Functional Analyses of Human Serum Paraoxonase1 (HuPON1) Mutants Using
Drop Coating Deposition Raman Difference Spectroscopy

THESIS

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Abstract

We present work on the structural implications of specific mutants of Paraoxonase1 (PON1) G2E6, and the turnover rate upon bonding of the enzymes with paraoxon when compared to the wild-type enzyme by using vibrational spectroscopy. A new Raman spectroscopy called Drop Coating Deposition Raman (DCDR) is utilized in our work. The Raman band changes in the paraoxon/H115W system are in good agreement with computational calculations and are strong evidence of the formation of the paraoxon hydrolysis product, p-nitrophenol in the reaction system. The corresponsive turnover rates of G2E6 wild-type and its two mutants, H115W and H115T, are also observed in DCDR spectra.

Dedication

This document is dedicated to my friends and family.

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CHAPTER 1 INTRODUCTION

1.1 Human Serum Paraoxonase1 (HuPON1)

The PON family is a group of mammalian enzymes that includes PON1, PON2 and PON3, which share ~ 60% sequence identity.¹ Human serum paraoxonase1 (HuPON1), as the best-studied member of PON family, is synthesized in the liver and then secreted into the human blood plasma where it is tightly bound to high-density lipoprotein, HDL, commonly known as good cholesterol.²⁻⁴ The enzyme, which derived its name from its ability to hydrolyze paraoxon, can catalyze the hydrolysis of a broad range of substrates, including arylesters, carbamates and lactones.⁵ The ability of inactivating various organophosphates, including nerve agents sarin and soman, makes PON1 the subject of many studies, since organophosphates present an environmental risk as well as terrorist threat worldwide.⁵⁻⁹

PON1 is a 354 amino acid glycosylated protein of 43-kDa. The polymorphic Q/R 192 residue display marked differences in catalytic activity towards some substrates in human blood.⁵ PON1, which shares a similar structure with diisopropylfluorophosphatase (DFPase), is a six-bladed β -propeller, each blade contains four strands and the bladed regions are arranged radially around a central core. There are two calcium ions in the central tunnel of the propeller, one (Ca1) at the top and the other (Ca2) in the central region [Figure 1.1].¹ Ca2 is referred to as the

‘structural calcium’ while Ca1 is assigned as the ‘catalytic calcium’.⁴ Recently, Harel et al. revealed that PON1 has a unique closed active site [Figure 1.2a], in which helices H2 and H3, together with the loops that connecting these helices to the β -propeller scaffold, form an active site canopy.^{1,10} Due to the difference in PON1 and DFPase’s overall architecture and active site structure, very different catalytic mechanisms have been observed.

Figure 1.1 Overall structure of PON1. a) View of the six-bladed β -propeller from above. Two calcium atoms in the central tunnel of the propeller (Ca1, green; Ca2, red). b) A side view of the propeller, including the three helices at the top of the propeller (H1-H3).¹

A recent computational modeling study found that the active site of PON1 is characterized by two distinct regions responsible for substrate interactions: the hydrophobic binding site for arylesters/ lactones, and the paraoxon binding site for phosphotriesters [Figure 1.2a,b].¹⁰ The region for paraoxon binding is composed primarily of polar residues, e.g. R192 and S193 from helix H2 and Y71 from the

flexible loop L1, and the postulated catalytic residues H115 and H134 (the His-His catalytic dyad) are located at the bottom.¹⁰ Harel hypothesized that the His-His catalytic dyad, which is near both Ca1 and the phosphate ion, is responsible for the enzymatic activities of PON1 with paraoxon. H115 (the closer nitrogen of which is only 4.1 Å from Ca1) acts as Bronsted base to deprotonate a water molecule generating the attacking hydroxide, meanwhile H134 is involved in a proton shuttle mechanism to increase H115's basicity.¹

Figure 1.2 a) A schematic representation of the overall architecture of the active site of HuPON1. The ester/lactone binding region (in cyan) and paraoxon binding region (in yellow) are shown as circles, and the calcium-ligating center is shown in the red region. b) Superposition of three substrates phenylacetate (cyan), d-valerolactone (green), and paraoxon (yellow) bound in the active site of HuPON1. The proposed catalytic residues, His dyad are shown in blue.¹⁰

The histidine (His) residues, especially H115 and H134, are identified to be essential for the activity of PON1 [Figure 1.3].^{1,2,11} Substituting either of the histidine residues should change the enzymatic activities of PON1 towards paraoxon

significantly. However, numerous studies showed that several different H115 mutations only exhibit a moderate decrease in paraoxonase affinity.^{1,3,10} Yeung et al. suggested that H115 is a residue near or within the active site and functions as a substrate-selective residue rather than being directly involved in the catalytic mechanism.⁴ To further examine if H115 plays a mechanistic role in PON1 catalysis mechanism, Raman spectroscopy was used to study a series of H115 mutations reacting with paraxon.

Figure 1.3 Structure of G2E6 PON1. Residue H115 is proximal to the “catalytic” Ca^{2+} and phosphate ion found in the crystal.¹¹

1.2 Paraoxon

Organophosphorus compounds (OPs) are neurotoxic chemical warfare agents that inhibit cholinesterases.¹ They were initially developed as insecticides, but have

been used as warfare agents due to their extreme toxicity that will result in severe symptoms, including death.¹² Paraoxon is an organophosphate oxon that is approximately 70% as potent as the nerve agent sarin, and also is one of the most potent acetylcholinesterase-inhibiting insecticides available^{13,13}.

Paraoxon contains a phosphoryl (P=O) bond, two alkyl substituents and a nitrophenyl substituent. The nitrophenyl group is commonly referred to as the “leaving group”, because it is more susceptible to hydrolysis than the alkyl groups. The hydrolysis reaction of paraoxon is illustrated in Figure 1.4.

Figure 1.4 The hydrolysis reaction of paraoxon.

Using computational methods, Hu et al. determined that the binding site of paraoxon in HuPON1 is mainly composed of helix H2 and loop L1, with the His-His dyad located at the bottom of the binding site.¹⁰ When the paraoxon substrate is situated in the binding pocket, the phosphate group is directed towards the catalytic calcium and facing residue D269. This orients the nitrophenyl leaving group to be directed towards residue R192 [Figure 1.5].¹⁰ In this work, we have employed a novel

type of Raman spectroscopy, Drop Coating Deposition Raman (DCDR), to monitor the enzyme-catalysis reaction and the hydrolysis products of paraoxon in the paraoxon/HuPON1 interaction.

Figure 1.5 The binding conformation of paraoxon with HuPON1 wild-type.¹⁰

1.3 Raman Spectroscopy in Enzymology

Understanding the catalytic mechanisms of enzymes is essential in protein engineering experiments directed towards creating novel catalysts and for designing drugs with enhanced enzymatic reactivity and selectivity.¹⁴ Different chemical and biochemical methods have been developed to elucidate the basic mechanisms of enzymatic catalysis in terms of detailed structural information of the active-site and bound substrates. Among all these techniques used in structural biology, high-resolution X-ray crystallography and NMR serve as the two most powerful biophysical methods of helping to identify the enzymes residues and the interactions

with substrates at the active-site of enzymes.¹⁵ These methods provide information such as bond lengths, bond angles and torsional strains of bound substrates within enzymes. However, some frequently asked questions are beyond the reach of these macromolecular techniques, such as quantization of small geometric changes which are less than 0.01 Å in bond length or the distribution of electrons in the substrates bonds.¹⁵⁻¹⁷ Vibrational spectroscopy, which is able to quantize very small changes in the overall structure, provides information on molecular geometry, ionization state, the extent of interaction between molecules, protein secondary structures and aromatic side chain environment. Between the two major vibrational spectroscopic methods, Raman and infrared (IR) spectroscopy, Raman spectroscopy has distinct advantages over IR spectroscopy for the study of biological systems. These include a lower Raman cross section of water and a large amount of information in the fingerprint region.¹⁸ Compared with X-ray crystallography, the picoseconds time scale of Raman spectroscopy allows chemical reactions in crystals to be monitored and can identify reaction intermediates.¹⁹ Furthermore, Raman spectra are not prone to line broadening due to the rapid chemical exchange or relaxation effects observed in NMR. Nevertheless, the use of Raman for the study on enzymes is less common than NMR and X-ray crystallography. Because of this, the databases available for Raman spectra interpretation are limited. Recent developments in computational methods in biochemistry offers a non perturbing approach to assess the electrostatic environment within the active site.²⁰ When coupled with computational approaches, Raman

spectroscopy is able to provide structural information on active-sites of enzyme-substrate complexes with high sensitivity, precision and with quantitative information.^{19,20}

1.4 Raman Difference Spectroscopy

Vibrational spectroscopy can monitor very small changes in molecular geometries.¹⁵ However, spectra of macromolecules like proteins are very complicated since many vibrational modes contribute to the spectrum at any given frequency.²¹ Unlike NMR or X-ray crystallography, the interpretation of Raman data is less software driven, and interpretation involves more human input and is time consuming.¹⁹ These factors hinder the accuracy of the interpretation of Raman spectra for biological systems. In order to overcome these challenges, selective measurements are used to interpret protein Raman spectra.

There are multiple types of Raman spectroscopy. One commonly used form is resonance Raman spectroscopy. In resonance Raman spectroscopy, the excitation wavelength is chosen to be within a region of the electronic absorption. In protein or DNA this region is in the UV, below 300 nm. The spectrum will exhibit Raman bands only from the particular chromophore in a protein, which shows resonance Raman scattering intensities many orders of magnitude greater than those of off-resonance Raman scattering.^{14,16-18,22} Carey et al. employed resonance Raman in a catalytic reaction which generated a chromophore dithio ester $RC(=S)SR'$ at the place of catalysis, and successfully obtained an abundant amount of information of the scissile

bonds from the resonance Raman spectrum.¹⁶ This approach, however, usually requires a “friendly” system, one containing a chromophore, thus its application is limited.^{17,22}

A very accurate nonresonance Raman difference spectroscopy (RDS) has been developed over the past two decades. It becomes a general method to solve spectral crowding of large macromolecules and can be applied to most protein systems. In a Raman difference experiment, a protein is tagged in some way, such as complexed with ligands, labeled with isotope or mutagenesis. The small differences between the protein and its modified version can be detected by subtracting the modified protein spectrum from the original protein spectrum. Thus, the RDS spectrum will have an interpretable number of bands, which contain useful information directly related to the modification in the protein system.¹⁸ Callender et al. have used RDS extensively to study the structure of proteins and protein complexes with small molecules. For instance, the structure information of the S-peptide region of RNase was obtained by Raman difference spectroscopy.^{21,23}

1.5 Drop Coating Deposition Raman (DCDR) Spectroscopy

Ben-Amotz and co-workers developed the drop coating deposition Raman (DCDR) method to collect high-quality Raman spectra of proteins from low-concentration solutions and serve as an effective method for spectroscopic proteomic separation.^{24,25} This method requires simply depositing 1 to 10 μL of a protein solution on a hydrophobic substrate and, after solvent evaporation, collection

of Raman spectra from the purified analyte deposits using an epi-illuminated microspectrometer [Figure 1.6].^{25,26} The optimum DCDR substrate has high optical reflectance and very low background signals. Common substrates are SpectRIM slides (polished stainless steel plate coated with 50 nm Teflon), CaF_2 slides and gold slides. The low water affinity of the substrate confines the solution drop to a small area, thus the protein deposit becomes thicker and more concentrated compared to other surfaces like glass.²⁴ During the evaporation process, the proteins are automatically separated from other solution components including salts, less hydrophobic buffer, and fluorescent impurities. Therefore, a higher concentration and purer protein sample is localized on the substrate due to the coffee ring effect.²⁵ As a result, high quality Raman spectra of more concentrated proteins are obtained, and interference from buffer and fluorescent impurities is minimized.

Figure 1.6 A 10x microscopic image of the DCD of G2E6 H115K.

This method was validated by Ben-Amotz's group, and has been determined that the protein deposits remain partially hydrated, the proteins largely retain their native structures from solution. Furthermore, minimal thermal or photochemical damage is induced from the laser allows for highly reproducible DCDR spectra, making DCDR a convenient and versatile method for biomedical spectroscopy studies.²⁶ Otiz et al. applied DCDR to the identification of peptide and protein structural changes induced by phosphorylation, fibrillation and ligand binding. The results demonstrated exceptional resolution and high performance of this method.²⁷

CHAPTER 2 EXPERIMENTAL

2.1 Samples

Recombinant PON1 G2E6 and mutants were synthesized by Dr. Magliery's group. The stock solutions of enzymes G2E6 wild-type, G2E6 H115W and G2E6 H115T were originally buffered with 50 mM Tris-HCl at pH 8 containing 50 mM NaCl, 1 mM CaCl_2 , 0.1% Tergitol and 10% Glycerol, and then were buffer exchanged with 50mM Tris-HCl containing 50 mM NaCl and 1 mM CaCl_2 at pH 8 to remove the glycerol in the solutions. The concentrations of enzymes were determined by a Bradford standard curve using BSA as protein standard. Paraoxon was purchased from Sigma-Aldrich and mixed with 100% methanol to make 0.13 M stock solution. Each enzyme and paraoxon were mixed at 1:50, 1:500 and 1:5000 ratio, and the 1:500 ratio was chosen for the best results. Lysozyme from chicken egg white was purchased from Sigma-Aldrich. The 50 mM saturated lysozyme solution sample was prepared by mixing the lysozyme crystal with 50mM Tris-HCl at pH 8. The SpectRIM substrates were purchased from Tienta Sciences, Inc.

2.2 Instrument setup

2.2.1 Raman Microscope Setup

Raman Spectra were acquired using an inVia Raman microscope (Renishaw).

The setup is shown in Figure 2.1. The 632.8 nm output from a He-Ne laser (~ 17 mW), which was used in all Raman experiments in this work, was passed through an interference filter to eliminate the plasma lines of the laser. Subsequently, the output was reflected by a notch filter and directed to the sample. The laser power at the sample was approximately 5.5 mW and the laser spot size is $2\mu\text{m}$. The Raman signal was collected at 180° backscattering geometry by $50\times$ objective lens (NA 0.75), passed through a notch filter to reject the Rayleigh line and directed through a $200\mu\text{m}$ confocal slit. The signal passed through a $200\mu\text{m}$ slit of the spectrograph (800 mm focal length) then dispersed by 1800 grooves / mm grating and detected by a CCD. The grating was automatically calibrated using the built-in silicon band.

Figure 2.1 Microscope Raman setup.

2.2.2 Solution Raman Microscope Setup

Solution Raman microscope spectra were acquired by making a slight

modification of the inVia Raman microscope (Renishaw) setup. A special platform was employed to hold the vials for solution sample, and the objective lens was changed to 30 $\times$. Other instrumental setups are all remained the same as for DCDR and single crystal Raman Microscope.

2.3 Procedure

2.3.1 Drop Coating Deposition Raman (DCDR) Spectroscopy

All the experiments were performed at room temperature. For paraoxon/enzyme samples, the enzyme and paraoxon were mixed at the indicated ratio, and the mixture was allowed to sit for 5 mins, then 2 μ L of the mixture was deposited on a SpectRIM substrate and the solvent was evaporated without further treatment [Figure 2.2]. The dried spot had a diameter of less than 2 mm approximately 25 mins after deposition. The spectra were acquired via the inVia Raman microscope (Renishaw). Raman spectra of protein samples were obtained by focusing the laser on the protein ring which is at the outer edge of the crystals area and about 100 μ m away from the edge of the spot. The exposure time was 60 seconds for 1:50 and 1:500 samples and 30 seconds for 1:5000 samples. Twenty acquisitions were averaged for all samples. The enzyme samples were mixed with 100% methanol at the indicated ratio then directly deposited on the substrate and the solvent evaporated for approximately 25 mins. The ligand sample was mixed with Tris-HCl buffer (50 mM NaCl and 1 mM CaCl₂ at pH 8) and prepared in the same manner. All the spectra were obtained the

same way as for the complex samples.

Figure 2.2 A 50x Microscope image of DCD paraoxon/G2E6 H115W system at ratio 1:500.

Figure 2.3 a) A 5x microscope image of DCD 5 mM lysozyme. The crystal of salt in buffer is observed. b) The DCD Raman spectrum of Tris-HCl buffer. Asterisks indicate the major bands of buffer.

A buffer sample was also prepared in the same way. The buffer spectrum was obtained from the film-like portion of the deposit rather than the crystals formed at the

center of the spot in order to achieve higher signal-to-noise ratio [Figure 2.3a]. The exposure time was 60 seconds and 5 acquisitions were averaged [Figure 2.3b].

2.3.2 Single crystal/ Solution Raman Spectroscopy

The single crystal lysozyme sample was obtained by directly putting the lysozyme crystal on the SpectRIM slide without further treatment [Figure 2.4]. The spectra were acquired via the inVia Raman microscope (Renishaw). The exposure time was 300 seconds and 5 acquisitions were averaged.

Figure 2.4 A 20x microscope image of the single crystal of lysozyme.

The solution lysozyme sample was held in a Fisherbrand glass vial (15×45 mm) that was free of fluorescence under our experimental conditions. The spectra were acquired using approximately 2.7 mW of laser power. The exposure time was 1 hour and 5 acquisitions were averaged.

2.4 Spectra Smoothing

All the spectra were smoothed using the smoothing function in Igor Pro (Version 6.0.0.0, WaveMetrics, Inc.) before subtraction, and a binomial algorithm and smoothing factor of five were used.

2.5 Spectra Subtraction

The difference spectrum that contains information of paraoxon hydrolysis products and changes in the enzyme conformation was obtained by subtraction of the enzyme spectrum from the spectrum of the enzyme-ligand complex. e.g. $\text{Spectrum}[\text{difference 1}] = [\text{complex}] - [\text{enzyme}] \times f1 - [\text{buffer}] \times f2$, where $f1$ and $f2$ are scaling factors. The spectrum of the buffer was also subtracted or added since the contribution of the buffer signals in each spectrum varies slightly. Scaling factors for the enzymes and buffer spectra were chosen to achieve flat baselines. A further difference spectrum was obtained by subtracting the spectrum of paraoxon (in 100% methanol) to clearly indicate the band changes of the hydrolysis products. e.g. $\text{Spectrum}[\text{difference 2}] = [\text{difference 1}] - [\text{paraoxon}] \times f3$, where $f3$ is scaling factor to achieve flat baselines. In the final spectrum (difference2), the positive bands and band shifts can be due to the formation of paraoxon hydrolysis products, and the negative bands can result from the breakup of certain bonds upon the hydrolysis of paraoxon.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 Comparison of Solution, Single Crystal and DCD Raman Spectroscopy

The Raman spectra of chicken egg white lysozyme crystal, lysozyme in buffer solution and DCD method are shown in Figure 3.1. Trace a is the spectrum of 50mM lysozyme in buffer (50mM Tris-HCl containing 50 mM NaCl and 1 mM CaCl_2 at pH 8). Trace b is the spectrum of lysozyme single crystal. And trace c is the spectrum of 50mM lysozyme in buffer (50mM Tris-HCl containing 50 mM NaCl and 1 mM CaCl_2 at pH 8) using DCDR method.

Figure 3.1 Comparison of different Raman spectra of lysozyme. a) The spectrum of 50 mM saturated lysozyme in 50mM Tris-HCl buffer pH=8. b) The spectrum of lysozyme single crystal. c) The spectrum of DCD lysozyme in 50mM Tris-HCl containing 50 mM NaCl and 1 mM CaCl_2 at pH 8.

Comparing these three raw data, they all very similar to each other, of which the major bands are retained at the same wavelength in all spectra, despite some different band shapes and intensities. Apparently, the DCDR spectrum shared more analogy with the solution spectrum than with single crystal spectrum. The difference spectra d (difference between solution spectrum and DCDR spectrum) and e (difference between single crystal spectrum and DCDR spectrum) supported this observation by showing more band changes in spectrum e than in spectrum d.

In spectrum e, the negative bands around 1466, 1064 and 756 cm^{-1} are caused by the buffer signal, since there is no buffer used in single crystal sample. Other than that, the band changes around 1446, 1346 and 786 cm^{-1} are probably caused by different environments between the single crystal and DCDR methods, because these bands are mainly assigned to amide III bands, which are sensitive to changes in the protein secondary structure. The single crystal spectrum requires the simplest instrument setup and relatively short acquisition time compared to the other methods, and it is helpful in studying the structural information about the protein system. Many single crystals of protein or protein-ligand complexes have been studied.²⁸ However, due to the absence of buffer, the information concerning the reactions in the biological system cannot be accurately obtained. Further, when the subject of single crystal is protein-ligand complexes or some macromolecule, making a pure single crystal sample can be very difficult.

In spectrum d, the band changes around 1461, 1052 and 765 cm^{-1}

corresponding to the buffer bands are also observed. This validated that in the DCDR method, the proteins remain partially hydrated, since the buffer signal is kept in the spectrum. However, it is not possible to determine accurately the degree of protein hydration within each deposit as shown in the study by Ortiz et al..²⁶ The other band changes are observed are changes in the relative intensity, while most band shapes in solution sample are retained well in DCD sample.²⁹ The solution system mimics natural biological environment the most accurately. Nevertheless, the acquisition time is very long as compared to DCD and single crystal Raman methods. Moreover, a long acquisition time results in a more complex spectrum due to cosmic ray interference. Additionally the signal-to-noise ratio is much lower in solution than with DCDR or single crystal Raman techniques.

The DCDR spectra are more similar to the solution Raman spectra than to the single crystal Raman spectra. Other than that, the better resolution, higher signal-to-noise ratio, shorter acquisition time all make the DCDR method preferred for biological systems. After comparing the spectra of all three Raman methods presented here, DCDR is chosen as the main study method for its convenient and informative application to biological systems.

3.2 Reproducibility of DCDR Method

Figure 3.2 demonstrates the high reproducibility obtained with the DCDR method. Trace d is a buffer spectrum. Trace a and b are the raw spectra of G2E6 H115W from different deposits. Trace c is the difference between a and b. Comparing

the difference spectrum c with the buffer spectrum d, the major bands around 1450, 1060 and 780 cm^{-1} resulted from the different contribution of the buffer signal. Similar reproducibility was obtained for other G2E6 wild-type and mutants samples.

Figure 3.2 Reproducibility of DCD protein Raman spectra. a) and b) Average spectrum of 5 acquisitions of two different G2E6 H115W depositions. c) Difference spectrum between trace a and b. d) Spectrum of buffer 50mM Tris-HCl containing 50 mM NaCl and 1 mM CaCl_2 at pH 8.

Figure 3.3 is a raw spectrum of paraoxon (0.13 M in 100% methanol and mixed with buffer used in enzyme samples by 1 to 500 ratio), and the difference spectrum between two trials. The environment of small molecules is simpler than in protein systems, thus the spectra of paraoxon are characterized by less band overlapping and sharper band shapes, making each vibrational mode more evident and distinct. In addition, the high reproducibility of the DCDR spectra is indicated by the

difference spectrum trace c. Generally speaking, the difference spectrum validates the high reproducibility of the paraoxon DCDR spectra.

Figure 3.3 Reproducibility of DCD small molecules Raman spectra. a) and b) Average spectrum of 5 acquisitions of two different paraoxon depositions. c) Difference spectrum between trace a and b. d) Spectrum of buffer 50mM Tris-HCl containing 50 mM NaCl and 1 mM CaCl_2 at pH 8.

3.3 Paraoxon/PON1 System Studies

3.3.1 Raman Band Assignment of the Spectra of G2E6 H115W

In Figure 3.4 we present the Raman spectrum of G2E6 mutant H115W. The approximate assignments of the major bands are listed in Table 3.1. The Raman bands in the region of $1800\text{--}400\text{ cm}^{-1}$ of the enzyme spectra can be ascribed to two

fingerprint band groups: bands arising from the peptide backbone signals $-C_{\alpha}-CO-NH-C_{\alpha}-$ and from the amino acid side chains signals.

Figure 3.4 The DCD Raman spectrum of G2E6 H115W without buffer.

The Peptide Backbone

Two useful regions in the enzyme spectrum are assigned to amide I and amide III modes. Amide I is primarily a carbonyl stretching mode, which is between $1640-1680\text{ cm}^{-1}$. The amide III band combines both in-plane N-H bending and C-C stretching motions, which is expected in the interval from $1230-1310\text{ cm}^{-1}$. The Raman frequencies and band shapes of amide I and amide III bands are very sensitive to the protein secondary structure. Therefore, important information about the protein secondary structure can usually be obtained through these two band regions. In the

spectrum of H115W, the amide III bands around 1242 cm^{-1} (β -sheet) were much stronger than the bands around 1267 cm^{-1} (random) and 1300 cm^{-1} (α -helix). This matches with the recent structural study of G2E6, a six-bladed β -propeller, which indicates that the β -sheets predominate the overall backbone conformation of the enzyme. In addition, the amide I bands also showed a similar relationship. The intensity of the band at 1669 cm^{-1} (β -sheet) is higher than those of bands around 1656 cm^{-1} (α -helix or random). Furthermore, the band around 971 cm^{-1} (the C-C stretching vibration associated with β -sheet conformation) was also strong for H115W. These spectral properties all confirm the β -sheet domain structure in G2E6 and are in good agreement with the crystal structure in the literature.^{1,4}

The Amino Acid Side Chains

The Raman bands of the amino acid side chains can be roughly grouped into three types, those are relating to aromatic amino acids, aliphatic amino acids and amino acids containing disulphide bridges and sulphur. The bands at 1367, 1335, 1134 and 766 cm^{-1} were all assigned to the aromatic amino acid tryptophan (Trp). The band at 1335 cm^{-1} and a weak shoulder at $1369\text{-}1365\text{ cm}^{-1}$ were assigned to the Fermi resonance vibration of Trp residues. The intensity of the band around 1367 cm^{-1} is usually used as a marker for the hydrophobicity of the indole ring microenvironment. The band at 766 cm^{-1} involves an indole ring-breathing vibration and is a potentially useful analytical indicator of tryptophan in proteins.

The bands at 1603, 1206 and 1001 cm^{-1} are assigned to phenylalanine (Phe).

The intense band centered at 1001 cm^{-1} is assigned to the ring-breathing vibration. This band can be used as an internal standard, since it is not sensitive to the conformational change with the protein structure under most circumstances.

The bands of tyrosine (Tyr) include those around 1603, 1206, 971, 846 and 825 cm^{-1} . The Fermi resonance doublet of Tyr around 850 and 830 cm^{-1} is sensitive to the changes of the local environment of the phenol ring (specifically by the hydrogen bonding state of the phenolic OH group). Thus the ratio of intensities of bands (I_{850}/I_{830}) can be used as an indicator of the hydrogen bonding interactions of the phenolic OH in the Tyr residues.

Several bands in region $1104\text{--}1054\text{ cm}^{-1}$ are assigned to CC and CN vibrations of aliphatic amino acids. Shoulders around 1384 cm^{-1} were assigned to the symmetric stretching vibration of COO^- of carboxylic amino acids. The stretching vibration band of C-S side chain in cysteine appeared at 672 cm^{-1} indicating that the C-S bond conformation is gauche in cysteine (Cys) residues. The weak band at 525 cm^{-1} indicated a trans-gauche-trans geometry of the SS stretching vibrations of disulphide bonds in the Cys-SS-Cys residues.³⁰⁻³³

3.3.2 Raman Band Assignment of Paraoxon and Its Hydrolysis Products

Figure 3.5 shows the DCD Raman spectrum (trace a) and the computational Raman spectrum (trace b) of paraoxon. The major bands in the two spectra are well matched. Not only do the major bands, such as bands around 1600, 1350, 1240, 1120 and 900 cm^{-1} , all show up in both Raman spectrum, but also the relative intensities of

the bands agree.

The approximate assignments of the major bands in DCDR paraoxon spectrum are listed in Table 3.2.³⁴ The strongest bands around 1345 and 857 cm^{-1} are assigned to the NO_2 stretching and bending vibrations. The band near 1230 cm^{-1} is assigned to the C-O stretching vibration of the hydroxyl group. The band around 1293 cm^{-1} is assigned to C-H bending vibration of benzene ring.³⁵

Figure 3.5 Comparison of DCD and computational Raman spectra of paraoxon. a) DCD Raman spectrum. b) Computational Raman spectrum.

Figure 3.6 is the computational Raman spectrum of paraoxon (trace a) and its hydrolysis products p-nitrophenol (trace b) and diethyl phosphate (trace c). The reaction mechanism for paraoxon hydrolysis is presented in [Figure 1.4]. The band

near 1256 cm^{-1} of paraoxon spectrum is assigned to the C-O stretching vibration of the hydroxyl group, thus it will be weakened when paraoxon is hydrolyzed into p-nitrophenol and diethyl phosphate due to the substitution of the hydroxyl group by phenyl group.

Figure 3.6 The computational Raman spectra of paraoxon and its hydrolysis products. a) Paraoxon. b) p-Nitrophenol. c) Diethyl phosphate .

3.3.3 Reaction Ratios

The spectra of different reaction ratios of H115W vs. paraoxon are shown in Figure 3.7. Three different reaction ratios (H115W:paraoxon) were chosen, i.e. 1:50, 1:500 and 1:5000.

Figure 3.7 Comparison of DCD Raman spectra at different reaction ratio of paraoxon/H115W system. a) 1:50. b) 1:500. c) 1:5000. d) DCD Raman spectrum of paraoxon without buffer.

When compared with the spectrum of paraoxon (bottom), an obvious difference between these reactions spectra is observed. Trace a, the 1:50 spectrum, is dominated by Raman bands of the enzyme, even the band around 1350 cm^{-1} , which is the band of paraoxon with highest intensity, is not observed. When the concentration of paraoxon is increased by three orders in the reaction, trace c, the 1:5000 spectrum, is dominated by the paraoxon bands. There are two bands with almost the same intensity around 1350 cm^{-1} assigned to the NO_2 stretching vibration in which one is from paraoxon and the other is from its hydrolysis product p-nitrophenol. The presence of the double bands indicates that a large quantity of paraoxon still remained in the deposition after the hydrolysis reaction at reaction ratio 1:5000. The intensities

of the Raman bands from the enzyme H115W are relatively low, and contribute little useful reaction information about the enzyme itself. The 1:500 reaction ratio (trace b) is chosen for all the enzyme vs. paraoxon reactions due to the best band information provided by their spectra. The bands from paraoxon hydrolysis products are most distinct and with relatively higher intensities among all three ratios. Furthermore, the major bands of the enzyme are all maintained with proper intensities and band shapes to give useful information about the enzyme during the reactions.

3.3.4 Paraoxon/G2E6 H115W system

A difference spectrum was produced by subtracting the enzyme H115W spectrum from the complex spectrum as seen in Figure 3.8 ([Difference trace c] = [complex] – [H115W] × f1, where f1 is a scaling factor). The spectrum contains information on the paraoxon hydrolysis reaction as well as the conformational changes of the enzyme upon the reaction. Further difference subtraction is performed between trace c and the paraoxon spectrum ([Difference trace e] = [trace c] – [paraoxon] × f2, where f2 is a scaling factor), thus the hydrolysis products can be clearly observed without the overlap from the bands of paraoxon.

The Raman band at 1227 cm⁻¹ decreased in intensity and several bands near 1320, 859 and 1287 cm⁻¹ changed in trace e, when compared with the spectrum of paraoxon only. The band near 1227 cm⁻¹ is assigned to the C-O stretching vibration of the hydroxyl group. The disappearance of this band indicates that the hydrolysis of paraoxon happens in the enzyme-ligand complex, after which the hydroxyl group on

the benzene ring is substituted by a phenol group and the vibrational mode devoted to the previous C-O band is changed. The bands at 1320 cm^{-1} and 859 cm^{-1} are assigned to the NO_2 stretching and bending vibrations, respectively. The red shift of the band at 1320 cm^{-1} and slight blue shift of the band at 859 cm^{-1} are observed in the complex spectrum. The intensity of the band around 1287 cm^{-1} , which is assigned to C-H bending vibration of benzene ring, increased significantly. These band changes are in good agreement with the RB3LYP simulations (performed by the Hadad group, private communication), which are strong evidence of the paraoxon hydrolysis products p-nitrophenol.

Figure 3.8 DCD Raman difference spectrum of paraoxon/H115W system.

- a) Paraoxon/H115W at 1:500 with buffer.
- b) G2E6 H115W with buffer.
- c) [Difference trace c] = [complex] – [H115W] $\times$ f1.
- d) Paraoxon without buffer.
- e) [Difference trace e] = [trace c] – [paraoxon] $\times$ f2.

These band changes reflected in the difference spectrum are in good agreement with theoretical calculations (see Figure 3.6). The formation of the paraoxon hydrolysis product, p-nitrophenol is observed using the DCDR method.

As indicated by Khersonsky et al., PON1 is actually not a phosphotriesterase but a lactonase. Since paraoxon has a leaving group pK around neutral pH (7.14), PON1 possesses no mechanism for stabilization of the negative charge of the leaving group.³⁶ Further, the phosphotriester/PON1 enzymatic systems are diffusion-controlled reactions, in which the rates of phosphotriester substrates hydrolysis are dependent upon the pK of the leaving group.³⁷ Paraoxon does not bind to PON1 in reactions.^{36,37} Therefore, no protein conformational changes were expected, and the difference spectra of G2E6 wild-type and mutants were not subtracted in this case.

3.3.5 Comparison of G2E6 wild type, H115W and H115T

The difference spectra ($[\text{Difference}] = [\text{complex}] - [\text{enzyme}] \times f_1 - [\text{paraoxon}] \times f_2$, where f_1 and f_2 are scaling factors) of wild-type G2E6 (trace b) and two of its mutants H115W (trace a) and H115T (trace c) were compared with the spectrum of paraoxon in Figure 3.9.

The band near 1229 cm^{-1} that had the highest intensity in each difference spectrum was not present in the paraoxon/H115W system. The paraoxon/H115W system had a characteristic of the paraoxon/wild-type system and showed relatively higher intensity in the paraoxon/H115T system. Since the bands are assigned to the C-O stretching vibration of the hydroxyl group, the turnover rate is inversely

proportional to the intensity of the band, indicating that a significant concentration of paraoxon remains after the reaction. In other words, the lower the intensity of the band after enzymatic reaction, the higher the turnover rate the enzyme has. Thus the order of the corresponding complex turnover rate: H115W > wild-type > H115T is evident when the spectra are compared.

Figure 3.9 Comparison of DCD Raman spectra of paraoxon with different G2E6 enzymes. a) Paraoxon/H115W. b) Paraoxon/G2E6 wild-type. c) Paraoxon/H115T. d) Paraoxon without buffer.

Furthermore, the relative intensities and the band shapes of the bands related to the hydrolysis products also reflect the same trend. Changes of the bands near 1345 cm^{-1} are most evident [Figure 3.9]. The relative intensity of the band is strong and the

band shape is sharp in the H115W-paraoxon complex (trace a). In contrast, the relative intensities decreased in the order of in wild-type-paraoxon (trace b) and in H115T-paraoxon complexes (trace c). The band shapes also broaden in the same order.

Figure 3.10 The binding conformation of paraoxon with a) HuPON1 wild-type. b) H115W mutant.¹⁰

The Raman spectra obtained via DCDR show the same results as indicated by enzyme kinetic experiments.^{4,11} H115 is considered to play a central role in the catalytic mechanism towards OPs. However, the mutants at this residue do not all eliminate the paraoxonase activity. The replacement of histidine by tryptophan increased the turnover rate towards paraoxon significantly.^{1,2} One possible explanation to this observation is the steric effects of the side chains. The tryptophan residue (W), which is larger than the histidine residue (H), prevents paraoxon from accessing the catalytic site of the enzyme [Figure 3.10], while the smaller size

threonine residue (T) does not [Figure 3.11].⁴ Hu et al. also predicted that there is a cluster of strong aromatic stacking interactions formed between the nitrophenyl group of paraoxon and the indole side chain of the mutated residue of H115W.¹⁰

Figure 3.11 The molecule structures of His, Trp and Thr residues.

The results of our studies support that H115 residue is important for paraoxonase activity study of PON1, but it is not directly involved in the catalytic mechanism of PON1 towards OP substrates. Most likely, H115 functions as a substrate-selective residue near the active site of PON1, and is essential for proper positioning and orientation of the substrates in the catalytic hydrolysis reactions.^{4,6,36}

CHAPTER 4 CONCLUSIONS

4.1 Paraoxon/PON1 System Studies

Recombinant PON1 G2E6 wild-type and two mutants H115W, H115T reacting with paraoxon were studied by DCD and difference Raman spectroscopy. The changes of Raman bands at 1227, 1320, 859 and 1287 cm^{-1} are consistent with the formation of the paraoxon hydrolysis product, p-nitrophenol, and in good agreement with theoretical calculations.

The turnover rate for H115W increased upon mutation and H115T decreased. The bands near 1229 cm^{-1} , which are assigned to the C-O stretching vibration of the hydroxyl group, disappear in H115W-paraoxon complex, show weak intensity in wild-type-paraoxon complex, and show relatively higher intensity in H115T-paraoxon complex. Furthermore, the relative intensity of the bands related to the hydrolysis products, such as bands near 1345 cm^{-1} , leads to the complex turnover rates of: H115W > wild-type > H115T.

The results of our studies support that H115 is not directly involved in the catalytic mechanism of PON1 towards OP substrates, and it functions more likely as a substrate-selective residue near the active site of PON1.

4.2 Future Directions

Additional experiments are necessary to support the above findings. More

G2E6 mutants can be studied under the same conditions. The steric effects controlled by different mutations at H115 residue should be examined more thoroughly. Moreover, the inhibitors of PON1, like DcMPA and DcPPA, which bind to the enzyme during the reactions, should be employed in the work. Thus the conformational changes of the enzyme as well as the bonds appearing upon binding can be detected.

APPENDIX TABLES OF BAND ASSIGNMENTS

Table 3.1 Approximate band assignments of G2E6 H115W mutant.³⁰⁻³³

Band (cm ⁻¹)	Assignment
1669	Amide I(β -sheets)
1656	Amide I(α -helix, random)
1603	Try, Phe
1464	δ (CH ₂)
1384	ν_s (COO ⁻)
1367	Trp(Fermi doublet)
1335	Trp(Fermi doublet)
1310	Amide III(α -helix)
1297	Amide III(α -helix)
1267	Amide III(random)
1248	Amide III(random)
1242	Amide III(β -sheets)
1206	δ (CH)-Phe, Tyr
1134	Trp
1104	ν (C-C), ν (C-N)
1083	ν (C-C), ν (C-N)
1053	ν (C-C), ν (C-N)
1001	Ring breathing-Phe
971	ν (C-C)(β -sheets), Tyr
846	Tyr(Fermi doublet)
825	Tyr(Fermi doublet)
766	Trp
672	ν (C-S)-Cys
525	ν (S-S)tgt

Table 3.2 Approximate band assignments of paraoxon.^{34,35}

Band (cm ⁻¹)	Assignment
1591	$\gamma(\text{C-C})$
1521	$\gamma(\text{NO}_2)$
1463	$\delta(\text{CH}_2)$
1345	$\gamma(\text{NO}_2)$
1293	$\delta(\text{CH}_2)$
1228	$\gamma(\text{C-O})$
1157	$\delta(\text{O-H})$
1109	$\delta(\text{CH}_2)$
1046	Ring breathing
856	$\delta(\text{NO}_2)$

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