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ORAL PRESENTATION

D. Small Molecules (O_2) Activation

D-02

Ribonucleotide reductases—essential radical enzymes

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Ribonucleotide reductases (RNRs) catalyse the reduction of ribonucleotides to their corresponding deoxyribonucleotides, providing the sole biological means for de novo synthesis of the building blocks of DNA. Ribonucleotide reduction has evolved only once during evolution and all RNRs make use of a common thiyl radical-based mechanism for catalysis. The three currently known classes of RNR each operate under a distinct set of biochemical and environmental conditions [1]. Class I RNRs contain a diiron center that via oxygen-mediated oxidation generates a protein-based tyrosyl radical that is a prerequisite to catalysis. Class II enzymes generate a radical via cleavage of vitamin B_{12} coenzyme (5′-deoxyadenosylcobalamin). Class III enzymes generate via *S*-adenosylmethionine cleavage (by action of an activase protein) a stable protein-based glycyl radical that is sensitive to oxygen. These differences form the basis for the classification of RNRs and result in distinct operational constraints (anaerobicity, iron/oxygen dependence and cobalamin dependence). While the propensity to synthesise deoxyribonucleotides is an essential function, the operational differences suggest that the type(s) of RNR(s) present in an organism will have an impact on the environmental conditions in which it can grow and reproduce. The effect of environmental parameters such as iron, cobalt and oxygen availability on the biochemistry of ribonucleotide reduction will impact our understanding of the adaptability of microorganisms to a range of environments.

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D-03

Concerted iron and oxygen detoxification at the tri-nuclear Fe site of bacterial ferritin from *Desulfovibrio vulgaris* Hildenborough

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Ferritins are ubiquitous and can be found in practically all known organisms ranging from bacteria, plants to vertebrates. They are 24-mers forming a hollow sphere with an inner cavity of ~80 Å in diameter. The main function of ferritin is to concentrate and oxidize the cytotoxic Fe^{2+} ions and store the oxidized Fe in the inner cavity. In anaerobic bacteria, ferritin also serves an additional function of oxygen scavenging using molecular oxygen as the oxidant. It has been shown that rapid oxidation of Fe^{2+} (ferroxidation) by H-type ferritins, found in vertebrates, occurs at a binuclear Fe site. In this paper, we report a spectroscopic (EPR and Mössbauer spectroscopy) and kinetic (stopped-flow absorption and rapid freeze quench technique) study of the Fe and O_2 detoxification mechanism of the bacterial ferritin from *Desulfovibrio vulgaris* (Hildenborough). The results indicate that, distinct from H-type ferritins, the *D. vulgaris* ferritin employs a novel trinuclear Fe site for rapid oxidation of Fe^{2+}. Also, in addition to the peroxodiferric intermediate, which was detected in the ferroxidation reaction of H-type ferritins, a mixed-valence $Fe^{II}Fe^{III}$ intermediate and a transient tyrosyl radical are observed in the bacterial ferritin reaction. Involvement of a third Fe site is demonstrated unambiguously by the absence of the $Fe^{II}Fe^{III}$ intermediate and tyrosyl radical, and by the stabilization of the peroxodiferric species in the E130A variant, in which the third Fe site has been removed. On the basis of these data, a minimal mechanistic scheme is proposed for the bacterial ferritin.

D-04

Formation and function of the heterodinuclear Mn(IV)/Fe(III) cofactor of *Chlamydia trachomatis* ribonucleotide reductase

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Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to deoxyribonucleotides, the precursors for DNA synthesis and repair. This reaction is initiated by abstraction of the C3′-H bond of the substrate by a cysteine thiyl radical (C·). In a class I RNR, the C· is generated by a stable tyrosyl radical (Y·), which is located in close proximity to a non-heme diiron(III/III) cluster, via a long-distance (~35 Å), conformationally gated, proton-coupled electron transfer (PCET) reaction that involves several conserved, redox-active amino acids along the PCET pathway. The recently discovered class I RNR from *Chlamydia trachomatis* (Ct) has all elements of the proposed PCET pathway conserved, except for the radical-harboring tyrosine, which is replaced with the non-oxidizable phenylalanine (F). This suggests that Ct RNR employs a different cofactor for generating the C·. The Y·-less RNRs have been termed class Ic RNR. We recently demonstrated that Ct RNR harbors a heterodinuclear Mn(IV)/Fe(III) cluster to generate the C·, presumably via a PCET mechanism. Thus, the Y· of a regular class I RNR is functionally replaced with a high-valent Mn(IV) site [1–3].

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D-05

Two-site competitive inhibition in hemoglobin-dehaloperoxidase A from *Amphitrite ornata*

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The enzyme dehaloperoxidase A (DHP A) from the marine worm *Amphitrite ornata* is a unique hemoglobin that functions as a peroxidase, capable of converting 2,4,6-trihalophenols (2,4,6-TXP, X = B, C, F) into the corresponding 2,6-dihalogenated quinines as well as other products. We have determined that 4-halophenols are inhibitors that bind in the distal pocket and displace the distal histidine (H55) based on X-ray crystal structures with the series of inhibitors (4-XP, X = I, Br, Cl, and F) in a unique binding interaction for a hemoglobin will be presented. The published X-ray crystal structures [1] of DHP A reveal that H55 is flexible and has two major conformations as observed in the closed (Fig. 1a, PDB 2QFK) and open (Fig. 1b, PDB 3DR9) forms of Sperm Whale myoglobin (SWMb). Figure 1c shows that binding of 4-XP inhibitor is consistent with the open conformation of H55. The 2,4,6-TXP substrates substrate affects H55 and none of the internal residues. The distal histidine acts to stabilize bound O_2 in hemoglobins and as an acid–base catalyst in peroxidases. Thus, in a dual-function protein the regulation of these functions is effected by changes in the geometry of the distal histidine. Bound phenols are simultaneously substrate and inhibitor of an enzyme and allosteric effectors of O_2 binding.

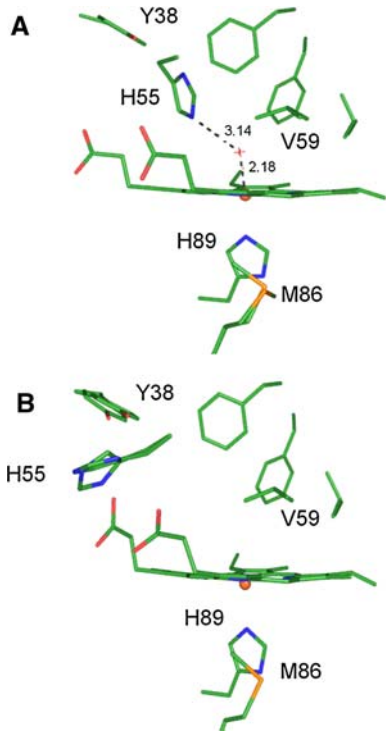

Fig. 1 X-ray structures of DHP

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D-06

Methane hydroxylation at the iron and copper sites of methane monooxygenase

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DFT studies on methane hydroxylation by enzymes and related metal-oxo species are presented. We propose a non-radical mechanism for methane hydroxylation by soluble and particulate methane monooxygenase having the reaction by the bare FeO^+ and CuO^+ complexes in mind. This mechanism is applicable when a metal-oxo species is coordinatively unsaturated. Direct interaction between methane and a metal active center can form a weakly bound methane complex in the initial stages of this reaction. Subsequent C–H bond cleavage to form an intermediate with an HO–Fe–CH_3 moiety in a non-radical manner and recombination of the resultant OH and CH_3 ligands take place at a metal active center to form a final methanol complex. Thus, this is a non-radical, two-step reaction. The fact that methyl radical is 10–20 kcal/mol less stable than secondary and tertiary carbon radicals and benzyl radicals leads us to propose this mechanism.

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D-08

The oxoiron(IV) reaction landscape

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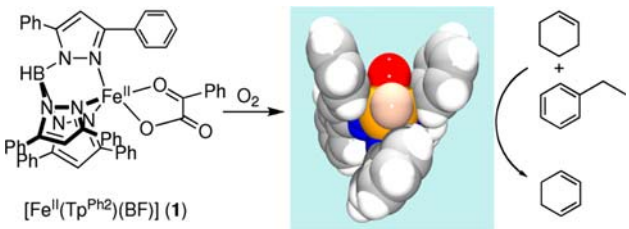
Oxoiron(IV) centers are involved in the oxygen activation chemistry of nonheme iron enzymes. Experimental evidence for the generation of oxoiron(IV) species from many synthetic iron(II) precursors has been obtained. Such biomimetic complexes serve as useful models for the proposed enzymatic oxidants. However, almost all of these synthetic complexes have an $S = 1$ spin state, while the iron(IV) centers in oxidizing intermediates of nonheme iron enzymes have an $S = 2$ spin state. Design strategies to achieve the latter will be discussed. Of particular interest is a functional model for the α-ketoglutarate-dependent iron enzymes. This iron(II) complex (**1**) activates O_2 to generate an oxidant that can oxidize hydrocarbon substrates shape selectively.



D-09

Non-heme iron model complexes as models of superoxide reductase: Fe^{III}-OOR formation, characterization and dependence on ligand environment

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The synthesis, spectroscopic characterization and reactivity of non-heme iron peroxo, oxo, and related species is an area of intense interest. This talk will describe our recent efforts in the synthesis of small-molecule models of such species. The active site of the iron enzyme superoxide reductase (SOR) is our specific target. A new series of non-heme iron(II) complexes with N_4S(thiolate) ligation will be described, approximating the His_4Cys coordination observed in the reduced, iron(II) form of SOR. These complexes have been shown to react with alkylhydroperoxides (e.g. tBuOOH) to give metastable iron(III)-alkylperoxo species that can be trapped at low-temperature. The spectroscopic characterization (UV–Vis, EPR, RR, EXAFS) of these Fe^{III}–OOR complexes, combined with a systematic variation of the S and N donors, has allowed us to correlate important spectroscopic features with the properties of the ligand environment at iron. One trend that arises from these data suggests a role for the unusual thiolate donor found in the active site of SOR.

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D-10

Understanding how thiolates contribute to the function of non-heme iron enzymes

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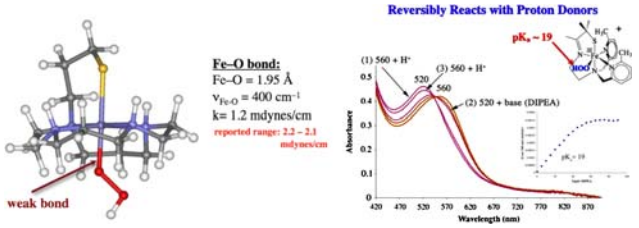
The cysteinate-ligated non-heme iron enzyme superoxide reductase (SOR) has been shown to selectively reduce superoxide to afford hydrogen peroxide via an iron–peroxo intermediate that undergoes preferential Fe–O, as opposed to O–O, bond cleavage [1]. The orientation of the sulfur has been proposed to play an important role in promoting the catalytic reaction [1]. Biomimetic analogues [2, 3] of this site will be described which provide insight as to why nature utilizes a *trans* thiolate [1] to promote SOR function. A new series of coordinatively unsaturated pyridine/thiolate-ligated synthetic models will be described which reduce O_2^- via metastable intermediates that, in some cases, can be reversibly protonated affording an equilibrium acidity constant (K_a) for the exchangeable proton. By tuning the electronic properties of the metal ion, we will show that we can alter the preferred reaction pathways.



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D-11

New pathways of nonheme-iron-catalyzed oxidation processes

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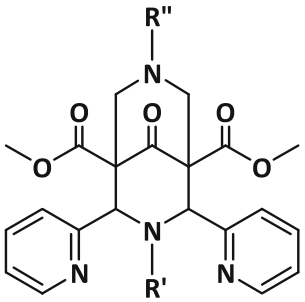
Bispidine ligands are extremely rigid, easy to synthesize and available in a large variety. They enforce coordination geometries derived from *cis*-octahedral, and the two vacant coordination sites with the tetradentate ligand systems are sterically and electronically distinct. Reasons are thoroughly analyzed on the basis of computational work as well as experimental structural data, thermodynamics and reactivities, specifically for the corresponding iron complexes. As a consequence, we are able to directly influence the electronic properties and reactivities of the corresponding catalyst systems, and this has lead to interesting and novel observations with respect to the oxidation power, reaction mechanisms, spin states of the high-valent complexes and catalytic efficiencies.



R', R" = CH_3 or $(CH_2)_n$-pyridine (n=1,2)

D-12

Oxidation reactivities of peroxo-diiron(III) and -dicopper(II) complexes: functional models for dioxygen activating dimetalloenzymes

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Reactivities of peroxo-diiron(III) and -dicopper(II) complexes are of particular interest as functional models for the dioxygen activating diiron and dicopper metalloenzymes such as methane monooxygenase, toluene monooxygenase, and tyrosinase. We have synthesized two types of peroxodiiron(III) complexes, $[Fe_2(L^{Ph4})(RCO_2)(O_2)]^{2+}$ (R = Ph_3C (oxy-**1**) and Ph (oxy-**2**)), the former performs regioselective hydroxylation of a phenyl group of L^{Ph4} and the latter exhibits reversible dioxygen binding (L^{Ph4} = *N*,*N*,*N*′,*N*′-tetrakis-[(1-methyl-2-phenyl-4-imidazolyl)methyl]-1,3-diamino-2-propanolate) [1]. The reactions mimic toluene monooxygenase and hemerythrin reactivity, respectively. We have also succeeded in preparation of (μ-η^2:η^2-peroxo)dicopper(II)$_2$ complexes, $[Cu_2(O_2)(R\text{-}L)]^{2+}$ (oxy-**R-1**: R = H, MeO, *t*-Bu, and NO_2), where R-L = 1,3-bis[bis(6-methyl-2-pyridylmethyl)aminomethyl]-5-R-benzene, which can perform not only

hydroxylation of the *m*-xylyl linker of R-L, but also epoxidation of styrene via an electrophilic addition of the peroxide to the C=C bond [2]. In addition, oxy-**H-1** can oxidize a variety of aliphatic C–H bonds which have the bond dissociation energies (BDE) ~ 75 - ~92 kcal mol^{-1} via H-atom abstraction. A good correlation between the second order rate constants of oxidation of the C–H bonds of the substrates and their BDEs was observed.

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D-13

Push effect in mononuclear nonheme iron complex

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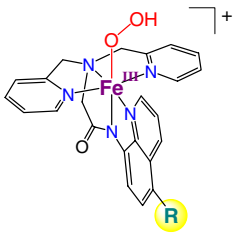
It has been well accepted that an axial thiolate ligand to heme iron induces the O–O heterolysis of iron(III)-OOH species in the reaction cycle of cytochrome P450s, which is further assisted by a hydrogen bonding network to the distal oxygen atom of iron(III)-OOH species. A similar mechanism is operative in heme-dependent peroxidases, such as horseradish peroxidase, which is known as the push–pull mechanism. In the case of peroxidases, both the imidazolate ligand to the heme iron (push effect) and a protonated His residue in the distal heme pocket (pull effect) polarizes the O–O bond to promote heterolysis of the O–O bond. Recently, we started a research program to develop bio-inspired oxidation catalysts based on the abovementioned reaction mechanisms of heme-dependent oxygenases. As a part of this program, we have synthesized a series of mononuclear nonheme iron(III) complexes supported by amidate-based ligands $[Fe^{III}(dpaq^{R})(H_2O)]^+$ ($\mathbf{1^R}$), where R = NO_2, Cl, H, and Me. The amidate coordination was introduced to mimic "push effect". We found that its methoxide complex $[Fe(dpaq)OMe]^+$ ($\mathbf{2^R}$) reacts with hydrogen peroxide to afford the corresponding iron(III)-OOH species ($\mathbf{3^R}$) at low temperatures. Complex $\mathbf{3^R}$ showed an amidate-to-iron(III) LMCT band at around 500 nm, which gradually decayed even at 233 K, Such instability cannot be observed with other iron complexes supported by neutral ligands, i.e., iron-N4Py complex. The order of the instability of $\mathbf{3^R}$, R = Me > H > Cl > NO_2, clearly demonstrates that the amide anion coordination facilitates the decomposition of iron(III)-OOH species via "push effect".



R = Me, H, Cl and NO_2

$[Fe^{III}(dpaq^{R})OOH]^+$ ($\mathbf{3^R}$)

D-15

The effects of the secondary coordination sphere on metal-mediated oxidations

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Hydrogen bonds stabilize and direct chemistry performed by metalloenzymes. With inspiration from enzymes, we have utilized an approach that incorporates intramolecular hydrogen bond donors to determine their effects on the stability and reactivity of metal complexes. Our premise is that control of secondary coordination sphere interactions will promote new function in synthetic metal complexes. For instance, we have developed a series of complexes with terminal oxo and hydroxo ligands, which are surrounded by intramolecular hydrogen bond networks. Our work has established that hydrogen bonds can compete with pi-bonds to stabilized monomeric oxometal complexes. This talk will discuss our recent efforts in understanding dioxygen activation by metal complexes and the reactivity of resultant oxometal complexes, in which the oxo ligands are highly basic. Our results indicated that mechanistic changes are correlated with the basicity of the oxo ligands.

D-16

Striking lessons from nickel-superoxides and related systems

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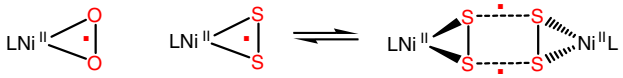
Diatomic chalcogen molecules X_2 in the coordination spheres of transition-metals can play an eminent role in oxidation processes in biological and catalytic systems. While peroxo and persulfido ligands and their suitability for X-atom transfer reactions have been studied in great detail, much less is known about paramagnetic superoxo (O_2 radical anion) and supersulfido (S_2 radical anion) complexes. Moreover, the heavier analogues (Se_2 and Te_2 anions) are hitherto unknown. We now learned that using a β-diketiminate nickel(+1) precursor complex gives facile access to the isolable and surprisingly stable superchalcogenido complexes **1** (X=O) and **2** (X=S) [1, 2] (Fig. 1).



1 **2** $\mathbf{2_2}$

Unexpectedly, complex **2** exists as a diamagnetic dimer $\mathbf{2_2}$ with a four-sulfur two-electron bond. The peculiar structure–reactivity relationships of **1** and **2** and related studies on the corresponding Se and Te systems [3] as potential building blocks for metalloenzyme models will be discussed in my talk.

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D-17

Bio-inspired alkane oxidation catalysts based on nickel complexes

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Development of the efficient oxygenation processes for hydrocarbons (especially alkanes) is still one of the challenging subjects from the

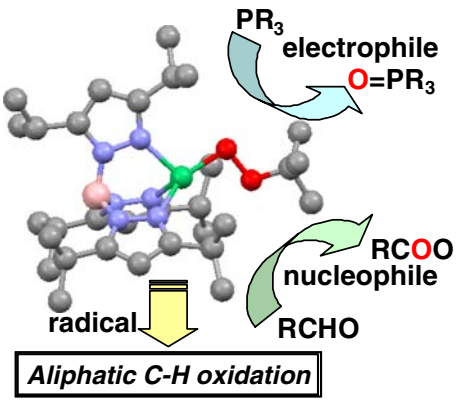

Fig. 1 Rectivity of **1**

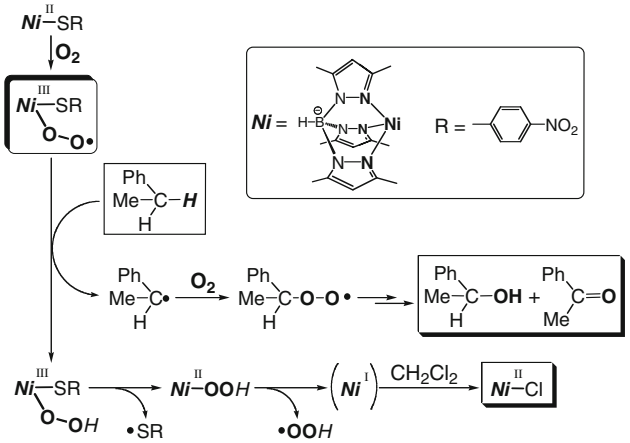

Fig. 2 Alkane oxygenation mediated by **3**

viewpoints of green and sustainable chemistry. In this work, oxidizing catalyses of Tp^R-supported nickel complexes with peroxides as well as O_2 were investigated. An alkylperoxo complex of nickel(II), $[Ni^{II}(OOtBu)Tp^{iPr2}]$ (**1**) exhibited the substrates-depending reactivity as shown Fig. 1. The thermal decomposition of **1** might proceed through O–O and Ni–O homolysis, and the resulting radical species would contribute to the catalytic cyclohexane oxygenation with TBHP [1]. In contrast, in situ generated acylperoxonickel analogues **2** exhibited high selectivity for the cyclohexane oxygenation to the corresponding alcohol. We also investigated O_2 oxidizing capability of a thiolato complex, $[Ni^{II}(SC_6H_4NO_2)Tp^{Me2}]$ (**3**). Complex **3** reacted with O_2 and abstracted a benzylic H atom of ethylbenzen (Fig. 2).

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D-18

Contribution of the supporting ligands to NiO_2 intermediates

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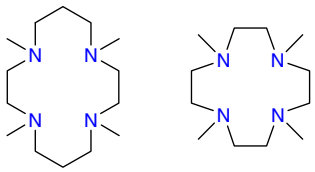
The binding and activation of dioxygen at transition metal centers are of great importance for understanding the reaction mechanisms of metalloenzymes and utilizing metal complexes as oxidation catalysts. NiO_2 intermediates have been developed and investigated to elucidate highly active species in the oxidative functionalization of organic substrates [1]. The synthesis and spectroscopic characterization of a NiO_2 complex with a 14-membered macrocyclic ligand were reported previously [2]. Herein, we report the formation, characterization, and reactivity of a novel NiO_2 complex having a 12-membered macrocyclic ligand, $[Ni(12\text{-TMC})(O_2)]^+$ (12-TMC = 1,4,7,10-tetramethyl-1,4,7,10-tetraazacyclododecane).



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D-19

Biomimetic and bioinspired oxidation by ruthenium and osmium oxo complexes

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Nature makes use of iron oxo species to carry out various oxidation reactions. In this presentation the kinetics and mechanisms of the oxidation of various inorganic and organic substrates by ruthenium and osmium oxo complexes will be discussed. These substrates include alkanes, arenes, NO_2^-, I^-, NCS^- etc. The formation of metal-oxo species is an important step in many enzymatic and chemical oxidation processes. In this presentation the kinetics of the oxidation of *trans*-$[Ru^{IV}(tmc)(O)(solv)]^{2+}$ to *trans*-$[Ru^{VI}(tmc)(O)_2]^{2+}$ (solv = H_2O or CH_3CN) by MnO_4^- in aqueous solutions and acetonitrile will also be described. We provide evidence that the initial rate-limiting step in water occurs by hydrogen atom transfer, while that in acetonitrile occurs by oxygen atom transfer.

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D-21

Human P450 heme–oxygen intermediates

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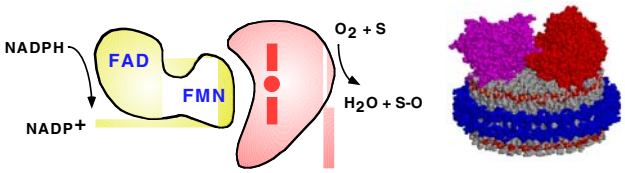
We have examined the electronic, vibrational and magnetic properties of the intermediate states of cytochrome P450 using a combination of cyrogenic optical, EPR and Raman spectroscopy and documented the channels leading to productive and uncoupled pathways. We extended our initial studies using the bacterial P450 CYP101 to the human enzymes involved in drug metabolism (CYP3A4) and steroid hormone biosynthesis (CYP19, CYP17 and CYP11A1) by using self-assembled nanoscale lipid bilayer "Nanodiscs". In these membrane-bound systems we document redox potentials, autoxidation rates, heme–oxygen intermediates and the existence of a substantial "oxidase" activity represented by water production from a putative "Compound I" state and will discuss these results in terms of the chemical reactivity of peroxo and iron–oxo intermediates.



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D-22

Substrate recognition and conformational stability of the active site in cytochrome P450cam

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The cytochrome P450cam from *P. putida* is studied as a model of the heme mono-oxygenases that are involved several vital biochemical processes such as drug metabolism, detoxification against xenobiotics and biosynthesis of steroid hormones. The active site of these enzymes consists of a deeply buried heme center anchored through a cysteine residue of the enzyme and the intervening amino acid residues control the entry and binding of the substrate at the active site of the enzyme. Site-specific mutation at the surface near the substrate entry channel of the enzyme showed distinct variations in the substrate association rate constants, suggesting the existence of a recognition site for the substrate at the enzyme surface. The structure of the enzyme indicated presence of a unique potassium ion binding site near the substrate access channel of the enzyme. Binding of the potassium ion not only facilitated substrate binding but also stabilized the active form of the enzyme. Moreover, substitution of the potassium ion by calcium ion was found to cause inactivation of the enzyme forming a P420-type species that was reversibly converted to the active P450 form on addition of potassium ion. The present talk will describe some of these results in the light of understanding the conformational properties of the heme pocket and the effects of substrate binding to the active site in cytochrome P450cam.

D-23

How nature uses oxygen—lessons from enzymes and model compounds

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The central paradigms of aerobic life processes derive from our understanding of how the redox chemistry of oxygen is manipulated and controlled by iron- and manganese-containing proteins. Nature has exploited the large, intrinsic driving force for the reduction of oxygen to water in a variety of intriguing ways. Now that the core chemistry of these processes has been revealed, they can be seen as a set of remarkably similar themes involving oxo- and peroxo-transition metal complexes. In this lecture we compare and contrast those mechanistic strategies looking alternately at heme proteins such as cytochrome P450, non-heme iron proteins such as AlkB and NDO and models metalloporphyin complexes containing iron and manganese. The oxygen activation and transfer reactions catalyzed by the heme and non-heme families of iron-containing oxygenase enzymes produce reactive metal–oxo intermediates. We find that a full understanding of the nature of these reactions requires consideration of metal ligation and peripheral substitution effects [1], spin-state crossing effects [2] and the stochastic effects of in-cage recombination and cage escape [3]. The lecture will describe recent experimental results and some new insights regarding these fascinating and important reactions.

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Acknowledgments

We thank the NIH (R37 GM36298, MERIT award), the NSF (CHE-0316301; Special Creativity Award) and the NSF-EMSI, CEBIC, at Princeton University, (CHE-9814301) for generous support of this research.

D-24

Human indoleamine 2,3-dioxygenase: new inhibitors and their functional consequences

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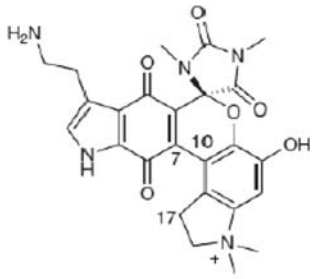
The role played by indoleamine 2,3-dioxygenase (IDO) in the immune escape mechanism of solid tumors has made it an appealing target for therapeutic intervention [1]. Using high throughput screening assays, we have identified several new and potent inhibitors of human IDO in extracts prepared from marine invertebrates and from microbes cultured from marine sediments. The structures of several of these new inhibitors have been determined. Their mechanism of inhibition and of some synthetic analogues related to exiguamine A [2] are uncompetitive with respect to Trp and exhibit submicromolar inhibitory constants. To evaluate the effects of these inhibitors on the interaction of the enzyme with diatomic gaseous

ligands, we have begun to study their effect on the affinity of the enzyme for dioxygen and on the binding of CO. The results indicate that these new inhibitors may be useful mechanistic probes as well as potential therapeutic leads.



Exiguamine A

Acknowledgments
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D-25

Crystal structures of the substrate-free and the decoy molecule-bound forms of cytochrome $P450_{BS\beta}$

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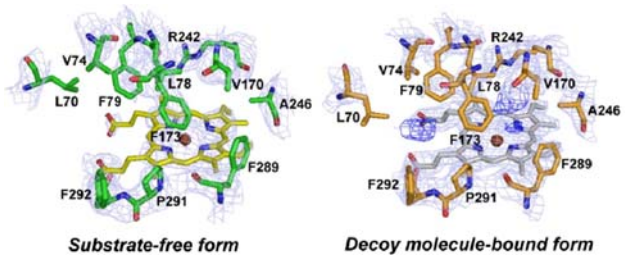
In contrast to most P450s, $P450_{BS\beta}$ (CYP152A1) isolated from *Bacillus subtilis* utilizes hydrogen peroxide to catalyze the hydroxylation of long-alkyl-chain fatty acid. Recently, we have reported that $P450_{BS\beta}$ can catalyze oxidation of a variety of non-natural substrates, such as hydroxylation of ethylbenzene, by employing a simple substrate trick: a series of short alkyl-chain carboxylic acids are misrecognised as substrates [1]. In order to examine possible structural changes induced by the substrate and decoy molecule binding, the crystal structural studies of the substrate-free form and a decoy molecule-bound form were carried out and compared those structures with that of the palmitic acid bound form reported (1IZO).



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D-27

The oxidation of L-tryptophan in biology

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The L-kynurenine pathway—which leads to the formation of NAD—is the major catabolic route of L-tryptophan metabolism in biology. The initial step in this pathway is oxidation of L-tryptophan to *N*-formyl-kynurenine (Scheme 1). In all biological systems examined to date, this is catalysed by one of two heme enzymes, tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO), in a reaction mechanism that involves binding of O_2 to ferrous heme. We have examined the activity of three heme dioxygenases (human IDO, human TDO and *X. campestris* TDO) with 1-Me-L-Trp. In contrast to previous work, we find that 1-Me-L-Trp is a slow substrate. These observations are inconsistent with current proposals in the literature for the mechanism of substrate oxidation, and we propose an alternative.

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D-28

How do novel functions evolve from existing protein scaffolds?

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Understanding the relationship between protein structure and function is one of the greatest challenges in structural biology. In this lecture, I will discuss our recent efforts to gain molecular insights into how new biosynthetic pathways evolve. Specifically, my talk will focus on the generation of oxygenated lipid mediators by the cytochrome P450 superfamily. Lipid mediators constitute a broad spectrum of molecules, including vasoactive substances in mammals and volatile organic compounds that confer characteristic flavors to fruits and vegetables. Plant oxylipins (such as jasmonates) and animal prostaglandins are short-lived but potent peroxide-derived lipid mediators that share strikingly similar biological activities, including metabolic regulation, reproduction, and host defense. Their biosynthesis involves extraordinary rearrangements of labile organic peroxides by a novel group of heme thiolate enzymes belonging to the cytochrome P450 superfamily. In spite of three decades of intense research, it has been difficult to unravel how some of these molecules are produced. Equally unclear is the evolutionary origin of the enzymes that synthesize these diverse groups of signaling molecules. Here, I will offer an atomic description of the enzymes involved in oxylipin biosynthesis. I will also elaborate on how our structural efforts led to the discovery of new oxylipin signaling pathways in both bacteria and marine invertebrates [1].

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D-29

Mechanisms, kinetics and thermodynamics of halide oxidation by human heme peroxidases

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The recently proposed peroxidase-cyclooxygenase superfamily includes enzymes from all living kingdoms with a broad spectrum of enzymatic features and physiological roles [1]. These proteins have differentiated very early in the evolutionary history as cornerstone of the innate immune defence system. Before organisms have developed an acquired immunity, their antimicrobial defence depended on enzymes that were recruited upon pathogen invasion and produced microbicidal reactive oxidants and diffusible radical species. A unique activity of these enzymes is their ability to use hydrogen peroxide and halides (Cl^-, Br^-, I^-) and/or thiocyanate as two-electron donors to generate halogenating oxidants such as hypohalous acids [2]. Here, we discuss the mechanisms, kinetics and thermodynamics of halide oxidation by mammalian peroxidases. It is presented how structural differences in their active sites (e.g. heme to protein linkages, heme asymmetry and distortion) are reflected by distinct thermodynamic and kinetic features [3].

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D-30

Conformational dynamics in the reductase domain of nitric oxide synthase

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As in P450s electrons in nitric oxide synthase (NOS) are delivered from a FAD/NADPH/FMN reductase domain to the heme, a required reaction for proper O_2 activation and substrate hydroxylation. In NOS, however, the heme and reductase domains are linked together as a single polypeptide chain and calmodulin binding to NOS regulates electron flow from the reductase to heme. It is generally thought that the reductase domain is quite flexible and that large movements must occur for the FMN domain to pick up an electron from FAD and then undergo a large repositioning required for proper alignment with the heme domain. Calmodulin binding favors the "output state" wherein the FMN is in position to reduce the heme. The crystal structure of the reductase domain [1] shows that, like in P450 reductase, the FMN and FAD are quite close. We now have shown that mutations at the FMN/FAD interface are critical in controlling the FMN redox potential and the ability of calmodulin to promote the "output state". The changes are subtle indicating that the dramatic effects observed when calmodulin binds are due to small changes in free energy.

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D-31

Theoretical studies on short-lived intermediates in the catalytic cycle of cytochrome P450 and their reactivity patterns

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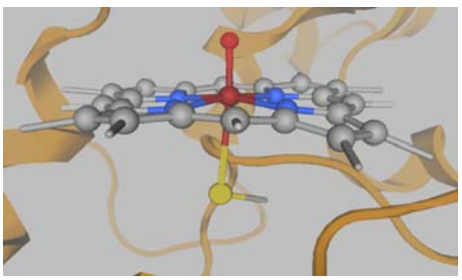
The cytochromes P450 are important heme enzymes in biosystems involved in drug metabolism and detoxification processes in the body. in spite of extensive experimental and theoretical studies there are still controversies regarding the nature of the active oxidant that performs the substrate monoxygenation. We have done a series of density functional theory and quantum mechanics/molecular mechanics calculations on cytochrome P450 enzymes and active site mimics to elucidate the properties of potential oxidants and their reactivity patterns [1, 2]. In this presentation we will highlight the latest achievements of our group using DFT and valence bond methods and thermodynamic cycles to explain the fundamental differences between heme and nonheme monoxygenases [3].



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D-32

Structural studies of the intermediates in the reaction between myoglobin and peroxides: effects of cryoradiolytic reduction

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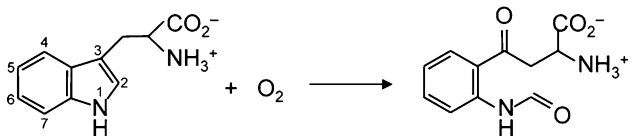
The intermediates generated in the reaction between myoglobin and peroxides mimic the intermediates found in many peroxidases, oxygenases and catalases [1, 2]. These myoglobin intermediates are also relevant because myoglobin is proposed to take part as scavenger of reactive oxygen species during oxidative stress. We have in this study combined crystallography and single-crystal light absorption spectroscopy (microspectrophotometry). Radiation-induced changes of the different intermediates in this reaction cycle have been observed and followed by microspectrophotometry [2–5]. We have been able by cryoradiolytic reduction of an oxymyoglobin equivalent (compound III) to generate and trap the so-called peroxymyoglobin intermediate,



Scheme 1 Reaction catalysed by IDO and TDO

a Fe(II)-superoxide form indicated by quantum refinement analysis [2, 4]. By annealing of this compound the oxygen–oxygen bond is broken and the reaction propagates to the compound II intermediate [3–5]. The structures have further been refined with quantum refinement [1, 4, 5] together with Ulf Ryde.

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D-33

Metallocorroles as models of high-valent heme protein intermediates

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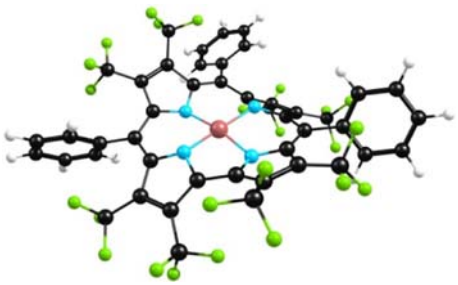
Metallocorroles are intriguing as stable models of heme protein compound I and compound II intermediates. They exhibit an amazing range of electronic structures, spectroscopic and electrochemical properties. Here we will present our findings on β-octakis(trifluoromethyl)corrole complexes (see diagram below) recently prepared in our laboratory [1], focusing on copper, manganese, and iron derivatives. Although chloroiron corrole derivatives have long been recognized as noninnocent [2], copper corroles have until now been thought of as true Cu(III) complexes. Combined crystallographic, spectroscopic, electrochemical and high-level theoretical (CASPT2) studies now indicate that even copper corroles are best viewed as antiferromagnetically coupled Cu^{II}-$corrole^{\cdot 2-}$. This antiferromagnetic coupling, mediated by a specific $Cu(d_{x2-y2})$-corrole(π) orbital interaction, is so strong that it results in a buckling of the corrole macrocycle, i.e. even sterically unhindered copper corroles are inherently buckled, a situation that is very different from cobalt corroles, which are planar even for highly hindered corrole ligands. We will attempt to find parallels between these and other intriguing aspects of corrole chemistry and spectroscopic features of metalloenzyme intermediates such as chloroperoxidase compound II and the ox1 state of methylcoenzyme M reductase.



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D-35

Exploring the role of the active site methionine residue in PHM catalysis

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Methionine 314 plays a critical role in PHM catalysis. No clear role for this residue has emerged but a number of proposals suggest that it may (i) stabilize the Cu(II)-superoxo intermediate (ii) stabilize the reduced form of the enzyme and (iii) provide a fluxional coordinate for H tunneling. For WT PHM the intensity of the Cu–S interaction in the Cu(I) EXAFS data is inversely proportional to catalytic activity over the pH range 3–8. At pH 8, the reduced Cu(I) spectrum can be simulated by replacing the Cu_M Cu–S(Met) interaction with a Cu–N/O. However, the pH 3.5 data still show the presence of a strong Cu–S interaction, and establish that the form observed at low pH in WT cannot be due to a M314 "on" conformer, but must arise from a different Cu–S interaction. These data are confirmed by studies of CO binding which show that M314H does not bind CO at pH 8, but that both WT and mutant exhibit a new FTIR band at 2,111 cm^{-1} assignable to the low-pH S-containing species. Therefore, lowering the pH causes a conformational change at one of the Cu centers which brings a new Cys or Met residue into a favorable orientation for coordination to the metal center generating an inactive form. These data, together with parallel results on tyramine β monooxygenase, are discussed in relation to the PHM catalytic mechanism (Fig. 1).

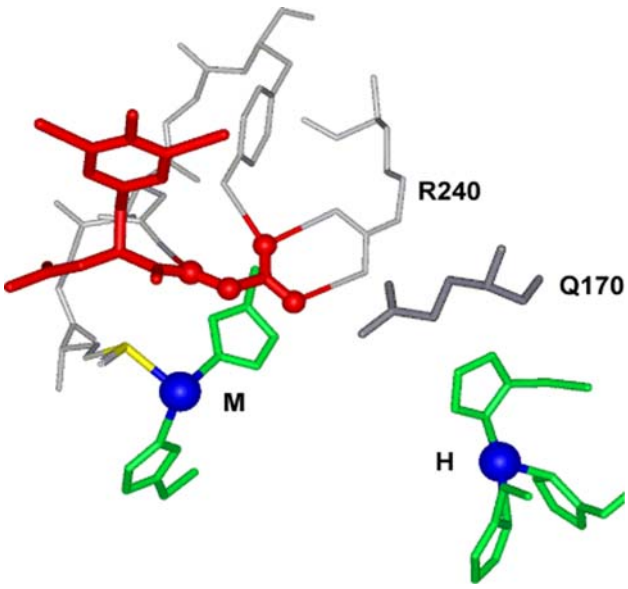

Fig. 1 Structure of the PHM Active Site. The YVG substrate is shown in red. M314 is shown coordinated to CuM as a yellow side chain

D-36

Peptidylglycine α-hydroxylating monooxygenase (PHM): oxygen activation and small molecule binding by a copper center

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Peptidylglycine α-hydroxylating monooxygenase (PHM) catalyzes the first step in the activation of peptide hormones and neurotransmitters: the stereospecific hydroxylation of the C_α of a C-terminal peptidylglycine. The enzyme uses for catalysis two separate Cu sites (Cu_M and Cu_H) located ~11 Å from each other; Cu_M is the metal involved directly in catalysis while Cu_H is associated with electron transfer. Using X-ray diffraction, site-directed mutagenesis and kinetic characterization, we identify important intermediates in the reaction mechanism. In addition, we proposed a path for the transfer of an electron from Cu_H to the site of Cu_M. To determine whether only oxygen does not bind to Cu_H or this lack of binding is a general feature of Cu_H, we determined the structures of the complexes of PHM with other small molecules such as nitrite (NO_2^-) and azide (N_3^-). Nitrite anion coordinates Cu_M but, surprisingly, not Cu_H despite the high concentration of nitrite used in the experiments (nitrite/protein > 1,000). Similarly, azide binds Cu_M but not Cu_H. The lack of binding of small molecules to Cu_H may be correlated with its functional role as an electron-transfer site.

D-37

Identifying the active site of particulate methane monooxygenase

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Particulate methane monooxygenase (pMMO) catalyzes the oxidation of methane to methanol in methanotrophic bacteria. The 300 kDa pMMO enzyme is a trimeric integral membrane protein, comprising three copies each of three subunits: pmoB, pmoA, and pmoC. In spite of extensive spectroscopic characterization and the availability of two crystal structures [1, 2], the location and metal composition of the pMMO active site remain unknown. We have detected three different metal centers crystallographically: a highly conserved dinuclear copper center, a nonconserved mononuclear copper center, and a site that can be occupied by zinc or copper. On the basis of spectroscopic data, other researchers have reported the presence of trinuclear copper center or a dinuclear iron center, of which each was proposed to be the active site. We present here new activity, biochemical, and spectroscopic data that directly address the validity of these models.

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D-38

Copper-dioxygen and cupric-hydroperoxo mononuclear complexes: formation, characterization and substrate reactivity

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For a number of copper metalloenzymes, copper(I)-dioxygen adducts or derived species are oxygenating (oxygenase) or oxidant (oxidase) active species. Mononuclear Cu^I/O_2 activation chemistry is considered to be important and of considerable recent interest. Here, we will describe: (i) Cu^I/dioxygen chemistry involving tripodal tetradentate ligands which lead to end-on bound superoxo-copper(II) complexes. Their kinetics of formation, spectroscopic and structural characterization along with reactivity traits will be presented. (ii) The chemistry of copper(II) or dicopper(II) hydroperoxo complexes, i.e., $Cu^{II}_n(-OOH)$ ($n = 1$ or 2), will also be described and we show that these can effect substrate C–H activation/oxygenation chemistries, such as N-alkyl hydroxylation or oxidative N-dealkylation. (iii) Chemistry of a new system where two-electron reduction of O_2 is effected at a single copper ion ligand complex.

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D-39

Copper chemistry within biomimetic chambers

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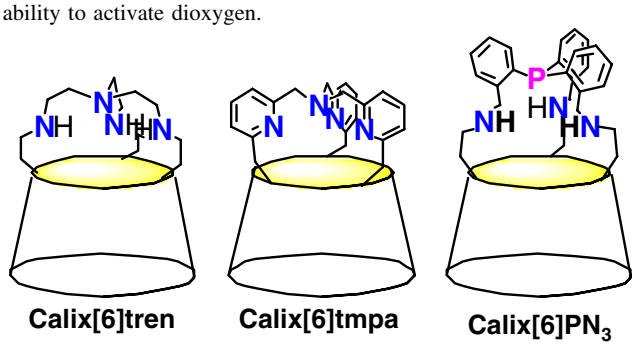
We are interested in modeling mono-nuclear active sites of copper-enzymes with ligands reproducing not only the first coordination sphere (a poly-histidine core by an aza-site), but also the hydrophobic funnel controlling the substrate binding. For this purpose, we have designed a ligand family, the calix[6]aza-cryptands, based on the calix[6]arene core onto which a poly-aza cap has been grafted at the small rim. The resulting capped structures provide a strong chelate effect, preclude any bimetallic interaction and enforce exogenous ligation exclusively through the funnel. The corresponding Cu(I) and Cu(II) complexes, their coordination properties and supramolecular behavior will be presented, together with recent insights into their ability to activate dioxygen.



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D-41

Activation and catalysis of inactive phenoloxidase and hemocyanins

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Among type 3 copper proteins hemocyanins (Hc) serve as oxygen carrier, phenoloxidase (PO) comprising tyrosinase (Ty) and catecholoxidase (CO) are enzymes starting the biosynthesis of melanin being involved in innate immunity, wound healing, coloring of hair and eyes, browning of fruit and plants. We suggest molecular mechanisms for the activation and catalysis [1–5]. Our model explains why oxidation process turns hair grey [6].

Acknowledgment
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D-42

Tyrosinase: mechanistic studies and new reactivity

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Tyrosinase is a ubiquitous dinuclear copper enzyme catalyzing the efficient oxidation of phenolic and catecholic substrates to quinones, and is therefore classified as an internal monooxygenase. The activity and mechanism of tyrosinase have been the focus of enzymatic and model studies in our laboratory for almost two decades [1]. A recent X-ray structure determination of the enzyme from *S. castaneoglobisporus* clarified important details about the dinuclear copper active site [2]. Important aspects of the enzyme reactivity remain not known, including the mode of substrate binding and dioxygen activation at the dinuclear copper center. According to biomimetic studies, three types of copper-dioxygen species are apparently suitable for the reaction: a μ-η^2:η^2-peroxidodicopper(II), a bis(μ-oxido)dicopper(III), or a μ-hydroperoxido-dicopper(II) species [1, 3]. Though, the activation parameters which characterize the biomimetic phenol hydroxylations are quite different from those we found for mushroom tyrosinase [4]. These data were obtained from enzymatic studies performed at variable temperature in a mixed aqueous-organic solvent. Other recent developments enabled us to set up enzymatic and model studies where tyrosinase and dinuclear copper complexes perform the sulfoxidation of organic sulfides. In this case, the reaction requires the presence of a co-substrate, and therefore the enzymatic activity becomes equivalent to those of external monooxygenases like cytochrome P450 [5].

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D-43

O_2-activation at an asymmetric dicopper center

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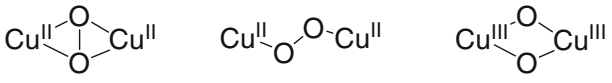
Understanding the intimate details of O_2 binding/activation at copper sites is of interest because of the relevance of such reactions in biological and technological processes [1]. For the particular case of dicopper sites, three basic Cu_2O_2 core structures have been widely described [1].



$Cu^{II}_2(\mu\text{-}\eta^2\text{:}\eta^2\text{-}O_2)$ *trans*-$Cu^{II}_2(\mu\text{-}\eta^1\text{:}\eta^1\text{-}O_2)$ $Cu^{III}_2(\mu\text{-}O)_2$

Each specific Cu_2O_2 core determines particular spectroscopic and chemical properties [3]: while end-on *trans*-$Cu^{II}_2(\mu\text{-}\eta^1\text{:}\eta^1\text{-}O_2)$ species exhibit nucleophilic–basic behavior, side-on $Cu^{II}_2(\mu\text{-}\eta^2\text{:}\eta^2\text{-}O_2)$ and bis-μ-oxo dicopper(III) cores show an electrophilic–acid oxidant character. The rich and subtle chemistry exhibited by Cu_2O_2 cores makes asymmetric options interesting, but Cu_2O_2 species in systems containing distinct copper sites have been seldom observed [2], and a unique example of an asymmetric $Cu^{II}_2\text{-}\mu\text{-}\eta^1\text{:}\eta^2\text{-}O_2$ core has been reported [3]. Herein we describe the O_2 chemistry of a novel unsymmetric dicopper complex. The chemistry of the corresponding Cu_2O_2 species offer new insights that call into revision our current understanding of the mechanism of O_2 activation at dicopper sites.

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D-44

Tyrosinase reactivity in a model complexes: an alternative hydroxylation mechanism through a Cu(III)-bis-oxide intermediate

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The dinuclear copper enzyme tyrosinase activates dioxygen to form a side-on peroxodicopper(II) complex, which is capable of oxidizing phenols to catechols. Several synthetic side-on peroxodicopper(II) complexes created with simple diamine ligands will be discussed that faithfully reproduce the spectrum of oxygenated tyrosinase, yet converted to Cu(III)-bis-oxide species upon phenolate addition at extreme temperatures (153 K). These species decay with hydroxylation of the aromatic ring by a mechanism that shares the hallmarks of an electrophilic aromatic substitution mechanism, as seen with the enzyme. DFT calculations on this system support strongly that the bis-m-oxodicopper(III) species can serve as the electrophilic agent in this oxidation. Overall, the evidence for sequential O–O bond cleavage and C–O bond formation suggests an alternative mechanism to the concerted or late-stage O–O scission generally accepted for phenol hydroxylation by tyrosinase.

D-45

Reduction of dioxygen to water by the multicopper oxidases

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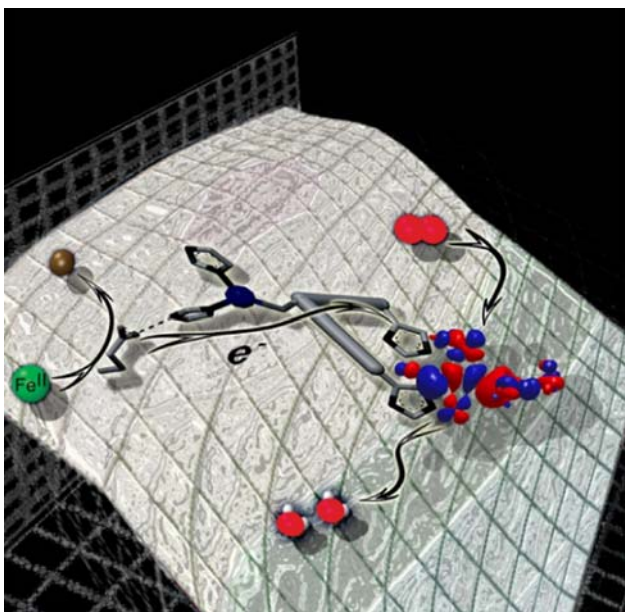
In nature the four electron reduction of O_2 to H_2O is carried out by Cytochrome *c* Oxidase (CcO) and the multicopper oxidases (MCOs). In the former, Cytochrome *c* provides electrons for pumping protons to produce a gradient for ATP synthesis, while in the MCOs the function is the oxidation of substrates, either organic or metal ions. In the MCOs the reduction of O_2 is carried out at a trinuclear Cu cluster (TNC). Oxygen intermediates have been trapped which exhibit unique spectroscopic features that reflect novel geometric and electronic structures. These intermediates have both intact and cleaved O–O bonds, allowing the reductive cleavage of the O–O bond to be studied in detail both experimentally and computationally. These studies show that the topology of the TNC provides a unique geometric and electronic structure particularly suited to carry out this key reaction in nature.



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D-46

Formation of a bridged butterfly dicopper core and its relevance to stepwise O_2-activation in biological systems

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In biological systems, the active dimetal center acts for dioxygen uptake and activation. In type III copper proteins, dioxygen binds to the dicopper(II) center in the μ-η^2:η^2-type side-on mode for its transportation and substrate-oxygenation, respectively. Recently, we succeeded in synthesizing a μ-η^2:η^2-peroxodicopper(II) complex, $[Cu^{II}_2(\boldsymbol{\alpha Sp})_2(\mu\text{-}\eta^2\text{:}\eta^2\text{-}O_2)(\mathbf{Bz}^-)]SbF_6$ ($\boldsymbol{\alpha Sp}$ = α-isosparteine, $\mathbf{Bz}^-$ = benzoate), and revealed its crystal structure by X-ray diffraction analysis (Fig. 1) [1]. The new butterfly-type μ-peroxo dicopper(II) complex can be readily formed by oxygenation of the copper(I) state in the presence of $\mathbf{Bz}^-$ or conversion of the corresponding bis(μ-oxo)dicopper(III) species with axial coordination of $\mathbf{Bz}^-$ in organic solution at −80°C. These reactions may be related to the controlled stepwise reduction of dioxygen in non-heme diiron proteins and oxidation of water to evolve dioxygen in manganese cluster of photosystem II.

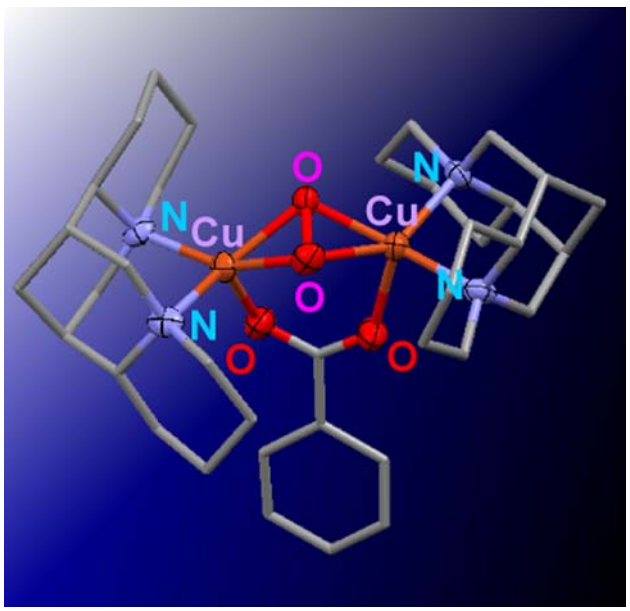

Fig. 1 Structure of the "*Bridged-Butterfly* core" of a O_2-binding dicopper(II) complex, **1**

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