by introducing a metal ligating cluster of three His residues in the core adjacent the Cys rich loop. This enabled the Fe₄S₄ cluster to bridge to a Ni atom upon addition of NiCl₂ (Laplaza & Holm, 2001, 2002) to make a cofactor analogous to carbon monoxide dehydrogenase.

Fe₄S₄ clusters insert into de novo designs with single-strand beta-sheet structures analogous to rubredoxin (Nanda et al., 2005). Redox potential of these clusters are relatively high, about 55 mV. Even though most FeS clusters in nature form in loops or beta structures, FeS clusters also assemble in the core, as seen in a computationally designed single-chain 4-helix bundle (Grzyb et al., 2010). A pair of core Fe₄S₄ clusters also form in dimeric 3-helix bundle designs (Roy, Sarrou, Vaughn, Astashkin, & Ghirlanda, 2013; Roy et al., 2014). When the designed edge-to-edge distance between FeS clusters is narrowed to around 9 Å, Fig. 6 suggests intraprotein electron-tunneling time in this dimer should be about 100 μs, fast enough in principle for two-electron transfer to an appropriate acceptor. Intraprotein electron transfer is observed on mixing with the single-electron acceptor oxidized cyt c₅₅₀ (Roy et al., 2014).

8. REDOX METAL BINDING SITES

Besides binding FeS clusters, Co and Ni, Cys and His groups buried in close proximity in de novo helical bundles can bind a host of other redox active metals (for a review, see Tegoni, 2014). Single-chain 4-helix bundle copper centers are promising for future in vivo work. Using a combinatorial approach of template-assisted synthetic protein (TASP) in which four alpha helices were assembled on a cyclic peptide base with various combinations of a Cys and several His, it was found that about a third of the combinations bound Cu²⁺ and most bound Co²⁺ (Schnepf et al., 2001). Spectroscopic evidence suggested a Cu ligation geometry intermediate between the tetragonal type 2 natural copper proteins and the trigonal type 1 copper proteins, called type 1.5. Subsequently, a single-chain 4-helix coiled coil with two buried *a* position His and one *d* position Cys binds the Cu of added CuCl₂ under anaerobic conditions, displaying a thiolate to Cu charge transfer absorption responsible for the blue color of type 1 copper centers (Shiga et al., 2010). Nearby core Leu residues replaced Ala to

provide space for the metal ions. This redox center has a K_D of $\sim 2 \mu M$ and a midpoint potential of 328 mV. In another variant of this frame, the burial of four α and δ position His on two helices four residues apart and two Cys on a third helix also four residues apart, lead to the binding of Cu in a binuclear purple copper center (Shiga et al., 2012) akin to the Cu site of cytochrome *c* oxidase.

Besides His and Cys, Glu is an effective ligand for redox active metals in a de novo helical bundle core. For example, a His-His-Glu variant of the blue copper protein just described binds Cu with similar μM affinity in an apparently distorted equatorial geometry (Shiga et al., 2009). Burial of two His with four Glu in a dimeric 4-helix bundle supports the assembly of a dimetal binding site (Faiella et al., 2009), including redox active Fe. Electron-transfer activity is evident as diferrous centers bind and reduce O_2 through an oxobridged intermediate. However, stability and activity of this liganding assembly requires relatively sophisticated design of second shell residues capable of hydrogen-bonding interactions with the primary liganding residues.

9. FLAVIN COFACTOR BINDING AND ELECTRON TRANSFER

Quinones and flavins are common one- or two-electron redox cofactors in natural biological systems. In natural quinone and flavin redox proteins, these organic redox cofactors are usually noncovalently bound. Binding interactions often employ nonredox active portions of the cofactor, such as the quinone polyisoprene tail or the bases, sugars, or phosphates of natural flavins FAD or FMN. Quinones have been covalently secured in de novo helical bundles by means of native chemical ligation using an unnatural quinone amino acid (Lichtenstein et al., 2015). However, most de novo quinoprotein designs exploit the ready ability of the chemically active unsubstituted ring carbons of p-benzoquinones and naphthoquinone to form a covalent thioester bond with Cys residues (Snell & Weissberger, 1939). These include a TASP bundle (Li, Hellwig, Ritter, & Haehnel, 2006) and a 3-helix bundle (Hay et al., 2007). Yet in natural proteins, covalently attached quinones are not anchored via thioester bonds in natural proteins. Instead covalent quinones are usually formed by modifications of Tyr or Trp, such as the tryptophan tryptophylquinone cofactor formed by cross-linking two Trp residues in methylamine dehydrogenase. In situ generation of quinone or perhaps specific incorporation of quinones via orthogonal tRNA-aminoacyl tRNA (Alfonta, Zhang, Uryu, Loo, & Schultz, 2003; Wang & Schultz, 2005) may be the best path for trying to engineer in vivo biogenesis of de novo quinone proteins.

On the other hand, some natural flavoproteins do covalently attach flavins via His, Tyr, or more commonly, a thioether linkage with Cys at one of several positions on the flavin cofactor (Edmondson & Newton-Vinson, 2001). For example, monoamine oxidase has a thioether linkage at the flavin 8 α methyl position while dimethylamine dehydrogenase has a thioether linkage at the C6 position (Mewies, McIntire, & Scrutton, 1998). These patterns suggest that biogenesis of de novo thioester flavoproteins may be tractable. Flavins display a rich variety of natural reactions, including light-activated electron-transfer reactions in catalysis and light and magnetic field sensing (Conrad, Manahan, & Crane, 2014) which make them attractive cofactors for incorporating in de novo designs.

We have experience with the thioester linkage of flavins to Cys residues in de novo helical bundles both directly to the flavin ring at the 8 position (Farid et al., 2013) and via an acetyl at the flavin C7 position (Sharp, Moser, et al., 1998) (Fig. 7). These two covalent linkages generate flavins with conspicuously different optical properties. Linkage via an acetyl at the 7 position of the flavin is achieved by brominating 7-acetyl-10-methylisoalloxazine (Levine & Kaiser, 1978; Sharp & Dutton, 1999). Maquettes with an *a* position Cys in the core are added to a fivefold excess of brominated flavin while dissolved in 50% DMF in water for 3 h and then dialyzed to remove DMF and unreacted flavin, purified by reverse-phase HPLC (C18 column, H₂O +0.1% TFA aqueous phase and acetonitrile +0.1% TFA organic phase), flash frozen in liquid nitrogen and lyophilized (Sharp & Dutton, 1999). Bis-His sites spaced one heptad away from the Cys are free to bind DMSO solubilized hemes or other tetrapyrroles.

Light-activated flavin is a powerful oxidant. It can extract electrons from Trp, Tyr, or amines such as EDTA to form one-electron reduced flavin semiquinone. Under prolonged illumination, the flavin becomes fully reduced. However, because the redox midpoint potential of the coupled seven acetyl flavin (−95 mV) is more positive than the midpoint potential of ordinary heme (−153 mV), in order to observe intraprotein photoreduction of heme by flavin, the bis-His sites are bound with a higher potential heme, 1-methyl-2-oxomesoheme XIII (−30 mV) (Sharp, Moser, et al., 1998). Upon light-induced abstraction of an electron from EDTA, the flavin semiquinone intermediate reduces the heme in 100 ns (Sharp, Moser, et al., 1998). This time is consistent with the expected second-order interaction between excited flavin triplet and EDTA while significantly slower than the ~1 ns electron tunneling expected for a heme and a flavin packed in the bundle core.

Direct attachment of a maquette bundle *a* position Cys sulfur to the flavin ring at position 8 is accomplished by first synthesizing 8-bromo-riboflavin (Mansurova, Simon, Salzmann, Marian, & Gärtner, 2013). A typical 5 ml coupling reaction contains ~300 μM maquette that was expressed in *E. coli* and cleaved of its His tag after Ni-NTA resin purification in 20 mM NaH₂PO₄, 0.5 M NaCl, 10 mM imidazole buffer. A 3–5 M excess of 8-bromoriboflavin (in DMF, final DMF concentration not to exceed 20%) is added with TCEP at a final concentration of 1.25 mM. Cys reductants such as beta mercaptoethanol or dithiothreitol are avoided as they displace bromine and couple directly to flavin. The pH of the solution was adjusted to nine with HCl to assure Cys deprotonation, and stirred overnight at 50°C while protected from light. The flavomaquette is purified by reverse-phase HPLC (C18 column, H₂O +0.1% TFA aqueous phase and acetonitrile +0.1% TFA organic phase), flash frozen in liquid nitrogen and lyophilized. After dissolving in 20 mM KH₂PO₄, 100 mM KCl, pH 7.5, flavin incorporation is confirmed by mass spectrometry and UV-visible spectroscopy (absorption maximum at 475 nm). Typical redox potentials of these flavins are around −100 mV. These flavomaquettes show a multitude of electron-transfer reactions. They are reduced by NADH and catalyze electron transfer from NADH to O₂ under aerobic conditions. In the presence of bound heme and EDTA they also photoreduce heme under continuous illumination (Farid et al., 2013). If Trp residues are placed close (~7 Å) to the light-activated flavin, they can be photooxidized in tens of ps (Fig. 7).

10. TRANSLATING AQUEOUS REDOX MAQUETTE DESIGNS INTO MEMBRANES

Redox maquette designs can be adapted for a membrane environment. This offers the possibility to integrate with bioenergetic membranes in synthetic biology or act as novel neuronal membrane potential reporters. However, it is a challenge to translate the method of binary patterning to drive aqueous single-chain 4-helix bundle assembly into membrane spanning electron-transfer proteins (Discher, Moser, Koder, & Dutton, 2003; Discher et al., 2005). The exterior polar interactions that stabilize the water-soluble four-helix bundles are energetically very unfavorable within the hydrophobic core of lipid membranes and need to be replaced by hydrophobic interactions. Compared to water-soluble proteins, membrane spanning residues between helices tend to be small; without an added hydrophobic effect of core burial within the membrane, the entropic advantage of packing small side chains with few rotatable bonds becomes more conspicuous (Walters & DeGrado, 2006).

The most common helical assembly motif in natural transmembrane proteins is an antiparallel packing with a small left-handed crossing angle between helices (Walters & DeGrado, 2006). Hydrogen bonding plays a role in stabilizing transmembrane helical bundles (Adamian & Liang, 2002) but not a dominant one (Bowie, 2011). Membrane protein design often relies on computational optimization of tight interhelical packing (Bender et al., 2007) or molecular simulations (Korendovych et al., 2010). However, if additional helix associating design elements are added (Goparaju et al., 2016), the transmembrane helices can be simply built from “generic” transmembrane leucine rich sequences with a 4:1 ratio of Leu to Ala and with aromatic residues that have lower insertion energies (Wimley & White, 1996) placed at the membrane interface. Our helix associating elements are a binary patterned extramembrane region to constrain the helical assembly and the enthalpic forces of redox cofactor covalent binding, such as bis-His ligation of heme (Goparaju et al., 2016) (Fig. 8).

For ease of purification, the desired sequence for each maquette is usually extended at the N-terminus with 6×-Histag and TEV protease cleavage site. Sequence encoding a GGDG loop is typically added between the His-tag and TEV protease cleavage sites to enhance TEV cleavage efficiency. The final amino acid sequence then undergoes DNA 2.0 algorithms for codon optimization, back translation, and primer design for insertion into plasmids. We select plasmids that produce fusions with maltose-binding protein (MBP) (pMal vectors) or ⁵-3-ketosteroidisomerase (KSI) (pET31 vectors). Fusing transmembrane maquettes with KSI results in aggregation of the fusion protein into inclusion bodies and leads to high expression yields. MBP-maquette fusion results in maquette expression within the bacterial membrane, although with low expression yields.

Membrane localization of de novo redox proteins provides a good opportunity for integrating with the oxidants and reductants of natural bioenergetic pathways of respiration and photosynthesis located in cell and organelle membranes. However, some care may have to be taken with maquette designs intended to indiscriminately promote heme-mediated transmembrane electron transfer as there are indications that expression of some of these designs can become deleterious to cell health.

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Fig. 1.

Alpha helical heptad repeat binary patterning leads to 4-helix bundle association with two distinct topologies: a fully antiparallel arrangement (*left*) or a combination of parallel and antiparallel adjacent helices (*right*) with positive and negatively charged K and E residues shown in *blue* (*light gray* in the print version) and *red* (*gray* in the print version), respectively. 4-Helix bundle topology is viewed from the amino (N₁) end of the first of four helices connected by loops (*black lines*) with the heptad positions *a*, *d*, and *e* comprised predominantly of nonpolar residues (*purple* (*gray* in the print version)) that self-associate to form the bundle core. Bottom right: an X-ray crystal structure of an extended antiparallel

bundle for four redox centers based on a LQQLQX motif; left a mixed parallel/antiparallel packing based on an LEELLKK motif (Huang, Gibney, Stayrook, Dutton, & Lewis, 2003).

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Fig. 2. Negative design, such as electrostatic clash between nearby similarly charged Lys (K) or Glu (E) residues energetically disfavors bundles shown in Fig. 1 from threading into alternate helical bundle topologies shown here.

Fig. 3.
In mixed parallel/antiparallel bundles, helices are rotated to orient *a* position histidines for bis-His ligation of heme iron. Some binary patterned residues are partly buried while others are partly exposed, putting strain on the iron ligating histidines.

Fig. 4.

Maquettes ligate heme B in various ligation schemes, using both amino acids and diatomic ligands. (*Left*) Spectra of oxidized heme B ligated proximally with His and distally ligated His, Cys, Met, Tyr, or Lys in the antiparallel bundle of Fig. 1. For comparison, unbound heme spectrum is dashed. (*Right*) Spectra of reduced heme B proximally ligated with Cys with the distal position occupied by Ala, a nonligating amino acid residue, binds a range of diatomic ligands as in natural protein gas sensors; shown here are CO and O₂.

Fig. 5. Cyclic voltammetry of an O_2 binding bis-His heme maquette immobilized on an electrode reveals a catalytic wave of O_2 reduction near the redox midpoint potential of the heme after air is introduced. Here dithiobismaleimidoethane (DTME) is used to anchor a Cys in the heme maquette loop to a 1 cm^2 gold electrode surface. The sweep rate is 0.01 V/s .

Fig. 6.

Expected characteristic times for intraprotein electron tunneling for a range of driving forces, given by the difference in redox midpoint potentials between donor and acceptor, and for a range of edge-to-edge distances between redox cofactors, which is modulated in maquettes by the positions of the cofactor anchoring amino acids along the alpha helix (*bottom*). Here the reorganization energy for electron transfer is a typical 0.9 eV.

Fig. 7.
 (Left) Oxidized lumiflavin or riboflavin with a thioether linkage to a core Cys at either a C7 (blue (gray in the print version)) or C8 (red (dark gray in the print version)) position display distinct UV-vis spectra. (Right) Light activation of flavin allows for oxidation of a core Trp or the sacrificial electron donor EDTA. The subsequent flavin semiquinone can reduce neighboring heme.

Fig. 8.

To aid in helical bundle assembly in a transmembrane design, a binary patterned single-chain maquette is extended with a generic, Leu and Ala rich sequence including helix associating bis-His heme binding sites. Hemes are shown as *dark brown squares* (*gray* in the print version) and the membrane spanning region is *light brown* (*dark gray* in the print version).

Table 1

A Single Maquette Frame Allows Change of Heme Ligating Amino Acid While Leaving the Rest of the Sequence Unchanged

Ligation Pattern	Natural Protein	Abs Peaks Natural (nm)	Abs Peaks Maquette (nm)	Em (mV)
His His	Neuroglobin	426, 560 (red) 413, 529 (ox)	426, 560 (red) 412, 529 (ox)	–260 to –150
His Cys	Rr CooA, Cysathione b-synthase	422, 540, 570 360, 424, 535	421, 540, 570 360, 424, 535	–290 to –150
His Met	b562 Cyt c	430, 562 406, 534	429, 562 409, 529	70
His Tyr	Bovine liver catalase	436, 558 406, 534, 620	427, 558 407, 530, 615	–180
His Lys	Cyt f (amino)	421, 554 412	427, 532, 560 414	–65
His Ala	Myoglobin	435, 555 409, 505, 635	428, 530, 562 416, 531	–110
Cys Ala	P450, NOS	410, 551 391, 635	430, 555 387, 635	–190

Several natural protein heme iron ligation motifs are compared with maquette counterparts. The His-Lys pattern resembles the His-amino terminus pattern of cytochrome *f* (Ponamarev & Cramer, 1998).