Iron Metabolism in Eukaryotes: Mars and Venus at It Again

Jerry Kaplan and Thomas V. O'Halloran

The chemistry of iron and copper has been well known since classical times. Iron chemistry has been associated with Mars (♂), an embodiment of the arts of war, and the chemistry of copper with Venus (♀), a personification of love. While tales of their intertwined fates and amorous travails pervade the older literature, two more recent publications provide genetic and structural linkages between the biological chemistry of these paramours. In the first issue of a new journal published by the Society of Biological Inorganic Chemistry, Zaitseva and co-workers report the structure of human ferroxidase (E.C. 1.12.3.1), better known as the multicopper oxidase ceruloplasmin (*1*). In a separate report in this issue of *Science*, Stearman and coworkers (*2*) describe a connection between a new iron transport gene in yeast, *FTR1*, and a multicopper oxidase related to ceruloplasmin, namely, FET3.

Iron and copper are essential cofactors in a variety of enzymatic reactions, but they are also substrates of cation-specific storage, transport, and sequestration systems. In a biological milieu, complex molecules containing iron or copper can readily gain and lose electrons. Although this property makes such molecules useful, it also makes them dangerous and capable of generating toxic organic and oxygen-based radicals. The concentration of these free transition-metal ions in biological fluids is therefore under tight control. In the past few years there has been an explosion of information regarding the regulation and biochemistry of iron transport in that paradigmatic eukaryote, *Saccharomyces cerevisiae*, as a result of genetic screens designed to identify genes that regulate intracellular iron concentration. Indeed, with the report by Stearman *et al.* (*2*) of an iron transporter, many if not most of the critical genes required for iron transport across the yeast plasma membrane have likely been described.

Budding yeast have two distinct transport systems for elemental iron, a low-affinity system and a high-affinity system. The low-affinity system [Michaelis-Menten constant (K_m), 40 μM], defined by the *FET4* gene, can also transport other metals, such as manganese and cadmium (*3*). The high-affinity transport system (K_m, 0.15 μM) is specific for, and is transcriptionally regulated by, iron (*4*). An essential component of the high-affinity transport system is the FET3 protein. This copper-containing protein has an external multicopper oxidase domain tethered to the plasma membrane by a single transmembrane unit (Fig. 1). Multicopper oxidases catalyze the sequential oxidation of four molecules of substrate coupled to the reduction of molecular oxygen to water. The ability of FET3 to catalyze the conversion of Fe(II) to Fe(III) is necessary for high-affinity iron transport to take place (*5*). The only other multicopper oxidase known to metabolize iron is the mammalian plasma glycoprotein ceruloplasmin.

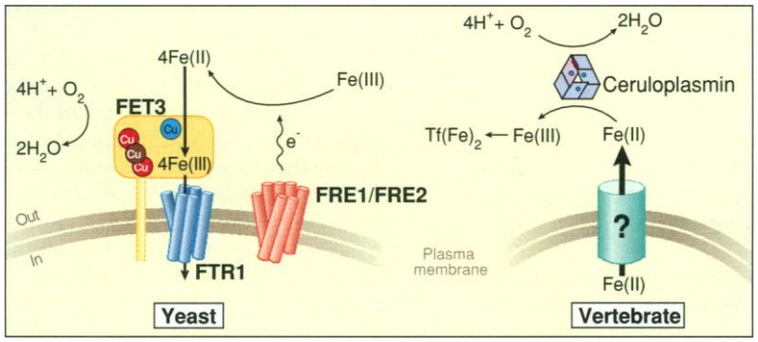

Fig. 1. Moving iron. In yeast, extracellular ferric iron chelates are reduced by metalloreductases, the products of the FRE1 or FRE2 genes or both. The resulting ferrous iron is a substrate for the multicopper oxidase FET3. Four molecules of Fe(II) are oxidized to Fe(III) with the concomitant reduction of molecular oxygen to water. Ferric iron may be directly transferred to FTR1 for transmembrane transport. In vertebrate plasma, the multicopper oxidase ceruloplasmin converts Fe(II) into Fe(III) which binds to the iron-binding protein transferrin (Tf). Ceruloplasmin is critical for iron egress from some cell types. The transport system responsible for iron release into plasma has not been identified.

Although FET3 is essential for iron uptake (*4–6*), the fact that it only has a single transmembrane domain suggests that another molecule must also be required to transfer iron across the membrane. The report of Stearman *et al.* (*2*) identifies that second molecule. These authors (who in the past have identified other yeast genes for copper and iron metabolism) found a gene, *FTR1*, that is also required for high-affinity iron transport. This gene encodes a polytypic membrane protein, suggesting that it may well be the transmembrane transporter. Mutations in this protein prevent iron uptake, whereas overexpression of this protein, in concert with overexpression of FET3, allows for supernormal rates of iron uptake. In a set of clever genetic experiments, the authors demonstrate that this protein is required for FET3 to obtain its ferroxidase activity. Further, FET3 is required to transport FTRI to the cell surface. These results provide genetic evidence that a complex between the two proteins is required for proper assembly and function. FTR1 may also participate in iron transport in addition to assisting in the proper assembly of FET3 oxidase. Mutants of FTR1 can allow the proper assembly and localization of FET3 but not iron transport. The authors have identified pairs of aspartate residues on FTR1 that may bind iron, in a way similar to a motif implicated in the binding of iron to ferritin L chains. Mutations of these potential metal-binding residues allow for normal FET3 oxidase activity but not for iron transport. Thus FTR1 has two roles: It is required for FET3 to obtain oxidase activity, and it may directly mediate iron transport.

These results are both satisfying and provocative—satisfying in that they help complete the picture of iron transport in yeast, and provocative in that they focus attention on how FTR1, in concert with FET3, effects transmembrane iron transport, particularly since the product of the ferroxidase reaction is Fe(III), the aquo complex of which is essentially insoluble at physiological pH. This system is unique: The only other elemental iron transporters that have been identified, FET4 in yeast (*3*) and *feoB* in *Escherichia coli* (*7*), appear to be Fe(II) transporters. The discovery of FTRI by Stearman *et al.* (*2*) makes the hunt for a mammalian equivalent of FTRI even more exciting.

These results also focus attention on the role of ceruloplasmin in vertebrate iron transport. Evidence that ceruloplasmin, the principal copper-binding protein in serum, contributes to mammalian iron transport antedates the studies on yeast by almost 40 years. Pigs made copper-deficient by dietary restriction had low levels of ceruloplasmin and could not release tissue iron to plasma (*8*). Individuals with mutations in the ceruloplasmin gene have a disease called

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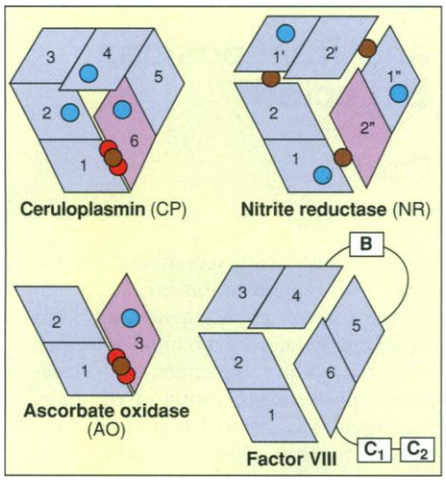

Fig. 2. Relatives of ceruloplasmin from (*1*). The small circles represent different copper environments: navy, type I; red, type II; and maroon, type III. Domains 1, 2, and 6 of ceruloplasmin correspond to domains 1, 2, and 3 of AO. Domains 1 and 2 of NR are spatially equivalent to domains 1 and 2 of CP, but NR has a type I copper in domain 1, CP does not. Factor VIII has parallels with CP domains 1 to 6 and is expected to exhibit three additional domains.

aceruloplasminemia and cannot mobilize iron from tissues (*9*, *10*). These observations, complemented by biochemical studies that show that ceruloplasmin has ferroxidase activity (*11*), prove ceruloplasmin's role in mammalian iron physiology. Other suggested functions, including amine oxidase, antioxidant, and copper transport activities, are controversial (*12*, *13*). In yeast, FET3 and FTR1 control iron entry into cells. In vertebrates, ceruloplasmin controls iron egress from cells. In bacteria, an ascorbate oxidase (AO) analog encoded in a copper resistance operon (*14*) also is involved in copper egress (*15*). How do these multicopper oxidases facilitate the transfer of iron or copper substrates to an acceptor? The answers are not yet in hand, but the recent resolution of the crystal structure of ceruloplasmin is informative (*1*).

The crystal structure of this large (1046–amino acid) glycoprotein confirms many expectations, such as the presence of six tightly bound copper ions, but reveals several surprises. The active sites are unexpectedly clustered together near one face of the molecule next to a negatively charged patch, an arrangement with important mechanistic implications. At the current resolution of 3.1 Å some of the attributes of interest to spectroscopists, coordination chemists, and biochemists are still unresolved.

Ceruloplasmin resembles an aggregate of several β-barrel domains, like those first observed in plastocyanin (*16*), but the enzyme is more similar to another plant protein, the multicopper enzyme AO (*17*, *18*). Ceruloplasmin consists of six homologous domains related by a pseudo-threefold axis with every other domain (2, 4, and 6 in Fig. 2) containing a type I or "blue" copper center. In addition to these electron-transfer sites, three more copper ions (one type II and two type III) are in a trinuclear cluster sandwiched between domains 1 and 6. The orientation of the trinuclear center and the mononuclear copper in domain 6 is essentially the same as that in the plant enzyme AO (*17*, *18*). In contrast, nitrite reductase monomers consist of two β-barrel domains that aggregate to form a copper-bridged trimer similar to the shape of ceruloplasmin but lacking the pair of type III copper ions (*19*). Indeed, on the basis of sequence similarities between ceruloplasmin and these structurally characterized cupredoxins, Messerschmidt and Huber (*17*) and Fenderson *et al.* (*20*) predicted many features of ceruloplasmin. Zaitseva *et al.* (*1*) suggest that all of these proteins may have evolved through gene duplication of an ancestral two-domain cupredoxin (Fig. 2). Such interrelations have allowed construction of a useful model of the more distantly related blood-clotting protein factor VIII (Fig. 2), mutations of which lead to severe forms of hemophilia (*21*). With the addition of the ceruloplasmin structure to the phylogenetically distant set of β-barrel copper protein structures, even more precise models for factor VIII and perhaps factor V and FET3 are anticipated.

The new structural data also provide a framework for understanding the chemistry of catalysis in this system. On the basis of studies of other multicopper oxidases, such as AO and laccase, the trinuclear center of ceruloplasmin is the site where the best understood substrate, dioxygen, binds to the enzyme. But how do electrons get into this buried site and where do they come from? The three mononuclear type I copper centers may accumulate electrons from appropriate substrates and shuttle them to the O_2 binding site. The type I sites are separated by about 18 Å, a distance well within range for effective electron transfer. Several potential electron transfer pathways similar to those proposed for the type I copper protein azurin (*22*) are also apparent in ceruloplasmin (*23*). Reduced type I coppers in domains 2 and 4 could plausibly relay electrons to the trinuclear active site by way of the closest type I copper, that in domain 6. But what are the penultimate electron donors, and where are the binding sites for the putative substrates that yield them? In this instance, the structure reveals features that were not elucidated by spectroscopists or predicted in advance of the crystallographic results.

Potential binding sites for cationic substrates are clearly apparent in a relatively small area on one side of the disk-shaped protein between domains 2, 4, and 6 (*23*) (Fig. 3A, red area). This region of ceruloplasmin contains clusters of aspartate, glutamate, and histidine side chains observed, and preliminary studies of Co(II)-soaked crystals reveal significant cation occupancy at these sites. The latter are within 10 Å of the tightly bound type I copper sites and are thus at a reasonable distance for outersphere electron transfer. All six of the copper cofactors lie toward the top face of the molecule, proximal to the putative substrate binding sites (Fig. 3). In this regard, the structure is reminiscent of cytochrome c oxidase, another multicofactor oxidase and an

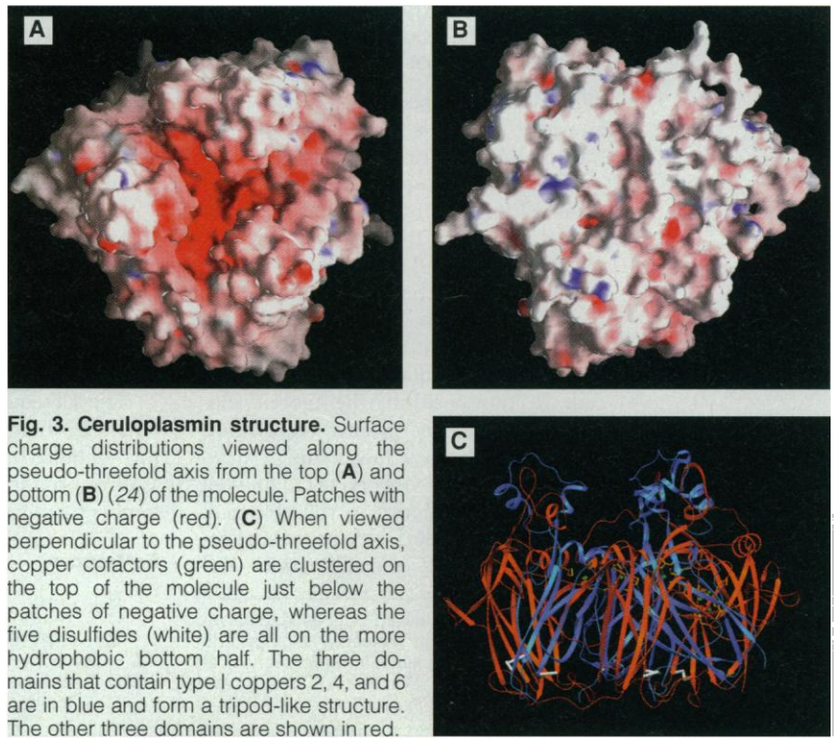

Fig. 3. Ceruloplasmin structure. Surface charge distributions viewed along the pseudo-threefold axis from the top (**A**) and bottom (**B**) (*24*) of the molecule. Patches with negative charge (red). (**C**) When viewed perpendicular to the pseudo-threefold axis, copper cofactors (green) are clustered on the top of the molecule just below the patches of negative charge, whereas the five disulfides (white) are all on the more hydrophobic bottom half. The three domains that contain type I coppers 2, 4, and 6 are in blue and form a tripod-like structure. The other three domains are shown in red.

intrinsic membrane protein that contains multiple metal cofactors.

These studies resolve many outstanding issues, but, as with any breakthrough, they lead to several conundrums: Does FET3 or ceruloplasmin transfer the product of the enzymatic reaction, Fe(III), to the acceptor site under physiological conditions, or are these biochemical activities more indirectly related to metal-ion movement? If they are the key physiological reactions, how—and more importantly, why—is catalytic oxidation of Fe(II) to Fe(III) necessary. Having the structure may allow identification of the substrate for the ferroxidase reaction: Is it an aquo iron complex, a protein-bound Fe^{2+}, or an iron complex with a low molecular weight ligand? If it is free Fe(II), how does the ferroxidase transfer the oxidation product to the physiologically relevant acceptor protein, such as transferrin or FTR1? These naïve questions indicate that the relation between cell biology and transition metal chemistry, like that between war and love, is still enigmatic.

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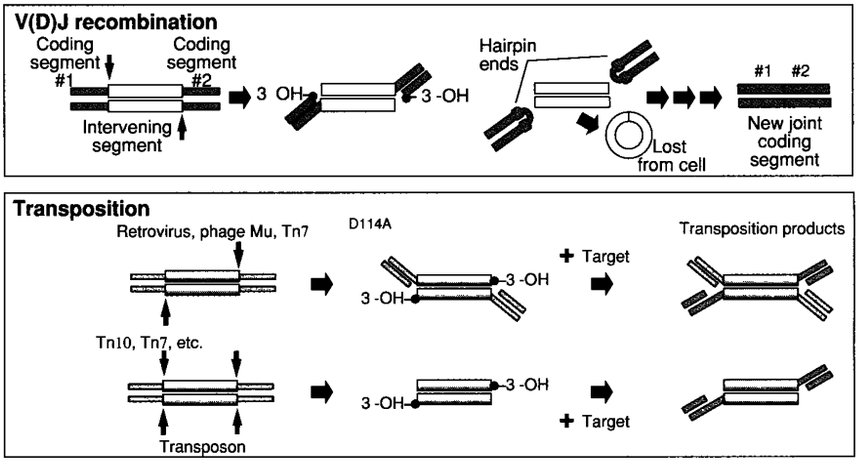
UPDATE

V(D)J Recombination and Transposition: Closer Than Expected

Nancy L. Craig

In a recent Perspective, I outlined the similarities among different transposition reactions—the movement of bacterial DNA segments and the replication and integration of certain bacteriophages and of retroviral DNA (*1*). All are mediated by recombinases of related structure that execute DNA breakage and joining by transesterification reactions. Now, in a report in this week's issue (*2*) V(D)J recombination, which underlies the ability of the immune system to assemble many different antigen-specific genes from separate gene segments, joins the family of transposition reactions.



Similarities. In V(DJ) recombination flanking DNA is joined to create a novel coding joint; in transposition the mobile segment is joined to a new target site. In retroviruses the flanking donor sequences are only several nucleotides in length.

The recombinases for both V(D)J recombination and transposition find their targets by recognition of specific DNA sequences. In V(D)J recombination, these sequences define the ends of a DNA segment that is excised to allow formation of a "coding joint"; the excised DNA segment is subsequently lost from the cell. In transposition, the mobile element, bounded by specific recognition sites, moves to a new target site.

Van Gent and co-workers (*2*) found a fundamental similarity in the chemical mechanisms of the DNA processing reactions of V(D)J recombination and strand nick, exposing a 3′-OH, which in the second step performs an intramolecular attack on the complementary strand of the nicked duplex to generate the hairpin.

This intramolecular attack proceeds by a direct transesterification: The 3′-OH exposed by the breakage step is the nucleophile that directly attacks the other strand. This reaction—direct transesterification—is the mechanistic link between V(D)J and transposition,(*3*).

The subsequent steps of V(D)J recombination—hairpin processing and joining—require other cellular proteins (*4*). Will transposition-like reactions also assemble these new coding joints?

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