SPECTROSCOPIC CHARACTERIZATIONS OF THE COMPOUND II INTERMEDIATE OF SOYBEAN PEROXIDASE FROM SOYBEAN SEED COATINGS

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SPECTROSCOPIC CHARACTERIZATIONS OF THE COMPOUND II INTERMEDIATE OF SOYBEAN PEROXIDASE FROM SOYBEAN SEED COATINGS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at Virginia Commonwealth University

By

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By

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2009

Director: Dr. James Terner, Professor, Department of Chemistry

Spectroscopic characterization of ferric soybean peroxidase with peroxides were studied to determine the ligand coordination and to characterize the structure of the heme active site and its intermediates (ferryl species). The lifetime, chemical reactivity and distinctive colors of the ferryl species (FeIV) formed during the oxidation of peroxidase (FeIII) by peroxides enabled structure, dynamics and reaction mechanisms to be studied.

Resonance Raman spectroscopy was used as a means of characterizing the structure of the soybean peroxidase and its intermediates. Excitation in the Soret absorption band at 406.7nm with 2-5mW laser power was used for this study. Resonance Raman spectra in the 200 to 1700 cm\(^{-1}\) region were obtained for the soybean peroxidase. However, the focus of this study was on the vibrational region of the resonance Raman
spectra from 900 to 500 cm\(^{-1}\) where the Fe\(^{IV}\)=O stretching frequencies for heme compound II intermediates are expected. Several pH and pD (deuterium substitution) samples of the soybean peroxidase were analyzed using resonance Raman spectroscopy.

The vibrational stretching frequencies of the ferryl peroxidases varied with varying pH/pD were observed within the 773–787 cm\(^{-1}\) range. From the deuterium experiment, accompanied with changes in the vibrational frequencies of the iron-ligand, a 3 cm\(^{-1}\) upshift and intense resonant enhancement of the peaks, we observed the ferryl nature of compound II intermediate for soybean peroxidase.

Badger’s rule was used to estimate the bond distances that existed within Fe-O which offers additional insight into the structure of the ferryl species. The estimated bond distance for the soybean peroxidase was significantly less than Fe-O bond distances proposed by X-ray crystallographers for other peroxidases in the same family. Comparing the vibrational frequencies of the ferryl intermediates in soybean peroxidase to that in heme proteins portrayed the effect the protein environment has on the vibrational frequencies.
Chapter 1: Introduction

Peroxidases are hemeproteins that contain iron III protoporphyrin IX active sites. They are widely known for their ability to catalyze a variety of substrates in the presence of peroxides.[1] Peroxidase has multiple biotechnological applications[2]. Some of the applications of peroxidases include testing for glucose and cholesterol in urine and blood, removal of phenolic compounds from wastewater, polymer synthesis, decolorization of synthetic dyes, enzyme immunoassays and biosensors[2, 3]. Peroxidases such as horseradish peroxidase have played a key role in the understanding of mechanism of heme enzymes which involve oxidation of the heme group to higher oxidation states. The catalytic cycle of peroxidases involve two spectroscopically distinct intermediates called compound I and II[4].

Compounds I and II have long been used as models for other heme enzymes. [1] Ferryl species are transient species normally formed during the oxidation reactions of peroxidases with peroxides resulting in a two-electron oxidized intermediate called compound I, a π-cation radical ferryl complex [5-7]. Reduction of compound I by an oxidizable substrate yields compound II believed to be a ferryl complex. Compound II is reduced to the ferric resting state by an oxidizable substrate. The formation of ferryl species or compound II intermediates has been studied in heme[1, 8] and non-heme[9] proteins. Investigations of ferryl species have contributed to an improved understanding of the catalytic pathway of numerous enzymatic systems[5, 6]

Among all the other peroxidases, horseradish peroxidase (HRP) is the most widely studied readily available peroxidase for commercial use and is prepared from the root of horseradish. HRP was believed to be the most stable peroxidase until recent
findings in soybean peroxidase (SBP) from soybean seeds coating appeared promising in peroxidases production, and was identified as a more cost effective alternative to HRP [10-12]. SBP has not been studied as comprehensively as HRP but it has gained a lot of interest due to its high activity at low pH and high thermal stability, after McEldoon and Dordick’s [12] findings on SBP’s unusual properties.

This research focused on the use of spectroscopic methods to investigate the changes in the structure and environment during the reaction of soybean peroxidases with both hydrogen and deuterium peroxide. The main focus of this was on SBP whilst data from Mb and Hb were used in comparative analysis. SBP was used in this work because of its high thermal stability and stability within a wide pH range; availability and low-cost. SBP may be a hopeful substitute for HRP. [11, 13-16].

SBP was isolated from the seed coatings of soybean and purified as described by Shannon et al and Barrett et al[17-19]. The peroxidase was purified using ammonium sulfate precipitation and column chromatography, resulting in increased peroxidative activity. The molecular weight of SBP was estimated to be 40KDa with SDS-PAGE. We also observed the melting temperature at 90.0°C as described by McEldoon and Dordick[12]. The kinetic parameters associated with the enzymatic reaction of the soybean peroxidases, myoglobin and hemoglobin with peroxides, were measured using absorption spectroscopy. The Soret absorption band of SBP did show some similarities to that of HRP. Some of these similarities include the iron (III) protoporphyrin IX active site and catalytic mechanism [20, 21]. Just like HRP, SBP has seen widespread application in areas such as food and industrial wastewater treatment, biosensing and biotechnological applications[22-24].
Resonance Raman spectroscopy was used as a means of characterizing the structure of the soybean peroxidase and its intermediates. Excitation in the Soret absorption band at 406.7nm with 2-5mW laser power was used. Resonance Raman spectra in the 200 to 2000 cm\(^{-1}\) region were obtained for soybean peroxidase.

The structure of soybean peroxidase compound II and the pH dependent protein interactions with the heme complexes were examined. To understand the protein dependency, the interactions with the heme prosthetic group were investigated by monitoring the effects of pH and pD on the porphyrin and ligand vibrations. Deuteration effects were monitored by verifying changes in vibrational frequencies in experiments performed with D\(_2\)O in D\(_2\)O and H\(_2\)O\(_2\) in H\(_2\)O. Iron ligand stretching frequencies were observed between 767 cm\(^{-1}\) and 787 cm\(^{-1}\) for the ferryl SBP, giving direct evidence of the formation of a Fe\(^{IV}=O\) (oxyferryl) heme structure. In this research, we observed a switching of the ferryl stretching frequencies of soybean peroxidase from 787 cm\(^{-1}\) at alkaline pH to 767 cm\(^{-1}\) at acidic pH. Also the resonance Raman spectra of SBP compound II was compared with other six coordinated low spin SBP derivatives. This resulted in the affirmation of the SBP compound II structure.

Badger’s rule was used to estimate the bond distances that existed within Fe-O bond offering additional insight into the structure of the ferryl species[25]. The estimated bond distance for the soybean peroxidase was significantly less than Fe-O bond distances proposed by X-ray crystallographers for other peroxidases in the same peroxidase family[25-27]. Comparing the vibrational frequencies of the ferryl intermediates in peroxidase to that of Mb and Hb, portrayed the effect the protein environment has on the vibrational frequencies.
1.1. Heme Proteins

Heme proteins are a class of biological macromolecules containing an active site or prosthetic group of an iron porphyrin complex[28]. Most heme proteins are known to have a protoporphyrin IX ring structure as shown in Figure 1. The octahedral ring structure of the heme has a centrally located iron atom bonded to four pyrrole nitrogen atoms of a flat porphyrin and connected by methane bridges, while the other two coordination sites (5th and 6th positions) lie perpendicular to the plane of the porphyrin. The 5th position is normally occupied by an amino acid whereas the 6th position of the iron remains available to bind with electronegative atoms of various ligands. The nature of the amino acids or substrates at the axial positions influences the electron configuration of both the iron and the heme, ultimately affecting the solubility, spectrum and reactivity of the protein[29, 30].

Heme proteins perform vital functions in biological systems[31]. Table 1 presents the different heme proteins and their functions. The type of interaction of iron porphyrin with other groups in the two axial positions determines the functions the heme performs[28]. Myoglobin and hemoglobin function as reversible oxygen transport proteins[32]. Peroxidases, catalases, cytochrome P-450s, and nitric oxide synthases are involved in electron abstraction processes, disproportionation of hydrogen peroxide, monooxygenations, nitric oxide synthesis, and nitrous oxide reduction respectively[33-37], while cytochromes serve as reversible electron transfer agents[38].

One important function of heme proteins in mammals is the reduction of dioxygen to water which constitutes the terminal step in the respiratory electron transport chain. However, dioxygen metabolism can result in the formation of the partially reduced
species. These species, hydrogen peroxide and superoxide anion radical (O$_2^-$), have potential toxic effects which, when not eliminated, can cause harm to the body. Catalases and peroxidases are heme enzymes notable for catalyzing the degradation of hydrogen peroxide. With the exception of the globins the remaining proteins shown in table 1 thus have the Fe$^{3+}$ protoporphyrin IX in their native state.

Figure 1: Molecular Structure and Labeling Scheme of Iron Protoporphyrin-IX[39]
<table>
<thead>
<tr>
<th>Heme</th>
<th>Distal ligand</th>
<th>Proximal-5th position</th>
<th>Functions in the body</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globin–Hemoglobin, Myoglobin</td>
<td>Phe, His, Val</td>
<td>Histidine</td>
<td>Transports oxygen from the lungs and muscle tissues</td>
</tr>
<tr>
<td>Peroxidase (HRP, SBP)</td>
<td>Phe, His, Arg</td>
<td>Histidine</td>
<td>Uses hydrogen peroxide (H₂O₂) as the electron acceptor to catalyze a number of oxidative reactions</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>Phe, Glut, Phe</td>
<td>Thiolate Cysteine</td>
<td>Catalyzes the halogenation of a number of aliphatic substrates</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>Thr, Asp</td>
<td>Thiolate Cysteine</td>
<td>Involves in metabolism of both exogenous and endogenous compounds</td>
</tr>
<tr>
<td>Catalase</td>
<td>Asp, His, Phe</td>
<td>Tyrosine</td>
<td>Catalyzes the decomposition of hydrogen peroxide to water and oxygen</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>His</td>
<td>Histidine</td>
<td>Transports electrons to the inner mitochondrial membrane</td>
</tr>
</tbody>
</table>
1.1.1. Peroxidases

Figure 2: X-ray crystal structure of HRP (Adapted from N.C. Vietch Phytochemistry 2004)

Peroxidases are heme enzymes which catalyze the degradation of hydrogen peroxide. Peroxidases couple the reduction of hydrogen peroxide (alkyl peroxides) to water (alcohols) and oxidation of substrates by hydrogen peroxide or organic peroxides.

\[
\begin{align*}
    \text{H}_2\text{O}_2 + 2\text{H}^+ + 2\epsilon & \rightarrow 2\text{H}_2\text{O} & \text{Eqn. 4} \\
    2\text{H}_2\text{O}_2 & \rightarrow 2\text{H}_2\text{O} + \text{O}_2 & \text{Eqn. 5}
\end{align*}
\]

Horseradish peroxidase (HRP), one of the most extensively studied heme peroxidases, is a glycoprotein (Figure 4). It is the most readily available peroxidase used worldwide in commercial scale for glucose testing. It has molecular weight of approximately 40 KDa and contains a single iron (III) protoporphyrin IX as its prosthetic group[6]. The first four coordination sites of this heme are occupied by the pyrrole nitrogens of the iron porphyrin ring. The fifth (axial) position of the heme iron is occupied by an imidazole side chain of a histidine amino acid residue. Seven isozymes of
HRP (A, B, C, D, and E) have been isolated and characterized by chromatographic, electrophoretic and spectrophotometric techniques[18, 40]. Chance[4] showed that there were two distinct intermediates of horseradish peroxidase, compound I and II. Unlike Compound I, the rate of formation of compound II was independent of peroxide concentration. Chance assumed that these intermediates were enzyme-substrate complexes of the enzyme and hydrogen peroxide[4]. Subsequent studies proved that the intermediates involve higher oxidation states than the enzymes and that a variety of substrates will lead to the formation of these intermediates. The ferryl structures of the intermediates is now widely accepted and supported by spectroscopy, electrochemical and magnetic susceptibility data.

The catalytic cycle of peroxidases involve oxidation of the heme prosthetic group to produce reaction intermediates. The iron atom in the resting enzyme is usually Fe$^{3+}$ with the fifth coordination proximal site occupied by a histidine imidazole. Peroxidase was previously known to have its sixth coordination position of the heme iron, distal to the heme surface, either empty or occupied by a water molecule. The reaction of ferric peroxidase with H$_2$O$_2$ results in a heterolytic cleavage of the oxygen–oxygen bond to produce an oxo-ferryl species (Fe$^{IV}$=O) paired with a porphyrin cation radical. Two electron transfers (oxidation) to the resting iron generates a green intermediate commonly referred to as compound I and one electron transfer (reduction) results in a red compound II intermediate. Compound I contains a porphyrin π -cation radical where an electron has been removed from the bonding orbitals of the porphyrin π-system. Compound II is formed as it accepts electron from compound I to fill its π orbital vacancy.
For peroxidases, peroxide oxidation is believed to depend on the distal histidine working as an acid–base catalyst. The mechanism (Figure 4) that has been proposed for the oxidation reaction of \( \text{H}_2\text{O}_2 \) with peroxidase is that the distal histidine first functions as a base to abstract a proton from hydrogen peroxide to allow the binding of the hydroperoxy anion to the heme iron. The protonated histidine then serves as an acid to facilitate the heterolytic O-O bond cleavage through the release of a water molecule. The ferryl peroxidase formed from the reaction of hydrogen peroxide and ferric peroxidase has low activity. The strength of the interaction (hydrogen bonding interaction) and reactivity may be explained by the difference in the vibrational frequencies of the ferryl species and the vital role the surrounding amino acid residues play during the peroxidative reaction.
Raman studies have been extensively done on peroxidase heme proteins. The fast formation and short lifetime of compounds I and II make it difficult to stabilize and characterize these compounds using traditional instrumental methods [1, 41, 42]. Further, instrumental characterization of compound I of porphyrin II-cation radical is considered more troublesome with regards to Raman Spectra studies in particular[43-46]. The results of recent studies on the resonance Raman signals for compound I intermediates of the oxo-iron (IV) porphyrin II-cation radical type are contradictory as a result of complications of photolability (light sensitivity of the unstable intermediate) that makes compound I signals appear to be similar to those of compound II or other forms.

Terner et al[1] showed that clear resonance Raman signals from in-plane skeletal modes for HRP-I could be obtained with near-ultraviolet excitation rather than with 406.7 or 413.1 nm deep violet krypton ion laser lines that usually provide the strongest
resonance enhancement of any wavelength for porphyrin systems [47]. Terner et al [48] also demonstrate that it is possible to identify HRP-I signals with deep violet excitation, and that these signals coincide with those from near-ultraviolet.

It is observed that features of Raman spectra at 1377, 1428, 1509, 1584 and 1633 cm\(^{-1}\) that had previously been attributed to HRP-I are actually prominent features of HRP-II. These bands are recognized to be the most prominent features of an HRP II-like photoproduct generated by in situ photolysis[48]. When the incident laser power was reduced to sufficiently low level, the most prominent bands begin to appear. A long searched for characteristics of HRP-I was that \(v_4\) is indeed lowered from 1379cm\(^{-1}\) of HRP-II, to 1359cm\(^{-1}\). A pseudo-Jahn Teller effect has been given as the basis for the observation of \(a_{2g}\) modes near 1000 cm\(^{-1}\) with anomalous frequencies in metalloporphyrin p-cation radicals[49, 50]. Figure 4 is a comparison of the near-ultraviolet resonance Raman spectra (356.4nm excitation) of HRP compound I, compound II, and the resting ferric and dithionite reduced ferrous enzyme[51].
Figure 4: Resonance Raman spectra of HRP: (a) resting horseradish peroxidase; (b) compound I; (c) compound II; (d) ferrous horseradish peroxidase; 356.4 nm excitation, pH 6.8\cite{1}
HRP intermediates and their reactions have been known to be pH dependent. The pH changes as a result of the changes in pKa have been attributed to ionization of amino acid residues (histidine) near the active site of the enzyme. It is known that the heme iron of HRP exists predominately as penta-coordinate at neutral pH. As the pH of the enzyme solution is raised from neutral to alkaline, the heme iron undergoes a reversible transformation from five to six coordinate, called alkaline transition. The alkaline transition of HRP is evident by a change in color from brown to red with concomitant change in optical absorption spectrum and a switch from high to low spin heme iron. A number of studies have shown that the ionization and coordination of amino acid side chain results in alkaline transitions in the heme iron resulting in a protein conformational change[52]. Dunford et al assumed the presence of water molecule in the sixth coordination position and proposed that an ionization of the water molecule to a hydroxide ion at alkaline pH, in analogy to the ionization of water molecule to hydroxide ion observed in the Fe$^{3+}$ heme of aquo metmyoglobin[53].

Terminal metal-oxo groups occurring in transition metal compounds are known to exist in high oxidation state[54]. The intensities of metal-oxo vibrations in metalloporphyrins can be enhanced by resonance Raman effect[55] and generally show up as distinctive polarized bands ($I_\perp/I_\parallel = 1/8$) characterized by $^{18}$O-sensitivity[56, 57]. While researchers agree that the iron-oxo group in synthetic ferryl porphyrin complexes is double bond, the consensus is less clear for the oxo-iron (IV) structures for the heme proteins. Badjor and Nakamoto[58] first identified an Fe$^{IV}$=O stretching vibration in synthetic iron porphyrin for oxoiron(IV) tetraphenylporphyrin (O= Fe$^{IV}$TTP) at $852\text{cm}^{-1}$. Similarly, Fe$^{IV}$=O vibrations in proteins were first detected in compound II intermediates
of isozymes of horseadish peroxidase (HRP) [59] and confirmed by several laboratories [43, 60, 61]. The vibrational frequencies of the oxo-iron bonds of HRP compound II were found to be weaker in intensity than for O= Fe$_{IV}$TTP and other meta-oxo vibrations, and those vibrations occur at lower frequencies, near 775 cm$^{-1}$. The lower frequencies of the protein relative to those of synthetic models are due to impurities in the protein environment.[1]

Figure 5 shows the Fe$_{IV}$=0 stretching vibration with 406.7 nm for HRP-II that is formed from the preparations containing HRP-A1 and A2 isozymes[61, 62]. It is shown that the ν Fe (IV)=0 frequencies are pH-dependent. Further, $^{18}$O-substitution ν Fe(IV)=0 bands occur at 779 cm$^{-1}$ for lower pH, and 789 cm$^{-1}$ at higher pH (Figure 5a), shifting to 745 and 756 cm$^{-1}$ respectively during $^{18}$O substitution (Figure 5b). In similar studies, pH-dependent frequencies were observed for other isozymes of HRP and turnip peroxidase [63]. The low pH band for compound II of HRP occurs at 775 cm$^{-1}$, slightly lower than the corresponding band of A1 and A2 isozymes. The low pH band for HRP isozyme C occurs at neutral pH. The pKa values of the transitions observed by Raman spectra for isozymes A1 and A2 are 6.9 and 8.6 respectively which correspond to those of the pH activity profiles of HRP-II for those isozymes[62-64]. It is noteworthy that the low pH ν Fe(IV)=0 frequencies are more sensitive to deuteration than their higher pH counterparts (Figure 5c), exhibiting intensity enhancement of full order magnitude, along with small though reproducible 3 cm$^{-1}$ [62, 65]. Basically, identical frequencies, and effects of pH and deuteration have also been reported for compound II of bovine liver catalase [64].
Figure 5: pH-dependence of Resonance Raman spectra of Horseradish Peroxidase compound II, 406.7nm Excitation: (a) Formed with H$_2$O$_2$; (b) Formed with H$_2$O$_{18}$O$_2$; (c) Formed with H$_2$O$_2$ in 2H$_2$O. (Adapted from Ref 1. pD values quoted according to pD = pH$_{measured}$ + 0.4)[1]
1.2. Raman Spectroscopy

Raman spectroscopy is one of the analytical technologies for molecular structure identification. Raman spectroscopy provides similar information as infrared spectroscopy, i.e., the energies of molecular modes of vibration. Like infrared, Raman spectroscopy probes the molecular structure of compounds using electromagnetic radiation, which interacts with molecular bonds to produce a unique spectral fingerprint of the compound[66]. The two methods however, differ fundamentally in mechanism and selection rules. Infrared involves a direct absorption of radiation at the frequency of a particular molecular vibration. Raman spectroscopy, on the other hand, involves the inelastic scattering of monochromatic light (laser), from the molecules in a sample.

Raman spectroscopy is useful for elucidating the structural characterization of compounds as it identifies analyte, characterizes sample matrices and provides molecular spectroscopic information. The Raman spectrum provides information specific to the molecules present in the sample which enables the analyte identification. Raman measurements can be fast, with acquisition times of very few seconds. In addition to haven a rapid acquisition time in comparison to other spectroscopic methods such as FTIR, Raman spectroscopy together with sample preparation methods such as micro-extractions, fraction collections, and small particle analysis, can be used to gather a large amount of information about a compound.

Raman spectroscopy as an inelastic scattering technique can be used to analyze compounds in all states of matter. Raman measurements require minimal sample preparation making the technique simple and extremely flexible. Advanced instrumentation has led to greater reliability, reproducibility, a much higher level of
automation and greater ease of use. Raman spectroscopy is suitable for characterizing aqueous samples that are difficult to characterize with other techniques because of water interference. It is a powerful technique with a promise of providing quick solutions to some of the most complex analytical problems.

As the electromagnetic radiation using a monochromatic light interacts with the molecular bonds, a small fraction of the scattered photons loses some energy to a vibrational mode of the molecule resulting in either loss or gain in energy. This is normally referred to as inelastic scattering (Raman scattering). As a result of the radiation, the energy of the system is given as $h (v_f) = h (v_o \pm v_i)$ where $h v_o$ is the incident energy, $h v_i$ is the final energy and $h v_f$ is the energy change in the system. In Figure 6, Rayleigh scattering occurs when the photon energy of scattered radiation $h v_f$ is the same as the incident photon $h v_o$. This elastic scattering (Rayleigh scattering) accounts for over 99.99% of all the scattered photons[67].

The Raman consists of two inelastic scattering processes, Stokes and Anti-Stokes. Stokes scattering process occurs when the radiation results in a decrease in energy ($v_f > v_o$ — long wavelength) while Anti-Stokes results when the scattered photons gain some energy and have a shorter wavelength ($v_f < v_o$) than the incident light. The difference in energy between the incident radiation and the Raman scattered photon is equal to the energy of a vibrational mode of the molecule. During the Raman scattering, photons interact and exchange energy with molecules. This exchange of energy results in a change in the wavelength of the photons due to interactions with bond vibrations within the molecules. The molecular vibrations can be determined by observing the frequency changes of the photons. Since Raman is a form of vibrational spectroscopy, the energy
transitions arise from molecular vibrations. Because these vibrations involve identifiable functional groups, when the energies of these transitions are plotted as a spectrum, they can be used to identify the molecule.

Figure 6: The Different Possibilities of Visual Light Scattering[68, 69]

A Raman spectrum is a plot of the intensity of Raman scattered radiation as a function of its frequency difference from the incident radiation (usually in units of wavenumbers, cm\(^{-1}\)). This frequency difference is called the Raman shift. The Raman shift is independent of the frequency of the incident radiation because it is a difference value. Typically, only the Stokes region is used (the anti-Stokes spectrum is identical in pattern, but much less intense).
The intensity of Raman scattered radiation is given by:

\[ I \alpha \nu^4 I_0 N f(\alpha^2) \]  

Eqn 6

Where \( I_0 \) is the intensity of the exciting light (laser), \( N \) is the number of scattered molecules in a given state, \( \nu \) is the frequency (Hz) of the exciting light, and \( \alpha \) is the polarizability of the sample.

For a normal stokes scattering, which originates from the ground state, \( N \) will be the number of molecules in the ground state. Thus \( N \) for anti-stokes is the number of molecules in the excited state. According to the Boltzmann’s law (equation 7), the number of molecules depends on the absolute temperature of the sample and on the energy difference between the ground state and the excited state for a given vibrational coordinate.

\[ \frac{N_i}{N_o} = e^{-(E_i-E_o)/kT} \]  

Eqn 7

The Boltzmann’s equation shows that the intensity of the anti-Stokes scattering decreases rapidly with increasing temperature and with increasing frequency shift from the exciting wavelength. Figure 7 shows a typical Raman spectra of CCl\(_4\) using a mercury arc lamp source which decline in intensity at the anti-Stokes region as a result of increasing frequency.
Figure 7: Raman Spectrum of CCl\textsubscript{4} using a Mercury Lamp Source 488nm [66]
The number of photons scattered in Raman measurements is quite small making Raman signals relatively weak. The sensitivity of a Raman signal has being enhanced by different types of Raman spectroscopy such as surfaced enhanced Raman spectroscopy, confocal Raman micro spectroscopy, coherent anti-Stokes Raman scattering and resonance Raman spectroscopy.

This study uses resonance Raman spectroscopy. Resonance Raman can be used when the laser wavelength utilized is close to the absorption wavelength of the molecule. By irradiating the sample with a wavelength close to the incident wavelength, the Raman signals are enhanced to an order of magnitude where they can be detected. Not all samples will show resonance enhancement with common Raman lasers, but generally species such as porphyrins and those with a heavy central atom can show such an enhancement. Unfortunately, resonance Raman spectroscopy is sometimes hindered by fluorescent interference. In this study, the fluorescent interference problem is addressed by purifying the protein samples prior to the Raman measurement.

1.2.1. Raman Theory

The theory of Raman spectroscopy is quite complex. Both group theory and high order perturbation theory are used in discussing this phenomenon. The Raman effect of the oscillating electric field on a molecule can be described classically and macroscopically. The classical view assumes that a molecule placed in an electromagnetic field, with an electric field $E$, will induce a dipole moment $\mu$, since the electromagnetic field will exert a force on the charged particles of the molecule. This induced dipole moment is proportional to the field strength, the proportionality constant,
\( \alpha \) (equation 8). \( \alpha \) is normally referred to as the polarizability of the molecule or polarizability tensor.

\[
\mu_{\text{ind}} = \alpha E \quad \text{Eqn 8}
\]

Considering the molecule behaving in wave like manner, whose electric field oscillates in space with frequency \( \nu \), the electromagnetic field \( E \) of a light wave can be written as

\[
E = E_0 \cos(2\pi\nu t) \quad \text{Eqn 9}
\]

Where \( E_0 \) is the maximum value of the electric field and \( t \) is time. The induced dipole moment of this molecule in this oscillating field obtained by substituting equations 9 into equation 8 is

\[
\mu_{\text{ind}} = \alpha E_0 \cos(2\pi\nu t) \quad \text{Eqn 10}
\]

The polarizability of a molecule is valuable in the theory of Raman effect and can be used to derive a number of off-resonance effects that occur in the interaction of light and matter. Polarizability can be visualized as the response of the molecule to electromagnetic field; the least tightly bound outer electrons in a molecule would easily respond to the force exerted by the electromagnetic field followed by oscillation of the light. Hence, polarizability is a molecular property whose magnitude varies as the molecules oscillate at the natural frequency of the bond. Thus,

\[
\alpha = \alpha_0 + \sum_n \alpha_n \cos(2\pi \nu n t) \quad \text{Eqn 11}
\]
Where $\alpha_0$ is the equilibrium polarizability, $\alpha_n$ is its maximum variation and $\nu_n$ is the natural frequency. Substituting equation 11 into equation 10,

$$\mu_{\text{ind}} = E_0\alpha_0 \cos(2\pi vt) + E_0 \sum_n \alpha_n \cos(2\pi nt) \cos(2\pi \nu_n t)$$  \hspace{1cm} \text{Eqn 12}$$

Using the trigonometric identity, \(\cos \theta \cos \phi = \frac{1}{2} [\cos(\theta + \phi) + \cos(\theta - \phi)]\), equation 12 can be rearranged as,

$$\mu_{\text{ind}} = E_0\alpha_0 \cos(2\pi vt) + \frac{1}{2} E_0 \sum_n \alpha_n [\cos 2\pi (\nu + \nu_n)t + \cos 2\pi (\nu - \nu_n)t]$$  \hspace{1cm} \text{Eqn 13}$$

Equation 13 predicts the major qualitative features of the Raman effect using the classical theory which predicts that the induced dipole moment will oscillate with components of frequency $\nu$, $\nu + \nu_n$ and $\nu - \nu_n$. The first term which accounts for the Rayleigh scattering, describes the component of the polarization having the frequency of the exciting field. The second component shows the Raman scattering frequencies $\nu + \nu_n$ (Anti-Stokes) and $\nu - \nu_n$ (Stokes).

Although the classical theory predicts the existence of the Raman effect and describes its dependence on the frequency of the exciting radiation, it fails to envisage that some of the normal frequencies do not give rise to Raman scattering (selection rules). The classical theory also neglects the tensor properties of the polarizability. Experimentally, measurements of polarization properties of Raman lines of heme proteins provide valuable information about the symmetry properties of normal vibrations. For instance the quantum theory explains the different intensities that exist between the Stokes and anti-Stokes Raman scattering. The quantum theory considers the
energy level of molecules as light interact with the molecule and the emission of a photon from a higher energy level to a lower energy level. According to quantum mechanical calculation, the application of second order perturbation theory to time dependent Schrödinger equation for the total intensity $I_{mn}$ of Raman scattered light at m and n transition states can be expressed as:

$$I_{mn} = \frac{128\pi^5 (\nu_i \pm \nu_{mn})^4}{9c^4} I_i \sum_{\rho\sigma} |\sigma_{\rho\sigma}|^2$$

Eqn. 14

Where from the above, the Raman scattering intensity is proportional to the intensity of the incident light $I_i$, frequency to the fourth power $\nu_i \pm \nu_{mn}$ and the square of the polarizability $\sigma_{\rho\sigma}$

$$\alpha_{\rho\sigma} = \frac{1}{\hbar} \sum_r \left[ \frac{M_{mr}M_{rn}}{\nu_{rm} - \nu_o} + \frac{M_{mr}M_{rn}}{\nu_{rm} + \nu_o} \right]$$

Eqn. 15

1.2.3. Resonance Raman Theory

Resonance Raman is used for characterizing the environmental structures of heme proteins by monitoring vibrational bands[67]. With visible laser excitation, the porphyrin skeletal and ligand metal vibrations are strongly enhanced over the remaining amino acid vibrations. This enhancement is an advantage since heme proteins have high molecular weights (some > 100,000 g/mole). In the absence of resonance enhancement, the Raman spectra for most biological molecules will be very tedious to interpret.
A molecule containing N atoms has 3N-6 (3N-5 for linear molecules) normal modes of vibration. The macromolecules of biology contain thousands of atoms and have far too many vibrational frequencies to be resolved, let alone assigned in a normal Raman or infrared spectrum. Fortunately, these frequencies tend to group themselves into more-or-less discrete bands, which can be identified as certain classes of structure. These bands are used to monitor changes in conformation. The Resonance Raman technique has been fruitfully applied to proteins, nucleic acids, and lipids[70].

A normal Raman spectrum is obtained by illumination of the sample in a transparent region of its spectrum. In resonance Raman spectroscopy, the illumination is within an absorption band. This effect is due to a coupling of electronic and vibrational transitions, and the vibrational modes which are subject to enhancement, are localized on the grouping of atoms which give rise to the electronic transition, i.e., the chromophore[71]. Resonance Raman spectroscopy therefore provides a means of monitoring vibrational frequencies of a chromophore, independent of its (nonabsorbing) matrix. For instance, metalloporphyrins, carotenoids and several other classes of biologically important molecules have strongly allowed electronic transitions in the visible region. The spectrum of the chromophoric moiety is resonance enhanced and that of the surrounding protein matrix is not. This helps to probe the chromophoric site (often the active site) without spectral interference from the surrounding protein[72]. Resonance Raman spectroscopy is also a major probe of the chemistry of fullerenes, polydiacetylenes and other "exotic" molecules which strongly absorb in the visible region. Although many more molecules absorb in the ultraviolet, the high cost of lasers and optics for this spectral region have limited UV resonance Raman spectroscopy to a
small number of specialists. Unlike other spectroscopic methods such as x-ray absorption spectroscopy, resonance Raman spectroscopy can be used to investigate samples of low concentration (< 50 μM).

Resonance enhancement of Raman bands comes into play when the energy of the incident light approaches that of an electronic transition. The theory of resonance Raman intensities is known to be proportional to the change in the square of that part of the molecular polarizability associated with a particular vibration. Kramers-Heisenberg-Dirac modified the Raman polarizability tensor by introducing a damping term $i\Gamma$ as shown in equation 16. The damping term shows specifically the initial and final states of a molecule and also gives information as to which vibrations were subject to resonance enhancement. This damping term is the lifetime of the intermediate state and ensures that increase in the scattering intensity to infinity does not occur as the denominator approaches zero.

$$\alpha_{mn} = \frac{1}{\hbar} \sum_r \left[ \frac{M_{mr}M_{mr}}{\nu_{rm} - \nu_o + i\Gamma} + \frac{M_{mr}M_{nr}}{\nu_{rm} + \nu_o + i\Gamma} \right]$$

Eqn 16

From equation 16, if $\nu_{rm} \ll \nu_o$, both terms give comparable contributions to the Raman intensity and the polarizability is independent of the incident frequency. When $\nu_{rm} - \nu_o$ becomes very small, (prevented from reaching zero by damping constant) Resonance enhancement is decreased. As $\nu_o$ approaches $\nu_{rm}$, the first term dominates and is responsible for resonance effects making the second term negligible (small contribution). Since resonance enhancement occurs within the electronic transition, molecular vibrations which modulate the electronic transition are selectively enhanced.
The transition moment $M$ is composed of purely electronic and vibrational parts (consisting of both rotational and vibrational contributions) which can be separated using the Born-Oppenheimer approximation.

$$M_{me} = \langle m | \mu | r \rangle \quad \text{and} \quad M_{re} = \langle r | \mu | n \rangle$$ \hspace{1cm} \text{Eqn 17}

$$\langle m | \mu | r \rangle \approx \langle j | e | \mu | e \rangle | v \rangle = \langle j | M_e | v \rangle$$ \hspace{1cm} \text{Eqn 18}

$$\langle r | \mu | n \rangle \approx \langle v | e | \mu | e \rangle | i \rangle = \langle v | M_e | i \rangle$$ \hspace{1cm} \text{Eqn 19}

Where $|i\rangle$ and $|j\rangle$ are the initial and final vibrational wavefunctions of the ground electronic state, $|v\rangle$ is a vibrational wavefunction of the excited electronic state and $M_e$ is the pure electronic transition moment between states $n$ and $r$.

$$|\Psi_{ele,vib,rot}\rangle = \langle ele | vib | rot \rangle$$ \hspace{1cm} \text{Eqn 20}

Each wave function comprises of electronic, vibrational and rotational contributions. $M_e$ is a function of nuclear coordinates, and may be expanded about the equilibrium geometry using the Taylor series.

$$M_e = M_e^0 + \left( \frac{\partial M_e}{\partial Q} \right)^0 Q \ldots .$$ \hspace{1cm} \text{Eqn 21}
Where, Q is a normal mode of the molecule. The first two terms in the expansion can be rewritten to express polarizability as

\[ \alpha = A + B \]  
Eqn 22

Where,

\[ A = (M_v^o)^2 \frac{1}{\hbar} \sum_v \frac{\langle j|v\rangle\langle v|i\rangle}{\nu_{vi} - \nu_o + i\Gamma_v} \]  
Eqn 23

\[ B = M_e^o + \left( \frac{\delta M}{\delta Q} \right)^o \frac{1}{\hbar} \sum_v \frac{\langle j|Q|v\rangle\langle v|i\rangle + \langle j|v\rangle\langle v|Q|i\rangle}{\nu_{vi} - \nu_o + i\Gamma_v} \]  
Eqn 24

The A term corresponds to the leading term in the expansion series, which contributes strongly to the scattering intensity. As shown in equation 23, the A-term varies with the strength of the electronic transition moment \( M_e \). A term scattering by a complex molecule shows a strong enhanced mode which has totally symmetric vibrations. The normal modes that experience the strongest resonance enhancement via A-term are those with coordinates that closely correspond to the distribution experienced by the molecule in the resonance excited state. Thus the \( \pi-\pi^* \) transitions provide large resonance enhancement for modes involving the stretching of the \( \pi \)-bonds, but little enhancement for carbon-hydrogen stretching modes. Raman scatter from totally symmetric vibrations will be strongly polarized parallel to the plane of polarization of the incident light. The scattered intensity from totally symmetric vibrations is \( 3/4 \) as strong in the plane perpendicular to the plane of polarization of the incident light as in the plane.
parallel to it. Nontotally symmetric modes gain intensity through the B term, because of the Q-dependent vibrational integrals. B-term involves vibronic mixing of two excited states, e and f. The active vibrations may have any symmetry which is contained in the direct product of the two electronic transition representations. The B enhancement is also responsible for resonance effects associated with the forbidden transitions such as d-d bands in transition metal complexes.

1.2.4. Resonance Raman Scattering in Heme proteins

Resonance Raman spectroscopy has been used as a probe of heme structure for both the stable and intermediate states in heme proteins and enzymes. The theory and practice of resonance Raman on heme proteins has developed into a major research field, since the pioneering work of Spiro and many others[73-75]. Several comprehensive reviews on this subject have been written[1, 71, 72, 76].

Absorption spectra of heme proteins (Figure 8) consists of an intense near ultraviolet band at 400-450nm, normally called the Soret or B band and a weak Q band in the visible region around 550-600nm. The B and the Q bands both arise from \( \pi-\pi^* \) transitions and can be explained by considering the four frontier orbitals (HOMO and LUMO orbitals). These bands tend to vary considerably with slight variation in the core of the heme (Fe atom) such as spin and oxidation states and substituents on the porphyrin ring.
Figure 8: Absorption Spectra of Human Hemoglobin at pH 7.4
Excitation in the Soret region leads to large enhancement of totally symmetric vibrations of the heme which can be observed at 1360 cm\(^{-1}\) and 674 cm\(^{-1}\). The enhancement is attributed to the A-type mechanism and the polarized enhanced vibrations. Whereas excitation in the Q region also produces enhanced vibrations, the Raman bands are both polarized and depolarized. This excitation results in a B type mechanism which involves the mixing of electronic states through the vibrational transition.

Detection of resonance Raman signals generated by the intermediates from the reaction of hydrogen peroxide with heme proteins can be explained using the Gouterman orbital scheme. The four-orbital scheme of Gouterman\(^\text{[77]}\) indicates electron removal from a porphyrin macrocycle to form a porphyrin π-cation radical which occurs from either of two highest occupied molecular orbitals \(a_{1u}\) or \(a_{2u}\) (Figure 9). Heme proteins produce electronic spectra in the visible and near ultraviolet regions that are the result of two \(\pi - \pi^*\) transitions of the aromatic porphyrin ring. Under \(D_{4h}\) point group symmetry, the highest filled orbital pair is split into \(a_{1u}\) or \(a_{2u}\) orbitals of slightly different energies\(^\text{[78]}\). However, the lowest unfilled pair of orbitals \(e_g\) remains degenerate. The electronic transition corresponding to \(a_{1u} \rightarrow e_g\) and \(a_{2u} \rightarrow e_g\) transitions which are polarized in the plane of the heme, are of the \(E_u\) symmetry due to the strong interaction. Some of the key features of metalloporphyrin resonance Raman spectra involve bands sensitive to spin state, coordination number, oxidation state, and the nature of the axial ligands\(^\text{[78]}\).
Figure 9: Orbital Diagrams Showing Possible Transitions for Porphyrins[77]
Applications of resonance Raman spectroscopy to the structural dynamics of myoglobin have been described by researchers including Rousseau and Friedman [72], Spiro and coworkers [79], and Kitagawa’s group [80]. In those studies, attention was focused on frequencies, so called marker modes, that describe the structure and oxidation state of the heme [81]. The nature of these frequency structure-sensitive modes has been elucidated by isotopic labeling studies and normal mode calculations [30] that catalogued the skeletal modes and saw an existence pattern of structure-dependent frequencies for both synthetic and heme porphyrin. A key step in gaining deeper understanding of the nature of these modes, i.e. to establish which particular molecular fragments participate in a given normal mode, is to test the sensitivity of the given modes to selective isotopic labeling, deuteration being the most accessible form of labeling.
Chapter 2: Experimental Design

Experimental data were generated from the resonance Raman spectroscopy of compound II intermediates formed from pH-varied heme proteins (Mb, Hb, and SBP) and peroxides: $\text{H}_2\text{O}_2$ and $\text{D}_2\text{O}_2$. Isotopically labeled $\text{H}_2\text{O}_2$ was prepared by modifying $\text{H}_2\text{O}_2$ preparation protocols[82, 83]. The proteins were purified and their activity tested periodically as required by the specific protein preparation procedure. Raman analyses were done on the protein samples at varying pH were performed by buffering the protein samples in these buffers: 0.01M sodium acetate, 0.01M MOPS, 0.01M MES, 0.01M tris acetate, 0.01M sodium borate, and 0.01M sodium carbonate for pH 4-5, 6-7, 8-8.5, 9.0, 10 and 11.5 respectively. Visible absorption spectrum of each protein was obtained using HP8452A diode array spectrophotometer at 280nm for protein content, with the heme (Mb and Hb) content of 409nm and SBP content of 403nm.

2.1. Protein Purification

Myoglobin and hemoglobin were purchased from Sigma. Myoglobin was used as is but hemoglobin was purified before use to reduce fluorescence which occurs during Raman data collection. The Whatman protocol for DEAE and carboxymethyl (CM)-cellulose ion exchange was used for Hb purification. SBP was purchased from the local farmers market. SBP was purified using various chromatographic steps. Proteins were centrifuged on a Sorvall RC-5B refrigerated super speed centrifuge by Dupont Instruments.
2.1.1. Hemoglobin

Lyophilized horse hemoglobin was purchased from Sigma and purified using DEAE-cellulose (Peterson and Sober 1962)[84] and CM-cellulose (Schroeder and Huisman 1976)[32]. DEAE-cellulose ion exchanger was washed in water to remove fine particulates. The slurry was suspended in NaOH and HCl and water flushed through to bring the slurry to neutral pH. DEAE-cellulose columns (30 X 0.9 cm) were equilibrated at room temperature with 0.01M Tris-HCl buffer at pH 7.9. Approximately 60 mg of hemoglobin, after overnight dialysis against the same buffer at 4°C, were loaded onto the column. The pH gradient for elution was produced by passing 500ml 0.01M to 0.1M Tris-HCl buffer with 0.1M NaCl over constant mixing with the column flow rate of 12 to 20 ml per hour.

Likewise, 50g of CM-cellulose (CM-52, microgranular and pre-swollen; Whatman, Clifton, NJ., U.S.A.) was washed in water to remove fine particulates and the slurry suspended in HCl and NaOH. Water was then flushed through to bring the slurry to neutral pH. The slurry was poured into a 30 X 0.9 cm column and equilibrated with 0.03M Bis-tris-HCl with 0.03M NaCl at pH 6.1 for several hours. The protein was loaded onto the column and eluted with a linear gradient from a two Erlenmeyer flasks. Flask 1 contained 500ml of 0.03M Bis-tris-HCl with 0.03M NaCl at pH 6.1. Flask 2 contained 0.03M Bis-tris-HCl with 0.12M NaCl. As flasks 1 and 2 were continuously mixed, pH gradient for elution was established with a column flow rate of 50ml per min. Different fractions of the eluted protein were collected.

The absorbance of each fraction was measured at 280nm and 410nm and the Reinheitzahl (Rz) value (A\textsubscript{410}/A\textsubscript{280} nm) of the fractions calculated. Fractions with Rz values exceeding 2 were pooled. The purified protein was concentrated using Amicon
ultra filtration set up. Sample concentration was estimated using the molar absorptivity for methemoglobin at 500nm ($\varepsilon_{500} = 9.04\text{mM}^{-1}\text{cm}^{-1}$).

### 2.1.2. Soybean Peroxidase

Soybean seeds were purchased from the local farmers market (Ellenwood, Richmond VA). Soybean coatings were obtained by soaking whole soybean seeds in distilled water for sometime till the coatings were wrinkled. The coatings were removed from the seeds and dried. The soybean seeds were pulverized in liquid nitrogen using mortar and pestle. The pulverized seeds coatings were dissolved in a 5mM tris-Acetate buffer at pH 8.4 overnight. The soybean extract was collected by filtering the mixture through two layers of cloth and the filtrate centrifuged (10,000rpm, 10min). The residue was reextracted via pulverizing, filtering and centrifuging. The centrifugates were concentrated by ultrafiltration on Amicon with 10KDa molecular weight cutoff membrane. The crude extract (centrifugates) were pooled and assayed for peroxidases activity and protein content. The resulting concentrate was brought to 45% saturation with solid ammonium sulfate and allowed to stand overnight in a refrigerator. The concentrate was centrifuged and the centrifugate was brought to 80% saturation with solid ammonium sulphate. The precipitate from ammonium sulphate, which is the soybean peroxidase, was dissolved in 5mM Tris-Acetate, pH 8.4 ($R_Z = 0.04$).

SBP was first purified as described by Shannon et al and Barrett et al [17-19]. The peroxidase was first purified by DEAE–Sepharose ion exchange chromatography. The DEAE-Sepharose column was equilibrated with 5mM Tris-acetate buffer, pH 8.4. Soybean extract was loaded onto the column and washed with the equilibrating buffer at
a flow rate of 100mL/h. The retained protein was eluted at the same flow rate using a linear 250ml gradient of 0.0-0.5 M NaCl in the same buffer. Fractions of 50 mL were collected; the protein and heme absorbance was monitored at 280 nm and 403nm respectively. Fractions from the eluted DEAE-Sepharose column that showed high peroxidase activity were pooled and concentrated using Amicon stirred ultrafiltration cells with 10 KDa molecular weight cutoff membranes. The DEAE-Sepharose column was regenerated and reequilibrated for reuse. The UV-vis absorption spectra of the main fractions pooled after the DEAE–Sepharose chromatography portrayed a significant change in the Rz value from 0.00-0.5.

The fractions were concentrated and dialyzed against 10mM sodium acetate buffer, pH 4.7. The sample was loaded onto a column packed with CM column which was previously equilibrated with 10mM sodium acetate, pH 4.7. The sample was then washed with the equilibrating buffer. The retained protein was eluted at the same flow rate using a linear 250ml gradient of 0.0-0.5 M NaCl in the above buffer. Fractions of 50 mL were collected and their protein content analyzed. All chromatographic steps were performed at temperatures of 4-5°C. The Rz value shown by the UV-vis absorption spectra recorded on a HP-8452A diode array spectrophotometer increased to 1.5.

Peroxidases underwent further purification using Concavalin-A (Con-A) Sepharose and Phenyl Sepharose to help curb the fluorescence in the protein. Con-A Sepharose column was equilibrated and the SBP was concentrated on the Amicon ultrafiltration with 20mM tris-HCl buffer at pH 7.4. SBP samples were loaded on to the column and eluted with 0.5M α-D-methylmannoside. Fractions collected were dialyzed.
in 5mM sodium citrate buffer pH 6.0 with 0.5M ammonium sulphate. The phenyl sepharose column was equilibrated in the same buffer.

The Bradford method[85, 86] was used to quantify the concentration of the peroxidase using bovine serum albumin as standard. In this method, the binding of the Coomassie brilliant blue dye to protein caused a shift in the absorption maximum of the dye from 465 to 595 nm, and the increase in absorption at 595 nm was monitored. A standard calibration curve was generated using triplicate assays of different concentrations (0-15uM) of bovine serum albumin. The concentration of the peroxidase was estimated from the calibration curve.

Peroxidase activity was determined by the change in absorbance at 510 nm due to 4-aminoantipyrine (4-AAP) and phenol oxidation in the presence of H$_2$O$_2$, at 25 °C as described by Bewtra et al[87]. The reaction mixture consisted of 100µL of 100mM phenol, 250µL of 9.6mM 4-AAP, 100µL of 2 mM H$_2$O$_2$, 450-500µL of 10 mM potassium phosphate buffer, pH7.4 and 50- 100 µL of enzyme. The rate of reaction was measured by monitoring the rate of formation of the products at 510nm. Enzyme activity was estimated with molar absorptivity of 4-AAP at 6000M$^{-1}$cm$^{-1}$ at pH 7.4 and 25°C. Table 2 shows the summary of purification procedure for pooled SBP fractions. The precipitation of the crude extract with 45-80% ammonium sulfate saturation increased the peroxidases purity and the column chromatography step helped improved the purity of Rz = 0.05 to Rz =4.0.
Purity and molecular weight of the purified protein was analyzed using SDS-polyacrylamide gel electrophoresis under reducing conditions. The NuPAGE® Bis-Tris Electrophoresis System is a discontinuous SDS-PAGE, pre-cast polyacrylamide gel system at neutral pH placed in an invitrogen mini cell from Invitrogen, Carlsbad, CA. This was conducted using a Mini-Protean II system from Bio-Rad Laboratories, Hercules, CA with runs performed at constant current (30mA/plate). The following molecular weight markers used for electrophoresis were obtained from Bio-Rad: myosine (200 kDa), α-galactosidase (116 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and

<table>
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<th>Sample</th>
<th>Enzyme activity (units/mL)</th>
<th>Total Protein (mg/mL)</th>
<th>Specific Activity (units/mg)</th>
<th>$R_z = \frac{A_{403}}{A_{280}}$</th>
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Table 2: Soybean Peroxidase Purification
aprotinin (6.5kDa). Protein bands were stained with Coomassie brilliant blue R250. After staining and destaining, the gel showed a single band with it molecular masses at 40kDa.

The purity of SBP was examined spectroscopically by monitoring the changes in absorbance in the Soret and visible regions. Spectra were collected digitally with HP8453 diode array spectrophotometer equipped with a thermostatically controlled cuvette holder using a cuvette with 1cm path length. The ferryl (FeIV) intermediates of soybean peroxidase formed by reacting peroxidase (Fe^{3+}) with peroxide. All assays were performed with 2.0mL final volume of the cuvette. Figure 10 shows a typical SBP spectrum and its intermediates as SBP reacts with peroxides with a Soret band shifting from 403nm to 414nm for soybean peroxidase. Simultaneously, the visible region saw new bands at 548 and 584nm accompanied by a decrease of the 503 and 631 bands of ferric SBP. These characteristic bands show the formation of the ferryl species.
Figure 10: Absorption spectra of 10 uM Soybean peroxidase in 10mM MOPS buffer at pH 7.4 with Hydrogen peroxide (1:5 molar ratio)
2.2. Deuterium Peroxide Preparation

Isotopic labeled hydrogen peroxide in aqueous solution is frequently used in the study of the catalytic decomposition mechanisms of various enzymes. A number of syntheses for preparing hydrogen peroxide such as autoxidation method, heating method and the industrial preparation procedure were examined. In the autoxidation method, deuterium peroxide is prepared by placing a small aliquot of unlabeled hydrogen peroxide in deuterium oxide and allowing sufficient time (2-7 days) for the exchange to occur. Engdahl et al[88] have also described how deuterium peroxide can be prepared using 30% hydrogen peroxide solution and deuterium oxide. In this approach, “the heating method”, hydrogen peroxide is first concentrated by slow evaporation at about 40°C until it is reduced to about one-third its initial volume. The concentrated hydrogen peroxide is diluted to its original volume with 99.9% deuterium oxide (D₂O) and evaporated to about half the initial volume. This step is repeated (dilution and evaporation).

A modified hydrogen peroxide preparation procedure was used to prepare deuterium peroxide. Several hydrogen peroxide preparation methods were considered. The criteria used in selecting a procedure are the concentration and purity of the hydrogen peroxide produced. In this study, the industrial method was selected as it generated “reasonably” high concentrated hydrogen peroxide with the least impurity. The procedure consists of catalytic hydrogenation of anthraquinone and autoxidation of anthraquinol as described by Sitter et al[89] and first introduced by Riedl and Pfleiderer[82]. Hydrogen peroxide production involves the sequential hydrogenation and oxidation of an alkylanthaquinone precursor dissolved in a mixture of organic solvents.
followed by liquid extraction to recover the \( \text{H}_2\text{O}_2 \). Deuterium peroxide is prepared through the catalytic deuteration of 2-ethylantraquinone as shown in equations 5 and 6. This method is a modification of the procedure for preparing hydrogen peroxide. In the modification, hydrogen is replaced with deuterium in the hydrogenation step and oxidized in air to produce deuterium peroxide which is extracted and purified.

A 6% solution of 2-ethylanthraquinone in 1-decanol, with 10% palladium on alumina, is deuterated for 12 hours in a catalytic hydrogenation setup (Parr Corp., Moline, IL) at room temperature under 2atm deuterium gas. Upon completion of the reaction, which is evident by a change in color from yellow to fluorescent green, the mixture is quickly removed from the catalytic hydrogenation setup and placed in a glove bag. The mixture is then filtered through a fritted glass funnel to remove the palladium catalyst, which might cause decomposition of the peroxide. The resulting solution of 2-ethyl anthrahydroquinol is poured into the Erlenmeyer flask. The autoxidation step is
accomplished by adding oxygen into the flask and stirring for 60 minutes in the air proceeding through a brown intermediate. Once the reaction mixture returned to the bright yellow color of 2-ethylanthraquinone in solution, the mixture was then transferred to a separatory funnel. The deuterium peroxide (D₂O₂) sample was extracted from the decanol-2-ethylanthraquinone mixture with four equal volumes of chilled (4°C) high purity D₂O. The aqueous extract was purified in a rotary evaporator (Buchi rotavap R-200) for efficient and gentle evaporation of the solvent. The samples were characterized using FTIR (Nicolet 670 ESP) and titration methods. FTIR was used to investigate and identify the functional groups of the deuterated sample. Titration of the deuterium peroxide produced with 0.01M permanganate was used to determine the concentration of D₂O₂ based on the volume of KMnO₄ used to reach the endpoint.

2.2.1. D₂O₂ Results

The spectra of deuterium peroxide samples were analyzed using spectroscopic techniques such as FTIR and titrimetric methods using potassium permanganate. Spectroscopy and titrimetry respectively, provide instrumental and non-instrumental means of characterizing hydrogen peroxide/deuterium peroxide. From the titrimetric analyses, the percent composition of hydrogen peroxide/deuterium peroxide is estimated with 0.00961M solution of KMnO₄. KMnO₄ solution was standardized with 0.1M Oxalic acid. The concentrations of D₂O₂ samples are shown in Table 3. D₂O₂ results using Engdahl’s method[88] (heated D₂O₂) yielded high concentration as compared to that of the industrial production procedure which is also called catalytic hydrogenation. However, the infrared spectra showed the presence of H₂O₂ impurities in the D₂O₂
samples produced with the heating method. Thus for much better deuterium samples with fewer impurities, the catalytic hydrogenation method is preferred.

### Table 3: $\text{D}_2\text{O}_2$ Concentrations from Different Preparation Procedures

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Volume of $\text{D}_2\text{O}_2$ (L)</th>
<th>Average Volume of KMnO$_4$ used (ml)</th>
<th>Mole of $\text{D}_2\text{O}_2$ (moles)</th>
<th>Concentration of $\text{D}_2\text{O}_2$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated $\text{H}_2\text{O}_2$</td>
<td>$6 \times 10^{-4}$</td>
<td>14.4</td>
<td>$3.46 \times 10^{-4}$</td>
<td>0.577</td>
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<tr>
<td>Heated $\text{H}_2\text{O}_2$ and Rotavap</td>
<td>$6 \times 10^{-4}$</td>
<td>19.2</td>
<td>$4.61 \times 10^{-4}$</td>
<td>0.969</td>
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<tr>
<td>$\text{D}_2\text{O}_2$</td>
<td>$6 \times 10^{-4}$</td>
<td>4.27</td>
<td>$1.03 \times 10^{-4}$</td>
<td>0.171</td>
</tr>
<tr>
<td>$\text{D}_2\text{O}_2$ Rotavap</td>
<td>$6 \times 10^{-4}$</td>
<td>7.37</td>
<td>$1.77 \times 10^{-4}$</td>
<td>0.295</td>
</tr>
<tr>
<td>Deuterated $\text{D}_2\text{O}_2$ and Rotavap</td>
<td>$6 \times 10^{-4}$</td>
<td>11.57</td>
<td>$2.78 \times 10^{-4}$</td>
<td>0.863</td>
</tr>
</tbody>
</table>

To increase the concentration of the deuterium peroxide made from catalytic hydrogenation, the decanol was deuterated before use. Deuteration of 1-decanol ($\text{C}_{10}\text{H}_{21}\text{OD}$) was prepared by mixing 100ml of 1-decanol and 10mL of deuterium oxide ($\text{D}_2\text{O}$). The mixture was then placed in a round bottom flask and heated with 10$\mu$L of concentrated HCl on a sand bath with boiling chips to ensure smooth and efficient boiling. After boiling for 30 minutes, the solution was cooled to room temperature. An additional 10ml of $\text{D}_2\text{O}$ was added to the mixture and heated; this procedure was repeated several times. Finally, the mixture was cooled for an hour and the organic phase was extracted using a separatory funnel. The H-NMR spectra of the deuterated decanol was obtained and compared with the H-NMR spectra of original 1-decanol. The spectra of the
deuterated decanol shows the absence of terminal hydrogen, confirming the OH group on the decanol deuterated. The deuterated decanol was used in the preparation of deuterium peroxide. Table 3 shows an increase in D₂O₂ concentration with deuterated decanol.

During the catalytic hydrogenation procedure, infrared spectroscopy was used to monitor the compounds formed. i.e., The conversion of 2-ethylanthraquinone to deter-2-ethylanthrahydroquinol and the autooxidation of 2-ethylanthrahydroquinol to ethylanthrahydroquinone. A peak at 2800 cm⁻¹ confirmed the conversion of 2-ethylanthraquinone to 2-ethylanthrahydroquinol. A peak at 1600 cm⁻¹ confirmed the autooxidation of deter-2-ethylanthrahydroquinol to ethylanthrahydroquinone.

Regions A and E in Figure 11a show strong, broad bands for both the symmetric and antisymmetric stretching at 3205.8 cm⁻¹ and 2470.1 cm⁻¹ for O-H and O-D stretching frequencies respectively. Regions B and F demonstrate O-H and O-D bending at 1633.8 cm⁻¹ and 1204.7 cm⁻¹ respectively. OOH and OOD stretching frequencies can be found at regions C and G (1371.4 cm⁻¹ and 1043.9 cm⁻¹). Region D describes the O-O stretching frequency at 800 cm⁻¹. The D₂O₂ spectra show isotopic effects with a decrease in frequencies and bandwidth in the approximate ratio of 1.35 as proposed by Giguere and Chen[83]. This characteristic band shift observed in the D₂O₂ spectra can be attributed to the fact that vibrational frequency is inversely proportional to the square root of the reduced mass implying that increasing reduced mass will result in a decrease in frequency. From Table 4, the experimental results shown have proven to be comparable to results showed by Giguere and Chen. Figure 12 reveals the differences in infrared spectra for different D₂O₂ preparation methods.
Figure 11: (A) IR Spectra of Deuterium Peroxide and Hydrogen Peroxide solution in water and deuterated water respectively. (B) IR spectra differential.

Table 4: Frequencies of H$_2$O$_2$ and D$_2$O$_2$ Molecules (cm$^{-1}$)

<table>
<thead>
<tr>
<th></th>
<th>H$_2$O$_2$ $\nu$ (cm$^{-1}$)</th>
<th>D$_2$O$_2$ $\nu$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
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<td>Vibrations</td>
<td>IR</td>
<td>Raman</td>
</tr>
<tr>
<td>A</td>
<td>O-H Stretching</td>
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</tr>
<tr>
<td>B</td>
<td>O-H Bending</td>
<td>1636</td>
</tr>
<tr>
<td>C</td>
<td>OOH Stretching</td>
<td>1377</td>
</tr>
<tr>
<td>D</td>
<td>O-O Stretching</td>
<td>735</td>
</tr>
</tbody>
</table>
2.3 Data Acquisition

2.3.1. Instrumentation

The experimental setup as shown in Figure 13 includes an excitation source using a Coherent Innova Sabre DBW krypton laser. The samples were analyzed with a transverse excitation laser with light of power less than 5 mW. Raman spectra were obtained at a wavelength of 406.7 nm. The scattered light from the sample were collected at 90° to the incident beam by a lens, focused onto a 0.5 m spectrograph fitted with 1800 line/mm holographic grating, and detected by an optical multichannel analyzer /diode array detector. The optical multichannel analyzer is capable of recording spectra at
sampling rates up to 100 kHz. The analyzer can record simultaneously the spatially distributed Raman intensity and the frequency distributed Raman spectrum with 0.01 nm or 0.12 nm resolution. The Raman band frequencies were calibrated using known frequencies of indene, fenchone and cyclohexane. The band assignments and numbering system follow modes described by Abe et al[90] and Choi et al[73, 74]. The energy difference between the initial and final levels, $\nu$, or Raman shift in wave numbers (cm$^{-1}$), is calculated as:

$$\nu = \frac{1}{\lambda_{\text{incident}}} - \frac{1}{\lambda_{\text{scattered}}}$$  
Eqn. 27

Figure 13: Experimental Setup for Resonance Raman Spectroscopy
2.3.2 Resonance Raman Spectroscopy of Heme Intermediates

Compound II intermediates were formed by mixing heme protein and H$_2$O$_2$ in H$_2$O, and D$_2$O$_2$ in D$_2$O. The D$_2$ substitution for H$_2$ could reveal the source of the substituted hydrogen on the heme and help describe the reaction mechanism of the ferryl intermediates. Raman measurements were done in a spinning quartz cylindrical cell. In order to obtain a satisfactory compound II resonance Raman spectra, the intensity of the laser were kept low. Resonance Raman spectra in the 1300 to 1700 cm$^{-1}$, 1000 to 1300 cm$^{-1}$, 600 to 900 cm$^{-1}$, and 200 to 600 cm$^{-1}$ regions for heme proteins were obtained at pH 8.7. However, the focus of this study was on the vibrational region of the resonance Raman spectra from 900 to 400 cm$^{-1}$ where the Fe$^{IV}$=O stretching frequencies for heme compound II intermediates are expected.

Protein samples were dissolved in 10mM tris acetate buffer pH 7.5. Ferryl heme proteins (Compound II) was formed by mixing one to five fold excess of D$_2$O$_2$ and 50uM ferric SBP at 20°C which results in a color change from brown to red.
Chapter 3: Determination of Ferryl Structure of Soybean Peroxidase Using Resonance Raman Spectroscopy

3.1 Overview
Resonance Raman spectra obtained with Soret band excitation is reported for compound II intermediates of the soybean peroxidase. Ferryl intermediates of soybean peroxidases were produced by 2-5 mW laser excitations with Soret absorption band at 406.7 nm. The vibrational modes of the ferryl intermediates were examined. The vibrational frequencies and depolarization data obtained for soybean peroxidases signify strong similarity between the resting state of myoglobin, hemoglobin, and horseradish peroxidase. This similarity thus imply the presence of protoporphyrin IX active site with iron III high spin five coordinated with histidine as the distal ligand.

Ferryl stretching frequencies formed from pD show an intense peak enhancement with a 3cm\(^{-1}\) up shift from 774cm\(^{-1}\) to 785cm\(^{-1}\) compared with that from hydrogen peroxide. The estimated bond distance for ferryl soybean peroxidase using Badger’s rule was significantly less than those observed by X-ray crystallographers. Thus, through deuterium sensitivity, accompanied with changes in the vibrational frequencies of the iron-ligand, we have shown that the intermediates formed during the reaction of the hemeproteins with peroxides have Fe\(^{IV}\) =O structures.
3.2 Introduction

Peroxidases have enjoyed a central position as model systems for investigating protein dynamics. The structure of the peroxidase and their intermediates has been extensively investigated\cite{34}. Peroxidase possesses wide substrate specificity and has been shown to catalyze peroxides and other organic compounds. Peroxidases play a key to understanding reaction mechanisms in heme proteins as catalyst in the oxidation of a wide variety of substrates, using $\text{H}_2\text{O}_2$ or other peroxides.\cite{1, 25, 29}

It has been suggested over the past fifty years that ferryl species contain an oxo-ferryl complex among other possibilities, consisting of an iron in the $+4$ state (Fe$^{\text{IV}}$=O). This structure has been confirmed by studies such as Mossbauer\cite{6, 91}, resonance Raman, EXAFS\cite{92}, NMR\cite{93}, DFT\cite{94} calculations and X-ray\cite{26, 27, 95} crystallography\cite{1}. However, recent theoretical and experimental findings have questioned the oxo-ferryl nature of compound II in heme proteins suggesting that compound II intermediate could be protonated\cite{94}.

Motivated by the recent debate on the subject of protonation of compound II in heme proteins, this chapter discusses the use of resonance Raman spectroscopy to investigate the changes in the structure and environment of soybean peroxidase, as it reacts with hydrogen and deuterium peroxide and compares the ferryl SBP vibrational frequencies with other heme proteins. Resonance Raman spectroscopy, is a means of characterizing the active site structure of heme proteins. Resonance Raman spectroscopy was used to help characterize the axial ligation of compound II. Spectral frequencies in the 200-1700 cm$^{-1}$ range gave vital information on the iron oxidation state, spin state and heme core size. The difference in vibrational stretching frequencies and bond energies as the hydrogen peroxide substrate was substituted with its isotope deuterium was used to
determine the ligand at the sixth position. Heme skeletal vibrational frequency modes were assigned based on resonance Raman spectral catalogue provided by Kitagawa et al[80], Abe et al[90] and Choi et al[69] for heme proteins. Assignment of the metal-ligand vibrations provides additional experimental evidence to the authenticity of the oxo nature of compound II ferryl complex.

3.3 Experimental
SBP samples were obtained and purified as described in section 2.1. The protein samples were concentrated by ultrafiltration and suspended in 0.01 M tris-acetate buffer at optimal pH: pH 8.4 for myoglobin, and pH 7.5 soybean peroxidases. Deuteration experiments were done using ultrafiltered protein samples suspended in 10mM deuterated buffers. Compound II species were produced by reacting the peroxidase with hydrogen peroxide or deuterium peroxide. The experimental (resonance Raman) setup and conditions for this study are presented in section 2.3. Protein sample concentrations ranged from 50 to 100 µM.

30% H$_2$O$_2$ was purchased from Sigma. As illustrated in section 2.2, deuterium peroxide was produced by the sequential deuteration and oxidation of alkylanthaquinone dissolved in deuterated decanol. The D$_2$O$_2$ samples were extracted from the deuterated decanol and 2-ethylanthraquinone solution with chilled 99.9% D$_2$O. Rotary evaporator (Buchi rotavap R-200) was used to concentrate D$_2$O$_2$ by evaporating the solvent from the aqueous extract. The purified sample was characterized using FTIR (Nicolet 670 ESP) and titration methods. FTIR was used to investigate and identify the functional groups of
the deuterated sample. Titration with 0.01M KMnO₄ was used to determine the concentration of the deuterium peroxide produced.

Samples were placed in 0.01M tris-acetate buffer for the met heme protein measurements. Ferryl heme was prepared by mixing hydrogen peroxide and 50 µM hemeprotein in 0.01M tris-acetate buffer at optimal pH. Spectrophotometric measurements of the protein were performed using a HP8452A diode array spectrophotometer to evaluate the protein concentration and determine the optimal hydrogen peroxide needed for the ferryl conversion. Ferryl formation resulted in a shift in the Soret and visible regions. Heme concentrations were estimated using the molar absorptivity at 409 nm (ε=188 mM⁻¹cm⁻¹) for myoglobin and hemoglobin, and 403 nm (ε= 90 mM⁻¹cm⁻¹) for soybean peroxidase. Ferryl species were generated from buffered Mb and Hb at pD 8.8 and buffered SBP at pD 7.9 with 0.01 M sodium acetate and deuterated acetic acid. Each ferryl heme Raman spectrum was collected by rapid mixing of ferric heme with peroxides in a spinning quartz cylindrical cell. The pH and pD of the protein solutions were measured with Orion pH meter model 420 A. The pD values for deuterated samples were calculated by adding 0.4 to the apparent pH. i.e. pD = pH_{apparent} + 0.4.[62]

### 3.4 Results

#### 3.4.1 Soybean Peroxidase

Figure 14 illustrates spectra of SBP in parallel and perpendicular polarization. Polarized SBP spectra were obtained by placing a polarizer between the analyzer and in front of the spectrograph. The depolarization ratio \( \rho = \frac{I_{\text{parallel}}}{I_{\text{perpendicular}}} \) of SBP was
measured at pH 7.5. The depolarization ratio was used to determine the symmetry of the vibrational modes in the resonance Raman spectrum. Resonance Raman spectra were recorded for both the hydrogenated and deuterated samples. For the hydrogenation experiment, ferric soybean peroxidase was buffered in H$_2$O and its ferryl myoglobin formed with H$_2$O$_2$. In the deuteration experiment, ferric soybean peroxidase was buffered in D$_2$O and its ferryl formed with D$_2$O$_2$. Figure 15 compares a Soret excitation at 406.7 nm of ferryl SBP in H$_2$O$_2$ and ferryl SBP in D$_2$O$_2$. Table 5 illustrates vibrational modes assignment for Figure 15 in the high frequency regions while Table 6 lists vibrational modes assignment for Figures 16 in low frequency regions. Tables 5 and 6 are summaries of frequencies, polarizations data, relative intensities of the bands and the symmetry species for each mode. The vibrational mode assignments were made in reference to the normal mode description of NiOEP based on the depolarization ratios and isotopic shifts [62, 74, 75, 96, 97].
Figure 14: Parallel and perpendicular polarized resonance Raman spectra of Ferric Soybean Peroxidase pH 7.5 at Soret excitation of 406.7nm
Figure 15: Resonance Raman Spectra of 50 μM Soybean Peroxidase using 406.7 nm Excitation
Table 5: Frequencies, Polarizations and Assignments of Resonance Raman Bands in High Frequency Region for SBP

<table>
<thead>
<tr>
<th>Assignment Mode Symmetry</th>
<th>Polarization Assignment</th>
<th>Met SBP in H$_2$O (cm$^{-1}$)</th>
<th>Ferryl SBP with H$_2$O$_2$ (cm$^{-1}$)</th>
<th>Ferryl SBP with D$_2$O$_2$ (cm$^{-1}$)</th>
<th>Mode Composition</th>
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</thead>
<tbody>
<tr>
<td>$v_{10}$ $B_{1g}$</td>
<td>dp</td>
<td>1632</td>
<td>1632</td>
<td>1633</td>
<td>C$\alpha$-C$_m$</td>
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<tr>
<td>$v_{37}$ $E_u$</td>
<td>p</td>
<td></td>
<td>1599</td>
<td>1599</td>
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<tr>
<td>$v_{2}$ $A_{1g}$</td>
<td>p</td>
<td>1573</td>
<td>1583</td>
<td>1584</td>
<td>C$\beta$-C$\beta$</td>
</tr>
<tr>
<td>$v_{11}$ $B_{1g}$</td>
<td>dp</td>
<td>1546</td>
<td>1560</td>
<td>1560</td>
<td>C$\beta$-C$\beta$</td>
</tr>
<tr>
<td>$v_{3}$ $A_{1g}$</td>
<td>p</td>
<td>1503</td>
<td>1508</td>
<td>1509</td>
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<td>-</td>
<td>C$\alpha$-C$_m$</td>
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<tr>
<td>$\delta$$_s$-CH$_2$(1)</td>
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<td>dp</td>
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<td>$v_{14}$ $A_{1g}$</td>
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<td>1181</td>
<td>1180</td>
<td>1181</td>
<td>C$\beta$-vinyl</td>
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</table>
Figure 16: Lower Frequency Region of Resonance Raman Spectra of 50 uM Soybean Peroxidase using 406.7 nm Excitation
Table 6: Frequencies, Polarization and Assignments of SBP Resonance Raman Bands at Lower Frequency Region

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Symmetry</th>
<th>Polarization</th>
<th>Met SBP in H$_2$O (cm$^{-1}$)</th>
<th>Ferryl SBP in H$_2$O$_2$ (cm$^{-1}$)</th>
<th>Met SBP in D$_2$O (cm$^{-1}$)</th>
<th>Ferryl SBP in D$_2$O$_2$ (cm$^{-1}$)</th>
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<tbody>
<tr>
<td>$\gamma_{CH=}$</td>
<td>-</td>
<td>dp</td>
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<td></td>
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<tr>
<td>$v_{FeIV=O}$</td>
<td>-</td>
<td>p</td>
<td>-</td>
<td>772</td>
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</tr>
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<tr>
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<td>p</td>
<td>715</td>
<td>711</td>
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<tr>
<td>$2v_{8}$</td>
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<td>$v_{49}$</td>
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<td>-</td>
<td>590</td>
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<td>594</td>
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</table>
Figure 15 shows a typical transition of the iron core from Fe$^{III}$ to Fe$^{IV}$ resulting in high frequency shifts for both ferryl SBP formed in H$_2$O$_2$ and D$_2$O$_2$. Resonance Raman spectra for ferric SBP, ferryl SBP buffered in H$_2$O and ferryl SBP buffered in D$_2$O show series of bands in 1000-1750 cm$^{-1}$ (Figure 15). This region was assigned to the porphyrin macrocyclic vibrational modes whose frequencies are dependent upon the spin state of the heme iron. The oxidation state marker polarized band ($\nu_4$) was seen at 1373 cm$^{-1}$, which shifted up to 1379 cm$^{-1}$ upon reacting with deuterium peroxide; this is the most intense mode below 1450 cm$^{-1}$. The $\nu_4$ mode is mainly dependent on the Ca-N stretching which in turn depends on the polarization of the pyrrole. The Raman spectrum of ferric SBP (region above 1450 cm$^{-1}$) shown in Figure 15 has a single $\nu_2$ vibration at 1573 cm$^{-1}$, indicative of an iron (III) heme. These spectra exhibited a reduced band at 1573 cm$^{-1}$ and a new polarized band at 1584 cm$^{-1}$, depicting a well characterized spin transition from Fe$^{III}$ to Fe$^{IV}$. The depolarized bands at 1632 cm$^{-1}$ ($\nu_{10}$) and 1560 cm$^{-1}$ ($\nu_{11}$) result from the low spin iron species. The ferryl spectra in both hydrogen and deuterium peroxide-induced sample show considerable changes in the 1400 to 1650 cm$^{-1}$ range, as the ferric SBP clearly transitions from FeII$^{III}$ to FeIV. Using the spectral assignments (Table 5) derived from Abe et al[90], Choi et al[73], Spiro et al[69] and Sitter et al[97],

Figure 16 shows Soret band excited resonance Raman spectra below 1000 cm$^{-1}$ for ferric SBP, ferryl SBP formed from H$_2$O$_2$ and D$_2$O$_2$. The band assignments presented in Table 5 are based on the spectral catalogs by Abe, Spiro, Choi and Kitagawa. Resonance Raman enhancement in this region are less intense since these modes involve ring deformation and bond stretching. However, the region is of much interest because it depicts the chemical changes (interactions) at the heme. Although the bands are relatively
weak (compared with those in the high frequency region), a new broad band between 773 and 785 cm\(^{-1}\) was observed in the ferryl SBP spectra. The spectra of the ferryl formed with deuterium peroxide showed a 3 cm\(^{-1}\) upshift \(\nu_{\text{Fe}^{\text{IV}}=\text{O}}\) stretching frequency at 774 and 788 cm\(^{-1}\). Although, the stretching frequency of the ferryl SBP compound II was lower than that of O=Fe(IV)TPP proposed by Badjor and Nakamoto\[58\]. The weak stretching frequency in SBP just like other heme proteins is due to the influences from the protein environment as compared to synthetic ferryl models. The vibrational frequency of the ferryl SBP within 773-785 cm\(^{-1}\) was considerably higher than the stretching frequency of the ferriperoxidase hydroxide at 505 cm\(^{-1}\) and oxyperoxidase at 550 cm\(^{-1}\)[98, 99].

3.5. Discussion

Resonance Raman spectra for the ferryl group in the oxidative reaction of peroxide and the heme were analyzed. The oxidant was mixed with buffered heme of known concentration at specific pH 7.5 and pD 7.9 for \(\text{H}_2\text{O}_2\) and \(\text{D}_2\text{O}_2\) experiments respectively. Resonance Raman spectra were taken in the range 1750-250 cm\(^{-1}\). Figures 15-16 describe the resonance Raman spectra of ferric SBP and SBP compound II formed with \(\text{H}_2\text{O}_2\) and \(\text{D}_2\text{O}_2\) in vibrational region between 1750 to 1000 cm\(^{-1}\) and 1000 to 400 cm\(^{-1}\). The high frequency regions (1000-1750 cm\(^{-1}\)) revealed the marker bands of the heme core size and the electron density in the porphyrin \(\pi\) orbitals which are useful in monitoring the oxidation and spin states of the heme. Heme proteins spectra in this region displayed strongly enhanced Raman bands dominated by the Soret electronic transitions which arise from in plane ring modes coupled to \(\pi-\pi^*\) excitations. Although, those in the
lower frequency region saw less intense marker modes, they provide information that
occurs within the heme pocket such as protein interactions, chemical changes at the heme
and the type of ligand at the sixth position. Cataloging of the skeletal modes for
vibrations revealed the frequency dependence on the heme structure and the structural
correlations with the porphyrin core size.

The resonance Raman spectra of the soybean peroxidase in the region 1750 to
250 cm\(^{-1}\) showed transition of the iron core from Fe\(^{III}\) to Fe\(^{IV}\) resulting in high frequency
shifts with well characterized spin marker bands. The shift in frequencies during the
transition of Fe\(^{III}\) to Fe\(^{IV}\) is attributed to the \(\pi\)-back bonding between iron and the
porphyrin ring, and the differences in the iron-ligand interactions at the sixth position. The
\(\text{D}_2\text{O}_2\)-induced spectra showed a frequency shift from 774 to 788 cm\(^{-1}\) indicating some
similarities to those produced in H\(_2\)O\(_2\)-induced. However, from mass defect calculation,
for the hydrogen bonding to occur there should be lowering of the vibrational frequencies
but in this case there is rather an upshift. The deuteration upshift of the ferryl SBP can be
attributed to the decrease in the strength of the hydrogen bond between the ferryl –
oxygen and the Arg-38 as proposed by Sitter et al and Feis et al\([62, 100]\). Thus, our
\(\text{D}_2\text{O}_2\) experiment helps in clarifying the issue on ferryl protonation and confirming the
oxoferryl nature of heme compound II intermediates,

Resonance Raman measurements performed on SBP compound II intermediates
show Fe-O stretching frequencies ranging from 773 - 787 cm\(^{-1}\). The vibrational stretching
modes were assigned for ferriperoxidase hydroxide (\(\nu\text{Fe}^{III}\)-OH) at 505 cm\(^{-1}\) and
oxyperoxidase (Fe\(^{II}\)-O\(_2\)) at 550 cm\(^{-1}\) while the Fe\(^{IV}\)=O modes have been assigned values
higher than 700 cm\(^{-1}\) for most heme proteins. Due to the distinct difference in frequencies
between the intermediates, deuterium experiment can best trace the protons linked with
the intermediates. Hence deuteration substitution is a better approach than hydrogen for
examining the structure of the ligand at the sixth position.

The ferryl oxo frequencies observed in the heme compound II intermediates as
shown in Table 7 depict the effect of isotopic substitution effects on the vibrational
frequencies of the protein. A similar blue shift has been observed in synthetic ferryl
porphyrin with vibrational frequencies at 807-852cm\(^{-1}\) upon reacting with hydrogen
peroxide, whereas the \(^{18}\text{O}\) substitution yielded a red shift. However, the ferryl vibrational
frequency for the hemeproteins does occur at lower frequencies. The lowering of the
ferryl frequencies compared to their synthetic counterparts thus show the variations in the
strength of the hydrogen bonding or intermolecular interactions that exist between the
ferryl group and the protein environment.
### Table 7: Comparison of Ferryl Frequencies obtained from Isotopic Substitution

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\text{H}_2\text{O}_2$ (cm$^{-1}$)</th>
<th>$\text{D}_2\text{O}_2$ (cm$^{-1}$)</th>
<th>$\text{H}_2^{18}\text{O}_2$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mb</td>
<td>797</td>
<td>794-806</td>
<td>771</td>
</tr>
<tr>
<td>Hb</td>
<td>805</td>
<td>807</td>
<td>761</td>
</tr>
<tr>
<td>SBP</td>
<td>773</td>
<td>775</td>
<td></td>
</tr>
<tr>
<td></td>
<td>785</td>
<td>787</td>
<td>-</td>
</tr>
<tr>
<td>$^a$HRP-C</td>
<td>789</td>
<td>790</td>
<td>756</td>
</tr>
<tr>
<td>$^a$Synthetic (O=Fe$^{IV}$TPP)</td>
<td>852</td>
<td>-</td>
<td>818</td>
</tr>
</tbody>
</table>

(a denotes data adapted from Terner et al[1])

### Table 8: Comparison of Vibrational Frequencies of Heme Proteins

<table>
<thead>
<tr>
<th>Proteins</th>
<th>pD</th>
<th>Distal Ligand</th>
<th>Resting State (cm$^{-1}$)</th>
<th>Ferryl (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\nu_4$, $\nu_2$, $\nu_{10}$</td>
<td>$\nu_4$, $\nu_2$, $\nu_{10}$, $\nu_{\text{Fe}^{IV}=\text{O}}$</td>
</tr>
<tr>
<td>Mb</td>
<td>8.8</td>
<td>Phe, His, Val</td>
<td>1372, 1565, 1620</td>
<td>1381, 1642, 1589</td>
</tr>
<tr>
<td>Hb</td>
<td>7.8</td>
<td>Phe, His, Val</td>
<td>1373, 1564, 1621</td>
<td>1382, 1643, 1582</td>
</tr>
<tr>
<td>SBP</td>
<td>7.9</td>
<td>Phe, His, Arg</td>
<td>1374, 1573, 1633</td>
<td>1379, 1584, 1633</td>
</tr>
<tr>
<td>$^a$HRP-C</td>
<td>7.4</td>
<td>Phe, His, Arg</td>
<td>1372, 1575, 1631</td>
<td>1379, 1602, 1639</td>
</tr>
</tbody>
</table>

(a denotes data adapted from Terner et al[1])
Table 8 is a summary of the changes in vibrational frequencies as heme proteins migrate from its resting state through the ferryl and back with the help of an oxidizable substrate. The vibrational frequencies for the resting hemeproteins do indicate some similarities among the globins and peroxidases. This can be attributed to the fact all these hemeproteins have an iron protoporphyrin core with the iron in the +3 state, and fifth coordinated high spin with histidine at the axial position. However, the ferryl intermediates of these proteins show some differences as they react with peroxides. The differences found between the globins and peroxidases can be explained by the differences in the proteins at the distal position. The distal ligand in the peroxidases is arginine as compared to valine in globins. During the peroxidative reaction, the distal His assumed to function as an acid/base catalyst to facilitate the heterolytic cleavage by accepting a proton from oxygen ligated to the iron and then donating it to the outer (leaving) oxygen. The negative charge on the leaving hydroxide during bond cleavage is then stabilized by the distal Arg in HRP (not found in Mb). Yamazaki et al [101] proposed that the low peroxidative activity in myoglobin results from the weak interaction of the imidazole at the distal base with the sixth ligand unlike that of peroxidase[102]. The strength of the interaction (hydrogen bonding interaction) may explain the difference in the vibrational frequencies of the ferryl species. Site mutagenesis studies have also shown that imitating the His distance and replacing the valine with arginine does increase the peroxidative activity in myoglobin [28, 103, 104].

Using the correlations of the frequencies modes $\nu_{10}$, $\nu_2$, $\nu_{11}$ and $\nu_3$ (as illustrated in Tables 5 and 8) to porphyrin core size according to the relation $\nu = K(A-d)$ proposed by Spiro et al and Choi et al[73]. The bond distance from the porphyrin center to pyrrole
nitrogen was estimated to have an average core size of 2.01 Å for ferric SBP and 1.99 Å for ferryl SBP. This indicates the heme core size contraction which is due to the decrease in the back bonding of the heme dπ electrons to the porphyrin anti-bonding orbitals as the heme is oxidized from FeIII to FeIV. Comparing the bond distances of the porphyrin center to the pyrrole nitrogen of peroxidases which is 1.99 Å to 1.98 Å for the globins, we realized the smaller the bond distance the higher the vibrational frequency. These observations can be seen in the stretching frequencies as resting SBP converts to ferryl SBP-II and also between the peroxidases and the globins.

The equilibrium bond distances of the hemeproteins were estimated using Badger’s rule[105] as illustrated by Green et al[25]. Equation 29 shows the Badger’s rule[105] as an empirical formula that relates bond distance and vibrational frequency.

\[ r_e = \frac{C_{ij}}{\nu_e^{2/3}} + d_{ij} \]

Eqn 29

Where \( r_e \) is the equilibrium bond distance, \( \nu_e \) vibrational frequency and \( C_{ij} \) and \( d_{ij} \) are the empirical constants. The values \( C_{ij} \) and \( d_{ij} \) (55.70 and 1.003 respectively) were derived from the slope and intercept of the plot of \( r_e \) versus \( \nu_e^{2/3} \). The ferryl stretching frequencies shown for the heme proteins from resonance Raman measurements indicate ferryl proteins had bond distance of 1.65 Å for Mb (pD 8.8), 1.65 Å for Hb (pD7.8) and 1.66 Å for HRP-C (pD 7.4).

The long Fe–O bond lengths found by X-ray crystallographers for myoglobin, hemoglobin and HRP-C deviated substantially from the values estimated from the Badger’s rule. Our study confirms other studies that have suggested that the compound II
intermediate have the ferryl structure. The deuterium substitution methods used to investigate these systems have allowed direct observation of protons. In each case, the ferryl state of the protein was inferred from the measured Fe–O bond length (1.65–1.68 Å), which was deemed to be too short for a Fe-OH bond.

3.6 Conclusion

Resonance Raman measurements performed on SBP compound II intermediates from reacting heme with deuterium peroxide, show Fe-O stretching frequencies ranging from 773 to 788 cm\(^{-1}\). Due to the distinct difference in frequencies between the intermediates, deuterium experiment can trace the protons linked with the intermediates. Hence deuteration substitution is a better approach than hydrogen for examining the structure of the ligand at the sixth position. Although the D\(_2\)O\(_2\)-induced spectra showed a frequency shift from 774 to 788 cm\(^{-1}\) from this study indicating some similarities to those produced in H\(_2\)O\(_2\)-induced, our D\(_2\)O\(_2\) experiment helps in clarifying the issue on ferryl protonation and confirming the oxoferryl nature of heme compound II intermediates.

Experimental studies have given conflicting results for the ferryl species. In particular, crystallographic and extended x-ray absorption fine-structure data have shown either a short (≤1.70Å) or a longer (≥1.90Å) Fe–O bond, indicating either a double or a single bond. Through deuterium substitution, vibrational frequencies of the iron-ligand, and estimated bond distances, this study has shown that the intermediates formed in heme compound II thus have Fe\(^{IV}\)=O structure.
Chapter 4: Observation of Fe$^{IV}=0$ Stretching Vibrations of pD-induced Soybean Peroxidase using Resonance Raman Spectroscopy

4.1 Overview
The pH and pD dependence of the ferryl forms of soybean peroxidases SBP was examined by resonance Raman spectroscopy. The effects of pH or pD on the ferryl intermediates were the primary focus. There were alterations of the pH/pD spectral signatures for ferryl SBP. The ferryl vibrational stretching band remained within the range of 773 cm$^{-1}$–787 cm$^{-1}$ irrespective of the pH or pD. We observed the changes in the ferryl frequency as the pH/pD is changed from alkaline to acidic. The ferryl vibrational frequencies above neutral pH were higher than 780 cm$^{-1}$ whilst those below the neutral pH were about 14 cm$^{-1}$ lower. Ferryl frequencies were sensitive to deuterium exchange at the lower pH but not in the alkaline pH. This observations can be attributed to deprotonation of the distal histidine for pH greater the pKa and protonation of the distal histidine for pH less than the pKa. The pH/pD induced changes for the ferryl SBP vibrational frequencies supports the proposal the perturbation of the distal hydrogen bonding network by the protonation status of $N_{ε2}$, orientation of a bridging distal water molecule and an indirect effect on the ferryl band. In view of this evidence, we conclude that the ferryl forms of SBP compound II possess the Fe$^{IV}=O$ structure and do change with variation in pH.

4.2 Introduction
Efforts have been made in determining the molecular properties of heme proteins due to their involvement in diverse biological functions[106, 107]. The interaction of peroxides with the respiratory pigments hemoglobin and myoglobin has been studied for
so many years. Studies on ferryl intermediates produced during the peroxidative reaction of heme proteins have contributed to an improved understanding of the catalytic pathway of numerous enzymatic systems[1, 5, 6, 107]. Ferryl intermediates possess the capacity to initiate oxidative damage in a variety of biological systems[32, 108]. Various analytical techniques have been used to understand the intricacies of the peroxidative reaction with heme proteins.

The traditionally accepted configuration of compound II (Fe$^{IV}$=O) has recently been questioned. Experimental and theoretical results have the proposed the compound structure to be hydroxyl ferryl instead of the traditional oxyferryl structure.[26, 27, 94, 109]. In chapter 3, we discussed how the substitution of deuterium for hydrogen atom in the peroxidative reaction provides information about the ligand in the 6th position which helps clarify the controversy on ferryl protonation. Previous evidence on spectroscopic techniques supports the view that ferryl protonation does not occur within physiologically relevant pH ranges with histidine ligated heme proteins, whilst cysteine or tyrosine ligated heme proteins may possess protonation. Optical absorption studies [110-113] have shown that ferryl heme proteins can exist in two forms depending on the pH (red at alkaline pH and green at acidic pH). Despite these conspicuous differences, Mossbauer spectroscopy[91, 114, 115] shows that the electronic structure of heme iron is the same in the red and green higher oxidation states of peroxidases. In this chapter, we examine the effect of pH/pD variations on the ferryl structure of soybean peroxidase.

pH and pD effects were studied for H$_2$O$_2$-induced and D$_2$O$_2$-induced experiments using Raman spectroscopy. The vibrational stretching frequencies of the ferryl intermediates formed by the reaction of soybean peroxidase reacting and peroxide at
different pH/pD were observed within 773 cm\(^{-1}\) – 787 cm\(^{-1}\). Resonance Raman scattering technique was used to determine ligand coordination and the structure of the active site in heme proteins. Varied pH/pD induced resonance spectra showed the presence of the Fe\(^{IV}=O\) structure and frequency switching. Deuterium exchange measurements were used to probe the path of the peroxide hydrogens and monitor the structural changes in heme proteins. Deuteration effects were monitored by verifying vibrational frequency changes in experiments performed in D\(_2\)O in D\(_2\)O and H\(_2\)O\(_2\) in H\(_2\)O.

4.3 Materials and Methods

Soybean peroxidases were isolated from soybean seeds coating, purified and characterized in the laboratory (see sections 2.1 and 2.2). All water was deionized to 18 MΩ/cm by a Milli-Q deionizing system (Millipore Corp.). 30% H\(_2\)O\(_2\) was purchased from Sigma. Deuterium peroxide (D\(_2\)O\(_2\)) was produced by the sequential deuteration and oxidation of an alkylanthraquinone dissolved in a deuterated decanol as illustrated in section 2.3.

Ferryl species formed from pD-varied experiments were generated by the reaction of deuterated ferric soybean peroxidase with deuterium peroxide. A similar setup was used for the pH-induced experiments. The proteins were buffered with 0.01 M sodium acetate, 0.01 M MES, 0.01 M tris acetate, 0.01 M sodium carbonate, and 0.01 M glycine for pH 4-5, 6-7, 8-8.5, 9-10 and 11.5-12 respectively. To minimize the hydrogen atoms interference, the pD buffers were adjusted using deuterated acetic acid. The pH and pD of the protein solutions were measured with an Orion pH meter model 420A equipped
with a silver reference electrode. The pD values for deuterated samples were calculated by adding 0.4 to the apparent pH (pD = pH_{apparent} + 0.4).

The concentration of the protein samples at various pH values were examined using a HP8452A diode array spectrophotometer. Spectroscopic measurements were also used to determine the optimal peroxide needed for the ferryl conversion. Ferryl formation was monitored by shifts in the Soret and visible regions. Heme concentrations were determined using the molar absorptivity at 409 nm (ε=188 mM$^{-1}$cm$^{-1}$) for myoglobin, 406 nm with molar absorptivity of 169 mM$^{-1}$cm$^{-1}$ for hemoglobin and 403nm at 90 mM$^{-1}$cm$^{-1}$ for Soybean peroxidase.

Resonance Raman experimental setup used in this chapter is as described in section 2.3.

### 4.4 Results

In an attempt to contribute to the reports regarding two pH-dependent structural forms of the ferryl SBP, resonance Raman spectra of ferryl SBP in deuterated buffer were obtained at pD 4.9 – 11.8. Figure 17 is an expansion of the lower frequency region of the ferryl SBP in deuterated buffer. The ferryl SBP resonance spectra at different pD with $v_4$ at 1371 cm$^{-1}$, $v_{10}$ at 1641 cm$^{-1}$ and $v_2$ at 1584 cm$^{-1}$. The high frequency region showed no significant changes in the spectra at both low and high pDs. Resonance Raman spectra in the lower frequency region thus possess the ferryl vibrational frequency band at 773-786 cm$^{-1}$. Ferryl band was found in compound II intermediates at all pDs with stretching frequencies switching between two pDs. The pD spectra also showed a 14 cm$^{-1}$ shift from the alkaline region to the acidic for SBP compound II intermediates (786 to 773 cm$^{-1}$).
However, pH above neutral pH (alkaline) did not show any deuteration effect but from neutral to acidic pH, a shift to higher frequencies is seen (774 to 777 cm\(^{-1}\)).

Figure 18 also show resonance spectra of ferryl SBP at pH 4.5 - 11.4 for lower frequency region. The spectra showed identical vibrational bands for both the high and low frequency regions as the pH of the protein was varied. The vibrational frequencies above the neutral pH moved to a higher frequency as the pH was varied. Below the neutral pH, the vibrational frequencies changed from 786 cm\(^{-1}\) to 773 cm\(^{-1}\).
Figure 17: Resonance Raman Spectra of Ferryl SBP at different pH $pD = pH_{\text{apparent}} + 0.4$ using 406.7nm Excitation within the 500 – 900 cm$^{-1}$ Region
Figure 18: pH Dependence of the Resonance Raman Spectra of Ferryl Soybean Peroxidase 406.7nm with Hydrogen Peroxide with 406.7nm Excitation from 500-900cm$^{-1}$ Region
4.5 Discussions

Resonance Raman spectra for SBP compound II intermediates at high frequency region is known to be sensitive to oxidation, ligand coordination and spin state of the iron in hemeproteins. Excitation within the Soret region enhances symmetric modes of vibration within the heme pocket. The modes $\nu_3$, $\nu_{10}$ and $\nu_2$ are the primary resonance Raman marker bands that characterize spin state, a measure of pyrrole nitrogen–carbon stretching frequencies within the porphyrin core that respond to changes in the heme spin. As the pH/pD was varied very subtle differences were observed in the high frequency region of the resonance Raman spectra. The oxidation state marker band at $1375\text{cm}^{-1}$ and a ferryl band at $774$ and $787\text{cm}^{-1}$ exhibited by SBP is typical of the Fe$^{3+}$ heme transition to Fe$^{4+}$.

The resonance Raman for ferryl SBP incubated in both deuterium and hydrogen ligated heme iron were very similar, indicating that the formation of the ferryl compound II does not disturb the porphyrin core as the pH or pD was varied. Incubation of the proteins in deuterium buffers and reacting with deuterium peroxide resulted in a $3 – 6\text{cm}^{-1}$ upshift with peak and band narrowing. Figures 17 and 18 illustrated the ferryl band of SBP existing as either of two frequencies depending on the pH.

Varying the pH or pD above or below the acceptable stable pH or pD (6.4 and 6.8) of ferryl SBP, resulted in $12\text{cm}^{-1}$ downshifts for both pH or pD in the ferryl vibrational frequencies over the range of 4 to 12 being observed for ferryl SBP in contrast to shifts found in turnip and horseradish peroxidase compound II. In addition, the observed vibrational frequency in both ferryl hemoglobin and ferryl myoglobin was higher than that of the peroxidases, 794 to 806$\text{cm}^{-1}$ and 788$\text{cm}^{-1}$ respectively.
The vibrational frequencies of heme peroxidase in previous studies have shown a shift to higher energy with increasing pH. The up shift in frequency upon increasing pH of the protein solution occurs with a characteristic pKa. The pKa is a pH dependent shift in equilibrium between the different heme states. Sitter et al[97] performed measurements in D$_2$O on horseradish peroxidase isozymes. Their results showed an up shift in vibration frequency at pD values below the pKa but no significant deuterium sensitivity for pD values above the pKa. In supporting their claim, they used earlier proposals on peroxidase models that suggest that the deuterium and pH induced frequency up shift in the resonance Raman spectrum of compound II intermediates result from the interactions with imidazole proton from the histidine residue to the ferryl oxygen. At pH values below the observed pKa, the hydrogen bond from the imidazole nitrogen’s proton destabilizes the oxo-ferryl bond creating the lower stretching frequency. Similarly, the protein in D$_2$O at pD values below the imidazole pKa resulted in an up shift in vibrational frequency due to the alteration of the hydrogen bond interactions within the heme pocket. As the pH increases above the imidazole pKa (8.5 ≤ pKa ≤ 6.5), the interacting proton ionizes breaking the hydrogen bond which resulted in the vibrational frequencies up shift.

Several interpretations of the changes in the pH activity of the ferryl intermediates have been proposed. The shift in the ferryl stretching frequencies for HRP compound I (793 cm$^{-1}$) to HRP compound II (775 cm$^{-1}$) at pH 7 was first proposed to be dependent on the type of radicals (a$_{1u}$ or a$_{2u}$) formed. Crystallographic data has also shown that the 18cm$^{-1}$ down shift is due to the movement of a water molecule 0.1Å closer to ferryl oxygen bond during the reduction of HRP compound I to HRP compound II. Others have provided ferryl-oxygen bond lengths to explain the shift as an increase in the
strength of hydrogen bonding as a result of the protonation of nitrogen in the distal histidine during the formation of HRP–II. The ferryl bond distance was then accepted by researchers and confirmed using different techniques. Berglund et al[26] reported a high ferryl oxygen bond distances for HRP-I at 1.7 Å and HRP-II at 1.8Å whereas Chance et al[4] and Chang et al[116] show that HRP-II moved from 1.9 Å at pH 7 to 1.72 Å at pH 10. Chang et al’s proposal that the pH activity (bond lengthen and pH dependence) of the HRP could be attributed to the donation of hydrogen bond to the distal histidine was supported by Hastimoto et al[117], Oertling et al[118] and Sitter et al[119]. Teraoka et al[120] studied Fe II HRP at different pH and did see similar shifts (20cm$^{-1}$) in vibrational frequency for Fe II histidine linkage (Fe-N) at the proximal position as compared that of myoglobin and hemoglobin. The higher Fe-N frequencies were attributed to the imidazolate character induced by the hydrogen bond between the proximal histidine (His 170) and the Asp-247. Terner et al[1] proposed that the switching in the vibrational frequencies at different pH can be related to the compound II heme ionization as shown in Figure 19. Figure 19 show the distal hydrogen bond network due to the protonation of the distal histidine and the orientation of the water molecule. The lack of arginine in the heme cavity of myoglobin and hemoglobin can be attributed to the low peroxidative activity. From our study, the observed changes at varied pH\pD for SBP can also be attributed to the heme–linked ionization of the histidine as shown in Figure 19.
4.6 Conclusion

We present resonance Raman iron-ligand stretching frequencies for the reaction intermediates of SBP. The trend of pD induced changes was found to be similar to that of the pH. However, the stretching frequencies of the SBP in pD experiments did show an upshift of 3 – 6cm$^{-1}$ with resulting peak and band narrowing. The resonance Raman for the SBP incubated in both deuterium and hydrogen ligated heme iron were very similar, indicating that the formation of the ferryl compound II does not disturb the
porphyrin core as the pH or pD was varied. Incubation of the proteins deuterium buffers and reacting with deuterium peroxide resulted in changes in the acidic pH region but not in the alkaline region. These results were interpreted as a result of the protonation and deprotonation of the distal histidine in the acidic and the alkaline region respectively. Deprotonation of the distal histidine disrupts the hydrogen bonding network around the oxyferryl center resulting in an increase in the vibrational stretching frequency.
Chapter 5: Comparison of the Compound II Intermediate with other derivatives of Soybean peroxidase

5.1 Overview

Resonance Raman spectra of the various six-coordinate low-spin forms of Soybean peroxidase were studied in the lower frequency region (600 to 1000 cm\(^{-1}\)). The ferryl vibrational frequency of SBP compound II was found at 773 cm\(^{-1}\) confirmed by a shift to 775 cm\(^{-1}\) upon reaction of ferric SBP with deuterium peroxide. Our resonance Raman data support the Fe(IV)=O compound II structure by the direct observation of a vibrational band in a region known to contain double-bonded metal-oxide stretching frequencies.

5.2 Introduction

Soybean peroxidase belongs to a family of peroxidases which are known to catalyze a variety of hydrogen donors in the presence of hydrogen peroxide. Soybean peroxidase was isolated from soybean seed coatings and contains iron III protoporphyrin IX ring as the prosthetic group with histidine at the distal position. Resonance Raman spectroscopy has been used extensively to examine the ligand coordination at the sixth position. In this chapter we compared the different six coordinate low spin derivatives of soybean peroxidase and distinguishes the vibrational frequency of SBP compound II structure from that SBP compound III, thereby confirming the structure of SBP compound II intermediate to be ferryl.

5.3 Materials and Methods

Soybean peroxidases were isolated from soybean seed coatings, purified and characterized in the laboratory (see sections 2.1 and 2.2). All water was deionized to 18
MΩ/cm by a Milli-Q deionizing system (Millipore Corp.). The alkaline form of soybean peroxidase was prepared by buffering the ferric SBP in 10mM sodium carbonate buffer at pH 11.4 and concentrated with amicon ultrafiltration setup. The same procedure was used for the deuterium samples. SBP samples were buffered in deuterated 10mM sodium carbonate buffer at pH 11.9. The ferricyanide oxidation alkaline form was prepared by reacting the ferric SBP with 50 molar excess of potassium cyanide.

Compound III was formed by rapid mixing of SBP with 100 fold excess peroxides in a spinning quartz cylindrical cell. The reaction assays were performed at 25°C using peroxidase at different pH. An Orion digital pH meter model 420A was used to measure the pH of the buffers. The peroxidase was buffered in 0.01M sodium acetate, 0.01M MES, and 0.01M sodium citrate for pH 4-5, 6-7, and 3-4 respectively.

Raman instrumental conditions were the same as described for compound II in section 2.4. Resonance Raman spectra of SBP were measured at room temperature by using 90° scattering in a cylindrical cell. The resonance Raman spectra were acquired using a Coherent Sabre krypton laser as described in section 2.3. Each ferryl Raman spectrum was collected by rapid mixing of SBP with peroxides in a spinning quartz cylindrical cell.

**5.4 Results**

Figures 20 and 21 show a resonance Raman spectra of low spin SBP derivatives. These derivatives are resting SBP enzyme at pH 7.0, resting SBP at pH 11.4, ferryl SBP and ferricyanide SBP. The resonance Raman spectra in the high frequency region as show in Figure 20 illustrates some similarities in the spectra. This region is known to be
sensitive to coordination, spin states and porphyrin core size. These similarities in the resonance Raman spectra of the SBP derivatives can be attributed to the fact that they are all six coordinated and in the low spin state. Comparing the resting SBP with the other derivatives we realized that differences in the 1500-1650 cm$^{-1}$ region was as a result of the change in the resting SBP from a five coordinated high spin to a low spin six coordinated state. Likewise in the case of the resting SBP at neutral pH compared to resting state at pH 11.4, the spectra the differences in the coordination of the SBP as the pH was varied. This shows the SBP undergoing an alkaline transition as seen in the other peroxidases. The alkaline transition was proposed to due to the ionization of the water molecule at the sixth position as the pH of the SBP was changed from neutral to alkaline.

Table 9 shows the assignment of vibrational modes, observed polarization and frequencies for the resonance Raman spectra shown in Figure 21 for SBP. The most intense peak of the resonance Raman spectra region from 500-900 cm$^{-1}$ is the $\nu_7 (A_{1g})$ at 677 cm$^{-1}$. The next intense peak is a shoulder on $\nu_7$ which been assigned as a vinyl mode. This vinyl mode band represents the interaction of the surrounding with the heme in the SBP derivatives. $\nu_{47} E_u$ mode is a strong polarized infrared active mode at 715 cm$^{-1}$ which normally occur under 406.7 nm excitation. $\nu_{15}$ mode is depolarized band at 757 cm$^{-1}$. The Fe(IV)=O stretch of compound II is a weak band which occur at 774 cm$^{-1}$ in the resonance Raman spectra of compound II and 783 cm$^{-1}$ for pH 11.4.
Figure 20: Resonance Raman spectra of Soybean peroxidases (SBP) using 406.7 nm excitation in the 1000 to 1650 cm$^{-1}$ frequency region.
Figure 21: Resonance Raman spectra of Soybean peroxidases (SBP) using 406.7 nm excitation in the 600 to 1000 cm$^{-1}$ frequency region.
Table 9: Frequencies, Polarization and Assignments of SBP Resonance Raman Bands at Lower Frequency Region

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Symmetry</th>
<th>Polarization</th>
<th>SBP pH 6.4 (cm(^{-1}))</th>
<th>SBP pH 11.4 (cm(^{-1}))</th>
<th>SBP Cpd II (cm(^{-1}))</th>
<th>SBPCN (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\gamma_{\text{CH}=})</td>
<td>-</td>
<td>dp</td>
<td>828</td>
<td>828</td>
<td>828</td>
<td>825</td>
</tr>
<tr>
<td>(\nu_{\text{FeIV}=O})</td>
<td>-</td>
<td>p</td>
<td>805</td>
<td>804</td>
<td>805</td>
<td>801</td>
</tr>
<tr>
<td>(\nu_{16})</td>
<td>B(_{1g})</td>
<td>dp</td>
<td>756</td>
<td>753</td>
<td>753</td>
<td>760</td>
</tr>
<tr>
<td>(\nu_{47})</td>
<td>E(_{u})</td>
<td>p</td>
<td>715</td>
<td>712</td>
<td>713</td>
<td>712</td>
</tr>
<tr>
<td>(\gamma_{\text{as CH}_2=}) (vinyl)</td>
<td></td>
<td></td>
<td>694</td>
<td>691</td>
<td>690</td>
<td>689</td>
</tr>
<tr>
<td>(\nu_{7})</td>
<td>A(_{1g})</td>
<td>p</td>
<td>677</td>
<td>675</td>
<td>679</td>
<td>675</td>
</tr>
<tr>
<td>(\nu_{49})</td>
<td>E(_{u})</td>
<td>-</td>
<td>590</td>
<td>587</td>
<td>587</td>
<td>587</td>
</tr>
</tbody>
</table>
Figure 23 shows a resonance Raman spectra comparing the structures of compound II and III at pH 4.5. Compound II was generated by reacting resting SBP with five molar excess peroxide and whilst compound III was with the resting SBP and hundred molar excess peroxide. Although they were all formed by reacting with peroxides, the structures of compound II and compound III differ slightly in the lower frequency region of the resonance Raman spectra. The high frequency region has some similarities within the spectra validating the structure of compounds II and III to be sixth coordinated and in low spin state. Figure 24 highlights on the lower frequency region for SBP compound III, SBP compound II in H₂O and SBP compound II in D₂O. The spectra show all the prevalent bands seen in the lower frequency region (chapter 3). Comparing the compound II and compound III, we observed a new band at 550 cm⁻¹ for compound III and 773 cm⁻¹ for compound II. The band at 550 cm⁻¹ is Fe(II)-O₂ whilst 773 cm⁻¹ band is the Fe(IV)=O. This observation clearly distinguishes the structure of SBP compound II form that of SBP compound III. The deuteration experiment shows an upshift of the ferryl stretching frequency from 773 to 775 cm⁻¹.

Figures 24 and 25 show resonance Raman spectra depicting the pH dependence of compound III at higher and lower frequency region respectively. The high frequency region spectra did not show any change even as the pH was varied. This is indicative of the fact that the heme remained sixth coordinated and in the low spin state. Figure 25 shows the pH dependence in compound III with slight changes in the FeII-O₂ vibrational stretching frequency. The resonance vibrational frequency downshift for FeII-O₂ band from neutral pH to acidic pH could be due to the heme-linked ionization of the distal histidine on the heme as discussed in chapter 4.4.
Figure 22: Comparison of the resonance Raman spectra of Soybean peroxidase compounds II and III at 500-1650 cm$^{-1}$ frequency region.
Figure 23: Comparison of the resonance Raman spectra of Soybean peroxidase compounds II and III at 500-900 cm\(^{-1}\) frequency region
Figure 24: pH dependence of compound III at higher frequency region of the resonance Raman Spectra
Figure 25: pH dependence of compound III at lower frequency region of the resonance Raman spectra
5.5. Discussions

Plant peroxidases are known to possess the ability to oxidize a wide range of substrates both organic and inorganic. Peroxidases such as HRP and recently found SBP have seen wide practical applications in natural products, bioengineering, waste water processing, chemical synthesis and medical diagnostics. The belief that HRP was the typical plant peroxidases with such great capabilities was distracted since the discovery of SBP by McEldoon and Dordick[12, 121]. SBP has shown to have better thermal stability and wider pH range than HRP-C, proven to be a better alternative to HRP.

We demonstrated that resonance Raman spectra of intermediates and derivatives of Soybean peroxidase, with sixth coordinate low-spin hemes, were very similar to the stretching frequency region from 1300 to 1650 cm$^{-1}$. The resonance Raman spectra in this region is found to be sensitive to the coordination and spin states, as well as porphyrin core size, whilst 500 to 900cm$^{-1}$ are sensitive to factors, such as the identity of the sixth ligand, and differences between iron oxidation states III and IV. Recent crystal structure and resonance Raman studies have demonstrated that the heme cavity of SBP is five coordinate and exist as quantum mixed spin and high spin forms[19-21, 122]. The heme coordination feature of SBP is similar to that of HRP and many other plant peroxidases. The absorption spectra also show a shift in 403nm to 414nm in the Soret region just like it is in HRP. The lower frequency region as shown in Figures 23 and 25 depict resounding differences between SBP compound II and the various SBP derivatives. The optical spectra of SBP compound II at different pH show the stability of SBP at lower pH (pH< 3.0). SBP undergoes a Soret shift from 403nm to 407nm at pH 2.5 and its stable for days whilst HRP shift from 403nm to 383nm with increasing
absorbance for a few hours. The 383nm shift indicates the inactivation of HRP in acidic conditions as result of the rupturing of the heme core from the protein.

Figure 25 confirms the pH dependence of the SBP compound III just like lactoperoxidase and the other ferric heme proteins[123]. The FeII-O2 complex is known to have short lifetime and with increased reactivity as pH was lowered below the pKa of SBP (pKa< 9.7)[124, 125]. The resonance Raman spectra as seen in Figures 24 and 25 show weak band at 550cm⁻¹ which undergo a downshift by varying pH. This observation can be attributed to the protonation of the distal histidine, and the extended hydrogen bonding network in the heme especially in acidic pH medium.

Comparing the reactivity of both SBP compound II and compound III to other class III peroxidases in extremely low pH such as pH 2.4, thus confirm the stability of the SBP and the unique properties at high temperature and wide pH range. The catalytic activity and conformational stability of SBP have been proven to be pH dependent with the difference in pH stability between SBP and HRP attributed to the structural differences. The main contribution factor is relatively due the more solvent exposed δ-meso heme edge enhancing the interaction of the substrates with the heme pocket.
5.6. Conclusion

We demonstrated that resonance Raman spectra of intermediates and derivatives of Soybean peroxidase with six-coordinate low-spin hemes, were very similar in the vibrational region from 1300 to 1650 cm\(^{-1}\). The resonance Raman spectra were sensitive almost exclusively to the coordination and spin states, as well as porphyrin core size whilsts lower frequency region is sensitive to other factors, such as the identity of the sixth ligand, and differences between iron oxidation states III and IV. Resonance Raman spectra of the various six-coordinate low-spin forms of soybean peroxidase at the lower frequency region (600 to 1000 cm\(^{-1}\)) proves the ferryl nature of SBP compound II. The ferryl vibrational frequency of SBP compound II was found at 773 cm\(^{-1}\) confirmed by a shift to 775 cm\(^{-1}\) upon reaction of ferric SBP with deuterium peroxide. Comparing SBP compound II to SBP compound III, thus confirm the authentic nature of the SBP compound II ferryl structure. Our resonance Raman data on the various SBP derivatives support the Fe(IV)=O compound II structure by the direct observation of a vibrational band in a region known to contain double-bonded metal-oxide stretching frequencies.
REFERENCES

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EDUCATION
PhD in Physical Chemistry, August 2009
Virginia Commonwealth University, Richmond, Virginia
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Master of Science in Chemistry (December 2001)
North Carolina Agricultural and Technical State University, Greensboro, North Carolina
Thesis Title: Monitoring the degree of polymerization of Diglycidyl ether of Bisphenol-A (DGEBA) with Aniline
Area of Specialization: Physical Chemistry (Polymer)

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TEACHING EXPERIENCE
Research/Teaching Assistant (August 2002 – August 2008)
Virginia Commonwealth University, Richmond, Virginia
- Taught Introductory and General Chemistry (Laboratory and Recitation)
- Taught Organic Laboratory

Research/Teaching Assistant (August 1998 – December 2001)
North Carolina A & T State University, Greensboro, North Carolina
- Taught Introduction and General Chemistry laboratory
- Supervised Physical Chemistry Laboratory

RESEARCH/PROFESSIONAL EXPERIENCE
Doctoral Research: Department of Chemistry, Physical Chemistry, Virginia Commonwealth University, Richmond, Virginia 2002 – 2009
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- Focuses on contributing to the enhanced understanding of the catalytic cycle intermediates of heme peroxidases which serves as models for intermediates of heme proteins, such as the terminal oxidases and cytochrome P450 enzymes
  o Examine the ferryl structure in heme proteins by monitoring the changes in vibrational stretching frequency
  o Identify ligand structure at sixth coordination site
  o Examine pH effects on ferryl structure
  o Use Badger’s rule to estimate bond lengths of the ferryl intermediates
• Isolation, purification and characterization of Soybean peroxidases which is found to have vital properties making it an important alternative to Horseradish peroxidases, the most readily used peroxidase with wide commercial applications
• Spectroscopic and calorimetric methods used to investigate the changes in the heme structure and environment during the reaction of heme proteins with peroxide

**Contract Chemist:** Analytical Chemistry Services department, Research Triangle Institute – RTP, Durham, North Carolina January 2002 – August 2002
- Provided analytical chemistry support for drug formulation, drug metabolism, and drug discovery and development studies
- Responsible for experimental design, performance of a variety of techniques for sample preparation and analysis, data processing and interpretation, documentation of procedures and results, report writing, and routine maintenance of laboratory equipments and instrumentation
- Developed analytical methods for quantitative analysis of test chemicals in dosing formulations or biological matrices
- Performed validation of analytical methods
- Performing stability studies of test chemicals in dosing formulations or biological matrices, analysis of test chemicals in dosing formulations or biological matrices, and identification of drug metabolites

**Masters Program Research:** Department of Chemistry, Physical Chemistry, North Carolina A & T State University, Greensboro, North Carolina 1998 –2001
(Research advisor: Dr. Alvin P. Kennedy)
- Investigation of the changes in the dielectric behaviors during the isothermal cure reaction of the epon 828 – aniline formulation
- Determination of the correlation between the neat DGEBA molecular weight distribution and network morphology to dielectric loss peaks during cure
- Polymerization of DGEBA polymers with aniline in solution to determine the effects convection, sedimentation and phase separation have on molecular weight distribution and network morphologies in gravity

**Contract Chemist:** Quality Control Laboratory, Proctor and Gamble Company, Greensboro, North Carolina  June 2000 – August 2000
- Performed routine chemical analysis on finished products and bulk chemicals
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- Performed studies to support ongoing stability programs on test chemicals and finished products
- Documented, organized and maintained analytical data

**Shift Chemist:** R&D Laboratory, STP, Ashanti Goldfields Company Limited, Obuasi, Ghana 1994 –1997
- Performed wet chemical analysis of samples from the world’s largest bacteria gold recovery plant
- Analyzed data and used problem solving skills to initiate corrective actions for out–of–specifications conditions
- Developed extraction method for analyzing solid gold
- Carried out routine testing and research activities leading to the development and validation of analytical test methods
Interfaced with contract manufacturers, consulting and testing laboratories to ensure consistency in reagent purity and concentration

- Assisted senior chemists with research activities and troubleshooting assignments
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- Trained and supervised junior chemists and technicians
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**Undergraduate Honors Research:** Department of Chemistry, Organic Chemistry, Kwame Nkrumah University of Science and Technology 1991-1994
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- Isolation and spectral analysis of the oil from the leaves Nicotinia Tabacum
- Investigation of the toxicity level of the oil of Nicotinia Tabacum on plant pesticides and mycobacterium ulcersis

**PUBLICATIONS/PRESENTATIONS**


**AWARDS**

- Merit Graduate Student Award, College of Arts and Sciences, North Carolina A & T State University, Greensboro, North Carolina, 2000
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- Atomic Absorption Spectroscopy (AAS)
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- Differential Scanning Calorimetry (DSC)
- Gas Chromatography (GC)
- Gel Electrophoresis (SDS-PAGE)
- High-Performance Liquid Chromatography (HPLC)
- Infrared Spectroscopy (FTIR)
- ICP-MS
- Isothermal Calorimetry
- Fluorescence Spectroscopy
- Leco (Sulfur) Analyzer
- Mass Spectroscopy (MS) – GCMS, LCMS
- Nuclear Magnetic Resonance (NMR)
- Tube furnace
- FT-Resonance Raman Spectroscopy
- Ultraviolet Spectroscopy (UV)
- X-ray fluorescence Spectroscopy (XRF)

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- Team-oriented
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