Chapter II

Oxidation of Paraoxonase in serum differentially modifies its substrate specificities
**Introduction**

The fundamental property of enzymes that makes them important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

- **Absolute specificity** - the enzyme will catalyze only one reaction.
- **Group specificity** - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
- **Linkage specificity** - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
- **Stereo chemical specificity** - the enzyme will act on a particular stearic or optical isomer

In fact, greater the importance of an enzyme to a pathway greater is its specificity (Worthington CE 1972). Enzymes often show promiscuity, i.e. to catalyze more than one reaction (catalytic promiscuity) or to show broad substrate specificity (substrate promiscuity). Enzyme promiscuity has been related to the evolvability of proteins, as a mechanism that can be traced in the divergence of enzyme families and super families. Inspired by this mechanism of natural evolution, enzyme promiscuity can serve in the lab as a starting place for directed evolution of enzymes capable of new functions, such as in bio catalysis, degradation of novel synthetic chemicals, or in other innovative ways in the context of synthetic biology. However, the mechanism by which a single catalytic site binds promiscuously to diverse substrates is not well understood (Ben-David M et al 2012). Since a large number of enzymes are known to bind to promiscuous substrates, promiscuity may be an inherent property of enzymes. Although promiscuous activities are supposed to be less efficient than the primary activity, they are known to match or even exceed the native activity (Garcia-Seisdedos et al 2012).
The enzyme that has received most attention for its promiscuous substrates is Paraoxonase (PON1). In fact it is named for its promiscuous activity and not its primary activity which is lactonase activity (David A Stoltz et al 2009). PON1 has been found to possess arylesterase activity and is shown to act on a wide variety of unrelated substrates. For example, PON1 has been shown to have peroxidase activity on hydrogen peroxides and lipid hydroperoxides (Yilmaz 2012). It hydrolyzes aryl esters, phosphate esters and lactones. The interest in the study of PON1 is not only because of its promiscuous activity on organophosphates, but also because of its association with the cardioprotective function of HDL.

The antioxidant properties of HDL are attributed to a large extent by the PON1 associated with it. Although other enzymes and proteins have been shown to have antioxidant activities attributed to PON1, PON1 knock out animals were more prone to LDL oxidation compared with control animals (Litvinov 2012), while mice over expressing PON1 are much more resistant to atherosclerosis development than wild-type. When PON1 was transfected to mice deficient in PON1, it decreased the levels of peroxides, lowered the release of superoxide and increased the intracellular levels of reduced glutathione. HDL in the presence of PON1 inhibitors loses its capacity to inhibit the accumulation of lipid peroxides under oxidizing conditions (Aviram M et al 1998).

Attempt to correlate the activities of PON1 with the cardio protective functions of HDL were not successful since the PON1 activity and concentration are highly variable in humans. The quality and quantity of enzyme in serum is likely to be more important in an individual for risk of developing cardiovascular disease. There is a wide variation (up to 13-fold) of PON1 serum concentration and activity by about 40 fold between individuals and even within the same genotype groups (Richter RJ and Furlong CE 1999). In addition to genetic polymorphisms, PON1 activity can be modified by acquired factors such as diet, lifestyle and disease. It is likely to be the functionality of the enzyme and not simply the genotype that is important in the interaction of PON1 with Coronary artery disease (CAD) and it is essential that PON1 serum concentration and/or activity (PON1status) also be measured in addition to gene polymorphism while evaluating its association with CAD. A few studies, which
have included PON1 concentration and/or activity data, have found that PON1 concentration was reduced in CAD patients and that this reduction was independent of PON1 genotype. Various polymorphisms attributes to their variation in activity. The PON1 gene coding region polymorphism affects the catalytic activity of PON1 whereas that of promoter region affects the PON1 gene expression. The association of these polymorphisms and factors modulating serum PON1 activity determine the PON1 status. PON1 activity can also be modified by variety of physiological modulators such as oxidation and non-enzymatic glycation resulting in a loss of catalytic activity. In this chapter, we have assayed the PON1 activity in 242 subjects to find out whether the three substrate activities correlate within themselves. We have also modified the PON1 activities by oxidation to investigate whether the oxidation modified the substrate binding and catalysis of the three substrates equally or differentially.

**Materials and Methods**

Phenyl acetate, Paraoxon, Cibacron Blue 3GA Sepharose and Deoxycholate were purchased from Sigma Aldrich, USA. Homocysteine Thiolactone (HTL) from Himedia, DTNB (Ellman’s reagent, 5’, 5-dithiobis (2-nitrobenzoic acid) and all other chemicals were of analytical grade purchased from SRL Chemicals.

**Serum**

Blood from 242 subjects recruited in this study was collected by vein puncture. It was allowed to clot and centrifuged at 3000 rpm for 10 mins. Serum was separated and stored at -20°C until use.

**Preparation of HDL**

Human serum HDL was isolated by density gradient ultracentrifugation using a Sorvall WX100 Ultracentrifuge. The centrifugation was carried out in a fixed angle rotor T-1250 (SL. 6102823) with vertical tubes of 36 ml capacity. Appropriate amount of KBr was added to the serum to get a density of 1.3 g/ml. Normal saline of density 1.006 g/ml was taken in the centrifuge tubes and the KBr added serum sample was layered below the saline layer without disturbing the two layers. Centrifugation was carried out at 45,000 rpm for 3 hrs at 4°C in a vertical tube rotor. The HDL
fraction was collected from the top by aspiration. Fractions were pooled and dialyzed to remove KBr (Naidu Ak and Thippeswamy NB 2002).

**Elution of PON activities from Cibacron Blue 3GA Sepharose column**

Nonspecific affinity chromatography Cibacron Blue 3GA Sepharose was washed and equilibrated in buffer A (50mM Tris, 3M NaCl, 1.5mM CaCl$_2$, 20 µM EDTA pH 8.0). The ratio of gel to serum should be 4:5 and serum was mixed with an equal volume of buffer A. The serum-salt buffer mixture was incubated for 1 hr at 4°C and then loaded on cibacron blue gel column, the eluate was recycled through the column twice. The eluate was then collected. The column was washed with Buffer A solution which prevents or greatly reduces albumin or other soluble proteins from binding to Cibacron blue gel and thus most of the soluble proteins; other lipoproteins are washed out of column. Next the column was washed with Buffer B which contained 50mM Tris, 1mM CaCl$_2$ pH8.0. This was collected as one fraction. Then the column was eluted with 25mM Tris, 20% Glycerol, 1mM CaCl$_2$, with 0.1% Deoxycholate and collected as one fraction. OD of the eluted fractions was measured at 280nm (Gan KN et al 1991). PON activities in the eluted fractions were monitored by assaying the Aryl esterase, Paraoxonase and Thiolactonase activities.

**Paraoxonase assay with phenyl acetate as substrate**

PON1 activity towards phenyl acetate (PA) was measured spectrophotometrically at 270nm in an automated Shimadzu UV-1601 UV-Visible spectrophotometer. The assay mixture included 2mM phenylacetate, and 10mM Tris HCl buffer, pH 8.3 containing 2mM CaCl$_2$. The reaction was initiated by adding serum or HDL. Absorbance was monitored continuously for 3 mins. Nonenzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The molar extinction coefficient, $e_{270}$, for the reaction was 1,310 M$^{-1}$cm$^{-1}$. One unit of paraoxonase activity is defined as 1µmol of phenylacetate hydrolyzed per minute under the assay conditions described above (Eckerson HW et al 1983).
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Paraoxonase assay with paraoxon as substrate

PON1 activity towards paraoxon (PON) was quantified spectrophotometrically using 10 mM Tris-HCl buffer, pH 8.3 containing 2mM CaCl$_2$ and 2mM paraoxon. The reaction was initiated by adding serum or HDL and monitored continuously for 3 minutes at 25°C by measuring the appearance of p-nitrophenol at 405 nm in an automated recording Shimadzu UV-1601 UV-Visible spectrophotometer. The blank (incubation mixture without serum) was run simultaneously to correct for spontaneous breakdown of substrate. One unit of PON1 activity is defined as 1µmol of p-nitrophenol formed per minute. The amount of p-nitrophenol was calculated from the molar extinction coefficient at pH 8.3, which was 18.05x10$^3$ M$^{-1}$ cm$^{-1}$ (Furlong CE et al 1989).

Thiolactonase Activity with Homocysteine Thiolactone (HTL) as substrate

Solution of Homocysteine Thiolactone and DTNB (Ellman’s reagent, 5’, 5'-dithiobis (2-nitrobenzoic acid) in methanol was prepared fresh daily. Thiolactonase activity was determined by the following method. The cuvette contained 0.5 mM Homocysteine Thiolactone in PON buffer (10mM Tris HCl, 2mM CaCl$_2$) and DTNB (0.5 mM) in a total volume of 1 ml. The reaction was initiated by the addition of enzyme (either serum or HDL or purified protein) and increase in absorbance at 412 nm was recorded. Blanks without enzyme were used to correct the spontaneous hydrolysis of Homocysteine Thiolactone. An e412nm = 7000 OD/M value was used to calculate the activity (Khersonsky O et al 2006).

Assay of HDL-Cholesterol

HDL-Cholesterol was assayed by the Randox two point direct assay kit according to the instructions given in the kit.

Oxidation of PON1 by Copper sulphate

Oxidation of HDL was carried out according to the procedure of Aviram et al (2000) with slight modification. Briefly, 1mg/ml of HDL protein was oxidized by incubating in 1 ml of Phosphate buffer saline pH 7.4 containing 1µmol/L CuSO$_4$ at room temperature for 1 hr.
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PON activities in serum samples

Serum paraoxonase activities using Phenyl acetate, Paraoxon and Homocysteine thiolactone as substrates were determined as described above.

Statistical Analysis

Results are expressed as Mean ± SD. Pearson’s Product-moment correlation was carried out for correlation studies. The Paraoxonase activities with the three substrates were sorted from the lowest to the highest and divided into 9 class intervals. The frequency of the activity in each class interval was plotted against the class interval.

Results

The Paraoxonase activity of serum and HDL isolated by ultracentrifugation for the three substrates is shown in Table 2.1.

Table 2.1 Paraoxonase activity for different substrates in serum and HDL

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Serum (µmol/min/ml)</th>
<th>HDL (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyl acetate</td>
<td>1.02 ± 0.10</td>
<td>2.23 ± 0.39*</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>0.14 ± 0.07</td>
<td>2.57 ± 0.25</td>
</tr>
<tr>
<td>Homocysteine thiolactone</td>
<td>0.14 ± 0.02</td>
<td>1.25 ± 0.34</td>
</tr>
</tbody>
</table>

* µmol/min/mg

Paraoxonase activity in serum and HDL were determined for the three substrates as described in the methods. Results are Mean ± SD n=3

When purified HDL was subjected to oxidation, the arylesterase activity and paraoxonase activity decreased to 10-20% of the original activity. However the Homocysteine Thiolactonase activity (HTase) increased to almost two fold (Figure 2.1).
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Figure 2.1. Effect of copper catalysed oxidation of HDL on PON activity

HDL was oxidized using copper ions as described in methods. The PON1 activity of unoxidized and oxidized HDL was determined using Phenyl acetate (PA), Paraoxon (PON) or Homocysteine thiolactone as substrates.

The elution of Arylesterase, Paraoxonase and Homocysteine Thiolactonase (HTase) activity from cibacron blue sepharose column is summarized in Table 2.2.

Table 2.2. Elution of Paraoxonase from Cibacron blue sepharose column

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Arylesterase</th>
<th>Paraoxonase</th>
<th>Thiolactonase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Unbound</td>
<td>87.8</td>
<td>97</td>
<td>86.5</td>
<td>89.3</td>
</tr>
<tr>
<td>Wash</td>
<td>12.0</td>
<td>2.3</td>
<td>12.3</td>
<td>10.1</td>
</tr>
<tr>
<td>Elute</td>
<td>0.49</td>
<td>0.7</td>
<td>0.5</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Serum was fractionated on cibacron blue sepharose column as described in the methods. Results are expressed as percent protein and activities. Results are mean of two independent experiments.

All the three enzyme activities were largely eluted in the unbound fraction. Paraoxonase and HTase activities were also eluted in the wash fraction. Only a very small fraction of the total activity was bound on the column. The distribution of PON activities in the serum of 242 subjects is shown in Figure 2.2.
Figure 2.2. Frequency distribution of paraoxonase activities for the different substrates and for HDL-C

*PON* activity was determined using PA, PON or HTL as substrates. The frequency distribution of activities was tabulated as described in methods and compared with the distribution of HDL-C in these subjects.

The activities of paraoxonase for the three substrates and for HDL-C were divided into nine class intervals and the frequencies were plotted against class intervals. The three enzyme activities were independently distributed in the serum. The HDL-C distribution paralleled the PA activity, but not PON, or HTase activities. Correlation coefficient of three activities in the serum of 242 subjects is show in Table 2.3.

**Table 2.3. Correlation coefficient r for PON activities for different substrates in Serum**

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA vs. PON</td>
<td>0.178</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PA vs. HTL</td>
<td>0.104</td>
<td>NS</td>
</tr>
<tr>
<td>PON vs. HTL</td>
<td>0.053</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Pearson’s correlation coefficient for the correlation of PA activity with PON and HTL activities as well as correlation of PON with HTL were determined as described in methods.*
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Only PA and PON correlated. Whereas, there was no correlation between PA and Thiolactonase or PON and Thiolactonase. The correlation of HDL-C levels and the catalytic activities are shown in Table 2.4.

Table 2.4. The correlations of HDL-C levels with PON activities

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>P&lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-C vs. PA</td>
<td>0.215</td>
<td>Significant</td>
</tr>
<tr>
<td>HDL-C vs. PON</td>
<td>0.122</td>
<td>Significant</td>
</tr>
<tr>
<td>HDL-C vs. HTL</td>
<td>0.0937</td>
<td>NS</td>
</tr>
</tbody>
</table>

Pearson’s correlation coefficients for the correlation of HDL-C with PON1 activities using PA, PON and HTL as substrates were determined as described in methods. HDL-C correlated with PA and PON but not with thiolactonase.

Discussion

Paraoxonase is a calcium dependent hydrolase. The name paraoxonase refers to its promiscuous activity rather than its main physiological activity which is lactonase (Ben-David M et al 2013). The enzyme has been reported to be a six-blade β propels with two calcium ions in its central cavity (Michal Harel et al 2004). The structural calcium is at the bottom of the active site (Akhileshwar Khanal 2009). In its hydrolysis of aromatic esters and lactones, the catalytic histidine dyad, His115-His134, deprotonates a water molecule to generate the hydroxyl anion. Calcium stabilizes resulting tetra hydroxy anion intermediate (Khersonsky O and Tawfik DS 2009). Cleavage of Paraoxon differs from this mechanism. The P-0 bond cleavage occurs via a pentavalent intermediate. It has been shown that mutations of His 115 decreases both lactonase and aryl esterase activity but enhances the paraoxonase activity. The enzyme uses different active site conformation to catalyze these multi substrate reactions (Ben-David M et al 2012).

In our study, we found that the oxidation of PON1 resulted in a loss of both arylesterase activity and paraoxonase activity but enhanced the Thiolactonase activity suggesting that oxidative modification of amino acids in the active site residues may affect the catalysis of these three substrates.
In addition to catalytic histidines, tryptophan residues have also been shown to be essential for PON activity. We have observed that the paraoxonase activity is lost by specific modification of Lysine, tyrosine and tryptophan (data not shown). Cys, Met, Tyr and Lys have been identified in proteins to be highly susceptible to oxidation. These amino acids are also present in PON1 (Shacter E 2000).

The distribution of paraoxonase and thiolactonase activities in the serum of subjects in the study showed a non overlap of activities. PON activity was skewed to the lower activity levels. This is consistent with our earlier observations (Prasad MJ et al 2009). The lack of correlation between the activities measured with three substrates for PON1 in serum may be because of endogenous oxidation of the PON1 to different extents in different individuals. Our earlier studies have shown that PON in our population is modified even at a relatively young age (Prasad MJ et al 2010).

The cibacron blue sepharose column chromatography is a method for the purification of hydrophobic molecules and can be applied for the purification of HDL particle. However in our study bulk of the enzyme activities associated with paraoxonase eluted in the unbound fraction. Only less than 1 percent of the total activity was held in the column. Since all the paraoxonase in the serum is expected to be tightly associated with HDL, why bulk of the activities were eluted in the unbound fraction is not known. In a proinflammatory stress it has been shown that the paraoxonase is displaced from HDL (Ansell BJ et al 2007). Whether the paraoxonase in the unbound fraction represents this displaced fraction of PON1 is not known. Interestingly the HDL purified by ultracentrifugation did not have the three enzyme activities in the same proportion as that in the serum (Table 2.1). This suggests that there may be an independent pool of paraoxonase not associated with HDL in the serum. Since PON1 activity is associated with HDL in the blood of humans and since HDL-C negatively correlates with risk of cardiovascular diseases, attempts have been made to correlate the HDL-C with risk of CVD. Our studies have shown that even when serum is oxidized the HDL-C does not change (Mamatha AM et al 2012) but the PON activity measured by PA or PON as substrate is decreased. Hence it appears that the functionality of HDL and its cholesterol content are not correlated. Since PON1 is easily measurable in a clinical laboratory by a simple colorimetric assay and
also susceptible to modification, it appeared as a promising candidate to assay the functionality of HDL. However PON1 activity has been shown to vary as much as 40 fold in the serum (Jyothi M 2011). PON1 exists in at least two known polymorphic forms namely the M55L and the Q192R. These polymorphisms have their effects on the activity of PON1. We do not know the distribution of such polymorphic forms in our population. Studies from our laboratory have shown novel polymorphisms in the promoter region of PON1 gene which also affect the PON1 activity (Jyothi M 2011). Our studies are in agreement where we have shown differential modification of the three enzyme activities. This may be the reason why PON activity and HDL did not correlate in our studies. The negative skewness of distribution of PON activities suggests that the PON activities are modified in the serum.

It is interesting to note that the thiolactonase activity of the PON1 had increased after oxidation, whereas the promiscuous activities had decreased by a large extent. The question as to why the thiolactonase activity should increase during oxidation is intriguing. It is possible that oxidation may give rise to potential lactones which could be toxic, add hence, PON1 would act as a detoxifying enzyme.

Oxidation by the Baeyer-Villiger reaction can produce lactones by the Bayer-Villiger monooxygenases, which converts ketones to lactones (Schulz et al 2005). Hence it is possible that the oxidation that generates the lactones also activates the PON1 enzyme for its detoxification.

**Conclusion**

PON1 activity in the serum reflects the activity left over after differential inactivation of the different substrate specificities by endogenous modifying agents. Oxidative stress and glycate stress are the two potential and prominent modifying agents that are relevant to PON1.
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References


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Oxidation of Paraoxonase in serum differentially modifies its substrate specificities


