2251-Pos Board B221
Resonance Raman Probes of the Internal Binding Pocket of Dehaloperoxidase from Amphitrite ornata
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Using Resonance Raman spectroscopy and activity assays we have determined that there are potent inhibitors of DHP activity and these inhibitors play a major role in the biology of DHP. Resonance Raman spectroscopy can detect substrate or inhibitor binding by monitoring shifts in the core size marker modes, ν_2 and ν_3, and the vinyl substituent mode, $\nu_{C=C}$, of the heme. Para-halogenated phenols bind internally, displacing the sixth position water molecule of the iron (in agreement with X-ray crystallography) resulting in a 5-coordinate high spin heme. Tri-halogenated phenols bind externally, forcing the distal histidine into the closed conformation (also in agreement with X-ray crystallography) resulting in a 6-coordinate high spin heme. The internal and external binding sites are supported by NMR data. X-ray crystallography shows when para-halogenated phenols bind internally, the halogen plugs into a hydrophobic cavity just above the back edge of the distal pocket that is analogous to the xenon 4 binding site in Sperm whale met-myoglobin often referred to as a ''packing defect.'' Analysis of the X-ray crystal structures of the 4-iodo-, 4-bromo-, and 4-chlorophenol/DHP complexes show a general trend in the position of the halogenated phenol with 4-iodophenol binding farthest into the cavity followed by 4-bromophenol, and 4-chlorophenol. Using RR spectroscopy, we demonstrate that the dissociation constants of the para-halogenated phenols mimic the trend observed in X-ray crystallography with 4-iodophenol being the most tightly bound in the distal pocket. DHP clearly demonstrates the importance of the hydrophobic cavity as an inherent selector for substrates entering the distal pocket by making use of the cavity-halogen hydrophobic interaction concomitantly preventing non-halogenated substrates from binding. Using activity assays, we demonstrate that internal binding of mono-halogenated phenols inhibits the function of DHP on the externally bound tri-halogenated substrates.

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Spectroscopic Probes of the Reactive Intermediates of Dehaloperoxidase from *Amphitrite ornata*
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The enzyme intermediates of dehaloperoxidase (DHP) from the marine worm *Amphitrite ornata* are unique within both the globin and cytochrome c peroxidase superfamilies. DHP has been shown to oxidize trihalophenols to dihaloquinones in a dehalogenation reaction that uses hydrogen peroxide as a substrate. We show that the initially formed heme intermediate in this reaction is not Compound I as is often the case in peroxidases, but rather is a combination of Compound II and a tyrosyl radical that has similarity to the Compound ES intermediate of cytochrome c peroxidase. Using stopped-flow UV-visible spectroscopy, we provide a detailed kinetic description for the reaction of pre-formed DHP Compound II and tyrosyl radical with the substrate 2,4,6-trichlorophenol, and demonstrate the catalytic competency of this intermediate in generating the product 2,4-dichloroquinone. Furthermore, using rapid-freeze-quench electron paramagnetic resonance spectroscopy, we detected a signal at $g \approx 2.0058$, confirming the presence of a protein radical in DHP Compound II, and assign it as a tyrosyl radical based upon mutagenesis studies and structural arguments. In the absence of a halophenol substrate, the DHP Compound II + tyrosyl radical intermediate decomposes to a new and significantly less active species, termed Compound RH, which is unique to dehaloperoxidase. We propose that this intermediate plays a protective role against heme bleaching. While unreactive toward further oxidation, Compound RH can be reduced and subsequently bind dioxygen, generating oxyferrous DHP, which may represent the catalytic link between the peroxidase and oxygen-transport activities in this bifunctional protein.

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Substrate binding triggers a switch in the iron coordination in dehaloperoxidase from *Amphitrite Ornate*
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We have explored the effect of substrate binding on the heme iron conformation in the enzyme dehaloperoxidase (DHP). DHP is a dimeric hemoglobin that also has significant peroxidase activity under physiological conditions and has been shown to oxidize trihalophenols to dihaloquinones in a dehalogenation reaction that uses hydrogen peroxide as a cosubstrate. Hyperfine sublevel correlation spectroscopic (HYSCORE) analysis of the ferric form of DHP was carried out to characterize effects of the substrate 2,4,6-trifluorophenol (TFP) binding on the iron coordination in order to elucidate molecular mechanisms responsible for switching the protein function from a globin to a peroxidase. The CW EPR spectrum shows that at pH 6.0 DHP heme iron exists in a highly axial high spin (HS) state that could be interpreted as arising from two different populations of the HS iron centers. Substrate binding does not change the spin state at pH 6.0, however, affects the magnetic parameters of the signal. HYSCORE spectra recorded at magnetic field corresponding to $g = 2$ revealed the presence of exchangeable protons with hyperfine coupling of ca. 6 MHz, consistent with a water molecule being the sixth ligand in the iron coordination. These protons' spectral features disappeared upon substrate binding. At pH 9.6 the EPR spectrum from heme iron of DHP shows the presence of both high- and low-spin states with the low spin signal characteristic of hydroxyl form. Upon TFP binding the low spin signal disappears. HYSCORE spectra at pH 9.6 also show the presence of exchangeable protons that disappear upon substrate binding. This observation highlights the proposed role of molecules in the distal pocket to control the peroxidase function of DHP.
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Dynamics And Energetics Associated With Ligand Photodissociation From Co Bound Chloroperoxidase
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Chloroperoxidase (CPO) is the most versatile heme peroxidase that can function as peroxidase, catalase, halogenase/dehalogenase and monooxigenase. The active site of CPO strongly resembles that of cytochrome P450 since the heme iron in both proteins is coordinated with a Cys axial ligand. Two hydrophobic channels that connect the heme binding cavity with the surrounding are likely to provide ligand access into the active site. At alkaline pH the protein undergoes a conformational transition resulting in the population of six coordinated heme iron with the histidine residue in the position of the sixth ligand. Here we report the results of the photoacoustic study of ligand dissociation from CPO. The ligand photodissociation and migration out of the protein matrix occurs within 50 ns and no additional kinetics was determined (detected?) between 50 ns and 5 μs. At acidic pH ligand dissociation is associated with a positive volume change ($\Phi\Delta V = 10.9 \pm 0.9$ mL mol^{-1}) and enthalpy change ($\Phi\Delta H = 24 \pm 5$ kcal mol^{-1}), strongly indicating that the ligand release from the protein matrix does not result in significant changes in protein conformation and CO is likely to migrate through one of the hydrophobic channels. On the other hand, the thermodynamic parameters associated with CO photorelease at pH 10 are significantly smaller than those determined at acidic pH ($\Phi\Delta V = 3.2 \pm 1.1$ mL mol^{-1} and $\Phi\Delta H = -12.1 \pm 5.3$ kcal mol^{-1}) due to changes in heme coordination upon CO dissociation.

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Hierarchical Structures of Natural Acellular Polymeric Hemoglobins: Model for Design of a Therapeutic Hemoglobin-Based-Oxygen-Carrier (HBOC)
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Natural acellular polymeric hemoglobins provide oxygen transport/delivery within many terrestrial and marine invertebrate organisms. It has been our premise that these natural acellular Hbs can serve as models for development of therapeutic hemoglobin-based oxygen carriers (HBOC). Understanding of how organisms utilize acellular oxygen carriers and maintain their structural integrity and redox stability within their circulatory systems is vital for the design of a safe and effective red cell substitute. Biophysical characterizations of naturally available acellular Hbs are warranted to determine unique structural and chemical properties that contribute to their success as acellular oxygen carriers. Our attention has focused on acellular Hbs from a terrestrial (*Lumbricus terrestris*) and marine (*Arenicola marina*) invertebrate, testing the hypothesis that their unique hierarchical structure and functional properties are significant in their success as a natural oxygen carrier. Structural and redox properties of these polymeric invertebrate Hbs were studied. Structural stability was evaluated by: 1) alkaline pH dissociation of the intact duodecamer (*in vivo* molecular hierarchy) by gel chromatography (pH 7.0-9.1 range), and 2) isothermal urea unfolding studies. Redox properties were examined by a comparison of the rates of autoxidation (heme-$Fe^{+2} \rightarrow$ heme-Fe^{+3}) of each of these invertebrate Hbs and compared to a unique zero-linked high M. wt. polymeric hemoglobin (OxyVita^{TM}) developed for application at the veterinary and clinical levels. This ''super polymer'' has an average M.wt.$=17 \times 10^6$ Da and a viscosity at 6g% comparable to plasma. Initial pre-clinical studies using the liquid or powder form of OxyVita^{TM} clearly demonstrate no increase in mean arterial pressure (MAP) upon injection, indicating that its molecular size prevents vascular extravasation.