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The Fox1 Ferroxidase of *Chlamydomonas reinhardtii*: A New Multicopper Oxidase Structural Paradigm

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Abstract

Multicopper oxidases (MCO) contain at least four copper atoms arrayed in three distinct ligand fields supported by two canonical structural features: 1) multiples of the cupredoxin fold; and 2) four unique sequence elements that include the 10 histidine and one cysteine ligands to the four copper atoms. Ferroxidases are a sub-family of MCO proteins that contain residues supporting a specific reactivity towards ferrous iron; these MCOs play a vital role in iron metabolism in bacteria, algae, fungi and mammals. In contrast to the fungal ferroxidases, *e.g.* Fet3p from *Saccharomyces cerevisiae*, the mammalian ceruloplasmin (Cp) is twice as large (six *versus* three cupredoxin domains) and contains three type 1, or “blue” copper sites. *Chlamydomonas reinhardtii* expresses a putative ferroxidase, Fox1, which has sequence similarity to human Cp (hCp). Eschewing the standard sequence-based modeling paradigm, a function-based model of the Fox1 protein has been constructed which replicates hCp's six Cu-site ligand arrays with an overall root mean square deviation of 1.4 Å. Analysis of this model has led also to assignment of motifs in Fox1 that are unique to ferroxidases, the strongest evidence to date that the well-characterized fungal high-affinity iron uptake system is essential to iron homeostasis in green algae. The model of Fox1 also establishes a sub-family of MCO proteins with a non-canonical Cu-ligand organization. These diverse structures suggest alternate mechanisms for intramolecular electron transfer and require a new trajectory for the evolution of the MCO superfamily.

Keywords

homology model; molecular modeling; electron transfer; metallocenter assembly

Introduction

There are only two enzyme classes that include members catalyzing the 4-electron reduction of dioxygen to water [1]; these enzymes are the copper and heme-containing terminal oxidases (EC 1.9.3.X) [2] and the multicopper oxidases (EC 1.10.3.X) [1,3]. MCO proteins contain at a minimum four copper atoms in three well-characterized ligand fields provided by the imidazole and thiol side chains of a quartet of conserved histidine and cysteine-containing sequence motifs [3,4]. Two examples of these four motifs are shown in the first two entries in Table 1, the first set for the fungal MCO, Fet3p from *Saccharomyces cerevisiae*, the second set for human ceruloplasmin, hCp.

The four copper atoms in MCOs represent all three spectroscopic types of copper in biology, type 1 (T1), type 2 (T2) and type 3 (T3) [3,4]. The T1 Cu(II) participates in the outersphere

electron transfer (ET) that results in the oxidation of the reducing substrate, that is, the properties of this site determine MCO substrate specificity [5-7]. The T3 site consists of a binuclear Cu cluster in which the two copper atoms are bridged by an exogenous oxygen atom. The T2 and T3 Cu atoms together form what is referred to as the trinuclear cluster (TNC) with Cu-Cu distances of ~ 4 Å. The innersphere 4-electron reduction of dioxygen to $2\text{H}_2\text{O}$ occurs at the TNC [1,8,9]. The T1 Cu and TNC are located ~ 13 Å apart within the MCO structure; in effect, MCOs are bifunctional enzymes with one active site catalyzing substrate oxidation (the T1 site) and another catalyzing substrate reduction (the TNC). The connectivity between the T1 Cu and TNC is illustrated by reference to the structure of Fet3p (Fig. 1) [10].

MCO proteins and this ligand set arise from the replication of a core folding domain with a conserved Greek key connectivity [11-15]. This fold is characterized by a 7- or 8-strand mixed parallel and anti-parallel β -sheet. This fold and the T1 Cu site in MCO proteins is found also in the so-called “blue” copper proteins, *e.g.* azurin, plastocyanin and stellacyanin. These small proteins (~ 150 residues in length) are known as cupredoxins; thus, this Greek key motif is known as the cupredoxin fold [16]. It is generally accepted that MCO proteins like Fet3p and hCp evolved from an ancestral cupredoxin by a series of gene duplication events combined with specific amino acid substitutions that resulted in the array of ligands represented by the motifs in Table 1 [11-15].

The majority of MCO proteins recognized in archived databases are proteins of *ca* 550 amino acid residues and three cupredoxin domains; fungal proteins like Fet3p and the laccases represent this group. A smaller number are proteins twice this size composed of six domains; hCp is a member of this MCO cohort. In either case, the ligand motifs as in Table 1 (first two entries) are arrayed within the overall sequence in a canonical fashion with the two HXH elements found in the N-terminal cupredoxin domain and the HXXHH and HCHXXXXH motifs found in the C-terminal domain. This Cu-site connectivity in relation to the peptide strand one is illustrated for Fet3p in Fig. 2. This distribution of ligand sets within the sequence of the “typical” MCO places the T1 Cu site wholly within the carboxyl-terminal cupredoxin domain and the T2 and T3 Cu atoms at the interface between first and last domains of the protein, *e.g.* between D1 and D3 in Fet3p, and between D1 and D6 in hCp (where D refers to domain).

This arrangement of Cu sites, which is a direct reflection of the order of the Cu ligand sets within the sequence, is generally recognized as the canonical MCO array. Thus, proteins possessing all four ligand motifs but in a different order within the sequence have been excluded from phylogenetic comparisons because of their “ambiguity in alignment” [12]. Among these are the Fox1 protein from *Chlamydomonas reinhardtii* [17-19] and homologous proteins in other alga [20], and proteins from marine *Bacillus* species [21] and *Nitrosomonas europaea* [22]. All three are proteins with ≥ 1100 residues and are likely composed of six cupredoxin domains as in hCp. The non-canonical, “inverted” order of the proto-typical MCO ligand motifs in these proteins is shown in Table 1, last three entries. These ligand motifs suggest that these proteins are MCOs but how their different sequence pattern would impact on the Cu site arrangement has not been examined.

Fox1 and the MnxG protein from *Bacillus* sp. are of particular interest since both have been implicated in organismal metal metabolism. Fox1 and its algal orthologs have been linked to high-affinity Fe-uptake [17-19] while MnxG is associated with the deposition of the manganic oxide capsule that protects *Bacillus* spores [21,23]. Consequently, these MCO species are suggested to be members of a sub-group of MCO proteins that serve as metallooxidases in their respective organisms. Fet3p and hCp are members of this group also, each playing essential roles in Fe-metabolism, serving as ferroxidases [24-26].

Ferroxidases are MCO proteins with a specific reactivity with Fe^{II} as reducing substrate in the 1-electron transfer reaction that reduces the T1 Cu(II) [4-6,26]. Structural analysis of hCp [27,28], and structure and function analysis in Fet3p [7,10,27-29] have identified the motifs that confer on these MCOs this unique ferrous iron reactivity. Broadly, they include a cohort of carboxyl side chains from D and E residues that are outersphere to the T1 Cu site. Whether such motifs are found in Fox1, for example, is not known; this knowledge would serve to support the current inference that Fox1, like Fet3p in yeast [24,30], for example, is directly involved in algal Fe-uptake.

In this paper we have constructed a knowledge-based model of the structure of Fox1. We eschew the standard approach which threads a sequence as a whole through an extant α -carbon backbone; this common algorithmic approach would completely ignore the fact that the ligand cohort in Fox1 is *not* arrayed as it is in Fet3p or hCp, for example (*cf* Table 1). Rather, we have built our model by aligning domains in hCp and in Fox1 that are homologous with respect to the function they play in assembling the putative Cu sites in Fox1. By assembling Fox1 *in silico* we have identified a novel MCO folding paradigm; our analyses confirm the presence of the first ferroxidase known in the Kingdom Plantae; and they establish the blueprint for function-based protein modeling that succeeds where a sequence alignment approach demonstrably fails to generate the structure of a *functional* protein. Our method is essentially topology-independent structural alignment [31] and is complementary to algorithms designed to identify circular permutations of domain shuffling in the evolution of multi-domain proteins [32,33], a mechanism of MCO evolution that has been ignored in phylogenetic studies to date.

Methods

The *Chlamydomonas* Fox1 protein sequence (XP_001694585) was used for all sequence analysis. Topology prediction was performed using the HMMTOP and TopPred programs through the ExPASy Proteomics Server at <http://ca.expasy.org/> [34,35]; the domain searches were performed in the InterPro Scan program [36]. Each domain predicted by the InterPro Scan was aligned with each cupredoxin domain of hCp using the MUSCLE multiple sequence alignment programs with default settings [37]. The predicted domains of Fox1 were paired with hCp domains according to either the copper ligands present in each or according to its place in the sequence from N-terminus to C-terminus. Models for each predicted cupredoxin domain based on its corresponding partner in hCp were constructed using the Modeller program at the Max-Planck Institute for Developmental Biology website (<http://toolkit.tuebingen.mpg.de/modeller#>) [38]. The input for each model was the MUSCLE sequence alignment of the Fox1 and hCp domain pair using the Protein Data Bank sequence for hCp (1kcw) as template. The InsightII software package (Accelrys Software, formally Molecular Simulations) was used for subsequent Fox1 modeling and analysis. Each resulting Fox1 domain model was energy-minimized using the CV (Constant Valence) ForceField with 5000 iterations and a CG (conjugate gradient) convergence of 1.0. The minimized domains were superimposed onto the corresponding domain in the crystal structure of hCp and the root mean square deviation (RMSD) was determined. Once each domain was superimposed onto hCp, all six domains were merged together into one unit and the unit was energy minimized. Assembling a model from the individual domains allowed us to compare Fox1_{Seq} with Fox1_{Cu}. However, this approach resulted in a coordinate file in which residues are not numbered in a uniform, consecutive manner; this file is readable in programs such as InsightII and Sybyl (Tripos), but not by freeware such as DeepView and PDBViewer. This file is available by request to ajt8@buffalo.edu and also is freely accessible at <http://www.smbs.buffalo.edu/bch/Labs/Kosman/publications>.

Results and discussion

Sequence analysis

An InterPro Scan domain search of the Superfamily database predicted that Fox1 contains six cupredoxin domains. Table 2 shows the predicted locations of each domain within the Fox1 sequence; the first of these begins at T87 *ca* 20 residues carboxyl-terminal to the predicted stop anchor sequence. This latter motif, residues 46–68, is the only transmembrane domain predicted by four of seven algorithms and is recognized as an uncleaved stop anchor sequence by Signal-P. We conclude that as in *S. cerevisiae*, where the MCO/ferroxidase domain of Fet3p is completely extracellular and interacts with its partner permease, Ftr1p [30], the six cupredoxin domains of Fox1 form an extracellular ferroxidase tethered to the plasma membrane via the transmembrane element provided by the amino-terminal stop anchor sequence. This Type II membrane protein topology would be consistent with Fox1 delivering Fe^{III} to the *Chlamydomonas* Ftr1 for plasma membrane iron permeation, a model inferred previously from gene expression studies [17–19].

Multiple sequence alignments of the six domains with one another reveal T1 ligand residues (His, Cys, and Met) present in D2, D4, and D6 (Fig. 3a, blue boxes); these are conserved in domains four and six of hCp (Fig. 3b, blue boxes). In all MCOs, the TNC histidine ligands appear in the sequence as four HXH-containing motifs (*cf* Table 1), where one of these motifs is the HCH bridge that couples ET from the T1 site to the TNC; this is the crucial and distinguishing sequence/ligand element in the MCO structure and definitively identifies the single domain that contributes to both of the enzyme's two active sites (illustrated for Fet3p in Fig. 1). The four HXH-containing motifs in hCp are found within D1 and D6, with the HCH bridge located in D6 (Fig. 3b, green boxes) [39]. In Fox1, these four HXH are found in D2 and D3 indicating that the TNC must reside between these two domains with the HCH bridge located in D2 (Fig. 3a). In all MCOs known to date, the TNC lies between the N-terminal and C-terminal domains [12–15]. The fact that in Fox1, the TNC must reside between D2 and D3 indicates that Fox1 has a novel organization of cupredoxin domains and is likely evolutionarily distinct from hCp. Reasonably, this structure obtains for all MCO proteins with this sequence motif display, *e.g.*, Fox1 orthologs and the bacterial proteins (and their homologs) listed in Table 1.

Model construction

Since the cupredoxin domain organization in Fox1 is different than in hCp, two approaches to building a Fox1 homology model were taken. In both cases, Modeller was used to construct a structural model of each Fox1 cupredoxin domain based on a domain in hCp. In one case, each Fox1 domain model was based on the hCp domain it matched in order of sequence, the standard sequence alignment approach: Fox1 D1 was modeled based on the structure of hCp D1, Fox1 D2 was based on hCp D2, and so on. The domains modeled in this manner are referred to as the Fox1_{Seq} domains. Alternatively, the domains from Fox1 were paired with hCp domains based on the type of copper site each contained, a general approach referred to as topology-independent alignment [31]. For instance, Fox1 D2 and hCp D6 were paired because both contain the canonical HCH motif unique as ligands that couple the T1 and T3 Cu atoms in the same domain. The domains modeled in this approach are referred to as the Fox1_{Cu} domains.

Using InsightII, each Fox1 Modeller domain was superimposed onto its assigned domain in the crystal structure of hCp. A comparison of the RMSD values resulting from the superimpositions generated by each of the two approaches is shown in Table 3. Focus first on the Fox1 modeling in relation to hCp D6, the unique, HCH-containing domain that supports the T1–T3 electronic coupling element. The RMSD value for Fox1_{Seq} D6 was approximately 7 Å lower than for Fox1_{Cu} D2, suggesting Fox1 D6 is a better match to hCp D6 than is Fox1

D2. However, closer inspection reveals that Fox1_{Cu} D2 is actually a better *functional* match to hCp D6 with respect to the Cu sites each contains (Fig. 4a). hCp D6 has a T1 site and two HXH motifs, where the four histidines of these motifs are liganded to the TNC (Fig. 4b). Sequence analysis of Fox1 indicated that Fox1 D2 contains ligands for a T1 site and two of the TNC HXH motifs. The superimposition of Fox1_{Cu} D2 on hCp D6 shows that the T1 site and the TNC ligands are in the same conformation in both domains. Although Fox1_{seq} D6 has a T1 site and a low RMSD value when superimposed onto hCp D6, it lacks any TNC ligands; in other words, it *couldn't* function together with another domain to assemble a TNC as D6 assembles with D1 in hCp (Fig. 4c). We suggest that this makes Fox1_{Cu} D2 a better homolog of hCp D6 indicating that the two domains are evolutionarily related in terms of function although not in overall protein sequence. Once each domain was superimposed onto its corresponding domain in hCp, each set of domains was merged to form one unit. To obtain the final homology models of Fox1, the merged domains were energy-minimized.

Model comparison

The two Fox1 homology models generated in this comparative fashion, referred to as Fox1_{seq} and Fox1_{Cu}, are shown in Figure 5 in comparison to hCp. Although the domain organization of Fox1 is slightly different from hCp in that the TNC lies between D2 and D3, the overall arrangement of the domains in each model is similar to hCp. One can think of the six-domain ferroxidases as forming an isosceles triangle where D1, D3, and D5 are the points and D2, D4, and D6 make up the sides (Fig. 5). When the Fox1_{seq} model is superimposed onto hCp, all sides of the Fox1_{seq} triangle match the corresponding sides of the hCp triangle. In contrast, if the Fox1_{Cu} model is superimposed onto hCp, D1, D6, and D5 compose its base, whereas D5, D4, and D3 make up the base of hCp.

Although the domain arrangements in each Fox1 model are similar, the difference in copper site structure demonstrates that only the Fox1_{Cu} model is that of a functional MCO; this underscores the emphasis we've placed here on our function-based modeling approach. In all ferroxidases, the three Cu atoms of the TNC are coordinated by four His ligands of one domain and four His ligands of a neighboring domain. In hCp, the TNC coordination sphere is composed of ligands from both D1 and D6, with Cu-N distances of approximately 2.1 Å; the ligand-to-ligand distances range from 2.8–4.1 Å (Fig. 6) [39]. If Fox1 is an MCO, the proposed His ligands of D2 and D3 must be close enough to one another to support a TNC with a similar ligand field. The putative TNC His ligands in Fox1 are residues 355, 357, 394, and 396 in D2 and 539, 541, 599, and 601 in D3. In the Fox1_{Cu} model, the TNC His ligands from D2 and D3 superimpose on the corresponding ligands in hCp with an RMSD of 0.999 Å; contrast this highly significant fit to the weaker overall match of Fox1 D2 to hCp D6 noted above. The distance between the putative T2 copper ligands is 5.9 Å, while the distances between the T3 site ligands range from 3.7–6.2 Å (Fig. 6). In the Fox1_{seq} model, the TNC His ligands superimpose on the hCp ligands with an RMSD of 4.5 Å and the distance between the two putative T2 copper ligands, His601 and His355, is 16.2 Å (Fig. 6). At this separation, these two histidines could not support a T2 Cu site which is coordinately under-saturated to begin with [1,40].

In summary, the Fox1_{seq} structure, in which the domains were modeled on hCp domains in a sequential manner, cannot support TNC formation. In contrast, the Fox1_{Cu} structure, in which each domain was modeled on an hCp domain with homologous ligands, supports a likely TNC and replicates the coordination at all six Cu atoms with an overall RMSD of 1.4 Å. In other words, although the energy-minimized Fox1_{Cu} structure differs from hCp overall (*cf* the RMSD values for the six *protein* domains, Table 3) the predicted Fox1 Cu ligand sets essentially replicate the hCp conformational array without inclusion of force fields due to coordinated Cu

atoms. The inference is that metal binding in an MCO may be more lock-and-key as opposed to induced fit.

T1 Copper sites and substrate specificity

Although each MCO domain is comprised of the same basic cupredoxin fold, not all cupredoxin domains contain a T1 site. hCp has a T1 site in domains two, four, and six. The Cu is coordinated by two histidines, a cysteine, and a methionine residue at the sites in domains four and six; the T1 Cu in D2 lacks the methionine ligand and is only 3-coordinate [39]. In contrast, all T1 Cu sites in our Fox1 model include the methionine ligand and are four-coordinate; Fig. 7 shows the positioning of the conserved T1 residues in each. The copper atom shown in blue in each case is the superimposed T1 copper atom from the hCp structure; our software did not allow us to model the copper ions into our structure of Fox1. However, this model clearly shows these four-ligand T1 copper sites and a TNC, compelling evidence that Fox1 is an MCO. Fox1 specificity towards iron, however, has only been indirectly suggested by the fact that Fox1 expression is regulated by cellular iron concentrations [19] and that Fe-dependent growth is reduced in parallel with knock-down of Fox1 mRNA [17]. Our modeling results (below) provide somewhat more compelling evidence as to the substrate specificity of this enzyme.

Ferrous iron specificity in MCO proteins has been attributed to carboxylate side chains located close to the T1 site(s); they play three essential roles in ET from Fe^{II}. First, they provide an anionic electric field gradient promoting ferrous iron binding [10,28,39]. Second, the carboxylate ligands set up a coordination field that reduces the Fe^{II} reduction potential, increasing the driving force for ET to the T1 Cu(II) [5-7,29]. Third, at least one of the carboxylate groups forms an H-bond with the ligand imidazole Nε2 atom that provides the coupling pathway for outersphere ET [5-7]. For example, at the T1 Cu sites in D4 and D6 of hCp, the Fe^{II} binding residues include one aspartate, one histidine, and two glutamates, where one of the glutamates comes from a different domain [27,28]. In hCp D6, E272 likely binds Fe^{II} and forms an H-bond with T1 ligand H1026. These acidic side chains and the H-bonds they make to coordinating imidazoles at T1 Cu atoms are the defining structural motif of MCOs as metalloxidases [4-6].

The only domain in Fox1 with a carboxylate-rich residue set comparable to those mapped in hCp is in D6, where D1006, E1039, D1077 and D1127 could contribute to Fe^{II} binding (Fig. 7a). The red atom is a Cu^I from the hCp structure superimposed on the Fox1_{Cu} D6 model; in hCp, this copper is proposed to be a bound substrate analog of Fe^{II} [28]. The carboxylate groups of D1077 and D1127 are positioned to make putative H-bonds with the imidazoles of H1079 and H1128, respectively, the two His ligands at the T1 Cu in Fox1_{Cu} D6 (Fig. 7a); an H-bond network like this could support Fe^{II}→T1 Cu(II) ET at this site. The Fe^{II} binding surface in Fox1_{Cu} D4 could include D647, M679, H695 and E727; at a distance of 4.8 Å in the model, the carboxylate side chain of the latter residue could be H-bonded to the Nε2 atom of putative T1 ligand H778 (Fig. 7b). In comparison, while the Fe^{II} binding pockets in Fox1_{Cu} D4 and D6 include at least four “ferroxidase” residues each, Fox1_{Cu} D2 contains only E349 (Fig. 7c). However, at a distance of 3.5 Å the carboxylate side chain of E349 is positioned to make an H-bond with the imidazole ring of H400, a ligand to the T1 Cu in this domain. The fact that the substrate binding pockets in the three T1 Cu domains in Fox1_{Cu} vary in their composition in comparison to T1-containing domains in hCp indicates that the intramolecular ET between T1 sites as characterized in hCp [41] could follow a different pathway in Fox1.

In particular, the aspect of our Fox1 model most critical to overall electron transfer from Fe^{II} to dioxygen is the relative paucity of putative iron binding residues at the T1 Cu site in Fox1_{Cu} D2; this is the *only* T1 Cu coupled directly to the TNC through the HCH bridge and therefore is kinetically the preferred route of intramolecular ET from a reduced T1 Cu to the site of O₂ reduction, the TNC. In hCp, electron transfer from the “remote” T1 Cu in D4 to the

TNC is proposed to be through an electronic matrix coupling pathway over a distance of 17.9 Å [41]. The question is whether our first-generation Fox1 model offers any clues as to how intramolecular e-transfer from the protein's T1 sites might occur. Fig. S1 illustrates an example of one such ET pathway from substrate bound in Fox1_{Cu} D6 to the T3 Cu atoms in D2 that is suggested by our Fox1 model.

Evolutionary considerations

There is general agreement that the evolutionary precursor to the MCO superfamily is a cupredoxin, perhaps rusticyanin (Fig. 8, first entry) [12-15]. These evolutionary models posit that following gene duplication, the two-domain product acquired the additional 2 His residues found in nitrite reductases. NiR proteins are trimers of this two-domain motif in which T2-like Cu sites assemble between the C-terminal domain of one monomer and the N-terminal domain of the next [11]. We suggest, however, that another two-domain protein family represents the evolutionary precursor to true MCO of proteins; in these proteins, *each* cupredoxin domain contains *all* of the Cu-ligands now found in the HCH-containing MCO domain irrespective of domain number (Fig. 8, third entry). In Fig. 8, Cu ligands are color-coded; T1 Cu ligands are blue, T2 Cu ligands are green, and T3 ligands are orange. Cupredoxin domains are color-coded, also. T1 Cu-containing domains (with the essential Cys residue) are in blue; these represent the blue copper, cupredoxin progenitor domain and in MCO proteins, they contain the His-Cys-His motif that couples the T1 Cu to the TNC. Domains that have lost the T1 ligands of the ancestral protein are colored red; they retain ligands that support the TNC *only*.

This domain color-coding emphasizes our hypothesis that this red domain evolved from a blue one, a model that requires the presence of a two-domain protein with comparable sets of T1, T2, and T3 Cu ligands in both domains. This protein exists in the genomes of halobacteria, members of the phylum *Euryarchaeota*; the ligand sequences in Fig. 8, third entry, are for a protein from *Halobacterium salinarium* commonly designated as Pan1 [43]. In our model, these Pan1 family members resulted from the gene duplication of a cupredoxin that had acquired the ligand set of the HCH-containing domain unique to MCO proteins (Fig. 8, second entry). This ancestor is not known in archived databases but the striking conservation of the presequence to the HC-containing motifs in both Pan1 domains (G followed by three or more bulky hydrophobic residues as is found in all MCO proteins) strongly indicates that the two domains in the Pan1 family members had a common, single domain predecessor with a fully-evolved set of Cu-site ligands.

Thus, our model is different from published evolutionary pathways since we suggest that the T2 and T3 copper ligands arose prior to gene duplication, while in previous models the ligands in each domain evolved independently and subsequent to domain duplication. The evolutionary pathway in our model from a two-domain protein to the three- and six-domain MCOs is congruent with other pathways, however [12-14]. In our model the precursors common to all MCOs are the two-domain proteins shown in Fig. 8, UniProtKB/TrEMBL entries Q9HQF4 and Q89T40. First is the one noted above (Pan1 proteins); the second is one in which D2 retains the full set of T1 and TNC ligands while D1 retains only the latter (Fig. 8, fourth entry). Based on structural modeling of these two-domain proteins, the TNC ligand set(s) is oriented outward such that a cluster cannot be formed within a monomer, but only as a result of dimer formation, as in NiR proteins [11,44]. For example, we have modeled Pan1 using *N. gonorrhoeae* NiR as template [44] and then built a hexamer of this protein on our model of Fox 1. This model has the overall architecture of a six-domain MCO (compare to Fox1 and hCp in Fig. 5) and has TNC ligands between two of the Pan1 monomers that closely overlap the TNC ligands found in Fox 1 between D2 and D3 (Fig. 9).

These relationships indicate why a TNC never exists in an MCO at the interface of a red and blue domain when the red domain precedes the blue domain topologically. That is, if Fet3p

evolved from the putative precursor Q89T40 (the product of the *bl2210* locus in *Bradyrhizobium japonicum*) by duplication of the “blue,” HCH-containing domain (not of the whole gene) and subsequent ligand loss in D2 of the resulting three-domain product, the only location a TNC could be formed is between the N-terminal red domain and the C-terminal blue domain as is observed in Fet3p and in all other three-domain ferroxidases and laccases (Fig. 8, entries five and six). The six-domain proteins, hCp and Fox1, evolved from this two-domain precursor, also, probably by triplication of the entire gene as has been suggested [13-15]. This triplication results in three predictable MCO motifs, two of which have now been established. Upon triplication, the even numbered domains would or could have a T1 copper site (the blue domains) while TNC sites would or could form between domains 2 and 3, 4 and 5, or 6 and 1 (indicated by the arrows in Fig. 8). hCp and Fox1 conform to this hypothesis since in both proteins all even-numbered domains contain T1 copper sites (Fig. 3) while the single TNC each protein possesses is found at the first (Fox1) and the last (hCp) of the predicted domain interfaces. The existence of a six-domain MCO in which the TNC resides between domains 4 and 5 (or at multiple locations) would help to confirm this evolutionary hypothesis; no such protein exists in any archived database, however.

Summary

Fox1 is likely involved in high-affinity uptake at the *Chlamydomonas* plasma membrane where it oxidizes iron for delivery to Ftr1, which channels Fe^{III} into the cell. While its cellular function may be similar to the function of Fet3p in yeast, its catalytic mechanism is likely more similar to hCp based on its homology to the more complex ferroxidase. The model of Fox1 indicates that Fox1 is a six cupredoxin domain-containing protein that has three T1 copper sites and a trinuclear cluster. While each domain is homologous to domains in hCp, the organization of the domains is unique to a new family of MCO proteins. In addition, the T1 sites in the Fox1 model differ from hCp in regards to the substrate binding environment, indicating Fox1 ferroxidation may have a mechanism unique from other ferroxidases known to date. The *in vitro* analyses required to determine the catalytic mechanism of Fox1 can be guided by the model presented here. A major question that remains in studies on the MCO superfamily is the relative selective advantage of a 6-domain MCO as in humans in comparison to 2- or 3-domain containing proteins. Further studies of Fox1 and the other non-canonical MCO proteins, and on their 2-domain progenitors, are required to obtain a more sophisticated understanding of the domain organization and function of the ferroxidases and laccases that make up this physiologically essential family of metalloproteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MCO, multicopper oxidase; (h)Cp, (human) ceruloplasmin; T1, Cu Type 1 copper; T2, Cu Type 2 copper; T3, Cu Type 3 copper; ET, electron transfer; TNC, trinuclear cluster; RMSD, root mean square deviation.

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Fig. 1.

Cu-site connectivity in Fet3p. The HCH motif that *defines* an MCO provides the electronic matrix coupling pathway that supports efficient intramolecular, outersphere electron transfer from the T1 Cu to the TNC. Note that H483 is ligand to the crystallographic Cu2 (often designated Cu3B) while H485 is ligand to Cu3 (Cu3A), *i.e.* the “wire” from the T1 Cu through the Cu-S bond (C484) connects to both of the T3 Cu, which are themselves coupled by their bridging oxygen ligand. The structure is from 1ZPU, Fet3p [10].

Fig. 2.

Cupredoxin domains in Fet3p and distribution of Cu-site ligands. Cu1 is the “blue,” T1 copper atom; Cu2 and Cu3 comprise the binuclear T3 Cu pair; Cu4 is the T2 copper. The latter 3 copper atoms make up the TNC. Note that the ligands to the TNC Cu atoms are distributed equally between D1 and D3 while the ligands to the T1 Cu are solely from D3.

Fig. 3.

Multiple sequence alignments of cupredoxin domains in Fox1 (**a**) and in hCp (**b**). T1 ligand residues are highlighted blue; TNC ligands are green; disulfide bridge cysteines are magenta. In Fox1 and hCp, the T1 ligands are found in domains 2, 4, and 6, however the organization of the cupredoxin domains is different. In Fox1 (**a**), the HXH motifs are found in domains 2 and 3, indicating the TNC residues between these domains; in hCp (**b**), the HXH motifs are within the TNC-forming domains 1 and 6. In Fox1, the conserved cysteine residues in domains 1, 3, and 5 likely form interdomain disulfide bridges with those in 2, 4, and 6, respectively. An additional intra-domain bridge is likely formed in domain 5. All disulfide bridges in hCp (**b**) are intradomain [17].

Fig. 4.

Comparison of Fox1_{Cu} D2 and Fox1_{Seq} D6 with hCp D6. **a** Fox1 D2 was modeled on the structure of hCp D6 (shown in **b**) since both domains contain the HCH motif that couples the T1 to the T3 Cu atoms. This Fox1_{Cu} model has the T1 and TNC ligands oriented in a conformation similar to the ligand set in hCp D6. Although the fit of the whole domain onto hCp D6 had RMSD of 13.1 Å, the Cu ligands of Fox1_{Cu} D2 superimpose onto the corresponding ligands of hCp with an RMSD of 0.999 Å. **b** Cupredoxin domain 6 of hCp displaying the T1 site and TNC. **c** Fox1_{Seq} D6 was modeled on hCp D6. Despite the low RMSD value (Table 3), this Fox1 D6 model lacks any TNC ligands.

Fig. 5.

Comparison of Fox1_{Cu} and Fox1_{Seq} models with hCp. Each domain is colored according to the domain in hCp upon which it was modeled. Little difference between Fox1_{Cu} and Fox1_{Seq} is apparent, *e.g.* if Fox1_{Cu} is rotated 60°, it mimics Fox1_{Seq}. Closer inspection of the interface of the TNC-forming domains highlights a critical difference between the models, however. In hCp, the β -barrel of D1 lies perpendicular to the β -barrel of D6, placing the TNC ligands in the correct orientation. Fox1_{Cu} D2 and D3 exhibit this critical conformational relationship while in Fox1_{Seq} these two domains do not; in this model there is minimal interfacial domain interaction providing no scaffold for the assembly of a TNC.

Fig. 6.

Comparison of the TNC of Fox1_{Cu}, Fox1_{Seq}, and hCp. A functional TNC is composed of eight histidine residues, which are evenly distributed between two neighboring domains. In hCp, the greatest distance between histidines from different domains is 4.1 Å, in Fox1_{Cu} this distance is 5.9 Å. In striking contrast, this separation in Fox1_{Seq} is 16.2 Å.

Fig. 7.

T1 and substrate binding sites of D2, D4, and D6 in Fox1_{Cu}. The MCO catalytic mechanism begins with outer-sphere ET from the bound substrate to the T1 copper site. In a ferroxidase, acidic residues adjacent to the T1 site not only participate in Fe^{II} recruitment, but also make H-bonds to the His ligand Nε2 atoms, bonds that are essential for ET to the T1 Cu. In Fox1, D2, D4, and D6 the four potential His, Cys, and Met residue ligands are arrayed in a geometry consistent with a T1 site. **a** The environment surrounding the T1 site in D6 is rich in acidic residues that likely participate in Fe^{II} binding. The T1 Cu superimposed from hCp is blue; the red ball is Cu^I bound as a substrate analog. **b** Fox1 D4 also contains several residues adjacent to the T1 site that may contribute to substrate recruitment and ET. This domain was modeled on hCp D2, which lacks a bound substrate; the red Cu atom is missing in this case. **c** Fox1 D2 contains the essential HCH motif that couples the T1 Cu to the TNC. Hydrogen bond(s) from the Nε2 hydrogen of a ligand imidazole to a spatially-adjacent carboxylate is found at each of the Fox1 T1 sites; these could support outersphere ET from a bound Fe^{II}.

Fig. 8.

Proposed evolutionary trajectory for three- and six-domain MCO proteins. The color coding is: T1 Cu ligands are in blue; T2 Cu ligands are in green; and T3 Cu ligands are in orange. Domains that contain a T1 Cu Cys residue (and thus, in MCO proteins, the HCH T1-T3 connectivity) are in blue while domains which have lost this Cys residue and contain only T2 and T3 Cu ligands are in red. This model serves to highlight three conclusions about MCO proteins drawn from our Fox1 model: 1) the domain duplication and gene-triplication that resulted in three- and six-domain MCOs occurred in an ortholog of the extant two-domain MCOs represented by Q89T40; 2) that this protein evolved from the Pan1 family of proteins (Q9HQF4); and 3) in six-domain MCO proteins a TNC can form only between an even-numbered, HCH-containing domain and an odd-numbered HXH-containing one while in three-domain family members it must reside between the N- and C-terminal domains (arrows). Note the conserved GN_n motif that marks the “blue” domain origin of the HXH T3 Cu ligand motif found in MCO “red” domains. Although there is no genomic evidence for this, we suggest that Pan1 proteins resulted from the duplication of the gene for a one-domain cupredoxin that contained the ligand sets found now in “blue” MCO domains (second entry). This hypothetical protein is, therefore, an intermediate between the ancestral cupredoxin (rusticyanin) and the MCO protein family.

Fig. 9.

Model of the Pan1 (Q9HQF4) trimer. The domain color-coding is as in Fig. 8. The model was based on Fox1; the “D1/2” domain designations are in reference to that template. The TNC ligands indicated at the blue-red domain interface at the upper left had an $\text{RMSD}_{\text{ave}} = 0.9 \text{ \AA}$ with respect to the TNC ligands between Fox1 D2 and D3. Note that this TNC is associated with a blue domain that precedes a red domain topologically. TNC ligand sets appear also at the other two blue-red domain interfaces (not shown).

Table 1

Cu-binding motif organization in canonical and "inverted" MCO proteins. The four motifs listed include the 10 His, 1 Cys ligand set minimally required for a 4-Cu MCO. In Fet3p and hCp, these are distributed between the N-terminal and C-terminal cupredoxin domains; this "canonical" organization, found in the majority of archived MCO proteins, places the TNC at the interface of these two domains. The last three entries illustrate the "inversion" of this order, placing the TNC at the interface of two internal cupredoxin motifs; in Fox1, we show here these two are domains 2 and 3. Identifiers in parentheses are UniProtKB/TrEMBL designations. Sequence numbering is from the initiating methionine.

Fet3p (P38993)	hCp (P00450)	Fox1 (A8IZT9)	mnxG protein (A7KBU7)	<i>N. europaea</i> MCO (Q82XF9)
⁸¹ HFH	¹²⁰ HSH	³⁵³ HVFHYH	²⁸¹ HVFHYH	⁴⁵¹ HVFHLH
¹²⁶ HSH	¹⁸⁰ HSH	³⁹⁴ HCHLYPH	³³⁴ HCHLYPH	⁵²¹ HCHLYPH
⁴¹³ HVFHLH	⁹⁹⁴ HTVVFH	⁵³⁹ HIH	⁵²⁹ HIH	⁸⁷⁷ HIH
⁴⁸³ HCHIEWH	¹⁰³⁹ HCHVTDH	⁵⁹⁹ HDH	⁵⁷⁵ HDH	⁹⁹⁸ HDH

Table 2

Modeled cupredoxin domains in Fox1

Domain	Inclusive residues
D1	87–278
D2	279–420
D3	419–631
D4	634–789
D5	799–986
D6	993–1139

Table 3

RMSD values for Fox1, hCp domain models. All domains are numbered with respect to the primary sequence. The data in the second column result from modeling these sequence-based domains from Fox1 using the corresponding domains from hCp. The data in the third column result from modeling Fox1 domains on the domains in hCp with the same Cu-ligand set.

hCp domain	Sequence-based Fox1 domain model (RMSD, Å)	Copper site-based Fox1 domain model (RMSD, Å)
D1	D1 (9.4)	D3 (10.5)
D2	D2 (13.7)	D4 (11.4)
D3	D3 (19.0)	D5 (16.4)
D4	D4 (15.4)	D6 (8.3)
D5	D5 (11.3)	D1 (11.1)
D6	D6 (5.9)	D2 (13.1)