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Substrate entasis and electronic coupling elements in electron transfer from Fe^{II} in a multicopper ferroxidase

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Abstract

Outersphere electron transfer in multicopper oxidases occurs at the type 1, blue Cu^{II}. One class of MCO proteins exhibits a specificity in this reaction towards Fe^{II}. In work carried out in collaboration with the Solomon lab over the past 7 years, we have delineated the structural motifs that support this ferroxidase specificity and have quantified the contributions that each makes to this outersphere electron transfer reaction from Fe^{II} to the type 1 Cu^{II}. Two features of this electron transfer catalysis stand out. First, the protein provides a binding site for Fe^{II} that actually favors Fe^{III}; this coordination sphere places the bound Fe^{II} in a state of “entasis” that can be relieved by loss of an electron. In short, the E^0 of the bound Fe^{II} is lowered relative to that of aqueous ferrous iron making electron transfer *thermodynamically favorable*. Second, carboxylates within this coordination sphere provide an electronic coupling pathway for the electron transfer *via* their H-bond network with type 1 Cu histidine ligands thus making electron transfer *kinetically efficient*. This brief report breaks down these contributions to ferroxidase specificity in terms of the semi-classical Marcus equation describing outersphere electron transfer.

Keywords

Multicopper oxidases; Ferroxidases; Electron transfer; Fet3p; Marcus theory; Entasis

1. Introduction

Professor Edward Solomon has turned 60 in this academic year of 2006–2007 but another birthday is coming up in 2008, the 40th birthday of “entasis,” first described by R.J.P. Williams and Bert Vallee in 1968 [1]. On the occasion of Professor Solomon’s birthday it is certainly appropriate to base an article on the work and ideas of two scientists who were “present at the creation” of bioinorganic chemistry in the mid-20th century (Fig. 1).

Professor Vallee was at Harvard when Professor Williams spent a sabbatical from Oxford with him at the Peter Bent Brigham Hospital. Each had published numerous papers that had as a theme the extra(literally)-ordinary properties of metal ions bound as protein prosthetic groups in comparison to the same metal ion’s properties in flexible coordination complexes of typical poly-dentate ligands. This *entatic* state implied to them a “catalytically poised state *intrinsic to the active site* (their italics)” of the protein to which the metal ion was bound; the IUPAC definition very clearly puts an “entatic” metal ion in this context with “A state of an atom or group which, due to its binding in a protein, has its geometric or electronic condition adapted

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for function.” In other words, metal ions bound to specific sites on proteins found themselves in *reactant*-state coordination complexes that were anything but *ground*-state in energy.

Ironically, Drs. Vallee and Williams had less to say when it came to enzyme catalysis in the sense that their focus remained tightly on metal ion as prosthetic group, *e.g.* a heme or catalytic Zn^{II} , and not about how a metal ion bound as *substrate* might be in a state of entasis also. Coincidentally (or not) in the same period, to explain catalysis generally the notions of orbital steering, propinquity, and the chelate effect were being vigorously discussed among physical organic chemists-turned enzymologists (*e.g.* Drs. Jencks, Bruice and Koshland); while “entasis” was never used to rationalize the reactivity of amides in the active site of chymotrypsin, more recent discussions of these same concepts (*e.g.* the Circe Effect, Reactant State Stabilization) by computational chemists like Drs. Wolynes and Warshel have effectively brought the entasis story full circle. The specificity that the multicopper oxidase, Fet3p from *Saccharomyces cerevisiae*, exhibits towards Fe^{II} as substrate is arguably one of the clearest examples of *substrate* entasis in bioinorganic chemistry.

Professor Solomon shares his 60th birthday with another, the 50th “birthday” of Rudy Marcus’ first full paper (in 1956) [2] on what became the Marcus Theory of electron transfer [3], a theory that, in effect, tells us *what* entasis *on* Fe^{II} Fet3p *could* perform in order to make ferrous iron a better e^- -transfer partner in the ferroxidase reaction. In this brief story, then, the intent is to describe how the Fet3p reaction with Fe^{II} reflects these two, defining aspects of bioinorganic chemistry that were in place over the course of Ed’s first 32 years as a member of the club.

2. The Fet3 protein: function and structure

The Fet3 protein expressed by fungi is a prototypical three-cupredoxin domain multicopper oxidase with two additional structural motifs: an amino-terminal signal sequence that targets the nascent Fet3 polypeptide to the endoplasmic reticulum (ER) and a carboxyl-terminal transmembrane domain that tethers the chain to the luminal face of the ER and thence to the exocyttoplasmic face of the plasma membrane [4,5]. In the ER, the *apo*-Fet3p is core-glycosylated and associates with the Ftr1 protein, a ferric iron permease which together with Fet3p makes up the fungal high-affinity iron uptake complex in the yeast plasma membrane [6,7]; Cu is inserted into the protein’s three Cu sites in a later vesicular compartment [8]. In Fe-uptake, Fet3p oxidizes Fe^{II} generated by ferriredoxin (in yeast, by Fre1p) and traffics the Fe^{III} product to Ftr1p for membrane permeation. This iron metabolic process is required for respiratory growth of *Saccharomyces cerevisiae* [6] and for virulence of fungal pathogens like *Candida albicans* [9] and *Cryptococcus neoformans* [10].

The exocyttoplasmic MCO domain of Fet3p includes residues 22–555; by expressing a gene encoding only these residues, a soluble Fet3p is produced that is secreted into the growth medium from which it can be readily purified [4]. The ribbon diagram of this Fet3p species based on a 2.8 Å resolution structure is shown in Fig. 2 [11].

Also shown in Fig. 2 is a ribbon diagram of a laccase from the fungus *Trametes versicolor* (Tv) [12]. This comparison illustrates that the specificity Fet3p exhibits towards Fe^{II} as substrate and that laccases show towards aromatic amines and phenols is not readily understood by inspection of the overall folds of these two MCO classes; indeed, the panels in Fig. 2 are easily mistaken for a stereo image. As the site of intermolecular e^- -transfer from substrate to the MCO, the T1 Cu is the active site for substrate oxidation, much as the trinuclear cluster is the site for dioxygen reduction [5,13]. Fig. 3 highlights the principle structural differences second sphere to the T1 Cu atoms in a fungal ferroxidase in comparison to a fungal laccase.

There are two differences of note. First, in the laccase, the NE2 pyrrole NH group of T1 Cu ligand, H458, is exposed to solvent whereas in Fet3p the homologous residue, H489, is below the Connolly surface masked by the carboxylate side chains of E185 and D283. Second is the overall acidic protein surface at the T1 Cu site in Fet3p in comparison to the non-polar one found in the laccase; the third acidic residue in Fet3p is D409. E185 and D409 are found within the sequences $^{181}\text{PTGAEP}^{186}$ and $^{409}\text{DTGTHPFLH}^{418}$; these two sequence motifs *define* a fungal ferroxidase, readily distinguishing ferroxidases from the laccase class of MCOs. The remainder of this article summarizes the quantification of the roles these three ferroxidase specificity elements play in inducing entasis in the Fet3p•Fe^{II}, enzyme•substrate complex.

3. Reverse protein engineering in Fet3p: kinetic mapping of ferroxidase specificity motifs

3.1. Properties of ferroxidase mutants in Fet3p

Alanine substitutions were made at E185, D283 and D409; these three single mutants and the Fet3p(E185A/D409A) double mutant proteins were purified and characterized [14–16]. The X-band cwEPR and near-uv and visible absorbance spectra of all four mutants were quantitatively indistinguishable from wild type. One the other hand, while mutants containing the E185A or D283A mutations had the wild type E^{O} values (433 ± 3 mV), those containing the D409A one had $E^{\text{O}} = 550 \pm 3$ mV [16].

3.2. Steady-state kinetic constants for Fe^{II} and HQ oxidation by wild type and Fet3p mutants

Standard kinetic analysis of the steady-state turnover of Fe^{II} and hydroquinone (HQ) monitored by O₂-uptake yielded rate data that were fit directly to the Michaelis-Menten equation; the [O₂] was 240 μM , or ~ 200 -times K_{M} for O₂ which is 1.3 μM . The K_{M} values for Fe^{II} and HQ derived from this fit were 5 μM *versus* 25 mM (Table 1). HQ is a standard “laccase” MCO substrate.

3.3. Stoichiometry and rates of T1 Cu^{II} reduction by Fe^{II} and HQ

Anaerobic samples of wild type and mutant forms of Fet3p were titrated with Fe^{II} and HQ; after equilibration following each addition, the absorbance of the protein sample at 608 nm was quantified (for Fet3p, $\epsilon_{608 \text{ nm}} = 5500 \text{ M}^{-1} \text{ cm}^{-1}$). Taken as a measure of reduction of the four Fet3p Cu^{II}, these values were plotted *versus* the molar ratio of reductant added; data like these for Fe^{II} are shown in Fig. 4 and indicate that the reduction is stoichiometric.

The rate of this reduction for each protein species was obtained by stopped-flow kinetic analysis in which similar anaerobic protein samples were flowed against Fe^{II} or HQ following the reduction at 608 nm. Pseudo-first order conditions obtained in these experiments (excess of reductant); the velocity data were therefore fit to a simple first-order exponential function to yield values for a first-order e[−]-transfer rate constant (k_{ET}) from each reductant to each Fet3p species [16]. These values are given in Table 1 as well. Together the steady-state and stopped-flow data show that in replacing the three carboxylate side chains by a methyl group, Fet3p has been converted into a laccase in terms of substrate specificity, albeit a relatively inefficient one.

4. Marcus theory

The semiclassical theory for intermolecular electron transfer includes five factors characteristic of a given donor-acceptor pair [3]. The first two are the steric term, S , and the equilibrium constant for formation of the interaction complex between the electron donor and acceptor, K_{A} . The steric term represents that fraction of the complex that is competent for electron transfer, reflecting what the enzyme kineticist would refer to as productive binding; thus, the

product, SK_A , is equivalent to $1/K_M$, since K_M is the effective substrate dissociation constant from a *productive* Michealis complex. The electronic matrix coupling element, H_{DA} , provides a measure of the relative conductivity of the combination of through-bond and through-space jumps the electron must navigate in its path from donor to acceptor molecular orbital. The thermodynamic driving force for this electron transfer is given by ΔG^O , related to the difference in reduction potentials of donor and acceptor by the Nernst equation. Last, the reorganization energy, λ , is the energy released or absorbed by the T1 Cu coordination site upon change in redox state, from Cu^{II} to Cu^{I} . These terms are related to the rate constant for ET by Eq. 1.

$$k_{ET} = S K_A \left(\frac{4\pi^3}{h^2 \lambda k_B T} \right)^{1/2} (H_{DA})^2 e^{[-(\Delta G^O + \lambda)^2 / 4 \lambda k_B T]}$$

Eq. 1. The Marcus equation. The rate constant for outer-sphere electron varies as a function of four properties of the donor, acceptor pair: 1) the product SK_A , which quantifies the probability of a productive electron transfer complex; 2) H_{DA} , quantifying the efficiency of electronic coupling between the donor electron and acceptor “hole”; 3) ΔG^O , the thermodynamic driving force given by the difference between the E^O values of donor and acceptor; and 4) λ , the energy of ligand field reorganization due to the change in redox state.

5. Entasis in ET from Fe^{II} to Fet3p

Aqueous Fe^{II} has a redox potential of ~ 770 mV at pH 6.0, the conditions of the Fet3p reduction experiments described above; for comparison $E^O = 70$ mV for HQ. Thus, the Nernst equation predicts that the T1 Cu in Fet3p ($E^O = 430$ mV) should *not* be reduced by stoichiometric Fe^{II} while the Marcus equation predicts that k_{ET} for reduction by HQ should be significantly larger than k_{ET} for the Fe^{II} process. The data in Table 1 are contrary to both predictions.

In order for the Fe^{II} -Fet3p Cu^{II} couple to go to completion at stoichiometric ratios of reductant/Fet3p Cu^{II} (Fig. 4), a reasonable *upper* limit on the E^O of the reductant is 190 mV, or 240 mV below that of the T1 Cu; the reduction by HQ adheres to this limit. For Fe^{II} to exhibit an equivalent reducing power its reduction potential must be lowered ≥ 580 mV *upon binding at the T1 Cu active site*. This shift in potential can only be “paid for” by binding energy, specifically, that the Fe^{II} was binding to a site that actually favored Fe^{III} . In terms of entasis, the effective ground state energy of the Fe^{II} was raised by the equivalent of ~ 580 mV (or $\sim +40$ kJ) by binding to a site that preferred Fe^{III} by a factor of $\sim 10^{10}$ in K_A . This thermodynamic cycle is illustrated in Fig. 5.

The bioinorganic chemist would have little trouble designing a coordination sphere that would exhibit this selectivity; one need only to consider that the oxygen-rich coordination site offered by transferrin or a hydroxamate siderophore sets the E^O of the $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ couple ≤ -400 mV. Reasonably a site that included three carboxylates would be an appropriate compromise between stability of the Fe^{III} -product complex (making Fe^{II} a good reductant) and open (water) coordination sites available for the subsequent ligand exchange reactions required to move the Fe^{III} along to Ftr1p for Fe-uptake.

6. Making ET efficient

Making ET thermodynamically probable doesn’t make ET efficient; it’s the SK_A and H_{DA} terms in the Marcus equation that do. The 10^{-3} -fold difference in K_A for Fe^{II} in comparison to HQ is worth an equivalent factor in k_{ET} , favoring ET from Fe^{II} *independent of the driving force*. A similar linear relationship holds between k_{ET} and $(H_{DA})^2$; the question then becomes, how does the structure of the bound substrate complex maximize this electronic matrix coupling term.

This term represents the conductivity of the matrix to exchange of the electron on the reductant with the hole on the oxidant. In outersphere ET the matrix includes the covalent and non-covalent bonds that link the electron donor to electron acceptor and, if present in the otherwise shortest path, through-space jumps. The PATHWAYS program developed by Beratan *et al* uses protein structural data to estimate the relative conductivity (H_{DA} terms) of possible ET routes [17]; application of this program to a putative $\text{Fe}^{\text{II}}\cdot\text{Fet3p}$ complex yields the relative k_{ET} values given in Fig. 6.

7. Summing up: where does the Fe^{II} specificity in Fet3p come from?

The data in Table 1 show that alanine substitutions at E185, D283 and D409 have markedly different effects on the kinetic values for Fe^{II} versus HQ as substrate: K_{M} values for Fe^{II} strongly increase while those for HQ are little changed; correspondingly, Fe^{II} k_{ET} values decrease while those for HQ increase. As noted, qualitatively the Fet3p(E185A/D409A) mutant is a better laccase than a ferroxidase. The increase in HQ k_{ET} could be due in part to the $\sim +115$ mV increase in the T1 Cu redox potential in D409A mutants, but this increase in driving force wasn't apparent with Fe^{II} as substrate. Part of the reduced ET from Fe^{II} can be ascribed to the increase in K_{M} value (decrease in K_{A}), but this term can't explain the increase in k_{ET} from HQ (Table 1). Another factor, then, in ET rate is a change in H_{DA} : decreasing for Fe^{II} , increasing for HQ. The values in Fig. 6 indicate the fold-change that might be ascribed for ET from Fe^{II} ; consideration of the ET pathway that likely obtains in a laccase could similarly explain the reactivity of HQ with the mutant Fet3 proteins.

Table 2 breaks down the Marcus equation and, based on the changes in K_{M} for Fe^{II} in steady-state turnover, the T1 Cu E^{O} , and the H_{DA} value calculated by the PATHWAYS program [17], then lists values for a predicted k_{ET} due solely to the fold-change in each term. For example, the decrease in e^- -transfer complex stability (K_{A}) in the double mutant can account for $\sim 25\%$ of the overall fold-change (decrease) in k_{ET} . The loss of coupling (decrease in H_{DA}) by itself can account for less than 1% of the change in electron transfer in this mutant. Taking into account the increase in driving force due to the 100 mV volt increase in T1 Cu E^{O} in D409A mutants, one can come reasonably close to reproducing the observed value for k_{ET} for this mutant. It is likely that compensating changes in electronic matrix coupling, S , and E^{O} for the bound Fe^{II} are masked by this simple calculation however satisfying that it comes close to recapitulating the experiment result.

The structure of the complex of TvLac with a substrate analog, 2,5-xylydine, is shown in Fig. 7. In this complex, the substrate $-\text{NH}_2$ group is H-bonded to the NE2 NH of H458, the otherwise solvent-exposed T1 Cu ligand noted in regards to Fig. 3; this H-bond is likely an essential component in the efficient ET from substrate to the T1 Cu^{II} in a laccase. Also shown in Fig. 7 is the T1 Cu in Fet3p with the carboxylate $\rightarrow$ alanine substitutions modeled in (along with three other hydrophilic $\rightarrow$ alanine ones); it's not hard to imagine 2,5-xylydine binding to this protein much as it does to TvLac. We expect that protein engineering approaches like this one will result in facile interconversion of the substrate specificity of MCO proteins, thus both confirming our inferences about what makes a ferroxidase and possibly affording MCOs with novel activities.

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Biographical Sketch

A Ph.D. physical organic chemist (University of Chicago) Professor Kosman has focused on the structure and function of copper proteins since 1970 when he joined the Department of Biochemistry at the University at Buffalo. Currently, his primary research is on the mechanisms of trafficking iron in cells with particular emphasis on the role played in these pathways by multicopper ferroxidases. Much of this work is in collaboration with Dr. Solomon and his students.

Fig. 1.
Three who were present at the creation of metallo-enzyme structure and reactivity; from the left: Bert Vallee, R. J. P. Williams, Rudy Marcus.

Fig. 2. Fet3p (1ZPU) (a) and *Trametes versicolor* laccase (1GYC) (b); T1 Cu (blue), T2 Cu (to the back, green) and T3 binuclear Cu cluster (yellow).

Fig. 3.
The substrate binding sites at the Tl Cu in Fet3p (a) and *Tv* laccase (b); H458 in *Tv* Lac is exposed to solvent whereas the homologous His (489) in Fet3p is shielded from solvent by E185 and D283.

Fig. 4.
Fet3p is reduced stoichiometrically by anaerobic addition of 4-protein equivalents of Fe^{II} .

Fig. 5. Thermodynamic cycle in which $K_A(\text{Fe}^{\text{III}}) > K_A(\text{Fe}^{\text{II}})$ stabilizes Fe^{III} over Fe^{II} reducing the effective E° for the $\text{Fe}^{\text{II/III}}$ redox couple.

Fig. 6. Relative matrix coupling efficiencies for outersphere e-transfer from bound Fe^{II} to the T1 Cu^{II} in Fet3p; values calculated from PATHWAYS estimates of H_{DA} terms.

Fig. 7. *TvLac*IIIB bound with 2,5-xylydine (1KYA) (a) and *ScFet3p* (1ZPU) with E/D to A and additional substitutions chosen to mimic the more accessible, non-polar surface presented to substrate in Lac.

Table 1

Kinetic constants for Fe^{II} and HQ reaction with wild type and D/E→A Fet3p mutants

Fet3p species	Fe ^{II} (μM)	K _M	HQ (mM)	Fe ^{II}	K _{ET} (sec ⁻¹)	HQ	Fe ^{II} specificity
WT	5		25	≥1500		0.02	375000
D283A	19		19	682		0.07	9000
E185A	36		18	2.5		0.10	23
D409A	19		30	62		1.12	75
E185A/D409A	4000		30	0.41		2.12	0.006

Fe^{II} specificity is given by $\frac{k_{ET}/K_M(Fe^{II})}{k_{ET}/K_M(HQ)}$

Table 2

Correlation between observed and predicted k_{ET} values

	Observed k_{ET} , s ⁻¹	Predicted k_{ET} based individual Marcus terms (fold-change due to each term) $1/K_M = SK_A$	ΔG^0 (eV)	H_{DA}	Calculated k_{ET} , s ⁻¹
WT	≥1500				
D283A	682	375 (0.25)	NC	WT	375
E185A	2.5	210 (0.14)	NC	165 (0.11)	22
D409A	62	390 (0.26)	18,000 (12.2)	660 (0.44)	165
E185A/D409A	0.4	1.5(0.001)	18,000 (12.2)	75 (0.05)	0.08

The values compare the predicted k_{ET} and fold-change (in parentheses) for the individual Marcus terms (Eq. 1) relative to the observed wild type k_{ET} with Fe^{II} as substrate. The calculated values for k_{ET} were determined as the sum of the individual terms *as per* the Marcus equation. The values H_{DA} were given by the PATHWAYS program and the coordinates for Fet3p (1ZPU).