Design and Fine-tuning Redox Potentials of Metalloproteins Involved in Electron Transfer in Bioenergetics

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Abstract:

1. *Introduction*

A significant portion of biological processes are involved with providing vital energy sources such as ATP, and controlling the flow of energy through living systems. These bioenergetics processes, the most important of which are photosynthesis and respiration, require electron transfer (ET) between different redox partners. Metalloproteins are one of the most widely used ET centers in biology. They can be classified into three major classes: cupredoxins, which include type 1 copper (T1Cu) proteins and Cu_A centers [1-10], cytochromes [10-17], and iron-sulfur (FeS) proteins [10,18-24]. Each class of ET proteins transfer electrons between different redox partners which possess different reduction potentials (E°) (Figure 1). Therefore, the ET centers need to adjust their E° in a way that matches those of their redox partners. No single class of protein can cover the entire range of physiological E°, which is between ~ -1V, at which protons are reduced to H_2, and 1V, at which water is oxidized to O_2. Cupredoxins usually function at the high end of the E°, while FeS proteins are mostly involved in ET reactions possessing relatively low E° [10]. The E°'s of FeS proteins overlap significantly with those of cytochromes that often have intermediate E° among the three classes of ET proteins.

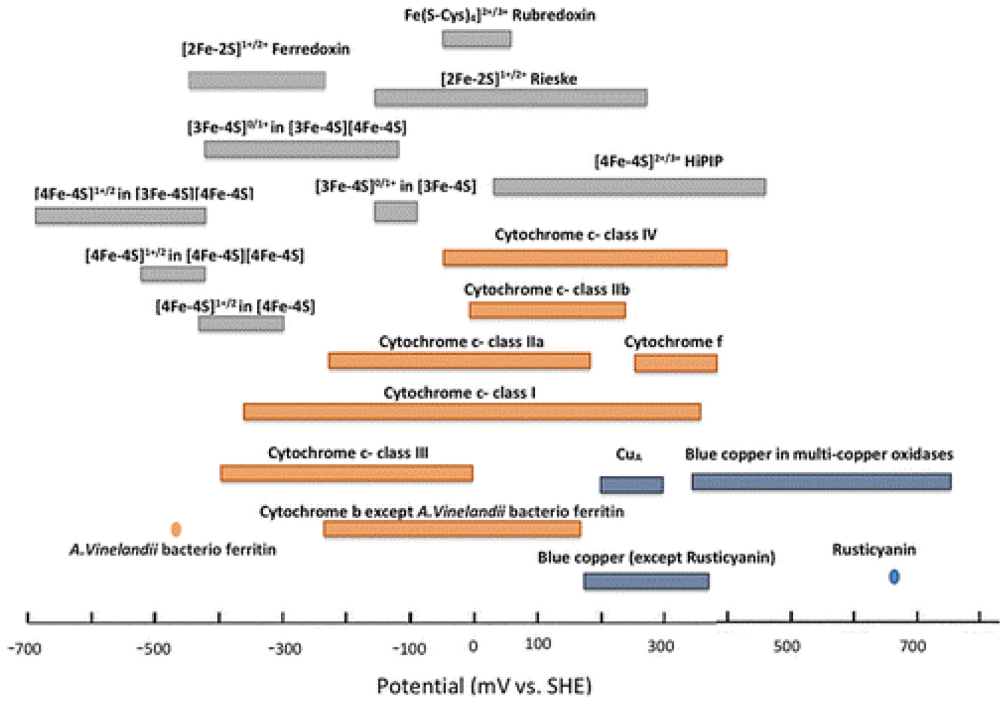


Figure 1. Reduction potential range of metal centers in electron transfer metalloprotein. Adapted from ref. [10]

In this review, we first describe the importance of tuning E° of ET centers, including the metalloproteins described above. The major focus of this review is to summarize recent work in designing the ET centers, the factors that affect redox potentials of ET centers, and strategies to fine-tune them, including several case studies that illustrate recent successes in this area. Outlooks for future endeavors are also provided.

1.1. The importance of tuning reduction potentials of ET centers

The reduction potential (E°) of a metal center is best described as the tendency of the center to acquire electrons and thus to be reduced (Eq. 1). The more positive the value of E°, often called high E° centers, the more favorable the reduction of the metal center becomes, and thus it is called an oxidant [25]. Similarly, centers with less positive values of E° are often referred to as low E° centers and their oxidation is more favorable, hence are better reductants [25].

$$M^m + ne^- \rightarrow M^{m-n} \qquad \text{Eq. 1}$$

As can be deduced from the definition, the value of E° is relative. The most common standard used for defining the E° value is the standard hydrogen electrode (SHE) which is the potential of a platinum electrode in a theoretical electrode/solution interface, where the concentration of H^+ is 1 M. The value of SHE has been arbitrarily assigned to be 0.0 mV. All E° values mentioned in this review are referred vs. SHE. Any factor that stabilizes a higher oxidation state or destabilizes a lower oxidation state of the metal ion will decrease the E°, i.e. the metal ion is easier to be oxidized or more difficult to be reduced. Similarly, factors stabilizing lower oxidation states of the metal ion will increase E°, causing the metal to be reduced more easily.

Another important factor to consider when discussing ET is the rate at which the process occurs. Several factors can influence the rate of ET, based on Marcus equation (Eq. 2).

$$k_{ET} = \frac{2\pi}{\hbar} |H_{AB}|^2 \frac{1}{\sqrt{4\pi\lambda k_B T}} \exp\left(-\frac{(\lambda+\Delta G^\circ)^2}{4\lambda k_B T}\right) \qquad \text{Eq. 2}$$

where k_{ET} is the rate constant of electron transfer, H_{AB} is the electron coupling between the initial and final states, λ is the reorganization energy, and ΔG° is is the total Gibbs free energy change for the ET reaction, often called the driving force of the reaction [26].

Among the factors that influence the ET rate, E° can play a major role, as it provides the driving force (ΔG°) for the ET reaction. By tuning E° of different metal centers within a protein, the ET rate can be precisely controlled. A primary example of such tuning is observed in ET reactions in photosynthesis and respiration.

In photosynthesis, the reaction center, also called light harvesting complex, is responsible for harvesting the energy in the form of light through several chlorophylls and pigments to ensure the maximum capture of visible light [27,28]. In the light-dependent stage of photosynthesis, the excited chlorophyll in photosystem II (PS-II) loses one electron, which is first transferred to a quinone (plastoquinone in plants) and then to cytochrome b_6f, an ET protein (plastocyanin in plants), and finally photosystem I

(PS-I). Upon additional exposure to light, the electron is excited and transferred to another molecule, usually a membrane bound FeS protein, then to ferredoxins, and through the action of ferredoxin-NADPH reductase (FNR), to its ultimate acceptor, $NADP^+$. The electron that left PS-II is restored through oxidation of water [27,28]. Through these series of reactions electrons flow thermodynamically downhill. The energy provided by these electrons results in the generation of a proton gradient across the membrane, which is later used to produce ATP through the action of ATP synthase. The newly produced NADPH will then take part in the next stage of photosynthesis, called the dark cycle, to help store the energy in the form of C-C bonds via a process called carbon fixation [29,30]. Special requirements for proteins that are involved in photosynthetic reaction centers have been discussed by Dutton and coworkers in detail [27,31]. The production of a proton-motive force, and subsequently ATP, is dependent on the downhill movement of electrons, which in turn is controlled by the E° of the redox partners involved in each reaction (Figure 2).

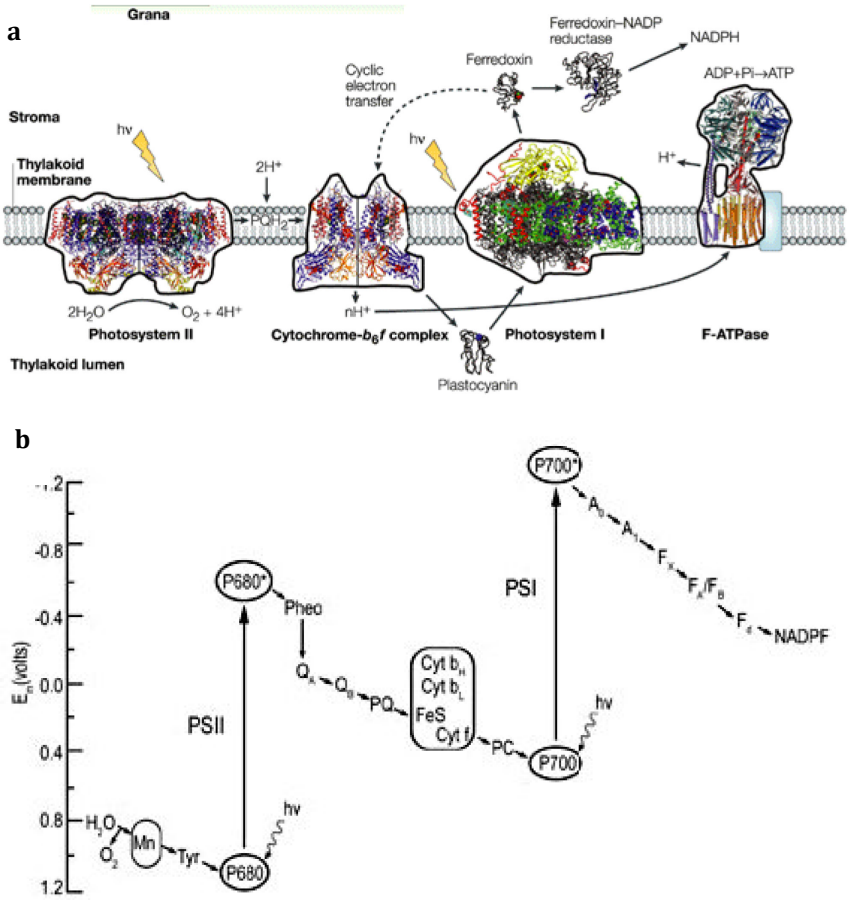
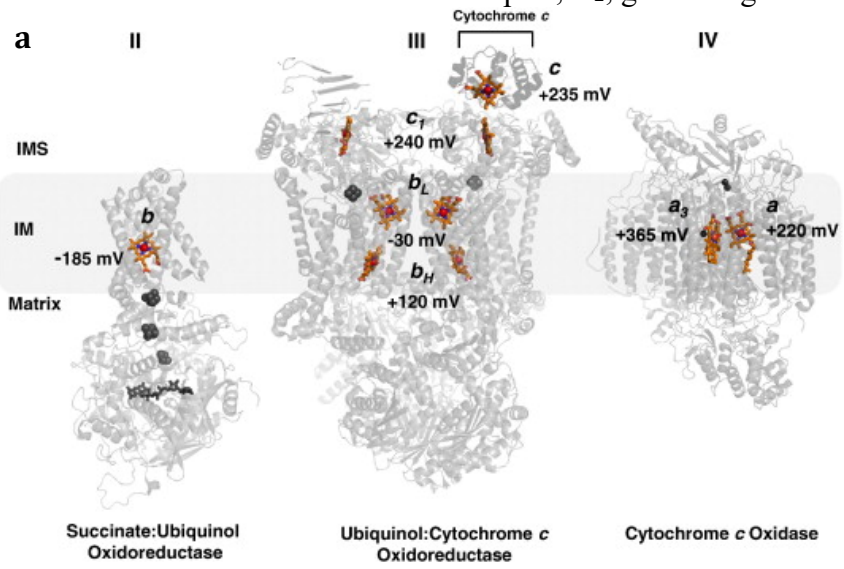


Figure 2. (a) Schematic representation of molecules involved in light stage of photosynthesis. (b) Z-scheme of photosynthesis showing the electron flow and the potential of redox pairs involved. Figures adapted with permission from Ref. [29] (Copyright © 2004, Rights Managed by Nature Publishing Group under license number 3636630566455) and [32] (Copyright © 1992, Kluwer Academic Publishers. Under license number 3636630756533), respectively.

Similar tuning of the E° is also observed in the respiratory electron transport chain (Figure 3). In aerobic respiration, electrons generated from the oxidation of NADH to NAD^+ are transferred to complex I (NADH-coenzyme Q reductase), and then to ubiquinone (coenzyme Q). Ubiquinone can also accept electrons from complex II (succinate dehydrogenase) that are generated through conversion of succinate to fumarate. Reduced ubiquinone will then pass its electrons to complex III (cytochrome bc_1), then to cytochrome *c*, and finally to complex IV (cytochrome *c* oxidase) which transfers electrons to their ultimate acceptor, O_2, generating water in the process. [33-37].



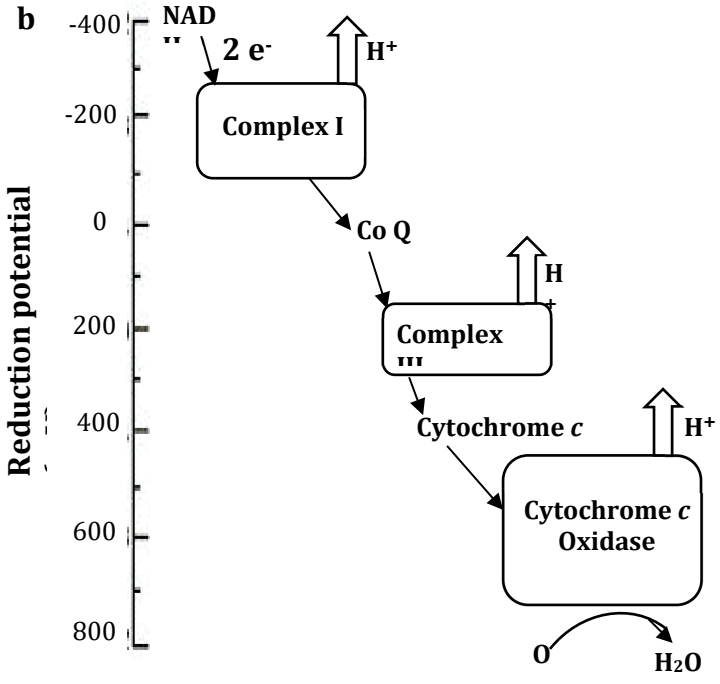


Figure 3.(a) Schematic representation of molecules involved in aerobic electron transport chain. Figure adapted from Ref. [35] Copyright © 2012 Elsevier B.V. under license number 3644871014372 (b) Associated redox potentials.

In addition to controlling rates of ET, tuning E° can regulate biological reactions. In several cases, the E° of a metalloprotein is changed upon binding to a substrate or to its cognate redox partner in a manner that facilitates the reactions. For example, cytochrome P450s are a class of enzymes that catalyze activation of O_2 and then transfer the oxo group to many organic substrates. In the absence of the target, the enzyme has a low E° which prevents its reduction by its redox partner and is thus stable. The binding of the target substrate into active site, however, raises the E°, making the enzyme reducible by its redox partner, and allowing the reduced enzyme to bind O_2, activating it for oxo-transfer reactions such as epoxidation [38-45].

Similarly, the reduction processes in cytochrome bc_1 are tightly regulated in order to avoid the formation of dead-end products. As a result, the system incorporates a way to ensure that cytochrome *c* is not reduced by the Rieske center (see Section 3.2) before heme b_L is reduced. To achieve this goal, E° of the Rieske center is regulated by binding to quinone. The principle of regulation is based on the finding that semiquinone has a strong affinity for the reduced Rieske center and the strong binding increases the E° of Rieske center, due to presence of a cation radical (semiquinone). Before the ET reaction, deprotonated hydroquinone binds the oxidized Rieske center and reduces the Reiske center by giving an electron and changing to semiquinone in the first ET step. However, this reduction resulted in reduced Rieske that has a strong affinity for the semiquinone, and hence its redox potential will increase. The increase in E° does not allow the reduced Rieske center to transfer electrons to cytochrome *c*. In the second ET step, on the other hand, the heme b_L will oxidize the semiquinone to quinone, which has a lower affinity for Rieske protein Consequently, the E° of Rieske becomes low again, enough to transfer electrons to cytochrmome *c* [46-48]. Finally, interaction between plant-type ferredoxins and their redox partner ferredoxin/NADPH oxidoreductase (FNR) cause a change in E° of both proteins, facilitating ET between the two redox partners [49,50].

2. Design of ET centers

ET proteins are of interest to a wide community of researchers not only because understanding them will shed light on essential biological reactions such as photosynthesis and respiration, but also due to their importance in designing biocatalysts, solar fuels, and artificial photosynthesis systems. In this section, we provide an overview of the efforts on designing metalloproteins as ET centers, as well as designing artificial networks and pathways involving metalloproteins. More in-depth discussions are summarized in other reviews in this field [51-55].

2.1. Design of Cu proteins

There are two classes of copper-containing proteins that are solely dedicated to transferring electrons: type 1 copper centers (T1Cu) and Cu_A centers, which are collectively given the name of cupredoxins. T1Cu proteins are widely used as model

proteins to study ET reactions and the factors determining the ET rates (see case study 1). The T1Cu proteins can be further classified into blue, green, and red centers based on their electronic structural differences, reflected in their UV-vis absorption spectra (Figure 4b). Interestingly, there are not many reports of a designed T1 site in either native or *de novo* scaffolds. Initial attempts involved replacement of Zn with Cu in a previously existing Zn-binding site in proteins such as horse liver alcohol dehydrogenase [56-59] or insulin [60-62]. Later, a T1Cu site was designed in a four-helix bundle using 2 His and 1 Cys ligands [63] (Figure 4a). A red Cu center has been engineered into the type 2 copper (T2Cu) site of Cu-Zn superoxide dismutase (SOD) by mutation of His46 or His120 to Cys [64-66]. Replacement of the Cys ligand in the Zn-binding site of the same protein to a His resulted in a green T1Cu site [64,65,67]. Both green and red T1Cu sites were engineered in the blue T1 site of azurin (Az) through incremental increase of strength to the axial Met by mutating it to a Cys or unnatural Hcy (homocysteine), respectively [68,69] (Figure 4b).

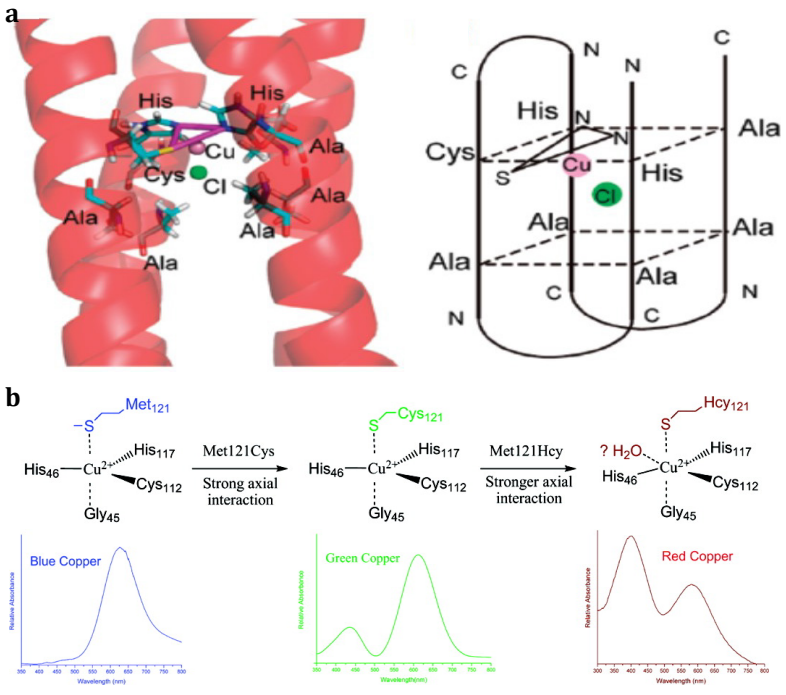


Figure 4. (a) Design of a T1 Cu site in a *de novo* four helix bundle. Figure adapted with permission from Ref. [63] Copyright © 2010, American Chemical Society. (b) Redesign of blue T1 Cu site in Az to green and red T1 Cu site through mutation of axial Met. Figure reprinted with permission from Ref. [68] Copyright © 2010, American Chemical Society.

Several reports are available on engineering a Cu_A center in different proteins and much insight has been provided about the native system by studying these models [9,70-73]. A Cu_A site was first designed into a quinol oxidase through mutating residues in quinol oxidase to their corresponding Cu ligands in subunit II of cytochrome *c* oxidase (C*c*O), guided by structure-dependent sequence alignment [74]. Later, the Cu_A site was introduced into the T1 Cu proteins amicyanin and azurin (Figure 5a) through loop-directed mutagenesis, a strategy in which the Cu binding loop in the T1 protein is

replaced with the loop from the Cu_A binding site in native C*c*O [75,76]. Similarly, a Cu_A site has been designed in a four helix bundle [77] (Figure 5b).

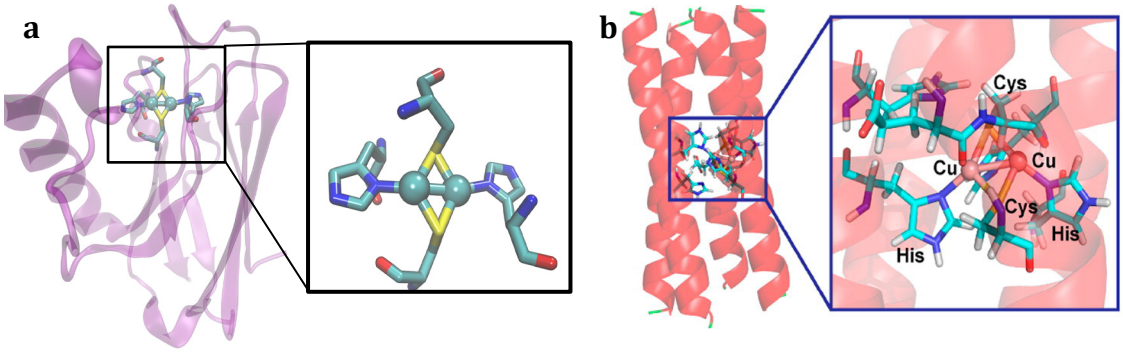


Figure 5. (a) X-ray structure of the designed Cu_A center in Az. (PDB ID: 1CC3). (b) *De novo* designed Cu_A center in a four helix bundle. Figure adapted wiwth permission from Ref. [77]. Copyright © 2012, American Chemical Society.

2.2. Design of heme proteins

Heme proteins are among the most prevalent electron carriers in biology. Cytochromes are widely used across kingdoms to transfer electrons in electron transport chains in order to generate energy. Several groups have engineered heme-containing ET centers in different protein-based scaffolds. These scaffolds include native proteins, *de novo* designed helical bundles, and peptides.

Rational design of bis-His heme *b* or *c* in four-helix bundles is among the most successful examples of *de novo* generation of ET centers (Figure 6a). Such bundles can bind to more than one heme molecule [78,79]. These models have been extended to bind to two different types of heme mimicking the structure of ba_3 oxidase [80], to study effect of mutagenesis on redox potential [78,79,81-83], and to demonstrate the coupling between ET and protonation [84-86]. Interestingly, one of these models showed cooperativity between heme potentials [78,79], a well-known feature of multi-heme proteins. The CORE algorithm was developed to aid the design of native-like bundles that can bind to heme [87,88]. Another example of a *de novo* heme binding protein is the design of sequences that can fold into a globin fold and bind to heme in a 6-coordinated manner [89,90] (Figure 6b). Successful semi-rational methods have been developed to overcome the potential limitations of rationally guided design that mostly arise from lack of prior knowledge about the features required for heme binding [91-96]. One successful example is achieving ET rate constants comparable to native cytochromes *c* [97,98].

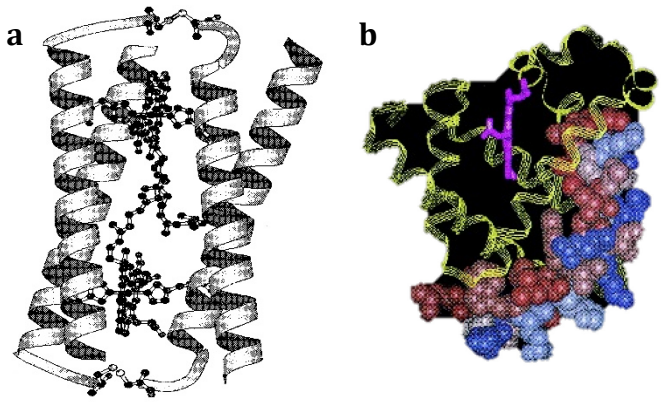


Figure 6. (a) Molecular model of a 4-helix bundle with two types of hemes (hemes *a* and *b*) bound to it. (Figure adapted with permission from Ref. [80] Copyright © 2000, American Chemical Society. (b) *De novo* designed globin fold. Blue, white and red colors show the hydrophilic, neutral, and hydrophobic residues in domain I of the globin fold. Adapted with permission from Ref. [89]. Copyright © 1999, American Chemical Society.

Peptide models of cytochromes have been extensively studied as well. These models are usually based on short peptides of 12-15 amino acids in length that either have a helical structure (such as those designed based on heme binding motif of myoglobin) [99] or change from a somewhat coiled state to a helical structure upon heme binding [100,101]. Some of these peptides have the heme moiety covalently attached to them, usually via amide bonds. In other cases, the heme is not covalently attached to the peptide [102-106]. It has further been shown that addition of Phe and Trp increases heme binding to His and enhances helix stability [107]. Further stabilization can be achieved through linking the two ends via disulfide bonds [98,105].

Another way to design cytochrome-type centers is to use native proteins as scaffolds. Taking advantage of the large repertoire of native proteins that are already evolved to be stable and foldable would offer a higher selection of possible scaffolds to choose from without being concerned if the protein will fold correctly. The most straightforward way to design cytochromes is through mutagenesis of a heme-containing protein. For example, several studies have demonstrated conversion of myoglobin, which has a proximal His and water, to a bis-His axial ligated protein, similar to cytochrome b_5 [108-110] (Figure 7a). In addition to proteins that naturally bind heme, heme-binding motifs have also been inserted into proteins that do not bind heme naturally, such as glycophorin A [111-114] (Figure 7b).

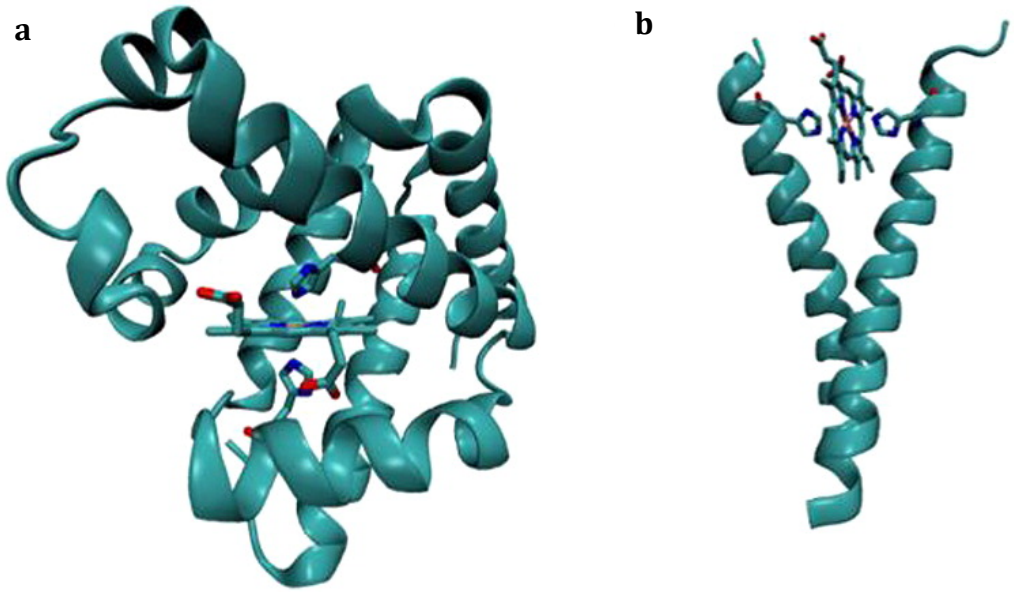


Figure 7. (a) X-ray structure of (a) redesigned pig myoglobin (PDB ID: 1MNI) and (b) redesigned glycophorin A, that bind to heme through bis-His axial ligation. Figures are adapted from Ref. [10]

Another way to design heme containing cofactors is through the conversion of one type of cytochrome to another through mutagenesis [115-119]. Since the major difference between the *c*-type cytochrome to *b*-type, is the presence of covalent linkage to the heme by two Cys residues in cyt *c*, through a conserved Cys-Xxx-Xxx-Cys-His motif, replacing the Cys residues in the motif with Ala resulted in conversion of a *c*-type cytochrome to *b*-type [118]. Conversely, a *b*-type cytochrome was converted to a *c*-type cytochrome upon introducing into a cyt *b* of a motif containing two Cys residues that is similar to the conserved motif mentioned above [116,120].

2.3. Design of FeS proteins

Iron-sulfur (FeS) containing proteins are the most versatile class of ET proteins in that they offer a wide range of oxidation states and redox potentials for a wide variety of functions in biology (Figure 8). Interestingly, different types of FeS clusters can convert into each other within the same protein scaffold via a process called cluster interconversion. The most common type of interconversion is from [4Fe-4S] to [2Fe-2S], which is observed in nitrogenase [121,122], ribonucleotide reductase [123], pyruvate-formate activating enzyme [124], and fumarate nitrate reduction transcription factor [125-128]. Interconversions between [4Fe-4S] to [3Fe-4S] [129-131] and between [2Fe-2S] to [4Fe-4S] have also been reported [132].

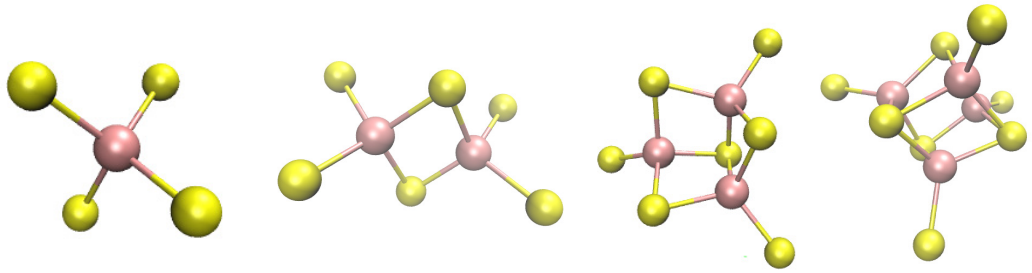


Figure 8. Ball and stick model of different FeS clusters.

In addition to such physiological interconversions, several methods have been applied to design FeS centers in proteins. Several peptide models have been reported that have been modeled to contain different FeS clusters. [133,134]. Both a solitary [4Fe-4S] cluster, and a [4Fe-4S] with heme has been engineered into *de novo* designed 4-helix bundles [135,136]. Aided by computational techniques, a rubredoxin-like site has been incorporated into thioredoxin [137]. Similarly, a minimal rubredoxin mimic was built from a domain-swapped dimer fused with a Trp zipper motif that can fold in β-strands with or without the metal [137]. In a more recent study, a [4Fe-4S] cluster was built into an α-helical coiled-coil structure using a metal-first approach [138] (Figure 9).

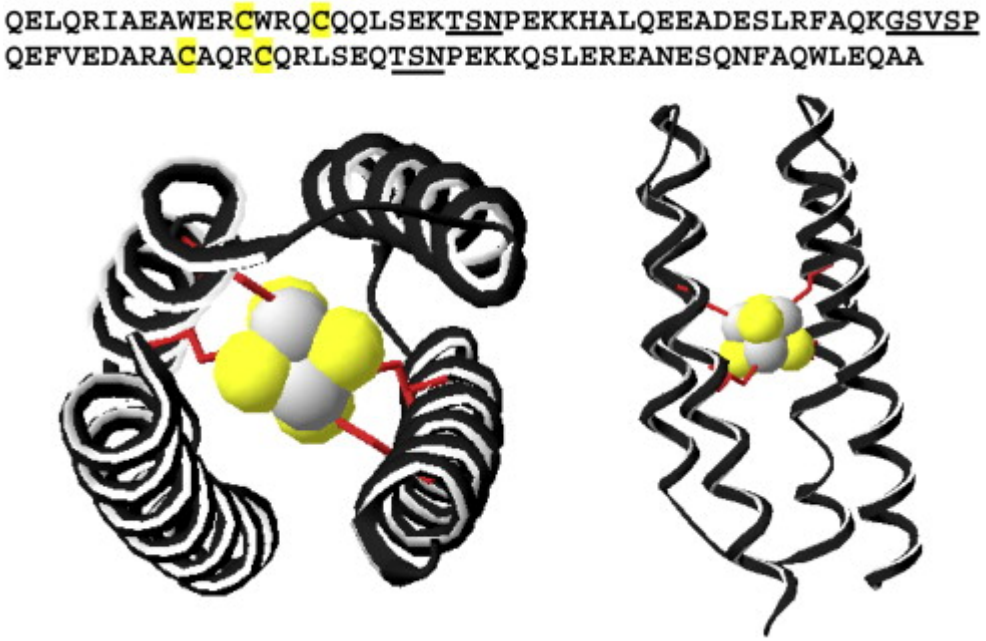


Figure 9. Sequence and structural model of a colied-coil helical structure with [4Fe-4S] binding site. The Cys residues are shown as red sticks in the structure and are highlighted in yellow in the sequence. Figure is reprinted with permission from Ref. [138] Copyright © 2009 Elsevier B.V. Under license number 3636621075094.

2.4. Design of artificial networks and pathways

While design of ET cofactors and ET proteins is challenging on its own, design of protein networks involved in ET is even more challenging. In order to have a functional

ET network, the ET partners should be placed in an appropriate distance from each other, and/or redox partners should have enough affinity for each other to transfer electrons [27]. The potential of the site should also be tuned in a way that ensures rapid ET in the right direction. While several small molecule ligands and cofactors have been designed to mimic important ET centers in biology, such as PS-II [28], the design of their protein counterparts is more complicated.

There are several ways to arrange proteins in a controlled fashion to design the required network of interactions. Perhaps one of the most successful and widely used methods is the use of an electrochemical set up. Inspired by biology, self-assembled monolayers (SAMs) of quinone/hydroquinone on gold have been used to study electron transfer kinetics [139]. In an inspiring study, Lisdat and coworkers designed a multicomponent protein system on the electrode surface consisting of laccase, cellobiose dehydrogenase, and cytochrome *c* embedded in carboxy-modified nanoparticles. The electrode force controls the redox state of cytochrome *c* and was used to switch between two different ET cascade reactions for the detection of O_2 or lactose (Figure 10) [140].

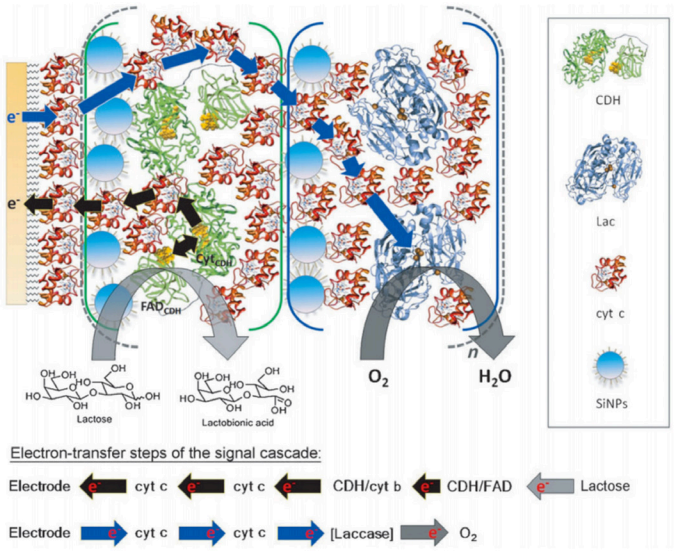


Figure 10. An electrochemical set up to provide control over ET between two systems, O_2 detection and lactose detection. Reprinted with permission from Ref. [140]. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim under license number 3636620458132.

Another common way to create networks is through the placement of the desired proteins on a membrane. One interesting avenue is the *de novo* design of amphiphilic redox-active helical bundles that can be incorporated into membranes, and the coupling of ET to other reactions such as generation of proton motive force (Figure 11) [85,141]. The precise immobilization of proteins with fixed distances on different nanomaterials has also been reported [142,143]. Of particular interest is the use of DNA as a conduit for electron transfer [144,145] or DNA-based structures such as DNA origami as a scaffold for such purposes [146-148].

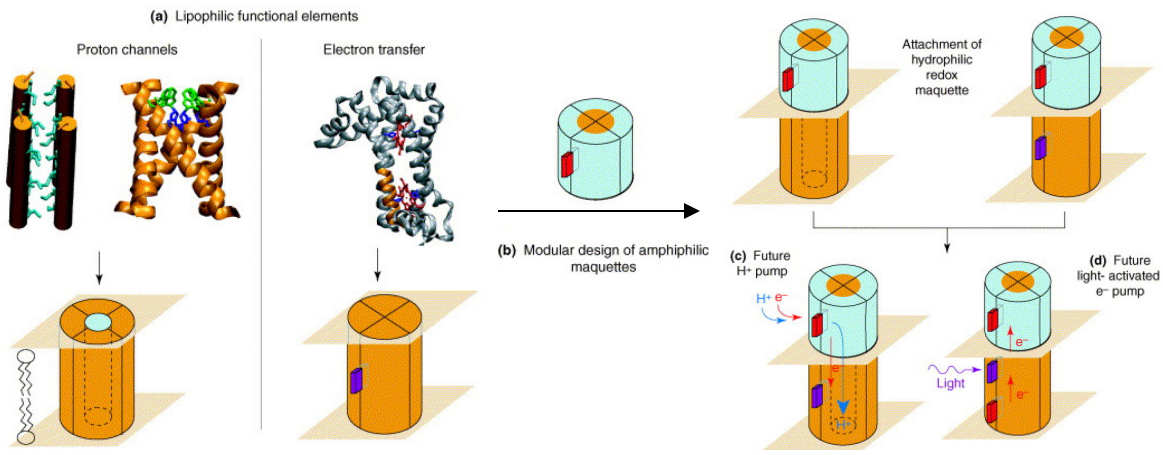


Figure 11. De novo design of amphiphilic helical bundles and their future application in proton or electron transfer. Adapted with permission from Ref. [141] Published by Elsevier Ltd. Under license 3636620217012.

Another area whose progress will significantly enhance the design of protein networks is of the design of protein-protein interactions, especially those involved in ET. There are several examples of designed protein-protein interfaces and protein interactions, and a detailed description of the subject is beyond the scope of this review [149-155]. Here we describe one relevant example of such designs in a redox pair, myoglobin and its physiological redox partner cytochrome b_5. The interaction between these two proteins is usually very weak and transient, mostly following a dynamic binding pattern. Hoffman and coworkers replaced three negatively charged carboxylates on the surface of Mb to positively charged Lys (a change of +6 in net charge on protein surface) to favor the binding between Mb and the negatively charged surface of cytochrome b_5. They further showed that the binding between new pair follows a simpler docking pattern which enables ultrafast ET in order of 10^{10} per second (Figure 12) [156,157].

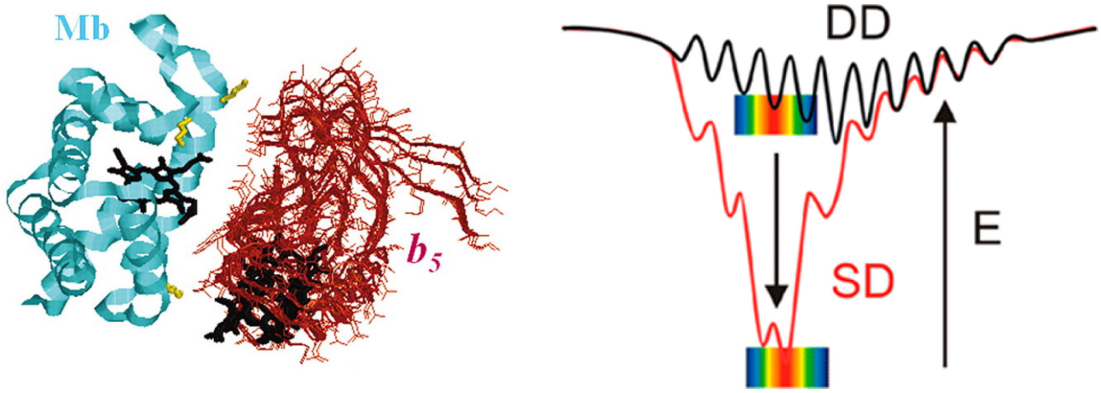


Figure 12. (a) The interaction between cytochrome b_5 and Mb(+6) (reprinted with permission from Ref. *[156]* Copyright © 2010, American Association for the Advancement of Science under license number 3636620002029) . (b) Representative models of binding of Mb and Mb(+6) to cytochrome b_5 (reprinted with permission from Ref. *[157]* Copyright © 2012, American Chemical Society).

Silver and Agapakis showed the design of an orthogonal ET pathway in *Escherichia coli*. To do so, they used pyruvate-ferredoxin oxidoreductase as the source for generating

electrons, ferredoxin as the electron carrier and [Fe-Fe] hydrogenase as the final acceptor of the electrons [158].

3. Factors affecting redox potentials of ET centers and strategies to fine-tune them.

In this section we will provide an in-depth review about the factors involved in tuning E°, as one of the most practical ways of designing tailor-made ET proteins.

3.1. Metal ions

The role of metal ions in determining the range of reduction potential of a metalloprotein is known to be very important. Depending on the metal ion and the valent states it's going through, the value of E° can vary substantially. Not all potentials are accessible to metal pairs and, especially in biology with the limited ligands offered, not all transitions are possible. In general, copper is known to be on higher ends of redox potential range, while iron serves mostly as a low E° redox center [10]. The transitions that a metal center can go through are a major determinant of the overall range of E° for a metalloprotein. The main reason for the higher reduction potentials observed in the [4Fe-4S] clusters in high potential iron-sulfur proteins (HiPIPs) compared with those in ferredoxins (100-500 mV vs. -600-100 mV, respectively) [10] is the different transitions they go through ($[4Fe\text{-}4S]^{2+/3+}$ vs. $[4Fe\text{-}4S]^{1+/2+}$, respectively) [159-162].

The number of metal ions in the metal center is also an important determining factor for the redox potential of both ions. The presence of a positively charged metal ion will directly affect redox potentials of neighboring metals and more importantly, any changes in the redox state of one metal ion will influence overall redox state of the other ions. One such effect is observed in binuclear centers such as Cu_A or [2Fe-2S] clusters in which the orbitals of the metals are mixed, resulting in a spin paired system with half integer charges on each metal [9,163-166].

Even in systems in which the metal centers are not as close, one can see cooperativity between metal centers. Cooperativity of the nearby hemes in multiheme cytochromes is one such example [167-169]. It has been suggested that interactions between the hemes in cytochrome c_3 around 28 Å for heme I and II in tetraheme c_{554} can change the values of E° of other hemes within the protein by 50-60 mV [170-172]. Electrostatic interactions and local aromatic groups are proposed to be the main mediators of such redox-dependent effects [173] (Figure 13). Spin state of a metal ion and the transitions it goes through also have effects on its redox potential. For example, low spin heme sites have shown to have lower redox potentials [174]. In cytochrome $P450_{cam}$, the low spin heme has a potential of -270 mV while the high spin state has a potential of -170 mV [175].

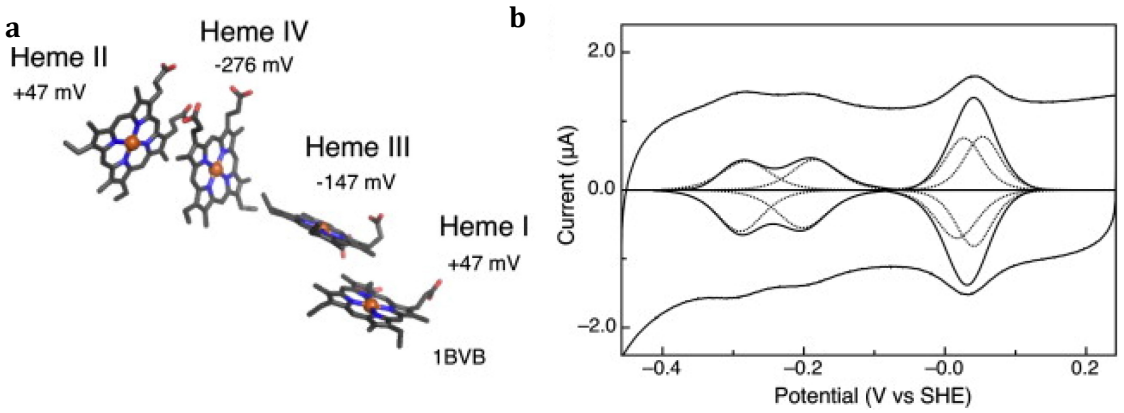


Figure 13. (a) The arrangement of hemes in tetraheme c_{554} with their corresponding redox potentials. (b) The redox potential windows of tetraheme c_{554}. Figures reprinted with permission from Ref. [176]. Copyright © 2013 Elsevier B.V. under license number 363611278854.

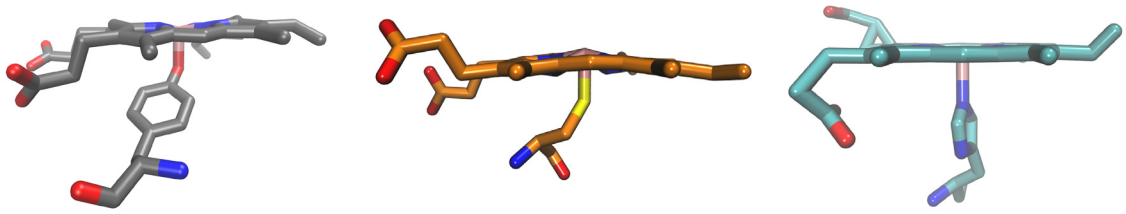
3.2. Ligands in the primary coordination sphere

In addition to the identity of the metal ion and its valent states, the ligands coordinated to the metal ions are major contributors to E° of that ion, conferring their contribution through changes in geometry and electronic properties. While a combination of all these effects determine the E° of a site, some are more prevalent in specific proteins, and we use those as examples to better explain such effects. Some metal ions have different preferences for certain geometries in their different oxidation states. Cu is one example of a metal ion which accommodates a tetragonal or square pyramidal geometry in its oxidized Cu(II) form while preferring a tetrahedral or linear geometry in its reduced Cu(I) form [177]. If the active site of a protein has a ligand geometry more similar to the tetragonal form, Cu(II) is stabilized and a decrease in E° is observed.

A metal ion's preference for soft vs. hard ligands can also be used as a means to tune the E°. In general, higher oxidation states of a metal ion are considered to be harder, due to higher charge and lower radii. Thus, the presence of a hard ligand would preferentially stabilize higher oxidation states, resulting in a decrease in E° values. For example, the Fe(II) heme is softer than Fe(III) heme, therefore softer ligands such as His would favor the lower oxidation state in comparison with a harder ligand such as ??, ultimately increasing E° [178].

Electron donating features of the primary ligands is also a major factor contributing to E° of the site. The proximal ligand of Tyr with high pKa is responsible for catalase to have a very low E° <-500 mV [179]. On the other hand, the proximal Cys with lower pKa than that of Tyr makes cyt P450 to have higher E° (E°= -170 mV in high spin and -270 mV in low spin p450 [175]). Finally the proximal His with even lower pKa caused the myoglobin to display even higher E° (E° = +50 mV) [180-182] (Figure 14). Mutation of the proximal His to either Cys or Tyr decreased the E° in Mb from +50 mV to -230 mV and -190 mV respectively, owing to the more electron donating ability of the new ligands [180]. Longer bond lengths between proximal His ligands and Fe in peroxidases is suggested to decrease electron density on heme, increasing the redox potential [183,184]. Similarly, it has been shown that one of the main reasons behind the higher potentials found in [2Fe-2S] Rieske centers compared to [2Fe-2S] ferredoxins is the replacement of Cys ligands in ferredoxin with less electron donating His ligands in

Rieske proteins [161,185]. Mutation of Cys ligands in FeS proteins to less electron donating ligands such as Ser resulted in an increased E° in cases where the cluster remained intact [47,186-188].



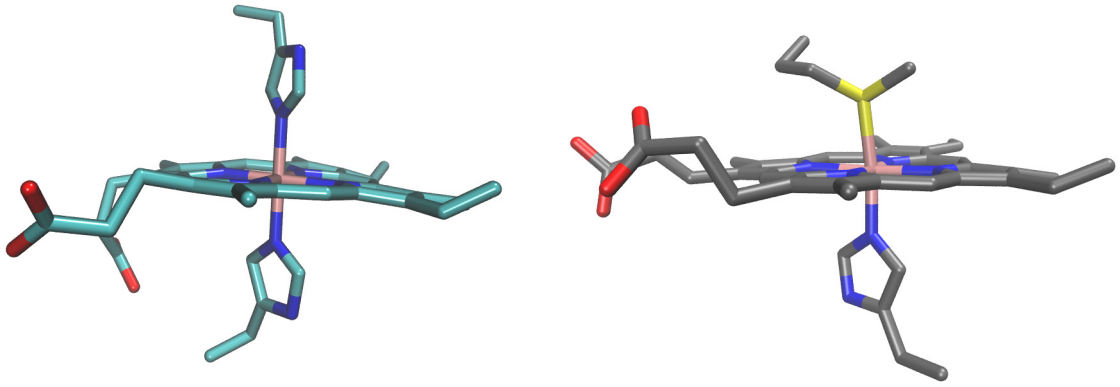
Catalase (E° <-500)
PDB ID: 4B7G

P450cam (E° =-170)
PDB ID: 3WRI

Myoglobin (E° = +50)
PDB ID: 1MBN

*Figure 14.*Effect of changing the proximal ligand in heme proteins. The more electron donating the proximal ligand is, the higher the redox potential will be.

Similar trends are observed with the electron donating nature of the distal ligand of heme proteins. In general, the more electron donating the distal ligand is, the more the higher redox states will be stabilized, increasing E°. Met ligation to heme results in an overall 100-150 mV increase in reduction potential compared with His ligation if all other factors remain the same [189-191] (Figure 15). Replacement of His with Met in cytochrome *c* results in an increased E° [192,193] while the Met to His replacement causes the reverse effect [190].



Cytochrome b_5 (E° = 3 mV)
PDB ID: 1CYO

Cytochrome b_{562} (E°= 168 mV)
PDB ID: 1QPU

Figure 15. Role of axial ligand in potential of cytochromes [194,195].

The electron donating or withdrawing features of the porphyrin functional groups also affect heme E° [178] due to delocalization of d orbitals into the π-system of the porphyrin ring [196]. Having more electron withdrawing groups on heme will create an

electron poor Fe ion, hence increasing the E° [181,182]. Detailed examples are provided in section 7.

Heme distortion or ruffling can also contribute to E°. The degree of ruffling will influence the electronic structure of the porphyrin [197,198] and has been shown to decrease delocalization of π electrons [199-205]. Inducing heme distortion by protein changes have shown that the E° can be changed by 170 mV in heme-nitric oxide/oxygen proteins [204] (Figure 16).

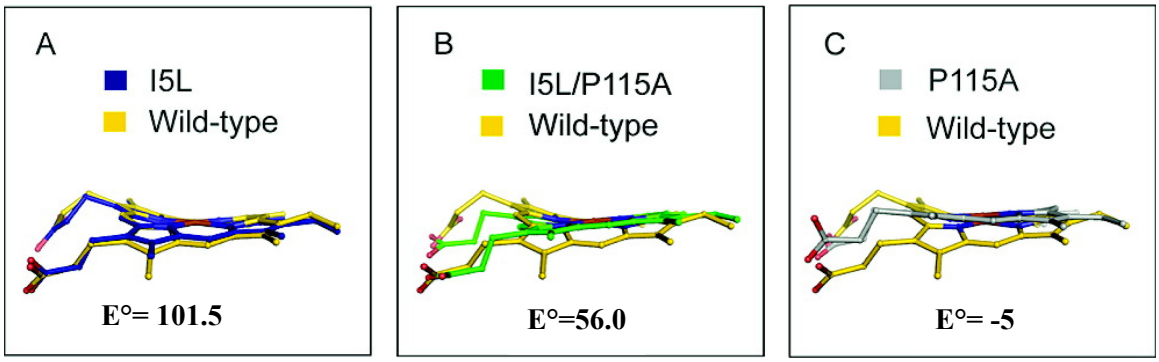
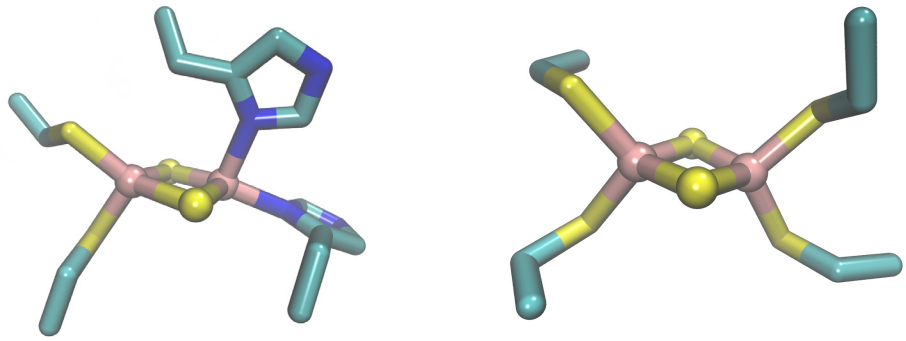


Figure 16. Changing the ruffling of the heme in H-NOX (heme nitric oxide-oxygen reductase) causes a change in the redox potential of the heme. Redox potential of the WT H-NOX is about 420 mV. (all values are reported vs. SHE). Figure is reprinted with permission from Ref. [204]. Copyright © 2010, American Chemical Society.

The presence of electron donating or withdrawing groups on the FeS proteins and the overall charge of the cluster have been shown to influence E° of the FeS proteins. The less negative overall charge of the [2Fe-2S] cluster in Rieske proteins (0/-1) vs. ferredoxins (-2/-3) is suggested as one of the main contributors to higher E° of former [10,47] (Figure 17). Slight changes in the structure of FeS clusters, such as differences in Fe-S-C_α-C_β bond angles [206-208] and distortions in the cuboidal structure of some 3Fe-4S centers can also cause E° changes [209].



Rieske [2Fe-2S] (E° = 312)
PDB ID: 3AV8

Plant-type [2Fe-2S] (E° ~ -400 mV)
PDB ID: 3AV8

Figure 17. Structure of (a) Rieske center in complex bc_1 (PDB ID: 1BE3) and (b) a plant-type [2Fe-2S] ferredoxin (PDB ID: 3AV8). As shown in the figure, while the clusters look very similar, the Rieske center has replaced two of its thiolate ligands with imidazolate. Values for redox potential are obtained from Ref. [210] and [211], respectively.

3.3. Secondary coordination sphere interactions

While primary ligands are the major contributors to the overall range of E° of a given metal center, secondary coordination sphere (SCS) interactions can fine-tune the E° within proteins that share similar primary coordination spheres (PCS). A quick look at different classes of ET proteins can clarify the essential role of interactions beyond the PCS of metal ions in modulating the E° to match that of the redox partner to ensure efficient and specific ET and activity (Figure 1) [10].

As an example, cupredoxins share the same geometry of Cu binding sites with two His and one Cys ligating to the Cu ion in a distorted tetrahedral geometry (Figure 18) [7,10,212]. Despite this similarity, their redox potentials span a range of over 500 mV [6,7,10,213-215]. Similar changes in range of E° are observed in cases of 2Fe-2S ferredoxins, and cytochromes (see Figure 1). The nature of these interactions and how they affect reduction potential is discussed in more detail in section 5.

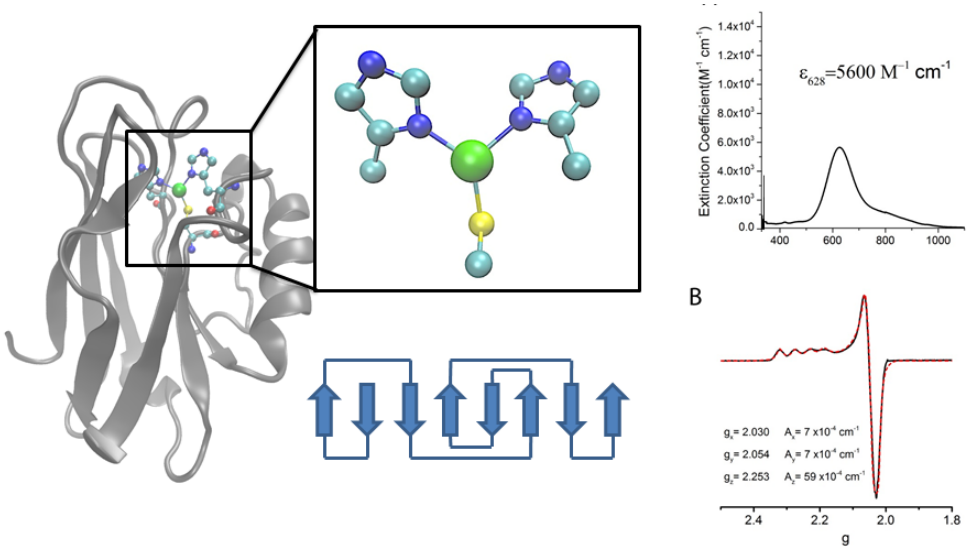


Figure 18. (a) Overall structure of a cupredoxins (azurin, PDB ID: 4AZU). (b) Representative active site structure of cupredoxins. (c) Overall scheme of greek key β-barrel fold of cupredoxins (adapted from [117]). (d) , (e) Representative UV-vis and EPR spectra of cupredoxins, respectively. (adapted from [21])

3.4. Longer range interactions

While not as prominent as factors mentioned above, longer range interactions within proteins and the overall structural features of protein scaffolds in which metal ions are residing can also influence the E° of the metal center. Hydrophobic environments provided by protein around the metal will increase the reduction potential of metal centers by disfavoring higher redox states [10]. This desolvation is the reason that metal complexes in organic model systems usually have lower E° compared with those of proteins [161]. Exclusion of solvent from the site will change the entropy and enthalpy of a redox transition. Solvent exclusion and heme burial is thought to be among the chief factors in controlling redox potential of cytochromes [182,193,216-222], with an effect of up to 240 mV [193].

In addition, there have been several reports of cases in which a change far away from metal site influences the overall arrangement of primary and secondary ligands, causing a shift in redox potential. This structural change is considered as one of the ways binding

to a redox partner can change E° and facilitate ET. Surface charge mutations in cytochrome b_5 [223] and C*c*P resulted in slight shifts in redox potential [224]. Net surface charges have been proposed to be important in determining E° of HiPIPs [160,225,226].

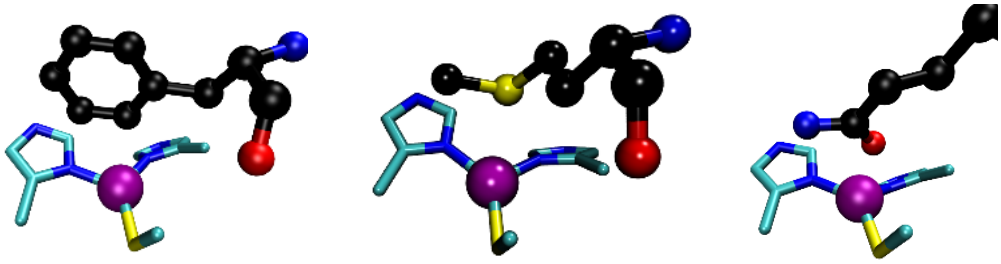
4. Tuning redox potential through secondary coordination interactions

Although metal ions and their primary ligands are the main contributors to the E°, these factors are highly evolved and optimized in natural ET proteins to ensure the lowest reorganization energy required for the rapid transfer of electrons. Such factors usually remain unchanged in different classes of ET proteins to avoid the need to re-optimize the sites, so accordingly most changes are exerted through SCS modifications. In this section, we will go through the ways by which nature tunes the ET proteins without changing the primary ligands.

4.1. Role of axial interactions

Axial interactions can have a great effect on the E° of the metal center. They are among the strongest SCS interactions and are usually closer to the metal center than the rest. Their effects can usually be directly transferred to the metal site. Several homology studies between proteins with the same metal center have sparked interest in investigating the role of the axial residue, showing a correlation between the nature of this ligand and the redox potential of the metal center. These observations further encouraged researchers to replace axial residues in several metalloproteins with other natural and unnatural amino acids.

Hydrophobicity of the axial residue has been shown to modify the metal center potential in different proteins. The more hydrophobic it is, the higher the E° of the site (Figure 19). Cupredoxins are the best demonstration of such an effect. While a Met is present in the majority of cupredoxins, there is a Gln in stellacyanin, which has a very low E° (190 mV) [10]. A Leu or Phe is found in laccase and ceruloplasmin, cupredoxins that are known to be in the higher extremes of the reduction potentials (790 mV for laccase) [10]. Replacement of the axial residue in several cupredoxins has confirmed that the hydrophobicity of this residue is directly related to E° values [227-229].



Laccase (E°= 790)
PDB ID: 3FPX

Azurin (E°= 310)
PDB ID: 4AZU

Stellacyanin (E°= 190)
PDB ID: 1JER

Figure 19. Role of hydrophobicity of the axial ligand in redox potential of cupredoxins. Moving from more hydrophobic to more hydrophilic (a to c), the redox potential will decrease [4,228,230]. Cu ion is shown as a purple ball, the primary ligands are shown in cyan and the axial ligand is shown in black stick and balls.

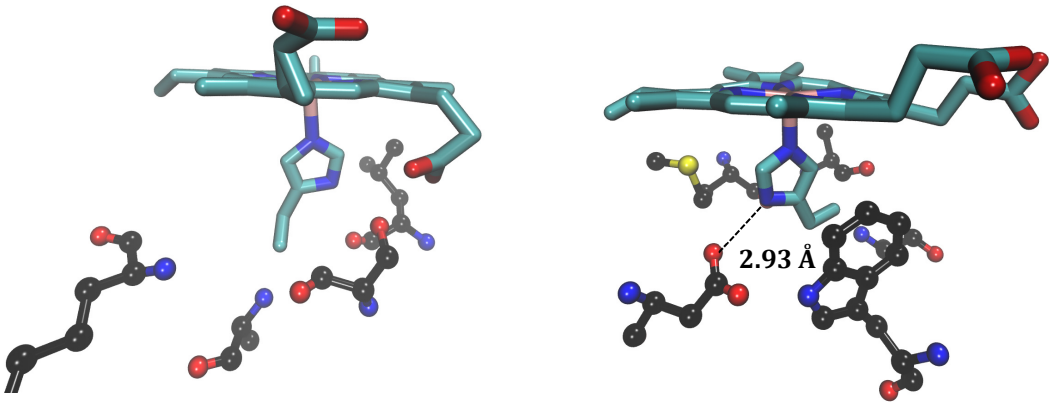
The presence of a carbonyl oxygen in azurin in a distal site of Cu is proposed to lower the redox potential of the site by stabilizing the Cu(II) form through increased electron density at the site [7,231].

4.2. Hydrogen bonding interactions

Despite the important role of the axial residue, it is not the sole contributor to E° fine-tuning as evidenced by cytochromes with similar primary and axial ligands, yet display a variety of E° values. There are known cases of cupredoxins with the same Met axial residues yet significantly different potentials, and FeS proteins with a range of E° values despite sharing similar Cys and inorganic S ligands [10]. In both these cases a prominent factor in tuning the E° is the hydrogen-bonding (H-bonding) network around the metal center. H-bonding interactions can lock the ligands in the proper position required for the redox activity, however, this is not the only role they play. H-bonding to primary ligands can alter their chemical properties resulting in altered reduction potentials.

H-bonding to the Cys ligand of cupredoxin is shown to decrease the electron-donating features of this residue. Removal of just one of these H-bond interactions from the backbone amide of Phe114 residue through a Phe114Pro mutation in azurin resulted in a decrease of 100 mV in the E° [232,233]. Asn to Ser mutation removes a H-bond between loops and has been shown to cause a 131 mV increase in E° in azurin [68], while changing Ser to Asn in rusticyanin decreases E° by 77 mV [234]. Decreasing the electron density on the Cu site by adding an H-bond to distal carbonyl of Gly45 through Phe114Asn mutation resulted in an increase in E° of azurin [235].

Another example can be found in the case of peroxidases. Heme *b* is found both in peroxidases and Mb, and both proteins have a His residue as their proximal ligand. However, peroxidases have significantly lower E°, as is required for their activity. This lowering of E° is mainly attributed to the presence of an Asp residue that H-bonds to a metal coordinated His ligand (Figure 20). This strong H-bond can partially deprotonate His, giving more imidazolate character to it [236,237]. Electrostatic stabilization of the positive charges in Fe(III) will lower the E° [238,239]. Removal of this bond will increase the reduction potential, drastically decreasing peroxidase activity. The presence of such H-bonds are indeed important, but they also require proper orientation to fully convey their role. These mutations are discussed in more details in section 5.4.2. Interestingly, strengthening the H-bond to the proximal His ligand of cytochrome *c* resulted in a 100 mV decrease in redox potential [240]. Disruption of H-bonds from Tyr to the Met axial ligand in yeast iso-1 cytochrome *c* resulted in a decrease of up to 56 mV in E°. This change in the E° was attributed to the increase in electron density on Met, stabilizing Fe(III) state [241,242].

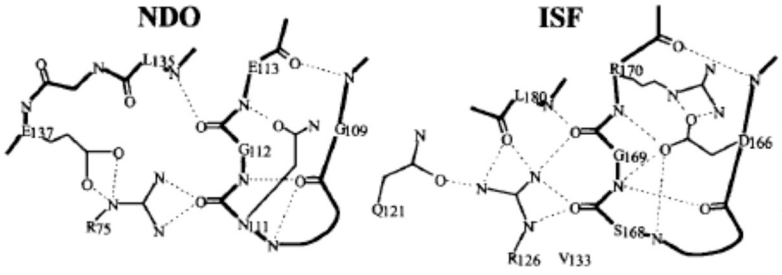


Mb (E° = 50 mV)
PDB ID: 1MBN

C*c*P (E° = -190 mV)
PDB ID: 2CYP

Figure 20. Comparison of the heme binding pocket of Mb and C*c*P. C*c*P has an Asp ligand that H-bonds to its proximal His and lowers the redox potential (potentials from Ref. [180] and [243], respectively)

H-bonding from backbone amides of SCS residues to FeS cluster ligands are shown to be an important feature in determining E° of FeS proteins [159,244]. More $NH_{amide}\cdots S$ H-bonds increase the E° by decreasing electron density on the sulfur ligand, thereby selectively stabilizing the reduced state. While ferredoxins have 11 conserved H-bonds, only 8 are observed in HiPIP proteins. This difference results in two distinct oxidation states of the cluster ($[4Fe\text{-}4S]^{1+/2+}$ for ferredoxins and $[4Fe\text{-}4S]^{2+/3+}$ for HiPIPs) [159,206,245-247]. Altering these H-bonds by replacing them with Pro residues resulted in a decrease in E° [47,248]. In Rieske proteins, several H-bonding interactions to Cys ligands are present from either main chain nitrogens or conserved residues such as Tyr or Ser. Mutagenesis studies have shown the importance of these H-bonds in maintaining high to Cys ligands of the Rieske center [47,249]. Interestingly, Rieske-type proteins have significantly lower E° values (between -150 to -100 mV) [47,248,250] than Rieske proteins (-100 to +490 mV) [47] despite their similarities in their primary ligands (Figure 21). This lower E° is partially attributed to lack of the H-bonds from conserved Tyr and Ser residues to the Cys ligands of FeS cluster [249].



NDO (E° = ~-100 mV)
PDB ID: 2NDO

ISF (E° = ~300 mV)
PDB ID: 1REI

Figure 21. Comparison of H-bonding network between a Rieske-type protein (naphthalene dioxygenase-NDO) and a Rieske protein (water soluble fragment of complex bc_1- ISF). The figure is reprinted with permission from Ref. [251].

4.3. Hydrophobicity and solvent exposure

In general, placing the metal center in a more hydrophobic environment and less exposure to water will result in an increase in E° due to greater destabilization of higher oxidation states compared with lower ones. The Cu sites in laccase and ceruloplasmin are surrounded by hydrophobic residues [230]. Placing Phe at several positions in SCS of Cu site in Az resulted in an overall increase in redox potential [252]. Increased hydrophobicity around the heme pocket is also shown to increase the redox potential in various heme proteins [80,81,100,196]. The most significant example of such hydrophobicity effect is seen in the case of HiPIPs and [4Fe-4S] ferredoxins. While the cluster is very similar between the two, the cluster's buried location resulted in a much higher E° in HiPIPs [206,225,253] (Figures 1, 22). Furthermore, the covalency of Fe-S bond can be altered by hydration, hence influencing the redox potential [254].

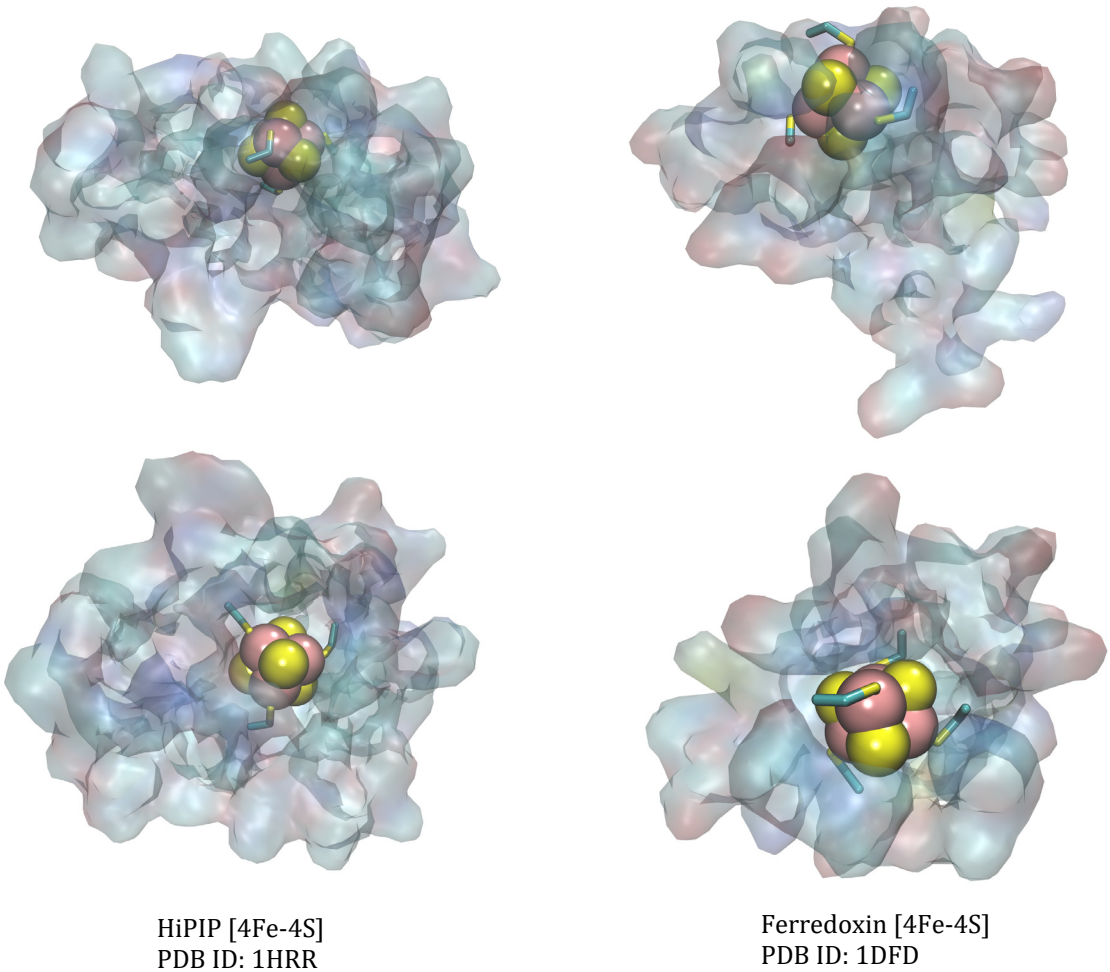


Figure 22. The [4Fe-4S] cluster is more buried in HiPIPS (PDB ID: 1HRR) vs. ferredoxins (PDB ID: 1DFD). One of the ligands in ferredoxin is completely exposed to water.

4.4. Net charge

While controversial in some cases, a net charge in the SCS of a metal center can influence the E°. Presence of a negative charge, or even a negative dipole, can stabilize higher oxidation states and decrease the E°. Precise positioning of Gln and the interaction of its dipole with heme in cytochromes c_6 and c_{6a} has been shown to raise the potential by 100 mV [255]. Mutation of Tyr to a Lys in cytochrome *c* resulted in 117 mV increase in the reduction potential, due to neutralization of negative charges in propionates by the positively charges Lys, resulting in less negative heme ring and hence destabilizing higher oxidation state of Fe [256]. Placement of polar or negative residues close to heme in Mb reduced the reduction potential by up to 200 mV [257]. Replacement of Leu by Glu in a *de novo* designed heme protein lowers the E° by 40 mV while introducing a Gln increased it from -156 to -104 mV [81]. Positive charges, on the other hand, will increase the E° by destabilizing these higher oxidation states. The presence of net positive amide dipoles is suggested to account for the differences between redox potentials of HiPIPs and ferredoxins [225,226,258].

4.5. Aromatic interactions and pi stacking

Although to a much lesser extent, aromatic interactions and π-stacking are known to influence the redox potential of certain metal centers. The majority of observed effects resulting from mutagenesis of aromatic residues are on the stability, rather than directly on the E° of the site [107,259-261]. However, there are cases in which an aromatic residue is shown to play a role in determining the E° of a metalloprotein, mostly through π–interactions. Effects due to increased hydrophobicity or H-bonding have been discussed in sections 5.2 and 5.3. Aromatic π-interactions between Tyr and proximal His in tetraheme cytochrome c_3 results in a 34-45 mV decrease in E° [262]. An aromatic residue is present in cyt b_6f that is in contact with heme *f* at position four. In algea cytochromes, there is a Trp present in this position, while a Phe in seen in cyanobacteria. Swapping these residues can result in a 70mV difference between the E° of the two proteins [263]. Another example is the Phe in cytochrome c_3 that is shown to be in contact with heme I, and is suggested to lower its E° through π-π interactions with porphyrin π system [173].

4.6. Other factors in the secondary coordination sphere

The overall composition of SCS ligands and the length of the loop between ligands have also been shown to be important in determining the E° of the metalloprotein. An interesting example of such effects is seen in a series of loop-directed mutagenesis studies performed on blue copper proteins, in which the ligand loop of different cupredoxins were swapped with one another [264-267]. The E° of the chimeric proteins were in between those of the loop donor and acceptor. A loop from a lower E° protein would decrease the E° of the acceptor and vice versa [265-268].

5. Tuning E° using unnatural amino acids and non-native cofactors

While native cofactors and amino acids are very effective for designing ET centers, the repertoire of redox active molecules can be further expanded by the introduction of unnatural amino acids (UAAs) or non-native cofactors. These molecules enable the deconvolution of features which contribute to ET rates.

One successful example is the use of unnatural derivatives of Met in Az to study the role of hydrophobicity, specifically, in tuning the potential of the T1Cu center. Upon incorporation of a series of UAAs (shown in Figure 23), Lu and coworkers unambiguously demonstrated a linear relationship between hydrophobicity (logP) of the axial ligand and reduction potential of T1Cu center [269].

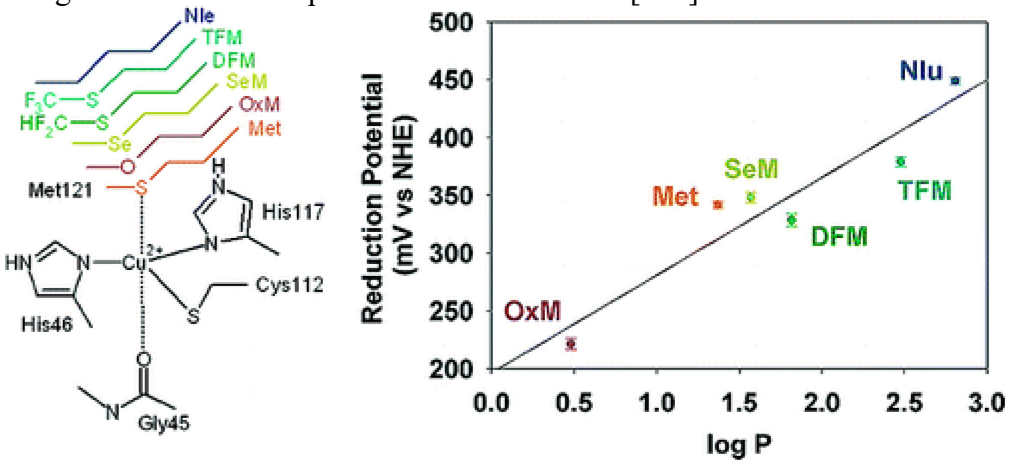


Figure 23. Unnatural Met derivatives that are incorporated into axial position of Az and their corresponding redox potentials. Figure is reprinted from Ref. [269].

Another example is the use of Tyr derivatives to probe ET. Tyr is a redox active amino acid that is shown to donate an electron and a proton during O_2 reduction by C*c*O. This Tyr is covalently attached to an adjacent His, which is a Cu ligand in the active site Cu_B center. In order to study the features of this Tyr that are important for ET and proton transfer, Lu and coworkers used derivatives of Tyr to study the role and function of this amino acid in the reaction mechanism of a functional model of C*c*O in myoglobin (Mb) (Figure 24). Using these derivatives, they showed the importance of the His-Tyr crosslink in increasing the activity and turnover of the enzyme [270]. They further confirmed the role of redox potential and pKa of the Tyr in the water production activity [271,272].

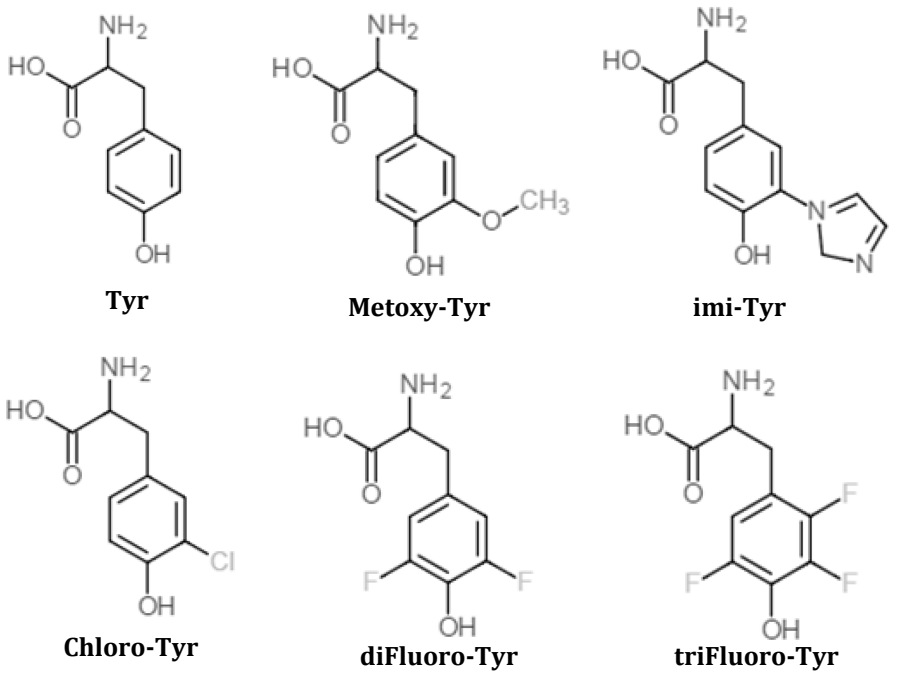


Figure 24. Redox active derivatives of Tyr used by Lu and coworkers to study water oxidation in mimics of C*c*O in Mb.

In order to study the role of H-bonds from backbone amide group in HiPIPs, Low and Hill used chemical synthesis to introduce O instead of N in the backbone of Val42 and Ala57 in *Rhodocyclus tenuis* HiPIP protein. They showed that removal of these H-bonds will result in a lower E° value as expected [273].

One class of nonnative cofactors that are widely used in design and study of ET proteins are modified porphyrins. Porphyrins with modified electron withdrawing groups were used to change the redox potential and hence ET rate in a Mb model of C*c*O and it was shown that an increase in ET rate corresponds to increase in activity [274] (Figure 25). Similarly, addition of a diacetyl group to the heme in horse radish peroxidase resulted in an increase in E° [275].

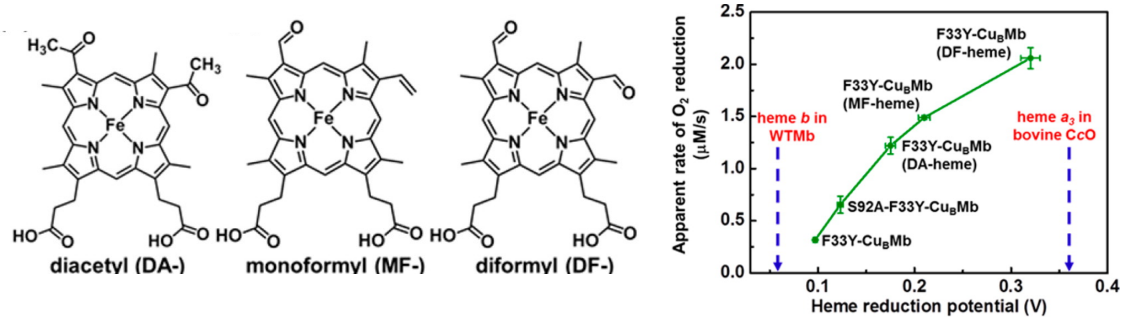


Figure 25. Addition of electron withdrawing groups to heme results in increase in reduction potential of the heme and in O_2 reduction activity in a Mb model of C*c*O. Figure adapted with permission from Ref. [274]

6. *Case studies*

6.1. Blue copper protein azurin as a platform for tuning redox potential

Azurin (Az) from *Pseudomonas aeruginosa* is one of the most well studied blue copper proteins. Several studies have been performed in order to understand the underlying role of different residues in determining the redox potential of Az and the ET rates in this protein [276-288]. Table 1 provides one of the studies in which E° of several Az variants were investigated under different pH conditions [289]. As shown in the table, replacement of the axial Met with hydrophobic ligands resulted in an increase in E° while addition of polar or charged ligands decreased it.

Table 1. Reduction potential of different Az variants. Reprinted from ref. [289]. Copyright © 2005, John Wiley and Sons under license number 3636610216032.

Protein	**pH**				
	4	5	6	7	8
Wild-type		346	333	310	293
His35Lys		344	337	317	296
Met44Phe	434	428	412	384	373
Gly45Ala		333	314	300	292
Asn47Asp				333	
Trp48Leu		352	345	323	306
Trp48Met		352	340	312	299
Lys85Glu		340	335	321	299
Ser89Gly	354	339	312	294	288
Glu91Gln		352	340	314	298
Phe114Ala		377	372	358	343
Phe114Val		346	340	324	314
Met121Ala				373	
Met121Asn				348	
Met121Asp			333	319	287
Met121Glu					184
Met121End				205	
Met121His				310	
Met121Ile				448	
Met121Leu			433	412	392
Met121Leu+Asn47Leu				510	
Met121Lys				318	
Met121Val				445	

In order to understand the role of different factors in SCS of T1Cu sites and their interplay in determining the E°, Marshall *et al*. used Az to incorporate several mutations in the SCS. As a result several variants were obtained that could span a range of ~700 mV in redox potential, never before reported in the cupredoxin superfamily (Figure 26) [235]. With only 3 mutations (Asn47Ser/Phe114Ans/Met121Leu) it was deomonstrated that the E° can increase beyond any reported E° values of mononuclear T1Cu sites. The mutant with the lowest E°, Phe114Pro/Met121Gln, had a redox potential very close to 0 at pH=9, never reported before in any natural cupredoxins or their variants [235]. The

authors further demonstrated that the effects of these specific mutations are additive in nature [235]. Replacement of the Met axial ligand with a more hydrophobic residue is accompanied with an increase in E°, while placing a more polar Gln residue at the same position decreased the redox potential, as explained in section 5.1. An Asn47Ser mutation also resulted in an increase in the reduction potential due to strengthening the H-bonds to a Cys ligand and rigidifying the site. The role of Phe114 is more complicated. Mutation of this residue to a Pro slightly disrupted the site, removing a H-bond to the Cys ligand, and hence decreased the E°. Mutation of Phe114 to Asn introduced an H-bond to backbone carbonyl of Gly45, and increased E° [235].

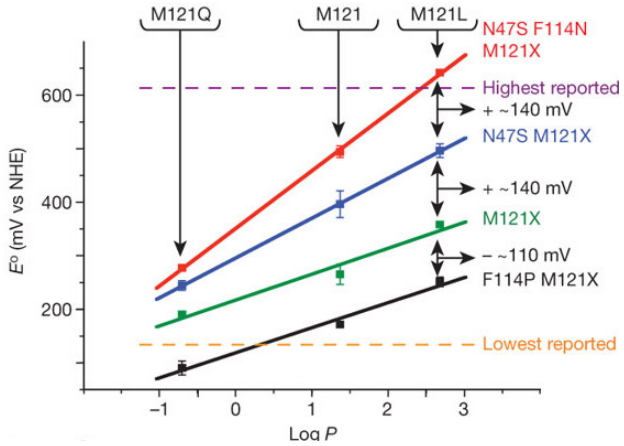


Figure 26. SCS mutants of azurin span a range of reduction potential never reported before. The effect of these mutations is additive. Adapted from [235]

A more in depth study on some of these mutants, Phe114Pro, Phe114Asn, and Asn47Ser, was performed to understand the underlying reason for their observed effects on E° [290]. Based on the experimental data that was obtained, Hadt *et al*. performed density functional theory (DFT) calculations on the mutants, and showed that the effects could be divided into covalent and nonlocal electrostatic contributions (Figure 27a) [290]. The covalent component arises from the changes in metal-ligand covalency, which can in turn influence the molecular orbitals. This component can be measured using S K-edge x-ray absorption spectroscopy studies. Active H-bonds (Figure 27b) within 5 Å of the metal site and carbonyl dipoles with a specific orientation towards the site, are the main SCS contributors to the covalent component. Nonlocal electrostatic interactions are another contributor to the changes observed in redox potential through affecting the enthalpy of the reaction. The effect of active H-bonds is additive. On the other hand, the effect of dipoles can be additive or oppose on other. The combination of these effects contribute to the fine-tuning of E° in azurin [290].

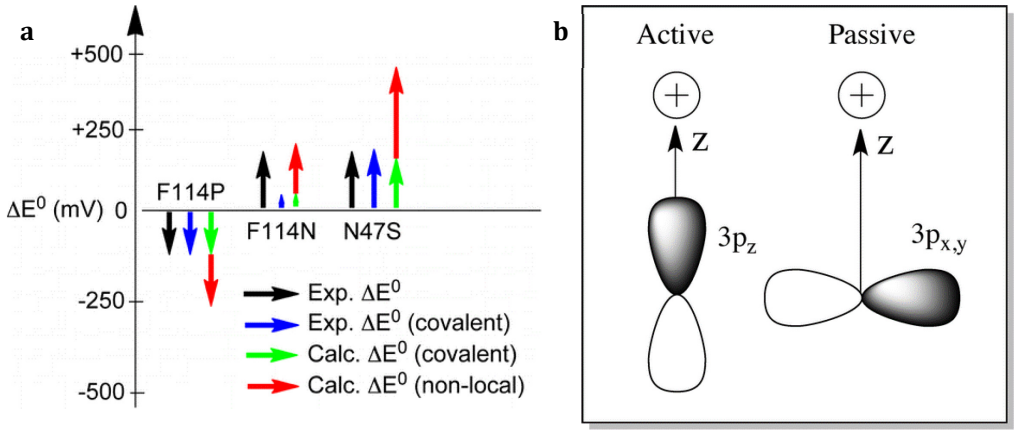


Figure 27. (a) Contribution of different factors in determining the E° in azurin mutants. (b) Active vs. passive H-bond. Reprinted with permission from [290].

Using this set of mutants, Farver *et al*. measured intramolecular ET rates from a disulfide to the copper site and showed that the ET rates increased upon increase in the redox potential, as predicted by Marcus theory of ET (Eq. 2) [291]. Interestingly they showed that the reorganization energy associated with redox transitions in these Az mutants is lower than that of WT-Az (Figure 28). It should be emphasized that the T1Cu site is already evolved to have very low reorganization energy, which is necessary for the rapid ET [291]. This lowering of reorganization energy is attributed to an increase in flexibility of the copper site for these mutants [291]. By considering more variants in SCS using the guidelines provided by previous studies, [235] they showed experimental evidence of the Marcus inverted region in a non-derivatized protein system [292] (Figure 28).

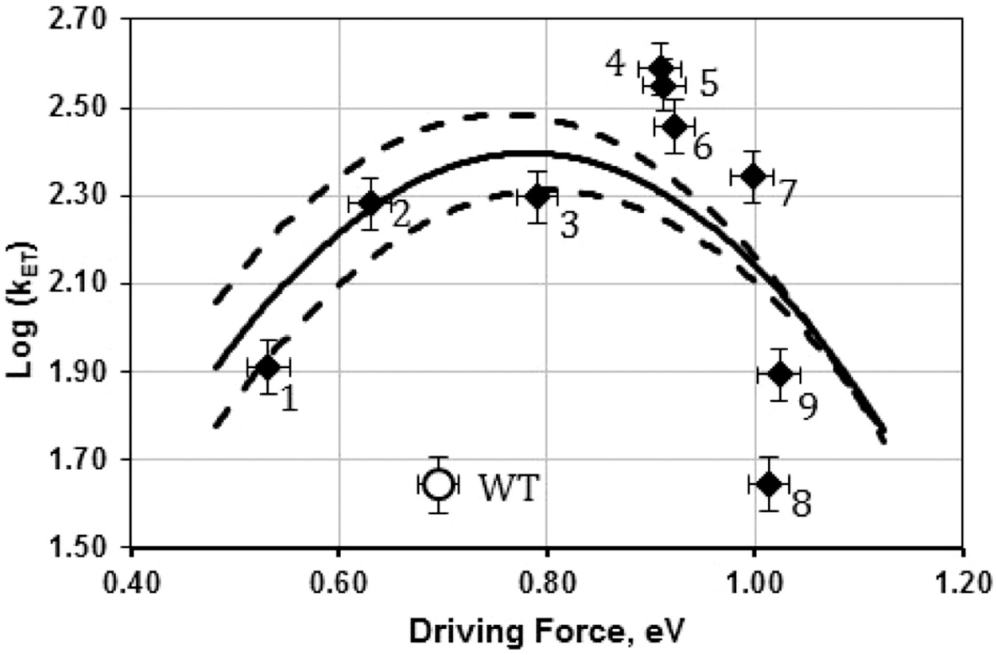


Figure 28. Plot of ET rate vs. driving force of intermolecular ET in several azurin mutants, Reprinted with permission from [292].

6.2. Heme b in myoglobin and cytochrome c peroxidase:

Heme *b* is one of the most versatile prosthetic groups found in nature. It is found in a series of proteins with a wide range of functionalities: from oxidation of molecules in P450s, to peroxidation in cytochrome *c* peroxidase (C*c*P), and to simply carrying oxygen or electrons in Mb and cytochromes. Some of the underlying reasons for their variety in activity lie within the differences in E° that result from the proximal ligand, and the H-bonding networks found in the distal and proximal sites. These interactions play a role in stabilizing different transition states of the heme iron. The E° of heme proteins span a range that is close to 1V, from -550 mV to +450 mV [181]. Several studies have been focused on understanding the mechanisms of E° tuning that govern their difference in activity. It has been shown that the E° is modulated by the overall protein fold (300mV range), type of the porphyrin (600 mV range), and axial ligands (800 mV range) [181]. Here we focus on the studies on Mb and C*c*P, both of which have a His as their proximal ligand to heme *b*.

6.2.1 Myoglobin:

Several residues in the SCS of Mb have shown to be important in tuning the E° of the heme center (Figure 29). One residue of interest in Mb is Val68 that is in van der Waals contact with heme *b*. Varadarajan *et al*. showed that mutating this residue to Asp, Glu, or Asn has drastic effects on the E°. As expected, removal of a hydrophobic residue resulted in a decrease in E°. The presence of negatively charged Asp or Glu residues decreased the E° by 200 mV while a polar residue like Asn decreased it by only 82.7 mV [257]. Several techniques including isoelectric focusing gel electrophoresis and UV-vis spectral inspection were used to confirm that Glu and Asp are ionized despite the fact that they are buried [257]. The corresponding residue in hemoglobin is Asp or Glu, and crystallographic studies have shown that this residue can weakly coordinate to heme, replacing the water ligand [293,294]. The crystal structure of the Val68Glu mutant in Mb also shows coordination of Val68Glu to the heme [295]. Replacement of heme with ZnPPIX (porphyrin that has Zn instead of Fe) resulted in proteins with same pI, suggesting that reduction of heme in Val68Asp/Glu is accompanied by a proton uptake from the aforementioned residue [257]. This observation was confirmed by the pH-dependent nature of the E°, which increased ~59 mV per unit decrease in pH.

Watanabe and coworkers studied a series of Mb mutants for investigating the heme degradation pathway and the role of His position in the regioselectivity of the reaction. Redox measurements of the mutants Phe43His/His64Leu and His64Leu showed a ~30 mV increase in E° values compared with WT-Mb, while a 10 mV increase was seen for Ile107His/His64Leu. Interestingly, Leu29His/H6is4Leu and Thr67Ala/Val68Ser had both reduction potentials of -22 mV, 70 mV lower than that of WT-Mb (52). The decrease in reduction potential is attributed to increased polarity of the heme environment, evidenced by the presence of two, rather than one, molecule of water in the active site [296]. Studies of a Thr67Arg mutant to mimic peroxidase activity suggested a higher reduction potential of compound II, consistent with the presence of a positively charged residue in the active site, which disfavors higher oxidation states more [297]. In a study on sperm whale Mb variants with peroxidase activity (Thr67Arg/Lys and Ser92Asp-Thr67Arg/Lys) it was shown that the reduction potential of heme was not

affected significantly. The reduction potential in these mutants had an enthalpic term disfavoring Fe(III) reduction and an entropic term selectively stabilizing the reduced state. The interplay between these two terms resulted in overall redox values similar to that of WT-Mb [298]. Ser92 is H-bonded to the proximal His in Mb. To study the effects of this H-bond, Ser92 was mutated to Ala or Asp. Surprisingly it was shown that the heme site structure of both WT and Ser92Ala were the same, with regards to orientation of the His imidazole. This indicates that the H-bond orientation from Ser to His was in a restricted conformation that was not observable with Asp [299]. Removal of an H-bond between the His93 proximal ligand and Ser92 in sperm whale Mb resulted in a 123 mV increase in the reduction potential by stabilizing the Fe(II) state more preferably over the Fe(III) state [274].

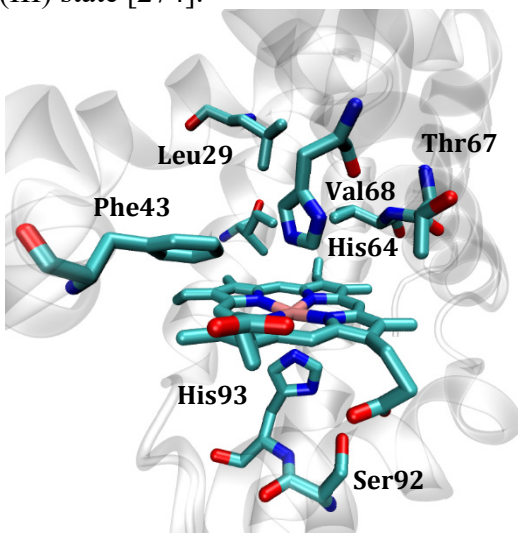


*Figure 29.*Several residues in primary and secondary coordination sphere of Mb that were discussed in terms of their effects on E° (PDB ID: 1MBN)

6.2.2. Cytochrome c peroxidase:

It has been shown that the E° of active species in heme peroxidases is closely related to E° of the Fe(II)/Fe(III) pair, which is easier to find experimentally [300]. Several studies have been performed to understand the effects of different residues (Figure 30) on tuning the E° of this enzyme, and on oxidizing substrates. C*c*P from yeast is by far the most well studied peroxidase. Table 2 shows several C*c*P mutants and their E° values, spanning a range of 113 mV [224]. Figure 30 of shows the positions of these residues in C*c*P. The residues in the distal pocket are Arg48, His52, and Trp51, all known to be well conserved among the peroxidases. While retaining the positive charge of Arg48 by replacing it with Lys didn't significantly change E°, mutating it to a nonpolar Leu residue increased it by 25 mV. As expected, positioning a negatively charged Glu at position 48 resulted in 50 mV decrease in the reduction potential. Replacement of His52 with Asn or Gln resulted in -70 mV and -35 mV change in reduction potential, respectively. This data is consistent with changing the ET center to a more polar environment. Placing a Lys residue at position 48 expectedly changed the E° in a positive direction. In the proximal pocket, Asp235 and Trp191 were analyzed. Removal of the conserved H-bond between Asp235 and proximal His175 by either Ala or Asn mutation resulted in 100 mV increase in the redox potential. Interestingly, even mutation to a Glu caused an increase in redox

potential, indicating the importance of optimal orientation and distance between H-bond donor and acceptor to exert the effect of the H-bond [301].

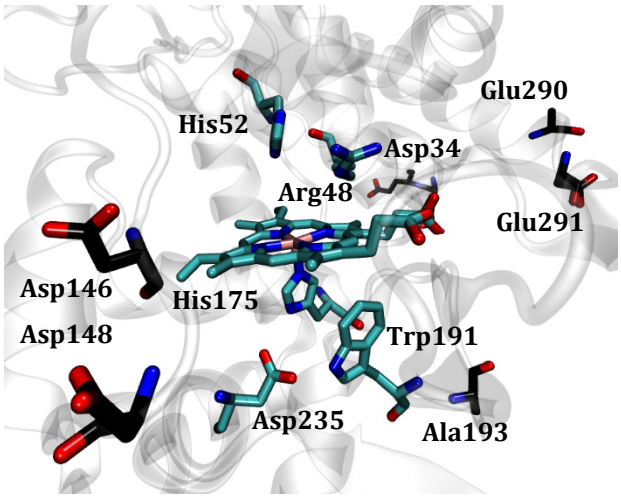


Figure 30. Several residues in primary and secondary coordination sphere of C*c*P that were discussed in terms of their effects on E_m. (PDB ID: 2CYP). Residues in black are surface charged residues.

Table2. Reduction potential of different C*c*P mutants. Adapted from [224] Copyright © 2006 Elsevier Inc. under license number 36636610646323.

Mutation	**E°**	**Mutation**	**E_m**
Wilde type enzyme		His52Lys	-157
Yeast C*c*P	-189, -194[a]	His52Leu	-170
MI-C*c*P	-194[b]		
MKT1-C*c*P	-183[c]	***Proximal pocket mutants***	
MKT2-C*c*P	-186[d]	His52Leu/Trp191Phe	-151
Distal pocket mutants		MI-Trp191Phe	-202[b]
Arg48Lys	-186	Ecoli-Trp191Phe	-187
Arg48Leu	-164	Asp235Ala	-78[c]
Arg48Glu	-179[d]	Asp235Glu	-113[c]
Arg48Ala/Trp51Ala/His52Ala	-163	Asp235Asn	-79[c]
Arg48Val/Trp51Val/His52Val	-150	**Surface mutants**	
Arg48Leu/Trp51Leu/His52Leu	-146	Glu32Gln	-168
Trp51His	-200	Asp34Asn	-175
Trp51His/His52Leu	-162	Asp146Asn	-173
His52Asp	-221	Asp146Asn/Asp148Asn	-173
His52Glu	-183	Ala193Phe	-170
His52Asn	-259	Glu290Asn	-177
His52Gln	-224	Glu291Gln	-162

a-data from ref. [243] of trans, b-data from ref. [302] of trans, c-data from [301]

6.3. MitoNEET as a case study for FeS proteins

In a mutational study on MitoNEET, a recently discovered unique 2Fe-2S protein, Zuris *et al*. could tune the redox potential in a range of ~700 mV, the largest range reported so far for any FeS protein [303]. One of the ligands to the FeS site (Figure 31a), His87 and two SCS residues, Lys55 and Asp84, were the targets of their study. A decrease in E° upon mutation of His to Cys agrees with previous reports on the role of electron donating features for the primary ligands in tuning the redox potential. Lys55 is a positively charged residue near the ligated His87, and can participate in H-bonding with the ligand. The authors showed that the primary role of this Lys residue is in changing the pKa of the His87. Replacement of the Lys with neutral Gln or negative Glu residues, respectively, resulted in an increase in pKa and hence an increase in the E°. Removal of Lys by a Lys55Met mutation in a variant without His87, however, confered no significant effect in E°, consistent with the known importance of interactions between Lys and His. Asp84 is in H-bonding distance to the inorganic S ligand of the FeS cluster. Removal of the negative charge from Asp84 by mutating it to Asn or Ser results in an increase in reduction potential, due to destabilization of the higher oxidation state. As shown in figure 31, the effects of mutations can be additive. An Asp84Gly mutation resulted in much higher increase in the E° due to changes in solvent accessibility and the overall organization of the site [303].

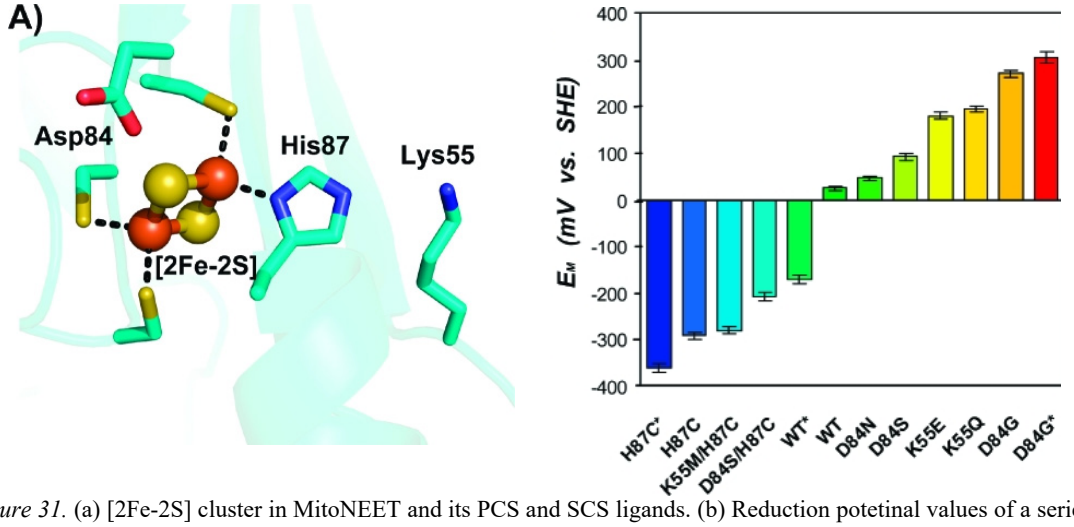


Figure 31. (a) [2Fe-2S] cluster in MitoNEET and its PCS and SCS ligands. (b) Reduction potetinal values of a series of MitoNEET mutants. * indicates extreme potentials. Adapted from ref. [303] copyright {2010} American Chemical Society.

6.4. Mn-Fe superoxide dismutase

An interesting case for the role of E° in tuning enzymatic functions is observed in superoxide dismutases (SODs). While FeSOD and MnSOD have very similar structures, metal substitution studies showed that switching of the two metals resulted in inactive enzymes, an observation that was attributed to differences in their E°. It has been shown that MnSOD proteins lower the E° of the metal site inherently much more than FeSODs. Hence positioning of an iron atom in MnSOD will result in Fe couples with much lower E° than is required for the activity. This lowering of the E° is required for the metal ions to be capable of performing their activity. It has been suggested that this lowering is

accompanied by the presence of a significant number of negatively charged residues in SCS of the metal binding site [304].

It was also proposed that the differences observed in suppression of metal ion E° is due to different uptakes of protons that are coupled to metal ion reduction [305,306]. The protonation in SODs occurs through acquiring a proton from the solvent, hence it has been postulated that MnSODs suppress the E° more than FeSODs by disfavoring protonation of the coordinated solvent molecule [305,306] (Figure 32a). A Gln residue in the SCS of MnSOD is shown to play an important role in tuning the E° of the site. The presence of this Gln residue preferentially destabilizes H_2O coordination compared with OH^- coordination, hence favoring the oxidized state of the metal ion [307]. Mutation of this Gln to Glu in FeSOD resulted in a higher E° compared to that of the wild type [308]. While Glu and Gln are isostructural, Glu can act as an H-bond donor, stabilizing the water bound Fe^{2+} form, hence increasing the E°. A combination of X-ray crystallographic studies, MCD, nuclear magnetic resonance (NMR), and DFT calculations were used to confirm this effect. It was shown that a combination of three effects contribute to a large (>600mV) increase in E° upon Gln69Glu mutation: loss of H-bond donation to solvent H_2O in the reduced state, a very strong H-bond acceptance from the solvent in the reduced state, and a subtle yet significant change in the origin of protons uptaken by the solvent [308,309]. Precise positioning of this SCS Gln is suggested as a mechanism by which SODs can tune their reduction potentials, resulting in increasing E° values with MnSOD< Fe(Mn)SOD<FeSOD). By changing this SCS ligand and metal ion, a 900 mV range of reduction potentials were obtained in a single protein scaffold [310] (Figure 30b).

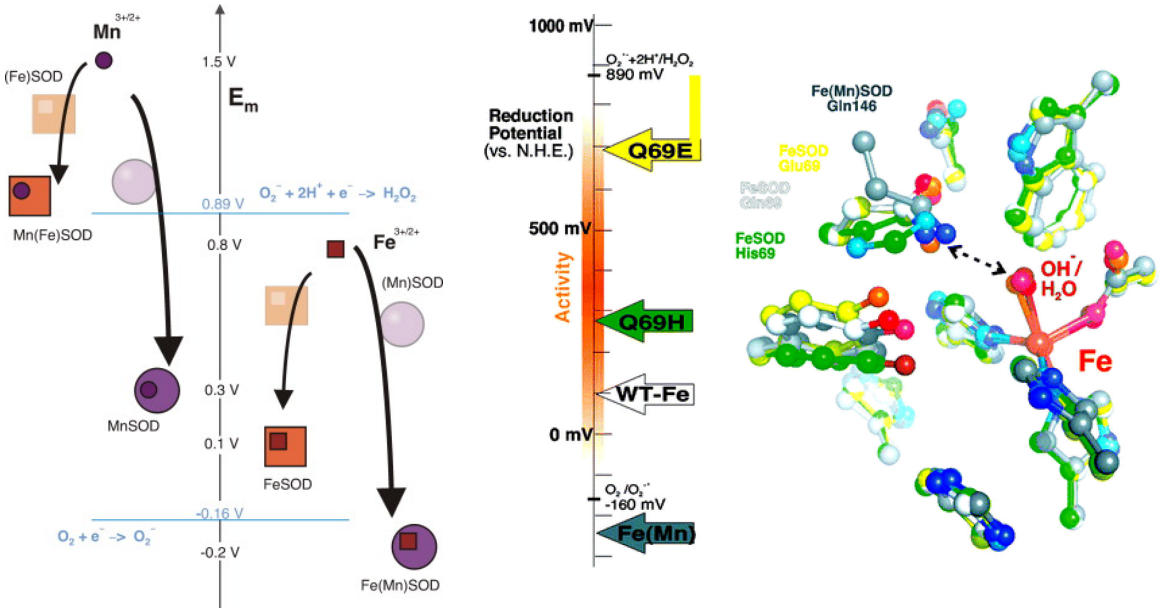


Figure 32. (a) Lowering the E° value in Fe-SOD vs Mn-SOD with different metal ions. (b) Tuning the E° value of SOD by SCS mutations. Figures adapted from ref. [310] Copyright © 2008, American Chemical Society.

7. Summary and outlook

Redox potentials are the major contributors to controlling the ET rates and thus regulating ET processes. To maximize the efficiency of the ET process, one needs to master the art of tuning of the E°, especially metalloproteins, as they represent major classes of ET proteins. While the roles of metal ions and the coordinating ligands cannot be underestimated as major contributors in determining the E° of these sites, these primary coordination spheres are evolved for many years to fit the functions they perform. For example, the primary coordination spheres of T1Cu proteins are known to have very low reorganization energy, which is critical to its ET function. It has been shown that any changes in the PCS, such as geometry or ligand will affect the reorganization energy, making the ET reaction less efficient. Therefore, within a specific class of proteins, one has to exploit SCS interactions to tune the E° of the metalloproteins in order to produce tailor-made redox reagents.

The most important contributor to reduction potential among SCS interactions is the axial interaction. The hydrophobicity of the axial interaction is shown to be important in tuning the E°: the more hydrophobic the ligand is, the higher the E° will be. The H-bonding interaction around the metal center will not only determine the correct positioning and geometry of the primary ligand, but will also cause subtle changes in the properties of these ligand through modulating the electron density on the metal center. The hydrophobicity of the site and nearby charges can also significantly change E°: the presence of either a positive charge or increased hydrophobicity of the site will result in an increase in E°. Other factors such as the presence of aromatic groups, and the overall constitution of residues in SCS can also affect E°, although usually less significantly.

While the general trends explained in this review are applicable to most systems, one should remember that these effects could work in the same direction or opposite to each other. Especially with amino acid substitution, it is usually very difficult to control one specific factor without affecting others. So one should be very careful in designing mutations. Using UAAs that are isostructural to the one being replaced with only a specific change can provide a unique opportunity to selectively tune E°. Another important fact is that the extent of these effects cannot be easily predicted in different proteins. An example of such a difference is seen in case of T1Cu in Az and Cu_AAz [311]. Hence, comprehending the results can be a daunting task in cases. It should also be noted that there are exceptions to these general rules. For example. there are reports of FeS clusters that are significantly more buried than their counterparts, but no significant redox potential difference is observed between them [162].

In general, using SCS interactions to tune the E° is a task frequently performed by nature to ensure efficiency and specificity within metal centers with similar primary ligands, which are fixed to have optimal activity. These features have only recently been recognized as a prime tool to design ET cofactors and proteins with tunable E° required to improve their functionalities. In comparison with mutations of PCS ligands, fine-tuning of the SCS interactions requires careful design and thorough investigation of the effects by spectroscopic and x-ray crystallographic studies to ensure the design has the intended efforts. With more and more studies devoted to SCS tuning and more tools to aid the endeavor, we will see more successful examples of designed ET centers and tuning of their redox potentials, some of which will see wide applications such as in solar energy transfer and water oxidation.

Abbreviations:

ET= electron transfer
PS-I/II= photosystem I/II
FeS= iron-sulfur
FNR= ferredoxin-NADPH reducatse
NAD= nicotinamide adenosine dinucleotide
HSAB= hard-soft acid-base
C*c*O= cytochrome c oxidase
T1/2Cu= Type 1/2 copper protein
SOD= superoxide dismutase
Mb= myoglobin
Az= azurin
SAM= self-assembled monolayers
E°= reduction potential
EPR= electron paramagnetoc resonance
C*c*P= Cytochrome *c* peroxidase
PCS=primary coordination sphere
SCS=secondary coordination sphere
SHE= standard hydrogen electrode
HiPIP= high potential iron-sulfur protein
H-bonding= hydrogen bonding
NMR= nuclear magnetic resonance
DFT= density function theory
H-NOX= heme nitric oxide/oxygen reductase

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