Interaction of Recombinant Paraoxonase-1 with Reconstituted High-Density Lipoproteins

THESIS

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Abstract

Overwhelming evidence suggests that low levels of high-density lipoproteins (HDL) increase one’s odds of developing coronary artery disease (CAD). This phenomenon could be due in part to the hydrolase activity of the enzyme human serum paraoxonase-1 (huPON1) that is found bound to the HDL in the circulatory system. Many studies have indicated lowered atherogenic effects due to raised huPON1/HDL levels. In order to fully understand these correlations, more insight into how the interaction of huPON1 and HDL may be delivering its beneficial properties is needed. In the current study, the interactions between reconstituted HDL (rHDL) particles and fluorophore-labeled variants of PON1 (G3C9-ybbR and five other variants) were probed via fluorescence anisotropy, effects on arylesterase and paraoxonase activities, and changes in arylesterase activity upon binding to rHDL. While the mutations to the hydrophobic signal sequence (L12A and L12K) did not appear to have significant effects on the arylesterase or paraoxonase activities, the mutations on the active site lid (W194A and W194K) caused increases in both activities. While the fluorescence anisotropy measurements were unable to reveal anything about the binding affinity, the arylesterase stimulation due to rHDL binding did.
Dedication

This document is dedicated to my loving and supportive husband, Aaron.
Acknowledgements

To begin, I would like to thank my Heavenly Father for the opportunities afforded to me. Without His Holy Spirit, my life would be aimless and confusing. I am thankful He has chosen me to represent Him in spite of my flaws.

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Chapter 1: Introduction

Pertinence of Paraoxonase-1’s Paraoxonase Activity

Beginning in 1946, the HDL-associated human serum enzyme paraoxonase-1 (PON1) was initially discovered to have the ability to hydrolyze organophosphorus compounds (OPs). Dr. Abraham Mazur performed experiments at Edgewood Arsenal in Maryland showing that human and rabbit tissue samples could hydrolyze diisopropyl fluorophosphate (DFP), an organophosphate insecticide known to be toxic to humans. His manometric assay revealed that the liver, kidney, and small intestine tissues, as well as the plasma had the highest activities against this OP (Mazur 1946). Mazur did not know the identity of the enzyme, but these studies were followed by Dr. W.N. Aldridge in the 1950’s. Aldridge identified the enzyme as PON1 and demonstrated its general characteristics, which included proposing that its A-esterase activity should be separated from B-esterases. His statement was based on the findings that diethyl p-nitrophenyl phosphate (paraoxon) could only slightly inhibit the A-type enzymes, whereas the B-types were completely inhibited (Adkins 1993, Costa 2002). This series of discoveries led to the clinical and biological interest in PON1’s A-esterase activity and its ability to metabolize toxic OPs, with both environmental and military implications.

When highly toxic derivatives of triesters of phosphoric acid, such as parathion (metabolized to paraoxon) and diazinon (metabolized to diazoxon), as well as nerve
agents like sarin and soman, are introduced into the body, many devastating reactions occur (James 2006). These compounds are capable of inhibiting the enzyme acetylcholinesterase (AChE). AChE is responsible for degrading acetylcholine, a neurotransmitter for both the peripheral nervous system and the central nervous system. The biological response to acetylcholine includes two major roles. First, when acting as an inhibitory neurotransmitter, it helps reduce a specimen’s heart rate. Second, when acting as an excitatory neurotransmitter, it increases the activities of neuromuscular junctions. In Figure 1, the overall process of synaptic response to acetylcholine can be seen. During AChE inhibition by OPs or nerve agents, AChE loses its ability to eliminate acetylcholine. The build-up of acetylcholine produces a cholinergic crisis, resulting in miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculation, behavioral incapacitation, muscular weakness and convulsions leading to death due to respiratory failure (Duysen 2011).

![Figure 1: Acetylcholine production and transfer at the synaptic cleft](image)

Figure 1: Acetylcholine production and transfer at the synaptic cleft
Clearly a medical response to severely reduce the effects of OP poisoning is needed. Currently, drugs are used to counteract the effects, such as atropine sulfate, oximes, and diazepam. While these treatments successfully prevent death, they are unable to stop post-exposure incapacitation, convulsions or permanent brain damage. By reversibly binding carbamates such as pyridostigmine and physostigmine to AChE, the OPs are unable to reach their target and irreversibly inactivate AChE, but these drugs also cause neurological effects. Alternatively, human proteins designed as bioscavengers are also being used to inactivate the OPs prior to AChE exposure. The short list includes butyrylcholinesterase (BChE), which binds to the OPs irreversibly. The main issue with this bioscavenger is the need for stoichiometric quantities, which can be rather large (Duysen 2011). Due to this, experimentation to produce a bioscavenger that is not consumed is currently the forefront of research. As mentioned above, the human serum paraoxonase-1 (huPON1) has become a viable candidate. Unfortunately, even though this enzyme is non-immunogenic and non-toxic, it has low activity against OPs, albeit some activity. Studies to genetically enhance PON1’s activity towards OPs and nerve agents are the next obvious step, but with a lack of structure-function studies and active site details, not much can be done.

**Pertinence of Paraoxonase-1’s Arylesterase and Lactonase Activities**

While the paraoxonase activity of PON1 is useful in the metabolism of toxic OPs and nerve agents, PON1’s chief biological function is still being debated. In 1993, Mackness et al. proposed that high-density lipoproteins (HDL) and PON1 appear to be protective against low-density lipoprotein (LDL) oxidation (Figure 2) (Mackness 1993).
Figure 2: LDL oxidation leading to atherosclerotic plaque

*In vivo,* the LDL structure can be altered due to oxidation by redox metals, macrophages, smooth muscle or endothelial cells. This modified structure binds to the acetyl low-density lipoprotein receptor of monocyte-derived macrophages. During the modification, lipoperoxides are formed from the LDL phospholipids containing unsaturated fatty acids. The unsaturated fatty acids are broken down to release reactive aldehyde compounds. Phospholipase A₂ converts the aldehyde compounds into lysophospholipids (Mackness 1991). The quick uptake of the oxidized LDL particles by the macrophage cells leads to foam cells and arterial fatty streaks. These are known to be precursors to more advanced lesions, leading to atherogenesis (Lusis 2000).
Many independent studies have shown an inverse relationship between high PON1 activity \textit{in vitro} and low amounts of oxidized lipid products (Mackness 1993, Aviram 1998). Other studies have shown this inverse relationship \textit{in vivo} (Shih 1996, Assmann 2003, Kontush 2003, Mackness 2006). Also, PON1 knock-out mice had high levels of atherosclerotic lesion development, and conversely over expression of PON1 in mice showed protection against lesion development (Shih 1998, Tward 2002). These studies coupled with the 1993 Mackness study mentioned above began an intense search for oxidized lipids that could act as substrates for PON1.

In 2005, two simultaneous publications claimed that PON1’s native function is that of a lactonase (Draganov 2005, Khersonsky 2005). This discovery opened the door for many diverse physiological substrates. The connection of PON1’s relevance to vascular disease was confirmed with activity against 5-hydroxy-eicosatetraenoic acid 1,5-lactone (5-HETEL) and 4-hydroxy-docosahexaenoic acid (4-HDoHE), which are oxidized fatty acid derivatives (Draganov 2005). Also, homocysteine thiolactone indicate thiolactonase activity, possibly a protective mechanism against atherogenic changes (Khersonsky 2005). Finally, homoserine lactones (HSLs), which are used in bacterial quorum sensing, can act as substrates which indicates a possibility of PON1’s protective feature against bacterial infections (Camps 2009).

The clinical relevance of PON1’s lactonase activity and its possible tie to coronary artery disease has become the primary focus of research for this protein. The connection between PON1 and its \textit{in vivo} shuttle, HDL, remains an important area of
interest. Unfortunately, very little is known about this association, and more knowledge is needed in order to better engineer drugs on the atherosclerotic front.

_Human Serum and Recombinant Paraoxonase-1 Characteristics_

Human serum paraoxonase-1 is one of three paraoxonases in the PON family, also containing PON2 and PON3. The genes of all three are found on the long arm of chromosome 7, with PON1 residing between q21.3 and q22.1 (Durrington 2001). While the structure of huPON1 has not been determined, a gene-shuffled bacterially-expressed variant of PON1 has been shown to be a six-fold beta-propeller protein (Figure 3), like that of the diisopropyl-fluorophosphatase (DFPase) found in squid _Loligo vulgaris_ (Harel 2004).

![Figure 3: Structure of gene-shuffled bacterially-expressed variant of PON1; rendered via PyMol from PDB entry 1V04](image)

Figure 3: Structure of gene-shuffled bacterially-expressed variant of PON1; rendered via PyMol from PDB entry 1V04
PON1 is a calcium-dependent glycoprotein with 354 amino acid residues, and has a molecular mass of approximately 45 kDa, depending on the degree of glycosylation. It is synthesized in the liver and bound to HDL in the bloodstream (Lenz 2010). The protein retains its signal sequence and has a unique active-site lid, both of which are thought to help bind PON1 to HDL (Sorenson 1999, Harel 2007). Unfortunately, little is known of its catalytic mechanism. Its human plasma concentration is near 50 mg L$^{-1}$ although this can vary significantly between specimens depending upon which isoform it contains. Two important polymorphisms, L55M and Q192R, are known, with the latter pertaining to paraoxonase activity and the Q-type having significantly greater activity (Gaidukov 2006). A dyad of histidines (H115 and H134) has been identified as possible active-site residues particularly for aryl esters and lactones, although experiments have not elucidated this specifically (Rosenblat 2006).

The promiscuity of this protein is its most intriguing quality. It can hydrolyze aryl esters, such as phenyl acetate (PhOAc), lactones, OPs, and chemical warfare agents. While the catalytic activity is low against OPs, this protein has been identified as a promising bioscavenger against such agents. Problems arise when stability and solubility are considered, hence, a recombinant PON1 (rePON1) was constructed via directed evolution. DNA-shuffling of the wild-type PON1 genes of human (R-form), mouse, rat and rabbit was completed in Escherichia coli (E. coli). Each new colony was screened against multiple substrates, and after three generations of shuffling, a rePON1 named G3C9 was discovered. It was purified at 12 mg L$^{-1}$, and was found to have a lysine at position 192, as is found in the rabbit PON1 (Aharoni 2004).
The problem of oligomerization and aggregation is likely due to the exposed hydrophobic residues on the surface of the protein (Figure 4). The initial signal sequence that is retained is highly hydrophobic and is part of an alpha-helix that points out towards the solvent. A second hydrophobic patch near the active-site lid also results in exposed hydrophobic residues (Harel 2004).

![Figure 4: rePON1 displayed in green with hydrophobic residues highlighted on H1 (blue), H2 (red), and H3 (orange); rendered via PyMol from PDB entry 1V04](image)

While these patches are problematic in the expression and purification of the rePON1, they may be necessary to maintain maximum catalytic efficiency. In other words, the hydrophobic HDL-association of PON1 may be required in order to engineer the most efficient PON1 variant.
**High-Density Lipoprotein Characteristics**

Low concentrations of high-density lipoprotein (HDL) particles, *in vivo*, have been shown to be a risk factor for coronary artery disease (CAD) (Miller 1987). HDL’s cardio-protective effects begin with its ability to transfer cholesterol from the non-hepatic peripheral cells to the liver in what is termed reverse cholesterol transport (RCT, Figure 5).

![Figure 5: Reverse cholesterol transport; used with permission from Segrest 2000](image)

The RCT begins with ATP-binding cassette transporter (ABCA1) assembling the lipid-poor apolipoprotein A-I (ApoA-I) into nascent discoidal HDL particles utilizing unesterified cholesterol and phospholipids like phosphatidylcholine (PC). The esterification of the cholesterol by lecithin-cholesterol acyltransferase (LCAT) converts the discoidal HDL particles to mature spherical particles. Finally, the circulating spherical HDL binds to scavenger receptor class B1 (SR-B1). The SR-B1 mediates the
uptake of the cholesteryl esters into the liver, and the cholesterol is excreted as bile (Segrest 2000). At this time, the lipid-free ApoA-I is regenerated, and the process begins again.

While lipoproteins are heterogeneous in size and density, HDL particles belong to the smallest and most dense class. This is due to the high proportion of proteins to lipids in the particles. The main protein components are ApoA-I and ApoA-II, although ApoA-IV, ApoC, ApoD, ApoE, and ApoM have also been noted as minor components. Other proteins such as PON1 have also been identified to bind to HDL (Davidson 2007).

The complexity of the HDL particles hinders the investigation of the mechanism used to reduce cardiovascular events. The major structural protein, ApoA-I, is being studied as a key component of PON1 association with HDL. ApoA-I is a structural protein with 243 amino acids at 28 kDa. It has no glycosylation, nor disulfide linkages. The conformation of ApoA-I on HDL is currently being debated in literature. While the non-lipidated conformation has been solved, no crystallography data is known for the lipidated form. The leading structure is known as the “double-belt” conformation, in which ApoA-I forms a dimer around the circumference of the discoidal HDL particles, as seen in Figure 6 (Segrest 2000).
Figure 6: Side view of ApoA-I monomers wrapped around the bilayer of phospholipids of an HDL particle as described in the “double-belt” model

It is proposed that the monomers are bound to each other through multiple salt bridges. The double-belt conformation has been supported by electron paramagnetic resonance (EPR) spectroscopy (Martin 2006), Förster resonance energy transfer (FRET) (Panagotopulos 2001, Tricerri 2001, Li 2002), infrared spectroscopy (Brouillette 1995), high-resolution mass spectrometry with cross-linking (Davidson 2003, Bhat 2005, Silva 2005), and H-D exchange mass spectrometry (Wu 2007), among others. Other conformations have been suggested, but most have flaws that cannot be reconciled. The “double-belt” model in its simplicity appears to be the conformation with the least doubt, although slight variations to this model are continually suggested. It is known via circular dichroism and fluorescence spectroscopy that while wrapped around the HDL particles, ApoA-I forms 10 amphipathic helices, 8 of which are 22-mers and 2 of which are 11-mers. Each of the 10 helices is punctuated with Pro (Segrest 1974, Segrest 1994). It is believed that ApoA-I is needed on HDL for ABCA1 and SR-B1 docking. ApoA-I is also suggested to be responsible for activating LCAT during the RCT process (Segrest
More importantly for this study, ApoA-I has been indicated in possibly interacting with PON1 on HDL particles, enhancing its atherogenic properties.

*High-Density-Lipoprotein-Associated Paraoxonase-1*

As indicated above, PON1 is synthesized in the liver and released into the bloodstream, where it is bound to HDL. The synthesis of the mature protein incorporates a rare occurrence: the hydrophobic N-terminal signal sequence is not removed. Typically in signal sequence peptides, the C-terminal polar region immediately following a hydrophobic core contains a cleavage site in which the -3 and -1 positions in this polar region require small, uncharged residues. Larger, polar residues, His and Gln, occupy the PON1 -3 and -1 sites respectively (Figure 7).

![Figure 7: Hydrophobic signal sequence of PON1](image)

This modification does not allow for the removal of the N-terminal signal sequence by signal peptidases, resulting in the mature PON1 with a hydrophobic tail exposed to its surrounding environment (James 2004).

The function of this retained N-terminal signal sequence is the subject of many intriguing studies. To begin, PON1 is shown to associate with HDL through its hydrophobic tail. ApoA-I knock-out mice have significantly reduced arylesterase
activity, but PON1 is still present in their HDL. This indicates that PON1 is associating with HDL through a hydrophobic tail and phospholipid interaction. A study with a cleavable N-terminal mutant soon revealed that this interaction is indeed the mechanism of binding, with ApoA-I in HDL particles stabilizing PON1 activity through a common binding of the phospholipids (Sorenson 1999).

In order to minimize exposure of this hydrophobic tail from the aqueous bloodstream, PON1 has been shown to attach to the external membrane of CHO cells via immunofluorescence (Deakin 2002, Deakin 2011). The circulation of PON1 in serum requires the release of PON1 from the cells, but an acceptable receptor is needed to protect the hydrophobic tail. HDL is the predominant, physiological acceptor in which its efficiency is influenced by its size and composition. In fact, the micellar size of the particle has direct affect on the PON1 activity. When the HDL volume is decreased, in other words the surface area is increased, the PON1 activity is increased, confirming that PON1 prefers the smallest lipoprotein particles, as opposed to LDL (Mackness 2011).

The desorption mechanism in which the PON1 is transferred from the cellular membrane to the HDL particles has been shown to be in a saturable, high-affinity manner, although the exact details, such as a possible HDL receptor, have not been determined (Deakin 2002).

The biological importance of the association between PON1 and HDL has been indicated in quite a few studies. PON1/HDL is protective against homocysteinylation, which is tied to atherosclerosis (Ferretti 2003). Lowered PON1/HDL activity is associated with oxidative stress in both LDL and HDL in obese patients, which could be
the cause of their greater risk for cardiovascular disease (Ferritti 2005). Diabetics also see this increase of oxidative stress and deterred PON1/HDL activity due to possible glycation of the HDL particles (Ferretti 2001).

The connections between PON1/HDL’s biological role and its anti-oxidant properties have been investigated by multiple studies. First, PON1/HDL has the ability to inhibit copper-induced HDL oxidation by extending its oxidative lag phase and reducing peroxide and aldehyde formation (Mackness 1993). Conversely, when PON1 was inhibited, the oxidative protection suffered significantly. Both free PON1 and PON1/HDL have the ability to inhibit LDL oxidation, which suggests PON1 may have peroxidase-like activity as well (Aviram 1998). Furthermore, another study showed that the hydrophobic tail is not needed to reduce copper-induced oxidation (Sorenson 1999). PON1/HDL is capable of inhibiting macrophage-mediated LDL oxidation and stimulating cholesterol efflux, when compared to PON1/phospholipid complexes, highlighting ApoA-I’s ability to stimulate PON1 activity (Aviram 1998). It has been suggested PON1/HDL’s anti-atherogenic properties involve lysosphatidylcholine (LPC) release with PON1’s ability to convert oxidized lipids into LPC in macrophages. A well-folded H115Q/H134Q mutant is also unable to complete this task, indicating that the His dyad is required for PON1’s oxidative protection (Rosenblat 2006).

Given the current understanding of PON1/HDL’s ability to protect the human body against many detrimental ailments, the details of the PON1 association to HDL are needed for a deeper comprehension of the mechanisms involved. Many of the above-mentioned studies indicate PON1/HDL as a possible key to reducing cardiovascular
events due to its ability to reduce oxidative damage. Although these conclusions have exciting implications to improve human health, they should be evaluated with restraint. Many of the results require experimentation to further isolate important variables to elucidate the exact mechanism. An important expansion of the role of the hydrophobic signal sequence and secondary hydrophobic active-site lid is needed. Even though the hydrophobic signal sequence has been identified as a major factor in the association, only a blunt-force method has been employed – the removal of the entire sequence. Finer details, like the specific residues, length of insertion into the HDL particle, and degree of association upon mutations are fundamental to the comprehension of the association mechanism.

**Methodologies Employed**

Previously, the PON1/HDL association has been studied utilizing surface plasmon resonance (SPR) with a streptavidin chip used to absorb the biotinylated reconstituted HDL (rHDL) particles. Following the injection of recombinant PON1 with or without the hydrophobic signal sequence over the chip, association and dissociation rates were determined through pseudo-first-order and first-order single exponentials, respectively. In the same study, the arylesterase, paraoxonase, and lactonase activities were investigated with and without rePON1 bound to rHDL (Gaidukov 2005). The enhancement of each activity was determined as an indication of how HDL influences the mechanism.

The current study focused on introducing a simpler and more sensitive methodology for the determination of PON1/HDL association. First, a PON1-fluorescent
variant was produced by tagging its C-terminal end with fluorescein-CoA utilizing Sfp phosphopantetheinyl transferase. Second, the PON1-fluorescent variant was subjected to fluorescence anisotropy experimentations in order to determine the degree of association to rHDL particles. Finally, arylesterase and paraoxonase activities were investigated with and without rHDL stimulation in order to try to elucidate the effect of mutagenesis.

Sfp phosphopantetheinyl transferase (Sfp) is used to transfer the 4’- phosphopantetheinyl (Ppant) group of coenzyme A (CoA) onto the conserved Ser residue of peptide carrier proteins (PCP) as seen in Figure 8. Studies identified a shorter fragment of PCP, a small 11-residue peptide (DSLEFIASKLA) that can be labeled by Sfp with high specificity and efficiency at the indicated Ser residue. Phage display of the genomic library of *Bacillus subtilis* yielded this tag, termed “ybbR tag”, due to its sequence derived from ybbR ORF (Yin 2005). The ybbR substrate is covalently attached to CoA via thioester, thioether, or disulfide linkages. It can also be easily fused to multiple proteins at either the N-terminal or C-terminal ends, as well as in flexible loop regions in the middle of the target protein. The tag has an α-helical conformation in solution, and helical wheel representations have indicated that it may be amphipathic with Ile, Leu, and Ala on one side and Lys, Glu, and Ser on the other. It has been shown that small-molecule Ppant conjugates are also transferable, with high diversity in the small-molecule moieties. These Sfp-catalyzed small-molecule CoA modifications of various proteins can be utilized to add small molecule probes with properties such as sugars, affinity and fluorescent probes. In the current study, fluorescein-CoA was utilized to produce fluorescent variants of rePON1.
The fluorescent-rePON1s were then subjected to fluorescence anisotropy (FA). FA measurements are dependent upon the fluorescence intensities emitted by fluorophores that have been excited with polarized light. As seen in Equation 1, dimensionless parameter $r$ indicates the rotational freedom of the fluorophore in the excited state, where $I_{VV}$ is the intensity of vertically polarized light emitted, and $I_{VH}$ is the intensity of the horizontally polarized light emitted.

Equation 1: Anisotropy measurement as it relates to the intensities of emitted polarized light

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$
This $r$ depends not only on intrinsic photophysical properties of the fluorophore, but also its volume and the medium’s viscosity. The signal collected in fluorescence anisotropy is derived from the ability of a small, unbound fluorophore to rotate and tumble rapidly. If it were completely immobilized the absorbed polarized light would be emitted as polarized light, but with rapid molecular movement, the emitted light is depolarized.

Thus, when the small fluorophore is bound to a receptor, the loss of depolarization can be attributed to the binding. By fixing the fluorophore concentration and varying the receptor concentration, a binding isotherm can be constructed with the anisotropy measurement at each point and the association and dissociation kinetic parameters can be determined through non-linear regression (Santos 2010).

The examination of the arylesterase and paraoxonase activities of PON1 variants is typically completed with phenyl acetate (PhOAc) and paraoxon, respectively. A chromophore is produced after the hydrolysis of PhOAc (phenol) and paraoxon ($p$-nitrophenol), with molar absorptivities of $1,310 \text{ cm}^{-1} \text{ M}^{-1}$ and $17,000 \text{ cm}^{-1} \text{ M}^{-1}$ at each absorbance wavelength (270 nm and 405 nm), respectively. Initial velocities are determined by monitoring the production of these chromophores spectrophotometrically. The enzymatic hydrolysis at various substrate concentrations is fit to Michaelis-Menten steady state kinetics to derive the $K_M$ and $V_{max}$ for each enzyme. If saturation is achieved, the catalytic parameters $k_{cat}$ and $K_M$ can be determined separately, but if saturation is not achieved, then only the apparent catalytic efficiency ($k_{cat}/K_M$) can be determined from the linear region of the Michaelis-Menten curve. Finally, the binding between PON1 and HDL has been shown to stimulate these catalytic activities. By
incubating the protein and particles together, binding is encouraged, therefore allowing for the investigation of HDL stimulation on PON1 arylesterase and paraoxonase activities.

**Conclusions**

In the present study, the binding between PON1 and HDL was investigated by incorporating a ybbR tag onto the C-terminus of the G3C9 rePON1. Sfp labeling of this tag with fluorescein-CoA yielded a fluorescent protein that was used to try to construct a binding isotherm with reconstituted HDL particles as the receptor. Site-directed mutagenesis helped determine which residues are required for PON1/HDL association by (1) removing the N-terminal hydrophobic signal sequence, (2) exchanging the nonpolar residue at the center of the N-terminal hydrophobic signal sequence, L12, to a smaller, nonpolar residue (Ala) and a larger, polar residue (Lys), and (3) exchanging the aromatic residue in the proposed second hydrophobic region at the active site lid, W194, to Ala and Lys.

In order to investigate the affect of the ybbR tag on the arylesterase and paraoxonase of the new variants PhOAc and paraoxon assays were utilized as mentioned above. rHDL stimulation of the arylesterase activity was also investigated with the new variants.
In this study, the buffers were made from salts purchased from Fisher Scientific, Sigma-Aldrich, or Acros Organics. The media utilized Bacto tryptone, Bacto casamino acids, and Bacto yeast extract from Becton, Dickinson and Company. Electrocompetent *E. coli* strains of BL21 (DE3) and DH10B were prepared in house. The majority of the chemical and biochemical reagents were purchased from American Bioanalytical, Acros Organics, Thermo Scientific, Fisher Scientific, and Sigma-Aldrich, unless otherwise stated. Also, electrophoretic molecular weight standards for proteins were purchased from USB or GE Healthcare.

All minipreps and agarose gel purifications completed in this study followed the QIAprep protocol, using standard in house buffers. Plating was accomplished with Luria broth agar plates supplemented with 1X ampicillin, which was prepared at 1,000X (100 mg mL\(^{-1}\)) and sterile-filtered (Millipore; 0.2 μm syringe filter). The water utilized in the experimentations was purified to 18 MΩ cm using a Barnstead NANOpure Diamond system.

Oligonucleotides were ordered from Sigma-Genosys and resuspended to 100 μM in water. Deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) at 100
mM each were purchased from American Bioanalytical. Any restriction endonuclease used was purchased from New England Biolabs along with the Klenow DNA polymerase, and λ DNA-BstEII ladder. The Pfu polymerase was purified in house. Agarose GPG/LE, ethidium bromide (EtBr), and Tris/Borate/EDTA buffer (TBE) allowed for agarose gel purification, while phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7), chloroform:isoamyl alcohol (24:1), 3 M sodium acetate, and EtOH allowed for EtOH precipitation, or PCR clean-up.

_Apolipoprotein A-I Production and Quantification_

The pNFXex plasmid that coded for the production of ApoA-I was obtained from the M. Oda group at the Children’s Hospital Oakland Research Institute (Oakland, CA). Transformation by electroporation (Bio-Rad Micropulser) of the plasmid into BL21 (DE3) *E. coli* cells allowed for the expression of the protein.

One liter of autoclaved NZCYM media made from pancreatic digest of casein (tryptone), casamino acids, yeast extract, sodium chloride, and magnesium sulfate heptahydrate was used as a rich media. An overnight seed culture (50 mL, at 37 °C, aerated) inoculated the remaining media (950 mL); both cultures had been supplemented with 1X ampicillin. The culture was grown to a 600 nm optical density of 0.6 (UV/Vis, Agilent 8453) at 37 °C with continuous aeration.

Induction of the ApoA-I protein with a His<sub>6</sub>-affinity tag was started with the addition of 0.5 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG). The induction was carried out for 3 h (37 °C, aerated). The cells were harvested by centrifugation (Sorvall; 4 °C, 6,000 g, 10 min) and were stored at -80 °C.
Purification began with lysis of the thawed bacterial cells. The lysis buffer, B-PER reagent (Pierce; 40 mL), helped retain the protein in the soluble fraction during sonication (Misonix Ultrasonic Liquid Processor). Centrifugation (4 °C, 16,000 g, 40 min) separated the soluble fraction, known as the cleared lysate, from the unwanted cellular debris. The cleared lysate was equilibrated with nickel nitrilotriacetic acid agarose resin (Qiagen; NiNTA agarose) in 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 3 M guanidine hydrochloride (GdnHCl) for 1.5 h (4 °C) to allow the His$_6$-tag of the protein of interest to bind to the NiNTA agarose. After equilibration of the ApoA-I (His$_6$-tagged) with the NiNTA agarose, the solution was delivered onto a chromatography column (Bio-Rad) to allow any non-bound proteins to elute. This first elution, or “flow-through,” was followed by two subsequent washes and two elutions. The first wash buffer contained the same concentrations of sodium phosphate buffer, NaCl, and GdnHCl as the equilibration buffer. The second wash buffer contained the same concentrations of sodium phosphate buffer and NaCl, but omitted the GdnHCl. The elution buffer contained 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, and 500 mM imidazole. Following the elution of the His$_6$-tagged ApoA-I protein (~8 mL), the buffer was exchanged (overnight, 4 °C) into 2 L of 20 mM Tris buffer (pH 8), 150 mM NaCl, and 1 mM EDTA via Slide-A-Lyzer dialysis cassettes (Thermo Scientific).

The pNFXex plasmid coded for the His$_6$-affinity tag to be separated from the human ApoA-I protein by an Asp-Pro acid labile bond (Figure 9).
This allowed the His6-tag to be removed from the ApoA-I protein by incubating the tagged protein with 45% formic acid (Fluka; 60 °C, 5 h) (Ryan 2003). To significantly reduce the amount of formic acid, and raise the pH back to 8, the incubation solution was buffer-exchanged three times (>2 h each, 4 °C) into 2 L of 20 mM Tris (pH 8) and 150 mM NaCl.

Quantitation of the ApoA-I (non-His6-tagged) was completed with the Bradford assay utilizing bovine serum albumin standards (Bio-Rad; BSA) and an UV/Vis spectrophotometer (Agilent).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) allowed for the visualization of the purification fractions (flow-through, wash 1, wash 2, elution 1, and elution 2), the investigation the His6-cleavage products, and the confirmation of the quantitation via Bradford. Each sample was mixed in a 1:1 ratio with SDS-PAGE loading buffer containing 200 mM dithiothreitol (RPI Corp.; DTT). The samples were denatured at 90 °C for 10 min before loading. The gel was stained with Coomassie brilliant blue R-250 and destained with 10% acetic acid and 10% ethanol (Decon; EtOH).

Once the quantitation was confirmed, the cleaved ApoA-I protein was aliquotted into batches of ~2.4 mg. These batches were frozen at -80 °C and lyophilized.
(Labconco) in preparation for the production of reconstituted high density lipoproteins (rHDLs).

Reconstituted High-Density Lipoproteins Production and Characterizations

Discoidal rHDLs containing ApoA-I and small unilamellar vesicles (SUVs) were produced via the cholate dialysis method (Matz 1982, Nichols 1983). Glass disposable culture tubes (Fisherbrand) were cleaned with detergent and water then flamed with ethanol. Lipid films were made by adding 5.0 mg 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Inc; POPC, 50 mg mL\(^{-1}\) in 2:1 CHCl\(_3\):MeOH) and either 0.19 mg or 0.38 mg free cholesterol (Sigma; 10 mg mL\(^{-1}\) in 2:1 CHCl\(_3\):MeOH) into the cleaned glass tubes. The tubes were rotated by hand under a stream of argon gas for approximately 15 min to allow the chloroform (CHCl\(_3\)) and methanol (Mallinckrodt; MeOH) to evaporate, leaving a smooth, uniform layer of lipids at the bottom. The tubes were immediately placed under vacuum for at least 20 h to remove any residual organic solvents.

The previously prepared ApoA-I was prepared for introduction into the rHDL particles by first denaturing the protein. The lyophilized protein (2.4 mg) was reconstituted in GdnHCl (6 M, 2 mL) in 20 mM Tris (pH 8), 150 mM NaCl for 3 h at room temperature. Following denaturation, the solution was dialyzed against 2L of 20 mM Tris (pH 8) and 150 mM NaCl for 4 h at room temperature.

While the ApoA-I was being prepared, the aforementioned lipid films were suspended into deoxycholic acid (DCA, 30 mg mL\(^{-1}\) in 20 mM Tris, pH 8) in the following manner. After the films were removed from the vacuum, DCA was added in a
2:3 molar ratio of POPC:DCA. The solution was vortexed thoroughly until the lipid film was removed from the tube walls, yielding a hazy suspension. Immediately, the suspension was placed into a water bath sonicator (room temperature, 40 min) until the solution appeared clear, indicating that the lipids were well-dispersed. The well-dispersed solution was allowed to shake for 1 h at 37 °C.

For the rHDL particles, the denatured ApoA-I was transferred into the lipid suspension to allow for the complexation of ApoA-I with the lipids. The final volume for this reaction was brought up to 4.0 mL with 20 mM Tris (pH 8) and 150 mM NaCl to yield a final ApoA-I concentration of 0.6 mg mL⁻¹. The final molar ratios of the rHDL particles were either 80:1:6:120 (POPC:ApoA-I:cholesterol:DCA) or 80:1:12:120 depending on the starting amounts of cholesterol. For the SUVs, no ApoA-I was transferred into the lipid suspension. Instead, the final volume was brought to 4.0 mL with 20 mM Tris (pH 8) and 150 mM NaCl.

Following the complexation with or without ApoA-I (rHDL or SUV, respectively), the deoxycholic acid was removed slowly, in order to yield the final discoidal particles and spherical SUVs. The samples were injected into Slide-A-Lyzer dialysis cassettes (molecular weight cut-off of 7,000 for rHDLs, 3,500 for SUVs) and subjected to extensive buffer dialysis (20 mM Tris, pH 8, 150 mM NaCl, 4 °C). The buffer was changed a total of 8 times after equilibration was completed (at least 4 h each). Following multiple days of buffer exchange, the samples were removed and stored at 4 °C in Teflon-capped glass vials with 0.02% sodium azide.
The quantitation of the rHDL particles in 20 mM Tris (pH 8), 150 mM NaCl, 0.02% sodium azide was completed via UV/Vis absorption at 280 nm. Each sample (100 μL) was loaded into a quartz absorption cuvette (Starna Cells, Inc). The molar absorptivity of ApoA-I (1.15 mL mg⁻¹ cm⁻¹; Jonas 1990) was used along with its molecular weight (28,000 g mol⁻¹) to calculate the concentration of rHDL particles, assuming two ApoA-I molecules per rHDL particle.

The rHDLs and SUVs were characterized by Transmission Electron Microscopy (TEM) and native polyacrylamide gel electrophoresis (Native PAGE). To complete the TEM, Richard Montione at the Campus Microscopy and Imaging Facility (The Ohio State University, Columbus, OH; OSU-CMIF) glow-discharged Formvar carbon film copper grids (Electron Microscopy Sciences, 300 square mesh). The samples were allowed to adsorb onto the grids, and then were negative-stained with the aid of 1% phosphotungstic acid (PTA) and observed using an FEI Tecnai G2 Sprint transmission electron microscope. To complete the Native PAGE, the samples of interest were mixed in a 1:1 ratio with non-denaturing loading buffer made of 300 mM Tris (pH 6.8), 0.05% bromophenol blue, and 50% glycerol. The samples were loaded onto a 4-20% gradient Ready Gel Tris-HCl Precast Gel (Bio-Rad; 40 μL loaded). Non-denaturing run buffer (Tris, pH 8.3, with Glycine) was poured over the gel apparatus and 150 V were applied for approximately 2 h. The gel was stained with Coomassie stain and destained with 10% acetic acid and 10% EtOH.
Recombinant Paraoxonase-1 Production and Characterization

For rePON1 production, a series of “null” vectors were constructed in order to yield an easy DNA scaffold for any future mutants. The first null vector (Null-ybbR-1) was formed using three-piece ligation. Cleavage of the pET32b-Null vector (Figure 10) from XhoI to NcoI yielded the first of the three pieces following agarose gel purification of the 5,369 bp DNA.

Figure 10: Plasmid maps of pET32b-Null, Pinpoint Xa-1, and pET32b-G3C9 with important restriction enzyme sites indicated

The 5’ phosphate group of this DNA fragment was removed by Antarctic Phosphatase (37 °C, 1 h) to prevent self-ligation. The second piece (560 bp) was purified from the cleavage of the plasmid Pinpoint Xa-1 (Promega) from NcoI to NotI (Figure 10). Finally,
a polymerase chain reaction (PCR) with Pfu polymerase, Primer C and Primer D (Table 1), resulted in the third piece containing a NotI-Gly,Ser,Ser,Gly-ybbR-Gly,Ser,Ser,Gly-His<sub>6</sub>-XhoI sequence.

The PCR protocol included 25 cycles of (1) denaturation at 96 °C for 30 s, (2) annealing at 57 °C for 45 s, and (3) elongation at 72 °C for 60 s (Bio-Rad; C1000 Thermo Cycler).

Following digestion with NotI and XhoI of the third piece, the three-piece ligation with T4 DNA ligase was transformed into DH10B cells which were grown overnight. Plasmid recovery was completed via a miniprep protocol (Figure 11).

### Table 1: Primers utilized in cloning schemes

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Resultant Vector</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer C</td>
<td>Null-ybbR-1</td>
<td>gcctg acgc gac gat ctc gtc aat tta tta ttc aac gtc tca aag cgg gc</td>
</tr>
<tr>
<td>Primer D</td>
<td>Null-ybbR-1</td>
<td>attact cgc gcg tct ggt aat gtc tgg tgg tgg tga ttc gtc aac gcg cgg aag ctt act act ac</td>
</tr>
<tr>
<td>Primer G</td>
<td>Null-ybbR-1</td>
<td>aacatc gcgc gcgc gtc tcg gcc gta cgc gtc gtc gtc gtc gtc ctc tca aag act act act aag</td>
</tr>
<tr>
<td>Primer F</td>
<td>Null-ybbR-2</td>
<td>attact ctc gac gct ttc aat gtc ttc gtc tgg ggg gga aag cgg cg</td>
</tr>
<tr>
<td>Primer I</td>
<td>G309-ybbR</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
<tr>
<td>Primer J</td>
<td>G309-ybbR</td>
<td>attattt ccc gca ctc tca cag tca cag tca aac tga c</td>
</tr>
<tr>
<td>Primer K</td>
<td>G309-ybbR</td>
<td>attattt ccc gca ctc tca cag tca cag tca aac tga c</td>
</tr>
<tr>
<td>L12A_Forward</td>
<td>G309-ybbR-L12A</td>
<td>ggc ctc aca ctc tgg ggg gca gga ttc gca ctc ttc gat g</td>
</tr>
<tr>
<td>L12K_Forward</td>
<td>G309-ybbR-L12K</td>
<td>ggc ctc aca ctc tgg aag gga ttc gca ctc ttc gat g</td>
</tr>
<tr>
<td>L12&quot;_Forward</td>
<td>G309-ybbR-L12A</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
<tr>
<td>W194A_Forward</td>
<td>G309-ybbR-W194A</td>
<td>gac cct tac tta aaa ttc ggc gaa atg cat ttc gga tga tta g</td>
</tr>
<tr>
<td>W194A_Reverse</td>
<td>G309-ybbR-W194A</td>
<td>cta ttc cca aat gca ttt cgg aag atg tta aag ggt c</td>
</tr>
<tr>
<td>W194K_Forward</td>
<td>G309-ybbR-W194K</td>
<td>gac ttt ttg ctc ccc aag gaa atg cat ttc gga tga tta g</td>
</tr>
<tr>
<td>W194K_Reverse</td>
<td>G309-ybbR-W194K</td>
<td>c taa ttc cca aat gtc ttc ctc act ttt taa gta ggg gtc gcg aac ata gty</td>
</tr>
<tr>
<td>Gene_Forward</td>
<td>G309-ybbR-W194A</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
<tr>
<td>Gene_Reverse</td>
<td>G309-ybbR-L12A</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
<tr>
<td>Gene_Reverse</td>
<td>G309-ybbR-L12K</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
<tr>
<td>Gene_Reverse</td>
<td>G309-ybbR-W194A</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
<tr>
<td>Gene_Reverse</td>
<td>G309-ybbR-W194K</td>
<td>attattt ccc gca ctc tca cag tca aac tga c</td>
</tr>
</tbody>
</table>

Blue highlights indicate restriction enzyme sites
Green letters indicate mutations
Red letters indicate overlap regions
The sequence of this new Null-ybbR-1 vector (Figure 12) was confirmed via restriction digest (AlwNI and HindIII) and sequencing (GeneWiz).

Following sequence confirmation, a few errors were noticed. First, the new restriction site, NotI, introduced a frame shift that was not intended and detrimental. Second, Primer
D introduced a His\textsubscript{3}ThrHis\textsubscript{2}-tag instead of a His\textsubscript{6}-tag. Thirdly, no stop codon was incorporated before the XhoI site in Primer D. (Note, the ybbR-tag DNA sequence is 5’-GAT TCT CTT GAA TTT ATT GCT AGT AAG CTT GCG-3’; Yin 2006)

The “stuffer” region in Null-ybbR-1 came from the Pinpoint Xa-1 DNA cleaved from NcoI to NotI (560 bp). Unfortunately, this segment carried an additional XhoI site, yielding two XhoI sites in the Null-ybbR-1 vector. This was problematic for future cloning, so the 1,099 bp segment from pET32b-G3C9 (Figure 10; NcoI to NotI) was placed into the Null-ybbR-1 vector, replacing the Pinpoint Xa-1 segment. This was done via digestion of the plasmids, agarose gel purification of the fragments, ligation with T4 DNA ligase, and transformation into DH10B cells. The bacterial cells were grown overnight, miniprepped, and the new sequence was confirmed by restriction digest (HindIII) and sequencing (GeneWiz). The final Null-ybbR-X vector contained the G3C9 “stuffer” and the ybbR sequence with the three previously mentioned errors (Figure 13).

**Figure 13:** Plasmid map of pET32b-Null-ybbR-X with NcoI, NotI, XhoI, and ybbR sequence indicated
In order to fix the frame shift, His6-tag, and to introduce a stop codon before the XhoI site, PCR was used on Null-ybbR-1 to produce the “corrected” ybbR sequence. Primer G and Primer F (Table 1) were mixed with Null-ybbR-1 and Pfu polymerase. The denaturation occurred at 96 °C for 30 s, the annealing at 60 °C for 45 s, and the elongation at 72 °C for 20 s (25 cycles). Following the PCR, EtOH precipitation, digestion with NotI and XhoI, and agarose gel purification yielded an insert ready for ligation. The scaffold was prepared by digesting Null-ybbR-X with NotI and XhoI and retaining the large DNA fragment (6,467 bp). This fragment was phosphatased in order to prevent self-ligation in the same manner as pET32b-Null. Finally, the “corrected ybbR” insert and the digested scaffold were ligated and transformed into DH10B cells. Following a miniprep, restriction digestion (SacII and AlwNI), and sequencing (GeneWiz), the new Null-YbbR-2 vector was confirmed (Figure 14).

Figure 14: Plasmid map of pET32b-Null-ybbR-2 with NcoI, BamHI, NotI, XhoI, and ybbR sequence indicated
This new vector contained a G3C9 “stuffer” sequence, followed by a NotI site, ybbR sequence, His6-tag, stop codon and XhoI site. The only downfall of this vector was that the “stuffer” was almost identical to any of the variants that would be placed into it, rendering restriction digest useless to help confirm sequences.

In order to resolve the problem with Null-ybbR-2, a restriction site (BamHI) was removed. The Klenow fragment, a DNA polymerase, was introduced to the digestion product of Null-ybbR-2 with BamHI-HF (1 h, 37 °C). The reaction produced a linear Null-ybbR-2 fragment without a BamHI site. Following ligation and transformation, the sequence was confirmed as Null-ybbR-F (Figure 15).

![Figure 15: Plasmid map of pET32b-Null-ybbR-F with NcoI, BamHI (absent), NotI, XhoI and ybbR sequence indicated](image)

The six variants used in this study included the “wild-type” rePON1-G3C9-ybbR, and 5 mutants; Δ17-G3C9-ybbR, G3C9-ybbR-L12A, G3C9-ybbR-L12K, G3C9-ybbR-W194A, and G3C9-ybbR-W194K (Figure 16).
First, the insert for the plasmid, G3C9-ybbR, was constructed by PCR with pET32b-G3C9, Pfu polymerase, Primer I and Primer J (25 cycles: 96 °C for 30 s, 60 °C for 45 s, 72 °C for 20 s). Following EtOH precipitation and digestion with NotI and NcoI, the PCR product was purified by agarose gel. The scaffold, Null-ybbR-2, was digested with NotI and NcoI yielding the fragment of 6,467 bp and phosphatased to remove the 5’ phosphate group. The insert and scaffold were ligated, transformed, miniprepped, and sequenced to confirm the construction of pET32b-G3C9-ybbR.

For the Δ17-G3C9-ybbR plasmid, the insert was constructed by PCR with pET32b-G3C9, Pfu polymerase, Primer K and Primer J (25 cycles: 96 °C for 30 s, 56 °C for 45 s, 72 °C for 20 s). The scaffold was the same as the G3C9-ybbR production.

| Figure 16: Sequence alignments of all 6 variants with G3C9 sequence (black), mutations (red), linkers (purple), ybbR sequence (blue), and His6-tag (green) indicated |
|---------------|---------------|---------------|---------------|---------------|---------------|
| G3C9-ybbR     | MAIWLTLTLGLAIFDGDQISQFQRTRNVHRREVTPVLPSNCLVKGVDGSEDELIELPNLGAIFGSKYGGSMFDPDKSILLMDLEDEPPVVELGFTGTNTLDSLSPFPHGST |
| Δ17-G3C9-ybbR | MAIWLTLTLGLAIFDGDQISQFQRTRNVHRREVTPVLPSNCLVKGVDGSEDELIELPNLGAIFGSKYGGSMFDPDKSILLMDLEDEPPVVELGFTGTNTLDSLSPFPHGST |
| G3C9-ybbR-L12A| MAIWLTLTLGLAIFDGDQISQFQRTRNVHRREVTPVLPSNCLVKGVDGSEDELIELPNLGAIFGSKYGGSMFDPDKSILLMDLEDEPPVVELGFTGTNTLDSLSPFPHGST |
| G3C9-ybbR-L12E| MAIWLTLTLGLAIFDGDQISQFQRTRNVHRREVTPVLPSNCLVKGVDGSEDELIELPNLGAIFGSKYGGSMFDPDKSILLMDLEDEPPVVELGFTGTNTLDSLSPFPHGST |
| G3C4-ybbR-W154A| MAIWLTLTLGLAIFDGDQISQFQRTRNVHRREVTPVLPSNCLVKGVDGSEDELIELPNLGAIFGSKYGGSMFDPDKSILLMDLEDEPPVVELGFTGTNTLDSLSPFPHGST |
| G3C4-ybbR-W154E| MAIWLTLTLGLAIFDGDQISQFQRTRNVHRREVTPVLPSNCLVKGVDGSEDELIELPNLGAIFGSKYGGSMFDPDKSILLMDLEDEPPVVELGFTGTNTLDSLSPFPHGST |
| G3C3-ybbR     | FLDNDTPIYLVVDPSSSVTRKIQESEKLFIHLTLTIRHLKPSLIAVGYEHPHATLTHYFAADPYIKILWENHHLNLALSWPYYSSNYVRVVAEGDFRGANSVSPGKV |
| Δ17-G3C3-ybbR | FLDNDTPIYLVVDPSSSVTRKIQESEKLFIHLTLTIRHLKPSLIAVGYEHPHATLTHYFAADPYIKILWENHHLNLALSWPYYSSNYVRVVAEGDFRGANSVSPGKV |
| G3C3-ybbR-L12A| FLDNDTPIYLVVDPSSSVTRKIQESEKLFIHLTLTIRHLKPSLIAVGYEHPHATLTHYFAADPYIKILWENHHLNLALSWPYYSSNYVRVVAEGDFRGANSVSPGKV |
| G3C3-ybbR-L12E| FLDNDTPIYLVVDPSSSVTRKIQESEKLFIHLTLTIRHLKPSLIAVGYEHPHATLTHYFAADPYIKILWENHHLNLALSWPYYSSNYVRVVAEGDFRGANSVSPGKV |
| G3C3-ybbR-W154A| FLDNDTPIYLVVDPSSSVTRKIQESEKLFIHLTLTIRHLKPSLIAVGYEHPHATLTHYFAADPYIKILWENHHLNLALSWPYYSSNYVRVVAEGDFRGANSVSPGKV |
| G3C3-ybbR-W154E| FLDNDTPIYLVVDPSSSVTRKIQESEKLFIHLTLTIRHLKPSLIAVGYEHPHATLTHYFAADPYIKILWENHHLNLALSWPYYSSNYVRVVAEGDFRGANSVSPGKV |
| G3C3-ybbR     | YAEALAFKHLYEKhHANHTLTUPIKFDLFDLVDINIDPVD60LWVGCMGMPRFRYPDPDNSPPSEVIRQGDILEEKPVTIVYVH6ENY121Y7GSTTAAVYIKYSLUSLTFH4KALY |
| Δ17-G3C3-ybbR | YAEALAFKHLYEKhHANHTLTUPIKFDLFDLVDINIDPVD60LWVGCMGMPRFRYPDPDNSPPSEVIRQGDILEEKPVTIVYVH6ENY121Y7GSTTAAVYIKYSLUSLTFH4KALY |
| G3C9-ybbR-L12A| YAEALAFKHLYEKhHANHTLTUPIKFDLFDLVDINIDPVD60LWVGCMGMPRFRYPDPDNSPPSEVIRQGDILEEKPVTIVYVH6ENY121Y7GSTTAAVYIKYSLUSLTFH4KALY |
| G3C9-ybbR-L12E| YAEALAFKHLYEKhHANHTLTUPIKFDLFDLVDINIDPVD60LWVGCMGMPRFRYPDPDNSPPSEVIRQGDILEEKPVTIVYVH6ENY121Y7GSTTAAVYIKYSLUSLTFH4KALY |
| G3C3-ybbR-W154A| YAEALAFKHLYEKhHANHTLTUPIKFDLFDLVDINIDPVD60LWVGCMGMPRFRYPDPDNSPPSEVIRQGDILEEKPVTIVYVH6ENY121Y7GSTTAAVYIKYSLUSLTFH4KALY |
| G3C3-ybbR-W154E| YAEALAFKHLYEKhHANHTLTUPIKFDLFDLVDINIDPVD60LWVGCMGMPRFRYPDPDNSPPSEVIRQGDILEEKPVTIVYVH6ENY121Y7GSTTAAVYIKYSLUSLTFH4KALY |
| G3C3-ybbR     | CELAAASGSGSGSSSAKLAGSGLHHH |
| Δ17-G3C3-ybbR | CELAAASGSGSGSSSAKLAGSGLHHH |
| G3C4-ybbR-L12A| CELAAASGSGSGSSSAKLAGSGLHHH |
| G3C4-ybbR-L12E| CELAAASGSGSGSSSAKLAGSGLHHH |
| G3C3-ybbR-W154A| CELAAASGSGSGSSSAKLAGSGLHHH |
| G3C3-ybbR-W154E| CELAAASGSGSGSSSAKLAGSGLHHH |
The two DNA fragments were ligated, transformed, miniprepped, and sequenced to confirm the construction of pET32b-Δ17-G3C9-ybbR.

For the G3C9-ybbR-L12A plasmid, the first, incomplete piece of the insert was constructed by PCR with pET32b-G3C9-ybbR, Pfu polymerase, and primers L12A_Forward and Gene_Reverse (25 cycles: 95 °C for 30 s, 63.3 °C for 45 s, 72 °C for 140 s). Following EtOH precipitation, agarose gel purification, DpnI digestion, and another agarose gel purification, this PCR fragment was subjected to another sequential round of PCR. Using the primers L12*_Forward and Gene_Reverse, along with Pfu polymerase, the full length insert was produced (25 cycles: 95 °C for 30 s, 63.3 °C for 45 s, 72 °C for 140 s). Following EtOH precipitation and agarose gel purification, the insert was prepared for ligation. The scaffold, Null-ybbR-F, was digested with NcoI-HF and XhoI yielding the fragment of 6,467 bp following agarose gel purification. The insert and scaffold were ligated, transformed, miniprepped, and sequenced to confirm the construction of pET32b-G3C9-ybbR-L12A.

The pET32b-G3C9-ybbR-L12K plasmid was produced in a very similar manner to the L12A plasmid. The only differences occurred in the production of the first, incomplete PCR product. First, the primers used were L12K_Forward and Gene_Reverse. Secondly, the PCR protocol was 25 cycles at 95 °C for 30 s, 61.4 °C for 45 s, 72 °C for 140 s. All other steps were followed as mentioned above.

For the G3C9-ybbR-W194A plasmid, overlap PCR was utilized. The first portion of the insert was constructed by PCR with pET32b-G3C9-ybbR, Pfu polymerase, and primers W194A_Forward and Gene_Reverse (25 cycles: 95 °C for 30 s, 59 °C for 45 s,
72 °C for 80 s). Following EtOH precipitation, agarose gel purification, DpnI digestion, and another agarose gel purification, this first PCR product was ready for the overlap PCR. The second portion of the insert was constructed by PCR with pET32b-G3C9-ybbR, Pfu polymerase, and primers Gene_Forward and W194A_Reverse (25 cycles: 95 °C for 30 s, 65 °C for 45 s, 72 °C for 80 s). Following EtOH precipitation, agarose gel purification, DpnI digestion, and another agarose gel purification, this second PCR product was also ready for the overlap PCR. Using the primers Gene_Forward and Gene_Reverse, along with Pfu polymerase, the full-length insert was produced (25 cycles: 95 °C for 30 s, 63.3 °C for 45 s, 72 °C for 140 s). Following EtOH precipitation and agarose gel purification, the insert was prepared for ligation. The same digested scaffold utilized in the production of L12A and L12K was used here. The insert and scaffold were ligated, transformed, miniprepped, and sequenced to confirm the construction of pET32b-G3C9-ybbR-W194A.

The pET32b-G3C9-ybbR-W194K plasmid was produced in a very similar manner to that of the W194A plasmid. The differences occurred in the production of the first and second PCR products as well as the overlap PCR protocol. The primers used for the first piece were W194K_Forward_SM (SM because it contained silent mutations) and Gene_Reverse (25 cycles: 95 °C for 30 s, 60.4 °C for 45 s, 72 °C for 80 s). The primers used for the second piece were Gene_Forward and W194K_Reverse_SM (25 cycles: 95 °C for 30 s, 60.4 °C for 45 s, 72 °C for 80 s). Finally, the overlap PCR protocol included 25 cycles of 95 °C for 30 s, 57.3 °C for 45 s, 72 °C for 80 s. All other steps were followed as mentioned above.
All six pET32b-G3C9-ybbR plasmids were transformed into BL21 (DE3) *E. coli* cells for expression. To begin, one liter of 2YT media made from tryptone, yeast extract, and NaCl was utilized as a rich media for each variant. An overnight seed culture (50 mL, at 37 °C, aerated) inoculated the remaining media (950 mL); both had been supplemented with 1X ampicillin and 1 mM calcium chloride (CaCl$_2$). The cultures were grown to a 600 nm optical density of 0.8 at 30 °C with continuous aeration.

Induction of the G3C9 proteins started with the addition of 0.1 mM (final concentration) IPTG. The induction was carried out for 3.5 h (30 °C, aerated). The cells were harvested by centrifugation (4 °C, 6,000 g, 10 min) and were stored at -80 °C.

Purification began with the lysis of thawed and resuspended bacterial cells. The lysis buffer (40 mL for G3C9-ybbR and Δ17-G3C9-ybbR, 20 mL for L12A, L12K, W194A, and W194K) was made with 50 mM Tris (pH 8), 50 mM NaCl, 1 mM CaCl$_2$, and 0.1 mM DTT. Following sonication, 0.1% Tergitol® (Sigma; Cat: NP-10) was added in order to recover the proteins (4 °C, 2.5 h). After centrifugation (4 °C, 15,000 rpm, 30 min), the cleared lysate was collected and equilibrated with NiNTA agarose in activity buffer (50 mM Tris (pH 8), 50 mM NaCl, 1 mM CaCl$_2$, and 0.1% Tergitol®) for 3 h (4 °C) to allow binding of the His$_6$-tag of the G3C9 proteins to the NiNTA agarose. After equilibration with NiNTA agarose, the solution was delivered onto a chromatography column to allow any non-bound proteins to elute. The “flow-through” was followed by three subsequent washes with increasing concentrations of imidazole in the activity buffer (5 mM, 10 mM, 25 mM) to remove any unwanted proteins with a slight affinity to the NiNTA agarose due to their native His residues. Activity buffer with 150 mM
imidazole (elution buffer) released the protein of interest in two elution steps. The first elution fraction of each sample was dialyzed against 2 L of storage buffer (50 mM Tris (pH 8), 50 mM NaCl, 1 mM CaCl₂, 0.1% Tergitol®, 50% glycerol, 1 mM DTT) overnight (4 °C) via Slide-A-Lyzer dialysis cassettes. Each sample was removed and stored at -20 °C.

Quantitations of the G3C9 proteins were completed with the Bradford assay utilizing bovine serum albumin standards and a Molecular Devices SpectraMax M5. SDS-PAGE (12.5%) allowed for the visualization of the purification fractions (flow-through, wash 1, wash 2, wash 3, elution 1, and elution 2) and the confirmation of the quantitation via Bradford. Each sample was mixed in a 1:1 ratio with SDS-PAGE loading buffer containing 200 mM DTT. The samples were denatured at 90 °C for 10 min before loading. The gel was stained with Coomassie brilliant blue R-250 and destained with 10% acetic acid and 10% ethanol.

The arylesterase and paraoxonase activities of each mutant were tested against phenyl acetate (PhOAc) and paraoxon-ethyl (paraoxon). The concentration of each substrate was determined via base hydrolysis (0.25 M NaOH), followed by UV/Vis absorption for phenol (λ₂₇₀ nm) and p-nitrophenol (λ₄₀₅ nm). A range of enzyme concentrations was tested against either 0.06 mM PhOAc or 0.03 mM paraoxon to determine what concentration would yield a linear response for the kinetics. Once the desired concentration of the enzyme was determined, the kinetics were followed for a range of substrate concentrations (3.3 mM to 0.06 mM for PhOAc; 2.6 mM to 0.03 mM for paraoxon). For each substrate, the UV/Vis absorption at 270 nm or 405 nm was
collected for 5 min. The initial rates were plotted against substrate concentrations. Each kinetic result was fit to the Michaelis-Menten equation where $V_{\text{max}}$ is equal to $k_{\text{cat}}$ multiplied by enzyme concentration (Equation 2).

Equation 2: Michaelis-Menten kinetics relating the initial rate with substrate concentration

$$V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]} = \frac{k_{\text{cat}} [E][S]}{K_M + [S]}$$

None of the variants reached saturation, so the linear region of the Michaelis-Menten plot was utilized to find the apparent $k_{\text{cat}}/K_M$ (Equation 3). Relationship of apparent $k_{\text{cat}}/K_M$ with the slope of the linear region of the Michaelis-Menten equation and enzyme concentration

Equation 3: Relationship of apparent $k_{\text{cat}}/K_M$ with the slope of the linear region of the Michaelis-Menten equation and enzyme concentration

$$\frac{k_{\text{cat}}}{K_M} = \frac{slope}{[E][S]}$$

The change in the arylesterase activity of each G3C9-ybbR variant due to rHDL stimulation was also studied. To begin, each variant was incubated (3 h, 37 °C) with freshly produced rHDL particles to allow for binding. The concentration used for each enzyme was determined from the arylesterase activity mentioned above. A range of rHDL concentrations resulted in ratios of 0.5, 2.5, 5.0, 10, and 50 (rHDL/variant).
Following the binding, the arylesterase assay was completed at a single PhOAc concentration (0.06 mM). The absorbance of phenol ($\lambda_{270\text{nm}}$) was monitored every 20 s for 3 min immediately following the addition of PhOAc. The apparent $k_{\text{cat}}/K_M$ was calculated from the initial rate (slope converted to concentration per time) using the enzyme concentration and substrate concentration (Equation 4). Relationship of initial rate to apparent catalytic efficiency, enzyme concentration, and substrate concentration

Equation 4: Relationship of initial rate to apparent catalytic efficiency, enzyme concentration, and substrate concentration

$$rate = \frac{k_{\text{cat}}}{K_M}[E][S]$$

The enhancement of this $k_{\text{cat}}/K_M$ was determined as a proportion to the $k_{\text{cat}}/K_M$ from the variant’s arylesterase activity without rHDL binding (with rHDL/without rHDL). It was also compared to that of the arylesterase activity without incubation or stimulation.

**Fluorescence Anisotropy of Recombinant Paraoxonase-1 Samples**

To begin the process of labeling the G3C9-ybbR samples with fluorescein-CoA, the protein samples were buffer-exchanged into 100 mM Tris (pH 7.4). Approximately 100 µL of each sample (G3C9-ybbR and Δ17-G3C9-ybbR) in the storage buffer (Tris, NaCl, CaCl$_2$, Tergitol®, DTT, 50% glycerol) were loaded onto centrifugal filter units (Millipore; 0.5 mL capacity, 30K molecular weight cut-off). Buffer (100 mM Tris, pH 7.4; 400 µL) was loaded onto the sample, inverted, and spun down (4 min, 13,200 rpm, 4
°C) to 100 μL (Eppendorf Centrifuge 5415R). This was repeated three times in order to reduce the percentage of storage buffer (~0.2% remained).

Following the buffer exchange, 10 mM magnesium chloride hexahydrate (MgCl₂), 2 mM 2-mercaptoethanol, Sfp phosphopantetheinyl transferase (2 μL), and fluorescein-CoA (20 μL) were added to the samples (47 μL). The labeling reaction was allowed to proceed overnight at room temperature in the dark. (Note: Sfp and fluorescein-CoA were produced according to the published protocol (Yin 2006) by post-doctoral student Lihua Nie.)

In order to purify the newly labeled proteins, the sample volumes with additional 100 mM Tris (pH 7.4) were loaded onto NiNTA agarose spin columns (Qiagen). After centrifugation (1 min, 1,600 rpm, 4 °C), the flow through was collected. The columns were loaded three times with 400 μL of activity buffer with increasing amount of imidazole (5 mM, 10 mM, 25 mM) and each fraction was collected. Finally, the columns were loaded two times with 400 μL and 200 μL of activity buffer plus 150 mM imidazole to collect the two elution fractions. The purified labeled proteins were then buffer-exchanged into 20 mM Tris (pH 8) and 150 mM NaCl in the same manner that the samples were exchanged into 100 mM Tris (pH 7.4). This new buffer matched that of the rHDL particles.

Each purified, labeled and exchanged sample was diluted 10-fold and submitted to UV/Vis absorption (Agilent) at 280 nm to quantify its concentration using a 50 μL quartz absorption cuvette (Starna Cells, Inc). The molar absorptivities (44,400 M⁻¹ cm⁻¹;
both) for both samples were determined via Scripps Protein Calculator
(www.scripps.edu/~cdputnam/protcalc.html).

Both G3C9-ybbR and Δ17-G3C9-ybbR proteins were bound to 80:1:6:120 rHDL
particles via incubation. Each protein (200 nM) was incubated with either 0, 200, 1,000,
or 2,000 nM of rHDL particles for 3 h at 37 °C in a hot water bath in the dark.

Each sample was placed into the fluorimeter (Olis DM45P) in a 50 μL quartz
fluorescence cuvette (Starna Cells, Inc). Fluorescence scans were collected with an
excitation wavelength of 480 nm and an emission scan from 500 nm to 560 nm. Fifty
increments at 0.5 s integration time were taken with a 5 nm slitwidth. A monochromator
was used to select the wavelength of interest. Fluorescence anisotropy data was collected
with an excitation wavelength of 480 nm and emission wavelength of 520 nm. After 50
increments at 2.0 s integration time with a slitwidth of 6 nm, the data was tabulated into
Excel (Microsoft). The vertical and horizontal polarizers were in place, and a filter (495
nm) was used instead of the monochromator. For both the fluorescence scans and
fluorescence anisotropy, the cell temperature was held at 20 °C.
Chapter 3: Results and Discussion

Apolipoprotein A-I Production and Quantification

The 249 amino acid protein, His₆-tagged ApoA-I, was expressed in BL21 (DE3) *E. coli*. Following this expression, the NiNTA agarose purification of the affinity tagged protein yielded a relatively pure sample. The purification flow-through, first elution, and second elution fractions were visualized on an SDS-PAGE (Figure 17).

![Figure 17: His₆-tagged ApoA-I purification as visualized on SDS-PAGE (12.5%)](image)

The flow through (FT) contained ApoA-I (28 kDa), which was likely due to the overloading of the column with the His₆-tagged protein. The first elution (E1) contained at least 95% ApoA-I with a few negligible unwanted proteins. More importantly, E1 contained a rather large quantity of ApoA-I, with E2 only containing a small amount.
(Note: the ApoA-I appeared abnormally small, with a band below the expected size of 28 kDa.)

In order to facilitate the removal of the N-terminus affinity tag, formic acid incubation was used to cleave the His$_6$-tag fusion. The acid labile peptide bond was created by mutating the second amino acid (Glu) to Asp to yield the NH$_2$-$^1$DDPPQ$^4$ sequence (Ryan 2003). This engineered peptide bond between the second and third amino acid allowed for specific chemical cleavage. For the current study, optimization of the reaction temperature was needed.

Figure 18: Non-cleaved His$_6$-tagged ApoA-I as compared to the cleaved products of 55, 60, and 65 °C incubations with 45% formic acid

In Figure 18, the non-cleaved ApoA-I, at decreasing quantities, can be seen in the first five lanes following the marker. The cleavage reactions at 55, 60, and 65 °C immediately follow, respectively. As seen, the 55 °C reaction did not completely remove the His$_6$-tag, and at 65 °C the cleaved protein appeared to begin to degrade. The 45% formic acid incubation at 60 °C for 5 h yielded the best results.
Discoidal rHDL particles and SUVs were produced by the cholate-dialysis method. Following the last buffer-exchange, the particles were taken to the OSU-CMIF for TEM images.

Figure 19: TEM image of rHDL particles (80:1:6:120) by Richard Montione

Figure 20: TEM image of rHDL particles (80:1:12:120) by Richard Montione
As seen in Figures 19 through 21, the 80:1:6:120 and 80:1:12:120 rHDL particles and SUVs, respectively, were produced as expected. The stain-driven hydrophobic stacking of the discoidal rHDL particles can be seen throughout the image as well as a large distribution of diameters. Typically, following purification via analytical ultracentrifugation, discrete sizes of approximately 7.6 nm and 9.8 nm are seen (Chen 2009). In the current study, no purification methods were utilized, so a range of approximately 5 nm to 20 nm can be seen in the image. The same can be seen with the 80:1:12:120 particles, although the stacking was not as evident (Figure 20). This was expected due to the additional cholesterol packed into the interior of the particle. The small unilamellar vesicles (SUVs) also have a large distribution in diameters as seen in the TEM image. The rHDL particles were also subjected to Native PAGE to further characterize their sizes.
As seen in Figure 22, both the 80:1:6:120 ("6x") and 80:1:12:120 ("12x") particles appear to have large distributions in size, although the average size is similar in both. This corresponds well with the results of the TEM images.

Recombinant Paraoxonase-1 Production and Characterization

The expressions and purifications of rePON1-G3C9-ybbR (GY) and its mutants, rePON1-Δ17-G3C9-ybbR (Δ17), rePON1-G3C9-ybbR-L12A (LA), rePON1-G3C9-ybbR-L12K (LK), rePON1-G3C9-ybbR-W194A (WA), and rePON1-G3C9-ybbR-W194K (WK) resulted in good yields for 1 L preparations (26, 20, 20, 24, 24, 20 mg L\(^{-1}\) for GY, Δ17, LA, LK, WA, and WK, respectively) and good purity (all >95%).
Figure 23: G3C9-ybbR purification as visualized on SDS-PAGE (12.5%)

Figure 24: Δ17-G3C9-ybbR purification as visualized on SDS-PAGE (12.5%)

Figure 25: G3C9-ybbR-L12A purification as visualized on SDS-PAGE (12.5%)
In Figures 23 through 28, G3C9-yybR and its mutants (~40 kDa) were seen in the insoluble pellet (P) fractions, but enough was found in the soluble cleared lysate (CL). A negligible amount of the protein is seen in the flow-throughs (FT). Very few proteins
were visualized in the three washes (W1, W2, W3), although a small amount of the rePON1 was seen in W3. The G3C9-ybbR and Δ17-G3C9-ybbR samples appeared to have an additional impurity in their first elutions, but a quick study showed that the bands near 80 kDa were oxidized dimers from improper handling before loading the gel (not shown). The remaining four mutants showed negligible impurities in their first elutions. None of the variants showed significant protein in their second elutions, indicating an efficient elution scheme.

Quantifications of the rePON1 proteins were completed utilizing the Bradford Assay with BSA. The concentrations were found to be 13, 10, 5, 6, 6, and 5 mg mL⁻¹ for GY, Δ17, LA, LK, WA, and WK, respectively.

Figure 29: SDS-PAGE (12.5%) to confirm Bradford assay concentrations of each variant

Assuming the above-mentioned concentrations were correct, 1 μg of each sample was loaded onto a 12.5% SDS-PAGE. By loading 5 μL of the USB Protein Marker (M) in the first lane (Figure 29), the assumed 1 μg of each variant loaded can be compared to that of the 50 kDa band, which is quantitatively 1 μg. With this visualization, it can be
determined that the LA, LK, WA, and WK variants have correct concentrations within error, but GY and Δ17 appear to be approximately 2-fold lower than calculated.

*Kinetic and Stimulation Assay Results*

The first step in determining apparent catalytic efficiencies of each variant included acquiring their initial velocities with respect to substrate concentration.

![Graph showing initial velocities (slopes) of G3C9-ybbR with various PhOAc concentrations.](image)

**Figure 30:** Initial velocities (slopes) of G3C9-ybbR with various PhOAc concentrations

As seen in Figure 30, the slope, or initial velocity, increased as substrate concentration increased. The enzyme concentration was held constant allowing for a relationship between the initial velocities and substrate concentration to be determined. This relationship followed Michaelis-Menten kinetics (Equation 2).
As seen in Figure 31, the $V_{\text{max}}$ was determined to be $0.071 \pm 0.001$ mM min$^{-1}$ and the $K_M$ was $3.0 \pm 0.1$ mM. Saturation is considered to be achieved when $K_M$ is greater than or equal to three times the maximum substrate concentration. Given that the resulting $K_M$ was on the order of the maximum substrate concentration (3.5 mM), the fit was said to be unsaturated. This required fitting only the linear region in order to calculate an apparent $k_{\text{cat}}/K_M$. 

Figure 31: Michaelis-Menten fit to G3C9-ybbR PhOAc assay results
As seen in Figure 32, the equation for the line was $y = 0.0196x + 0.0004$. In order to determine the apparent $k_{cat}/K_M$ (630 mM$^{-1}$ min$^{-1}$) the slope (0.0196 min$^{-1}$) was divided by the enzyme concentration (31 nM) (Equation 3). This process was completed for all six variants with both PhOAc as a substrate (arylesterase activity) and paraoxon (paraoxonase activity). A summary of the triplicated results for each assay can be seen below in Table 2 (± standard deviation).
Table 2: Kinetic results of G3C9-ybbR variants and the “wild-type” G3C9 enzyme (*acquired by graduate student David Mata*)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Arylesterase Activity (mM⁻¹ min⁻¹)</th>
<th>Paraoxonase Activity (mM⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-G3C9</td>
<td><em>7200 ± 2</em></td>
<td><em>61 ± 3</em></td>
</tr>
<tr>
<td>G3C9-ybbR</td>
<td>630 ± 10</td>
<td>1.89 ± 0.02</td>
</tr>
<tr>
<td>Δ17-G3C9-ybbR</td>
<td>284 ± 4</td>
<td>2.07 ± 0.01</td>
</tr>
<tr>
<td>G3C9-ybbR-L12A</td>
<td>1114 ± 4</td>
<td>3.79 ± 0.02</td>
</tr>
<tr>
<td>G3C9-ybbR-L12K</td>
<td>770 ± 10</td>
<td>4.65 ± 0.04</td>
</tr>
<tr>
<td>G3C9-ybbR-W194A</td>
<td>1360 ± 30</td>
<td>8.60 ± 0.03</td>
</tr>
<tr>
<td>G3C9-ybbR-W194K</td>
<td>1990 ± 40</td>
<td>19.5 ± 0.2</td>
</tr>
</tbody>
</table>

A single-point kinetic assay was used in order to study the stimulation of arylesterase activity due to the binding of the G3C9-ybbR enzymes to rHDL. First, each variant, at a constant concentration, was bound to a range of rHDL concentrations as discussed in the experiment methods. Following the binding step, each sample was diluted such that the final assay concentration of the enzyme would be similar to that of enzyme concentration for the arylesterase activity without rHDL stimulation. This was to ensure a linear response for each sample. The absorptions at 270 nm were collected as time progressed to yield an absorbance versus time plot (Figure 33).
Figure 33: Production of phenol by G3C9-ybbR when bound to rHDL (80:1:6:120)

The slope was converted to concentration (mM) per time (min) utilizing Beer’s Law, which resulted in the rate of the reaction. This slope was related to apparent catalytic efficiencies through Equation 4. Each slope (mM min⁻¹) was divided by the substrate concentration (0.06 mM) and enzyme concentration (mM) to yield a $k_{cat}/K_M$ for each variant and rHDL concentration. After plotting the apparent $k_{cat}/K_M$ for each concentration of rHDL an increase due to increasing rHDL could be seen for each variant (Figure 34).
The maximum increase for each variant’s $k_{cat}/K_M$ was taken as a ratio of the maximum value over the value at 0 mM rHDL. A summary of the triplicated results can be seen below in Table 3 (± standard deviation). Also, the recovered stability of the enzyme due to the rHDL particles can be seen in Table 3. This was calculated by comparing the ratio of arylesterase activity, without incubation, to the maximum enhanced arylesterase activity of each protein.
Table 3: The enhancement of arylesterase apparent catalytic efficiencies due to rHDL stimulation for each variant

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enhancement of $k_{cat}/K_M$</th>
<th>Recovered Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3C9-ybbR</td>
<td>8 ± 2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Δ17-G3C9-ybbR</td>
<td>20 ± 40</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>G3C9-ybbR-L12A</td>
<td>8 ± 2</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>G3C9-ybbR-L12K</td>
<td>8 ± 3</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>G3C9-ybbR-W194A</td>
<td>1,000 ± 38,000</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td>G3C9-ybbR-W194K</td>
<td>10 ± 10</td>
<td>1.26 ± 0.09</td>
</tr>
</tbody>
</table>

*Fluorescence Anisotropy Results*

The kinetics of binding between the G3C9-ybbR samples and rHDL particles were investigated with fluorescence anisotropy. To begin, the G3C9-ybbR and Δ17-G3C9-ybbR were labeled with fluorescein-CoA by Sfp overnight. The samples were purified to remove any non-bound free dye. At the completion of a buffer exchange into 20 mM Tris (pH 8) and 150 mM NaCl, the fluorescent samples were quantified at the 280 nm absorbance with a UV/Vis. The absorbance at 494 nm was also quantified in order to confirm fluorescein absorbance (Figure 35).
Following this quantification, mixtures of each enzyme (200 nM) and rHDL (0, 200, 1,000, and 2,000 nM) were made and allowed to bind via incubation in the same way as in the arylesterase activity stimulation by rHDL. Fluorescence scans of each free enzyme and bound mixtures were collected (Figure 36).
Figure 36: Fluorescence scans of G3C9-ybbR and Δ17-G3C9-ybbR with and without rHDL particles (80:1:6:120)

As seen above, each sample’s scan showed the expected emission peak near 520 nm. The addition of rHDL particles did not appear to affect the fluorescence in any biased manner. Finally, the fluorescence anisotropy of each sample was collected over approximately two minutes in order to acquire adequate data points. The average and standard deviation of each timed scan was calculated to indicate an overall anisotropy measurement for each mixture (Table 4).
Table 4: Fluorescence anisotropy data for each free and bound enzyme

<table>
<thead>
<tr>
<th>Enzyme (200 nM)</th>
<th>rHDL Concentration (nM)</th>
<th>Fluorescence Anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3C9-ybbR</td>
<td>0</td>
<td>-0.0009 ± 0.0011</td>
</tr>
<tr>
<td>G3C9-ybbR</td>
<td>200</td>
<td>-0.0015 ± 0.0009</td>
</tr>
<tr>
<td>G3C9-ybbR</td>
<td>1000</td>
<td>-0.002 ± 0.001</td>
</tr>
<tr>
<td>G3C9-ybbR</td>
<td>2000</td>
<td>-0.002 ± 0.001</td>
</tr>
<tr>
<td>Δ17-G3C9-ybbR</td>
<td>0</td>
<td>-0.0006 ± 0.0011</td>
</tr>
<tr>
<td>Δ17-G3C9-ybbR</td>
<td>200</td>
<td>-0.0007 ± 0.0016</td>
</tr>
<tr>
<td>Δ17-G3C9-ybbR</td>
<td>1000</td>
<td>-0.0019 ± 0.0008</td>
</tr>
<tr>
<td>Δ17-G3C9-ybbR</td>
<td>2000</td>
<td>-0.001 ± 0.001</td>
</tr>
</tbody>
</table>
Chapter 4: Conclusions

Production of ApoA-I Protein, rHDL Particles, and G3C9-ybbR Enzymes

As seen in the Chapter 3, the production of ApoA-I was rather straightforward. By receiving the pNFXex plasmid, the expression and purification were completed with very little optimization needed. The production of the rHDL particles involved more problem-solving, but in the end the particles were able to be produced multiple times with and without ApoA-I and at different concentrations of cholesterol. The cholate-dialysis method proved successful by TEM and Native PAGE. The results achieved with the particles appeared relatively good given that the rHDL particles were not subjected to purification such as analytical ultracentrifugation. No experiments were able to be completed based on the rHDL size due to the lack of purification, but the representation of a wide distribution of rHDL particles gave insights nonetheless.

The cloning of the multiple Null-ybbR vectors proved problematic, but the finalized Null-ybbR-F yielded an important scaffold for the G3C9-ybbR mutants produced. The G3C9-ybbR cloning was successful in producing plasmids that coded for variants that expressed well and were able to be purified.

Conclusions Drawn from Kinetic Assays

The arylesterase and paraoxonase kinetic assays revealed a few interesting conclusions for the variants in this study. First, when compared to the G3C9 “wild-type”
which has no ybbR tag at the C-terminus, all the variants lose significant activity against PhOAc and paraoxon. The ybbR tag may interfere in some manner during the enzymatic activity, although it appears the enzyme is well-folded, and still remains slightly active. This is surprising when compared to the C-terminal His6-tagged G3C9 “wild-type” without the ybbR tag. The His6-tag did not affect G3C9’s arylesterase or paraoxonase activity, suggesting that the ybbR tag causes significant issues.

Secondly, when the five mutations to G3C9-ybbR are compared to G3C9-ybbR a few more points of interest are highlighted. The deletion of the majority of the hydrophobic signal sequence (“H1”) significantly affected the enzyme’s arylesterase activity, but did not significantly affect its paraoxonase activity. This indicated the need for the signal sequence to be present for PhOAc turnover.

The mutation of the central Leu in “H1” to a smaller, nonpolar residue (Ala) only appears to recover some of the arylesterase and paraoxonase activities, while mutating it to a larger, positively charged Lys appeared to have no effect on both activities. This indicated that the central residue on H1 may not have a major effect on the either activity of the enzyme due to its third-dimensional separation of the active site.

The mutation to the surface Trp at position 194 had the most significant results. First, when mutated to Ala, the enzyme had an increase of about 2-fold in arylesterase activity and 4-fold in paraoxonase activity. Second, when mutated to Lys, the enzyme had an increase of about 3-fold in arylesterase activity and 10-fold in paraoxonase activity. Residue W194 is on the active site lid, so mutations in this region could greatly affect the accessibility of the substrate to the active site. Logically, the smaller residue
may allow for easier access of the substrate to the active site. The larger, positive residue is harder to explain, but it may be that the positive charge is attracted to the negatively charged residues near to it, allowing for an opening of the lid. Another simpler explanation could be that the charged Lys allows the G3C9 sample to be more soluble, hence readily more available for activity.

Most interesting were the results from the stimulation by rHDL on the arylesterase activity. The first thing noticed was that the incubation of the G3C9-ybbR variants without rHDL particles dropped their catalytic efficiencies possibly due to thermal inactivation. This was seen when comparing the arylesterase activities in the above-mentioned study to the activities of the enzyme in the stimulation study without any rHDL particles. Interestingly, their $k_{cat}/K_M$ values were recovered through binding to the rHDL. As seen in Table 3, Δ17-G3C9-ybbR did not recover as well as the others, probably due to its lack of binding affinity. The other enzymes appeared to return to their previous arylesterase activities, although G3C9-ybbR-L12A and G3C9-ybbR-W194K appeared to increase their activities ever so slightly. In vitro, the G3C9-ybbR variants can be affected by temperature, and this appears to be the case with the rHDL particles lending a hand in the G3C9-ybbR variants’ stabilities.

The relative increase of $k_{cat}/K_M$ with increased rHDL was significant. Specifically, the removal of the hydrophobic signal sequence (Δ17-G3C9-ybbR) increased the arylesterase activity by 2-fold when a high rHDL concentration was used. It is likely that the enzyme was still able to bind to rHDL, although to a much lesser extent. The residual binding of the rHDL particle to the enzyme had a larger effect on the
 enhancement than the L12 mutations. This may have been due to the additional need for the hydrophobic environment of the rHDL particle due to the lack of the hydrophobic sequence.

The mutation of position 12 to Ala or Lys did not appear to have an effect on the enhancement of the arylesterase activity. This indicated that the binding to rHDL may not have been affected by the mutations. The G3C9-ybbR-W194K mutant also had no effect on the enhancement indicating that the larger, positively charged residue also may only have a slight positive effect on the rHDL binding.

While the error is high, the G3C9-ybbR-W194A mutation had higher enhancement than any of the other variants. The replacement of the Trp with an Ala appeared to greatly increase the arylesterase activity of this enzyme with rHDL binding. With a smaller residue in place of a large aromatic residue, the binding may be better, or the accessibility of the substrate may play an important role or both.

Conclusions Drawn from Fluorescence Anisotropy Assays

The labeling of the enzymes with fluorescein-CoA was successful as seen in the UV/Vis absorption at 494 nm and the fluorescence scans of each mutant, but the lack of good fluorescence anisotropy data raised many questions. First, with anisotropy values basically zero, there was an indication that there may be free fluorescein-CoA in the samples. The quick tumbling rate (isotropy) indicated that this may be an issue. Controls were completed to show that the purification of the protein yielded a highly fluorescent flow-through with decreasing fluorescence in the subsequent washes (Figure 37).
The elution fractions regained the fluorescent character as expected. Another control allowed for the investigation of the free dye’s ability to make it into the elution fractions. This experiment included the free dye mixed with Sfp for overnight labeling without any protein present. The purification was completed the same way a labeled protein would be purified. The highly fluorescent flow-through and decreasingly fluorescent washes were similar to that of a labeled-protein purification. The important difference was when the no fluorescence was seen in the elution fractions of this negative control, indicating that the free dye could not independently make it through the purification process (Figure 37).

The only remaining source of free dye included the nonspecific attachment of the dye to the protein during labeling, and upon incubation the free dye being released. A silica thin layer chromatography (TLC) plate and solvent containing 85% chloroform, 15%
methanol and a drop of strong acid (8 M HCl) allowed for the separation of the protein and free dye. The protein remained at the bottom of the plate while a free dye control was seen higher up on the plate (Figure 38). Although it appears there may be some free dye released from the protein in the first lane, the free dye quantity appears insignificant when compared to the quantity of the protein.

![Figure 38: Thin layer chromatography of fluorophore-labeled protein and fluorescein-CoA (free dye)](image)

Given the control experiments, it is unlikely that non-bound fluorescein-CoA is the culprit for such anomalous fluorescence anisotropy measurements. The results indicate that the fluorescent molecule in the system, presumably the G3C9-ybbR samples, is tumbling much faster than that of a typical protein regardless of whether or not it is bound to a receptor like an rHDL particle. An explanation for this could be that the ybbR tag chosen is too flexible to accurately indicate the proteins tumbling rate. The ybbR tag is attached to the C-terminus end with a two very flexible linkers of Gly, Ser,
Ser, Gly on either side. This extra flexibility may allow for the tag to rotate freely and isotropically.

Summary of Conclusions and Future Work

In this study, the interaction between rePON1 variants and rHDL particles were investigated. First, the fluorescent tag incorporated at the C-terminus of the proteins was shown to be disadvantageous when considering arylesterase and paraoxonase activities, although it still allowed for comparisons of the mutations of interest. Interestingly, the placement of the Lys in position 194 allowed for the highest arylesterase and paraoxonase catalytic efficiencies. In the future, it would be desired to determine the change in arylesterase and paraoxonase activities of these mutations without the ybbR tag incorporated. Stimulation by rHDL particles on the arylesterase activity was seen for all six variants, with W194A having a larger enhancement. Again, the investigation of these results without the ybbR tag would be beneficial to know as well as the enhancement of the other variants’ activities.

Finally, the fluorescence anisotropy data was unable to elucidate association or dissociation rates for rePON1/rHDL binding. Choosing a different technique, like nonspecific binding of a fluorophore may be the next step in this endeavor.
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