CHARACTERIZATION OF HYDROLYTIC DEHALOGENASES:

SUBSTRATE SPECIFICITY AND CARBON ISOTOPE FRACTIONATION

A thesis submitted in conformity with the requirements for the degree of Master of Science.

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ABSTRACT:

Characterization of Hydrolytic Dehalogenases: Substrate Specificity and Carbon Isotope Fractionation:

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The first project is focused on kinetic analysis of two enzymes: Rsc1362 (Ralstonia solanacearum GMI1000) and PA0810 (Pseudomonas aeruginosa PA01). Rsc1362 had a $k_{cat}$ of 504±66 min$^{-1}$ and a $K_M$ of 0.06±0.02 mM, PA0810 had a $k_{cat}$ of 2.6±0.6 min$^{-1}$ and a $K_M$ of 0.44±0.2 mM. A lack of environmental context for a chloroacetate dehalogenase was noted in Pseudomonas aeruginosa PA01.

The second project focuses on kinetic and stable isotope fractionation of 1,2-dichloroethane by DhlA (Xanthobacter autotrophicus GJ10), and Jann2620 (Jannaschia CCS1). Although both enzymes had different kinetics (DhlA: $K_M = 4.8±0.6$ mM and $k_{cat} = 133±8$ min$^{-1}$, Jann2620: $K_M = 25.9±2.3$ mM and $k_{cat} = ~1.7$ min$^{-1}$), they fractionated similarly (ε values of -33.9‰ and -32.9‰ for DhlA and Jann2620, respectively). As calculated AKIE values were similar to the expected values of an abiotic reaction, it was determined that neither enzyme masks the intrinsic fractionation.
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1.0 INTRODUCTION:

1.1 Halogenated Organic Compounds
Halogenated organic compounds are an industrially relevant family of chemicals in the modern age, and they are found in a variety of commercial solvents, plastics, and pesticides. They are ubiquitous in global production lines; most primary examples are members of the chloro-organic compounds: 1,2-Dichloroethane (1,2-DCA), a precursor to PVC plastic, 1,1,2-trichloroethane (1,1,2-TCA) and 1,1,1-trichloroethane (1,1,1-TCA), which are used as industrial solvents, and dichlorodiphenyltrichloroethane (DDT), a well known insecticide.54 Brominated compounds are used in flame retardant materials, while fluorinated organics have been used in refrigeration (chlorofluorocarbons), stain-resistant coatings, non-stick coatings (polytetrafluoroethylene, or Teflon®), and lubricants.20,29,52

In a practical sense, these compounds are useful industrially due to a general rule of thumb: as a compound becomes more halogenated, there is an increase in stability of the molecule, as well as increased hydrophobicity, toxicity, and reactivity, while decreasing flammability, thus making them preferable building blocks for plastics and solvents, desirable for robust forms of pesticides, or intermediates in material pathways.

There is, however, a dichotomy to the production of these compounds. The same attributes important for their significance in industrial practices are also key factors in the environmental fate of these compounds; the added stability derived from the halogen bond increases the environmental half-life, while the relative hydrophobicity allows accumulation into organic phases, making it difficult to remediate.21 Chlorinated ethanes, for example, are known to accumulate and form dense non-aqueous phase layers (DNAPLs) in soil that slowly dissolve, providing continuous input of pollutant into groundwater aquifers that then contaminate drinking water.10 This is compounded by their relatively high water solubility, allowing for groundwater transport of these contaminants far from the primary site of entry.
The inherent toxicity of these compounds is also a concern; for example, many studies have shown a causal link between polychlorinated biphenyls and non-Hodgkin Lymphoma, a typically fatal form of cancer. A number of chlorinated compounds, including 1,2-DCA, 1,2-dichloropropane, and dichlorobenzenes are known or suspected causes of carcinogenic and hepatotoxic effects. It is no coincidence then, that of the 126 compounds classified as priority pollutants by the US Environmental Protection Agency (USEPA), 68 of them contain at least one halogen atom.

Due to their ubiquitous use and heavy production, environmental contamination of halogenated organics is widespread and significant. According to the USEPA, out of 1723 total National Priorities (NPL) sites, the United States alone currently (as of September, 2012) has 372 active sites containing 1,2-DCA, and 548 sites containing vinyl chloride (VC), both compounds that are known to be bioaccumulative, possibly carcinogenic, and manufactured in massive amounts. With respect to environmental and public health interests, removal and remediation of these contaminants are of vital interest.

1.2 Hydrolytic Dehalogenation
The breakage of the carbon-halogen bond is absolutely key in degradation of halogenated organic compounds, as the organic character of the halogen, which causes the toxicity, is lost. Cleavage of the carbon-halogen bond also encourages further downstream degradation, as the stability of the molecule is decreased. Enzymes (mostly microbial in nature) capable of catalyzing this reaction are termed dehalogenases, and interest has risen in these proteins due to their projected use in remediation or industry as a simple route to detoxification. Although there are multiple noted reaction mechanisms of aerobic and anaerobic dehalogenation (including reductive and oxidative), there is a focus on hydrolytic dehalogenases due to their relatively simple and robust reaction mechanism, which only requires water as a co-substrate (Eq. 1.1), and generates a halide ion, hydrogen ion, and an alcohol as products.
**Equation 1.1:** $RX + H_2O \rightarrow ROH + X^- + H^+$

Generalized hydrolytic dehalogenation reaction with a halogenated compound RX and water. The halide ion $X^-$ is replaced with an OH$^-$ group, and a proton is generated as a coproduct.

Hydrolytic dehalogenases are known to exist in two large enzyme superfamilies: the haloacid dehalogenase-like hydrolases (HAD) and the alpha/beta hydrolases (ABH)\textsuperscript{6,9,15,16,19} superfamilies. Additional information for both families are found in Chapters 1.6.1 and 1.6.2 respectively. The reaction mechanism for both families is similar. A conserved Aspartate (Asp) residue in the catalytic site serves as a nucleophile that forms a Michaelis complex with the substrate, followed by $S_N2$ displacement of the halide by nucleophilic attack, creating a covalent alkyl-enzyme intermediate\textsuperscript{9,16}. An H$_2$O molecule eventually hydrolyses this intermediate, resulting in the alcohol product (Figure 1.1).

![Figure 1.1: Hydrolytic dehalogenation mechanism of halide displacement.](image)

Conserved Asp nucleophile forms an ester intermediate with the substrate, driving out the halide via $S_N2$ displacement. The intermediate is then hydrolyzed, forming an alcohol product.

Both the HAD and ABH superfamilies are known to be involved in biodegradation of halocarbon pollutants, as many of the known examples have been isolated from environmental microbes capable of metabolizing toxic halogenated wastes as sole
sources of carbon and energy. The prime example is in *Xanthobacter autotrophicus* GJ10, a well known nitrogen-fixing bacterium capable of 1,2 Dichloroethane degradation, through the production of at least two hydrolytic dehalogenases (Figure 1.2)\(^4\). One is a haloalkane dehalogenase (HLD) known as DhlA\(^{22,28}\) from the ABH superfamily, which catalyzes the first dechlorination step, removing a chloride ion from 1,2-DCA. The second is a haloacid dehalogenase (HAD) known as DhlB\(^{61}\) from the HADSF, and it dechlorinates the subsequent chloroacetic acid, producing glycolic acid, which is then shunted into the metabolic pathways of the bacterium. In conjunction with two dehydrogenases, this allows Xanthobacter autotrophicus GJ10 to grow on 1,2 DCA as a sole carbon and energy source.

![Biodegradation pathway of 1,2-Dichloroethane by Xanthobacter autotrophicus](image)

**Figure 1.2: Biodegradation pathway of 1,2-Dichloroethane by Xanthobacter autotrophicus.**
This pathway highlights hydrolytic dehalogenation in an environmentally relevant setting. Degradation pathway involves two dechlorination steps, removing one halide from 1,2 DCA and Chloroacetic acid using DhlA (α/β hydrolase) and DhlB (Haloacid dehalogenase) respectively. The end product glycolate can directly enter the metabolic pathway of the organism. ADH = Alcohol dehydrogenase, ALDH = Aldehyde dehydrogenase. Figure is from Mena-Benitez et al, Plant Physiol. 2008\(^{45}\)

As the hydrolytic dehalogenation mechanism is inherently simple and robust in design, and requires only water as a cosubstrate, there is much interest in these enzymes for biotechnical applications, both in the environmental and industrial fields.

**1.3 Motivation and Goals**
The use of hydrolytic dehalogenases in environmental and biotechnological applications is suspected to have a strong positive impact due to the simplicity of the reaction mechanism, as well as the proven ability of these enzymes to cleave the highly stable carbon-halogen bond, a feat that is valuable not only in remediation of
toxic compounds, but may see use in industrial practices for the production of specialized chemicals. However, despite our current level of knowledge on enzyme mediated hydrolytic dehalogenation, there are many areas which require further understanding before specific application of these enzymes is achievable and their value maximized for societal use. This thesis will deal with two such areas.

The first area of interest is a more in-depth understanding of the structure-function relationship of substrate specificity within these enzymes; understanding the structural basis for controlling substrate specificity will be a boon for future applications of hydrolytic dehalogenase technology. In particular, the research of fluoro-organic specific dehalogenases is very appealing. As stated earlier, manufactured organofluorines are produced for a variety of purposes, and are significant due to their adverse global effects and environmental persistence. Natural hydrolytic dehalogenases capable of fluorocarbon degradation are rare; this is likely due to the large dissociation energy of the C-F bond rendering this bond less susceptible to cleavage than other halogen-carbon bonds. Working as part of a larger project, one research goal in this thesis is to obtain kinetic data of HAD hydrolytic dehalogenases, and add to a compendium of structural and kinetic data researched by others in parallel to investigate the structure-function relationship of fluorodehalogenases. The basis and relevant background information for this project can be found in Chapter 1.4.

The second area of interest focuses on understanding the relationship between hydrolytic enzymes and carbon isotope fractionation. A key aspect of many environmental studies is the ability to measure and quantify the level of degradation of a particular contaminant. The use of isotope fractionation has recently become regarded as a major tool for assessment of contaminant degradation, using a technique called Compound Specific Isotope Analysis (CSIA). There is debate however, whether or not the efficiency of an enzyme-mediated degradation pathway can affect isotope fractionation, by masking the expected fractionation effect. This is a significant point of interest, as understanding the nuances of fractionation variation may help increase the depth of information that can be
interpreted from CSIA analysis, which may lead to better conceptual understanding of a remediation site, and in turn, lead to better assessment and planning. Although there is evidence of masking in isotope fractionation during biodegradation, it is unsure whether these effects are at the enzymatic level, or due to some other biological effect, such as membrane transport of organic substrates. Thus, the second research goal of this thesis is to utilize purified hydrolytic dehalogenases of significantly different enzymatic efficiency to directly assess the effect, if any, of enzymatic efficiency on carbon isotope fractionation in 1,2-DCA. Two hydrolytic dehalogenase enzymes with known activity on 1,2-DCA and suspected to have significant variance in enzyme efficiency were chosen: DhlA from *Xanthobacter autotrophicus* Gj10 and Jann2620 from *Jannaschia* CCS1. The basis and relevant background information for this project can be found in Chapter 1.5.

The specific research objectives I addressed in this thesis for each project are presented below, followed by background information for both projects.

**The research goals for these projects are thus:**

**PROJECT 1: Adaptations for defluorination in HAD enzymes**

**Specific objectives:**

- Assessment of pH dependent activity for Rsc1362 and PA0810, using chloroacetate as a substrate
- Under optimal pH conditions, determine Michaelis-Menten Kinetic constants for Rsc1362 and PA0810 using chloroacetate as a substrate
- Integrate kinetic data with additional analyses from PhD student Peter Chan and Post Doc Pierre Petit to investigate the structural requirements for fluorodehalogenating activity, and attempt to illuminate features of the structure-function relationship in HAD superfamily members
PROJECT 2: Carbon Isotope Fractionation in Haloalkane Dehalogenase (HLD) enzymes

Specific Objectives:

• Assess pH dependent activity for DhIA and Jann2620 using 1,2-DCA as a substrate
• Under optimal pH conditions, determine the Michaelis-Menten kinetic constants for DhIA and Jann2620 using 1,2-DCA as a substrate, to confirm the difference in catalytic efficiency between the two enzymes
• Assuming that there is indeed a difference in catalytic efficiency, determine the isotope enrichment factors of both DhIA and Jann2620 on the degradation of 1,2-DCA (work done in conjunction with PhD student Lisa Douglas)

1.4 Background relating to Project 1: Structure/Function relationships between Defluorination and Dechlorination Specific Enzymes

1.4.1 Recent Genomic Screen reveals Novel Defluorinating Haloacid Dehalogenases
Although the general mechanism is known, not enough characterization data is available between different hydrolytic dehalogenases to provide a concrete understanding of the structure-function relationship of these enzymes, particularly with respects to substrate specificity. One goal of this project is to obtain structural data of known hydrolytic dehalogenases using X-ray crystallography and relate this data to enzyme kinetic analysis. In particular, we are interested in structural differences between a fluorodehalogenase vs a chlorodehalogenase.

A recent genomic screen by colleagues Wing Yiu (Peter) Chan, Max Wong and others uncovered new enzymes in both ABH and HAD superfamilies, including 4 new HADs with defluorinating capabilities, using fluoroacetate as a substrate. This is of interest due to two factors:
a) The Fluorine-Carbon bond is the strongest carbon halogen bond, making fluorodehalogenases rarer in comparison to other dehalogenases, and

b) defluorinating activity was previously thought to be exclusive to the ABH superfamily\textsuperscript{7,26,38}.

The large dissociation energy of the C-F bond (up to 130kcal/mol)\textsuperscript{55} renders this bond less susceptible to cleavage than other carbon halogen bonds. As a comparison, the dissociation energies in the series of halomethanes are 115, 83.7, 72.1, and 57.6 kcal/mol for F, Cl, Br, and I respectively\textsuperscript{3}. These novel enzymes represent the first HAD superfamily members to exhibit defluorinating activity\textsuperscript{31}. Interestingly, sequence similarity between the new HAD defluorinases do not significantly differ from non-defluorinating HADs; this similarity suggested parallel biochemical and structural characterization would be required to tease out important features specific to defluorination\textsuperscript{6}.

The collective discovery of these novel enzymes provided us with an interesting opportunity to perform comparative studies between enzymes that could defluorinate, and those that could not. To this end, four novel dehalogenase proteins from this screen were chosen for further characterization. Two defluorinating HADs discovered from the original screen: Bpro0530 from \textit{Polaromonas sp. JS666} and Rha0230 from \textit{Rhodococcus sp. RHA1}, and two dechlorinating (non-defluorinating) HADs: PA0810 from \textit{Pseudomonas aeruginosa PAO1} and RSc1362 from \textit{Ralstonia solanacearum}. It is noteworthy that these enzymes are specific for the dehalogenation of haloacids (fluoroacetate and chloroacetate Figure 1.3); no activity was recorded for haloalkanes or aromatic halocarbons.
1.4.2 Origins of the enzymes studied: Parent Species
The two defluorinating enzymes (Bpro0530 and Rha0230) were isolated from bacterial strains noted to have dehalogenase activity. *Polaromonas sp.* JS666 is a member of the family *Comamonadaceae* in the beta-proteobacteria, and is an aerobic, *cis*-dichloroethene (cDCE)-assimilating organism. It was isolated from a granular activated-carbon filter treating chloroethene-contaminated groundwater, and is capable of using cDCE as a sole carbon and energy source. *Rhodococcus sp.* RHA1 is a soil actinomycete that was isolated from lindane-contaminated soil and is best known for its potent ability to transform polychlorinated biphenyls (PCBs). Therefore, both of these defluorinating enzymes were isolated from strains noted for their ability to degrade chloro-organic compounds.

The two chloroacetate dehalogenating enzymes (Rsc1362 and PA0810) also come from similar parent strains. *Pseudomonas aeruginosa* PA01 and *Ralstonia solanacearum* GMI1000 are both well known pathogens to humans and plants respectively. *Ralstonia solanacearum* is an aerobic, non-sporing, Gram-negative plant pathogenic β-proteobacteria capable of colonizing the xylem and causing bacterial wilt to a widespread number of host plant species, including a variety of crop species such as potatoes, tomatoes, bananas, and tobacco. GMI1000 is a wide host range strain originally isolated from tomato in French Guyana in 1960. *Pseudomonas aeruginosa* is a Gram negative opportunistic pathogen with an extraordinary capacity to survive in, and adapt to, a wide range of environmental niches. It is the major cause of morbidity and mortality in human patients with
cystic fibrosis (CF). *P. aeruginosa* PA01 is a laboratory reference strain originally isolated from an infected burn/wound of a patient in Melbourne, Australia41.

1.4.3 Rationale and Breakdown of Project 1
As fluoroorganic compounds represent some of the most useful and diverse materials despite their adverse global impact and environmental persistence20,29,52, understanding the structural basis of defluorination is essential to the development of appropriate control and remediation technologies. The overall goal is to provide biochemical characterization of each enzyme (pH optimization and enzyme kinetics data), and compare them to structural data provided through X-ray crystallography. It is hopeful that this investigation will yield insight to the structure-function relationships within these enzymes. This project was a collaboration alongside Peter Chan (UofT, Dept. of Biochemistry) and Pierre Petit (UofT, Structural Proteomics in Toronto), who performed additional enzyme kinetics analysis and obtained the crystal structure data respectively. The work presented in this thesis will focus on my portion of the project: the kinetic analysis of the two chloroacetate dehalogenases Rsc1362 and PA0810 using isothermal calorimetry. However, I will be including the overall results and conclusions identified by the group as a whole.

1.5 Background relating to Project 2: Enzyme Efficiency and Carbon Isotope Fractionation

1.5.1 Compound Specific Isotope Analysis
Compound Specific Isotope Analysis (CSIA) is an increasingly utilized tool for assessment of biotic and abiotic degradation of organic contaminants. It has been used successfully to monitor the occurrence of biodegradation in the field, as well as a novel means of estimating the extent of biodegradation and quantifying biodegradation rates. CSIA has been used for a variety of organic contaminants including chlorinated ethenes/ethanes, petroleum hydrocarbons, and fuel additives such as methyl tert-butyl ether MTBE5,40,57. According to the USEPA, as of 2008, CSIA has been in transition from research tool to an applied method that would integrate itself into more comprehensive plans for management of contaminated sites60.
The concept of CSIA is based on Stable Isotope Fractionation, which will be reviewed in depth later on. Put simply, elements in a compound normally exists in a natural isotope ratio. When a compound undergoes degradation, these ratios will change in a predictable manner, depending on the type and extent of degradation. CSIA is able to detect these changes in isotope ratios precisely; given their predictability, these changes can be exploited to determine the fraction remaining of a compound, as well as information on the type of degradation pathway observed.

The technique involves collecting samples (for example, groundwater), which are prepared and then injected into a tandem GC-C-IRMS (gas chromatograph/combustion/isotope ratio mass spectrometer). The process involves (1) separation of individual carbon-bearing compounds on a gas chromatograph, (2) quantitative conversion of each compound to CO₂ in a high temperature combustion oven, and (3) removal of H₂O produced in combustion and introduction of the CO₂ derived from each compound into the mass spectrophotometer for isotopic analysis. After ionization of the CO₂, the IRMS separates ions using mass-to-charge ratios, allowing simultaneous measurement of the each isotope variant using fixed Faraday cups.

The high precision required in measuring isotope ratios at the natural abundance level of stable isotopes can only be achieved with this simultaneous ion measurement.
Figure 1.4: Schematic of the GC-IRMS and Overview of GC-IRMS Procedure in CSIA. The lower figure displays the instrumentation of CSIA, while the upper figure displays the respective output from each step. The figure showcases 1) the separation of individual carbon bearing compounds via GC, 2) conversion of selected compounds of interest to CO₂ in a high temperature combustion oven, and 3) ionization and mass spectrophotometry for isotopic analysis. Figure is from the USEPA guide for CSIA (2008)⁶⁰.

The main benefit of CSIA over traditional concentration-based monitoring techniques is its ability to distinguish between degradation and other forms of mass loss of a specific compound from a site. In the majority of remediation efforts, it is often difficult to unequivocally prove that a contaminant is being transformed, and not simply diluted or sorbed to the soil or sediment. For instance, a standard approach to characterizing degradation of a groundwater contaminant would require a dense network of monitoring wells, monitoring that extends for long periods of time, and mass balance calculations to estimate the extent of degradation. The level of effort and capital required in such an approach make CSIA more economically viable in many cases. In addition, any evidence of degradation given by concentration-based techniques is provided indirectly; these methods simply assess the concentration of a compound at discrete locations at a site. As CSIA does not measure the mass of a contaminant, but rather the isotope ratio, it can directly
assess the presence of degradation in addition to information on the type of degradation, and the fraction remaining from the initial population of contaminant, even if the contaminant is subject to dilution, for example from influx of rainwater or changing water table levels. CSIA in combination with concentration data provides a better understanding of the conceptual model of the site, and can lead to a more effective strategy for remediation.

1.5.2 Stable Isotope Fractionation

In brief, many compounds exist in a range of isotopic variants, with each variant differing in isotopic composition (or the number of neutrons), but not elemental composition. In analysis of halogenated organic compounds, it is usual to focus on the carbon isotopes: $^{13}$C and $^{12}$C. $^{12}$C is the most abundant isotope of carbon. About 1% of Carbon exists as $^{13}$C. This natural abundance is commonly represented as a ratio of heavy to light isotopes ($^{13}$C/$^{12}$C); drift away from this natural abundance ratio is known as fractionation.$^{18}$

It is known that for chlorinated hydrocarbons, nondegradative processes that can decrease the overall mass of the compound in a given space (volatilization, dissolution, and sorption) are nonfractionating under equilibrium conditions.$^{18}$ A chlorinated hydrocarbon such as 1,2-DCA therefore maintains a natural $^{13}$C/$^{12}$C isotope ratio under such conditions. In contrast, degradation processes, whether by biotic or abiotic means, are mass-discriminating due to inherent bond energy differences between heavy and light isotopes (the ratio between reaction rates of a heavy vs light isotope is called the Kinetic Isotope Effect, or KIE, and is elaborated upon in Chapter 1.5.3). Typically degradation processes enrich the relative concentration of the heavy isotope-containing compounds, as the light isotope-containing compounds are preferably degraded due to lower bond strength.$^{5,18,40}$ Thus, measurement of the $\delta^{13}$C (defined as the relative enrichment of $^{13}$C containing compounds, measured in ‰) of a compound found in the environment, when compared to a known standard, can give a quantitative evaluation of how much degradation of the compound has occurred within a system. The measure of $^{13}$C
enrichment between a sample undergoing degradation and a known standard can be defined as the delta value, or $\delta^{13}C$, using the equation:

$$\delta^{13}C \%_0 = 1000[R_{\text{sample}}/R_{\text{std}} - 1]$$

where $R_{\text{sample}}$ and $R_{\text{std}}$ represent the $^{13}C/^{12}C$ ratio in the sample and standard respectively, and $\delta^{13}C$ is given in units of permil.

Isotope fractionation based techniques such as CSIA involves measurement of the ratio of heavy to light isotopic elements (such as $^{13}C/^{12}C$) relative to a known standard to normalize instrument variability (defined as $\delta^{13}C$), and using this to quantify the fraction remaining of a compound undergoing degradation using $\epsilon$, the experimentally determined isotopic enrichment factor. The $\epsilon$ value defines the relationship between how much a compound is isotopically enriched as it is degraded, and the fraction remaining. The value is critical for CSIA, and calculation of $\epsilon$ is a key goal for many degradation studies.

The determination of the isotopic enrichment factor $\epsilon$ requires experimental data on the enrichment of the heavy isotope, and report of $\delta$ values in units of permil ($\%_0$). The relationship between fraction remaining and $C^{13}$ enrichment is represented by the following equation, known as the Rayleigh equation$^{5,18,40,57,62}$:

$$\frac{R}{R_0} = f^{(\alpha-1)}$$

Where $R$ is the isotopic composition ratio ($^{13}C/^{12}C$) of a substrate at any given time with respect to the initial isotope composition $R_0$ and the fraction of the substrate remaining ($f$). The fractionation factor, $\alpha$, is a measure of the difference in reaction rates of heavy versus light isotopic molecules, and is considered a constant. The fractionation factor, $\alpha$, is experimentally determined by plotting $\ln f$ vs $\ln(R/R_0)$ and determining the slope ($m$) of the linear regression, where $m=(\alpha-1)^{18,57,62}$. This
fractionation factor represents the difference in reaction rates of isotopically enriched versus isotopically depleted molecules, and can also be expressed through the equation:

\[ \varepsilon = 1000(\alpha - 1) \text{ [%]} \]

The \( \varepsilon \) value is critical for reliable use of CSIA. If fractionation is to be used to predict degradation, then the isotopic enrichment factor \( \varepsilon \) for a particular compound undergoing a particular degradation process or pathway must be reproducible. The Handbook on CSIA\(^56\), representing the USEPA’s opinion based on data from Elsner et al\(^{12}\), 2005; Mancini et al\(^{40}\), 2003; Meckenstock et al\(^{44}\), 2004; Morrill et al\(^{47}\), 2006; Schmidt et al\(^{56}\), 2004 on compound specific \( \varepsilon \) values determined for various biodegradation processes suggest that, in general, there is good agreement between enrichment factors of the same compound undergoing the same degradation process. There are known examples however, where the isotopic enrichment factor \( \varepsilon \) can change, due to a phenomenon known as the AKIE, which we will discuss next.

### 1.5.3 Apparent and Intrinsic Kinetic Isotope Effects

The Kinetic Isotope Effect (KIE) of a compound is described as the ratio of the reaction rates of two isotopically labeled molecules in a chemical reaction\(^12\). For isotopically differentiated 1,2 DCA in an enzyme catalyzed reaction, the KIE is described as:

\[ KIE = \frac{K_{12}}{K_{13}} \]

Where \( K_{13} \) and \( K_{12} \) are reaction rates for the dechlorination of the \(^{13}\text{C}\) and \(^{12}\text{C}\) variants of 1,2 DCA respectively.

If we observe the KIE in a manner removed from the complexities of enzymatic catalysis and environmental factors, then theoretically, the KIE is predictable based solely on bond theory. This predicted KIE is known as the *intrinsic* KIE\(^48\), and refers to the full effect originating from the single isotopically sensitive step of catalysis,
exclusive of all interference from isotopically insensitive steps. It therefore represents the theoretical maximum effect an isotopic variance can have on a kinetic reaction.

However, the apparent KIE (the observed difference in reaction rates, abbreviated as AKIE), may differ from the intrinsic KIE. The observation that enzyme catalyzed reactions can “mask” the effect of the KIE was theorized by Northrop in 1981. This change is attributed to the realization that the intrinsic KIE is only observable if the bond change is the sole rate-determining step in the overall reaction. In an enzyme-catalyzed process, however, this is rarely the case. Bond conversion can be preceded by a non or only slightly fractionating process such as transport to reactive sites, adsorption to reactive surfaces, or formation of enzyme-substrate complexes in biotransformations\textsuperscript{12}. These additional processes can “mask” the effect of the intrinsic KIE, and can be simplistically described by the following scenario described by Northrop\textsuperscript{48}:

\[
E + S \underset{k_{-1}}{\xrightarrow{k_1}} ES \underset{k_2}{\xrightarrow{}} E + P
\]

where E represents the enzyme, S represents the substrate, ES represents the enzyme-substrate complex, and P represents the product. \(k_1\) and \(k_{-1}\) are the first order rate constants for the formation and dissociation of the enzyme-substrate complex, respectively, and \(k_2\) is the rate constant for the chemical transformation of the substrate. If we assume that there are negligible isotope effects on \(k_1\) and \(k_{-1}\), the relationship between the AKIE, the KIE, and the individual rate constants during an enzymatic reaction can be described by the following equation\textsuperscript{12,48,57}:

\[
AKIE = \frac{KIE + C}{1 + C}
\]

Where \(C\) represents the “commitment to catalysis” which is defined as \(k_2/k_{-1}\), and represents the tendency of the formed enzyme-substrate complex to go forwards
through catalysis rather than dissociate back to the free enzyme and unreacted substrate, and is intended to describe the efficiency of forward catalysis in an enzyme already bound to a substrate. In cases where the efficiency of the enzyme is limited \((k_{-1} \gg k_2)\), the \(C\) value is negligible, and AKIE approaches the intrinsic KIE. In a case where the efficiency of the enzyme is high \((k_2 \gg k_{-1})\), however, there is a masking of the intrinsic KIE as AKIE approaches unity. This seems intuitive; an enzyme that catalyses a reaction strongly will less likely to discriminate between the bond strength of a \(^{12}\)C and \(^{13}\)C. Put in other terms, the more efficient an enzyme is at catalysis, the less of an effect the KIE has on fractionation, and the smaller the \(\epsilon\) value.

### 1.5.4 Rationale and Formation of Project #2

Masking of the KIE is a noted phenomenon within the literature. In an experiment by Sherwood Lollar et al, 2010\(^5\), the biodegradation of 1,1,1-TCA and 1,1-DCA by a mixed Dehalobacter culture presented \(\epsilon\) values of -1.8‰ and -10.5‰ respectively. These values were significantly lower than values reported for abiotic reductive dechlorination of the same compounds (-15.8‰ and -17.9‰), suggesting some form of masking was occurring due to biological effects. Similar results were obtained from experiments degrading the same contaminants using cell free extracts (-0.8‰ and -7.9‰ respectively). Comparison to previous studies demonstrates that these patterns of isotopic fractionation masking are not attributable to transport effects across the cell membrane. It was suggested that the results reflected significant differences in the kinetics of the enzymes catalyzing chlorinated ethane degradation. Similar conclusions of enzyme efficiency masking isotopic fractionation can be found in Chan et al, 2012\(^5\), and Mancini et al, 2006\(^4\).

These papers however, infer the effect of enzyme efficiency on fractionation masking using data that is indirect; they all utilized whole cell or cell extract assays to obtain data which complicate analysis of any enzyme mediated effect. To the best of our knowledge, there has not yet been a comprehensive study of stable isotope fractionation in 1,2 DCA biodegradation using pure enzymes. The assessment of \(\epsilon\) values in a “clean” assay is necessary for further investigation into the Kinetic
Isotope Effects (see section 1.5) within 1,2 DCA degrading enzymes, without the added complexity of live cultures (metabolic processes, cell barriers to substrate entry, etc...).

What is therefore missing from the discussion is a definitive experiment designed to investigate the effect of enzymatic efficiency on AKIE at the enzyme level. The overall research goal of this project is therefore to obtain direct information on enzyme mediated AKIE effects using purified protein that all catalyze the same reaction, with the same mechanism, but at different efficiencies.

To this end, the haloalkane dehalogenases DhlA and Jann2620 were proposed to be characterized for 1,2-DCA degrading activity, observing both enzyme kinetics and carbon isotope fractionation. 1,2-DCA (Figure 1.5) was chosen as the substrate as it was

a) a priority pollutant, providing relevant context in the field of environmental remediation  
b) a known substrate for DhlA and Jann2620, two enzymes we already had cloned and could readily purify  
c) a compound for which analytical methods of assessing activity, carbon isotope fractionation, and kinetics were already established on local equipment

![Figure 1.5: Structure of 1,2-Dichloroethane (1,2-DCA)](image)

DhlA is a well known haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10\textsuperscript{22}, while Jann2620 is a haloalkane dehalogenase isolated from *Jannaschia* sp.
CCS1, recently discovered to harbor 1,2-DCA degradation activity from a screen performed by Peter Chan et al., DhlA is an HLD-I haloalkane dehalogenase, which is in contrast to Jann2620, a member of the HLD-II subfamily (for additional information on HLD subtypes, please consult Chapter 1.6.2). Both enzymes are from the ABH superfamily, and both employ the same hydrolytic dehalogenase reaction mechanism. Structurally speaking, a catalytic Asp (DhlA) is switched for a glutamate (Glu) (Jann2620), and Jann2620 lacks a secondary halide binding tryptophan (Trp). Preliminary screenings have also suggested Jann2620 is much slower in 1,2 DCA degradation than DhlA.

With respect to the interests of this thesis, it was known from preliminary trials that DhlA and Jann2620 have vastly different catalytic potential for 1,2-DCA degradation; DhlA is greatly more efficient at 1,2-DCA dechlorination. This provides us with a unique stage to investigate Northrop’s theory on AKIE. If DhlA is indeed significantly more efficient than Jann2620, we should see a masking effect on KIE for DhlA, and thus an ε value closer to zero.

1.6 Additional Background Information on Haloacid and Haloalkane dehalogenases:
This section includes additional information not required for interpretation of the motivations of either project, but that helps discussion and understanding of the results. This section will contain background information on the known structural data for both HADs and HLDs, substrates used and their relevance, and a primer on the use of Isothermal Titration Calorimetry (ITC) on measuring enzyme kinetics.

1.6.1 Haloacid Dehalogenases
Haloacid dehalogenases (HADs) catalyze the dehalogenation of halocarboxylic acids. There are at least two phylogenetically distinct families of HADs: L-2-haloacid dehalogenases (L-DEXs; EC 3.8.1.2) which catalyze the transformation of L-2-haloacids to stereochemically inverted D-2-hydroxyacids, and D-2-haloacid dehalogenases (D-DEXs; EC 3.8.1.9), which catalyze the D-2-haloacids into L-2-
hydroxyacids. A third type of HAD, the DL-DEX (EC 3.8.1.10, 3.8.1.11) is capable of interacting with both enantiomers and appears to be phylogenetically related to D-DEXs\textsuperscript{27}. Of the three subfamilies, L-2-haloacid dehalogenases are the most studied and characterized.

The Haloacid Dehalogenase Superfamily (HAD) surprisingly comprises more of phosphatases (thought to be \textasciitilde 90\% of the family), P-type ATPases, and phosphoglucomutases rather than true haloacid dehalogenases \textsuperscript{1,2,4}. Indeed the majority of the 25,000 members handle phosphoryl transfer chemistry rather than halide transformation\textsuperscript{24}. Despite the larger range of substrates, all enzymes within the superfamily catalyze their respective transformations through a hydrolytic reaction mechanism. The structure of HADs includes a core domain in a Rossman fold arrangement with a six-stranded parallel \(\beta\) sheet flanked by \(\alpha\) helices, which contain 4 highly conserved sequence motifs on loop regions; residues on these motifs contribute catalytic features to the active site and are used to identify HAD superfamily members. The structure also features a variable cap domain responsible for substrate specificity composed entirely of \(\alpha\) helices\textsuperscript{1,2,4}.

HAD-mediated dehalogenation occurs via the standard hydrolytic dehalogenation mechanism described earlier. This mechanism is established from the functional and structural investigations of 3 model enzymes: L-Dex YL from \textit{Pseudomonas sp.} YL\textsuperscript{35,36}, DhIB from \textit{Xanthobacter autotrophicus} GJ\textsuperscript{50,51}, and DehIVa from \textit{Burkholderia cepacia} MBA\textsuperscript{49,55}. A conserved aspartate nucleophile attacks the halogenated \(\alpha\)-carbon via SN2 reaction, which creates an ester intermediate that is subsequently hydrolyzed by water, yielding the \(\alpha\)-hydroxyacid product and regenerating the enzyme. It is notable that L-DEXs are unique among HADs to require no cofactors for activity.
**Figure 1.6: General HAD mediated hydrolytic dehalogenation mechanism on haloacid substrates.** The haloacid substrate is recognized by the substrate recognition site, while the binding pocket stabilizes halide release. Glycolate and halide ion are the main products. Figure is courtesy of Peter Chan (UofT Biochemistry).

1.6.2 Haloalkane Dehalogenases

Haloalkane dehalogenases (EC 3.8.1.5) facilitate the removal of a halide from halogenated alkanes, such as 1,2-dichloroethane (1,2-DCA).

Haloalkane hydrolytic dehalogenases (HLDs) are from the ABH superfamily, a large group that spans a diverse set of hydrolytic functionalities, including esterases, thioesterases, epoxide hydrolases (epoxidases), and dehalogenases. To this date, only DhlA of *Xanthobacter autotrophicus* GJ1 and dhmA of *Mycobacterium avium* N85 have been characterized within literature as 1,2-DCA degrading enzymes. Other characterized haloalkane dehalogenases such as LinB (*Sphingobium japonicum* UT26), DrbA (*Rhodopirellula baltica* SH1) and DmbC (*Mycobacterium bovis* 5033/66) all have different substrate specificities (1,2,3 Trichloropropane, 1-iodobutane, and 1,3-diiodopropane respectively) and are unable to degrade 1,2-DCA.

The crystal structures of at least 3 ABH HLDs have been solved – DhlA, LinB, and DhaA (*Rhodococcus sp.*)

The structures reveal a two-domain tertiary structure similar to HAD hydrolases, involving a core domain composed of an 8 stranded mostly parallel β sheet surrounded by α-helices, and an α-helical cap domain that exhibits significant structural variability. The α/β fold of the core domain serves as a scaffold for the main catalytic residues, while the flexible cap domain has been shown to influence substrate specificity. A conserved Asp nucleophile, along with a catalytic acidic residue (Asp or Glu) and a His (base) constitute a conserved catalytic
triad, which along with two halide stabilizing residues (Trp and Trp or Asn) reside in a region between the core and cap domains. This interdomain region, which is comparatively hydrophobic, forms the active site cavity to bind haloalkanes\textsuperscript{25,39,64}.

Traditionally, HLDs were classified according to substrate specificity, but such classification is problematic due to insufficient biochemical characterization of the majority of the HLD family as a whole. Chovancova et al\textsuperscript{8} attempted to classify haloalkane dehalogenases using a phylogenetic approach. From their work, three subfamilies of HLDs can be described, denoted as HLD-I, HLD-II, and HLD-III. Most of the biochemically characterized HLDs are found in the HLD-II subfamily, although notably DhlA from \textit{Xanthobacter autotrophicus} GJ10 is classified as an HLD-I.

Between subfamilies, the composition of the catalytic pentad differ, and there seems to be variation regarding the substrate specificity of each group (Table 1.1). Enzymes within the HLD-I subfamily tend towards the dehalogenation of small, terminally halogenated substrates, while HLD-II enzymes lean towards larger, β-substituted substrates. The HLD-III subfamily was postulated to exist through phylogenetic analysis of existing genome databases, but currently no experimentally characterized proteins were available within this family, thus the substrate specificity of this subfamily is currently unknown.

\textbf{Table 1.1: Table of Experimentally Characterized Haloalkane Dehalogenases}. Known HLDs are phylogenetically placed into different subfamilies, and characterized with substrate specificities and important catalytic residues. Table is data summarized from Chovancova et al 2007.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Protein</th>
<th>Organism</th>
<th>GI number</th>
<th>Substrates</th>
<th>Catal. pentad</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLD-I</td>
<td>DhlA</td>
<td>\textit{Xanthobacter autotrophicus}</td>
<td>442872</td>
<td>Small, terminally halogenated</td>
<td>Asp-His-Asp-Trp-Trp</td>
</tr>
<tr>
<td></td>
<td>DhmA</td>
<td>\textit{Mycobacterium avium}</td>
<td>41408155</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DmbB</td>
<td>\textit{Mycobacterium tuberculosis}</td>
<td>15609433</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLD-II</td>
<td>LinB</td>
<td>\textit{Sphingobium japonicum}</td>
<td>4521186</td>
<td>Larger, β-substituted</td>
<td>Asp-His-Glu-Asn-Trp</td>
</tr>
<tr>
<td></td>
<td>DmbA</td>
<td>\textit{Mycobacterium tuberculosis}</td>
<td>13882401</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DmsA</td>
<td>\textit{Mycobacterium smegmatis}</td>
<td>16508080</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DhaA</td>
<td>\textit{Rhodococcus sp.}</td>
<td>7245711</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DjbA</td>
<td>\textit{Bradyrhizobium japonicum}</td>
<td>27349338</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DmA</td>
<td>\textit{Mesorhizobium loti}</td>
<td>13474464</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DatA</td>
<td>\textit{Agrobacterium tumefaciens}</td>
<td>16119878</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLD-III</td>
<td>DrbA</td>
<td>\textit{Rhodopirellula baltica}</td>
<td>32476333</td>
<td>Unknown</td>
<td>Asp-His-Asp-Trp</td>
</tr>
<tr>
<td></td>
<td>DmbC</td>
<td>\textit{Mycobacterium tuberculosis}</td>
<td>15608970</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.0 METHODS:

2.1 Recombinant Cell Lines:
Rsc1362 (*Ralstonia solanacearum*), PA0810 (*Pseudomonas aeruginosa* PAO1), DhlA (*Xanthobacter autotrophicus*), and Jann2620 (*Jannaschia* sp. CCS1) were all cloned into p15TV-L vectors (GenBank Accession Number EF456736). An N terminus His\textsubscript{6}-tag was Overexpression was achieved with *E. coli* BL21 CodonPlus (DE3) RP (*Lon* protease/OmpT deficient) cultures. Vector construction was performed with the InFusion ligation-independent method; negative selection was performed with 5% sucrose. These cell lines were made by Max Wong (University of Toronto, Dept. of Chemical Engineering) and Peter Chan (University of Toronto, Dept. of Biochemistry).

2.2 Recombinant Protein Purification:
Recombinant BL21 cells were grown overnight at 37°C in 20mL aliquots of Luria Bertani (LB) broth, with 100 ug/mL of ampicillin, and 50 ug/mL of kanamycin. This culture was used to inoculate 1L batches of Terrific Broth (TB), which also contained 100µg/mL of ampicillin, and 50µg/mL of kanamycin. The culture was grown at 37°C for 5-6 hours until an OD600 of 0.8 to 1.0 was reached, at which point the culture was inoculated with IPTG to a final concentration of 0.5mM to induce recombinant protein expression. Growth then proceeded at 16°C overnight (16-18 hours), and then culture was harvested through centrifugation at 4°C, 8,000 RPM for 25 minutes.

| Table 2.1: Composition of various buffer solutions created for purification of recombinant protein. All buffers maintained similar levels of salt, glycerol, and pH, but differed in Imidazole content. Buffers were generally made fresh 24 hours prior to purification. |
|----------------|----------------|--------------|----------------|
|                | Na\textsubscript{2}SO\textsubscript{4} | Imidazole    | Glycerol       | HEPES pH 7.5   |
| Binding Buffer | 300mM           | 5mM          | 10%            | 50mM           |
| Wash Buffer    | 300mM           | 50mM         | 10%            | 50mM           |
| Elution Buffer | 300mM           | 250mM        | 10%            | 50mM           |
The cell pellet was then resolubilized in 25-35mL of Binding Buffer (BB, Table 2.1) using a 50 mL Falcon tube and vortex mixing. The slurry was subjugated to sonication by rectangular waveform duty cycle (3 s on, 7 s off) for a total active time of 10 minutes. Sonication was performed in pre-chilled metal cups in ice water. The lysed cell slurry was then centrifuged at 4°C, 24,000 RPM for 40 minutes to pellet and remove cell debris from soluble protein.

All subsequent steps were performed at 4°C. The cleared cell lysate supernatant (containing soluble protein) was combined with 5mL of Nickel Nitrilotriacetate beads (Ni-NTA) and left to rotate for 15 minutes to establish binding between the Ni-NTA beads and His-tags of the recombinant protein. The lysate and bead mixture was slowly passed through a column until only a fraction (~1cm) of liquid remained above the collected beads at the bottom of the column. The flowthrough liquid (designated FT) was collected for SDS-PAGE.

Wash buffer (WB, Table 2.1) was then slowly added to the column to remove impurities bound to the Ni-NTA beads. A constant flow in and out of the column was maintained until flowthrough did not show any reaction with Bradford reagent, indicating the column was clean of non-His-Tagged protein. Flowthrough from this section was labeled Wash Flowthrough (WF), and saved for SDS-PAGE.

Elution buffer (EB, Table 2.1) was then passed through the column to remove the purified recombinant protein from the Ni-NTA beads. The EB was passed in 5 mL aliquots until the flowthrough did not show reaction with Bradford reagent. The elution was collected, quantified, concentrated (if necessary) prior to being flash frozen in liquid nitrogen and stored at -80°C.

2.2.1 Protein Quantification and Quality control:
Protein concentration was determined with a Nanodrop ND-1000 spectrophotometer (A280). The recombinant protein was concentrated using 10kDa Millipore Amicon Centrifugal Filter Units using the prescribed rotational speed at 4°C. Final protein concentration was aimed for over 25mg/mL.
2.3 pH Optimization Assays:
All enzymes (HAD and HLDs) were tested for optimal activity from a pH range of 7.5 – 10.0 (see Table 2.2). Buffer pH was controlled only using H₂SO₄ and NaOH to avoid background noise for halide quantification (no F⁻, Cl⁻, Br⁻, or I⁻ was added to the buffer via HCl, etc...).

Each trial was performed in 1mL aliquots within standard 1.5mL Eppendorf tubes. Assays contained a liquid concentration of 10mM of substrate (1,2 DCA for DhIA and Jann2620, or Fluoro/Chloro/Bromo/Iodo-Acetic Acid for Rsc1362 and PA0810), 25mM of the corresponding buffer solution, as well as 20µg/mL of dehalogenase enzyme.

All components other than the protein were added and vortexed to ensure proper dispersal of substrate. Protein was added immediately prior to incubation and vortexed to ensure consistency in timing. Assays were run at 30°C and shaken at 300 RPM for using an Eppendorf Thermomixer-R (1.5mL), and the reaction was quenched upon addition of 10µL of 2M H₂SO₄ (reduction of pH to ~1). Rsc1362 and DhIA were incubated for 20 minutes, PA0810 and Jann2620 for 3-5 hours. Negative control blank standards were created for each assay, each containing identical reagent concentrations including buffer, substrate, and protein, but quenched at time zero. Products of reaction were quantified using Ion Chromatography. Resulting data was analyzed for pH optimization.

Table 2.2: pH buffers utilized for pH optima studies of Rsc1362, PA0810, DhIA, and Jann2620. All pH stocks were created within 3 months of experimentation, and kept at 4°C in 15mL Falcon Tubes.

<table>
<thead>
<tr>
<th>pH</th>
<th>7.5</th>
<th>8.0</th>
<th>8.5</th>
<th>9.0</th>
<th>9.5</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>HEPES-Na</td>
<td>TRIS-SO₄</td>
<td>TRIS-SO₄</td>
<td>CHES-Na</td>
<td>CHES-Na/BORAX</td>
<td>CAPS-K</td>
</tr>
<tr>
<td>Stock Concentration</td>
<td>1M</td>
<td>500mM</td>
<td>500mM</td>
<td>500mM</td>
<td>500mM</td>
<td>500mM</td>
</tr>
<tr>
<td>Working Concentration</td>
<td>25 mM</td>
<td>25 mM</td>
<td>25 mM</td>
<td>25 mM</td>
<td>25 mM</td>
<td>25 mM</td>
</tr>
</tbody>
</table>
2.4 pH Optima Analysis and Quantification

2.4.1 Ion Chromatography (HAD pH optimization):
Ion chromatography allows separation and quantification of both product (glycolate, halide ion) and substrate (haloacid) in the expected reactions for Rsc1362 and PA0810. Quenched samples from pH optimization trials were centrifuged at 13,000 RPM for 10 minutes to remove particulates, then diluted 10X. Diluted samples were loaded into a Dionex AS40 autosampler in 0.5mL sample vials and injected using a 20μL injection loop.

A Dionex AS19 anion exchange column was used to separate compounds of interest. An elution gradient profile of 2mM NaOH (20mins) followed by 20mM NaOH (20 mins) was used to separate Fluoroacetate and Chloroacetate from Glycolate. An isocratic eluent of 20mM NaOH was used to separate Bromoacetate and Iodoacetate from Glycolate. Both elution profiles were run at 1mL per minute flow rate.

Integration areas of resolution peaks were transformed into calculated molar concentrations using standard curves. Blank subtraction of raw data was utilized to ensure proper measurement. Quantification and analysis of glycolate product formed determined the rate of reaction in each assay.

2.4.2 Ion Chromatography (HLD pH optimization):
Ion chromatography allows separation and quantification of product (Chloride ion) in the expected reactions for DhlA and Jann2620 with 1,2 DCA. As with the HAD pH optima samples, quenched reactions were centrifuged at 13,000 RPM for 10 minutes to remove particulates, particularly the precipitated acidified protein, and then diluted 10 fold. Samples were loaded into a Dionex AS40 autosampler (0.5mL vials) and injected using a 20μL injection loop.

A Dionex AS14 anion exchange column was used to separate compounds of interest. An isocratic bicarbonate mobile phase consisting of 3.5 mM Na₂CO₃ and 1mM NaHCO₃ was used to elute bound anions. Runs lasted 20 minutes at a flow rate of 1mL per minute. Quantification of chloride ion was used to assess enzyme activity.
2.5 Isothermal Titration Calorimetry (HAD Kinetic Analysis):
A TA Instruments TAM III Isothermal Calorimeter (TA Instruments, New Castle, USA) was used for all readings. Internal cell temperature for both the reference and analytical ampoules was kept constant at 25°C, and all reactions were run with 25mM CHES pH 9.5 as buffer. A gold plated propeller stirrer kept the reaction solution well mixed (25 RPM), and both ampoules had a maximum volume of 1.4mL. A single injection technique (see Calorimetry, Section 1.5) was used to determine the apparent enthalpy of the dechlorination reaction. An experimental set up included 5 titrations of 25µL of 100mM ClAc into a 1mL ampoule containing either no enzyme or 20µg/mL of Rsc1362/PA0810. Titrations were placed 1 hour apart to ensure total breakdown of the added substrate.

All data producing experimental runs were performed using the multiple injection technique (see Calorimetry section in background information). The reference ampoule contained 1mL of ddH₂O buffered at pH 9.5 (25mM CHES). The analytical ampoules initially contained 1mL of the enzymatic reaction assay; this buffered solution contained a starting concentration of either 0.15µg/mL of Rsc1362 or 5µg/mL of PA0810. Subsequent titrations of substrate (Chloroacetate; 5mM stock for Rsc1362 experiments, 25mM stock for PA0810 experiments) were added to the ampoule from a 250µL syringe over 19 injections (7 x 2µL, 6 x 8µL, and 6 x 31.5µL injections, in that order). Rsc1362 therefore was tested on a chloroacetate substrate range of 0.01mM to 1mM, and PA0810 had a substrate range of 0.05 to 5mM. Each enzyme was measured in triplicate.

Raw data obtained was converted from heat flow into reaction rates using the empirically determined enthalpy of reaction. Substrate concentrations were corrected for subsequent titrations and the corresponding volume changes, as well as correcting for dechlorinated substrate. Non-linear regression and kinetic analysis was performed with Graphpad Prism (La Jolla, California).
2.6 Enzyme Kinetics: Isothermal Titration Calorimetry
As use of titration calorimetry in measuring enzyme kinetics is not a widely used procedure, this section was added as existing literature is relatively sparse on the subject. It is meant to be a general primer for the use of anyone interested in replicating enclosed methods in this thesis.

Isothermal Titration Calorimetry (ITC) can be used as an alternative to Ion Chromatography or Spectrophotometry to measuring halocarbon degrading enzyme activity and kinetics\(^\text{44}\). This technique was used to determine the kinetic parameters for HADs Rsc1362 and PA0810, and was particularly useful as trials using ion chromatography were proving inconclusive. Put simply, ion chromatography was not sensitive enough to detect halide product at low substrate conditions (Rsc1362 in particular had a very low \(K_M\); substrate concentrations under 0.05mM were required for proper analysis).

The breakage of the carbon-halide bond is a thermodynamic reaction, and creates heat (~16kcal/mol for C-Cl to C-OH in CHES, quoted from Peter Chan, UofT Biochemistry), which can be measured as an indication of reaction activity. In simple terms, the ITC measures the heat produced by the reaction at any given time point of the experiment, and quantified heat data can therefore be converted into enzyme activity. The total heat of the reaction is measured as the comparison of the heat production from the analytical cell, or ampoule, to the reference cell, which is essentially a blank.
Figure 2.1: Schematic of a typical ITC calorimeter. A reference cell is shown on the right, and is typically filled with water to the same volume as the analytical cell. The analytical cell contains a non-reactive gold plated stirring propeller to keep the sample well mixed, and the system is kept constant at 25°C.

Technically speaking, the ITC measures the amount of power required to keep the analytical ampoule the same temperature as the reference. The raw data is thus a time plot of the experiment (Figure 2.1), showcasing the power (in J/s or W) or heat flow required at each time point to keep the analytical ampoule at the desired temperature.
**Figure 2.2:** Example of raw data output from a typical multiple injection experiment. The bottom axis is in units of time, while the vertical axis is in units of heat flow, or power. Note that although the ITC technically measures the heat flow required to keep the ampoule exactly at 25°C, the output display was customized to show the reverse negative values. Hence, this raw output is indicative of the heat flow produced by the reaction. The large peaks are caused by the heats of dilution, and indicate when a titration has occurred.

The rate of reaction is directly proportional to thermal power:

\[
\text{Power} = \frac{dQ}{dt}
\]

Where \( Q \) is heat, and \( t \) is time. Todd and Gomez\textsuperscript{55} have demonstrated that the amount of heat involved in converting \( n \) moles of substrate to product can be expressed by

\[
Q = n \cdot \Delta H_{\text{app}} = [P] \cdot V_0 \cdot \Delta H_{\text{app}}
\]

Where \( \Delta H_{\text{app}} \) is total molar enthalpy for the reaction in cal/mol of substrate, \([P]\) is concentration of product generated, and \( V_0 \) is the volume of the reaction. It then follows that thermal power generated by the enzyme during the reaction is:

\[
\text{Power} = \frac{dQ}{dt} = \frac{d[P]_{\text{total}}}{dt} \cdot V_0 \cdot H_{\text{app}}
\]
Since \( \frac{d[P]}{dt} \) is equal to the rate, the equation can be rearranged into:

\[
Rate = \frac{1}{V_0 \cdot \Delta H_{app}} \cdot \frac{dQ}{dt}
\]

\( V_0 \) is known, and \( \Delta H_{app} \) can be determined experimentally by ITC; thus only \( \frac{dQ}{dt} \) is required to determine the rate. Obtaining \( \frac{dQ}{dt} \) can vary depending on the type of calorimetry experiment; either single injection or multiple injection (Figure 2.3).

In single injection, a known amount of substrate is added once and allowed to react completely. Integration of the heat flow function will give the total heat produced, which can be divided by the time of reaction to obtain \( \frac{dQ}{dt} \). In a multiple injection method, a series of injections of substrate are performed, each injection occurring before significant substrate depletion can occur. \( \frac{dQ}{dt} \) can be measured simply by the difference of the current baseline signal to the original baseline.

![Diagram of theoretical outputs from a single injection experiment (left) and a multiple injection experiment (right).](image)

**Figure 2.3:** Diagram of theoretical outputs from a single injection experiment (left) and a multiple injection experiment (right). Integration of the heat rate/flow signal in the single injection experiment allows one to determine the total amount of heat produced from a single injection. Deviation of heat flow baseline from zero baseline in a multiple injection experiment allows one to determine the rate of reaction at any given titration.

### 2.7 Carbon Stable Isotope Fractionation and 1,2 DCA Kinetics of HLDs:

Individual enzyme assays were done in triplicate to assess enzyme kinetics and isotopic fractionation for each enzyme (DhlA and Jann2620) in 50mL aliquots. A 125mL screw cap amber bottle was used in conjunction with a PTFE Mininert cap
and rubber septa to house the enzymatic reaction. The headspace of the assay was thus around 75mL. The enzyme assay contained 35mM of TRIS pH 8.5 (as suggested through previous pH optima trials) and a 10mM liquid concentration of SIL 43 1,2 DCA, as well as purified enzyme from recombinant BL21 cell lines. DhlA was added to a 33.6µg/mL concentration, while Jann2620 was added to 1.45mg/mL. Assays that contained all components except dehalogenase protein were left overnight to allow equilibration of 1,2 DCA between liquid and gas phases.

The start of an assay coincided exactly with the addition of the dehalogenase protein. It was determined that at high enzyme concentrations (particularly with Jann2620), there was a tendency for bubbles to form from the initial injection of the enzyme using a disposable syringe. The bubbles were in high enough number and density to cause worry for efficient equilibration of the 1,2 DCA over the course of the experiment. Dehalogenase was thus added directly into the assay solution by turning the bottle upside-down, and injecting the enzyme within the body of the assay mixture. This procedure resulted in zero bubble formation and was adopted for all replicates.

DhlA replicates were run for 4 hours, while the Jann2620 replicates were observed for 16 hours. All samples were kept shaking at 400rpm in an effort to maintain real-time headspace equilibrium throughout the experiment. Regular headspace samples were taken to a Varian 3300/Finnigan MAT 252 GC-MS for isotope analysis, and a Varian 3380 GC for kinetic analysis. Carbon isotope analysis was performed using equations described in chapter 1.4, with Vienna Pee Dee Belemite as a carbon isotope standard. Kinetic analysis was performed using Graphpad Prism software. All studies were performed in partnership with Lisa Douglas from the Sherwood Lollar Lab, Dept. of Geology, UofT.
3.0 PROJECT 1 RESULTS: CHARACTERIZATION OF HADs Rsc1362 AND PA0810

3.1 Rsc1362 and PA0810 pH Optima
Use of Ion Chromatography to assess HAD pH dependent activity on haloacid substrates (Fluoro/Chloro/Bromo/Iodo-Acetic acid) resulted in very similar results for Rsc1362 and PA0810. Critically, the two enzymes displayed a pH optima at pH 9.5 for all haloacids except for Fluoroacetic Acid; no fluoro-dehalogenating activity was detected for either enzyme. These results are in line with existing HAD literature. Previous examples include L-DEX YL from Pseudomonas sp. (pH 9.5)\textsuperscript{37} and Dh1B from \textit{Xanthobacter autotrophicus} (pH 9.5)\textsuperscript{61}. Rsc1362, however, displayed a dehalogenating activity level about 10 times that of PA0810. Noticeable trends include a dip in activity at pH 9 compared to pH 8.5 and 9.5 for both Rsc1362 and PA0810. This dip deviates from the steady increase of activity towards the pH optima as one would expect. The relative strength of activity for Iodoacetic acid compared to the others seems to increase at higher pHs, ultimately resulting in both enzymes preferring this substrate at pH 10.0.

![Rsc1362 pH Dependent Activity](image)

**Figure 3.1:** Rsc1362 pH Dependent Activity across pH 7.5-10.0. No fluoro-dehalogenating activity was detected. Activity determined as a rate of reaction, measured by the amount of glycolate produced by a $\mu$g of protein per second. Glycolate was quantified using Ion Chromatography.
Figure 3.2: PA0810 pH Dependent Activity across pH 7.5 to 10.0. No fluoro-dehalogenating activity was detected. Activity determined as a rate of reaction, measured by the amount of glycolate produced by a µg of protein per second. Glycolate was quantified using Ion Chromatography. Note that the scale of activity is over 10x smaller than in Figure 3.1.

3.2: Isothermal Titration Calorimetry and Kinetic Analysis

3.2.1 Determining Apparent Enthalpy of Reaction:
The Apparent Enthalpy of Reaction was determined experimentally using a series of single injection experiments. An experimental set up included 5 titrations of 25µL of 100mM ClAc into a 1mL ampoule containing either no enzyme or 20µg/mL of Rsc1362/PA0810 (Figure 3.3). Titrations were placed 1 hour apart to ensure total breakdown of the added substrate, effectively producing 5 separate single injection experiments. Subtraction of the peak of dilutions from the positive enzyme experiment gives the total heat produced per injection. Given the total amount of heat of reaction, and a known amount of substrate, we calculate the apparent enthalpy of reaction using the formula:

$$\Delta H_{\text{app}} = \frac{Q_{\text{Total}}}{S_{\text{amt}}}$$

where $Q_{\text{Total}}$ is the heat produced, and $S_{\text{amt}}$ is the amount of substrate reacted. The final numbers give an experimental $\Delta H_{\text{app}}$ of 15.6 kcal/mol for both Rsc1362 and PA0810. This number is close to the expected 16kcal/mol (calculated from
previous experiments by Peter Chan), and was used for later titration experiment calculations. Note that the elongated peaks for each subsequent titration in the enzyme positive injections suggest a product inhibition effect; the substrate is being degraded at a slower rate, hence the stretching of the heat flow signal. This necessitates the use of multiple injection titration calorimetry, as opposed to single injection, to minimize product concentration buildup.

Figure 3.3: $\Delta H_{app}$ experimental Raw Data for Rsc1362. $\Delta H_{app}$ = Heat of reaction for every unit of product (kcal/mol). Measured using injection studies where a known amount of substrate is completely reacted. Subtraction of peaks of dilutions, and integration of the resulting area gives the total energy of the reaction. Appears to be product inhibition. $\Delta H_{app}$ of both Rsc1362 and PA0810 are around 15.6 kcal/mol. Experimental data for PA0810 not shown.

3.2.2 Enzyme Kinetics:
Data for kinetic activity was obtained via multiple injection titration experiments. Initial “gauging” runs were used to identify proper ranges for substrate concentration, with an ideal range of 0.1-10x the estimated Km value.
Table 3.1: Run parameters for multiple injection experiments

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[Enzyme]</th>
<th>Substrate Range</th>
<th>[Substrate] (Stock)</th>
<th>Time between injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsc1362</td>
<td>0.15µg/mL</td>
<td>0.01-1mM</td>
<td>5mM</td>
<td>5 mins</td>
</tr>
<tr>
<td>PA0810</td>
<td>5µg/mL</td>
<td>0.05-5mM</td>
<td>25mM</td>
<td>6.5 mins</td>
</tr>
</tbody>
</table>

The raw data (example in Figure 3.4) was analyzed for heatflow baseline deviation, and converted to enzyme activity. A Michaelis-Menton curve was formulated using linear regression from titration data that represented enzyme activity rate vs substrate concentration (See Figure 3.5). Linear regression analysis and kinetic parameter calculation was performed using the software package Graphpad Prism (La Jolla, California). The data was averaged from triplicate runs (Figures 3.6 and 3.7, and Table 3.2).

![Figure 3.4: Rsc1362 Run 1 titration experimental raw data.](image)

Figure 3.4: Rsc1362 Run 1 titration experimental raw data. Titration experimental goal is to perform injections of substrate faster than the enzyme can catalyze the reaction: result is a series of heat flow measurements with increasing substrate concentrations. Enzyme activity is monitored by increasing baselines, shown in green in the figure, which are indicative of equilibrated heat flow at a certain concentration, and thus [Substrate] dependent activity (recall Figure 3.3). Data on all runs can be found in Appendix.
Figure 3.5: Example of Michaelis-Menton curve of Rsc1362 Rate of Activity vs. Chloroacetate Concentration. Additional data for all runs can be found in Appendix.

Rsc1362 Runs

Figure 3.6: Replicate data for Multiple Injection ITC experiments for Rsc1362. Shown are the Michaelis-Menton plots for replicate runs.
Figure 3.7: Replicate data for Multiple Injection ITC experiments for PA0810. Shown are the Michaelis-Menton plots for replicate runs. Note that the PA0810 data shows considerable variability. This is attributed to the significant and immediate activity loss of PA0810 after purification.

Table 3.2: Rsc1362 Kinetic Data for all replicates. Rsc1362 has a Km of 0.06±0.015 mM, Vmax of 8.4±1.1 mol/sec*mol protein, and a Kcat of 504±66 min⁻¹.

<table>
<thead>
<tr>
<th></th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (mM)</td>
<td>0.05±0.015</td>
<td>0.06±0.0085</td>
<td>0.08±0.0065</td>
<td>0.06±0.015</td>
</tr>
<tr>
<td>Vmax (mol/sec*mol protein)</td>
<td>9.6±0.15</td>
<td>7.4±0.13</td>
<td>8.1±0.18</td>
<td>8.4±1.1</td>
</tr>
<tr>
<td>kcat (min⁻¹)</td>
<td>576±9</td>
<td>444±8</td>
<td>486±11</td>
<td>504±66</td>
</tr>
<tr>
<td>R²</td>
<td>0.9918</td>
<td>0.9892</td>
<td>0.9805</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: PA0810 Kinetic Data for all replicates. PA0810 has a Km of 0.44±0.23, Vmax of 0.043±0.01 mol/sec*mol protein, and a Kcat of 2.6±0.6 min⁻¹.

<table>
<thead>
<tr>
<th></th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (mM)</td>
<td>0.68±0.14</td>
<td>0.23±0.063</td>
<td>0.41±0.064</td>
<td>0.44±0.23</td>
</tr>
<tr>
<td>Vmax (mol/sec*mol protein)</td>
<td>0.040±0.0027</td>
<td>0.035±0.0024</td>
<td>0.055±0.0025</td>
<td>0.043±0.01</td>
</tr>
<tr>
<td>kcat (min⁻¹)</td>
<td>2.4±0.2</td>
<td>2.1±0.2</td>
<td>3.3±0.15</td>
<td>2.6±0.6</td>
</tr>
<tr>
<td>R²</td>
<td>0.9265</td>
<td>0.9375</td>
<td>0.8203</td>
<td></td>
</tr>
</tbody>
</table>
Final numbers for kinetic parameters of the two chlorodehalogenases were tallied and compared to kinetic analysis of the fluoroacetate dehalogenases Bpro0530 and Rha0230 (performed by Peter Chan). Table 3.4 summarizes the kinetic parameters determined for all four enzymes on chloroacetate substrate, and the kinetic parameters for Bpro0530 and Rha0230 on fluoroacetate substrate.

Table 3.4: Finalized kinetic parameters for PA0810, Rsc1362, Bpro0230, and Rha0230 with Chloroacetate and Fluoroacetate as substrates where applicable. Notably, PA0810 is significantly less efficient at chlorodehalogenation than Rsc1362, with a larger $K_M$ and $k_{cat}$ value. Both Bpro0530 and Rha0230 are better able at dehalogenating chloroacetate than fluoroacetate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bpro0530</td>
<td>13 ± 1</td>
<td>20.3 ± 0.3</td>
<td>10</td>
</tr>
<tr>
<td>Rha0230</td>
<td>17 ± 3</td>
<td>18 ± 3</td>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0810</td>
<td>2.6 ± 0.6</td>
<td>0.44 ± 0.2</td>
<td>100</td>
</tr>
<tr>
<td>Rsc1362</td>
<td>504 ± 66</td>
<td>0.06 ± 0.02</td>
<td>$1.5 \times 10^5$</td>
</tr>
<tr>
<td>Bpro0530</td>
<td>320 ± 20</td>
<td>0.0051 ± 0.0009</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>Rha0230</td>
<td>40 ± 4</td>
<td>18 ± 2</td>
<td>37</td>
</tr>
</tbody>
</table>

3.3 Structural Data:
The following data was obtained from experiments performed by Peter Chan (UofT Biochemistry) and Pierre Petit (UofT, Structural Proteomics in Toronto), and represents the bulk of the structural data obtained for the joint HAD project intended to investigate the structure/function relationship between chloro/fluorodehalogenases. All of the following data, as well as the previous kinetic data, are part of a manuscript that is currently being reviewed. The manuscript is titled “Structural adaptations by L-2-haloacid dehalogenases for hydrolytic defluorination”, and is authored by Peter W.Y. Chan, Terence K. W. To, Pierre Petit, Christopher Tran, Marielle A. Wailti, Alexei Savchenko, Alexander F. Yakunin, Elizabeth Edwards, and Emil F. Pai. The presented data is meant to be a skeletal but concise summary of the overall work, and is particularly relevant.
towards the general conclusions and discussion of structural adaptations for defluorination.

Table 3.5 showcases important catalytic residues and their functions, as inferred from studies on three model HADs: L-Dex YL, DhlB, and DehIva. This was used as a guideline in the analysis of subsequent X-ray crystallography data. Figure 3.8 reveals the monomeric structures of the 2 novel fluorodehalogenating HADs (Bpro0530 and Rha0230), as well as the 2 chlorodehalogenating HADs. Of particular interest to this thesis is the dynamic nature of the cap domain in PA0810. Variations in the α2 conformations hint at inherent flexibility within this region (shown boxed in Figure 3.8); repeated analysis suggests there is a favoured alternate conformation which retracts Tyr47 (important in halide binding, Table 3.5) away from the active site in 3 of 4 instances.

**TABLE 3.5. Primary roles of active site residues in HADs**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>L-Dex YL</th>
<th>DhlB</th>
<th>DehIva</th>
<th>Bpro0530</th>
<th>Rha0230</th>
<th>PA0810</th>
<th>RSc1362</th>
</tr>
</thead>
<tbody>
<tr>
<td>SwissProt ID</td>
<td>Q53464</td>
<td>Q60099</td>
<td>Q51645</td>
<td>Q12G50</td>
<td>Q0SK70</td>
<td>Q9I5C9</td>
<td>Q8XZN3</td>
</tr>
<tr>
<td>Nucleophile</td>
<td>Asp10</td>
<td>Asp8</td>
<td>Asp11</td>
<td>Asp10</td>
<td>Asp21</td>
<td>Asp7</td>
<td>Asp10</td>
</tr>
<tr>
<td>Halide binding</td>
<td>Arg41&lt;sup&gt;a&lt;/sup&gt; Gln44&lt;sup&gt;b&lt;/sup&gt; Asn119 Trp179</td>
<td>Arg39 Gln42 Asn115 Phe175</td>
<td>Arg42 Gln45 Asn120 Trp180</td>
<td>Arg41 Gln44 Asn119 Trp179</td>
<td>Arg58 Gln44 Trp179</td>
<td>Arg44 Tyr47 Asn122</td>
<td>Arg41 Gln44 Asn123 Trp183</td>
</tr>
<tr>
<td>Carboxylate binding</td>
<td>Ser118</td>
<td>Ser114</td>
<td>Ser119</td>
<td>Ser118</td>
<td>Ser138</td>
<td>Ser121</td>
<td>Ser122</td>
</tr>
<tr>
<td>Hydrolysis of covalent ester intermediate</td>
<td>Thr14&lt;sup&gt;d&lt;/sup&gt; Ser175&lt;sup&gt;de&lt;/sup&gt; Asp180&lt;sup&gt;efg&lt;/sup&gt;</td>
<td>Thr12 Ser171 Asp176</td>
<td>Thr15 Ser176 Asp176</td>
<td>Thr14 Ser175 Asp181</td>
<td>Thr25 Ala193&lt;sup&gt;g&lt;/sup&gt; His195&lt;sup&gt;de&lt;/sup&gt; Asp198</td>
<td>Thr11 Ala176&lt;sup&gt;g&lt;/sup&gt; His178 Asp181</td>
<td>Thr14 Ser179 Ala181 Asp184</td>
</tr>
<tr>
<td>Multifunction</td>
<td>Lys151&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Lys147</td>
<td>Lys152</td>
<td>Lys151</td>
<td>Lys169</td>
<td>Lys152</td>
<td>Lys155</td>
</tr>
<tr>
<td>Stabilizes Asp180</td>
<td>Tyr157</td>
<td>Tyr153</td>
<td>Tyr158</td>
<td>Tyr157</td>
<td>Tyr175</td>
<td>Tyr158</td>
<td>Tyr161</td>
</tr>
</tbody>
</table>

The functions of the catalytic residues inferred from studies on the model HADs L-Dex YL, DhlB and DehIva. <sup>a</sup>The conserved arginine is also proposed to recruit the substrate in a 'lockdown’ mechanism based on its highly dynamic nature. <sup>b</sup>At this position, the glutamine side chain invariably points away from the active site and only the tyrosine is expected to bind halide. <sup>c</sup>Rha0230 lacks the conserved aromatic side chain which interacts with the halide. <sup>d</sup>Forms the oxyanion hole. <sup>e</sup>Binds the catalytic water. <sup>f</sup>Activates the catalytic water. <sup>g</sup>Lacks the hydroxyl group of the conserved serine, binding of catalytic water may be compensated by the histidine found two residues downstream. <sup>h</sup>Orients the aspartate nucleophile, binds substrate and stabilizes Asp180 (L-Dex YL).
**Figure 3.8 Monomeric structure of HADs.** Different subunits of the same proteins are superposed when the crystal structure contains multiple protein subunits, using the Ca atoms only from the core domain to reveal any potential interdomain displacement. A) Bpro0530. Only a short loop fills the site of the subdomain insert. Superposition of the 2 subunits reveals no obvious interdomain displacement. B) Rha0230. This enzyme contains a ‘flap’ subdomain outside the active site entrance. The 8 cap domains display the largest positional difference at the tip of the protein, suggesting the possibility of hinge-like interdomain motion. C) PA0810. Its overall fold, including the ‘flap’ subdomain, closely resembles that of Rha0230. The positional differences of the 4 cap domains also hint similar hinge-like interdomain motions. Additionally, the variations in α2 conformations (Arg44-Ala50) reveal inherent flexibility in this region of the cap domain (boxed). D) RSc1362. This protein lacks the subdomain insert.

The final figure summarizes the findings on HAD active site geometries. Measurements were made to determine the distance between the halide pocket and the aspartate nucleophile, as well as the size of the halide pocket. Experiments were designed to identify structural differences between fluoro and non-fluoro dehalogenating enzymes. Attempts made to estimate the measurement cutoff for defluorination are also represented on the graph. These apparent cutoffs were a halide pocket size under 6.8 Å (measuring between Asn(Nδ2)--Arg(Nη1) and Asn(Nδ2)--Arg(Nη2)), and a distance between the halide pocket and aspartate nucleophile (measuring between Asp(Oδ1)--Asn(Nδ2) and Asp(Oδ1)--Arg(Nη2) distances) to be under 6.2 Å and 5.7 Å respectively.
Figure 3.9: Analysis of HADs active site geometries. The interatomic distances (Å) between the reactive Oδ1 atom of the aspartate nucleophile, the halide binding atoms in arginine (Nη1 and Nη2) and asparagine (Nδ2), and the hydroxyl group of the unique cap domain tyrosine (Oη). A) Asp(Oδ1)--Asn(Nδ2). B) Asp(Oδ1)--Arg(Nη2). C) Asn(Nδ2)--Arg(Nη1). D) Asn(Nδ2)--Arg(Nη2). E) Asp(Oδ1)--Tyr(Oη). In essence, A) and B) compare how close is the halide pocket to the aspartate nucleophile, and C) and D) reveal the size of the halide pocket. Triangles mark measurements in the defluorinating HADs Bpro0530 and Rha0230. Crosses show distances in the non-defluorinating HADs L-Dex YL, DhlB, DehIVa, PA0810 and RSc1362. Squares correspond to distances in ST2570, whose defluorination capability has not been examined previously. Multiple plots for each HAD represent measurements from different crystal structures or different active sites in a single crystal structure, and the distribution of the plot correlates with the dynamic nature of the active site structures. The dashed horizontal line in each panel represents the apparent measurement cutoff required for defluorination; it is set to the largest minimum distance among the defluorinating Bpro0530 and Rha0230. No other HAD besides ST2570 exhibits distances under every measurement cutoff. Distances to the aspartate nucleophile of Bpro0530 are estimated from the superposed aspartate in L-Dex YL (1ZRM).
4.0: PROJECT 1 DISCUSSION: HALOACID DEHALOGENASE CHARACTERIZATION

4.1: Advantages/Disadvantages of Isothermal Titration Calorimetry in Enzyme Kinetic Analysis
Overall, the use of Isothermal Titration Calorimetry (ITC) worked well in obtaining kinetic data for analysis, particularly as it was able to negate problems encountered previously with other analytical procedures. Silver halide spectrophotometry as performed in previous assays by other students was suggested to have low reproducibility. Ion chromatography (IC) was attempted as a means of quantifying enzymatic rates, but was unsuccessful due to insufficient sensitivity. Attempts to circumvent this problem via sample concentration using vacuum centrifuge evaporation proved unreliable. Likewise, increases in sample volume provided new problems; the peak sizes of unwanted compounds were increased alongside the compounds of interest glycolate and chloride, and separation of these peaks became an issue.

The core benefit of using ITC was the sensitivity to explore a preferred substrate range of $K_M/10$ to $10xK_M$, which equated to a minimum substrate concentration of 0.01 mM (Rsc1362 trials). However, this sensitivity was at the cost of qualitative detail; ITC only reports back in heat produced by a reaction, and there is no information on the type of reaction producing the heat. The process is also time intensive; normal preparation of the ITC for a single run usually takes 2-3 hours. For this reason, one should have estimates of desired experimental conditions prior to using the ITC, as it would be time inefficient to calibrate experimental parameters using this methodology. The use of ITC in biocharacterization can therefore only be practical when coupled with a method that can both assess the mechanism of reaction, and allow for quick, “ballpark” estimations of experimental conditions.

Prior IC trials were unsatisfactory in terms of sensitivity at low substrate levels, but provided valuable qualitative insight on enzymatic reactions at higher substrate
levels. Critically, glycolate and halide ion were produced stoichiometrically to haloacid degradation, validating the hydrolytic dehalogenase reaction mechanism expected of both enzymes, and confirming that this is the only degradative process occurring. In addition, the apparent enthalpy of reaction of Rsc1362 and PA0810 has been experimentally determined to be 15.6kcal/mol, similar to the expected value of 16kcal/mol derived from Peter Chan. This is further support that the correct reaction is observed within the ITC. The data obtained from failed IC kinetic analysis runs, as well as from the pH optima project, were invaluable in determining experimental parameters for the ITC such as substrate and enzyme concentrations. It is suggested then, that ion chromatography partners well with ITC, at least in the interest of characterizing hydrolytic dehalogenases.

4.2 Kinetic and pH optima Data Suggests Chloroacetate is not the Primary Substrate of PA0810
The pH optima of both Rsc1362 and PA0810 have been experimentally determined to be at pH 9.5. This result correlates well with known literature on previously assayed proteins in the HAD family, which generally have a pH optima between pH 9.0 and 10.0. Previously assayed proteins include L-DEX YL from Pseudomonas sp. (pH 9.5) and DhlB from Xanthobacter autotrophicus (pH 9.5). The correlation between high pH levels (relative to standard environmental conditions) and high activity is suspected to be due to the hydrolytic reaction mechanism. Higher pH environments are more conducive to the deprotonated state of the active site aspartate nucleophile, which is critical for reaction.

The “dip” in activity at pH 9.0 occurs for both Rsc1362 and PA0810, resulting in a pH dependence curve that deviates from the typical bell-shape encountered with most other enzymes. This deviation persists with a change of buffer from CHES pH 9.0 to CAPS pH 9.0 (data not shown), suggesting that CHES itself is not inhibiting reaction. Particularly, the subsequent use of CHES at pH 9.5 seems to yield no ill effects towards observed activity, further reinforcing the idea that enzymatic inhibition from CHES buffer is nonexistent. This particular trend of pH dependent activity, marked by a single, critical drop in enzymatic activity at a specific pH, is not
seen anywhere in HAD literature. Taken holistically, the concurrence of both enzymes sharing the same anomaly, as well as the lack of similar results in related literature, suggests that this dip in activity is more likely an artifact in the methodology rather than true data. However, a possible explanation is that pH 9.0 marks a threshold in which amino acids critical for reaction are protonated due to their respective pKa values. This may promote structural changes that decrease the activity of the enzyme at pH 9.0. An alternative explanation is that the activity potential for pH 8.0 and 8.5 is artificially enhanced by the TRIS buffer.

PA0810 has a marked affinity for Iodoacetic acid when compared to other substrates, and across parallel experiments with Rsc1362. This is most apparent at pH 9.5, where all substrates experience maximum activity, but iodoacetic acid surprisingly overtakes bromoacetic acid and even chloroacetic acid as the primary substrate when looking at dehalogenating activity alone. This may be indicative of PA0810 evolving not as a chloro-dehalogenating enzyme, but primarily as an Iodo-cleaving one.

Analysis of the raw ITC data gave us kinetic parameters for both chloroacetate HAD enzymes. Rsc1362 had a \( k_{\text{cat}} \) of 504±66 min\(^{-1}\) and a \( K_M \) of 0.06±0.02 mM, PA0810 had a \( k_{\text{cat}} \) of 2.6±0.6 min\(^{-1}\) and a \( K_M \) of 0.44±0.2 mM.

These results clearly show that Rsc1362 is significantly more effective than PA0810, exhibiting an impressively higher \( k_{\text{cat}} \) and lower \( K_M \). We must remember, however, that the data only assesses the kinetics of these enzymes within the boundaries of using chloroacetate as a substrate; as stated previously, data from the pH optima experiments suggest chloroacetate may not be the primary substrate for PA0810. From our assay, the most effective substrate for PA0810 in our tests was iodoacetate, though claiming that this is the primary substrate may be up for debate, as overall PA0810 activity on this compound is still quite low. Since the HAD superfamily has a large repertoire of known substrates, it may therefore be possible that the true primary substrate for PA0810 is currently unknown.
4.3: Environmental Background of Microbial Strains
Reviewing the background of the original microbial strains used to isolate these enzymes provides some interesting environmental context. Rsc1362 was isolated from *Ralstonia solanacearum* GMI1000, a soil plant pathogen capable of causing wilt in a number of crop species, and enriched from French Guyana tomato samples in 1960. Although many organochloride insecticides and pesticides are now banned from commercial use in favor of modern equivalents such as glyphosate, such compounds were heavily used in agriculture prior to the mid 1960s, including DDT, Dicofol, and Heptachlor. For instance, DDT was used as an agricultural insecticide after World War II, and was not banned in the US until 1972. Thus the particular strain of *Ralstonia solanacearum* that was used to isolate Rsc1362 has likely been exposed to one or more types of toxic organochloride based compounds.

In comparison, the background of *Pseudomonas aeruginosa* PA01 suggests no such environmental context, at least none which are easily discernable. To recapitulate, *Pseudomonas aeruginosa* is an opportunistic human and animal pathogen capable of surviving in a wide variety of environments. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments. The bacterium is preferentially pathogenic towards immunocompromised individuals, and commonly infects the pulmonary and urinary tracts, burns, and wounds. The *P. aeruginosa* PA01 strain was isolated from an infested wound of a burn victim in Melbourne, Australia in 1955. The wide range of environments in which the organism is found suggests an absence of any prolonged exposure to chlorinated organic compounds. This lack of environmental context with respect to chlorinated compound exposure correlates well with the severe disparity of dechlorinating activity we observe between Rsc1362 and PA0810.
4.4: Structural Explanations for the Difference in Activity Between Rsc1362 and PA0810

The structural differences between Rsc1362 and PA0810 may also give a clue to the disparity in dechlorination potential between the two enzymes. The marked difference may be attributed to the dynamic Tyr47 residue in PA0810. The residue is noted as an important component in halide binding but X-ray crystallography studies reveal it has a tendency to move away from the catalytic site (3 of 4 times during trials). This is another example of PA0810 essentially being ill designed for dechlorination. Interestingly, a dynamically moving residue critical in halide binding may be beneficial in forming complexes with larger substrates. Activity and substrate assays have shown PA0810 has a greater affinity for Iodo and Bromoacetate at the pH optima, both of which contain primary halides larger than chlorine.

4.5: Conclusions for the Overall Project: Structural Adaptations for Defluorination

Defluorination in HADs Bpro0530 and Rha0230 is significantly slower than dechlorination, which is consistent with the increased stability of the C-F bond, as well as the environmental history of the parent organisms Polaromonas sp. JS666 and Rhodococcus sp. RHA1, which suggest past significant and consistent interaction with various chlorinated organic compounds (See Chapter 1.4.2). This is in stark contrast with ABH fluoroacetate dehalogenases, which appear to be more specialized and defluorinate faster than they can dechlorinate. From this observation it seems that defluorination in HADs is a novel activity, and is still not optimized. The higher Km values observed for fluoroacetate compared to chloroacetate support this conclusion. Alternatively, the fluoro-dehalogenating HADs may have been under selective pressure to evolve chloro-dehalogenase activity; and merely retained a vestigial ability to defluorinate.

Structurally, there are a number of key adaptations for HAD defluorination. Firstly, the active sites of both defluorinating HADs contain a halide pocket smaller than their dechlorinating counterparts, conforming to the smaller fluorine atom, and facilitating effective interaction. The apparent requisite halide pocket size from
structural analysis is one under 6.8 Å (measuring between Asn(Nδ2)--Arg(Nη1) and Asn(Nδ2)--Arg(Nη2)); any larger and the halide pocket is too big to effectively accommodate the fluorine. A second structural adaptation is the placement of the halide pocket is closer to the aspartate nucleophile in Bpro0530 and Rha0230 compared to the chloro-dehalogenating enzymes. This is predicted to better stabilize the transition state of the SN2 cleavage of the C-F bond, which is expected to be more compact because of the shorter scissile bond and of the smaller halogen atom. The required cutoff in the distance between halide pocket and nucleophile, as measured between Asp(Oδ1)--Asn(Nδ2) and Asp(Oδ1)--Arg(Nη2), is under 6.2 Å and 5.7 Å respectively.
5.0 PROJECT 2 RESULTS: KINETICS AND ISOTOPE FRACTIONATION

5.1 pH Optima of HLD Enzymes
The pH optima of DhlA and Jann2620 were determined by quantification of chloride production using ion chromatography. DhlA exhibited expected pH-dependent activity as predicted from existing literature, clearly maximizing activity at pH 8.2. Jann2620 maximized activity at pH 9.5, although unlike DhlA, there is a broad spectrum of pH levels close to peak activity; Jann2620 seems to be active across pH 7.5 to 10.0. Comparatively, Jann2620 dechlorinates at a much slower rate than DhlA. CHES buffer seemed to prove inhibitory towards Jann2620 activity (See appendix), thus BORAX buffer was used as a replacement (see Methods).

Figure 5.1: The pH dependent activity for DhlA on 1,2-DCA. The pH range tested was between pH 7.5 to pH 10.0. The presented data is of triplicate runs. DhlA maximizes activity at pH 8.2, and maintains a standard bell shaped pH dependence curve.
Figure 5.2: The pH dependent activity for Jann2620 on 1,2-DCA. The pH range tested was between pH 7.5 to pH 10.0. The presented data is of triplicate runs. Jann2620 appears to maximize activity at pH 9.5, but has relatively similar levels of activity across the range tested. CHES buffer was inhibitory to the protein, so BORAX was used for pH 9.0-9.5. Jann2620 is notably less active on 1,2-DCA than DhlA.

Since both enzymes were relatively close to maximum activity at pH 8.5, this pH was deemed an acceptable “middle ground” for DhlA and Jann2620, and was used in subsequent studies for both enzymes to ensure as much similarity between enzyme assays as possible.
5.2: Degradation of 1,2-DCA by DhlA and Jann2620
For the enzyme DhlA, 90% of 10mM 1,2 DCA was degraded in 4 hours (Figure 5.3) using an enzyme concentration of 33.6 µg/mL. Despite using considerably more protein (1.45mg/mL), Jann2620 in contrast was only able to achieve ~80% degradation in 16 hours (Figure 5.4). The replicate data are in good agreement; the apparent differences between each of the Jann2620 assays may be attributed to slight changes in the starting concentrations of substrate, and these differences are accounted for in subsequent calculations. Note that in each case, only the three best replicate runs (Runs 4-6 for both enzymes) are shown; Runs 1-3 were preliminary trials aimed at determination of optimal protein concentrations.

![1,2-DCA Degradation by DhlA](image)

**Figure 5.3: 1,2 DCA depletion by DhlA over 4 hours.** 1.68mg (33.6µg/mL) of DhlA was used in a 50mL assay. All runs maintained high correlation with one another and suggested a high level of reproducibility. Pictured are the 4 replicate runs used for subsequent Isotope Analysis (previous runs D1-D2 were preliminary runs aimed at determining ideal protein concentration levels, and were not analyzed downstream). Only runs D4-D6 were used in kinetic analyses.
Figure 5.4: 1,2 DCA depletion by Jann2620 over 16 hours. 72.5 mg (1.45mg/mL) of Jann2620 was used in a 50mL assay. Despite a difference in starting concentration of 1,2-DCA, most likely from human error, rates of degradation across 3 replicates seem well correlated with one another. Previous replicates (J1-J3) were used as preliminary runs meant to determine ideal protein concentrations and run parameters, and were not used in downstream analysis. All 3 replicates shown here were used in both kinetic and isotope analysis.

5.3: Carbon Isotope Fractionation Data for DhlA and Jann2620
Figures 5.5 and 5.6 show the δ¹³C values observed over time for DhlA and Jann2620 respectively in the same experiments for which concentrations are shown in Figures 5.3 and 5.4. As expected, the δ¹³C value increases as the reaction continues, indicative of fractionation, and the enrichment of ¹³C molecular species. The J6 run with Jann2620 was slightly slower than Runs J4 and J5 (Figure 5.4); correspondingly, the isotope data for J6 is also a little behind J4 and J5. This may be due to slight loss of enzyme activity, or a difference in enzyme purity (J6 used a different batch of newly purified enzyme compared to the others). However, once the δ¹³C values were normalized to the fraction of 1,2 DCA remaining, all runs for the Jann2620 provided similar isotopic fractionation data (See figure 5.8).
**Figure 5.5:** $\delta^{13}$C values of DhIA degradation of 1,2 DCA over 3 hours. Sampling of DhIA degradation for $\delta^{13}$C data stopped after 3 hours as the concentration of 1,2 DCA decreased below the limit of detection for the GC-MS. The y-axis measures the relative enrichment of $^{13}$C in units of permil and is normalized to a known standard.

**Figure 5.6:** $\delta^{13}$C values of Jann2620 degradation of 1,2 DCA over 16 hours. Discrepancies between J6 and the other runs are due to differences in fraction remaining at similar timepoints (see Figure 5.4). The y-axis measures the relative enrichment of $^{13}$C in units of permil and is normalized to a known standard.
Figures 5.7 and 5.8 show the determination of the ε value using a ln plot with ln(f) vs ln(R/R₀). It shows high correlation between replicates, now that the raw δ¹³C values have been normalized to the fraction of substrate remaining. The isotopic enrichment factors (ε) are calculated as -33.9‰ and -32.95‰ for DhlA and Jann2620 respectively. Table 5.1 compares these numbers to previous studies performed on 1,2 DCA biodegradation.

**DhlA Rayleigh Determination In Plot**

**Figure 5.7: Rayleigh determination for DhlA.** Graph shows 1000ln(R/R₀) vs ln(f), and the slope of the line is equal to the ε value (isotopic enrichment factor) of the degradation. Effectively, this graph linearly represents the rate of enrichment as related to the fraction remaining f (and not against time).
Figure 5.8: Rayleigh determination for Jann2620. Graph shows $1000\ln(R/R_0)$ vs $\ln(f)$, and the slope of the line is equal to the $\epsilon$ value (isotopic enrichment factor) of the degradation. Effectively, this graph linearly represents the rate of enrichment as related to the fraction remaining $f$ (and not against time).

Figures 5.9 and 5.10 shows the observed data fit with the Rayleigh Model. The $\alpha$ values are determined from the $\epsilon$ values of figures 5.7 and 5.8. These serve mainly to show a good fit correlation between our calculated isotope fractionation constants and our observed data.
**Figure 5.9:** Rayleigh model of aerobic 1,2-DCA degradation by DhIA. Model was constructed using $\epsilon$ values obtained from $1000\ln(R/R_0)$ vs $\ln(f)$ plots. Error bars are 95% confidence intervals.

**Figure 5.10:** Rayleigh model of aerobic 1,2-DCA degradation by Jann2620. Model was constructed using $\epsilon$ values obtained from $1000\ln(R/R_0)$ vs $\ln(f)$ plots. Error bars are 95% confidence intervals.
Table 5.1: Recorded $\varepsilon$ values of this study compared to other studies dealing with carbon isotope fractionation of biodegradation of 1,2-DCA. All studies use *Xanthobacter autotrophicus*, but only this study observes the effect of pure protein rather than whole cell or cell free extract. All values were between -27 to 34‰; our values were quite similar to previous studies.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$\varepsilon$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell</td>
<td>-27 to -33‰</td>
<td>Hunkeler and Aravena, 2000</td>
</tr>
<tr>
<td><em>X. autotrophicus</em> Gj10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Cell</td>
<td>-32.3±1.8‰</td>
<td>Hirschorn et al., 2004</td>
</tr>
<tr>
<td><em>X. autotrophicus</em> Gj10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Cell</td>
<td>-28.6±0.3‰</td>
<td>Abe et al., 2009</td>
</tr>
<tr>
<td><em>X. autotrophicus</em> Gj10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Free Extract</td>
<td>-28.2±0.3‰</td>
<td></td>
</tr>
<tr>
<td><em>X. autotrophicus</em> Gj10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified DhlA (from <em>X. autotrophicus</em> GJ10)</td>
<td>-33.9±0.9‰</td>
<td>This Study</td>
</tr>
<tr>
<td>Purified Jann2620 (from <em>Jannaschia</em> sp. CCS1)</td>
<td>-33.0±0.8‰</td>
<td></td>
</tr>
</tbody>
</table>

5.4: Enzyme Kinetic Data
Kinetic analysis was performed on the substrate depletion data presented in Figures 1 and 2 using Michaelis Menten Kinetics. The standard Michaelis Menten equation is:

\[
\text{Equation 5.1: } V = \frac{V_{\text{max}} [S]}{K_M + [S]}
\]
where \((v)\) is equal to the observed rate of reaction, \(V_{\text{max}}\) and \(K_M\) are kinetic constants, and \([S]\) is the substrate concentration at any given time. The data collected does not contain data on the rate of reaction; there is only the collection of data points that follow the depletion of 1,2 DCA over time. It was thus necessary to utilize a different form of the MM equation, which was achieved through integration of Eq. 5.1 and the isolation and solving of \(t\) (Eq. 5.2-5.6).

**Equation 5.2:**

\[ v = -\frac{ds}{dt} = \frac{V_{\text{max}} [S]}{K_M + [S]} \]

**Solve for \(t\)**

**Equation 5.3:**

\[ V_{\text{max}} dt = -\left(\frac{K_M + [S]}{[S]}\right) ds = -\left(\frac{K_M}{[S]} + 1\right) ds \]

**Equation 5.4:**

\[-V_{\text{max}} \int_0^t dt = K_M \int_{S_0}^S \frac{1}{S} ds + \int_{S_0}^S ds \]

**Equation 5.5:**

\[-V_{\text{max}} t = K_M (lnS - lnS_0) + S - S_0 \]

**Equation 5.6:**

\[ t = \frac{K_M (lnS - lnS_0) + S - S_0}{-V_{\text{max}}} \]
Graphpad Prism (La Jolla, California) was used to non-linearly regress substrate depletion data using equation 5.6. The regression solves for $K_m$ and $V_{max}$, taking in known values $t$, $S$, and $S_0$ from the observed data (Figures 5.3 and 5.4). The non-linear regression fits are shown in Figures 5.11 and 5.12, and the calculated kinetic constants and relevant experimental data are shown in Table 5.2. Critically, we see that DhlA is indeed stronger at dechlorination than Jann2620, with significantly lower $K_m$ values and much higher $k_{cat}$ values.

**Figure 5.11: Substrate depletion kinetic analysis for DhlA (3 replicates)** with fitted curve for Michaelis Menten Kinetics using rearranged form of the original MM equation shown in Equation 5.6. Kinetic parameters determined using non-linear regression in Graphpad Prism.
Figure 5.12: Substrate depletion kinetic analysis for Jann2620 (3 replicates) with fitted curve for Michaelis Menten Kinetics using rearranged form of the original MM equation shown in Equation 5.6. Kinetic parameters determined using non-linear regression in Graphpad Prism. Fitted curves were non-linearly regressed by constraining $V_{\text{max}}$ to 0.058 mM/hour*mg.
### Table 5.2: Enzyme Kinetic constants calculated from substrate depletion data for DhlA and Jann2620.

Vmax values for Jann2620 (marked with *) were determined through a separate enzyme assay and Ion Chromatography, as it became apparent Jann2620 was not saturated at 10mM 1,2 DCA. These trials were performed in 80mM 1,2 DCA, and the resulting Vmax values were used to constrain the non-linear regression to determine the Km values.

<table>
<thead>
<tr>
<th>Run</th>
<th>DhlA</th>
<th>Jann2620</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Km (mM)</td>
<td>4.49±0.25</td>
<td>5.58±0.64</td>
</tr>
<tr>
<td>Vmax (mM/hour*mg)</td>
<td>4.59±0.14</td>
<td>4.80±0.35</td>
</tr>
<tr>
<td>kcat (min⁻¹)</td>
<td>134±4</td>
<td>140±10</td>
</tr>
<tr>
<td>R² values</td>
<td>0.9990</td>
<td>0.9971</td>
</tr>
</tbody>
</table>

Table 5.2 summarizes the kinetic data obtained from analysis. **DhlA exhibits a Km of 4.8±0.6 mM and a kcat of 133±8 min⁻¹. Jann2620 exhibits a Km of 25.9±2.3 mM and a kcat of ~1.7 min⁻¹.** These values are highly reproducible across three replicates, and have high correlation to the non-linear regression. Note that DhlA is much more adept at 1,2-DCA dechlorination than Jann2620, possessing a Km 5 times smaller and a kcat an order of magnitude higher.

### 5.5: Calculation of AKIE

AKIEs were also calculated for both enzymes. As 1,2-DCA molecules contain two carbon atoms with Cl bonds, and because of the low natural abundance of ¹³C, it is generally expected that only one of the carbon atoms, at most, will be ¹³C. Since the reaction only occurs at one of the carbon atoms, there will be competition for dehalogenation between the ¹²C and the ¹³C position within a single 1,2-DCA molecule. To correct for this, a factor of 2 must be introduced when converting ε values to the corresponding AKIE (Elsner et al, 2005)\(^\text{12}\). The equation used for AKIE for 1,2-DCA then is:

\[
\frac{^{12}k}{^{13}k} = \frac{1}{[1+(2\varepsilon)/1000]}
\]

where ε is the isotope enrichment value (in ‰) and \(^{12}k/^{13}k\) is the observed AKIE of the reaction. Calculated AKIEs for DhlA and Jann2620 are displayed in Table 5.3, along with Literature AKIEs and expected KIEs.
Table 5.3: Calculated AKIEs for DhlA and Jann2620 using data from this study. Literature AKIEs and KIE were taken from Elsner et al, 2005.

<table>
<thead>
<tr>
<th></th>
<th>DhlA</th>
<th>Jann2620</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKIE</td>
<td>1.073 ± 0.002</td>
<td>1.071 ± 0.001</td>
</tr>
<tr>
<td>Literature AKIEs*</td>
<td></td>
<td>1.057 to 1.068</td>
</tr>
<tr>
<td>KIE</td>
<td></td>
<td>1.057</td>
</tr>
</tbody>
</table>

5.6: Controls
Negative controls were performed using killed enzymes in assay replicates. Experimental parameters were replicated except the enzyme was treated with 2M H₂SO₄ prior to inclusion within the assay solution. About 30μL of acid per mL of stock protein was used; enough was added to inactivate and fully precipitate the protein based on visual inspection.

Figure 5.13: Degradation of 1,2-DCA by killed DhlA. There is no significant decrease.
**Figure 5.14:** $\delta^{13}C$ measurements of killed DhlA. There is no carbon isotope fractionation observed.

**Figure 5.15:** Degradation of 1,2-DCA by killed Jann2620. There is no significant decrease.
Figure 5.16: $\delta^{13}C$ measurements of killed Jann2620. There is no carbon isotope fractionation observed.

The controls show that as predicted, 1,2-DCA fractionation and degradation stops when the enzyme is inactivated. This confirms that the degradation observed in our experiment is enzyme mediated, and that there is no loss of 1,2-DCA due to sorbing, leakage, or some otherwise non-enzymatic process.
6.0 PROJECT 2 DISCUSSION: HALOALKANE DEHALOGENASE CHARACTERIZATION

6.1 General Discussion
Compound-specific isotope analysis (CSIA) is emerging as a powerful technique to monitor degradation in the field, due to its ability to distinguish between mass loss due to degradation versus mass loss due to physical processes such as dilution, sorption, and volatilization.

However, the practical use of CSIA is very much dependent on a sound understanding of the relationship between the isotopic ratio $R$ (between the heavy and light isotopes) and the fraction remaining $f$, and how these relationships are tied to respective degradation pathways. The comprehension of the nuances in carbon isotope fractionation variance amongst various types of degradation pathways will help provide more depth in CSIA analysis, and hopefully lead to better understanding and decision making within the field.

One of the main areas requiring investigation is the effect of biology on the masking of fractionation effects. Previous experiments by the Sherwood Lollar and Edwards lab have shown a significant masking of the $\epsilon$ value in the biodegradation of 1,1,1-TCE and 1,1-DCA in *Dehalobacter* cultures and cell free extracts when compared to abiotic trials. At the heart of this topic is the role of enzymatic efficiency, and whether this value has any bearing on the masking of carbon isotope fractionation at all.

One hypothesis that was held prior to this experiment was that the obvious kinetic differences and activity potential of DhlA and Jann2620 from initial assays, since they both utilized a hydrolytic dehalogenase mechanism, the carbon isotope fractionation would remain similar for both enzymes.
Such a hypothesis would be supportive of Northrop’s AKIE theory (Apparent Kinetic Isotope Effect, explained in detail within the background information), which states that the Kinetic Isotope Effect can be masked due to a given enzyme’s commitment to catalysis. According to Northrop, a fast enzymatic degradation of 1,2 DCA should have an isotopic fractionation ratio that is much closer to unity than a slow one, and thus, there should be a difference in the carbon isotope fractionation. The key point is that if our results were to show negligible changes between the isotope fractionation of 1,2-DCA of DhlA and Jann2620, we must be prepared to also show how this does not conflict with Northrop’s derivations, or be prepared to refute them.

Another key point to establish prior to a full discussion of the results is that this is the first experiment (as far as we know) observing the carbon isotope fractionation of 1,2 DCA degraded using purified enzymes. Previous experiments have tested either whole cell or cell free extracts, and with that comes various unknowns that may affect the overall reaction process. A focal point for debate in whole cell Xanthobacter autotrophicus experiments is the fact that the enzymes are cytosolic, forcing the 1,2 DCA to first traverse the plasma membrane. This uptake into the cytosol is an additional process which is non-degrading in nature, and can mask the fractionation if the diffusion through the membrane is the rate limiting step. This masking from membrane diffusion can of course, obscure results if one is interested in enzymatic KIE masking.

In purifying the enzymes, purposely choosing two that operate on identical reaction mechanisms, and observing both in identical conditions (similar temperature, pH), we are removing as many non-essential variables to the experiment as possible. In addition, shaking of each assay at 400rpm (something previously not performed) maintained constant equilibrium of 1,2-DCA between liquid and headspace, allowed for accurate real-time measurements in an experiment measured in hours instead of days. Without the interference of cellular membranes or other biological compounds present in whole cell or cell extracts, we are left only with observing the pure reaction. These results should therefore provide the most accurate and clear
picture of 1,2 DCA enzyme mediated carbon isotopic fractionation to date, and allow for specific interpretation of enzyme efficiency effects on fractionation.

6.2 Analysis of pH Optima Data
The pH optima experiments mark DhIA to be most active at pH 8.2, and Jann2620 at pH 9.5. This is consistent with literature hydrolytic dehalogenases; DhmA\textsuperscript{53} (\textit{Mycobacterium avium} N85, HLD-I subfamily) has a pH optimum of pH 8, and DmbA\textsuperscript{54} (\textit{Mycobacterium tuberculosis}, HLD-II subfamily) has a pH optimum of 9. Jann2620 notably has a wide pH range of activity, but with a severely low activity level compared to DhIA. This wide pH tolerance is also seen in DmbA, another HLD-II subfamily member\textsuperscript{54}. Using 1,2 dibromoethane as a substrate, DmbA had a maximal pH optimum at pH 9, but displayed more than one optimum and significant activity across a range of pH 5 to 10. It may be interesting then in future studies, to observe Jann2620 activity at pH levels lower than pH 7, and perhaps with a different substrate than 1,2-DCA. As both DhIA and Jann2620 both displayed near optimal activity at pH 8.5, this pH was chosen to be used for all remaining experiments. The goal was to ensure a level comparison between both proteins.

6.3 Analysis of Enzyme Kinetic Data
DhIA and Jann2620 possess vastly different kinetics on 1,2 DCA, with DhIA operating with significantly higher activity than Jann2620 (See 5.2). Raw data correlation to the non-linear regression in the kinetic analyses is over 98%, using the amended version of the standard Michaelis-Menten equation, and triplicate data for each enzyme has a standard deviation of up to 12\% ($K_M$ and $k_{cat}$ values). The data thus is reproducible, and conforms to first order kinetics. DhIA is shown to be the better 1,2-DCA degrading enzyme in two respects, both in a smaller $K_M$ value as well as a larger $k_{cat}$. This suggests that

a) DhIA possesses a much more specialized and efficient substrate recognition site, and is able to form the Enzyme-Substrate complex more efficiently than Jann2620, and

b) DhIA is better able to catalyze the actual substrate conversion (dechlorination) step of 1,2-DCA degradation (lower activation energy)
These results are not unexpected since we have established that DhlA, as a member of the HLD-I subfamily based on BLAST analysis, is likely to be better at dehalogenase activity on small, terminally halogenated substrates than Jann2620, a noted HLD-II member. How these kinetic results are related to structure and function is currently unknown, as there is no structure data on Jann2620 for direct comparison. However general differences in structures between HLD-I and HLD-II have been elucidated by Chovancova et al, 2007. A noted variation is the spatial arrangement of helices $\alpha_4$ and $\alpha_5$ is different between the cap domains of DhlA and HLD-II enzymes. As well, the second helix in the cap domain ($\alpha_5'$) is common to HLD-II subfamily members, but is not present in the structure of DhlA. As the cap domain is known to be influential in substrate specificity, these differences may be critically related to the difference in $K_M$ values reported for 1,2-DCA degradation between both enzymes. With respect to differences in $K_{cat}$, the rate of catalysis, Chovancova et al also reported different compositions of the catalytic pentad for HLD-I and HLD-II subfamilies (See Figure 1.4). In addition, there is a change in the type and location of one halide-stabilizing residue. HLD-I members employ Trp (W175 of DhlA) located in helix $\alpha_4$, whereas HLD-II members use Asn located in the loop following $\beta$-strand 3. Further experiments aimed at establishing a structure-function relationship between HLD enzymes should focus on these residues.

Past literature from Janssen et al22, 1985, reports $K_m$ and $V_{max}$ values for DhlA as 1.1mM and 10 umol/min per mg of protein respectively, which appears to be quite different from our readings of approximately 5mM and 3.8 umol/min per mg of protein (adapted to the change in units). These differences can be accounted for since Janssen et al performed their characterization assays at the peak conditions for DhlA activity: pH 8.2 and 37°C. In comparison our assays were performed at pH 8.5 and a more environmentally relevant 24°C, away from the optimal conditions.

Kinetic analysis for Jann2620 was complicated when it became apparent that the enzyme was not fully saturated at 10mM 1,2-DCA, the starting concentration of the assay. Attempted non-linear regression of the raw data at this stage resulted in
nonsensical $K_M$ and $V_{max}$ values, as the regression algorithm was unable to properly determine a maximum activity rate without data of the enzyme operating in a substrate saturated environment. Amending the current method and recollecting isotope and kinetic data was deemed too time consuming to be useful, particularly as Jann2620 required 16 hours to partially degrade 10mM 1,2 DCA; a study at saturated conditions would require more than 16 hours of observation. The non-linear regression was therefore constrained with an estimated $V_{max}$ value, determined experimentally in a separate assay at seemingly saturated conditions: 80mM 1,2-DCA, using the same concentration of protein (1.45mg/mL, see Appendix). Note that 80mM is the liquid phase saturation point of 1,2-DCA in water. The constrained regression was used to obtain the final numbers for Jann2620 kinetic analysis.

6.4 Carbon Isotope Fractionation Analysis
These experimentally derived kinetic parameters capably showcase the huge difference in activity between DhlA and Jann2620. Presumably, if there is any effect on isotope fractionation based on reaction speed, this is an ideal matchup. However, carbon isotope fractionation data for both DhlA and Jann2620 yield similar results ($\varepsilon$ values for DhlA: -$33.9\pm0.9\%$, Jann2620: -$33.0\pm0.8\%$), suggesting there is no such effect on isotope fractionation at all. The data compares nicely to previous estimates in literature, where $\varepsilon$ values of DhlA range from -$27\%$ to -$33\%$. Replicate data for both enzymes were well correlated, and $R^2$ fit values for both the Michaelis-Menten Kinetics and Rayleigh model were generally above 0.99, strongly suggesting reproducibility and accuracy of the data. Coupled with the efforts we put into the design of the experiment, we believe the reliability of the data to be strong.

Accordingly, there are multiple ways to view these results. First, one can simply choose to refute Northop's theory on AKIE. However this position feels premature given the small amount of data we have. This is only one study on two enzymes, and we have to be careful to ensure that Northrop's terminology and conditions for his derivations are properly understood and present in the data. The following figure
(6.1) summarizes Northrop’s equations, and highlights important aspects for discussion.

\[
\text{AKIE} = \frac{\text{KIE} + C}{1 + C} \\
\text{Where:} \quad C = \frac{k_2}{k_{-1}}
\]

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P
\]

\[
K_M = \frac{k_{-1} + k_2}{k_1} \quad k_{\text{cat}} = k_2
\]

**Figure 6.1:** Notable formulaic equations of AKIE theory, originally derived by Northrop. AKIE = Apparent Kinetic Isotope Effect. KIE = Intrinsic Kinetic Isotope Effect. C = Commitment to Catalysis. E, S, and P = Enzyme, Substrate, and Product respectively. Proportional relationships on the bottom row are simplified from the original Northrop equations for the sake of discussion. Derivations of \(K_M\) and \(k_{\text{cat}}\) assuming Pseudo Steady State kinetics are shown in the appendix.

What is critical to understand is that the commitment to catalysis \(C\) is the only factor that plays a role in altering AKIE from the intrinsic KIE, and that that \(C\) is not just simply based on the maximum rate of reaction \(k_{\text{cat}}\). \(C\) is proportional to the rate of substrate conversion \(k_2\) and inversely proportional to the rate of enzyme-substrate dissociation \(k_{-1}\). Therefore, an enzyme that is more active (ie. displays a greater \(k_2\))
does not necessarily possess a greater commitment to catalysis; one must also pay attention to the $k_1$ value.

From our data, we know that the carbon isotope fractionation of DhlA and Jann2620 are almost identical. Thus, following Northrop’s derivations, both enzymes must have similar AKIE and C values that are constrained. Our kinetic analysis determined that DhlA is faster than Jann2620 by virtue of a larger $K_{cat}$ value. However the $K_{cat}$ is essentially the $k_2$, and thus does not fully represent $C$. Note that the $k_1$ rate itself, for either enzyme, cannot be readily determined, as the term cannot be mathematically isolated from the given data ($K_M$ and $K_{cat}$) and equations. We know that the $k_2$ rates are different between the enzymes from the determined $K_{cat}$ values, but importantly we have no way of knowing what the $k_1$ rate is. It is possible then, though unlikely, that the $k_1$ numbers for both DhlA and Jann2620 just happen to be the right values in order to properly compensate for the difference in $k_2$, and allowing both enzymes to have a similar $C$ value. Again, this option is highly unlikely, and simply represents the possibility of a mere coincidence to explain the data.

A third option is to suggest that despite the high activity level of DhlA, its commitment to catalysis value $C$ is insufficient in masking the AKIE significantly far away from the original KIE. In other words, DhlA may be not be efficient enough to visibly affect isotope fractionation, which by extension infers Jann2620 is also incapable of such an effect. The experimentally determined AKIE values for DhlA and Jann2620 (1.073±0.002 and 1.071±0.001 respectively) are also larger to the calculated intrinsic KIE for 1,2 DCA, which is quoted at 1.057 (Elsner et al 2005); even if there isn’t a perfect match between numbers, the enzymes are clearly not expressing a commitment to catalysis strong enough to lower the intrinsic KIE towards unity. Out of all considered options, this position best and most simply explains the data while adopting the key conditions of Northrop’s AKIE theory.
6.5 Final Comments on Biotic Masking of KIE
The overarching conclusion is that these two proteins, despite vastly different enzyme efficiencies, displayed similar fractionation behaviour, and did not mask the kinetic isotope effect compared to the intrinsic value. The last point is key when we compare it to previous studies that suggest a biologic effect for the masking of the kinetic isotope effect. The lack of any significant masking suggests these enzymes innately possessed a commitment to catalysis value far too small to impart measurable changes to the KIE. It is therefore difficult to directly address the true effect of enzyme efficiency on the KIE, since there is no masking effect to be analyzed. Future studies should be focused on kinetic isotope enrichment studies on purified enzymes with predicted commitment to catalysis values strong enough to significantly mask KIE; these enzymes would theoretically be found in enrichment cultures of organisms that have previously been shown to possess a “biotic” masking. As an added note, the use of purified enzymes from “biotically masking” cultures referred to in previous experiments (cite references) was not a viable option here, due to the inability of recombinant expression of reductive dehalogenases in E. coli.

Another interesting point is that studies that observed carbon isotope fractionation in whole cell and cell extracts of Xanthobacter autotrophicus (the origin species of DhlA) report similar fractionation as observed with both purified proteins in this study (Table 5.1). This implies none of the biotic factors in whole cells or cell extracts of Xanthobacter autotrophicus significantly affect the carbon isotope fractionation of 1,2-DCA.

Lastly, our data suggests that there is no masking of the KIE, or in other words, the AKIE is equal to the KIE. This necessarily means the $C$ value must be zero, based on the Northrop defining equation for AKIE (Figure 6.1). As pointed out in Chapter 6.4, a $C$ value of zero makes it impossible to define the $k_1$ for either enzyme. However, we can give constraints to the $C$ value to determine a possible range for the $k_1$ value; since the maximum error for the calculation of AKIEs for both DhlA and Jann2620
was ±0.002, we can assume it to be the error of the experiment and use it to constrain $C$. Therefore:

$$C < 0.002$$

And since $C = k_2/k_1$

We can determine the range of $k_1$, as the $k_2$ values, which are equal to the $k_{\text{cat}}$ of each enzyme, are already known. Thus, the “ballpark” $k_1$ ranges of DhlA and Jann2620 are approximately $>66,500 \text{ min}^{-1}$ and $>850 \text{ min}^{-1}$. What is intriguing is that in both enzymes, these $k_1$ ranges are far in excess of the $k_2$ ($133 \text{ min}^{-1}$ and $1.7 \text{ min}^{-1}$ for DhlA and Jann2620 respectively). This suggests that the enzyme-substrate complex of both enzymes can undergo multiple dissociation and formation reactions for each catalysis.

A large $k_1$ constant relative to the $k_2$ may also be indicative of an enzyme that poorly masks the KIE. A high rate of dissociation of the enzyme-substrate complex suggests that the catalysis of preferred isotope species will be uninhibited. An analogy can be presented in the form of job applications. When the number of applicants are significantly higher than the number of jobs, the company or individual in charge of hiring must be increasingly stringent and picky with the desired qualifications. Minor differences between candidates now become significant, as there is much higher competition. In this scenario, there is no “masking” of the candidate’s qualifications; every tiny detail of the candidate’s resume is significant. When the number of applicants are low however, the company is forced to hire whoever applies in order to fulfill job requirements. In this scenario, lower qualified candidates may be equally likely to find a job; there is a “masking” effect on the qualifications here.

Likewise, when an enzyme is able to formulate/dissociate the enzyme-substrate complex at a rate much greater than it can catalyze the product reaction, there is little masking of the KIE. Here the substrate can be thought of as the “job applicant”, and the isotope makeup of the substrate can be thought of as its “job qualifications”,

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and the $k_2$ is the rate limiting step. The surplus of enzyme-substrate complexes encourages preferential catalysis of $^{12}$C isotope variants over $^{13}$C due to competition, in accordance to the intrinsic KIE value. In conditions where an enzyme’s ability to formulate/dissociate the enzyme-substrate complex is much slower, masking will occur, since the enzyme is in a sense, forced to catalyze whatever substrate is bound. The rate limiting step in this scenario is thus based on the relationship $k_1$ and $k_{-1}$, which do not take into account the KIE.
7.0 Conclusions:

7.1 Project 1 HAD conclusions:
The two haloacid dehalogenases Rsc1362 and PA0810 were assayed for activity against a variety of haloacid substrates. Although no activity against fluoroacetate was observed, activity for chloro/bromo/iodoacetates were determined for a pH range between 7.5 and 10.0. It was determined that the pH optima for both enzymes was pH 9.5, consistent for expected values from literature results.

Analysis of enzyme kinetics using Isothermal Calorimetry proved fruitful; enzyme kinetics of Rsc1362 and PA0810 were obtained using chloroacetate as a substrate. Rsc1362 has a Km of 0.06±0.015 mM, Vmax of 9.4±0.45 mol/sec*mol protein, and a \( k_{cat} \) of 562±27 min\(^{-1}\). PA0810 has a Km of 0.29±0.12 mM, Vmax of 0.033±0.004 mol/sec*mol protein, and a \( k_{cat} \) of 2.5±0.23 min\(^{-1}\). The variance in kinetic potential was attributed to a dynamic Tyr47 residue within PA0810 that was inferred from previous HAD studies to be critical for halide binding, but moved away from the catalytic site 3 of 4 times during crystallization. It was also apparent from these studies that PA0810 is not an efficient chloroacetate dehalogenase, and may have evolved to work with larger substrates.

Data in this thesis has also contributed to the larger investigation of the structural criteria in fluoro-dehalogenating HADs. As a group, we have been able to determine that two HAD defluorinating enzymes (Bpro0530 and Rha0230) have evolved a more compact active site and a smaller halide pocket than the non-defluorinating enzymes Rsc1362 and PA0810. These characteristics yield more effective reaction with the smaller fluorine atom, and result in a more efficient activation and cleavage of the C-F bond.

7.2 Project 2 HLD conclusions:
The haloalkane enzymes DhlA and Jann2620 were assessed for pH optima, enzyme kinetics, and carbon isotope fractionation, on 1,2-DCA as a substrate. DhlA has been previously characterized in prior literature, and our results seem in agreement if one concedes that different conditions were used. DhlA was found to have a pH
optimum of 8.2, and a $K_M$ and $K_{cat}$ of 4.8±0.6 mM and 133±8 min$^{-1}$ respectively. As Jann2620 has only recently been screened as a 1,2-DCA dehalogenase, all results for Jann2620 are novel. The enzyme was found to have a pH optima of 9.5, and a $K_M$ and $K_{cat}$ of 25.9±2.3 mM and ~4.2 min$^{-1}$ respectively.

Despite their large kinetic differences, carbon isotope fractionation was very similar between the two enzymes. DhlA fractionated with an $\epsilon$ value of -33.9±0.9‰, and Jann2620 with an $\epsilon$ value of -33.0±0.8‰. It was concluded that the commitment to catalysis values of both DhlA and Jann2620 were likely to both be too low to affect AKIE, as the calculated AKIE from both enzymatic reactions were similar to the expected intrinsic KIE values.

7.3 Contributions:

- Kinetic data for 2 chloroacetate dehalogenases: Rsc1362 and PA0810
- Kinetic data for 2 haloalkane (1,2-DCA) dehalogenases: DhlA and Jann2620
- Novel Carbon Isotope Fractionation data for pure DhlA and Jann2620 on the degradation of 1,2-DCA, without the use of whole cells or cell extract
- Novel pH optima profiles for Rsc1362, PA0810, Jann2620

7.4 Future Studies:

- Assess additional 1,2-DCA degrading hydrolytic dehalogenases (such as Dhma, Mycobacterium avium N85)
- Structure analysis on Jann2620 may be beneficial for future comparative studies that investigate the structure and function relationship of HLDs
- Perform carbon isotope fractionation studies with enzymes isolated from known “biotically masking” organisms
- Theoretically, point mutations that only affect the catalytic rate of product formation ($k_2$) should alter the AKIE, as the Enzyme-Substrate dissociation rate ($k_{-1}$) should not be affected. If it were possible to mutate only the catalytic site of DhlA or Jann2620 and maintain the same practical qualities of the substrate recognition and halide binding sites, we can more clearly investigate AKIE.
8.0 REFERENCES:
11. Dinglasan-Panlilio et al, 2006, Microbial oxidation of 1,2-dichloroethane under anoxic conditions with nitrate as electron acceptor in mixed and pure cultures, FEMS Microbiol Ecol, 2006 Jun;56(3):355-64


17. Hirschorn, S et al, Isotope analysis as a natural reaction probe to determine mechanisms of biodegradation of 1,2-dichloroethane, (2007), Environmental Microbiology, 9(7), 1651-1657

18. Hirschorn, S et al, Pathway Dependent Isotopic Fractionation during Aerobic Biodegradation of 1,2-Dichloroethane, (2004), Environmental Science and Technology, 4775-4781


26. Jitsumori, K., Omi, R., Kurihara, T., Kurata, A., Mihara, H., Miyahara, I., Hirotsu, K.,


37. Liu et al, Purification and characterization of thermostable and nonthermostable 2-haloacid dehalogenases with different stereospecificities from *Pseudomonas* sp. strain YL, Appl Environ Microbiol. 1994 July; 60(7): 2389-2393


43. McLeod et al, The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse, *PNAS October 17, 2006 vol. 103 no. 42 15582-15587*


80
54. Sax and Lewis, Hazardous chemicals desk reference, 1987, Published by Van Nostrand Reinhold (New York)


58. Silberstein, M et al, Exploring the Binding Sites of the Haloalkane Dehalogenase DhlA from *Xanthobacter autotrophicus* GJ10, (2007), Biochemistry 46, 9239-9249


APPENDIX:

PART A: Additional Data for HLD Experiments:
Preliminary experiments were performed with DhlA and Jann2620 to determine the following:

- Estimations of Kinetics for establishing experimental parameters in the primary carbon isotope fractionation experiment
- Substrate Inhibition
- Product Inhibition
- Ability of the enzymes to degrade Chloroethane, the product of 1,2-DCA degradation

All experiments were performed using 2mL glass screw cap vials with Teflon lined caps and analyzed for chloride production using Ion Chromatography using methodology described in the Methods section.

Preliminary Estimations of Kinetics in DhlA:
2μg/mL of enzyme was incubated at 30°C for 20 mins in each assay. Assays contained a starting liquid concentration of 1,2-DCA that varied between 0.1mM to 10mM. Estimated Km Value: 0.23 mM 1,2 DCA, Vmax is 3.8 umol per min per mg protein. Future runs should be from ~0.05mM to 5mM 1,2 DCA

Figure A1: Preliminary Estimates of DhlA Kinetics. Only one replicate was made for this estimate, hence the lack of error on the figure.
### Table of Data for Figure A1:

<table>
<thead>
<tr>
<th>mM Starting 1,2 DCA</th>
<th>Total Cl µMol</th>
<th>% 1,2 DCA degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.06</td>
<td>62.28</td>
</tr>
<tr>
<td>0.2</td>
<td>0.08</td>
<td>39.67</td>
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<td>0.09</td>
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<td>6.31</td>
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<tr>
<td>4</td>
<td>0.15</td>
<td>3.70</td>
</tr>
<tr>
<td>7</td>
<td>0.16</td>
<td>2.26</td>
</tr>
<tr>
<td>10</td>
<td>0.16</td>
<td>1.59</td>
</tr>
</tbody>
</table>

### Preliminary Estimations of Kinetics in Jann2620:
20µg/mL of enzyme was incubated at 30°C for 60 mins in each assay. Assays contained a starting liquid concentration of 1,2-DCA that varied between 0.1mM to 10mM. Estimated Km: 1.7mM 1,2 DCA  Estimated Vmax: 0.31 mM per min per mg protein. Estimated numbers are much slower than DhIA.

### Figure A2: Preliminary Estimation of Jann2620 Kinetics
Two replicates were made for the estimate, the error here is a measure of the range of data received. In retrospect, the odd shape of the curve is due to the lack of enzyme saturation.
Table of Data for Figure A2:

<table>
<thead>
<tr>
<th>mM Starting 1,2 DCA</th>
<th>Total Cl µMol</th>
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</thead>
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<tr>
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<td>0.08</td>
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<td>0.2</td>
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<td>0.08</td>
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<td>0.11</td>
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<td>10</td>
<td>0.32</td>
<td>3.18</td>
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</tbody>
</table>

**Product Inhibition:**

10mM liquid concentration of the product of the reaction (chloroethanol) was added to observe any effect on the degradation of 10mM liquid starting concentration of 1,2-DCA. 10mM of chloroethanol was chosen as a reasonable concentration to observe, as it would be the theoretical maximum of product produced from 10mM of 1,2-DCA, which at this point was assumed a saturating concentration of substrate for either protein.
Figure A3: Product Inhibition for DhlA. There is negligible effect from chloroethanol to the degradation of 1,2-DCA. Assay was 2μg/mL of protein at 20 mins.

Figure A4: Product Inhibition for Jann2620. There is negligible effect from chloroethanol to the degradation of 1,2-DCA. Assay was 20μg/mL of protein at 20
mins. Large discrepancy in error in the non-chloroethanol control was due to a skewed reading showing high activity, possibly due to improper quenching.

**Product Inhibition Data for DhlA:**

<table>
<thead>
<tr>
<th></th>
<th>μMol of Cl produced With Chloroethanol</th>
<th>μMol of Cl produced Without Chloroethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>5.51</td>
<td>6.07</td>
</tr>
<tr>
<td>Run 2</td>
<td>3.86</td>
<td>5.19</td>
</tr>
<tr>
<td>Run 3</td>
<td>5.40</td>
<td>3.91</td>
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</table>

**Product Inhibition Data for Jann2620:**

<table>
<thead>
<tr>
<th></th>
<th>μMol of Cl produced With Chloroethanol</th>
<th>μMol of Cl produced Without Chloroethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>0.674</td>
<td>0.639</td>
</tr>
<tr>
<td>Run 2</td>
<td>0.632</td>
<td>1.03</td>
</tr>
<tr>
<td>Run 3</td>
<td>0.642</td>
<td>0.577</td>
</tr>
</tbody>
</table>
**Substrate Inhibition:**
Various levels of substrate concentrations were tested for substrate inhibition. Substrate levels were all at concentrations thought to be saturating.

![DhLA Substrate Inhibition Experiment](image)

**Figure A5:** Substrate Inhibition for DhLA. There is negligible evidence of substrate inhibition.

![Jann2620 Substrate Inhibition Experiment](image)
Figure A6: Substrate Inhibition for Jann2620. There is negligible evidence of substrate inhibition.

Table A1: Substrate Inhibition Data for DhlA:

<table>
<thead>
<tr>
<th>Run 1 (mM Cl)</th>
<th>Run 2 (mM Cl)</th>
<th>Run 3 (mM Cl)</th>
<th>Average</th>
<th>STD</th>
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<td>1mM DCA</td>
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<td>1.16E-01</td>
<td>1.22E-01</td>
<td>1.21E-01</td>
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Table A2: Substrate Inhibition Data for Jann2620:

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<tr>
<th>Run 1 (mM Cl)</th>
<th>Run 2 (mM Cl)</th>
<th>Run 3 (mM Cl)</th>
<th>Average</th>
<th>STD</th>
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Table A3: Chloroethane Degradation Activity of DhlA and Jann2620:

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<th></th>
<th>Total Cl in 24 hours</th>
<th>Activity (mM/min*mg)</th>
<th>Estimated 1,2 DCA activity</th>
<th>Activity % (Chloroethane vs. 1,2-DCA)</th>
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<tbody>
<tr>
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There is therefore negligible degradation of chloroethane when compared to 1,2 DCA degradation.

### Table A4: Complete Dataset for ALL DhlA Runs

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<th>Gas Conc (uM)</th>
<th>Liquid Conc (mM)</th>
<th>Corr. Total Moles (umol)</th>
<th>Time (Hours)</th>
<th>$f$</th>
<th>$\ln(f)$</th>
<th>$\ln(R/R_0)$</th>
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<th>Liquid Conc (mM)</th>
<th>Corr. Total Moles (umol)</th>
<th>Time (Hours)</th>
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<th>$\ln(f)$</th>
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<th>Time</th>
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**Standard Curves for 1,2-DCA:**

20-Mar-12 Calibration Curve for 1,2-DCA the Varian 3380 GC by Headspace

\[ y = 4379x - 18019 \]
\[ R^2 = 0.99553 \]
22-Mar-12 Calibration Curve for 1,2-DCA the Varian 3380 GC by Headspace

\[ y = 4541.6x - 4167 \]

\[ R^2 = 0.98813 \]

16-Mar-12 Calibration Curve for 1,2-DCA the Varian 3380 GC by Headspace

\[ y = 4467.4x - 27523 \]

\[ R^2 = 0.99884 \]
13-Mar-12 Calibration Curve for 1,2-DCA the Varian 3380 GC by Headspace

\[ y = 4784.1x - 53455 \]
\[ R^2 = 0.99721 \]

7-Mar-12 Calibration Curve for 1,2-DCA the Varian 3380 GC by Headspace

\[ y = 6716.2x + 797.62 \]
\[ R^2 = 0.9903 \]
Part B: Additional HAD Data:

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**Example IC Standard Curves:**

![Glycolate Standard Curve](image)

$y = 8.834 \times 10^8 \pm 3.380 \times 10^7 \text{ Gly} \\
R^2 = 0.987$
**Chloroacetate Standard Curve**

\[ y = 1.365e+009 \pm 7.240e+007 \]

\[ R^2 = 0.978 \]

**Bromoacetate Standard Curve**

\[ y = 1.216e+009 \pm 2.629e+007 \]

\[ R^2 = 0.996 \]
PART C: PSEUDO STEADY STATE KINETIC DERIVATIONS

Let $k_1$ and $k_{-1}$ be the enzyme-substrate complex formation and dissociation rate constants respectively, and $k_2$ be the enzyme catalytic rate constant.

The rate of the reaction at any point is equal to the number of enzyme-substrate complexes times the rate of catalysis constant $k_2$. Thus,

Equation 1: \[ v = k_2[ES] \]

Assuming pseudo steady state, the overall $[ES]$ does not change over time, which means

Equation 2: \[ \frac{d[ES]}{dt} = 0 = k_1[E][S] - k_2[ES] - k_{-1}[ES] \]

In addition, the amount of enzyme not bound by substrate is equal to the initial amount of enzyme minus the amount of enzyme-substrate complexes.

Equation 3: \[ [E] = [e_0] - [ES] \]

Substitute Equation 3 into Equation 2 in order to isolate and solve for $[ES]$:

\[
0 = k_1(e_0 - [ES])[S] - k_2[ES] - k_{-1}[ES] \\
0 = k_1 e_0[S] - k_1[ES][S] - k_2[ES] - k_{-1}[ES] \\
k_1 e_0[S] = k_1[ES][S] + k_2[ES] + k_{-1}[ES] \\
[ES] = \frac{k_1 e_0[S]}{k_1[S] + k_2 + k_{-1}}
\]
Substitute new derivation of [ES] into Equation 1:

\[ v = \frac{k_2k_1e_0[S]}{k_1[S] + k_2 + k_{-1}} \]

We now get

\[ v = \frac{k_2e_0[S]}{[S] + \left(\frac{k_2 + k_{-1}}{k_1}\right)} \]

which is a rewriting of the Michaelis Menten Equation (written below), albeit with different constants.

Michaelis-Menten Equation: \[ v = \frac{V_{max}[S]}{[S]+K_M} \]

Thus:

\[ V_{max} = k_2e_0 \]

and

\[ K_M = \frac{k_2 + k_{-1}}{k_1} \]

Since \( k_{cat} \) is the enzyme turnover rate for one enzyme, it is equal to the \( V_{max} \) divided by the total enzyme concentration \( e_0 \). Therefore:

\[ k_{cat} = \frac{k_2e_0}{e_0} = k_2 \]