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The Rise of Radicals in Bioinorganic Chemistry

Harry B. Gray and Jay R. Winkler

Beckman Institute, California Institute of Technology, 1200 E California Boulevard, Pasadena, CA 91125, USA

Abstract

Prior to 1950, the consensus was that biological transformations occurred in two-electron steps, thereby avoiding the generation of free radicals. Dramatic advances in spectroscopy, biochemistry, and molecular biology have led to the realization that protein-based radicals participate in a vast array of vital biological mechanisms. Redox processes involving high-potential intermediates formed in reactions with O₂ are particularly susceptible to radical formation. Clusters of tyrosine (Tyr) and tryptophan (Trp) residues have been found in many O₂-reactive enzymes, raising the possibility that they play an antioxidant protective role. In blue copper proteins with plastocyanin-like domains, Tyr/Trp clusters are uncommon in the low-potential single-domain electron-transfer proteins and in the two-domain copper nitrite reductases. The two-domain multicopper oxidases, however, exhibit clusters of Tyr and Trp residues near the trinuclear copper active site where O₂ is reduced. These clusters may play a protective role to ensure that reactive oxygen species are not liberated during O₂ reduction.

Keywords

Electron transfer; radicals; tyrosine; tryptophan

Introduction

Arguably the most important chemical reaction on our planet is water oxidation to oxygen in green plants. The catalyst that makes this reaction run so well is a manganese-calcium cluster called the oxygen evolving complex (OEC), which is activated by multiple hole injections from a nearby tyrosyl radical. Solar photons provide the energy for tyrosyl radical generation, which occurs by hole injection from a photogenerated P680 chlorophyll cation radical. Amino acid radicals also play key roles in many other bioinorganic reactions, most notably involving redox enzymes such as ribonucleotide reductase and DNA photolyase.^[1]

The oxidizing equivalents needed for many substrate reactions often cannot be met by metal ions alone. A case in point is cytochrome P450, where porphyrin radicals function during substrate oxygenations; what is more, in the reactions of peroxidases, both porphyrin and tryptophan radicals have been shown to be functional intermediates.^[1] It would appear that radical enzymes dominate bioinorganic chemistry! Indeed, if we include all the radical S-

adenosylmethionine (SAM) enzymes, there are more redox enzymes that require radicals for function than ones that do not.

Two years ago, we advanced a hypothesis based on straightforward thermodynamics that high-potential oxidative bioinorganic chemistry cannot occur without the generation of amino acid radicals.^[2] We began a search of structural databases that led to the discovery of long chains of tyrosines and tryptophans in several classes of proteins, most notably in metalloenzymes that utilize oxygen for redox function.^[3] We further argued that these chains protect enzymes from oxidative destruction by steering highly oxidizing holes to regions where they can be rendered harmless by available reducing agents. We suspect that some of these radicals also could be players in active-site function, a hypothesis that we will advance for multicopper oxidases in this paper.

How did this rise of radicals come about? Who first detected an amino acid radical? Read on!

Free Radicals in Proteins

Leonor Michaelis argued in 1946 that “all oxidations of organic molecules, although they are bivalent, proceed in two successive univalent steps, the intermediate state being a free radical”.^[4] Influenced by Michaelis’ suggestion, Barry Commoner and coworkers in the early 1950s applied the newly developed technique of electron-paramagnetic resonance to probe for free-radical signals in plant and animal tissues.^[5] Lyophilized samples from plant leaves and roots, as well as from animal blood, muscles, and organs, exhibited large and persistent EPR signals indicative of free radicals. Fractionation of the tissue samples revealed that the free radicals tended to be found in protein components. Two years later, Commoner reported that EPR signals consistent with free radicals could be detected in isolated tobacco-leaf chloroplasts upon irradiation with an automobile headlamp.^[6] The EPR signals reached a maximum value after about 20 s of irradiation, then decayed exponentially with a 45-s time constant when the lamp was switched off.

Meanwhile, Helmut Beinert was studying intermediates formed in flavoprotein catalysis. He reported results from rapid-scan UV-vis spectrophotometry that suggested flavin semiquinone free radicals were involved in reactions catalyzed by pig-liver acyl-CoA dehydrogenase.^[7] EPR evidence for the intermediacy of flavin semiquinone radicals in flavoprotein catalysis was reported by several laboratories in the late 1950s and early 1960s.^[8]

Perhaps the earliest discovery of a stable amino acid radical in an enzyme was reported by Takashi Yonetani, Heinz Schleyer, and Anders Ehrenberg in 1966. They found an intense narrow signal at $g = 2.00$ in the EPR spectrum of the ES complex formed in the reaction of cytochrome *c* peroxidase (CCP) with one equivalent of C_2H_5OOH ; integration of the signal indicated the presence of 1 spin per heme group. Knowing that Complex ES was oxidized two equivalents above the ferric resting state of the enzyme, Yonetani and coworkers proposed that one of the oxidizing equivalents was “retained in the form of a stable and reversible free radical of an aromatic amino acid residue of the enzyme protein;” the second

equivalent was suggested to reside in the heme group.^[9] The identity of this residue remained a mystery for many years. Amino acid analyses of the enzyme after reaction with excess H₂O₂ at pH 4 and 7 revealed extensive Tyr decomposition, along with some Trp decomposition in the more acidic conditions.^[10] An analysis of the resting-state CCP X-ray crystal structure led Tom Poulos and Joe Kraut to propose in 1980 that the radical in Complex ES resided on Trp⁵¹, a residue situated about 3.6 Å from the distal side of the heme.^[11] At about the same time, ENDOR measurements on the CCP ES radical by Brian Hoffman and coworkers were interpreted in terms of a nucleophilically stabilized methionyl radical.^[12] The advent of site-directed mutagenesis helped resolve some of the uncertainty surrounding the identity of the CCP ES radical. Dave Goodin, Grant Mauk, and Michael Smith demonstrated that Met¹⁷² could not be the site of this radical,^[13] and measurements from Kraut's laboratory on the CCP Trp⁵¹Phe mutant demonstrated that this residue was not the source of the radical signal.^[14] The X-ray crystal structure of CCP ES identified a new possible locus for the radical site, near a cluster composed of the Met²³⁰, Met²³¹, and Trp¹⁹¹ sidechains, about 10 Å from the proximal side of the heme.^[15] The Trp¹⁹¹Phe mutant was found to be catalytically deficient,^[16] and finally, in 1989, high-field ENDOR measurements on specifically deuterated enzymes provided definitive evidence that Trp¹⁹¹ is the site of the CCP ES radical.^[17] It is now appreciated that heme Compounds I, the analogues of CCP ES in a vast array of peroxidases and heme oxygenases, typically contain a porphyrin or protein radical in addition to an Fe^{IV}-oxo heme.^[18]

Several new protein radicals were discovered while the hunt for the identity of the CCP ES radical was underway. In the late 1960s and early 1970s, AdoCbl-dependent (AdoCbl = adenosylcobalamin) enzymes were reported to exhibit EPR signals during turnover that were consistent with low-spin Co(II) and an organic (adenosyl) free radical.^[19] These observations provided compelling support for enzymatic mechanisms involving Co-C bond homolysis.^[20]

Ribonucleotide reductases (RNR) have long been a prolific hunting ground for radical intermediates. EPR signals indicative of radical intermediates in the Class II AdoCbl-dependent RNR from the facultative anaerobe *Lactobacillus leichmannii* were detected during ribonucleotide reduction by J. A. Hamilton and R. L. Blakley in 1969.^[19c] Subsequent freeze-quench EPR measurements on *L. leichmannii* RNR revealed a different radical signal that exhibited formation and disappearance kinetics consistent with a catalytic intermediate.^[21] Twenty years later, EPR measurements on a protein grown with β-(²H)-cysteine confirmed that the radical was located on the sidechain of Cys⁴⁰⁸.^[22] In 1972, Ehrenberg and Peter Reichard reported that the Fe-containing subunit of the Class I ribonucleotide reductase from *Escherichia coli* exhibited a stable radical EPR spectrum that correlated with a unique 410-nm absorption feature in the near-UV spectrum of the enzyme.^[23] To track down the identity of the radical, bacteria were grown in D₂O containing media and the resulting EPR spectrum of the enzyme exhibited a loss of hyperfine structure.^[24] Bacteria were then grown in D₂O media that were selectively enriched in non-deuterated amino acids. The proton hyperfine coupling in the EPR spectrum was restored only when non-deuterated Tyr was included in the growth medium. Site-directed mutagenesis studies confirmed the identity of the radical as Tyr¹²².^[25]

Thirty years after the discovery of a radical in AdoCbl catalysis, adenosyl radicals were found to be key intermediates in the reactions of a large superfamily of S-adenosylmethionine (SAM) dependent proteins known as radical SAM enzymes.^[26] Radical SAM enzymes employ a [4Fe-4S] cluster and a SAM cofactor to generate Ado[•] radicals that participate in an extraordinarily diverse array of chemical transformations. One particular radical SAM reaction leads to the production of a fourth type of stable amino acid radical (glycyl). Stable glycyl radicals are formed in the abstraction of an H-atom from glycine by Ado[•] in enzymes such as pyruvate formate lyase, anaerobic ribonucleotide reductase, benzylsuccinate synthase, 4-hydroxyphenylacetate decarboxylase, and glycerol dehydratase.^[26a]

Hole Tunneling Chains in Proteins

Protein radicals are not always isolated active sites; several examples are known in which radical-transfer chains transport oxidizing equivalents over long distances in proteins. The photoactivation of DNA photolyase described by Klaus Brettel and coworkers is a case in point.^[27] Upon photoexcitation, the flavin adenine nucleotide (FADH) cofactor in *E. coli* DNA photolyase abstracts an electron from the adjacent (4.2 Å) Trp³⁸² residue in 30 ps. The oxidizing hole in the Trp³⁸² cation radical transfers through Trp³⁵⁹ (5.2 Å) and reaches solvent-exposed Trp³⁰⁶ (3.9 Å) in under 10 ns. The Trp³⁰⁶ cation radical deprotonates in about 300 ns, forming the neutral radical. Recent research suggests that a 4-residue Trp chain in *Xenopus laevis* (6–4) photolyase conducts holes from the excited flavin to the surface in less than 20 ns.^[28] Tyr radicals also can participate in radical-transfer chains. In the Class I *E. coli* ribonucleotide reductase, JoAnne Stubbe and coworkers have characterized a chain of Tyr and Trp residues that conduct a hole over 35 Å from the stable Tyr¹²² radical in the β2 subunit to a transient Cys⁴³⁹ radical that drives ribonucleotide reduction in the α2 subunit.^[29]

In an effort to identify new radical-transfer chains, we searched the structural database (RCSB: <http://www.rcsb.org>) for proteins containing three or more redox-active Tyr or Trp residues separated from one another by ≤ 5 Å.^[3a] We found that Tyr/Trp chains comprised of three or more residues are present in about one-third of ~27,000 unique protein structures. The Tyr/Trp chains occurred with highest frequency in glycosylases and oxidoreductases. In the latter class, the chains were suggested to serve protective roles by guiding potentially damaging oxidizing equivalents away from active sites toward protein surfaces where they could be scavenged by cellular reductants. The evolutionary trajectory of blue copper proteins from small single-domain electron-transfer (ET) proteins to multidomain, multinuclear redox enzymes provides an interesting test case for this hypothesis.^[30]

Blue Copper Protein Evolution

Single-domain cupredoxins, exemplified by plastocyanin, are ET proteins characterized by an eight-stranded β-barrel fold with a type 1 copper binding site near one end of the barrel. The Cu ion is coordinated in a trigonal plane by two histidine imidazole ligands and a cysteine thiolate; a longer distance axial interaction with a methionine sulfur atom is commonly, although not exclusively, found. This coordination environment produces the

intense blue color of the Cu^{2+} forms, and leads to $\text{Cu}^{2+/+}$ formal potentials ranging from 184 mV vs. NHE for stellacyanin to 680 mV for rusticyanin. Analyses of genome sequences and three dimensional structures have revealed that the cupredoxin fold occurs as a structural element in many larger proteins, leading to speculation that these proteins share a common ancestor.^[30a–d] The reasoning is that the multidomain proteins evolved through a sequence of domain duplication coupled with active-site deletion and creation.

Two-domain blue copper proteins include copper nitrite reductases (NiR) and multicopper oxidases (2dMCO). These proteins have homotrimeric structures with a type 1 center in one or both cupredoxin domain, and a catalytic active site constructed in the interface between the two domains. NiR has a single type 1 center in the first cupredoxin domain, and a type 2 interdomain copper center coordinated by three histidine imidazole sidechains. The type 2 copper center is the locus of NO_2^- reduction to NO. The interdomain active site in 2dMCOs is composed of a type 2 copper atom closely coupled to a binuclear (type 3) site, creating a trinuclear catalytic center (TNC) where O_2 reduction occurs. Three classes of 2dMCOs have been characterized on the basis of their type 1 configuration: class A has type 1 centers in both domains; class B has the type 1 center in domain 2; and class C has the center in domain 1.

We searched for Tyr/Trp chains in the structures of 30 single-domain cupredoxins, 9 NiRs, and 8 2dMCOs and found that chains are uncommon in the single-domain proteins, occur away from the catalytic site in NiRs, and cluster around the TNC in the 2dMCOs.

Tyr/Trp Chains in Single-Domain Cupredoxins

The 30 single-domain cupredoxin structures included 13 plastocyanins, 8 azurins, 4 pseudoazurins, 2 amicyanins, 2 aurocyanins, and rusticyanin (Figure 1). Closely coupled Tyr/Trp chains occur infrequently in these structures. In no instance was a redox-active Tyr or Trp residue located within 5 Å of the type 1 copper center. The only Tyr or Trp residue located less than 7.5 Å from a Cu site was Tyr¹¹⁴ (5.03 Å) in the iso-2 azurin from *Methylobacter* sp. (strain J) (PDB ID 1CUO). Clusters of 2 to 3 Tyr and Trp residues separated by ~5 Å occur near the midpoint of the β -barrel axis in about one-third of the structures. The scarcity of closely coupled Tyr/Trp chains is possibly a consequence of the fact that the formal potentials of the Cu centers in these proteins are substantially lower than that required to oxidize a Tyr or Trp sidechain (E° ~1 V vs. NHE).

Tyr/Trp Chains in Copper Nitrite Reductases

Copper NiRs catalyze the two-proton, one-electron reduction of NO_2^- to NO in denitrifying bacteria.^[31] The type 1 centers in NiRs fall into two categories: Class I proteins are blue and Class II are green.^[32] The reduction potentials of the type 1 sites are in the 200–300 mV vs. NHE range at pH 7 (*Alcaligenes faecalis* S-6, 213 mV;^[33] *Achromobacter cycloclastes* IAM 1013, 240 mV;^[31] *Rhodobacter sphaeroides*, 247 mV;^[34] *Alcaligenes xylosoxidans* GIFU 1051, 280 mV;^[31] *Pseudomonas chlororaphis* DSM 50135, 298 mV^[35]) The type 2 copper centers, where nitrite binding and reduction occur, are characterized by potentials in a similar range (*Pseudomonas chlororaphis* DSM 50135, 175 mV;^[35] *Alcaligenes faecalis* S-6,

234 mV;^[33] *Alcaligenes xylosoxidans* GIFU 1051, 250 mV;^[31] *Achromobacter cycloclastes* IAM 1013, 240 mV;^[31]). The enzymatic mechanism remains a topic of discussion,^[31–32, 36] but the consensus is that reducing equivalents are delivered first to the NiR type 1 site by a pseudoazurin or azurin redox partner; subsequent electron transfer to the nitrite-bound type 2 site induces NO formation. Mutagenesis studies have implicated several acidic NiR residues (Glu¹¹⁸, Glu¹⁹⁷, Glu²⁰⁴, and Asp²⁰⁵) in the *Alcaligenes faecalis* S-6 enzyme where its basic pseudoazurin redox partner is thought to bind electrostatically.^[31, 37] Nitrite reduction catalysis in these enzymes operates at potentials that are too low to produce Tyr or Trp radicals, so closely spaced clusters of these residues likely arose from other exigencies.

Tyr/Trp chains occur in 9 structurally characterized copper NiRs (Figure 2) with substantially greater frequency than in the single-domain cupredoxins (Figure 1). Yet, closely spaced (~ 5 Å) Tyr and Trp residues are not found near the type 2 sites in the NiRs. Instead, Tyr and Trp residues tend to cluster near the type 1 Cu binding sites. In the *Alcaligenes faecalis* enzyme, for example, Tyr²⁰³ and Trp¹⁴⁴ are adjacent to two of the residues implicated in pseudoazurin binding (Glu²⁰⁴ and Asp²⁰⁵). Since high-potential intermediates likely are not formed during nitrite reduction, it is possible that the Tyr/Trp clusters near the electron entry point in NiR enhance superexchange coupling for electron transfer between the type 1 Cu centers of pseudoazurin and NiR.^[38]

Tyr/Trp Chains in Two-Domain Multicopper Oxidases

Multicopper oxidases catalyze the oxidation of organic substrates and metal ions by O₂.^[39] The consensus mechanism for this transformation involves substrate oxidation at the type 1 Cu and electron transfer to the TNC where O₂ is reduced to water. The 2dMCOs are believed to be evolutionary precursors to three-cupredoxin-domain monomeric MCO enzymes.^[30c, 30e] The eight reported X-ray crystal structures include 6 class B enzymes (5 *Streptomyces* enzymes, 1 *Amycolaptopsis* sp. ATC 39116 enzyme from a bacterium formerly classified as *Streptomyces setonii*), and 2 class C enzymes (*Nitrosomonas europaea*, *Arthrobacter* sp. FB24). The 6 class B enzymes are from soil bacteria implicated in biomass degradation, and recent work has suggested that the *Streptomyces* 2dMCOs participate in lignocellulose degradation.^[40] The handful of reported formal potentials for the type 1 sites in the 2dMCOs are below 400 mV vs. NHE (*S. viridochromogenes*, 350;^[41] *S. svicensis*, 375 mV;^[42] *S. coelicolor*, 378 mV;^[43]). Given their low potentials, the ability of these bacterial 2dMCOs to oxidize phenolic and lignocellulosic substrates at the type 1 copper site is remarkable, particularly in comparison to heme peroxidases that are thought to employ Trp radicals in lignin degradation.^[18c] The 6 structurally characterized class B 2dMCOs exhibit considerable sequence conservation (Supporting Information). In particular, all Tyr and Trp residues are strictly conserved save for one His replacement for a Tyr in the *S. viridochromogenes* enzyme, and just 3 of the usual 10 Tyr residues are exchanged in the *Amycolaptopsis* sp. (strain ATCC 39116) enzyme. The 5 Trp residues are fully conserved among the 6 class B proteins. Sequence conservation is much less extensive in the two structurally characterized class C enzymes (Supporting Information), as is the case for a larger set of class C 2dMCOs examined by Amy Rosenzweig and coworkers.^[44]

In 2009, Gerard Canters and coworkers reported the appearance of a 420-nm absorption feature in the *S. coelicolor* 2dMCO catalyzed oxidation of ascorbate by O₂ under continuous turnover conditions.^[45] In a type 1 Cu-depleted (T1D) form of the enzyme, the 420-nm band correlated with an unusual triplet biradical EPR spectrum. The two spectroscopic signatures suggested that a Tyr radical forms near the TNC during enzyme turnover. Structural and mutagenesis studies revealed that the optical and paramagnetic resonance signals arose from a Tyr¹⁰⁸ radical.^[46] The triplet biradical EPR spectrum in the T1D enzyme indicated exchange coupling to a nearby Cu²⁺ center; the type 2 Cu ion 4.3 Å from the Tyr¹⁰⁸ phenol O-atom is the likely candidate. Under single-turnover conditions in which fully reduced enzyme reacted with O₂, transient spectroscopy revealed that the Tyr¹⁰⁸ radical, as well as oxidized type 1 and type 3 sites, all appear with same kinetics; moreover, the observed rate constant (23 s⁻¹) is the same in the wild-type and T1D enzymes. Replacement of Tyr¹⁰⁸ with Phe or Ala, however, led to a threefold reduction in the k_{cat} for TMPD oxidation.^[46a]

Our survey of Tyr/Trp chains revealed that Tyr¹⁰⁸ is a member of a cluster of redox-active residues separated from one another by ~5 Å in the *S. coelicolor* 2dMCO; the cluster includes the three copper atoms of the TNC, Tyr¹⁰⁸, and Trp²⁸⁴ (Figure 3). Trp²⁸⁴ has been suggested as a source of a radical signal observed in the T1D-Tyr¹⁰⁸Phe mutant.^[46a] Sequence alignments (Supporting Information)^[46a] indicate that both residues are fully conserved in class B 2dMCOs. Given the extensive sequence homology, it is not surprising that a Tyr/Trp pair is found near the TNC in all of the *Streptomyces* 2dMCO structures (Figure 4). Canters suggested that the Tyr¹⁰⁸ might serve a protective role for the enzyme by providing reducing equivalents to the TNC under conditions where natural reductants were unavailable.^[46a] We have made similar arguments about Tyr/Trp chains found in heme oxygenases.^[3a]

Three pairs of Tyr residues in the *S. coelicolor* 2dMCO cluster near the type 1 Cu sites, a grouping reminiscent of the Tyr/Trp clusters found near the electron entry points in Cu NiRs. Similar clusters appear in all of the *Streptomyces* enzymes, but the Tyr residues in the structures did not always have the nearby proton acceptor that was imposed as a criterion of redox activity.

The class C *Nitrosomonas europaea* 2dMCO does not have a Tyr residue in a position analogous to that of Tyr¹⁰⁸ in the *S. coelicolor* 2dMCO. It does, however, retain a Trp²⁹¹ residue at a position analogous to that of Trp²⁸⁴ in the *S. coelicolor* enzyme. Situated about 8.1 Å from the type 2 center of the TNC, this residue also could play a redox role during times of reductant shortage. Rosenzweig's sequence alignment reveals that this site is occupied by a Tyr residue in 9 of the 10 other class C 2dMCOs that were examined. The *Nitrosomonas europaea* 2dMCO also has a 3-residue Trp chain that extends from one of the type 3 Cu centers in the TNC. The first Trp in the chain is just 3.5 Å from the Cu atom and may be susceptible to oxidation during turnover with oxygen.

Concluding Remarks

Many of the radicals that have been discovered in proteins can trace their origins to the ready availability of O₂ in the atmosphere. Clearly, enzymes that utilize the oxidizing power of O₂

run the risk of damage by highly oxidizing intermediates. The redox proteome evolved, in part, to help protect proteins from this damage.^[47] The antioxidant properties of cysteine and methionine are well recognized, and enzymes have been found that can reverse their oxidative transformations. In view of the facility with which Tyr and Trp residues can move high-potential oxidizing equivalents,^[27–29] as well as the preponderance of Tyr/Trp chains in protein structures,^[3a] it is possible that these aromatic amino acids play an antioxidant role complementary to that of the sulfur amino acids in the redox proteome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Polypeptide ribbon diagrams of single-domain cupredoxins highlighting locations of the Cu center (spheres) and Tyr/Trp chains with 5-Å (red) and 7.5-Å (green) ET cutoff distances: (a) *Synechococcus elongatus* plastocyanin (PC) (PDB ID 1BXV);^[48] (b) *Ulva pertusa* Pc (1IUZ);^[49] (c) *Ulva prolifera* PC (7PCY);^[50] (d) *Synechocystis* sp. PCC 6803 Pc (1PCS);^[51] (e) *Chlamydomonas reinhardtii* Pc (2PLT);^[52] (f) *Silene latifolia* Pc (1BYP);^[53] (g) *Populus nigra* Pc-B (4DP6);^[54] (h) *Populus nigra* Pc-A (4DPB);^[54] (i) *Spinacia oleracea* Pc (1AG6);^[55] (j) *Adiantum capillus-veneris* Pc (1KDJ);^[56] (k) *Nostoc* sp. PCC 7120 Pc

(2CJ3); (l) *Phormidium laminosum* Pc (2Q5B); (m) *Anabaena variabilis* Pc (2GIM);^[57] (n) *Paracoccus denitrificans* amicyanin (Am) (2OV0); (o) *Paracoccus versutus* Am (1ID2);^[58] (p) *Paracoccus pantotrophus* pseudoazurin (pAz) (3ERX);^[59] (q) *Alcaligenes faecalis* pAz (1PAZ);^[60] (r) *Methylobacterium extorquens* pAz (1PMY);^[61] (s) *Achromobacter cycloclastes* pAz (1BQK);^[62] (t) *Pseudomonas putida* azurin (Az) (1NWO);^[63] (u) *Pseudomonas fluorescens* Az (1JOI);^[64] (v) *Alcaligenes faecalis* Az (2IAA);^[65] (w) *Pseudomonas aeruginosa* Az (5AZU);^[66] (x) *Achromobacter xylosoxidans* Az-II (2CCW);^[67] (y) *Achromobacter xylosoxidans* Az-I (1RKR);^[68] (z) *Methylobacter* sp. J Az (1CUO);^[69] (aa) *Alcaligenes denitrificans* Az (1AZC);^[70] (bb) *Chloroflexus aurantiacus* auracyanin-A (2AAN); (cc) *Chloroflexus aurantiacus* auracyanin-B (1QHQ);^[71] (dd) *Acidithiobacillus ferrooxidans* rusticyanin (1RCY).^[72]

Figure 2.

Polypeptide ribbon diagrams of copper nitrite reductases (NiR) highlighting locations of the copper centers (spheres) and Tyr/Trp chains with 5-Å (red) ET cutoff distances: (a) *Achromobacter cycloclastes* (PDB ID 2BW4);^[73] (b) *Alcaligenes faecalis* (1SNR);^[74] (c) *Alcaligenes xylosoxidans* (1OE1);^[75] (d) *Geobacillus kaustophilus* (3WI9);^[76] (e) *Geobacillus thermodenitrificans* (4ZK8);^[77] (f) *Hyphomicrobium denitrificans* (2DV6);^[78] (g) *Nitrosomonas europaea* (4KNU);^[79] (h) *Neisseria gonorrhoeae* (1KBV);^[80] (i) *Rhodobacter sphaeroides* (1ZV2).^[81] The upper three Cu centers are the type 1 sites; the Cu atoms in the middle of the structures are the type 2 sites.

Figure 3. Polypeptide ribbon diagram of the small 2dMCO from *Streptomyces coelicolor* (PDB ID 3KW8).^[46b, 82] Copper centers are shown as spheres; Tyr/Trp clusters are shown in red (5-Å ET distance) and green (7.5 Å). The type 1 Cu centers are at the top of the structure; the TNC Cu centers are in the midlevel region. The Tyr¹⁰⁸/Trp²⁸⁴ clusters lie below the TNC, toward the periphery of the enzyme.

Figure 4. Polypeptide ribbon diagrams of copper two-domain multicopper oxidases (2dMCO) highlighting locations of the copper centers (spheres) and Tyr/Trp chains with 5-Å (red) ET cutoff distances: (a) *Amycolatopsis* sp. ATCC 39116 (PDB ID 3T9W);^[40a] (b) *Streptomyces viridosporus* (3TAS);^[40a] (c) *Streptomyces viridochromogenes* (4N8U); (d) *Streptomyces sviveus* (4M3H);^[42] (e) *Streptomyces lividans* (4GYB); (f) *Streptomyces coelicolor* (3KW8);^[46b, 82] (g) *Nitrosomonas europaea* (3G5W);^[44] (h) *Arthrobacter* sp. FB24 (3GDC). Type 1 Cu centers appear in the upper portions of the structures; the TNC Cu centers are in the midlevel region.