Molecular Mechanism of Bifunctionality in an Ancestral Apicomplexan Dehydrogenase

Master’s Thesis
Presented to
The Faculty of Graduate School of Arts and Sciences
Brandeis University

Department of Biochemistry
Douglas Theobald, Advisor

In Partial Fulfilment
of the Requirement for the Degree
Master of Science
in
Biochemistry

by
Miriam Hood
May 2018
Acknowledgements

This thesis and all the work it represents would not exist without the help of an amazing community.

To Professor Douglas Theobald, thank you for welcoming me into your lab and introducing me to the wonderful world of research. I could never have imagined how transformative and amazing my time in your lab would be.

To Brian, thank you for encouraging me to seek out this opportunity and the time you took to ensure that I had the practical knowledge and skills to succeed in my work.

To Jacob, thank you for your constant and consistent support, answering my endless questions, and the always surprising and entertaining music.

To John and Masha, thank you for your incredible guidance and help in collecting and understanding NMR.

To Rick, Molly, Charles, and Christine, thank you for making the lab such a delightful place to work. Thank you for all the insightful conversations on life, the universe, and everything – they made the bad science days not so bad at all.

To the Biochemistry Office, particularly Jennifer Roy and Maryanna Aldrich, thank you for making this process so smooth and seamless.

To my friends, thank you for listening to me chatter on at length about the lab, the late nights working on theses together, and for making Brandeis a home.

To my family, thank you for always believing in me and my dreams. I would have never made it this far without you and truly appreciate everything you have done for me.

Finally, I would like to dedicate this thesis to my grandparents, Gregory Haas, Tybe Haas, and Sondra Hood, who have encouraged my curiosity from the very beginning.
Abstract

Molecular Mechanism of Bifunctionality in an Ancestral Apicomplexan Dehydrogenase

A thesis presented to the Department of Biochemistry

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Miriam Hood

Malate dehydrogenases (MDH) and lactate dehydrogenases (LDH) are essential metabolic enzymes that share similar folds and mechanisms despite their strong substrate specificity. The unicellular eukaryotes of the Apicomplexa phylum, responsible for a variety of human diseases, have convergently evolved a unique and highly specific LDH from an MDH. The Apicomplexan LDH and MDH are an excellent model for the evolution of enzyme specificity due to the presence of a putative promiscuous intermediate in the evolutionary pathway. A six amino acid insertion into the active site conferred pyruvate activity by shifting the key catalytic residue from an arginine at position 102 in MDHs to a tryptophan at position 107f. However, the bifunctional intermediate contains both R102 and W107f. Though only one conformation is observed in x-ray crystallography data, I hypothesize that there are two active conformations of the intermediate. I used heteronuclear single quantum coherence nuclear magnetic resonance (HSQC NMR) to visualize a dimer of the promiscuous intermediate in the presence of each substrate. Loss of the tryptophan peak was seen in the presence of pyruvate at 25°C. I used intrinsic tryptophan fluorescence to monitor the catalytic tryptophan in the presence of each substrate. Pyruvate amplifies tryptophan fluorescence, but oxaloacetate demonstrates no
consistent trend. Mutation of Trp107f to an alanine results in the loss of LDH activity but does not affect MDH activity.
3.2 Tryptophan to Alanine Mutant Only Affects LDH Activity .................................................. 23
3.3 NADH and NADH₄ Demonstrate Similar Binding ............................................................. 24
3.4 HSQC NMR Reveals Two Different Bound Conformations .................................................. 27
3.5 Ancestral Enzymes are Highly Thermostable ........................................................................ 31

4 Discussion .................................................................................................................................. 33
4.1 Epistasis Matters ..................................................................................................................... 33
4.2 Arginine, not tryptophan is required for oxaloacetate turnover ............................................. 33
4.3 Elucidation of an MDH versus LDH conformation ................................................................. 34
4.4 Dimerization has an impact .................................................................................................... 34

5 Future Directions ........................................................................................................................ 35

References ...................................................................................................................................... 36

6 Appendix ..................................................................................................................................... 38

List of Tables

Table 1 NADH and NADH₄ for AncMDH²*-INS I193D I287D binding constants determined by ITC ........................................................................................................................................... 24
Table 2 NADH and NADH₄ for AncLDH* K102R binding constants determined by FRET and intrinsic tryptophan fluorescence ...................................................................................................................................... 25
Table 3 Steady-State Kinetics of ancestors turning over oxaloacetate. ........................................... 38
Table 4 Steady-state kinetics of ancestors turning over pyruvate ...................................................... 38
Table 5 Melting temperatures of ancestors from DSC experiments .................................................. 41

List of Figures

Figure 1 Phylogeny of MDH/LDH superfamily ............................................................................. 2
Figure 2 Scheme of MDH/LDH active site and catalytic mechanism ............................................. 2
Figure 3 Evolution of LDHs in Apicomplexa ................................................................................... 4
Figure 4 Ancestral Crystal Structures ............................................................................................. 5
Figure 5 Proposed Bifunctional Active Site Dynamics .................................................................... 6
Figure 6 Tetramer and Dimer Structures ......................................................................................... 10
Figure 7 NADH₄ Synthesis Scheme ............................................................................................... 11
Figure 8 Steady State Kinetic results for ancestral enzymes .......................................................... 23
Figure 9 Fluorescent profiles of NAD and NAHD₄ ........................................................................ 26
Figure 10 HSQC NMR Spectrum of AncMDH²*-INS I193D I287D, NADH₄, and Substrate at 45°C ...................................................................................................................................................... 28
Figure 11 HSQC NMR Spectrum of Tryptophan 107f in AncMDH2*-INS I193D I287D with NADH4 and substrate at 45°C .............................................................. 29
Figure 12 HSQC NMR Spectrum of AncMDH2*-INS I193D I287D, NADH4, and Substrate at 25°C .......................................................................................................................................................................................... 30
Figure 13 HSQC NMR Spectrum of Tryptophan 107f in AncMDH2*-INS I193D I287D with NADH4 and substrate at 25°C. .................................................................................................................................................................................. 31
Figure 14 Melting Points of ancestors from DSC analysis ........................................................................................................................................ 32
Figure 15 Size exclusion curve of AncMDH2*-INS I193D I287D ................................................. 39
Figure 16 ITC binding curves of NADH and NADH................................................................. 40
Figure 17 Sequence alignment of enzymes ................................................................................ 41
1 Introduction

1.1 Apicomplexan Dehydrogenases

Malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) are structurally and catalytically similar metabolic essential for a diverse range of organisms (1,2). MDH interconverts oxaloacetate and malate and LDH interconverts pyruvate and lactate by an oxidation/reduction reaction (1). Both utilize the coenzyme NADH (1). These enzymes are essential for core metabolic processes including the tricarboxylic acid cycle (TCA) and anaerobic respiration (1,3). Despite similarity in substrate structure, differing by only a carboxyl moiety on oxaloacetate and a methyl on pyruvate, modern enzymes have evolved high levels of substrate specificity (3). The Theobald lab has shown that LDH activity has evolved from MDH activity on four separate occasions (Figure 1) (4,5).

Primary sequence similarity in the dehydrogenases is low, at about 20% sequence identity, but amino acids around the catalytic site and cofactor binding site are highly conserved (1,6). Position 102 in MDH is a highly conserved arginine, which sits on a loop that closes over the active site and forms a salt bridge with the β-carboxyl group on oxaloacetate (1,3,4). Pyruvate lacks a charge due to the C3 methyl, thus in canonical LDHs position 102 is a conserved glutamine (1,4). Given the importance of position 102 in discriminating between substrates it has been dubbed the “specificity reside” (5).
Figure 1 Phylogeny of MDH/LDH superfamily: The tree is colored by function (MDH in blue, LDH in orange, HicDH in green). The LDH events are numbered: 1 – Canonical LDHs, 2 – Trichomonad LDHs, 3 – Apicomplexa LDHs, 4 – Cryptosporidium LDHs. Modified from DOI: 10.7554/eLife.02304.010

Figure 2 Scheme of MDH/LDH active site and catalytic mechanism: Substrate R groups are boxed (Methylene carboxylate in oxaloacetate/malate, Methyl in pyruvate/lactate). Conserved active site residues are in black and substrate is in blue. DOI: 10.7554/eLife.02304.003
The Apicomplexa phylum is predominantly made up of eukaryotic intracellular parasites responsible for several diseases, including malaria, toxoplasmosis, and babesiosis (5). Apicomplexan LDHs show up in the LDH-like MDH clade (Figure 1) as opposed to the larger canonical LDH clade where most eukaryotic LDHs are found and are the only eukaryotic enzymes to show up in the predominately bacterial clade (4,5,7). Apicomplexa likely acquired their MDH from a lateral gene transfer from α-proteobacteria and evolution of apicomplexan LDH activity is reasoned to be due to a gene duplication event followed by a functional change (4,7). Despite their shared structural and catalytic aspects modern apicomplexan MDHs and LDHs are highly specific (5). Additionally, Apicomplexa LDH is essential and has greater specificity than human LDH, thus it is of interest as a potential drug target (8-10).

While in canonical malate dehydrogenases the substrate specificity can be changed by substitution of a single amino acid, Apicomplexa have evolved LDH activity by a different mechanism (1). Apicomplexan LDHs have a lysine at position 102 maintaining the same charge as the arginine in MDHs, but a five amino acid insertion in the active site loop at position 108 changes the loop conformation (4,10,11). Due to the change in conformation tryptophan 107f is the LDH specificity residue in modern apicomplexan LDHs (5). The lysine at position 102 points away from the substrate and instead tryptophan 107f is positioned to interact with the C3 methyl of pyruvate (4). The Trp170f may recognize pyruvate either by hydrophobic interactions versus the negative charge on oxaloacetate or by steric due to the larger methylene carboxylate on oxaloacetate (4,5).

Two phylogenies were generated, an initial tree with sequences from a broader range of organisms and an alternative tree with fewer overall sequence, but a greater number of apicomplexan taxa (5). Ancestral sequence reconstruction shows that the loop insertion occurs
between the last common ancestor of Apicomplexa MDHs and LDHs and the last common of Apicomplexa, except *Cryptosporidium parvum* LDH (5). The enzymes from the alternative tree are respectively known as AncMDH2* and AncLDH*, my work primarily focuses on variations of these enzymes. The enzymes from the initial tree are known as AncMDH2 and AncLDH.

**Figure 3** Evolution of LDHs in Apicomplexa: The tree is colored by activity (MDH in blue, LDH in orange, Bifunctional in purple. The y-axis of the bar graphs represents log($k_{cat}/K_M$), with oxaloacetate in blue and pyruvate in orange. Modern sequences include *Plasmodium falciparum* (Pf), *Toxoplasmosa gondii* (Tg), *Cryptosporidium parvum* (Cp), and *Rickettsia bellii* (Rb). DOI: 10.7554/eLife.02304.016
1.2 Loop and Specificity Swap

Interestingly, introducing either the 5 amino acid insertion and R102K mutation into modern MDHs or reverting the lysine to an arginine and deleting the insertion in the modern LDHs does not swap the enzyme’s specificity. Rather it reduces the native activity without conferring any novel activity (5). The only differences between modern MDHs and LDHs within 6Å of the active site are the 6 amino acid changes responsible for LDH activity, while there are over 200 differences in total. This suggests that the inability to swap specificity is due to long range epistasis (5). However, AncMDH2*-INS, the ancestral MDH with the five amino acid loop insertion, has bifunctional capabilities and only differs from AncLDH* K102R by 52 mutations (5). Both AncMDH2* and AncLDH* demonstrate high levels of activity for the native substrates, just as the modern enzymes (5). The presence of this bifunctional intermediate in the evolutionary pathway makes theses enzymes a prime model for studying the evolution of substrate specificity.

Figure 4 Ancestral Crystal Structures: E) Superposition AncMDH2 (blue, 4plw, chain C), AncLDH* (orange, 4plg, chain A), AncMDH2-INS (purple, 4ply, chain F and 4plv, chain B). Ligands are in grey. F) Active site detail with catalytic side chains as sticks. DOI: 10.7554/eLife.02304.029
AncMDH2, AncLDH*, and AncMDH2-INS was crystallized with lactate, malate, or oxamate, as well as NADH (Figure 4), but crystal structures of AncMDH2-INS only reveal pyruvate and/or lactate in the active site and an LDH conformation with Trp107f occupying the specificity residue position and Arg102 solvent exposed (5). These structures offer no explanation of how AncMDH2*-INS is capable of turning over oxaloacetate, as tryptophan is too bulky and lacks the appropriate charge to accommodate oxaloacetate in the active site (5). I hypothesize that the two specificity residues, Arg102 and Trp107f, swap positions by flipping in or out of the active site depending on the substrate present (Figure 5).

![Figure 5 Proposed Bifunctional Active Site Dynamics: Arg102 in blue and Trp107f in orange.](image)

In order to determine if this is the case I used heteronuclear single quantum coherence nuclear magnetic resonance (HSQC NMR) to probe the active site. Tryptophan side chains have unique 1H15N signal on HSCQ and Trp107f is the only tryptophan in the enzyme, which allows it serve as an probe for the active site conformation (12). Provided Trp107f is flipping in and out of the active site, then there should be a shift in or disappearance of the HSQC signal (12,13). If
this is not the case, no change in the Trp107f signal should be seen. I also used intrinsic tryptophan fluorescence in the presence of iodide, a fluorescence quencher. If Trp107f is behaving as hypothesized, then in the presence of pyruvate it should be protected from iodide quenching and in the presence of oxaloacetate it should be subject to iodide quenching (14). If there is no difference in quenching behavior, then Trp107f is likely not changing conformation. Since Trp107f is essential for pyruvate turnover, I introduced the W107fA mutation into AncLDH* K102R to determine if the bifunctional enzyme could turnover oxaloacetate without Trp107f (5). If AncLDH* K102R W107fA can turnover oxaloacetate, then both Trp107f and Arg102 are necessary for bifunctional activity. This would support the existence of a second conformation as crystal structures only show Trp107f pointed into the active site.

There are few experimental challenges that face this project. There is an upper limit on the size of protein which has clear resolution in HSQC NMR (13). As the ancestors are larger tetramers, mutations to force dimerization were required. I used steady-state kinetics to ensure that the dimeric enzyme was still active. To ensure that the mutations resulted in a dimer and prevent association of the dimers into tetramers I ran size exclusion chromatography. Additionally, the high turnover rate of these enzymes means that capturing the substrate bound complex in NMR or fluorescence experiments is difficult if not impossible. This required the use of the cofactor analogue and inhibitor NADH₄, which has been shown to be a competitive inhibitor of NADH dependent dehydrogenases (15). I confirmed this with a steady-state kinetic assay. To confirm that NADH₄ does not bind in drastically different manner to NADH I used isothermal calorimetry and fluorescence binding assays. Both compounds should have similar KDs and binding curves if they bind similarly.
1.3 Experimental Background

1.3.1 Steady State Kinetics

Steady-state kinetics may be used to determine the efficiency and preference of an enzyme with respect to various substrates, a central question in this work. The Michaelis-Menten equation describes enzyme steady-state kinetics and yields two parameters, $k_{\text{cat}}$ and $K_M$:

$$v = \frac{V_{\text{max}}[S]}{(K_M+[S])} \quad (1.1)$$

$$v = \frac{E_T k_{\text{cat}}[S]}{(K_M+[S])} \quad (1.2)$$

$$\frac{v}{E_T} = \frac{k_{\text{cat}}[S]}{(K_M+[S])} \quad (1.3)$$

In equation 1.1, $V_{\text{max}}$ is the maximum velocity the enzyme can achieve at saturating substrate and a given enzyme concentration, $E_T$. $K_M$ is the substrate concentration, [S] at which the velocity is half of $V_{\text{max}}$. Further manipulation gives equation 1.3, where $k_{\text{cat}}$ can be extracted. From this we can determine the Michaelis constant, $k_{\text{cat}}/K_M$, with units of M$^{-1}$s$^{-1}$. This is a second order rate constant not dependent on enzyme concentration. This allows us to compare the efficiency of one enzyme with regard to several substrates or several enzymes with regards to one substrate.

The simplest model assumes two components, the enzyme and substrate, however malate and lactate dehydrogenases require an NADH cofactor as a hydride source in the oxidation/reduction reaction. In order to prevent any confounding results NADH is present in saturating conditions for all steady-state experiments. NADH has been shown to have a $K_d$ of 4.0 ± 0.8 μM and a $K_M$ of 11 ± 2 μM for the modern PfLDH (16). Using 200 mM of NADH, well
above its $K_M$, in all experiments allows for the reasonable assumption that the rate of reaction is solely a result of substrate concentration.

An additional complicating factor is substrate inhibition. MDHs, LDHs, and the bifunctional enzymes are subject to substrate inhibition at high levels of pyruvate and oxaloacetate (17). This requires slight modification of the Michaelis-Menten equation:

$$\frac{v}{E_T} = \frac{k_{cat}[S]}{K_M \left(1 + \frac{[S]}{K'_M} + [S] \right)} \quad (1.4)$$

Where $K'_M$ is the second substrate concentration at which the rate is half of $k_{cat}$.

1.3.2 HSQC NMR

Heteronuclear Single Quantum Coherence Nuclear Magnetic Resonance (HSQC NMR) is two-dimensional NMR experiment that correlates the shifts of two different nuclei. In the case of proteins $^1H^15N$ HSQC NMR is often the first step in investigating protein structure by NMR (13). HSCQ NMR is conducted in solution, and may provide information about protein dynamics that X-ray crystallography may not be able to access (13).

Each $^1H$-$^15N$ pair has a characteristic shift, including tryptophan side chain nitrogen, allowing easy identification of peaks and the ability to track changes of a particular residue (18). Each peak in the spectra represents $^1H^15N$ bonded pair through which magnetization is transferred from the hydrogen to the nitrogen (18). $^15N$ spectra can specifically be used to understand conformation changes through potentially conformation dependent $15N$ chemical shifts (12,18).
Larger molecules, such as a protein, must be labeled as the natural abundance of 15N is quite low and 15N can be readily incorporated into the protein through growth in minimal media supplemented with (15NH4)2SO4 (13,18). Even for labeled proteins there are limitations on size as the quality of spectra decreases with increasing size (13). AncMDH2*-INS approaches 150kDa in size, well over the size limit for HSQC. MDHs are multimeric, typically existing as homodimers or homotetramers, and LDHs exist primarily as tetramers with a few exceptions (1,4). In each case the subunits functions independently (1). Previously the lab dimerized AncMDH2*-INS by mutating two isoleucines to aspartates, AncMDH2*-INS I193D I281D (Figure 6), at the dimer-dimer interface to achieve higher resolution (19).

![Figure 6](image_url)

*Figure 6* Tetramer and Dimer Structures: (A) Tetramer of AncMDH2*-INS (4ply) with loop shown in yellow. The dimer-dimer interface is between the blue and green dimers. (B) Representation of dimer of AncMDH2*-INS with loop show in yellow.
One of the challenges in capturing the active and bound structure of the bifunctional ancestor is its rapid turnover and release of substrate. To prevent this rapid turnover of during data collection for NMR a known co-factor analogue and inhibitor, NADH₄, was used in place of NADH. NADH₄ has been used to prevent turnover of a dehydrogenase substrate, causing an enzyme-inhibitor-substrate complex to be formed and studied (20). This is precisely what was needed to observe the loop conformations. NADH₄ likely prevents hydride transfer due to the loss of resonance stabilization of the 5,6 carbon double bond in the reduced state, weakening their ability to react. Additionally the loss of the double bond changes the bond length and reduces the planarity of the co-factor analogue and potentially disrupting the placement of the hydride relative to the substrate (15). The apicomplexan malate and lactate dehydrogenases use an ordered mechanism with cofactor binding first (16). The use of NADH₄ should allow proper binding, prevent release of product, and lock the enzyme in the active conformation.

Figure 7 NADH₄ Synthesis Scheme

One of the challenges in capturing the active and bound structure of the bifunctional ancestor is its rapid turnover and release of substrate. To prevent this rapid turnover during data collection for NMR a known co-factor analogue and inhibitor, NADH₄, was used in place of NADH. NADH₄ has been used to prevent turnover of a dehydrogenase substrate, causing an enzyme-inhibitor-substrate complex to be formed and studied (20). This is precisely what was needed to observe the loop conformations. NADH₄ likely prevents hydride transfer due to the loss of resonance stabilization of the 5,6 carbon double bond in the reduced state, weakening their ability to react. Additionally the loss of the double bond changes the bond length and reduces the planarity of the co-factor analogue and potentially disrupting the placement of the hydride relative to the substrate (15). The apicomplexan malate and lactate dehydrogenases use an ordered mechanism with cofactor binding first (16). The use of NADH₄ should allow proper binding, prevent release of product, and lock the enzyme in the active conformation.
1.3.3 Isothermal Titration Calorimetry

Isothermal titration calorimetry is a simple experiment used to determine the binding affinity of a ligand for a given protein in addition to the corresponding thermodynamic properties, changes in enthalpy (ΔH), and entropy (ΔS) (21,22). The binding of a ligand to protein releases or absorbs heat in proportion to the amount of ligand bound to protein. ITC instruments can measure this by the amount of power required to maintain and constant temperature in the reaction cell (21).

The amount of heat each binding event releases or absorbs is calculated from the area under each power peak (21). As the titration continues less and less unbound protein is available, and the peaks decrease in magnitude until the protein is completely saturated (21). This results in the familiar sigmoidal binding curve, which can be fit to determine the $K_D$, enthalpy, and entropy of binding (22).

1.3.4 Differential Scanning Calorimetry

Differential scanning calorimetry is used to determine the thermodynamics and energetics, including melting temperatures, heat capacity, enthalpy, and entropy, of protein folding and unfolding (23). The thermodynamics of protein folding/unfolding can be influenced by oligomerization state and conformation of the protein (22). DSC instruments measure the difference in heat capacity between sample and reference cell by measuring how much energy is required to keep the difference in temperature at zero under adiabatic conditions (22,23). The collected data are normalized to the protein concentration and the apparent molar heat capacity and melting temperature are determined (23).
1.3.5 Intrinsic Tryptophan Fluorescence Spectroscopy

Ligand-dependent intrinsic tryptophan fluorescence quenching has been used in multiple cases to investigate conformational changes upon ligand binding \((14,24)\). The mechanism of quenching follows these scheme:

\[
T^* \rightarrow T + h\nu \quad k_1(T^*) \quad (2.1)
\]

\[
T^* \rightarrow T \quad k_2(T^*) \quad (2.2)
\]

\[
T^* \rightarrow T + X \quad k_3(T^*)(X) \quad (2.3)
\]

Scheme 2.1 is the release of light following the decay of the fluorophore, scheme 2.2 is when decay occurs but no radiation is released, and scheme 2.3 is in the presence of quencher \((14)\). Using steady-state approximations the quantum yield without quencher or the amount of measured fluorescence, \(F_0\), is given by:

\[
F_0 = \frac{k_1}{k_1 + k_2} = k_1\tau_0 \quad (1.6)
\]

Where \(\tau_0 = 1/(k_1 + k_2)\) and is the fluorescence lifetime when the quencher is not present. When the quencher is present the quantum yield becomes:

\[
F_0 = \frac{k_1}{k_1 + k_2 + k_3(X)} \quad (1.7)
\]

Combining equations 1.6 and 1.7 gives the Stern-Volmer law:

\[
\frac{F_0}{F} = 1 + K_Q(X) \quad (1.8)
\]

Where \(K_Q = k_3\tau_0\). As AncMDH2*-INS and AncLDH* K102R have a single tryptophan my experiments are simplified by not having to approximate the combined fluorescence of
several tryptophans as one fluorophore (14). The Stern-Volmer law can be modified to linearize the plot:

\[ \frac{F_0}{\Delta F} = \frac{1}{(x)f_0K_Q} + \frac{1}{f_0} \]  (1.9)

Where \( \Delta F = F_0 - F \) and \( f_0 \) is the maximum fraction of accessible fluorophore (14). This equation is particular to collisional quenching but static quenching, where a non-fluorescent complex forms, follows a similar law (14).

NADH₄ has been previously shown to quench tryptophan fluorescence by static quenching in other enzymes (15). This offers an alternative to FRET binding assays, as NADH₄ should not have any fluorescent behavior. A specific method of collisional fluorescence quenching, known as solute perturbation of protein fluorescence, makes use of potassium iodide to specifically probe the solvent exposure of a tryptophan (14). In the presence of a substrate that moves the tryptophan from an aqueous solvent exposed position to a position within the protein/hydrophobic region, such as pyruvate, the effect of iodide quenching will be reduced and the presence of a substrate/inhibitor that keeps the tryptophan exposed, such as oxaloacetate, will increase quenching affinity (24).

1.3.6 Förster Resonance Energy Transfer

Förster Resonance Energy Transfer (FRET) experiments can be used to determine the binding of a fluorescent ligand, such as NADH, that binds in close proximity to a fluorescent residue, such as tryptophan (25). FRET experiments require the presence of a donor and acceptor fluorophore with spectral overlap whose absorption and emission wavelengths lie in UV, visible, or IR ranges (25). Tryptophan and NADH are a perfect naturally occurring donor-acceptor pair: they require no incorporation of additional tags and overlap well with Tryptophan absorbing at
~280nm and emitting at ~340nm and NADH absorbing at ~340nm and emitting at ~450nm (25).

This pair has been used to measure NADH binding in PfLDH and was fit using a tight binding equation (16).
2 Materials and Methods

2.1 Plasmid Construction and Mutation

Protein sequences were codon optimized for E. coli expression and subcloned into pET-24a with a 6xHis-tag on the C-terminus. All genes bypassed the N-terminal T7-histags and 6 histidines were instead added to the C-terminus. All gene synthesis and subcloning was performed by Genscript (Piscataway, NJ).

2.2 Protein Expression

Plasmids were transformed in BL21 DE3 (pLysS) E. coli (Invitrogen, Grand Island, NY). Cells were grown at 37°C/225rpm in 2XYT media with 30mM potassium phosphate, 0.1% (w/v) glucose, 0.06 mg/mL chloramphenicol, 0.034 mg/mL kanamycin, ascorbic acid, pH 7.8. After cultures reached an OD$_{600}$ of 0.5-0.8, cells were induced with 0.5mM IPTG for 4 hours. Cells were pelleted with centrifugation at 8,000 rpm for 20 minutes and stored at -20°C.

2.3 N$^{15}$-Labeled Protein Expression

Plasmids were transformed in BL21 DE3 (pLysS) E. coli (Invitrogen, Grand Island, NY). Cell cultures were started in 5 mL of LB with 0.06 mg/mL chloramphenicol and 0.034 mg/mL kanamycin at 37°C/225rpm for 10 hrs. LB starter cultures were passaged to 100 mL minimal media with 1x M9 media (48mM Na$_2$HPO$_4$, 22mM KH$_2$PO$_4$, 8.6mM NaCl, 18mM N$^{15}$H$_4$Cl), 2mM magnesium sulfate, 0.1mM calcium chloride, 0.4% glucose, 0.06 mg/mL chloramphenicol, 0.034 mg/mL kanamycin and grown at 37°C/225rpm overnight.
Expression flasks of 1x M9 media, 2mM magnesium sulfate, 0.1mM calcium chloride, 0.4% glucose, 0.06 mg/mL chloramphenicol, 0.034 mg/mL kanamycin were inoculated with minimal media overnights to an OD600 of 0.04 and grown at 37°C/225rpm until the cultures reached and OD600 of 0.5-0.8. Cells were induced with 0.5mM IPTG and expressed at 20°C/225rpm for 18 hrs. Cells were pelleted with centrifugation at 8,000rpm for 20 minutes and stored at -20°C.

2.4 Protein Purification

Pellets were thawed on ice and resuspended in 15-20 mL of 50mM sodium phosphate, 300mM sodium chloride, 10mM imidazole, 300µM TCEP HCl, pH 8.00 buffer with 2 µL of Piece Universal Nuclease (Thermo Scientific, Rockford, IL). Homogenized lysate was sonicated on ice at 35% amplitude for 2 minutes (30s on, 20s off). Lysate was centrifuged for 20 minutes at 18,000 rpm and supernatant was collected.

Proteins were purified with nickel affinity chromatography. Supernatant was filtered and run over a 5 mL HisTrap FF column (GE Healthcare, Piscataway, NJ) and eluted with an imidazole gradient from 10mM to 50mM on an AKTA Prime (GE Healthcare, Piscataway, NJ). Collected fractions were monitored with UV absorbance and analyzed by SDS-PAGE. Fractions containing protein were pooled and concentrated with an Amicon Ultracel-10K centrifugal filter (Milipore, Billerica, MA). Concentrated protein was desalted into 50mM Tris base, 100mM sodium chloride, 0.1% EDTA, 0.02% sodium azide, 300µM TCEP HCl, pH 7.4 buffer with a PD10 column (GE Healthcare, Piscataway, NJ). Protein concentration was determined by absorbance at 280nm and extinction coefficients and molecular weights from ExPASy’s ProtParam tool.
2.5 Size Exclusion Chromatography

Concentrated protein in 50mM Tris base, 100mM sodium chloride, 0.1% EDTA, 0.02% sodium azide, 300µM TCEP HCl, pH 7.4 buffer was run over a HiPrep 16/60 Sephacryl S-200 High Resolution size exclusion column (GE Healthcare, Piscataway, NJ) on an AKTA Purifier (GE Healthcare, Piscataway, NJ). Fractions containing protein were pooled and concentrated with an Amicon Ultrace-10K centrifugal filter (Milipore, Billerica, MA). Protein elution volume was compared to a standard curve from Blue Dextran, Aldolase, Ovalbumin, Ferritin, and Conalbumin. The standard curve was fit with a single exponential and the molecular weight was determined by the elution volume.

2.6 NADH₄ Synthesis

Synthesis was carried out with the help of Masha Rosenberg. NADH₄ was prepared by hydrogenation in the presence of a palladium catalyst, Pd/C. NADH₄ (2.5g, mmol) and Pd/C (g, mmol) were dissolved in 60mL of MilliQ water, pH 8.5 and flushed with nitrogen gas. Hydrogen gas absorption took place for 4hr until absorbance at 340nm was completely reduced. The crude yield was lyophilized and dissolved in 10mL of water. The catalyst was removed by filtration through DEAE and run HiPrep DEAE FF 16/10 over a column (GE Healthcare, Piscataway, NJ) with a gradient from 20mM to 1M TEAB.

2.7 Steady State Kinetic Assay

Steady state kinetic parameters were determined for oxaloacetate and pyruvate on a CARY 100 Bio (Agilent, Santa Clara, CA) at 25°C at saturating NADH conditions in 50mM Tris Base, 50mM potassium chloride, 0.3mM TCEP HCl, pH 7.4. Substrate was titrated in from 0-10,000nM while NADH was held at 200µM. Protein concentration was 2.8nM. Kinetic data was
fit with a chi-squared estimate of either the Michaelis-Menten equation or a substrate inhibition equation using Kaleidagraph. Data was collected and fit in triplicate.

2.8 Stopped Flow Steady State Kinetic Assay

Steady state kinetic parameters were determined for oxaloacetate and pyruvate on a SX 20 Stopped Flow (Applied Physics, Leatherhead, Surrey, UK) at 25°C at saturating NADH conditions in 50mM Tris Base, 50mM potassium chloride, 0.3mM TCEP HCl, pH 7.4. Substrate was titrated from 0-10,000nm while NADH was held at 200µM and enzyme ranged from 50nM-500nM depending on the construct. Data was fit with a chi-squared estimate of either Michaelis-Menten equation or a substrate inhibition equation using Kaleidagraph. Data was collected and fit in triplicate.

2.9 HSQC NMR

All HSQC NMR were conducted in collaboration with John Stiller of the Kern Lab and Masha Rosenberg of the Theobald Lab at Brandeis University. Data of enzyme and substrate was collected at 45°C from 300µL sample with 0.5mM enzyme, 0.5mM NADH₄, 3mM oxaloacetate or 0.05mM pyruvate, and 10% D2O. Data of enzyme and substrate was collected at 25°C with 0.4mM enzyme, 0.5mM NADH₄, 5mM oxaloacetate, or 25mM pyruvate, and 10% D₂O. Data of enzyme with just cofactor was collected from 300µL sample with 0.5mM enzyme, 0.5mM NADH₄, and 10% D₂O. All enzyme samples were diluted from 1mM stocks in 50mM Tris Base, 100mM sodium chloride, 0.02% sodium azide, 0.1% EDTA, pH 7.4.

2.10 FRET

Förster Resonance Energy Transfer (FRET) data was collected on a Fluoromax 4 spectrometer (Horiba Jobin Yvon, Longjumeau, France) with a 1mL sample of 100nM NADH in
50mM Tris base, 50mM potassium chloride, 0.3mM TCEP HCl, pH 7.4. Enzyme was titrated in from 0-30mM in 1µL injections. The sample was excited at 250nm and emission was monitored at 450nm. Data was fit with a chi-squared estimate of a tight binding curve using Kaleidagraph:

\[
\theta = \frac{[P]_t + 1 + K_d - \sqrt{([P]_t + 1 + K_d)^2 - 4([P]_t)^2)}}{2[P]_t} A + B
\]

Where A is the amplitude, or the maximum fluorescent signal, and B is the y intercept, or the fluorescent signal with no fluorescence acceptor.

2.11 Intrinsic Tryptophan Fluorescence Assay

Intrinsic tryptophan fluorescence data was collected on a Fluoromax 4 spectrometer (Horiba Jobin Yvon, Longjumeau, France) with 1mL sample of µM enzyme and 200nM NADH in 50mM Tris Base, 50mM potassium chloride, 0.3mM TCEP HCl, pH 7.4. Substrate was titrated in from 0-30µM in 1µL injections. The sample was excited at 280nm and emission was monitored at 450nm. Data was fit with a chi-squared estimate of the Stern-Volmer equation using Kaleidagraph.

2.12 Differential Scanning Calorimetry

All differential scanning calorimetry (DSC) data was collected on a NanoDSC. Data was collected from 600µL samples of 25-50µM enzyme dialyzed into 50mM sodium phosphate dibasic anhydrate, 50mM sodium phosphate monobasic monohydrate, 100mM sodium chloride, pH 7.4. Scans were run up from 0C to 100C and back down from 100C to 0C. Data was analyzed in NanoAnalyze and fit with the NanoAnalyze General and Two-State models.
2.13 Isothermal Titration Calorimetry

All isothermal titration calorimetry (ITC) was collected on a NanoITC in the Kern Lab at Brandeis University. Data was collected from 100µL sample of 40µM enzyme dialyzed into 50mM Tris Base, 100mM sodium chloride, 0.02% sodium azide, 0.1% EDTA, pH 7.4 and 280µM of ligand in 50mM Tris Base, 100mM sodium chloride, 0.02% sodium azide, 0.1% EDTA, pH 7.4. Data was collected over 45 1µL injections, 180s/injection at 25°C/350rpm. All data was analyzed in NanoAnalyze.
3 Results

3.1 AncLDH K102R demonstrates less substrate preference than AncMDH-INS

AncMDH2*-INS has been characterized as a bifunctional enzyme, but size limitations on HSQC required us to force dimerization. Thus I wanted to confirm that the mutations introduced do not affect the kinetic behavior. Additionally, I was interested in how AncLDH* K102R would compare to AncMDH2*-INS I193D I287D as AncMDH2-INS-59mut, the equivalent ancestor from the initial tree, has a more balanced kinetic profile than AncMDH2-INS (5). I assayed the kinetics of these ancestors under steady-state conditions, turning over either oxaloacetate or pyruvate with an excess of NADH.

AncMDH2*-INS I193D I287D activity is not greatly affected by dimerization as compared to previously determined AncMDH2*-INS activity (5). AncMDH2*-INS I193D I287D demonstrates a slight preference for oxaloacetate over pyruvate, while both AncLDH K102R and AncLDH* K102R demonstrate little to no preference for one substrate over the other. There are 52 amino acid differences outside of the active site between AncMDH2* and AncLDH* which appear to play a role in improving LDH activity. These results also agree with the kinetic behaviors of ancestors from the original tree, further supporting that these are likely the way Apicomplexa’s ancestral MDH and LDH enzymes behaved.

Since NADH₄ is required to prevent substrate turnover in HSQC and fluorescence experiments, I also looked at AncMDH2*-INS I193D I287D activity while titrating in NADH₄. Inhibition is observed and NADH₄ has a mid-teens micromolar Kᵢ.
Figure 8 Steady State Kinetic results for ancestral enzymes: Each enzyme was assayed with pyruvate and oxaloacetate; no measurable rate could be obtained for AncLDH* K102R W107fA or AncMDH*-INS W107fA with pyruvate. INS refers to the 5 amino acid insertion. Relative specificity (RS) is the ratio of $k_{cat}/K_m$ pyruvate$/k_{cat}/K_m$ oxaloacetate where a positive log(RS) represents a preference for pyruvate and negative log(RS) represents a preference for oxaloacetate. The original data can be found in Appendix 6.1.

3.2 Tryptophan to Alanine Mutant Only Affects LDH Activity

I chose to mutate that tryptophan to an alanine in the bifunctional ancestors, as previous work has shown that the tryptophan was essential to LDH activity, but its role in MDH activity for the bifunctional ancestors had not been determined. As expected the W107fA mutants have no significant pyruvate activity but retain normal oxaloacetate activity. The $k_{cat}$ for both W107fA mutants increases 4 fold (Appendix 6.1), which may be due to increased freedom of movement on the active site loop due to the loss of steric hindrance from the tryptophan. This confirms that the tryptophan is not essential for oxaloacetate turnover, but the arginine is. As previous crystal structures have shown that the arginine is pointed out of the active site, away from the substrate,
when pyruvate is present, there is likely a second conformation where arginine is in closer proximity to the active site when oxaloacetate is present.

### 3.3 NADH and NADH₄ Demonstrate Similar Binding

I chose to investigate binding of NADH₄ versus NADH and the binding of substrate in the presence of NADH₄ using ITC to confirm that the two ligands do not bind in drastically different manners. Both NADH and NADH₄ were found to bind AncMDH2*-INS I193D I287D with single digit micromolar Kᵩₐₚ (Table 1), similar to published NADH binding constants for modern PfLDH (16). The shape of the binding curves for NADH and NADH₄ were similar and did not indicate any significant differences in binding behavior (Appendix 6.2). ITC data for substrate binding was inconclusive and protein precipitation was observed following the experiment.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kᵩ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>3.00 ± 0.39</td>
</tr>
<tr>
<td>NADH₄</td>
<td>2.72 ± 0.34</td>
</tr>
</tbody>
</table>

*Table 1 NADH and NADH₄ for AncMDH2*-INS I193D I287D binding constants determined by ITC*

Tryptophan 107f provides a unique probe for investigating the active site via fluorescence experiments. As NADH also has a fluorescent profile, I determined NADH binding for AncLDH* K102R using FRET experiments. NADH binds AncLDH* K102R with a low double digit micromolar Kᵩ. NADH₄ should not be fluorescent or capable of FRET due to the loss of aromaticity in the nicotinamide ring, which I confirmed by comparing excitation and emission scans NADH and NADH₄ (Figure 13). Since NADH₄ does not fluoresce, I used intrinsic tryptophan fluorescence to determine the binding of NADH₄. NADH₄ also binds AncLDH*
K102R with a low double digit micromolar $K_D$ (Table 2). NADH$_4$ quenches the tryptophan fluorescence signal upon binding, potentially due to the remaining conjugated double bonds absorbing the tryptophan’s emission without being excited.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_D$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>14.80 ± 2.49</td>
</tr>
<tr>
<td>NADH$_4$</td>
<td>38.08 ± 0.20</td>
</tr>
</tbody>
</table>

*Table 2* NADH and NADH$_4$ for AncLDH* K102R binding constants determined by FRET and intrinsic tryptophan fluorescence

I investigated pyruvate and oxaloacetate binding in the presence of saturating NADH$_4$ and iodine using intrinsic tryptophan fluorescence experiments. While the data is noisy, pyruvate consistently amplifies the tryptophan signal, suggesting that pyruvate binding pushes the equilibrium towards loop closure with the tryptophan in the active site where it may be protected from iodine quenching. Oxaloacetate does not produce a consistent trend, though preliminary results indicate similar signal amplification to pyruvate. The current results do not allow any conclusions about the conformation of the protein to be drawn based on fluorescence.
Figure 9 Fluorescent profiles of NADH and NAHD4
3.4 HSQC NMR Reveals Two Different Bound Conformations

I confirmed AncMDH2*-INS I193D I287D’s oligomeric state of with size exclusion chromatography (Appendix 6.2) and activity at elevated temperatures with steady state kinetic assays prior to conducting the NMR experiments (Appendix 6.1). I collected HSQC NMR data, in collaboration with John Stiller and Masha Rosenberg, in the presence of NADH₄ and oxaloacetate or pyruvate. HSQC NMR of AncMDH2*-INS I193D I287D is well resolved and shows a single tryptophan peak as expected (Figure 9). While a few changes in the spectrum are seen at 45°C between the oxaloacetate and pyruvate spectra, the tryptophan peak shows no significant changes – shifting only 0.025ppm along the N¹⁵ axis (Figure 10). The small number of shifts in the spectra could suggest a change in conformation upon binding of a pyruvate versus oxaloacetate, but the lack of significant change in the tryptophan signal provides no insight into how the structure of the active site changes to accommodate each substrate.

We initially chose to conduct the experiments at 45°C to improve the resolution, due to low resolution of earlier experiments with AncMDH2*-INS. While AncMDH2*-INS I193D I287D was still active at elevated temperatures, the pyruvate kcat was reduced (Appendix 6.1), and the elevated temperature may have contributed to protein precipitation during the 45°C experiments. Given this and that AncMDH2*-INS I193D I287D falls below the 70kDa, we conducted later experiments at 25°C – the same temperature as the steady-state kinetic assays. These spectra provide more insight into the enzymes’ dynamics. The spectrum of AncMDH2*-INS I193D I287D with NADH₄ and substrate versus AncMDH2*-INS I193D I287D with NADH₄ only show slightly different chemical shifts, suggesting the substrate is binding in the presence of NADH₄ (Figure 11A). In spectra with pyruvate the tryptophan signal disappears, while in the spectra with oxaloacetate the tryptophan does not disappear (Figure 12). This
suggests that there are two different conformations of the enzyme, with the tryptophan in different positions, depending on the substrate present.

Figure 10 HSQC NMR Spectrum of AncMDH2*-INS I193D I287D, NADH₄, and Substrate at 45°C: Spectra were collected for 300µL of 0.5mM protein, 0.5mM NADH₄, and either 3mM oxaloacetate or 50mM pyruvate over 4hrs. Oxaloacetate is shown in green and pyruvate is shown in purple.
Figure 11 HSQC NMR Spectrum of Tryptophan 107f in AncMDH2*-INS I193D I287D with NADH₄ and substrate at 45°C: Oxaloacetate shown in green and pyruvate shown in purple.
Figure 12 HSQC NMR Spectrum of AncMDH2*-INS I193D I287D, NADH₄, and Substrate at 25°C: Spectra were collected for 0.4mM protein, 0.5mM NADH₄, and either 5mM oxaloacetate or 25mM pyruvate over 4hrs. (A) Spectrum with NADH₄ (green), NADH₄ and oxaloacetate (red), and NADH₄ and pyruvate (blue). (B) Spectrum of NADH₄ and oxaloacetate (red) and NADH₄ and pyruvate (blue).
3.5 Ancestral Enzymes are Highly Thermostable

I used DSC to determine the melting temperatures of the bifunctional ancestors as compared to the ancestral state. All the ancestors demonstrated high thermostability in the ancestral and bifunctional states. The dimer of AncMDH2*-INS had a significantly reduced melting temperature. The mutations introduced to create the dimer are along the dimer-dimer interface of the ancestral state’s tetrameric form and resulted in no significant changes in activity, thus were thought not to have any significant impact upon the enzyme as a whole. The change in melting point may be due to change in polymeric state but may also suggest that the mutation have compromised the structural integrity of AncMDH2*-INS I193D I287D.
I also used DSC to determine if the melting temperature of enzyme in the presence of NADH was different and potentially to determine the thermodynamics of cofactor binding. There is no significant difference in the melting temperature of AncMDH2*-INS I193D 287D with and without NADH.
4 Discussion

4.1 Epistasis Matters

Evolution from AncMDH2* to AncLDH* involves 52 amino acid mutations outside of the active site in addition to the active site changes (Appendix 6.4). Through kinetic characterization I have found that oxaloacetate activity is not significantly different between AncMDH2*-INS I193D I287D and AncLDH* K102R, but the pyruvate $k_{cat}/K_M$ increases over 100-fold from the AncMDH2* to the AncLDH* background (Figure 8). The effect is primarily due to changes in $K_M$ (Appendix 6.1). Permissive mutations that stabilize new structural elements and give rise to novel functions are seen in other evolutionary pathways (26,27). These 52 mutations have significant effect on pyruvate activity through binding, suggesting that they may stabilize an LDH conformation of the enzyme and be an example of permissive mutations.

4.2 Arginine, not tryptophan is required for oxaloacetate turnover

In Apicomplexa Trp107f is the specificity residue for pyruvate and Arg102 is the specificity residue for oxaloacetate, the lack of either residue in monofunctional enzymes results in significantly reduced activity. The loss of Trp107f in the bifunctional ancestors results in a loss of pyruvate, but not oxaloacetate activity, suggesting the tryptophan is not essential for oxaloacetate turnover. Furthermore, apicomplexan enzymes only have significant oxaloacetate activity when Arg102 is present, suggesting it is necessary for MDH activity. The requirement of both specificity residues for bifunctional activity along with previous crystal structures that show
only one specificity reside (tryptophan) present in the active site (5), suggest that there is a
different conformation of the bifunctional enzyme for the turnover of oxaloacetate.

4.3 Elucidation of an MDH versus LDH conformation

While HSQC NMR of AncMDH2*-INS I193D I287D at 45°C shows no significant
differences, spectrum collected at 25°C show the disappearance of the tryptophan side chain
signal in the presence of pyruvate, but not in the presence of oxaloacetate. The difference in
tryptophan signal and additional shifts in the spectra in the presence of each substrate also
suggest two distinct substrate-bound conformations. This disappearance of the tryptophan signal
and earlier crystallography data does suggest that the tryptophan is buried in the active site in the
presence of pyruvate. Assignment of the spectra, particularly of Arg102, will create a clearer
picture of the differences between the two conformations.

While fluorescence data is not as clean as desired preliminary results show amplification
of the intrinsic tryptophan signal in the presence of pyruvate. This suggests an LDH
conformation with the tryptophan submerged in the active site, agreeing with the crystal
structures. Fluorescence data in the presence of oxaloacetate is inconclusive and further
experiments will be required to determine the conformation capable of turning over oxaloacetate.

4.4 Dimerization has an impact

AncMDH2*-INS I193D I287D maintains the same kinetic behavior as AncMDH2*-INS
but has a reduced melting temperature. The lower melting temperature may be due to the change
in oligomeric state, but it may also be due to destabilization of enzyme. While many MDHs are
found in the dimeric state, very few LDHs are, which suggests that enzymes with LDH activity
may not tolerate oligomeric states other than tetrameric.
5 Future Directions

The next immediate step in this project should pin down the conformation of the bifunctional ancestors capable of turning over oxaloacetate. While intrinsic tryptophan fluorescence has proven to be less conclusive than desired, fluorescence anisotropy experiments may allow for the detection of tryptophan dynamics and two different conformations. We also plan to assign the spectrum with each substrate, and in combination with crystallography data, determine the structures of each active enzyme conformation.

Earlier kinetics work has shown that modern Apicomplexa MDHs and LDHs are not capable of becoming bifunctional, despite few changes to the active site (19). My kinetics work suggests that there is long range epistasis involved in stabilizing LDH activity, which may in turn influence substrate specificity later on. We plan to continue to investigate how substrate specificity develops over the course of evolution and what causes the loss of promiscuity in the modern enzymes by characterizing more recent ancestors and their bifunctional derivatives.

The difference in LDH activity between AncMDH2*-INS and AncLDH* K102R suggests there is a difference in mechanism. Future experiments to better understand the differences between the two enzymes and identify the rate-limiting step(s) should include single turnover experiments, multiple turnover experiments, and potentially kinetic isotope effects.
References


6 Appendix

6.1 Steady State Kinetic Parameters

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AncMDH2*-INS I193D I287D</td>
<td>53.08</td>
<td>54.80</td>
<td>9.48 x 10$^5$ ± 2.09 x 10$^5$</td>
</tr>
<tr>
<td>AncLDH K102R</td>
<td>11.94</td>
<td>17.73</td>
<td>5.75 x 10$^5$ ± 2.93 x 10$^5$</td>
</tr>
<tr>
<td>AncLDH* K102R</td>
<td>203.58</td>
<td>465.19</td>
<td>5.42 x 10$^5$ ± 2.96 x 10$^5$</td>
</tr>
<tr>
<td>AncMDH2*-INS W107fA</td>
<td>216.68</td>
<td>50.28</td>
<td>4.30 x 10$^6$ ± 4.28 x 10$^5$</td>
</tr>
<tr>
<td>AncLDH* K102R W107fA</td>
<td>1284.55</td>
<td>873.54</td>
<td>1.50 x 10$^6$ ± 1.65 x 10$^5$</td>
</tr>
<tr>
<td>AncMDH2*-INS I193D I287D (RT Assay)</td>
<td>19.28</td>
<td>20.065</td>
<td>9.61 x 10$^5$</td>
</tr>
</tbody>
</table>

Table 3 Steady-State Kinetics of ancestors turning over oxaloacetate.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AncMDH2*-INS I193D I287D</td>
<td>43.55</td>
<td>4117.27</td>
<td>1.11 x 10$^4$ ± 1.49 x 10$^3$</td>
</tr>
<tr>
<td>AncLDH K102R</td>
<td>14.78</td>
<td>24.29</td>
<td>5.93 x 10$^5$ ± 8.03 x 10$^4$</td>
</tr>
<tr>
<td>AncLDH* K102R</td>
<td>77.395</td>
<td>300.32</td>
<td>2.61 x 10$^5$ ± 1.19 x 10$^4$</td>
</tr>
<tr>
<td>AncMDH2*-INS W107fA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AncLDH* K102R W107fA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AncMDH2*-INS I193D I287D (RT Assay)</td>
<td>9.74</td>
<td>1700.1</td>
<td>5.73 x 10$^3$</td>
</tr>
</tbody>
</table>

Table 4 Steady-state kinetics of ancestors turning over pyruvate.
6.2 Size Exclusion Chromatography

Figure 15: Size exclusion curve of AncMDH2*-INS H193D I287D
6.3 ITC Binding Curves
6.4 DSC Melting Point Parameters

<table>
<thead>
<tr>
<th>Ancestral State</th>
<th>AncMDH2* (°C)</th>
<th>AncLDH* (°C)</th>
<th>AncLDH (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancestral State</td>
<td>88.02 ± 1.51</td>
<td>77.50 ± 0.03</td>
<td>95.03 ± 0.06</td>
</tr>
<tr>
<td>Bifunctional State</td>
<td>88.68 ± 0.02</td>
<td>77.47 ± 0.10</td>
<td>95.77 ± 0.20</td>
</tr>
<tr>
<td>Dimer</td>
<td>66.73 ± 0.06</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5 Melting temperatures of ancestors from DSC experiments

6.5 Sequence Alignment

Figure 17 Sequence alignment of enzymes: Anc350 refers to AncMDH2*, Anc356 refers to AncLDH*, Anc2686 refers to AncLDH