Asymmetry in Bridged Binuclear Metalloenzymes: Lessons for the Chemist

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This microreview describes the structures, properties and mechanisms of three selected bimetallic enzymes: Cu-Zn superoxide dismutase (SOD), purple acid phosphatases (PAPs) and catechol oxidases (COs). The relevance of the unsymmetrical structures in the binucleating environment to the reaction mechanism and the respective roles of each metal center of these enzymes are discussed. Relevant model compounds are also considered.

Introduction

The active site of numerous homobinuclear and heterobinuclear metalloenzymes contains two metal centers, bridged by oxo, hydroxo, carboxylate or imidazolate groups. Even for homobinuclear sites the two metal centers usually reside in chemically different environments, an asymmetry that may also involve the coordination numbers and the geometry of the metal centers. One, two or three bridging groups may be encountered, for example: one imidazolate bridge in Cu-Zn superoxide dismutase, one oxygen and one carboxylate in the diiron enzyme ribonucleotide reductase B_2, one hydroxy and two carboxylates in the diiron enzyme deoxyhemerythrin.

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One challenging problem presented by these binuclear enzymes and their models is to elucidate the overall catalytic mechanism and to identify the roles of the metal ions. These may include:

– activation (fixation) of the substrate,

– stabilization of a transition state by metal ion coordination, hydrogen bonding or proton transfer,

– nucleophile generation via proton abstraction by a basic group,

– nucleophilic attack on the substrate by a functional group or by metal-coordinated hydroxide.

We will focus here on the respective roles of the two metal centers of the selected metalloenzymes (whose essential features are summarized in Table 1). The electronic structures of the metal centers of the enzymes have been described in recent reviews.[1−4] We also indicate selected synthetic models for these enzymes. The identification of small well-defined molecules with unsymmetrical bimetallic centers is

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Catherine Belle received her Chemical Engineering degree in 1985 in Marseilles. She obtained her PhD in 1988 at the University of Marseilles under the supervision of Pr. R. Gallo. After a two-year postdoctoral stay at the University of Firenze (Italy) with Pr. A. Guarna she joined the research group of Pr. J. L. Pierre in Grenoble in 1991, where she now works as a CNRS researcher (senior researcher). She has launched new research axes in biomimetic chemistry, especially on purple acid phosphatases and catechol oxidases models.

MICROREVIEWS: This feature introduces the readers to the author's research through a concise overview of the selected topic. Reference to important work from others in the field is included.

Table 1. Three selected enzymes with unsymmetrical binuclear sites

Enzyme	Function	Nature of the bridge(s) (Intermetallic distance)	Nature of the asymmetry	Ref.
Cu^{II}-Zn^{II} Superoxide dismutase (SOD)	O_2^- dismutation	Imidazolate (6.3 Å)	Heterobinuclear Cu^{II} ligands: four His and one H_2O removed by substrate Zn^{II} ligands: three His and one carboxylate	[5]
Fe^{II}-Fe^{III} or Zn^{II}-Fe^{III} Purple acid phosphatases (PAP)	Hydrolysis of phosphoric esters	One hydroxo and one monodentate carboxylate (3.31 Å [Fe^{II}-Fe^{III}]) (3.26 Å [Zn^{II}-Fe^{III}])	Mixed valence or heterobinuclear Fe^{III} ligands: one His, one Tyr, one hydroxo and one carboxylate (Asp) Fe^{II} (or Zn^{II}) ligands: two His, one carboxylate (Asp) and one H_2O	[4,6–10]
Cu^{II}-Cu^{II} Catechol oxidase (CO)	Oxidation of *o*-diphenols to quinones	One hydroxo (2.87 Å)	Cu_A ligands: three His, one of them covalently linked by an unusual thioether bond to a cystein Cu_B ligands: three His	[11–13]

relevant in catalysis and in understanding the enzymatic processes, and will increase our understanding of the advantages of asymmetry in binuclear metallocatalysts.

Cu-Zn Superoxide Dismutase: A Redox Heterobinuclear Enzyme with only One Metal Center Involved in the Catalytic Cycle

Structural and Functional Aspects of Cu-Zn SOD

Copper-zinc superoxide dismutase (SOD) is composed of two identical subunits, each containing in its active site an imidazolate-bridged bimetallic center with one copper(II) and one zinc(II) ion.[5] It occurs in the cytosol of eukaryotic cells and protects them from the toxic effects of superoxide ions. It catalyzes the dismutation of superoxide via two diffusion-controlled one-electron redox processes ("ping-pong" mechanism):

$$O_2^- + [Cu^{II}] \rightarrow O_2 + [Cu^{I}]$$

$$O_2^- + [Cu^{I}] + 2\,H^+ \rightarrow H_2O_2 + [Cu^{II}]$$

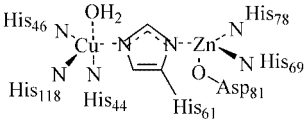


Figure 1. The active site in Cu-Zn SOD

In the metal-binding site (Figure 1) the Cu^{II} ion is coordinated by four histidines in a distorted square-planar coordination sphere and by a fifth axial water ligand, while the tetrahedral zinc ion is coordinated by three histidines, one of them bridging to the copper, and by a carboxylate group. The SOD Cu^{II} center exhibits type-2 copper protein characteristics: a d → d transition in the 500 to 1 μm region and normal EPR signals for tetragonal copper(II) ($g_{//} = 2.229$, $g_{\perp} = 2.041$, $A_{//} = 137 \times 10^{-4}$ cm^{-1}).[3]

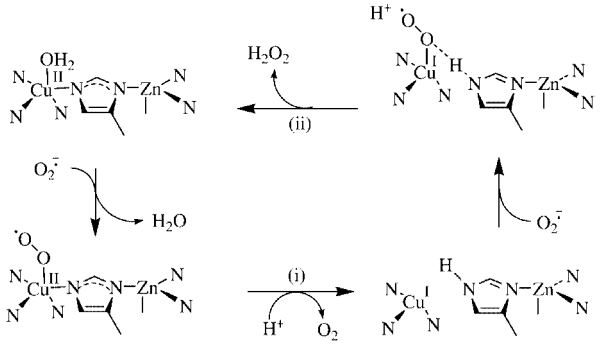


Figure 2. Mechanism for Cu-Zn SOD[5]

The superoxide ion is electrostatically guided by a deep (≈ 13 Å), narrow (≈ 4 Å) channel lined by positively charged amino acid residues. The zinc ion probably assists the protein in adopting the required coordination environment and confers stability to the protein, which is remarkably stable to heat and is active from pH 4.5 to 10. The catalytic mechanism is depicted in Figure 2. The oxidation step (i) is an inner-sphere electron transfer between the coordinated superoxide and the copper(II) center. The concomitant cleavage of the copper-imidazolate bond is assisted by protonation of the imidazolate by the solvent, and oxygen is released. Reoxidation of Cu^{I} to Cu^{II} by the next superoxide reforms the bridge with transfer of a proton to the peroxide ion.

A modified enzyme, having copper in both metal binding sites, Cu-Cu SOD, exhibits the same catalytic activity as native SOD,[14] demonstrating clearly that a copper ion located in the zinc site does not exert catalytic activity.

Cu-Zn SOD Models

Many low-molecular weight copper chelates exhibit, in vitro, superoxide dismutase-like activity. Most lose their activity in the presence of bovine serum albumin (BSA), which can mobilize the complexed copper(II) ions (BSA, a plasmatic protein, is one of the strongest biological chelators of cupric ions; log $K = 16.2$). One of us had described

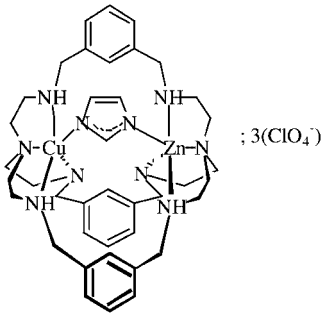


Figure 3. Biomimetic model for the Cu-Zn SOD active site[15]

a biomimetic model[15] that catalyzes the dismutation of superoxide at biological pH and retains its activity in the presence of BSA. Moreover this model shares many of the spectroscopic properties of the enzyme. It is an imidazolate-bridged heterobinuclear copper(II)-zinc(II) complex of a macrobicyclic ligand (Figure 3). The closed bicyclic structure of the ligand is more selective than monocyclic or open-chain ligands: only small ligands (e.g. O_2^-) can access the copper center and the bicyclic structure is expected to mimic the SOD protein channel.

X-ray diffraction studies show a Cu−Zn distance of 5.93 Å (6.3 Å for the enzyme). The EPR and electronic spectral parameters are close to those of the protein. The imidazolate bridge is remarkably stable over a large pH range (6 to 10.5), which is significantly better than other abiotic models that do not involve a protecting bicyclic structure,[16] and is close to that of the enzyme (pH: 4.5 to 10). This is a good structural and functional model, but it does not evidence cooperation between copper and zinc.

An imidazolate-bridged heterobinuclear Cu-Zn complex with a symmetrical ligand, exhibiting SOD activities, has been recently described by Fukuzumi et al.,[17] who tried to clarify the role of the zinc ion (Figure 4). The X-ray structure indicates that the Cu^{II} and Zn^{II} ions have a slightly distorted trigonal-bipyramidal coordination geometry. The cyclic voltammogram in acetonitrile exhibited only one reversible Cu^{I}/Cu^{II} redox couple, at $E_{1/2} = -0.03$ V (vs. Ag/AgCl). This is 0.2 V more positive than for a Cu^{II} mononuclear complex homologue. The authors ascribe this shift to the electronic effect of the imidazolate-bound zinc ion. They conclude that the zinc ion in the heterobinuclear complex may accelerate an outer-sphere electron transfer from superoxide to the Cu^{II}-Zn^{II} complex to produce the Cu^{I}-Zn^{II} complex. The free energy change of electron transfer for the complex is thermodynamically more favorable than that without the Zn^{II} ion. The Zn^{II} ion, which can act as a Lewis acid, may also accelerate electron transfer from the Cu^{I}-Zn^{II} complex to superoxide, since superoxide can form a complex with metal ions acting as a Lewis acid to accelerate the electron transfer reduction of superoxide. Thus, the essential role of Zn^{II} in SOD may be to accelerate both the oxidation and the reduction of superoxide by controlling the redox potential of the Cu^{II} ion and superoxide in the SOD catalytic cycle.

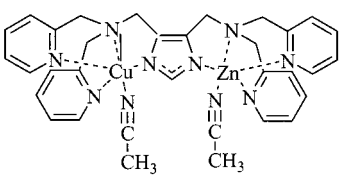


Figure 4. SOD model of Fukuzumi et al.[17]

Purple Acid Phosphatases: An Example of Non-Redox Bimetallic Catalysis

Structural and Functional Aspects of Purple Acid Phosphatases

Purple acid phosphatases (PAPs, Table 1) have been isolated from mammals, plants and fungal sources. They catalyze in vitro the hydrolysis of phosphate esters such as ATP, at a pH range of 4−7.[4,7−9] Their function in vivo is still subject to debate but has been proposed to involve fetal iron transport, control of bone resorption, and phosphate homeostatis in plants.[4] This class of acid phosphatases are purple due to a tyrosinate-to-Fe^{III} charge-transfer at about 560 nm ($\varepsilon \geq 3000$ $M^{-1}\cdot cm^{-1}$ per iron) for their inactive form. The crystal structure of the red kidney bean purple acid phosphatase (kbPAP)[8,10] displays a heterobinuclear $Zn^{II}Fe^{III}$ core, while those of the mammalian PAPs, determined from rat,[18] rat bone[19] and porcine uterus (UfPAP),[20] contain an $Fe^{III}Fe^{III}$ site in the oxidized non-active state.

The crystal structure of kbPAP, first determined at a resolution of 2.9 Å[10] and subsequently at 2.65 Å,[8] reveals a homodimeric 111 kDa enzyme, with two domains in each subunit. In the active site, located in the carboxyl-terminal domain, the two metal ions are 3.26 Å apart, and bridged monodentately by Asp164, each one involving octahedral coordination.

The iron is further coordinated by Tyr167, His325 and Asp135 residues, and the zinc by His286, His323 and Asn201 (Figure 5). Three exogenous ligands are also included in the X-ray model to complete the coordination sphere: one terminal hydroxo ligand to iron, one terminal water ligand to zinc, and one hydroxo bridge (also supported by physical measurements[21]) between the metal ions. In the μ-phosphato derivative structures of mammalian PAPs, the dimetallic ligation appears to be identical and confirms the similarities observed with kbPAP from sequence homologies and spectroscopic studies. The detailed electronic structure of the oxidized and reduced di-iron centers have been characterized by magnetic suscepti-

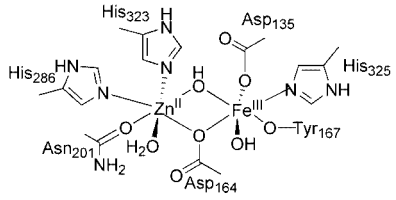


Figure 5. Active site in Zn-Fe kbPAP

bility and EPR measurements, which showed a strong antiferromagnetic interaction ($-2J > 80$ cm^{-1}) in the $Fe^{III}Fe^{III}$ ($S = 0$ ground state) forms and a weaker value in the reduced $Fe^{II}Fe^{III}$ ($S = 1/2$ ground state) forms (typically $-2J = 11$ to 22 cm^{-1}).[2,22] The active forms exhibit a pH-dependent rhombic EPR signal[23] ($g_{av} \approx 1.75$) whereas the inactive forms are EPR silent. Native kbPAP shows a signal at $g = 4.3$, which is characteristic for isolated high-spin Fe^{III} with a $S = 5/2$ ground state, as expected for the $Zn^{II}Fe^{III}$ center.[24]

The crystal structures of kbPAP complexed with μ-phosphate, the product of the reaction, (2.7 Å resolution) and with μ-tungstate inhibitor (3.0 Å) have also been determined, allowing a proposed mechanism for the hydrolysis of phosphomonoesters (Figure 6).[8] In this mechanism, which has not been experimentally demonstrated in such detail, the phosphate group of the substrate binds zinc in monodentate fashion by displacing the presumed water ligand. The terminal Fe^{III}-bound hydroxide ion attacks the phosphorus to form a pentacoordinate intermediate, and the P−O bond opposite the hydroxide ion attack breaks to form the leaving group and phosphate, which is consistent with the inversion at phosphorus. In addition, the three histidines (His202, His295 and His296) located near the bimetallic center can interact with the phosphate. In mammalian PAPs the corresponding residues are two histidines and a glutamate. No similar role is envisaged for the glutamate, which points away from the active site, but the histidines are hydrogen bonded to the bridging phosphate.[18−20]

This proposed mechanism underlines the respective roles of the two metal centers: The substrate first binds the Fe^{II} or Zn^{II} center by removal of the exchangeable water ligand, followed by intramolecular nucleophilic attack of the hydroxide group bound to the Fe^{III} center on the phosphorus P−O bond (activated by its binding to the electrophilic metal center). The well-fitted intermetallic distance allows the overall mechanism. The essential features of the respective roles of the two metal centers are: (*i*) a metal center (bearing an exchangeable ligand) that firstly binds the substrate; (*ii*) a second metal center bearing a reactive OH group that allows the assisted intramolecular reaction; (*iii*) a geometry that allows an intermediate in which the substrate is bound to both metal centers.

Alternative proposed reaction pathways of M^{II}/Fe^{III} purple acid phosphatases have been reviewed recently.[25] Merkx and co-workers propose that the terminal bound hydroxide deprotonates another water molecule from the second coordination sphere of the ferric ion, which then acts as an intramolecular base catalyst (Figure 7, A).[26,27]

As proposed for Fe^{II}/Fe^{III} uteroferrin,[28,29] the two metallic centers are bridged by the phosphate ester. The μ-OH may act as a nucleophile and attack the phosphate (Figure 7, B). When coordinated to one metal ion (Figure 7, A), the hydroxide is expected to be much more nucleophilic

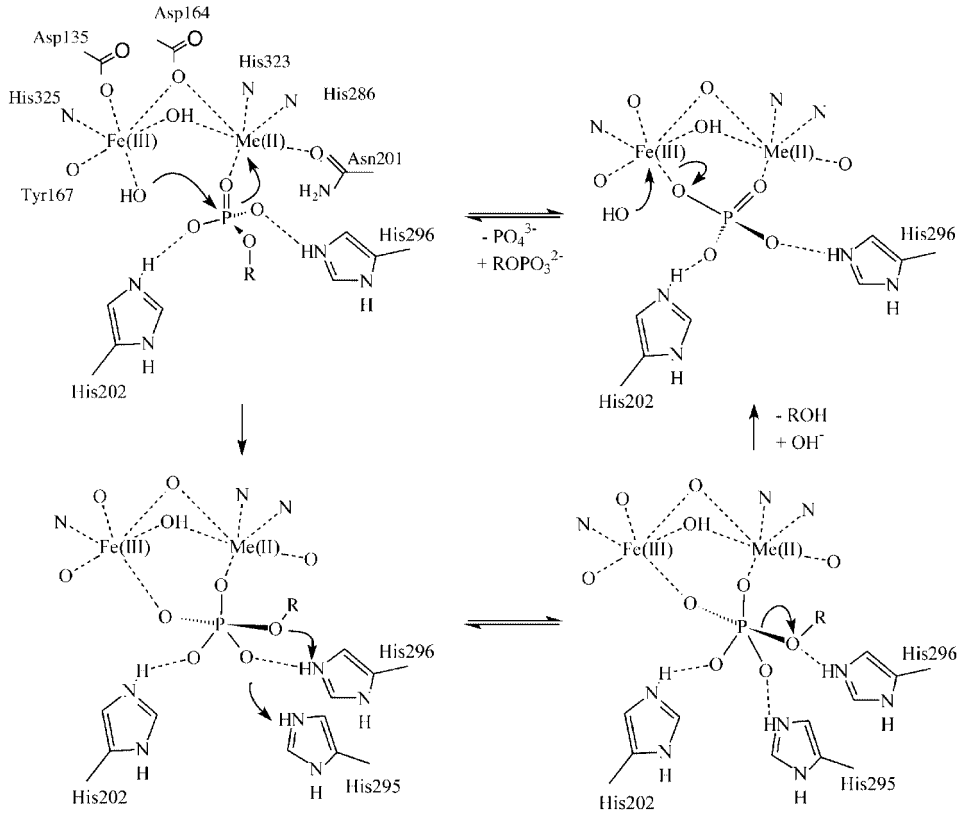
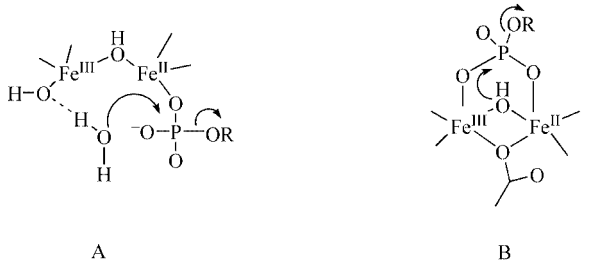


Figure 6. Proposed mechanism of phosphate ester hydrolysis in kbPAP (based on B. Krebs[8])



A B

Figure 7. Proposed reaction intermediates for the hydrolysis of phosphomonoesters by PAPs

than when it bridges two metal ions.[7] The second metal ion may help to generate the nucleophilic hydroxide by lowering the p*K*a of the bridging hydroxo and by converting the hydroxo bridge into a quasi-terminal $M^{II}-OH$ moiety.[29]

However, it is not known whether the phosphate ester binds to both metal ions in a bridging mode (as in the structurally characterized phosphato derivatives of PAPs)[8,18−20] or only to the divalent metal ion. For bovine spleen PAP (bsPAP), the bridging phosphate complex reportedly represents an inactive form of the enzyme−substrate complex.[27] No decisive evidence is available about the fixation mode of the substrate during the enzymatic cycle.

Although the exact mechanism has not been clearly elucidated, in all proposed reaction pathways the two metal centers act cooperatively, with a precise role for each. These proposed mechanisms are fascinating examples of bimetallic catalysis.

To explore the role of each metal center, the Fe^{II} has been selectively substituted with various divalent metal ions, leading to changes in catalytic activity[7] (e.g. the $Zn^{II}Fe^{III}$ Uf reacts with $H_2PO_4^-$ 35 times faster than does the native $Fe^{II}Fe^{III}$ form[30]). Conversely, replacing the Fe^{III} center with Ga^{III} results in small but consistent changes in catalytic activities.[31]

Purple Acid Phosphatase Models

A View on PAPs Models

Most reported model complexes of binuclear metallobiosites imply equivalent environments for the two metal ions, since symmetrical dinucleating ligands greatly simplify the synthetic problem.[32] The structural and functional model compounds of PAPs have been reviewed.[9] To mimic the reactivity of PAPs, two isostructural binuclear complexes, Zn^{II}-Fe^{III} and Fe^{II}-Fe^{III}, of the phenolate-hinged dinucleating ligand H-BPMOP [2,6-bis{[bis(2-pyridylmethyl)-amino]methyl}-4-methoxyphenol] have been prepared and studied to compare their activity towards phosphate ester cleavage, and to allow comparisons between kbPAP and bsPAP.[33] The pH dependence of the acceleration in cleavage of the activated phosphodiester 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP) was investigated in acetonitrile/water 1:1 (optimum pH 8.5 ± 0.2). The Zn^{II}-Fe^{III} complex of H-BPMOP shows a twofold higher rate acceleration than the complex with H-BPMP [2,6-bis{[bis(2-pyridylmethyl)amino]methyl}-4-methylphenol], and the di-iron complex from BPMOP is 10-fold more reactive than the homologous complexes from BPMP. As observed with the Zn^{II} substituted $Fe^{II}Fe^{III}$ UfPAP enzyme,[30,31] the heterobinuclear catalyst exhibits a higher reaction rate than the homobinuclear catalyst.

Unsymmetrical Models for PAPs

The rational design of dinucleating ligands that induce a controlled synthesis of the targeted unsymmetrical complexes is a prerequisite for a better understanding of spectroscopic and chemical properties related to metalloenzymes. The search for unsymmetrical dinucleating ligands has been reviewed and discussed by Fenton.[34]

Two essential factors in designing dinucleating ligands are:

– The ligand, which has to accommodate two metals with a well-suited metal−metal separation, allowing bridging by the designed ligand(s).

– The coordination environment, which dictates steric and electronic features, especially the redox properties of each metal center.

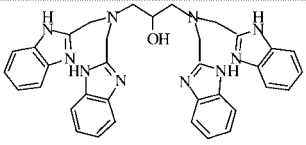
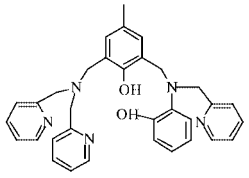
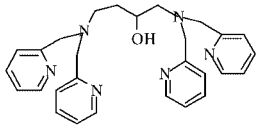
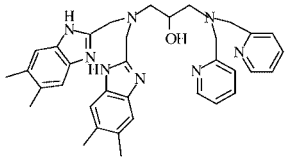
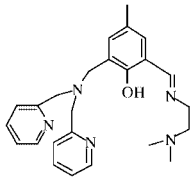
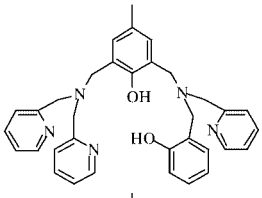
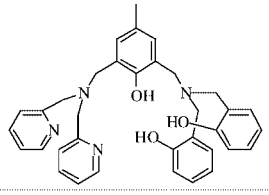
Table 2 shows some example ligands and their corresponding unsymmetrical complexes that have been characterized by X-ray structures.

An unsymmetrical binuclear iron complex from a symmetrical ligand, reported by Krebs et al.,[35] involves a monodentate coordinated phosphato ligand. Phosphate (and arsenate) anions have also been found as bidentate bridges between the two metal centers in other model complexes (Table 2). Thus, these biomimetic models do not allow decisive arguments regarding the substrate coordination to the enzyme.

In the complexes described by Latour et al.,[36] the unsymmetrical environment mimics the coordination of the tyrosine residue at the PAP active site. This work shows how the introduction in the iron environment of a single terminal phenolate influences the redox properties (i.e. noticeable destabilization of the diferrous state, less pronounced stabilization of the diferric one, and enhanced stability domain for the mixed valence state). A similar effect has been observed Neves.[42] The stabilizing effect of the mixed valence state compared to the symmetric ligands is attributed to a stereochemical effect of the ligand and to coulombic interactions.

We have described a zinc(II)-iron(III) complex involving two chemically distinct coordination sites, one suited for iron(III) complexation and bearing two phenolate nonbridging ligands, the other bearing pyridine nitrogen donor sites (half part of BPMP); the two sites being connected by the classical bridging phenolate group.[41] We used our previously described unsymmetrical ligand LH_3 (Table 2, last Entry).[43] A heterobinuclear Zn^{II}-Fe^{III} complex has been obtained by the addition (in methanol) of one equivalent of iron(III) perchlorate in the presence of triethylamine to a solution of the zinc complex, followed by the addition of two equivalents of diphenylphosphate. This complex in-

Table 2. Selected unsymmetrical complexes as models for PAPs

Ligand (LH_n)	Dimetallic core and Structural formulas	Bridging mode	Coordination mode	Ref.
	$Fe^{III}Fe^{III}$ $[Fe_2Cl_2(L)(O_2P(OPh)_2(MeOH)](ClO_4)_2$	μ-alkoxo	Fe^{III}: N3O2Cl Fe^{III}: N3O2Cl	[35]
	$Fe^{II}Fe^{III}$ $[Fe_2(L)(O_2P(mpdp)^{[a]}]BPh_4$	μ-phenoxo, bis μ-carboxylato	Fe^{II}: N3O3 Fe^{III}: N2O4	[36]
	$Fe^{II}Fe^{III}$ $[Fe_2(L)(C_6H_5CO_2)_2](ClO_4)_2$	μ-alkoxo, bis μ-carboxylato	Fe^{II}: N3O3 Fe^{III}: N3O3	[37]
	$Zn^{II}Fe^{III}$ $[ZnFe(L)(O_2AsMe_2)(MeOH)](ClO_4)_3$	μ-alkoxo, μ-dimethylarsinato	Zn^{II}: N3O2 Fe^{III}: N3O3	[38]
	$Zn^{II}Fe^{III}$ $[ZnFe(L)(AcO)_3)]BPh_4$	μ-phenoxo, bis μ-acetato	Zn^{II}: N3O3 Fe^{III}: N2O4	[39]
	$Zn^{II}Fe^{III}$ $[ZnFe(L)(AcO)_2)]ClO_4$	μ-phenoxo, bis μ-acetato	Zn^{II}: N3O3 Fe^{III}: N2O4	[40]
	$Zn^{II}Fe^{III}$ $[ZnFe(L)(O_2P(OPh)_2]$	μ-phenoxo,bis μ-diphenylphosphato	Zn^{II}: N3O3 Fe^{III}: NO5	[41]

[a] *m*-Phenylenedipropionate.

volves two phosphate bridges, as shown in the X-ray structure.[41] The main feature of the electronic absorption in CH_3CN, at 501 nm ($\varepsilon \approx 3077\ cm^{-1}$), is assigned to charge-transfer transitions from the terminal and the bridging phenolates to the iron(III). This complex can act as a structural model of the substrate bound to the active site of kbPAP (a phosphate-bridged species has been tentatively proposed during the catalytic cycle of kbPAP). Moreover, the NO5 coordination sphere around the Fe^{III} reproduces the kbPAP oxygen-rich NO5 coordination sphere of iron.

Functional models have been prepared by a combinational method using a mixture of distinct bidentate ligands, the best activity being obtained with unsymmetrical models.[44]

Catechol Oxidase: Two Copper(II) Ions with Distinct Roles for a Two-Electron Oxidation

Structural and Functional Aspects of Catechol Oxidase

Catechol oxidases (COs) are ubiquitous plant enzymes that catalyze the oxidation of a broad range of *ortho*-diphenols into *ortho*-quinones in the presence of oxygen.[4,11−13,45] They belong to the family of type-3 copper proteins, containing a binuclear copper active site that is EPR silent in the native *met* Cu^{II}-Cu^{II} form, due to a strong antiferromagnetic coupling between the copper(II) atoms. The other prominent type-3 copper proteins are hemocyanins (Hc), the dioxygen carrier for arthropods and mollusks, and tyrosinases (Tyr).[3] The crystal structure of

CO from sweet potatoes (*Ipomoea batatas*, ibCO) was resolved in 1998.[13] Both metal binding sites in the *met* form involve three histidine ligands. The two cupric ions, probably bridged by a hydroxide ion, are 2.87 Å apart in a trigonal pyramidal coordination sphere. Interestingly, the binuclear metal center has a covalent thioether bond between a carbon atom of His109 (one of the copper ligands) and the sulfur atom of Cys92 (Figure 8).

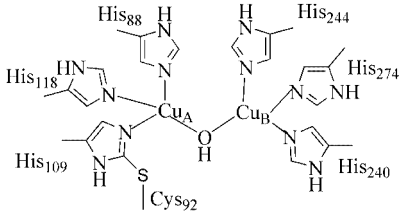


Figure 8. Active site of catechol oxidase from *Ipomoea batatas* (*met* form)[13]

The crystal structure of catechol oxidase reveals that this covalent bond puts additional structural restraints on the ligand sphere of the Cu_A center. Cys-His thioether bridges have been reported for the sequences of ncTyr (*Neurospora crassa* Tyr),[46] odgHc (*Octopus dofleini* Hc),[47] hpHc (*Helix pomatia* Hc)[48] and in all known COs. The absence of the covalent thioether bond in Hcs from arthropods and human Tyr does not support its direct involvement in the catalytic process.[4,11] A thioether bond between cysteine and tyrosine has also been reported for the mononuclear copper enzyme galactose oxidase.[49] In COs, this Cys-His unit may optimize the redox potential of the metal needed for the oxidation of the *ortho*-diphenol substrate and (or) prevents the displacement of His109 and therefore tunes the binding mode of the substrate.

The crystal structure was solved in the *deoxy* Cu^I Cu^I state and with a substrate analogue inhibitor (phenylthiourea, PTU) relevant to the mechanism.[13,45] In the reduced state a water molecule was coordinated to Cu_A. The coordination sphere is a distorted trigonal pyramidal whereas that of Cu_B (three histidine ligands) can be described as square planar with a vacant coordination site. The copper−copper distance is 4.4 Å. In the dimetallic center for the PTU-enzyme complex, the hydroxo bridge is replaced by a sulfur bridge from PTU, increasing the $Cu^{II}-Cu^{II}$ separation to 4.2 Å compared to the *met* form. The amide nitrogen interacts weakly with Cu_B, leading to still more unsymmetrical coordination spheres for the two copper centers.

A catalytic cycle for catechol oxidase activity (Figure 9) has been proposed from a combination of biochemical, spectroscopic, structural data[11] and by comparison with the tyrosinase-related cycle.[3] It begins with the native *met* form. The monodentate binding of the diphenolic substrate to the Cu_B is suggested from the crystal structure of the ibCO-PTU complex.[13] Nevertheless, a bidentate bridging of catechol has also been proposed.[3,45] Stoichiometric amounts of the quinone product are formed immediately after addition of catechol, even in the absence of dioxygen. This excludes the *oxy* form at the start of the catalytic cycle. The *oxy* form may arise from the *deoxy* form in the presence of oxygen. The *oxy* form has been experimentally obtained upon addition of H_2O_2 to the *met* form. In the proposed catalytic cycles, catecholate binds either monodentately or bidentately the *oxy* form, for a two-electron transfer

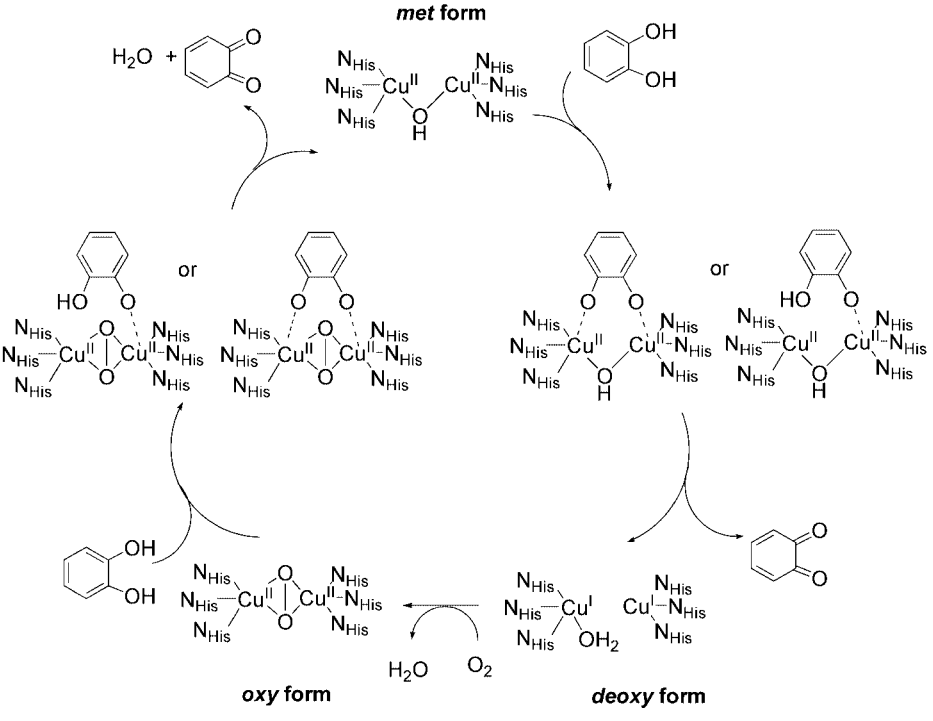


Figure 9. Proposed catalytic cycle for catechol oxidase activity[3,11,45]

leading to the release of the quinone product and to the *met* form.

Interestingly, Krebs et al.[11] have noted that in copper type-3 proteins the Cu_B site is always strictly conserved, while the varying Cu_A environment seems to tune the proteins to specific activities. Comparison of the metal coordination region from structural data on ibCO and Hcs evidences high similarities. The active site of odgHc has an identical coordination for Cu_A as in ibCO.[45] As no detailed structural information was available for any tyrosinase, it would be of great interest to explain the difference between CO and Tyr, which may be related to the respective roles of each metal center.

Catechol Oxidase Models

Catecholase activity on a model substrate (3,5-di-*tert*-butylcatechol, 3,5-dtbc) has been described, in particular for binuclear compounds.[50,51] A complex with a bridging tetrachlorocatechol between the two Cu^{II} ions has been characterized, by X-ray diffraction studies, and suggested as a model for a substrate-binding intermediate in catechol oxidation.[52] Our group has investigated a series of dicopper(II) complexes as model systems for the catechol oxidase active site. We have described the pH-controlled changes of the metal coordination in one such complex, with a H-BPMP ligand, and shown that its catecholase activity depends on the copper−copper distance, which was modified according to pH changes (Figure 10).[53]

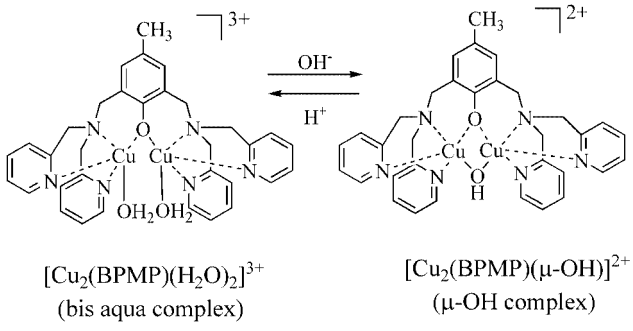


Figure 10. pH-Driven change in dicopper(II) complexes model of catechol oxidase[53]

The complex $[Cu_2(BPMP)(\mu\text{-}OH)](ClO_4)_2$ is EPR silent, in agreement with the antiferromagnetic coupling ($J = -112\ cm^{-1}$) between the two copper(II) atoms, with a very short Cu−Cu distance of 2.87 Å, similar to the enzyme in the *met* form.[12,13,54] Only the μ-OH complex $[Cu_2(BPMP)(\mu\text{-}OH)](ClO_4)_2$ exhibits catecholase activity compared to the bis aqua compound $[Cu_2(BPMP)(H_2O)_2](ClO_4)_3$. Substitution at the 4-position of the bridging phenolate with an electron-donating or an electron-withdrawing group drastically influences the catecholase activity, even though the structural properties are only slightly affected.[55]

These, models, and others in the literature, involve symmetrical ligands leading to the same coordination sphere for each metal center. Nevertheless, mechanistic studies with our model series have shown distinct roles for each metal center. First, a monodentate coordination on one copper center, associated with an interaction between the second phenol group of the catechol substrate and the hydroxy group bound to the second copper center (arising from cleavage of the initial hydroxo bridge). Then the proton transfer occurs followed by displacement of a water molecule and the bridging coordination of the catecholate (Figure 11).[56]

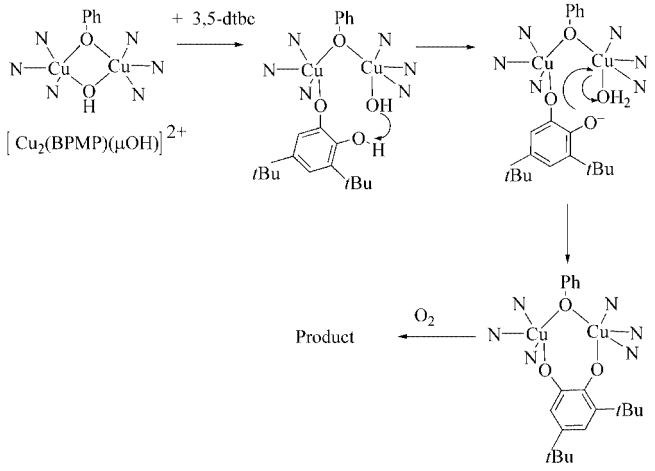


Figure 11. Proposed mechanism for the interaction between dinuclear copper(II) μ-OH complexes and the 3,5-dtbc substrate[56]

It is of interest to prepare CO models from unsymmetrical ligands, differentiating, in a controlled manner, the two metal centers as it is done in the enzyme. Few model complexes for catechol oxidase from unsymmetrical ligands have been reported (Figure 12).[51,57] With such models further studies to identify the roles of each metal ions could shed light on the likely behavior of the two copper atoms in dissimilar environments.

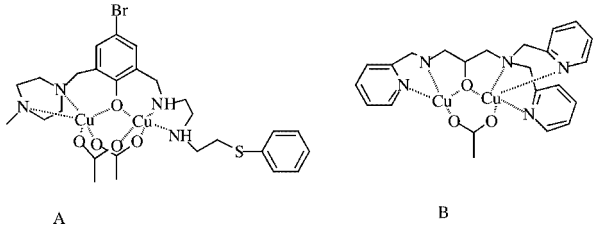


Figure 12. Unsymmetrical dicopper(II) complexes as models of catechol oxidase. (A) from,[57] (B) from[51]

Conclusion

From our briefly developed examples of μ-bridged binuclear metalloenzymes, focusing on the asymmetry of the two metal centers and their respective roles, we can underline the following aspects:

– One redox catalytic metal center; the second metal helps maintain the structural integrity and possibly accelerates the redox processes by controlling the redox potential of the first metal center. This has been observed for Cu-

Zn superoxide dismutase (moreover, the coordination of the bridging histidine nitrogen to the Zn ion lowers the p*K*a of the other nitrogen, which consequently prefers to bind to a proton rather than to Cu^{I} after the first step of the catalytic cycle).

– Coordination number asymmetry that presents the possibility of "open coordination sites" for direct interaction of one metal center with the substrate. The reactant can be bound to the second metal center. Alternatively, the second metal center can "assist" the reaction. This has been encountered with PAPs.

– In another possibility, the substrate is coordinated to both metal centers simultaneously in a bridging manner. The substrate is then activated and reacts with another molecule, either bound or not to one metal center.

– A very tenuous and subtle asymmetry with catechol oxidase (CO): a simple substitution of one ligand of one metal center may confer to CO a distinct reactivity compared with that of the closely related copper type-3 proteins.

Bimetallic catalysis is a fascinating aspect of chemical catalysis.[32] Chemists can even envisage the self-assembly of bimetallic sites during the catalytic event and, in this sense, outdo Nature. Of course, our limiting the discussion to the active site and to the first coordination spheres of the metal centers is an over-simplification. Nevertheless, this approach partly answers the question: Asymmetry in binuclear metalloenzymes: what and why?

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