Published in final edited form as:

Metallomics. 2011 February ; 3(2): 140–151. doi:10.1039/c0mt00061b.

Cupredoxins – A study of how proteins may evolve to use metals for bioenergetic processes.

Moonsung Choi, Victor L. Davidson*

Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216

Summary

Cupredoxins are small proteins that contain type I copper centers, which are ubiquitous in nature. They function as electron transfer shuttles between proteins. This review of the structure and properties of native cupredoxins, and those modified by site-directed mutagenesis, illustrates how these proteins may have evolved to specifically bind copper, develop recognition sites for specific redox partners, tune redox potential for a particular function, and allow for efficient electron transfer through the protein matrix. This is relevant to the general understanding of the roles of metals in energy metabolism, respiration and photosynthesis.

I. Copper in nature

While present in trace amounts, copper is a critical component of biological systems. Very little free copper is found in living systems; rather it is bound to proteins and enzymes. The functional copper-binding sites in proteins are classified as type 1, 2 or 3 (Figure 1).^{1, 2} This designation depends primarily the spectroscopic properties of the protein-bound copper which is a consequence of the number of coppers bound and the copper ligation geometry. Type I copper centers bind a single copper atom with distorted tetrahedral ligation geometry. The copper is coordinated by two nitrogens from two His residues, a sulfur from a Cys residue and typically a sulfur from a Met residue. Relatively small proteins with a single type 1 copper center and no other metal or cofactor are commonly called blue copper proteins or cupredoxins. In the oxidized state, cupredoxins exhibit an intense visible absorption centered near 600 nm and an EPR spectrum characterized by very narrow hyperfine splitting in the direction parallel to the magnetic field ($A_{||}$) (Figure 2).³ Type 1 copper sites function primarily as mediators of electron transfer. Type 2 copper centers bind copper in a square planar coordination with nitrogen ligands typically provided by His and oxygen ligands provided by Asp or Tyr residues. Type 2 copper proteins exhibit a weak visible absorption around 700 nm, and an axial electron paramagnetic resonance (EPR) spectrum with copper hyperfine splitting in the parallel region similar to that observed in inorganic copper coordination compounds. The type 2 copper centers occur in enzymes, where they assist in biological oxidations. Type 3 copper centers are binuclear in that they bind two copper atoms, each coordinated by three nitrogens from three His residues. Type 3 copper proteins exhibit no EPR signal due to strong antiferromagnetic coupling metal ions.

* Address correspondence to: Victor L. Davidson, Department of Biochemistry, University of Mississippi Medical Center, 2500 N. State St., Jackson, MS 39216-4505. Tel: 601-984-1516. Fax: 601-984-1501. v davidson@umc.edu.

The type 3 copper centers occur in enzymes which are oxidases and oxygenases, and in oxygen-transporting proteins. In addition, there are enzymes that have been characterized that use multiple type 1, 2 and 3 copper sites, sometimes in combinations of different types to catalyze reactions.⁴ As seen in Table 1, the functions of copper-containing enzymes and proteins are diverse and their functions are critical for respiration, photosynthesis and proper metabolism.

II. Type 1 copper proteins (cupredoxins)

Type 1 copper sites are ubiquitous in nature and typically function as mediators of electron transfer through and between proteins. Many cupredoxins have been structurally and spectroscopically characterized.⁵ The type 1 copper site always involves strong coordination from a Cys and two His residues in an approximately trigonal planar arrangement with a weaker axial ligand, usually provided by a sulfur of a Met residue, which displaces the Cu^{2+} from the trigonal plane. The strong interaction between the Cu^{2+} and the sulfur of the Cys ligand is responsible for the intense blue color that arises from a band at approximately 600 nm due to a $\text{S}(\text{Cys})\pi \rightarrow \text{Cu}(\text{II})d_{x^2-y^2}$ ligand-to-metal charge transfer transition.³ This distorted tetrahedral geometry of the type 1 copper site of amicyanin, which is typical of most cupredoxins, is shown in Figure 3. An example of a type 1 copper site in which Met does not provide the axial ligand is stellacyanin where the axial ligand is a side-chain oxygen of a Gln residue.⁶ This stronger axial ligation, as evidenced by the shorter bond length further displaces the Cu^{2+} from the trigonal plane yielding a slightly more rhombic geometry. A variation of the typical type 1 geometry is seen in azurin, where a second weak axial interaction with the copper is provided by a backbone carbonyl oxygen atom.⁷ Another variation in the type 1 geometry is seen in the type 1 copper site of ceruloplasmin, which is not a small cupredoxin but a multicopper eukaryotic protein that is comprised of six cupredoxin-like domains and contains multiple types of copper sites. In one of the type 1 sites, the position typically occupied by the axial ligand is a Leu residue which cannot provide a ligand, and the Cu^{2+} possesses only three ligands in a trigonal planar geometry,⁸ yet retains the spectroscopic features of a type 1 copper site.

While a general function for cupredoxins as electron transfer mediators has been well established, the specific physiological redox partner proteins for specific cupredoxins have only been identified in a few cases. This review will focus on studies performed on amicyanin from *Paracoccus denitrificans*,⁹ a representative cupredoxin. Its physiological electron transfer donor protein, methylamine dehydrogenase (MADH),¹⁰ and electron transfer acceptor protein, cytochrome *c*-551i,¹¹ have been identified, and the structure of the electron transfer complex of these proteins has been determined (Figure 4).¹² The binding and rate constants for the reactions between these proteins have been characterized,^{13–15} and extensive site-directed mutagenesis studies have elucidated a variety of structure function relationships. The results of these studies and those with other cupredoxins will be used to discuss four specific areas of cupredoxin structure, function and evolution. How did cupredoxin structure evolve to specifically bind copper? How did the relatively simple and highly conserved overall structure of cupredoxins evolve to react with a wide range of structurally diverse redox partners? How did cupredoxin structure evolve to tune the electronic properties of the highly structurally conserved copper site to span a wide range of

oxidation-reduction midpoint potential (E_m) values, thus expanding the range of roles it may play as a biological electron transfer mediator? Considering that different redox partner proteins of cupredoxins can bind to different sites on the surface of the protein, how did cupredoxins evolve to efficiently mediate electron transfer via different pathways through this conserved structure? The answers to these questions are relevant to understanding the roles of copper, and metals in general, in energy metabolism, respiration and photosynthesis.

III. How did metalloproteins arise?

It has been estimated that approximately one third of all enzymes contain metals. The choice of the metal depends on its ionic radius, on the reaction to be catalyzed, on the E_m value needed, on the type of ligands available and on the biological availability of the metal. How and why metalloproteins came to be are fundamental questions. It seems unlikely that a protein evolved *de novo* to bind a specific metal for a specific function. More likely a non-metal-containing protein, which evolved and persisted for some other function, fortuitously was suited to bind a metal. Binding of this metal endowed the protein with enhanced stability or a new function, or both. This initiated new lines of evolution which fine-tuned this metalloprotein for a variety of new functions.

Type 1 copper proteins are a straightforward system with which to consider these questions of metalloprotein evolution and metal-binding specificity. Cupredoxins are relatively small proteins of 10–20 kDa and possess relatively simple structures comprised primarily of beta sheets and turns (Figure 5).² The bacterial cupredoxins function in the periplasmic space of gram negative bacteria rather than in the cytoplasm, and there is no evidence for involvement of chaperones in cupredoxin assembly. One clear advantage for cupredoxins to bind a metal is to enhance their stability. Such an effect on thermal stability was demonstrated with *P. denitrificans* amicyanin. The folded apoamicyanin was prepared from the holoprotein by reduction with sodium dithionite followed by dialysis against 0.1 M KCN.¹⁶ The crystal structure of this apoamicyanin was identical to that of the holoprotein except for the absence of copper.¹⁷ Differential scanning calorimetry revealed that the midpoint temperature for thermal transitions (T_m value) for apoamicyanin was significantly lower for apoamicyanin than for the copper-containing holoprotein.¹⁸ This indicated significant structural destabilization in the absence of bound type 1 copper. It follows that during evolution a random mutation which allowed a pre-existing protein to bind a metal which enhanced the protein's stability could provide a strong selective advantage. If binding of a metal also endowed the protein with a new beneficial function, then this would be an even greater selective advantage. This new function need not be the one which exists today. For example, free copper in solution is potentially toxic as it can generate reactive oxygen species under certain circumstances. If a bacterial periplasmic protein acquired the ability to tightly bind copper, then this could conceivably serve as a defense mechanism against copper toxicity. The periplasmic location of cupredoxins could also provide an opportunity for a redox-active metalloprotein to acquire roles in electron transfer and respiration. The periplasmic space in bacteria is analogous to the mitochondrial intermembrane space where cytochrome *c* functions as an electron transfer mediator during respiration. Cupredoxins are known to interact with components of the bacterial membrane-bound respiratory chains and mediate the entry of electrons into the respiratory chain from periplasmic dehydrogenases. It seems

reasonable to believe that a stable redox-active metalloprotein could have adapted to perform these tasks and gain new physiological roles over time.

IV. What dictates the metal specificity for the type 1 copper site of proteins?

There is ample evidence that the type 1 center of cupredoxins can accommodate metals other than copper. It is possible to reversibly remove the copper to generate the metal-free apoprotein and then reconstitute the apoprotein with copper or some other metals. In particular cobalt- and nickel-substituted cupredoxins have been generated and studied.^{19–23} Furthermore, recombinant cupredoxins that are expressed in the cytoplasm of *E. coli* are sometimes isolated with zinc rather than copper occupying the site.²⁰ These zinc-containing proteins are inert but stable. Given the very low concentrations of free soluble copper in biological systems, and the ability of the type 1 site to accommodate other metals, one wonders what factors dictate the specificity for copper insertion into the type 1 site *in vivo*.

It was shown for amicyanin that the nature of the axial ligand exerts a strong influence over the specificity for metal incorporation. Recombinant *P. denitrificans* amicyanin which was expressed in the periplasm of *E. coli*²⁴ was isolated with full occupancy of the type 1 site by copper. Mutation of the axial Met98 ligand of this amicyanin to Gln, Ala, or Leu resulted in the synthesis and isolation of amicyanin which contained primarily zinc rather than copper bound to the type 1 site.^{25–27} The Zn²⁺ could be removed by unfolding the protein in the presence of EDTA. Then refolding the protein in the presence of Cu²⁺ generated the copper-containing mutant amicyanins whose properties were altered by the mutations but which did react with the physiological redox partner proteins.^{26–28} The influence of the nature of the axial ligand on the specificity of metal binding was studied by comparing the relative affinities of native and M98Q apoamicyanins for Zn²⁺ versus Cu²⁺ in the folded, partially folded and unfolded states.²⁵ Surprisingly, the influence of the axial ligand of the type 1 copper site on metal specificity was the strongest prior to the completion of protein folding and adoption of the final type 1 site geometry. Thus, at the point during the biosynthetic process when the type 1 copper site is not yet formed, the residue which ultimately provides the weakest ligand is a strong determinant of the specificity of the metal for incorporation. This also suggested that metal incorporation *in vivo* most likely occurs during protein folding in the periplasm and not to a preformed type 1 site.²⁵ It should be noted that not all type 1 copper proteins possess a Met axial ligand, and stellacyanin naturally has an axial Gln ligand yet contains copper (see Figure 3).⁶ So while the presence of methionine as an axial ligand is not the sole and absolute requirement for copper specificity, there is evidence that it is a very strong determinant.

V. What dictates the specificity for the redox partner proteins that interact with different cupredoxins?

Many biological processes involve highly specific interactions between proteins which either strongly or weakly associate. “Static” complexes are characterized by a strong binding affinity with slow dissociation (e.g., antibody-antigen interactions and signal transduction

protein complexes). “Transient” complexes have weaker binding affinity with a shorter lifetime. Electron transfer between soluble proteins typically involves formation of transient complexes. Readily reversible protein interactions allow continuous electron flow between redox partners. Specificity is also critical to ensure that electrons are not transferred to the wrong molecule which would waste energy and potentially lead to formation of damaging free radicals and reactive oxygen species. Since cupredoxins are electron transfer mediators, they must interact with two different proteins, one that donates electrons and one that accepts electrons. While few complexes of cupredoxins with their physiological redox partner protein have been structurally characterized, those that have provide insight into how the specificity of these interactions is achieved. Four examples are discussed below.

A. Amicyanin

Amicyanin⁹ serves as a mediator of electron transfer from methylamine dehydrogenase (MADH)¹⁰ to cytochrome *c*-551i.¹¹ These three proteins are isolated from *P. denitrificans* as individual soluble proteins but they have to form a ternary complex to catalyze methylamine-dependent cytochrome *c*-551i reduction.^{14, 29} The complex of MADH, amicyanin, and cytochrome *c*-551i is one of the best characterized physiological protein electron transfer systems (Figure 4).³⁰ The proteins have been structurally characterized by x-ray crystallography as the binary complex of MADH and amicyanin,³¹ and the ternary protein complex¹² which includes cytochrome *c*-551i. The protein complexes were shown to be catalytically active and able to perform electron transfer in the crystalline state.^{32, 33} Electron transfer from MADH to the cytochrome is thermodynamically favorable but does not occur because the proteins are unable to interact with other. The E_m value of amicyanin (+294 mV) is more positive than that of cytochrome *c*-551i (+190 mV).³⁴ This means that when amicyanin is free in solution and interacts with the cytochrome, the direction of electron transfer would be from the cytochrome to amicyanin, the reverse of the physiological direction.^{29, 35} On complex formation with MADH, the E_m value of amicyanin becomes more negative which allows the electron transfer in the physiological direction to become more favorable. This is because a redox state-dependent conformational change that is linked to the protonation of His95 of amicyanin is sterically constrained in the complex with MADH relative to free amicyanin.³⁶ Thus, formation of the ternary protein complex is critical for electron transfer from MADH to cytochrome *c*-551i.

The structure of the protein complex indicated that the interface between MADH and amicyanin is stabilized largely by hydrophobic interactions (Figure 6), and possibly one or two intermolecular salt bridges at the periphery of this hydrophobic interface.³¹ The specific contributions of these hydrophobic and electrostatic interactions were investigated using site-directed mutagenesis.²⁴ Conversion of Phe97 to a more polar Glu residue, increased the K_d for complex formation with MADH by two orders of magnitude, consistent with the importance of this hydrophobic patch. Arg99 and Lys68 of amicyanin were inferred from the structure to possibly form salt bridges with residues on MADH. While a K68A mutation did not affect binding, R99D and R99L mutations did significantly increase the K_d for complex formation and altered the ionic strength dependence of binding. These results demonstrate that a combination of specific hydrophobic and ionic interactions is required to stabilize complex formation between MADH and amicyanin, and that individual amino acid residues

on the protein surface are able to dictate very specific interactions between soluble redox proteins. Interestingly the structure of the ternary protein complex reveals that the interaction of amicyanin with cytochrome *c*-551i is stabilized a different set of interactions. That protein interface involves multiple van der Waals and hydrogen bonding interactions including some with bridging water molecules.¹²

B. Plastocyanin

Plastocyanin is critical for photosynthesis in plants and bacteria as it functions as a soluble mobile mediator of electron transfer from the membrane-bound cytochrome *f* of the cytochrome *bf* complex to the membrane-bound photosystem I.³⁷ The crystal structures of several plastocyanins from plants and algae revealed two potential molecular recognition sites for its redox partners (Figure 7).^{38, 39} One is a hydrophobic patch; similar to what is seen in amicyanin (Figure 6), which is a region of nonpolar residues around the solvent accessible, exposed His ligand of copper. The other, which is unique to plastocyanin is an acidic patch which was thought to interact with a basic Lys-rich region on the surface of cytochrome *f*.^{38, 40–42} No crystal structure of a complex of plastocyanin with a redox partner protein is available but the solution structure of the complex between plastocyanin and cytochrome *f* was determined by NMR.⁴³ This study confirmed the interaction between residues in the lysine-rich domain on cytochrome *f* with the acidic patch of plastocyanin, and interactions between the hydrophobic patch of plastocyanin and hydrophobic residues around the heme of cytochrome *f*. Given the close proximity of the copper and heme redox sites of the two proteins at the interface of the hydrophobic patches, this interaction is thought to be directly related to mediation of interprotein electron transfer. This has been confirmed by site-directed mutagenesis studies and the acidic patch appears to provide a docking site for the initial binding with cytochrome *f*.

The presence of the acidic patch is not common to all plastocyanins (Figure 7). Plastocyanin from plants actually have two acidic patches, one near the hydrophobic patch and the other one further away. Plastocyanin from algae has the one acidic patch, corresponding to the one that is further from the hydrophobic site. In addition to the eukaryotic plants and algae, plastocyanin is found in the prokaryotic cyanobacteria.⁴⁴ Interestingly, the acidic patch is absent in prokaryotic plastocyanin which only exhibit the hydrophobic patch.⁴⁵ Thus, plastocyanin provides an example of how residues on the surface of this protein, whose overall tertiary structure is highly conserved, have adapted during evolution to apparently enhance specificity for its redox protein partner by going from one to two to three distinct recognition sites for protein-protein interaction.

C. Azurin

Azurins are present in several bacteria and are located in the periplasmic space. Expression of azurin is typically constitutive and it is believed to serve a general function in mediation of electron transfer. A crystal structure is available for one complex of an azurin with its physiological redox partner, the complex of aromatic amine dehydrogenase (AADH) and azurin from *Alcaligenes faecalis*.⁴⁶ The structure reveals that the AADH-azurin interface is largely hydrophobic with one direct hydrogen bond.

It is of particular interest to compare the protein interactions in this complex with those in the MADH-amicyanin complex described earlier. AADH from and MADH are structurally highly conserved proteins which each possessing tryptophan tryptophylquinone (TTQ)⁴⁷ as its redox cofactor.^{46, 48} While the kinetic and structural properties of AADH and MADH are very similar,^{49, 50} the electron acceptor specificity is different; azurin rather than amicyanin.⁵¹ Neither copper protein can substitute for the other as an effective electron acceptor.⁵¹ The regions of MADH and AADH that bind their respective copper proteins are essentially the same. As does amicyanin, azurin also interacts with AADH via the hydrophobic patch near the copper site. However, the orientation of azurin with AADH is largely different from that of amicyanin with MADH being rotated by 90° degree with respect to azurin.⁴⁶ Also, the fraction of hydrophobic residues in AADH-azurin complex is larger than that in MADH-amicyanin.⁴⁶ This fact is consistent with solution studies which showed that the AADH-azurin complex was stabilized at high ionic strength,⁵¹ which is the opposite of what was observed for the MADH-amicyanin complex. Thus, subtle changes at the periphery of the hydrophobic patch, a hydrogen bond with azurin versus a salt bridge with amicyanin, allows these two cupredoxins to discriminate between two structural similar electron donor proteins.

D. Rusticyanin

Rusticyanin is localized in the periplasm of acidophilic bacteria and is involved in mediation of electron transfer from Fe^{2+} ions in the extracellular environment to the membrane-bound respiratory chain.⁵² The best studied rusticyanin is that from *Thiobacillus ferrooxidans*. Rusticyanin forms a complex with cytochrome c_4 to mediate electron transfer.⁵³ Rusticyanin is distinct from other cupredoxins in that it contains an N-terminal extension of 35 amino acids that protects the β -barrel core from its interaction with the solvent, maintaining a high degree of hydrophobicity of the protein.^{54, 55} No crystal or NMR structure of the rusticyanin-cytochrome c_4 complex is available; however, modeling studies revealed possible hydrogen-bond mediated recognition sites that included several water molecules that could stabilize this complex.^{56, 57}

VI. How do cupredoxins tune the redox potential of type 1 copper site?

Despite their highly conserved structures, type 1 copper sites in proteins exhibit E_m values that range from 184 mV for stellacyanin⁵⁸ to >1000 mV for type1 copper center in human ceruloplasmin.⁵⁹ A common feature of cupredoxins is that the E_m values are all more positive than that of the $\text{Cu}^{2+}/\text{Cu}^+$ aqua couple. This is likely due to structural constraints which are imposed upon the copper by the protein in the type 1 site. As discussed in the rack-induced concept of copper binding in cupredoxins, the type 1 copper protein forces the copper into a configuration intermediate between those preferred by Cu^{2+} and Cu^+ .⁶⁰ This could minimize the difference in relative stabilities of Cu^{2+} and Cu^+ in the type 1 copper site relative to those in solution which would account for the more positive E_m values of cupredoxins than aqueous copper complexes. The Cu^+ -axial ligand bond distance in the reduced cupredoxin also influences the E_m value.⁶¹ A normal Cu^+ - S_{met} bond length is about 2.3 Å, whereas in cupredoxins the observed bond length is about 2.9 Å. This increased distance would reduce the charge donation of the ligand to the copper ion, which would

increase the E_m value. Further variation in E_m value is also to some extent due the nature of the axial ligand; however, most cupredoxins possess a Met ligand so clearly other factors are in play. These factors include desolvation or hydrophobic effects, hydrogen bonding pattern around the copper site, and intraprotein electrostatic interactions.⁶²

A hydrophobic environment raises the E_m value of the $\text{Cu}^{2+}/\text{Cu}^+$ redox couple by preferential stabilization of the less charged Cu^+ oxidation state. All of the structurally characterized cupredoxins have their type 1 copper centers embedded in a hydrophobic region of nonpolar side chains. The highly positive E_m value of *T. ferrooxidans* rusticyanin (680 mV) has been attributed in part to its unusually high hydrophobicity due to an N-terminal extension of 35 amino acids that contributes to the rigid hydrophobic core.^{54, 63, 64} Numerous site-directed mutagenesis studies with different cupredoxins have demonstrated the relationship between E_m value and hydrophobicity of metal-binding site. When the residue providing the axial copper ligand is replaced with a hydrophobic amino acid such as Leu, the E_m value typically becomes ~130mV more positive (Table 2). For example, an M98L amicyanin mutation increases E_m value by 127 mV,²⁷ an M148L rusticyanin mutation increases E_m value by 131 mV,⁶⁵ and an M121L azurin mutation increases E_m value by 135 mV.⁶⁶ A clear correlation between the E_m value and amino acid side chain hydrophobicity of the axial ligands was also obtained using recombinant azurin in which the axial Met was replaced with unnatural amino acids which are iso-structural analogues of Met.^{66–68}

Site-directed mutagenesis studies of cupredoxins have clearly demonstrated that the hydrogen bonding pattern around the copper site, especially hydrogen bonds involving the thiolate of the Cys ligand, influences the E_m value (Table 3). In *P. denitrificans* amicyanin mutation of Pro94 to Phe or Ala increased the E_m value by 120 mV and 150 mV, respectively.⁶⁹ High resolution x-ray crystal structures of the mutants revealed that in each an electron-withdrawing hydrogen bond to the copper-coordinating thiolate sulfur of Cys92 is introduced by movement of the amide nitrogens of Phe94 and Ala94 much closer to the thiolate than the amide N of Pro94, which in native amicyanin is too far from the thiolate to form a hydrogen bond.⁷⁰ Similarly, a P80I mutation in *A. faecalis* pseudoazurin introduced an additional hydrogen bond from the protein backbone to the thiolate of the Cys ligand resulting in a 180 mV increase in E_m value.⁷¹ Conversely in azurin, a F114P mutation which resulted in loss of a hydrogen bond decreased the E_m value by 94 mV.^{6, 67}

Certain cupredoxins also exhibit a pH-dependent E_m value. The E_m value of amicyanin increases by approximately 60 mV per pH unit. This is because in reduced *P. denitrificans* amicyanin, a pH-dependent conformational change occurs on protonation of the His95 copper ligand with an associated pK_a value of 7.7.³⁶ When protonated, His95 rotates 180° about its $\text{C}\beta\text{—C}\gamma$ bond effectively removing it from the copper coordination sphere. A similar “histidine flip” is observed with plastocyanin,⁴² but it exhibits a more acidic pK_a value of 4.9 for spinach plastocyanin.⁷² It should be noted that these different pK_a values correlate approximately with the physiological pH of the periplasmic space and thylakoid space, respectively, where amicyanin and plastocyanin are localized and function.

VII. How do cupredoxins optimize rates of interprotein electron transfer reactions?

Cupredoxins have been extensively studied as model systems for protein electron transfer reactions. Azurin and plastocyanin, which were labeled at various sites on the protein surface with redox-active ruthenium complexes, have been used to study the distance-dependence of the rate of interprotein electron transfer to and from the copper.^{73–75} Using NMR it has also been possible to study electron transfer self-exchange reactions of cupredoxins.^{76, 77} These two approaches have yielded substantial information that provides insight into general mechanisms of protein electron transfer reactions. However, these excellent model systems do not describe physiological interprotein electron transfer reactions.

In physiological interprotein electron transfer reactions, the redox centers which serve as electron donor and acceptor are not in direct contact. They are not only separated by relatively long distances but also the gap between the two proteins. Furthermore, interprotein electron transfer is a bimolecular reaction which at a minimum requires a binding step to precede electron transfer and may also include additional non-electron transfer reactions steps such as protein rearrangements to optimize the orientation of the initial complex for the electron transfer reaction.⁷⁸ The physiological electron transfer protein complex is that of NADH, amicyanin and cytochrome *c*-551i from *P. denitrificans* has been extensively studied.^{12, 30} The rates of the individual electron transfer reactions that occur within the complex have been determined by monitoring characteristic changes in the absorption spectra of the proteins that occur during the redox reactions. Site-directed mutagenesis studies of amicyanin have identified specific amino acid residues that influence electron transfer parameters for the reactions that occur within the complex (Table 4). It was also possible to generate mutations of amicyanin that alter the kinetic mechanisms of electron transfer reactions within the complex by converting true electron transfer reactions to ones that are gated or coupled.³⁰ These studies showed that electron transfer rates may be significantly altered by subtle changes in protein structure by a variety of mechanisms.

Before giving specific examples of site-directed mutagenesis studies which have elucidated features of cupredoxin structure that influence electron transfer rates, a brief discussion of electron transfer theory is presented to provide a framework to understand how individual protein residues can influence rates of electron transfer. According to Marcus theory (eq 1)⁷⁹ which is the prevalent form of electron transfer theory, the rate of electron transfer (k_{ET}) depends on these factors; driving force ($-\Delta G^\circ$), reorganization energy (λ) and the donor-acceptor electronic coupling (H_{AB}).

$$k_{ET} = \left[4 \pi^2 H_{AB}^2 / h (4 \pi \lambda RT) \right]^{0.5} \exp \left[-(\Delta G^\circ + \lambda)^2 / 4 \lambda RT \right] \quad (1)$$

A. Activation energy

The activation energy for an electron transfer reaction is determined by both G° and λ . The G° for electron transfer reaction depends on the E_m for the electron donor and acceptor of redox centers. The mechanisms by which cupredoxins tune their E_m values were discussed earlier. λ is the energy difference between the reactant and product states at the equilibrium nuclear configuration of the reactant, and it is the energy required to bring the reactant and product states to this common intermediate in which electron transfer occurs. The type 1 copper site seems to have evolved to minimize λ , thus maximizing k_{ET} . Redox reactions of copper in aqueous solution can exhibit high λ values < 2.4 eV⁸⁰ due to the different ligation geometries preferred by Cu^+ and Cu^{2+} and influence of solvent. However, in cupredoxins copper is shielded from solvent and the copper site is constrained by the protein into a geometry intermediate between that preferred for Cu^+ and Cu^{2+} . This minimizes changes in copper site geometry and solvent effects during the redox reaction which would otherwise increase λ .⁶⁰

The importance of the geometry of the type 1 site in controlling λ was demonstrated with *P. denitrificans* amicyanin. It was shown that conversion of the axial Met ligand to Gln by site-directed mutagenesis decreased k_{ET} for the reaction between MADH and amicyanin by 45-fold due to a 0.4 eV increase in λ ²⁸ (Table 4). Structural studies indicated that this mutation increased the distance of the copper from the equatorial plane of the type 1 site and that this rhombic distortion of the type 1 geometry was responsible for the increase in λ .^{26, 28} This demonstrated that even subtle perturbation of the type 1 site geometry can significantly affect electron transfer rates by modulating λ .

B. Electronic coupling.

The rate of electron transfer also depends upon H_{AB} , which depends on the electron transfer distance and the nature of the intervening medium.^{75, 81} It is generally accepted that electron transfer through a vacuum is much less efficient than electron transfer through a covalent bond.⁸² It follows that efficient electron transfer through proteins would occur primarily through bonds rather than through empty space. An F97E mutation of amicyanin decreased k_{ET} for the reaction between MADH and amicyanin by 24-fold⁸³ (Table 4). The G° and λ associated with the electron transfer reaction were unaffected by the mutation and the decrease in k_{ET} was due solely to a decrease in H_{AB} . Phe97 is located at the MADH-amicyanin interface and had previously been shown to perturb the protein-protein interaction.²⁴ From structural and computational analysis it was concluded that the F97E mutation caused an increase in the interprotein distance of 0.9 Å which accounts for the observed decreases in H_{AB} and consequently k_{ET} . This illustrates the importance of the identity of surface residues at the protein interface and demonstrates that small (sub-angstrom) changes in the length of through-space segments of electron transfer pathways, particularly interprotein gaps, can significantly alter H_{AB} .

C. Kinetic regulation of observed electron transfer rates.

For interprotein electron transfer reactions the observed rate of the redox reaction is not always a true electron transfer rate constant (k_{ET}). Given the complexity of the biological electron transfer reactions,⁸⁴ the possibility exists that some non-electron transfer process

(e.g., a protein conformation change or reconfiguration of proteins after initial complex formation) is influencing the observed rate. This may give rise to what have been described as gated and coupled electron transfer reactions (Scheme 1).³⁰ Gated electron transfer occurs when the non-electron transfer reaction step (k_x) is slower than the true electron transfer reaction. If the rates of these two sequential reactions cannot be kinetically distinguished, then the observed rate constant (k_{obs}) for the redox reaction will be the rate constant for the preceding slower non-electron transfer reaction step rather than k_{ET} . Coupled electron transfer occurs when a true electron transfer reaction is preceded by a more rapid, but thermodynamically unfavorable non-electron transfer reaction (described by $K_x = k_x/k_{-x}$), which limits the availability of the optimized state. In this case the observed rate constant (k_{obs}) for the redox reaction is actually equal to the product of k_{ET} and the equilibrium constant for the preceding reaction step ($k_{\text{obs}} = K_x * k_{\text{ET}}$).⁸⁵ Two examples in which site-directed mutagenesis of amicyanin has been used to convert true ET reactions within the MADH-amicyanin-cytochrome *c*-551i complex to coupled and gated reactions (Table 5) are described below.

Pro94 which lies within the hydrophobic patch and the ligand loop of amicyanin was converted to Ala. The crystal structure of oxidized (Cu^{2+}) P94A amicyanin was relatively unaffected by the mutation but the structure of reduced (Cu^+) P94A amicyanin exhibited two alternate conformations with the positions of the copper 1.4 Å apart.⁷⁰ One was similar to that of the native protein. In the other a water replaced Met98 as the copper ligand and the electron transfer distance to the heme of the cytochrome was increased by 1.4 Å, which would make electron transfer from this conformation much less favorable. Analysis of the electron transfer reaction from amicyanin to cytochrome *c*-551i suggested that this true electron transfer reaction had been converted to a coupled electron transfer reaction.⁸⁶ A kinetic mechanism was proposed to explain these data in which after the reduction of Cu^{2+} by MADH, electron transfer from the favored conformation of Cu^+ is coupled to an unfavorable equilibrium with the unfavorable conformation, thus limiting the availability of the optimized state and decreasing k_{ET} .

In another study, mutation of Met51 which lies within the hydrophobic patch of amicyanin was converted to Ala.⁸⁷ Analysis of the electron transfer reaction from MADH to amicyanin revealed that the rate of the redox reaction had become much slower because the electron transfer reaction was now gated. Structural analysis revealed that intermolecular contacts between Met51 of amicyanin and MADH were lost as a consequence of the M51A mutation. The K_d for complex formation was not affected and it was concluded that the mutation slowed the rate of normally rapid conformational rearrangement that precedes electron transfer resulting in conversion of the true electron transfer reaction to a gated electron transfer reaction. It was subsequently shown using molecular dynamics simulations that the interactions between Met51 and MADH comprised a “molecular breakwater” that optimized the position of water molecules at the protein interface, and that the M51A mutation disrupted this breakwater resulting in a decreased availability of the optimum configuration for electron transfer.⁸⁸ These results highlight the fact that specific individual residues at the surface of redox proteins not only dictate specificity for initial binding to their redox protein partners, but also are critical to optimize the configuration of the redox centers and intervening media within the protein complex for the electron transfer event.

VIII. Perspective

Copper, as an element, has captured our imagination and found a variety of practical uses over the years. The alchemist's name for copper was Venus, the Roman goddess of love and beauty. Copper and its alloy, bronze, have been used by humans since ancient times. Today its electrical properties are exploited for use in electrical wiring and electromagnets, and its thermal conduction is exploited for use in heat exchangers. Cupredoxins have also intrigued and fascinated chemists and biochemists for many years due to their intense blue color. Cupredoxins were one of the first classes of metalloproteins to be studied in detail and at least in part contributed to the development of the field of bioinorganic chemistry. The roles of cupredoxins in nature are reasonably well-established. Perhaps because they are such well-defined proteins one is beginning to see reports of potential applications that employ cupredoxins in biotechnology and medicine. For example, cupredoxins may prove useful as components of bio-electronic devices,^{89, 90} and surprisingly the cupredoxin azurin has been shown to preferentially enter cancer cells and cause apoptosis.^{91, 92} Understanding how cupredoxins have evolved and adapted to perform their natural functions will hopefully provide insight into how protein engineering may be used to further adapt this class of metalloproteins for new applications.

Acknowledgements

Work from the author's laboratory was supported by NIH grant GM41574.

Abbreviations

AADH	Aromatic amine dehydrogenase
EPR	Electron paramagnetic resonance
E_m	oxidation-reduction midpoint potential
MADH	Methylamine dehydrogenase
NMR	Nuclear magnetic resonance
TTQ	Tryptophan tryptophylquinone

References

1. Solomon EI, Sundaram UM and Machonkin TE, Chem. Rev, 1996, 96, 2563–2606. [PubMed: 11848837]
2. Adman ET, Adv. Protein Chem, 1991, 42, 145–197. [PubMed: 1793005]
3. Solomon EI, Szilagyi RK, DeBeer George S and Basumallick L, Chem. Rev, 2004, 104, 419–458. [PubMed: 14871131]
4. Nakamura K and Go N, Cell Mol. Life Sci, 2005, 62, 2050–2066. [PubMed: 16091847]
5. Dennison C, Coord. Chem. Rev, 2005, 249, 3025–3054.
6. Hart PJ, Nersissian AM, Herrmann RG, Nalbandyan RM, Valentine JS and Eisenberg D, Protein Sci, 1996, 5, 2175–2183. [PubMed: 8931136]
7. Nar H, Messerschmidt A, Huber R, van de Kamp M and Canters GW, J. Mol. Biol, 1991, 221, 765–772. [PubMed: 1942029]

8. Zaitseva I, Zaitsev V, Card G, Moshkov K, Bax B, Ralph A and Lindley P, J. Biol. Inorg. Chem, 1996, 1, 15–23.
9. Husain M and Davidson VL, J. Biol. Chem, 1985, 260, 14626–14629. [PubMed: 2997215]
10. Davidson VL, Adv. Protein Chem, 2001, 58, 95–140. [PubMed: 11665494]
11. Husain M and Davidson VL, J. Biol. Chem, 1986, 261, 8577–8580. [PubMed: 3013855]
12. Chen L, Durley RC, Mathews FS and Davidson VL, Science, 1994, 264, 86–90. [PubMed: 8140419]
13. Davidson VL and Jones LH, Anal. Chim. Acta, 1991, 249, 235–240.
14. Davidson VL and Jones LH, Biochemistry, 1996, 35, 8120–8125. [PubMed: 8679563]
15. Brooks HB and Davidson VL, Biochemistry, 1994, 33, 5696–5701. [PubMed: 8180195]
16. Husain M, Davidson VL and Smith AJ, Biochemistry, 1986, 25, 2431–2436. [PubMed: 3718960]
17. Durley R, Chen L, Lim LW, Mathews FS and Davidson VL, Protein Sci, 1993, 2, 739–752. [PubMed: 8495197]
18. Ma JK, Bishop GR and Davidson VL, Arch. Biochem. Biophys, 2005, 444, 27–33. [PubMed: 16289023]
19. Carrell CJ, Wang X, Jones L, Jarrett WL, Davidson VL and Mathews FS, Biochemistry, 2004, 43, 9381–9389. [PubMed: 15260481]
20. Nar H, Huber R, Messerschmidt A, Filippou AC, Barth M, Jaquinod M, van de Kamp M and Canters GW, Eur. J. Biochem, 1992, 205, 1123–1129. [PubMed: 1576995]
21. Blackwell KA, Andersen BF and Baker EN, Acta Crystallogr. D Biol. Crystallogr, 1994, 50, 263–270. [PubMed: 15299437]
22. Moratal JM, Romero A, Salgado J, Perales-Alarcon A and Jimenez HR, Eur. J. Biochem, 1995, 228, 653–657. [PubMed: 7737159]
23. McMillin DR, Holwerda RA and Gray HB, Proc. Natl. Acad. Sci. USA, 1974, 71, 1339–1341. [PubMed: 4275396]
24. Davidson VL, Jones LH, Graichen ME, Mathews FS and Hosler JP, Biochemistry, 1997, 36, 12733–12738. [PubMed: 9335529]
25. Ma JK, Lee S, Choi M, Bishop GR, Hosler JP and Davidson VL, J. Inorg. Biochem, 2008, 102, 342–346. [PubMed: 17986390]
26. Carrell CJ, Ma JK, Antholine WE, Hosler JP, Mathews FS and Davidson VL, Biochemistry, 2007, 46, 1900–1912. [PubMed: 17295442]
27. Choi M, Sukumar N, Liu A and Davidson VL, Biochemistry, 2009, 48, 9174–9184. [PubMed: 19715303]
28. Ma JK, Mathews FS and Davidson VL, Biochemistry, 2007, 46, 8561–8568. [PubMed: 17602663]
29. Davidson VL and Jones LH, J. Biol. Chem, 1995, 270, 23941–23943. [PubMed: 7592588]
30. Davidson VL, Acc. Chem. Res, 2008, 41, 730–738. [PubMed: 18442271]
31. Chen L, Durley R, Poliks BJ, Hamada K, Chen Z, Mathews FS, Davidson VL, Satow Y, Huizinga E, Vellieux FM and et al., Biochemistry, 1992, 31, 4959–4964. [PubMed: 1599920]
32. Ferrari D, Di Valentin M, Carbonera D, Merli A, Chen Z-W, Mathews FS, Davidson VL and Rossi G-L, J. Biol. Inorg. Chem, 2004, 9, 231–237. [PubMed: 14735334]
33. Merli A, Brodersen DE, Morini B, Chen Z, Durley RC, Mathews FS, Davidson VL and Rossi GL, J. Biol. Chem, 1996, 271, 9177–9180. [PubMed: 8621571]
34. Gray KA, Knaff DB, Husain M and Davidson VL, FEBS Letters, 1986, 207, 239–242. [PubMed: 3021532]
35. Gray KA, Davidson VL and Knaff DB, J. Biol. Chem, 1988, 263, 13987–13990. [PubMed: 3170535]
36. Zhu Z, Cunane LM, Chen Z, Durley RC, Mathews FS and Davidson VL, Biochemistry, 1998, 37, 17128–17136. [PubMed: 9860825]
37. Hope AB, Biochim. Biophys. Acta, 2000, 1456, 5–26. [PubMed: 10611452]
38. Colman PM, Freeman HC, Guss JM, Murata M, Norris VA, Ramshaw JAM, Venkatappa MP, Nature, 1978, 272, 319–324.

39. Shibata N, Inoue T, Nagano C, Nishio N, Kohzuma T, Onodera K, Yoshizaki F, Sugimura Y and Kai Y, J. Biol. Chem, 1999, 274, 4225–4230. [PubMed: 9933621]
40. Gewirth DT and Moore PB, Nuc. Acids Res, 1988, 16, 10717–10732.
41. Guss JM and Freeman HC, J. Mol. Biol, 1983, 169, 521–563. [PubMed: 6620385]
42. Guss JM, Harrowell PR, Murata M, Norris VA and Freeman HC, J. Mol. Biol, 1986, 192, 361–387. [PubMed: 3560221]
43. Ubbink M, Ejdeback M, Karlsson BG and Bendall DS, Structure, 1998, 6, 323–335. [PubMed: 9551554]
44. Bertini I, Ciurli S, Dikiy A, Fernandez CO, Luchinat C, Safarov N, Shumilin S and Vila AJ, J. Am. Chem. Soc, 2001, 123, 2405–2413. [PubMed: 11456890]
45. Crowley PB, Otting G, Schlarb-Ridley BG, Canters GW and Ubbink M, J. Am. Chem. Soc, 2001, 123, 10444–10453. [PubMed: 11673974]
46. Sukumar N, Chen ZW, Ferrari D, Merli A, Rossi GL, Bellamy HD, Chistoserdov A, Davidson VL and Mathews FS, Biochemistry, 2006, 45, 13500–13510. [PubMed: 17087503]
47. McIntire WS, Wemmer DE, Chistoserdov A and Lidstrom ME, Science, 1991, 252, 817–824. [PubMed: 2028257]
48. Govindaraj S, Eisenstein E, Jones LH, Sanders-Loehr J, Chistoserdov AY, Davidson VL and Edwards SL, J. Bacteriol, 1994, 176, 2922–2929. [PubMed: 8188594]
49. Edwards SL, Davidson VL, Hyun YL and Wingfield PT, J. Biol. Chem, 1995, 270, 4293–4298. [PubMed: 7876189]
50. Hyun YL and Davidson VL, Biochemistry, 1995, 34, 12249–12254. [PubMed: 7547967]
51. Hyun YL and Davidson VL, Biochemistry, 1995, 34, 816–823. [PubMed: 7827040]
52. Blake RC 2nd and Shute EA, J. Biol. Chem, 1987, 262, 14983–14989. [PubMed: 3667619]
53. Giudici-Orticoni MT, Guerlesquin F, Bruschi M and Nitschke W, J. Biol. Chem, 1999, 274, 30365–30369. [PubMed: 10521412]
54. Jimenez B, Piccioli M, Moratal JM and Donaire A, Biochemistry, 2003, 42, 10396–10405. [PubMed: 12950166]
55. Grossmann JG, Hall JF, Kanbi LD and Hasnain SS, Biochemistry, 2002, 41, 3613–3619. [PubMed: 11888277]
56. Mukhopadhyay BP, Ghosh B, Bairagya HR, Bera AK, Nandi TK and Das SB, J. Biomol. Struct. Dyn, 2007, 25, 157–164. [PubMed: 17718594]
57. Mukhopadhyay BP, Ghosh B, Bairagya HR, Nandi TK, Chakrabarti B and Bera AK, J. Biomol. Struct. Dyn, 2008, 25, 543–551. [PubMed: 18282009]
58. Nersissian AM, Immoos C, Hill MG, Hart PJ, Williams G, Herrmann RG and Valentine JS, Protein Sci, 1998, 7, 1915–1929. [PubMed: 9761472]
59. Machonkin TE, Zhang HH, Hedman B, Hodgson KO and Solomon EI, Biochemistry, 1998, 37, 9570–9578. [PubMed: 9649340]
60. Malmstrom BG, Eur. J. Biochem, 1994, 223, 711–718. [PubMed: 8055947]
61. Guckert JA, Lowery MD and Solomon EI, J. Am. Chem. Soc, 1995, 117, 2817–2844.
62. Li H, Webb SP, Ivanic J and Jensen JH, J. Am. Chem. Soc, 2004, 126, 8010–8019. [PubMed: 15212551]
63. Donaire A, Jimenez B, Moratal J, Hall JF and Hasnain SS, Biochemistry, 2001, 40, 837–846. [PubMed: 11170402]
64. Donaire A, Jimenez B, Fernandez CO, Pierattelli R, Niizeki T, Moratal JM, Hall JF, Kohzuma T, Hasnain SS and Vila AJ, J. Am. Chem. Soc, 2002, 124, 13698–13708. [PubMed: 12431099]
65. Hall JF, Kanbi LD, Strange RW and Hasnain SS, Biochemistry, 1999, 38, 12675–12680. [PubMed: 10504237]
66. Berry SM, Ralle M, Low DW, Blackburn NJ and Lu Y, J. Am. Chem. Soc, 2003, 125, 8760–8768. [PubMed: 12862470]
67. Marshall NM, Garner DK, Wilson TD, Gao YG, Robinson H, Nilges MJ and Lu Y, Nature, 2009, 462, 113–116. [PubMed: 19890331]

68. Garner DK, Vaughan MD, Hwang HJ, Savelieff MG, Berry SM, Honek JF and Lu Y, J. Am. Chem. Soc, 2006, 128, 15608–15617. [PubMed: 17147368]
69. Machczynski MC, Gray HB and Richards JH, J. Inorg. Biochem, 2002, 88, 375–380. [PubMed: 11897353]
70. Carrell CJ, Sun D, Jiang S, Davidson VL and Mathews FS, Biochemistry, 2004, 43, 9372–9380. [PubMed: 15260480]
71. Libeu CA, Kukimoto M, Nishiyama M, Horinouchi S and Adman ET, Biochemistry, 1997, 36, 13160–13179. [PubMed: 9341204]
72. Sinclair-Day JD, Sisley MJ, Sykes AG, King GC and Wright PE, J. Chem. Soc. Chem. Commun, 1985, 1985, 505–507.
73. Gray HB and Winkler JR, Annu. Rev. Biochem, 1996, 65, 537–561. [PubMed: 8811189]
74. Bjerrum MJ, Casimiro DR, Chang IJ, Di Bilio AJ, Gray HB, Hill MG, Langen R, Mines GA, Skov LK, Winkler JR and et al., J. Bioenerg. Biomembr, 1995, 27, 295–302. [PubMed: 8847343]
75. Onuchic JN, Beratan DN, Winkler JR and Gray HB, Ann. Rev. Biophys. Biomol. Struct, 1992, 21, 349–377. [PubMed: 1326356]
76. Ma L, Philipp E and Led JJ, J Biomol NMR, 2001, 19, 199–208. [PubMed: 11330808]
77. Jensen MR, Hansen DF and Led JJ, J. Am. Chem Soc, 2002, 124, 4093–4096. [PubMed: 11942848]
78. Davidson VL, Acc. Chem. Res, 2000, 33, 87–93. [PubMed: 10673316]
79. Marcus R. A. a. N. S., Biochim. Biophys. Acta, 1985, 811, 265–322.
80. Winkler JR, Wittung-Stafshede P, Leckner J, Malmstrom BG and Gray HB, Proc. Natl. Acad. Sci. USA, 1997, 94, 4246–4249. [PubMed: 9113974]
81. Page CC, Moser CC, Chen X and Dutton PL, Nature, 1999, 402, 47–52. [PubMed: 10573417]
82. Regan JJ, Risser SM, Beratan DN and Onuchic JN, J. Phys. Chem, 1993, 97, 13083–13088.
83. Davidson VL, Jones LH and Zhu Z, Biochemistry, 1998, 37, 7371–7377. [PubMed: 9585551]
84. Davidson VL, Biochemistry, 1996, 35, 14035–14039. [PubMed: 8916887]
85. Davidson VL, Biochemistry, 2000, 39, 4924–4928. [PubMed: 10769151]
86. Sun D, Li X, Mathews FS and Davidson VL, Biochemistry, 2005, 44, 7200–7206. [PubMed: 15882058]
87. Ma JK, Wang Y, Carrell CJ, Mathews FS and Davidson VL, Biochemistry, 2007, 46, 11137–11146. [PubMed: 17824674]
88. de la Lande A, Babcock NS, Rezac J, Sanders BC and Salahub DR, Proc. Natl. Acad. Sci. USA, 2010, 107, 11799–11804. [PubMed: 20547871]
89. Shleev S, Tkac J, Christenson A, Ruzgas T, Yaropolov AI, Whittaker JW and Gorton L, Biosens. Bioelectron, 2005, 20, 2517–2554. [PubMed: 15854824]
90. Andolfi L, Bruce D, Cannistraro S, Canters GW, Davis JJ, Hill HAO, Crozier J, Verbeet MP, Wrathmell CL and Astier Y, J. Electroanal. Chem, 2004, 565, 21–28.
91. Punj V, Bhattacharyya S, Saint-Dic D, Vasu C, Cunningham EA, Graves J, Yamada T, Constantinou AI, Christov K, White B, Li G, Majumdar D, Chakrabarty AM and Das Gupta TK, Oncogene, 2004, 23, 2367–2378. [PubMed: 14981543]
92. Chaudhari A, Mahfouz M, Fialho AM, Yamada T, Granja AT, Zhu Y, Hashimoto W, Schlarb-Ridley B, Cho W, Das Gupta TK and Chakrabarty AM, Biochemistry, 2007, 46, 1799–1810. [PubMed: 17249693]
93. Brooks HB and Davidson VL, J. Am. Chem. Soc, 1994, 116, 11201–11202.
94. Sun D and Davidson VL, Biochemistry, 2003, 42, 1772–1776. [PubMed: 12578392]

true ET: $k_{ET} \ll k_X$ $K_X \gg 1$ $k_{\text{obs}} = k_{ET}$

gated ET: $k_X \ll k_{ET}$ $k_{\text{obs}} = k_X$

coupled ET: $k_{ET} \ll k_X$ $K_X \ll 1$ $k_{\text{obs}} = K_X^* k_{ET}$

Scheme 1.

Kinetic mechanisms of electron transfer reactions

Figure 1.

Examples of the three major classes of copper-binding sites in proteins. The structures were drawn using the structure coordinates from PDB files for the type 1 copper center of amicyanin from *Paracoccus denitrificans* (PDB code 2OV0), the type 2 copper center of Cu,Zn-superoxide dismutase from human bovine erythrocytes (PDB code 2SOD) and the type 3 copper center of hemocyanin from *Octopus dofleini* (PDB code 1JS8). The structures are presented as sticks colored gray for carbon, red for oxygen, blue for nitrogen and yellow for sulfur. The copper is displayed as a dark blue sphere.

Figure 2. Spectroscopic properties of amicyanin which are characteristic of cupredoxins. (A) The visible absorption spectrum of oxidized (Cu^{2+}) amicyanin. (B) X-band EPR spectrum of oxidized (Cu^{2+}) amicyanin.

Figure 3.

Variations in the ligation geometry of type 1 copper sites. The structures were drawn using the structure coordinates from PDB files for amicyanin from *Paracoccus denitrificans* (PDB code 2OV0), stellacyanin from *Cucumis sativus* (PDB code 1JER), azurin from *Pseudomonas aeruginosa* (PDB code 4AZU) and ceruloplasmin from human serum (PDB code 1KCW). Only the atom providing the copper ligand is shown with the one-letter amino acid code and residue number indicated. The plane described by the two His and one Cys ligand in each is shaded light green.

Figure 4.

The methylamine dehydrogenase-amicyanin-cytochrome c_{551i} complex. One half of the symmetrical complex of the crystal structure (PDB code 2MTA) is shown with the cytochrome colored tan, amicyanin colored blue, the MADH α subunit colored olive green, and the MADH β subunit colored light green. The TTQ in MADH, copper in amicyanin and heme in the cytochrome are colored black.

Figure 5.

Structures of representative cupredoxins. The secondary structures are highlighted with beta sheets colored light blue, alpha helices colored red, and unstructured loops and turns colored gray. The structures were drawn using the structure coordinates from PDB files for amicyanin from *Paracoccus denitrificans* (PDB code 2OV0), plastocyanin from *Ulva pertusa* (PDB code 1IUZ), azurin from *Pseudomonas aeruginosa* (PDB code 4AZU) and stellacyanin from *Cucumis sativus* (PDB code 1JER).

Figure 6. Space filling model of the structure of amicyanin from *Paracoccus denitrificans* (PDB code 2OV0). The MADH binding site (red) seen in the structure of the protein complex is comprised of residues Met28, Met51, Pro52, Met71, Pro94, Pro96 and Phe97. The cytochrome c-551i binding site (purple) is comprised of Asp24, Glu31, Glu32, Thr34 and Arg48. The copper is displayed as a blue sphere.

Figure 7.

Redox partner binding sites of plastocyanins. Space filling models of the structure were prepared using the coordinates from the PDB files of plastocyanins from a higher plant (*Silene* PDB code 1BYO), from a green alga (*Ulva pertusa* PDB code 1IUZ) and from a cyanobacteria (*Synechococcus sp.* PDB code 1BXU). The hydrophobic patch which is the site of interaction with cytochrome *f* and is present in all three is red. The acidic patch which also interacts with cytochrome *f* in the plastocyanins of plants and algae is purple. The additional acid patch found only in plastocyanin from plants is green. Copper is indicated as a blue sphere.

Table 1.

Examples of members of different types of copper proteins

	Examples (Source)
Type 1 copper proteins	Amicyanin (bacteria) Azurin (bacteria) Plastocyanin (plants, algae) Pseudoazurin (bacteria) Stellacyanin (plants) Rusticyanin (bacteria)
Type 2 copper proteins	Galactose oxidase (fungi) Cu-Zn Superoxide dismutase (yeast, mammals)
Type 3 copper proteins	Hemocyanin (arthropods, mollusks) Tyrosinase (fungi, mammals)
Multicopper oxidases	Ceruloplasmin (mammals) Laccase (fungi, plants, insects) Ascorbate oxidase (plants)

Table 2.

Influence of the axial copper ligand on redox potential.

Protein	E_m (mV)	E_m (mV)	Reference
Amicyanin	+ 294	–	27
M98L amicyanin	+ 421	+127	
Rusticyanin	+ 667	–	65
M148L rusticyanin	+ 798	+131	
Azurin	+ 315	–	66
M121L azurin	+ 450	+135	

Table 3.

Influence of Hydrogen bonding to the cysteine copper ligand on redox potential

Protein	H-bonds to cysteine ligand	E_m (mV)	E_m (mV)	Reference
Amicyanin	1	+ 294	–	
P94A amicyanin	2	+ 444	+150	69
P94F amicyanin	2	+ 414	+120	69
Pseudoazurin	1	+ 270	–	
P80I pseudoazurin	2	+ 450	+180	71
Azurin	2	+ 265	–	
F114P azurin	1	+ 171	–94	67

Table 4.

Alteration of rates of true electron transfer rates by site-directed mutagenesis

Electron transfer reaction	Reaction rate at 30° C (s ⁻¹)	G° (kJ/mol)	λ (eV)	H _{AB} (cm ⁻¹)	Reference
MADH to amicyanin	9.8	-3.2	2.3 ± 1	12 ± 7	93
MADH to M98Q amicyanin	0.2	-3.3	2.7 ± 1	12 ± 4	28
MADH to F97E amicyanin	0.2	-3.2	2.3 ± 1	3 ± 1	83
MADH to P94F amicyanin	53	-21.7	2.3 ± 1	5 ± 1	94
Amicyanin to cytochrome <i>c</i> -551i	87	+3.2	1.2 ± 1	0.3 ± 0.1	14
P94F amicyanin to cytochrome <i>c</i> -551i	0.6	+21.7	1.3 ± 1	0.3 ± 0.1	94

The reactions rates which were altered and the parameters which were altered that caused the change in rate are highlighted in red.

Table 5.

Alteration of kinetic mechanism of electron transfer by site-directed mutagenesis

Electron transfer reaction	Reaction rate at 30° C (s ⁻¹)	λ (eV)	H_{AB} (cm ⁻¹)	Kinetic mechanism	Reference
MADH to amicyanin	9.8	2.3 ± 1	12 ± 7	True	93
MADH to M51A amicyanin	1.3	3.1 ± 1	142 ± 20	Gated	87
Amicyanin to cytochrome <i>c</i> -551i	87	1.2 ± 1	0.3 ± 0.1	True	14
P94A amicyanin to cytochrome <i>c</i> -551i	0.4	2.3 ± 1	8.3 ± 5.5	Coupled	86

The reactions rates and the parameters which were altered by the change in kinetic mechanism are highlighted in red.