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Mechanistic Studies on Malate Dehydrogenase from Escherichia coli

S. Kirk Wright

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Mechanistic Studies on Malate Dehydrogenase

from *Escherichia coli*

(TITLE)

BY

S. Kirk Wright

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from *Escherichia coli*

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ABSTRACT

Kinetic studies and chemical modification studies were performed on malate dehydrogenase from *Escherichia coli* (eMDH). Chemical modification studies using diethylpyrocarbonate and iodoacetic acid, along with log V/K profiles indicate that one ionizing group with a pK_a between 7.9 and 8.5 acts as a general acid/base in the catalytic mechanism. Log V profiles suggest that malate binds to the correctly protonated form of the enzyme. These results imply that a histidine residue is required for catalysis.

A primary deuterium kinetic isotope effect of $1.43 (\pm 0.14)$ was observed on V/K using malate-2-d, while no isotope effect was measured on V_{max} . This implies that proton abstraction is partially rate determining under nonsaturating conditions. Small isotope effects were observed on V_{max} and V/K (1.22 ± 0.19 and 1.08 ± 0.06) when using NADD, indicating that the rate determining step is the release of the cofactor.

Other kinetic studies show that eMDH is inhibited by the substrates oxaloacetate and NADH at high concentrations. Through substrate analog inhibition experiments, using citrate, malonate, ketomalonate and ATP, it was found that eMDH is very specific for its substrates.

Allosteric interactions between ligand and substrate provide a means of regulation, since they allow the activity of an enzyme to be controlled by changes in the concentrations of other species (including the products). It was determined that eMDH is allosterically inhibited in the presence of 0.065 mM NADH

Other chemical modification reagents specific for arginine residues such as, 1,2-cyclohexanedione, indicate that arginine residues are important for catalysis and most likely play a role in binding the substrate.

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LIST OF ABBREVIATIONS

ARG.....	arginine
ASP.....	aspartate
ATP.....	adenosine triphosphate
BSA.....	bovine serum albumin
CHES.....	2-[N-Cyclohexylamino]ethanesulfonic acid
cMDH.....	cytosolic malate dehydrogenase
DEPC.....	diethylpyrocarbonate
DNA.....	deoxyribonucleic acid
DTT.....	dithiothreitol
EDTA.....	disodium ethyldiaminetetraacetate
eMDH.....	malate dehydrogenase from <i>E. coli</i>
HEPES.....	N-[2-Hydroxyethyl]piperazine-N'[2-ethanesulfonic acid]
HIS.....	histidine
KIE.....	kinetic isotope effect
KPi.....	K_2HPO_4
LDH.....	lactate dehydrogenase
MAL.....	malate
MDH.....	malate dehydrogenase
MES.....	2-[N-Morpholino]ethanesulfonic acid
mMDH.....	mitochondrial malate dehydrogenase
NAD ⁺	β -nicotinamide adenine dinucleotide

NADD.....dueterated β -nicotinamide adenine dinucleotide

NADH..... β -nicotinamide adenine dinucleotide, reduced form

PIPESPiperazine-N,N'-bis[2-ethanesulfonic acid]

SDS.....sodium dodecylsulphate

TAPS.....N-tris[Hydroxymethyl]methyl-3-amino-propanesulfonic acid

TCA.....tricarboxylic acid

Tris.....Tris((hydroxymethyl)-aminomethane

Chapter I

INTRODUCTION

Most every reaction that occurs in the cell is catalyzed by its own particular enzyme. In bacterial cells, such as *Escherichia coli*, it is estimated that there are 3,000 different proteins. In higher eukaryotic cells there are 50,000 proteins, the majority of which are enzymes. In the absence of enzymes, most of the reactions that occur in the cell would not occur even over a time period of years, and life as we know it would not exist. Thus, the ultimate goal of enzyme studies is to gain an understanding of the crucial role that these catalysts play in the metabolism of living organisms.

Enzymes are biological catalysts that increase the rate of chemical reactions without undergoing a permanent change themselves. Enzyme catalysis is usually comprised of three basic steps: 1) The substrates (reactants) bind to the enzyme in a specific order to form an enzyme-substrate complex. 2) Due to the catalytic activity of certain amino acid residues located in the active site, the substrates are converted to products. 3) The products then dissociate from the enzyme.

Enzymes display four remarkable characteristics; 1) high catalytic power, 2) mild reaction conditions, 3) great specificity, and 4) the capacity for regulation.

The rate of an enzyme catalyzed reaction may be as much as 10^{14} fold greater than the non-catalyzed reaction. Enzyme catalyzed reaction rates are typically 10^6 to 10^{12} greater than uncatalyzed reactions, and are several orders of magnitude greater

than the corresponding chemically catalyzed reactions. Furthermore, enzymatically catalyzed reactions occur under mild conditions; temperatures less than 100°C, atmospheric pressure and neutral pH, in contrast to chemical catalysis which usually requires elevated temperatures and pressures as well as extremes of pH.

Enzymes show great specificity with respect to both substrates and products. There are some enzymes that have low specificity; they will utilize a wide range of substrates as long as they contain the required chemical bond. A second class of enzymes exhibit group specificity; they will catalyze the reactions of a variety of similar molecules. The last group of enzymes show near-absolute specificity or absolute specificity. These enzymes will catalyze a reaction of a single substrate. Also, it should be noted that enzymes display distinct stereospecific requirements. This stereospecificity, by virtue of the inherent chirality (enzymes are comprised of only L-amino acids), happens due to enzymes forming asymmetric active sites.

The fourth important property of enzymes is that their catalytic activity may be regulated by substances such as small ions or molecules as well as the substrate(s). These regulatory processes include allosteric control, covalent modification, and the amount of enzyme synthesized [1].

A fundamental problem in enzymology is the determination of the enzymatic mechanism, and how to account for their astonishingly high and specific catalytic activity based on the chemical structure of the enzyme. Enzymes may be studied by a variety of biological, chemical and physical means, the most classical of which is kinetic study, which can be regarded as essential [11]. Inferences of the enzymatic

mechanism may be drawn from varying the substrate concentration, pH, ionic strength, and temperature. However, it should be noted that it is not possible to definitively determine how an enzyme works in chemical terms without a knowledge of the enzyme's kinetics [1].

Kinetic measurements and chemical studies of enzymatically catalyzed reactions are among the most powerful tools for elucidating the catalytic mechanisms of enzyme reactions. Therefore, the remainder of this chapter is devoted to describing the kinetic and chemical tools used in the determination of the enzymatic mechanism of eMDH.

The initial velocity equation for a two substrate reaction is:

$$v = V[A][B] / (K_m A[B] + K_m B[A] + [A][B] + K_i A K_m B) \quad (1)$$

When one of the substrate concentrations is varied and the other is fixed at a saturating level, the two substrate equation is simplified into the Michaelis-Menten equation:

$$v = VA / (K_m + A) \quad (2)$$

where A is the substrate concentration and K_m is the Michaelis constant [14].

There are many methods or tools available to the enzymologist which can be used to study the kinetic and chemical mechanisms of enzymes. In this study, many

classical tools such as inhibition experiments, isotope effects, and chemical modification experiments were employed.

Isotope effects were used to determine the rate limiting steps of the reaction. The catalytic group required for activity and the pK of that catalytic group was determined by the use of pH variation studies. Chemical modification studies were used to probe for the identity of the catalytic group in the active site and were also used to determine the role of other amino acid residues located in the catalytic site.

In the initial phase of enzyme study, it is important to be able to determine the kinetic order of a reaction. The kinetic order of an enzyme catalyzed reaction may vary considerably depending on the conditions. This may be demonstrated by an investigation of three kinetic experiments in which the enzyme concentration is held constant, but the initial substrate concentration is varied from low to high concentrations. If the initial substrate concentration is low, the reaction may appear to be a first-order process with respect to substrate loss. If the initial substrate concentration is high, the reaction rate is independent of substrate concentration which is a zero-order process. However, if the substrate is present in an intermediate concentration, the rate of conversion of substrate to product may be neither first-order nor zero-order. Thus, under the given set of conditions it may become very difficult to determine the rate law for the enzyme catalyzed reaction.

In order to avoid these difficulties, enzyme catalyzed reactions are generally conducted by measuring the initial velocities. Plotting substrate concentration versus time usually yields straight lines for the first 10 to 20% of the total reaction. The

slope of the line is equal to the initial velocity, which is a zero-order rate constant [12, 13].

In the second phase of enzyme study, the kinetic mechanism is determined. Generally, a mathematical model is developed based on the kinetic study to describe the kinetic mechanism. For a two substrate reaction, there are two general types of kinetic models. The first is a double displacement, or ping pong mechanism in which one or more products must be released before all substrates can react. The second model is a sequential mechanism, in which all reactants must combine with the enzyme prior to reaction. The sequential mechanism can be further subdivided into an ordered or random mechanism. In the ordered mechanism, the substrates combine and dissociate in a specific order. However, in the random mechanism the order of combination and dissociation is not obligatory [12].

The type of inhibition (product inhibition) shown by the products P and Q towards the substrates A and B can be used to propose a mechanism for the reaction (Scheme I). In the random mechanism, Q acts as a competitive inhibitor with respect to substrate B. Whereas in the ordered mechanism, Q acts as a mixed-type inhibitor with respect to substrate B. Thus, product inhibition can be used to distinguish between the ordered and random ternary complex by studying the inhibition caused by the product Q [1].

Isotope effect experiments can be used to aid in the determination of the rate-limiting step(s) of a reaction. There are two uses of isotope effects: 1) From the absolute values for the bond-breaking steps, clues of the chemical mechanism, and the

A = NADH

P = Malate

B = Oxaloacetate

Q = NAD⁺

Scheme I. The kinetic Mechanism of eMDH.

nature of the transition states may be ascertained. 2) The degree to which the absolute value is expressed in V or V/K tells where the rate limiting steps are in the mechanism. For a non-enzymatically catalyzed chemical reaction a full isotope effect is generally seen since the bond-breaking reaction is commonly rate determining. However, for an enzyme catalyzed reaction, steps other than the catalytic step are often partially rate determining. Therefore, the observed isotope effect is usually smaller than the true isotope effect on the bond-breaking step. If a normal hydrogen atom is replaced with a deuterium atom, the breakage of the carbon-deuterium bond is slower than that of a carbon-hydrogen bond and therefore an isotope effect is observed. Isotope effects are usually expressed as k_H/k_D and usually range from 5 to 15 for deuterium.

By using pH variations of the kinetic parameters, it is possible to determine which amino acid residues play a role in catalysis. The parameters of interest which may change with pH are V and V/K for each substrate. V can be defined as the maximum velocity of the reaction. K is $V/2$ and is also a measure of the affinity of the enzyme for its substrates. The Michaelis constants (K) of the substrates are of no interest since they are merely the ratio of V and V/K . These parameters are generally plotted as $\log V$ or $\log V/K$ vs. pH, thus any drop on the graph corresponds to a lower rate or lessened binding. Because these are log-log plots, they consist of straight lines with a whole number slope, which are connected by a curve that spans two pH units. From these plots, an estimation of the pK_a of the amino acid residue(s) and the number of groups that participate in catalysis may be determined.

The biological, chemical, and physical properties of proteins can be altered by chemical modifying reagents. The underlying principle of chemical modification is extremely simple. If an amino acid residue involved in catalysis is chemically modified, there will be a decrease in enzyme activity. Thus, the roles of the individual amino acid side chains may be determined by chemically modifying the amino acid residues in the active site.

Chemical modification poses one major problem, unspecific modification. Ideally, it is highly desirable that the modifying agent be specific for only one type of side chain and that only one of these amino acid side chains will have reacted. Therefore, any change in catalytic activity can be attributed to the modification. When changes in activity correspond to the modification of only one residue, it is unlikely that the loss of activity is due to a conformational change. This also implies that if there is no effect on catalytic activity, the modified residue(s) is not essential for the activity under investigation.

As stated earlier, when one substrate is varied and the other is fixed at saturating levels, the enzyme is expected to follow equation 2. However, in substrate inhibition, v only increases to a certain point and decreases at higher concentrations of A [14].

Many inhibitors are substances that structurally resemble the substrates, and typically react slowly or not at all. Inhibitors are commonly used to probe the chemical and conformational nature of the active site in an effort to determine the enzyme's catalytic mechanism. By varying one substrate, fixing the other, and fixing

the inhibitor concentration, inhibition patterns can be determined. Three basic types of inhibition are observed: competitive, noncompetitive (or mixed inhibition), and uncompetitive.

The first is competitive inhibition, which is characterized by slopes that are affected by an inhibitor, intercepts that are not, and a point of intersection on the vertical axis. This pattern indicates mutually exclusive binding of inhibitor and substrate to the same form of the enzyme. The second inhibition pattern is a series of parallel lines in which the intercepts are affected, but the slopes are not, and is characteristic of uncompetitive inhibition. This pattern demonstrates that the inhibition disappears as the substrate concentration is extrapolated to zero, and indicates that the enzyme form with which the inhibitor binds is not present when the variable substrate concentration is zero. The third form of inhibition is noncompetitive or mixed competition, where both the slopes and the intercepts are affected by inhibitor concentration. This pattern indicates that inhibitor and substrate bind to different forms of the enzyme and that the inhibitor can cause inhibition at very low or high substrate concentration.

One of the incredible characteristics of enzyme catalysis and thereby metabolism is the high degree of regulation. Allosterism, which is a form of regulation, is the cooperative interaction of one ligand (substrate) binding to a specific (active) site which is influenced by the binding of an effector ligand at an allosteric site on the enzyme. An allosteric effect is characterized by a sigmoidal curve in a plot of rate vs. [substrate].

A class of enzymes known as dehydrogenases catalyze the conversion of an alcohol to a keto-group. This study focused on malate dehydrogenase which catalyzes the oxidation of malate to oxaloacetate with the subsequent reduction of NAD^+ to NADH (Scheme II) and can be found in a variety of organisms. The equilibrium of this reaction is in favor of malate/ NAD^+ by a factor of 10^5 .

Malate dehydrogenase is found as the cytosolic and mitochondrial isozymes in eukaryotic organisms. The cytosolic form participates in the aspartate/malate shuttle, the function of which is to transfer cytosolic reducing equivalents across the mitochondrial membrane. Mitochondrial MDH participates on the other side of the aspartate/malate shuttle and also catalyzes the final step of the TCA cycle. The eukaryotic isozymes of MDH have been extensively studied and their chemical and physical properties have been well defined [2]. In most cases, the reaction catalyzed by the dimeric forms of MDH has been proposed to follow an sequential ordered Bi-Bi mechanism with the cofactor binding before the substrate [2-6]. However, the prokaryotic forms of MDH have been much less studied. Kinetic studies were performed by Sanwall in which it was determined that eMDH is allosterically regulated [7]. Murphey *et al.* described some of the physical properties of eMDH [8]. Recently, the crystal structure of eMDH was elucidated in the presence of citrate, a substrate analog, and NAD^+ [9, 10].

In the past 30 years much research has been conducted on lactate dehydrogenase, mitochondrial malate dehydrogenase, and cytosolic malate dehydrogenase. Thus determining that all of the above enzymes are homologous, and

Scheme II. The reaction catalyzed by eMDH.

that there is an invariant histidine residue in the active site of all three enzymes [15, 16].

Site directed mutagenesis studies on LDH and mMDH indicate that both enzymes have a histidine located in the active site which interacts with an aspartate, and have proton-donating and accepting capabilities [17, 18]. Furthermore, a comparison of x-ray crystal structure of cMDH and LDH indicate a His:Asp pair which may function as a proton relay system during catalysis [19].

The crystal structure of MDH from *Escherichia coli* strain HB101, indicates that eMDH is closely homologous with mMDH. Research has determined that *E. coli* MDH is a dimeric enzyme, and that His177 which is located in the active site, is found to be hydrogen bonded to an aspartate to form a classic His:Asp pair [19, 10]. A crystal structure, if available, is an excellent foundation for enzymatic study. However, as stated earlier, a crystal structure alone is not enough for the proposal of an enzymatic mechanism. Based on the information gleaned from the site-directed mutagenesis studies and from the crystal structure, an investigation of the importance of the invariant histidine residue (H177 in eMDH), which is proposed to be involved in catalysis was carried out by chemical modification.

The chemical modifying agent, diethylpyrocarbonate, reacts with the imidazole ring of histidine to yield a N-carbethoxyhistidyl derivative. This reaction may be conveniently followed spectrophotometrically by the increase in absorbance between 230 and 250 nm [20, 21]. The number of modified residues can be calculated from the molar absorption difference for N-carbethoxyhistidine at 240 nm: $\epsilon_{240\text{ nm}} = 3200$

$\text{M}^{-1} \text{cm}^{-1}$ [20]. Hydroxylamine removes the carbethoxy group from N-carbethoxyhistidyl residues [22]. The inactivation of an enzyme by DEPC may be correlated with the modification of a histidyl residue if treatment with hydroxylamine reactivates the enzyme [23]. The selective reaction of DEPC with imidazole groups and the ease with which this reaction can be quantitated, makes it a convenient probe for determining the accessibility and function of histidine residues.

Haloacetates, such as iodoacetate, are the most widely used protein alkylating agents. Under various conditions, they have been shown to react with sulfhydryl, imidazole, thioether, and amino groups of proteins. In several enzymes, histidine residues have been shown to have an unusually high reactivity, considerably more than even the imidazole group of histidine itself. At pH 5.5 and slightly above, imidazole groups are at least partially unprotonated, and their modification is fast enough to be the principle reaction in proteins lacking sulfhydryl groups [24].

Chemical modification studies have been conducted on porcine heart and bovine mMDH using iodoacetamide. The results of these studies were that a histidine was chemically modified, and that the histidine residue can be protected from modification by the presence of NADH in both cases. The results of protection against modification by NADH indicate that the histidine is located in the active site [25-27]. Chemical modification studies using haloacetates, such as iodoacetic acid, provided a second method to probe the importance of the histidine residue located in the active site.

Substrate recognition and binding plays a critical role in the effectiveness of an enzyme. In MDH, there are at least three arginine residues that participate in substrate recognition and binding. Arg81 is involved in binding the dicarboxylic acid substrate, and is located in the nucleotide binding region. Arg87 is located within a region of the enzyme that participates in conformational changes, which occur after the substrate has bound to the active site. The third arginine residue, Arg153, is located in the catalytic region of the enzyme. It participates in hydrogen bonding at the subunit interface and participates like Arg81, in substrate binding [10].

Certain 1,2-diketones, react in alkaline solution with the guanidino moieties of proteins. Of these 1,2-diketones, 1,2-cyclohexanedione, an arginine specific reagent, appears to be one of the most useful [24] and was used to verify the importance of arginine residues in eMDH.

Over the years, much research has been done on LDH and on the eukaryotic cytosolic and mitochondrial MDH isozymes, yet eMDH has been relatively unstudied. The goal of this research is to propose a mechanism of action for eMDH, and also to compare eMDH with other MDHs in order to discover the similarities and differences in enzyme function.

Chapter II

EXPERIMENTAL PROCEDURES

1. Materials and Instruments

a. Chemicals

All reagents were obtained from Sigma Chemical Company unless otherwise specified. *E. coli*, strain HB101 was purchased from American Type Culture Collection. Potassium phosphate dibasic was obtained from Aldrich Chemical Company Inc.. The Bradford dye reagent and Cibacron Blue (Affi-Gel Blue Gel, 100-200 mesh) were obtained from Bio-Rad. DTT was purchased from Boehringer Mannheim Biochemicals. Diethylpyrocarbonate was obtained from Aldrich Chemical Company Inc. Iodoacetic acid was obtained from Eastman Organic Chemicals.

b. Instruments

Centrifugation was performed with a Sorvall SS-4 Manual Superspeed Centrifuge or a Beckman Microfuge 11 at 4°C. The sonification of *E. coli* cell paste was performed using a Bronwill Scientific Biosonik III sonicator. A LKB Ultrospec 4050 (non-thermostated) was used for routine protein and activity assays. The final enzyme assays, kinetic studies and chemical modification studies were performed on a Shimadzu UV-3100 Spectrophotometer with a thermostated microprocessor controlled

cell holder.

2. Transformation of HB101 with pEM6 Plasmid by the Calcium Chloride Procedure

A HB101 colony was picked from a refrigerated agar plate and was used to inoculate 5 mL of LB medium, which was incubated at 37°C for 12 h with shaking. One milliliter of the overnight culture was used to inoculate 100 mL of LB medium. The 100 mL culture was grown with vigorous shaking at 37°C for 2 to 4 h (density = 5×10^7 cells/mL). The culture was chilled on ice for 10 min and then was centrifuged at 4,000 RPM for 5 min at 4°C. The supernatant was decanted and discarded. The cells were resuspended in 5 mL of an ice-cold, sterile solution of 50 mM CaCl_2 , 10 mM Tris, pH 8.0. The cell suspension was placed in an ice bath for 15 min and then centrifuged at 4,00 RPM for 5 min at 4°C.

The supernatant was discarded, and the cells were suspended in 1 mL of the ice-cold CaCl_2 solution. The cells were stored on ice for 24 h. The DNA (pEM6 plasmid; 10 ng) in TE buffer was added to the cell, mixed gently, and placed on ice for 30 min. The cells were then heat-shocked at 42°C. One milliliter of LB medium was added to the cells, which were then allowed to incubate at 37°C for 1 h without shaking. The cells then were spread onto an LB agar plate containing 1% ampicillin and incubated at 37°C for 14 h during which time the colonies appeared [28].

3. Enzyme Assays

The enzyme assays consisted of: 0.15 mM NADH, 0.10 mM OAA, 2 mM EDTA, and 50 mM HEPES buffer at pH 7.0. Routine activity assays were performed at room temperature while final assays, kinetic studies and chemical modification studies were performed at 25°C. The decrease in absorbance of NADH at 340 nm was measured.

4. Protein Concentration Determination.

Protein concentrations were determined by the method of Bradford [29], using bovine serum albumin as a standard. The protein concentration was also measured by absorbance at 205 nm in 5 mM K₂PO₄, 50 mM Na₂SO₄ buffer at pH 7.0. The extinction coefficient was determined by the formula:

$$E_{205}^{1 \text{ mg/ml}} = 27.0 + 120 * (A_{280}/A_{205}) \quad [30]$$

5. Enzyme Purification.

Malate dehydrogenase was isolated and purified from *E. coli*, strain HB101, containing the pEM6 plasmid which contained the MDH gene. HB101 cells containing the plasmid were plated out on a LB agar plate (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 15 g/L of agar) and incubated for 12 h at 37°C. Selected colonies were then replated onto LB agar plates that contained 1% ampicillin and were incubated at 37°C. Colonies from this plate were used to inoculate 4 test

tubes of 5 ml of LB medium containing 1% ampicillin which were allowed to grow with shaking at 37°C. The contents of the test tubes were added to four one liter Fernbach flasks (1 test tube per flask) of LB medium. The flasks were incubated at 37°C for 14 h with shaking at low speed on a orbital shaker located in a shaker room. The suspension was centrifuged in a GSA rotor at 12,000 RPM at 4°C. The cell paste was collected, weighed, and then stored at -20°C.

Approximately 0.1 g of the HB101/pEM6 cell paste was thawed and homogenized on ice with a magnetic stir bar in 10 mL of 50 mM KPi, pH 7.0. The homogenate was sonicated on ice at 55% of maximum power for 30 sec. The protein solution was allowed to cool for 1 min. This was repeated 10 times, the total sonication time was 3 min. The resultant light grey protein slurry was then centrifuged at maximum speed in a SS-34 rotor for 30 min at 4°C. The supernatant, which contains the MDH, was decanted and the pellet was discarded. The supernatant was then assayed for activity and protein.

Affi-Gel Blue Gel (15 mL) was washed with 500 mL of deionized water and was then equilibrated with 10 mM KPi buffer pH 7.4. The supernatant was mixed with the equilibrated blue agarose, and was allowed to stand for 30 min with periodic stirring. This allowed the MDH to bind to the blue agarose. A column (30 mm x 120 mm; 50 mL) was built with the MDH-bound blue agarose. The column was washed with pH 7.5 KPi buffer to remove any unbound protein until the absorbance at 280 nm was less than 0.10. Approximately 15% of the total units were lost in the wash. The MDH was eluted from the column by using a 0 to 2 M NaCl gradient in

the pH 7.4 KPi buffer (total gradient volume was 100 mL). Fractions (3 mL total volume) were collected and assayed for MDH activity. Those fractions that exhibited activity were combined and assayed for activity and total protein.

The protein concentration at this point was very low (0.4 mg/mL), therefore the eMDH was concentrated by using Centricon microconcentrators. The enzyme was loaded into the Centricons and was centrifuged at 3,500 RPM for 10 min at 4°C. The concentration process was continued until the protein concentration was greater than 1 mg/mL. The eMDH was sterile filtered using a 0.2 μ m pore size disposable sterile filter from Sigma Chemical Company. The final assay for activity and protein concentration was performed on a Shimadzu UV-3100 at 25°C. The protein concentration was determined by using the Bradford method and by absorbance at 205 nm. The purity of the enzyme was checked by running a SDS polyacrylamide gel.

6. Kinetic Isotope Studies.

a. Synthesis and studies with malate-2-d

Malate-2-d was synthesized by Dr. N.C. Furumo according to the article published by Viola, Cook, and Cleland [31]. Ethanol-d₆ (7.4 mM), NAD⁺ (0.20 mM), OAA (10 mM), 50 units of liver alcohol dehydrogenase, 250 units of aldehyde dehydrogenase, and 50 units of MDH were combined in 400 mL of PIPES at pH 7.0 at 25°C. The amount of malate-2-d formed was monitored by adding 0.2 mL of 60% perchloric acid and 1.8 mL of deionized water to 1 mL of the reaction mixture which quenched the reaction. The pH was adjusted to 8.0 with 1 M KOH and the volume

was brought to 10 mL.

The malic enzyme assay for malate consisted of 3 mL of 100 mM TAPS at pH 9.0, 2 units of chicken liver malic enzyme, 1mM NAD⁺, 2 mM MgSO₄, 1 mg/mL BSA, and 100 μ M DTT.

After the reaction was complete, the proteins and the PIPES were precipitated by adjusting the pH of the reaction mixture to 1 with perchloric acid. The mixture was then filtered and applied to a AG-1-X8, 200-400 mesh anion exchange column in the formate form. Malate-2-d was eluted using a 500 mL linear gradient from 0 to 4 M of formic acid .

The kinetic isotope effect was determined by assaying eMDH activity in the presence of either malate-2-d or malate. The assays contained 50 mM Taps buffer at pH 9.0, 100 mM KCl, 2 mM EDTA, 7.0 mM NAD⁺, 6.2 nM active sites, and malate-2-d or malate varying in concentration from 0.2 to 15 mM. The reaction was monitored by measuring the change in absorbance of NADH at 340 nm.

b. Synthesis and studies with NADD.

A-side NADD was prepared according to the published method by Viola, Cook and Cleland [31]. Ethanol-d₆ (2.8 mM), 5.6 mM NAD⁺, 50 units horse liver alcohol dehydrogenase, and 100 units of yeast aldehyde dehydrogenase were mixed in 10 mL of 10 mM Taps buffer, pH 9.0 at 27°C. The reaction was monitored by uv absorbance at 340 nm. The mixture was vortexed with several drops of CCl₄ to precipitate the proteins, The aqueous phase, which contained the NADD, was

removed.

The kinetic isotope effect was determined by assaying eMDH activity in the presence of either NADH or NADD. The assays contained 50 mM Kpi buffer at pH 7.2, 100 mM KCl, 2 mM EDTA, 0.15 mM NADH or NADD, 1.9 mM MDH, and OAA varying in concentration from 0.02 to 0.15 mM. The reaction was monitored by measuring the change in absorbance of NADH or NADD at 340 nm.

7. pH Studies

The buffers used for the pH studies are listed in Table 1:

Table 1. Buffers and respective pH	
Buffer	pH
MES	5.5, 6.0, 6.5
PIPES	7.0
HEPES	7.5, 8.0
TAPS	8.5, 9.0
CHES	9.5, 10.0, 10.5

The assays contained 60 μ M active sites. A 1000 dilution of 60 mM stock eMDH in 100 mM KPI/100 mM KCl was needed to obtain measurable rates. The reaction mixtures were incubated for 3 min at 25°C. The reaction was initiated by the addition of 10 μ L of diluted eMDH (62 nM active sites). Immediately after the addition of eMDH, the reaction mixtures were mixed well with a plunger and the initial rate (normally the first 5-20 sec) was recorded. The reaction mixtures consisted of 50

mM buffer, 100 mM KCl, 2.0 mM EDTA, and 6.2 nM active sites. The NAD^+ was held constant at 7.0 mM, while the malate concentration was varied from 1 to 15 mM. The rate of the reaction catalyzed by MDH was measured by monitoring the change in absorption of NADH at 340 nm at pH values listed in Table 1.

8. Substrate Inhibition

The substrate inhibition assays were composed of 50 mM PIPES, 2 mM EDTA, 100 mM KCl at pH 7.0 for OAA. The NADH concentration was held constant at 0.15 mM, while the OAA concentration was varied between 0.015 and 20 mM.

The malate substrate inhibition study was also performed in TAPS (50 mM), 2 mM EDTA, 100 mM KCl, pH 9.0. NAD^+ was fixed at 0.15 mM and malate was varied from 0.015 mM to 20 mM.

The cofactor substrate inhibition studies were carried out in either 50 mM PIPES, 2 mM EDTA, 100 mM KCl at pH 7.0 for NADH or 50 mM TAPS, 2 mM EDTA, 100 mM KCl, pH 9.0 for NAD^+ . For the cofactor substrate inhibition experiments, OAA or malate was fixed at 0.10 mM and NADH or NAD^+ was varied from 0.015 to 2 mM.

The reaction mixtures were incubated for 3 min at 25°C. The reaction began with the addition of 10 μL of diluted MDH (62 nM active sites). Immediately after the addition of the MDH, the reaction mixtures were mixed well with a plunger and the initial rate was recorded.

9. Inhibition Studies with Substrate Analogs

Four series of 5 assays were performed in which the citrate concentration was fixed from 0 to 4 mM (0, 1, 2, 4 mM). In each series, the enzymatic assays contained 50 mM TAPS buffer, 100 mM KCl, 7 mM NAD^+ and 2.0 mM EDTA at pH 7.0. Malate was varied from 0.1 to 2.0 mM, and there was 6.2 nM active sites MDH. The reaction mixtures were incubated for 3 min at 25°C. Immediately after the addition of the MDH, the reaction mixtures were mixed well with a plunger and the initial rate was recorded at 25°C. The assays were monitored by measuring the change in absorbance of NADH at 340 nm. This experiment was repeated using malonate as the inhibitor instead of citrate.

In order to determine if ketomalonate or malonate inhibited OAA, four series of 5 assays were performed in which the ketomalonate or malonate concentration was varied from 0 to 4 mM (0, 1, 2, 4 mM). In each series, the enzymatic assays contained 50 mM PIPES buffer, 100 mM KCl, and 2.0 mM EDTA at pH 7.2. NADH was fixed at 0.15 mM, OAA was varied from 0.1 to 2.0 mM, and there was 6.2 nM active sites MDH. The reaction mixtures were incubated for 3 min at 25°C. The reaction began with the addition of 10 μL of diluted eMDH.

A third inhibitor study was performed with ATP (0, 1, 3, and 5 mM) being the inhibitor. The MAL was fixed at 1.0 mM and NAD^+ was varied from 0.1 to 1.0 mM. All other aspects were the same as the above experiments.

10. Allosteric effects.

The enzyme assays contained 6.2 nM active sites. The reaction mixtures were composed of 50 mM TAPS buffer, 2.0 mM EDTA, and 100 mM KCl, pH 9.0. The NAD^+ and NADH concentrations were held constant at 0.7 mM, while the MAL concentration was varied around 0.9 mM (0.1 - 20 mM). The reaction mixtures were incubated for 3 min at 25°C. The reaction began with the addition of 10 μL of diluted MDH (62 nM active sites). Immediately after the addition of the MDH, the reaction mixtures were mixed well with a plunger and the initial rate was recorded.

11. Chemical Modification.

a. Treatment with diethylpyrocarbonate

Diethylpyrocarbonate (97%) was diluted to 100 mM in anhydrous acetonitrile (HPLC grade). The concentration of the stock solution was determined quantitatively by absorption at 230 nm. A 10 μL aliquot of the 100 mM stock DEPC solution was added 990 μL of 10 mM imidazole at pH 7.5 and 25 °C. The increase in absorbance at 230 nm, which was due to N-carbethoxyimidazole ($\epsilon = 3.0 \text{ mM}^{-1} \text{ cm}^{-1}$) formation was recorded.

The stock eMDH solution used in the chemical modification studies had a specific activity of approximately 665 $\mu\text{g}/\text{mg}$. The enzyme concentration was 72 μM . The modification was carried out in 50 mM KPi, 100 mM KCl, at pH 6.5. Dicarbethoxyhistidyl derivatives, which cannot be reversibly modified, are formed in the presence of excess DEPC. A concentration of 0.75 mM DEPC yielded the best

modification and yet was still reversible. The degree of modification was determined by a decrease in MDH activity and by UV spectroscopy

To quantify the number of modified histidine residues, a control was prepared by combining 50 μL of MDH (71 μM), 7.5 μL of anhydrous acetonitrile, and 943.5 μL of buffer (100 mM KPi, 100 mM KCl, pH 6.5) in a 1 mL quartz cuvette with a 1 cm path length. This was placed in the reference beam of the spectrophotometer. The modification reaction was composed of 50 μL of MDH (71 μM), 7.5 μL of DEPC (100 mM), 943.5 μL of buffer (100 mM KPi, 100 mM KCl, pH 6.5). The reaction mix was placed in a microfuge tube and incubated at 25°C for the duration of the experiment. At five minute intervals (including $t = 0$), 10 μL of the reaction mixture was removed and diluted 1/100 in an assay mix. A spectrum (320 -220 nm) was recorded every five minutes for 25 minutes and was used to quantify the number of histidine residues modified.

In order to determine the loss of eMDH activity as a function of time, the following experiment was performed. Both the control mixture and the reaction mixture were prepared as above. The control sample again was placed in the reference beam of the spectrophotometer in the constant temperature cell holder. The deactivation was performed at 25°C. At five minute intervals (including $t = 0$), 10 μL were removed and diluted 1/100, from this dilution, 10 μL were removed (total dilution was 1/10,000 including the 1/100 dilution into the assay mix) and then was assayed. The control was also assayed every five minutes.

The reactivation of MDH was done by again preparing the control and the reaction mixtures as described above. Both were incubated at 25°C for 25 minutes. Aliquots (310 μ L) of both the sample and the control were removed and mixed with 690 μ L of 1.45 M NH_2OH , pH 7.0, to give a final concentration of hydroxylamine of 1 M. An aliquot (10 μ L) of both the control and the sample were removed and diluted 1/100 in the assay mix and assayed at 340 nm every 15 min for 1 h.

In order to determine the pH dependence of the rate of inactivation, the pH was varied from 6.0 to 8.0 in KPi buffer. A control was prepared by combining 20 μ L of eMDH (19 μ M), 10 μ L of anhydrous acetonitrile, and 970 μ L of buffer (100 mM KPi, 100 mM KCl, pH 6.0 - 8.0) in a 1 mL quartz cuvette with a 1 cm path length. A 10 μ L aliquot was removed and assayed for activity. The activity assay conditions were as follows: 50 mM KPi at the various pH values specified above, 2 mM EDTA, 100 mM KCl, 0.10 mM OAA, and 0.15 mM NADH. The modification reaction was composed of 20 μ L of eMDH (19 μ M), 10 μ L of DEPC (100 mM), 970 μ L of buffer (100 mM KPi at the various pH values, 2 mM EDTA, and 100 mM KCl).

b. Treatment with 1,2-cyclohexanedione

A control for the 1,2-cyclohexanedione modification was prepared by combining 20 μ L of MDH in 980 μ L of 50 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ at pH 8.5. The control was incubated at 25°C. An 10 μ L aliquot was removed at 5 min intervals and assayed for activity at 340 nm for 1 hr. The assays were performed in 50 mM KPi, pH 7.2, 2 mM EDTA, 100 mM KCl, 0.10 mM OAA, 0.15 mM NADH, and 25°C.

1,2-cyclohexanedione (16 mM) was added to 8 μ M MDH in 50 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ at pH 8.5. The mixture was incubated at 25°C. A 10 μ L aliquot was removed at 5 min intervals and assayed for activity at 340 nm for 1 hr [32].

c. Treatment with iodoacetic acid

MDH was chemically modified with 10 mM iodoacetate at pH 5.5. A control was prepared by incubating 8 μ M MDH in 50 mM MES buffer, pH 5.5. An 10 μ L aliquot of the control mix was removed every 5 min and assayed for activity for 1 hr at 340 nm. The assay conditions were: 0.10 mM OAA, 0.15 mM NADH, 50 mM KPi at pH 7.2, 2 mM EDTA, 100 mM KCl and 25°C.

The chemical modification mix contained 8 μ M MDH, 10 mM iodoacetate, 50 mM MES buffer, pH 5.5. An aliquot was removed and assayed for activity for every 5 min for 1 hr.

12. Data Analysis

The kinetic data were analyzed by using the BASIC version of Cleland's FORTRAN programs [14]. Substrate inhibition data were fit to equation 3.

$$Y = V \cdot A / (K + A + A^2 / K_i) \quad (3)$$

The pH profile kinetic parameters were determined at each pH by varying the concentration of malate, and fixing NAD^+ . The initial rate data was fit to equation 4. The $V_{\max}$ and V/K values determined at each pH were fit to equation 5 and 6

respectively.

$$v = VA/(K+A) \quad (4)$$

$$\log Y = \log(C/1 + ([H^+]/K_a)) \quad (5)$$

$$\log Y = \log(C/(1 + K_b/[H]))) \quad (6)$$

The isotope effect data were fit to equation 7 or 8, where I is the inhibitor.

$$V/K = V^*A/(K(1 + I * VKI) + A) \quad (7)$$

$$V_{\max} = V^*A/(K + A(1 + I * VI)) \quad (8)$$

Chapter III

RESULTS

1. Purification of Malate Dehydrogenase

The protein concentration was determined by both the Bradford dye method and by absorbance at 205 nm [30]. The results of the purification process are shown in Table 2.

Table 2. The results of the purification of eMDH.			
Total protein (mg)	Total volume (mL)	Total Units (u)	Specific Activity (u/mg)
4.2 ^a	8.0	23,721	706
4.6 ^a 5.2 ^b	4.5	13,746	665

^a Protein concentration determined by method of Bradford [29].

^b Protein concentration determined by spectrophotometric method [30].

2. Kinetic Isotope Effect

Kinetic isotope effect studies were performed in order to determine the rate limiting step of the oxidation of malate to OAA. Initial velocities were obtained with substrates protio-malate or malate-2d which were varied from 0.2 to 15 mM, while

NAD⁺ was held constant at 7 mM. A double-reciprocal plot of 1/V versus 1/[protio-malate or malate-2d] is presented in Figure 1. A KIE of 1.43 (± 0.14) was obtained on V/K by using the BASIC version of the FORTRAN programs of Cleland [14]. No isotope effect was observed on V.

Table 3a. Kinetic isotope effect data.			
[Malate] (mM)	Rate (Δ OD/min)	[Malate-2-d] (mM)	Rate (Δ OD/min)
0.20	0.219	0.20	0.176
0.50	0.354	0.50	0.289
1.0	0.432	1.0	0.405
2.0	0.453	2.0	0.446

A kinetic isotope effect study was also performed using NADD. Initial velocities were obtained using NADH or NADD which were varied from 0.01 to 0.15 mM while the OAA concentration was held constant at 0.10 mM. For V, a double-reciprocal plot of 1/V versus 1/[NADH or NADD] is presented in Figure 2 and for V/K, a double reciprocal plot of 1/V versus 1/[NADH or NADD] is presented in Figure 3. A KIE of 1.22 (± 0.19) was obtained on V and a KIE of 1.08 (± 0.06) was obtained on V/K.

Fig. 1. The primary deuterium isotope effect on V/K was determined by plotting the inverse rate data as a function of the inverse substrate concentration. The substrate was either protio-malate (●), or malate-2-d (○).

Fig. 2. The primary deuterium isotope effect on V was determined by plotting the inverse rate data as a function of the inverse substrate concentration. The substrate was either NADD (●), or NADH (○).

Fig. 3. The primary deuterium isotope effect on V/K was determined by plotting the inverse rate data as a function of the inverse substrate concentration. The substrate was either NADD (●), or NADH(○).

Table 3b. Kinetic isotope effect data.			
[NADH] (mM)	Rate (Δ OD/min)	[NADD] (mM)	Rate (Δ OD/min)
0.01	0.277	0.01	0.237
0.03	0.646	0.03	0.831
0.05	0.934	0.05	1.120
0.10	1.712	0.10	1.507
0.15	2.053	0.15	1.467

3. pH Studies

In order to determine the identity of the active site residue whose ionization is necessary for catalysis, pH variation studies were performed. The initial velocity was determined as a function of malate and NAD^+ concentrations at various pH values. The kinetic parameters K_m and $V_{\max}$ and standard errors in K_m were obtained from the data and are presented in Table 4.

Table 4. The kinetic parameters obtained from initial velocity studies at various pH values. Error in parenthesis.		
pH	K_m (malate)	K_{cat}
10.0	2.81 ($\pm 6.4\%$)	1.37 ($\pm 2.0\%$)
9.5	1.97 ($\pm 18.5\%$)	1.48 ($\pm 5.2\%$)
9.0	0.877 ($\pm 16.0\%$)	1.11 ($\pm 3.0\%$)
8.5	0.488 ($\pm 12.2\%$)	0.712 ($\pm 1.7\%$)
8.0	0.418 ($\pm 11.2\%$)	0.461 ($\pm 3.8\%$)
7.5	0.407 ($\pm 20.6\%$)	0.114 ($\pm 6.9\%$)
7.0	0.708 ($\pm 11.9\%$)	0.0462 ($\pm 4.5\%$)
6.5	0.718 ($\pm 30.3\%$)	0.0232 ($\pm .2\%$)
6.0	1.25 ($\pm 23.4\%$)	0.0234 ($\pm 10.7\%$)
5.5	2.27 ($\pm 34.9\%$)	0.0207 ($\pm 18.1\%$)

The data were fit to a pH-profile kinetics program [14], from which a pKa value of 7.80 (± 0.08) and 7.91 (± 0.12) for V/K and V_{max} respectively. The V/K_{mal} pH rate profile is presented in Figure 4 and the V_{max} profile is presented in Figure 5. The break observed corresponds to a pK value of approximately 8.0. The data for the rate versus [malate] at various pH values is located in Table 5 and the pH profile data is located in Table 6.

Fig. 4. The data were plotted as a function of pH to determine the effect of pH on $\log(V/K_{\text{mal}})$. The line represents the best fit of the data to equation 5, from which a pKa of 7.8 was determined.

Fig 5. Plot of $\log V_{\text{max}}$ as a function of pH. Data were fit to equation 6 from which a pK_a of $7.91 (\pm 0.12)$ was determined.

Table 5. Rate versus [malate] at various pH values.

pH	[malate] (mM)	Rate (Δ OD/min)
5.5	0.10	0.0009
	0.25	0.003
	1.0	0.006
	3.0	0.012
6.0	0.25	0.004
	0.50	0.007
	1.0	0.010
	3.0	0.017
6.5	0.25	0.008
	0.50	0.009
	1.0	0.012
	3.0	0.019
7.0	0.25	0.013
	0.50	0.019
	1.0	0.026
	3.0	0.038
7.5	0.10	0.029
	0.25	0.044
	0.50	0.066
	1.0	0.076
8.0	3.0	0.104
	0.10	0.092
	0.25	0.176
	0.50	0.257
	1.0	0.308
	3.0	0.413

Table 5. Continued.

pH	[malate] (mM)	Rate (Δ OD/min)
8.5	1.0	0.485
	3.0	0.609
	5.0	0.629
	10.0	0.695
	15.0	0.693
9.0	1.0	0.585
	3.0	0.845
	5.0	1.01
	10.0	1.01
	15.0	1.03
9.5	1.0	0.452
	3.0	0.904
	5.0	1.11
	10.0	1.28
	15.0	1.24
10.0	1.0	0.366
	3.0	0.698
	5.0	0.893
	10.0	1.05
	15.0	0.17

Table 6. pH profile data.	
pH	log (V/K)
10.0	-0.3312
9.5	-0.1242
9.0	0.1023
8.5	0.1640
8.0	0.0425
7.5	-0.5527
7.0	-1.185
6.5	-1.491
6.0	-1.728
5.5	-2.040

4. Substrate Inhibition:

At high concentrations, both NADH and OAA display substrate inhibition. An apparent K_i value of -4.4×10^{-4} for NADH and 8.1×10^{-1} for OAA were obtained. Since the substrate inhibition patterns for NADH and OAA do not intersect the vertical axis, the inhibition by both of these substrates is complete at saturating levels (Figures 6 and 7). The products of the forward reaction, malate and NAD^+ , displayed no substrate inhibition.

Fig. 6. Substrate inhibition by NADH was determined by plotting the inverse of the rate data as a function of the inverse of NADH concentration. This plot shows that substrate inhibition occurs at high concentrations of OAA (above 10 mM).

Fig. 7. Substrate inhibition by OAA was determined by plotting the inverse of the rate data as a function of the inverse of OAA concentration. This plot shows that substrate inhibition occurs at high concentrations of NADH (above 10 mM).

Table 7. NADH substrate inhibition data.	
[NADH] (mM)	Rate (Δ OD/min)
0.015	0.043
0.05	0.068
0.10	0.101
0.25	0.203
0.50	0.302
0.70	0.161
0.80	0.136
0.90	0.034
1.0	0.022
1.5	0.012
2.0	0.006

Table 8. OAA substrate inhibition data.	
[OAA] (mM)	Rate (Δ OD/min)
0.015	0.109
0.05	0.518
0.10	2.07
0.25	3.00
0.50	3.22
0.60	2.60
0.70	2.69
0.80	2.76
0.90	2.64
1.00	2.36
1.50	2.27
2.00	1.62
5.00	0.546
10.0	0.627
15.0	0.714
20.0	0.544

5. Inhibition studies with Substrate Analogs

None of the substrate analogs (ATP, citrate, ketomalonate, or malonate) were bound tightly by eMDH as indicated by the relatively high K_i 's. The substrate analog inhibition data is presented in Table 9, and the calculated inhibition constants are located in Table 10.

Table 9. Substrate analog inhibition by various concentrations of substrate analogs.		
[malate] (mM)	[citrate] (mM)	Rate (Δ OD/min)
0.10	0	0.1626
0.50	0	0.4630
0.75	0	0.6229
0.90	0	0.6425
1.30	0	0.7952
1.70	0	0.8136
2.00	0	0.9098
0.10	0.1	0.1176
0.50	0.1	0.4423
0.90	0.1	0.5921
1.5	0.1	0.6998
2.00	0.1	0.7311
0.10	0.5	0.1535
0.50	0.5	0.4721
1.50	0.5	0.6534
2.00	0.5	0.6765
0.10	1.0	0.0614
0.50	1.0	0.2230
0.90	1.0	0.2773
1.50	1.0	0.3522
2.00	1.0	0.3707

Table 9. Continued.		
[malate] (mM)	[malonate] (mM)	Rate (Δ OD/min)
0.10	0	0.1626
0.50	0	0.4630
0.75	0	0.6229
0.90	0	0.6425
1.30	0	0.7952
1.70	0	0.8136
2.00	0	0.9098
0.10	0.10	0.1482
0.50	0.10	0.4699
0.90	0.10	0.6616
1.50	0.10	0.6612
2.00	0.10	0.7401
0.10	3.0	0.1322
0.50	3.0	0.3914
0.90	3.0	0.5502
1.50	3.0	0.6452
2.00	3.0	0.6695
0.10	7.5	0.0443
0.50	7.5	0.1571
0.90	7.5	0.1566
1.50	7.5	0.2622
2.00	7.5	0.2640

Table 9. Continued.		
[OAA] (mM)	malonate (mM)	Rate (Δ OD/min)
0.010	0	0.0686
0.033	0	0.1671
0.055	0	0.2564
0.078	0	0.2681
0.10	0	0.2475
0.010	1.0	0.050
0.055	1.0	0.1869
0.078	1.0	0.1864
0.10	1.0	0.2057
0.010	8.0	0.0800
0.055	8.0	0.1644
0.078	8.0	0.1782
0.10	8.0	0.1670
0.010	15.0	0.0489
0.033	15.0	0.0790
0.055	15.0	0.0900
0.078	15.0	0.1043
0.10	15.0	0.1282

Table 9. Continued.		
[OAA] (mM)	[ketomalonate] (mM)	Rate (Δ OD/min)
0.010	0	0.0686
0.033	0	0.1671
0.055	0	0.2564
0.078	0	0.2681
0.10	0	0.2475
0.010	2.0	0.1227
0.033	2.0	0.1852
0.055	2.0	0.2025
0.078	2.0	0.2058
0.10	2.0	0.2207
0.010	5.0	0.0926
0.033	5.0	0.1495
0.055	5.0	0.1601
0.078	5.0	0.1681
0.10	5.0	0.1655
0.010	8.0	0.0747
0.033	8.0	0.1213
0.055	8.0	0.1396
0.078	8.0	0.1469
0.10	8.0	0.1705

Table 9. Continued.		
[NAD ⁺] (mM)	[ATP] (mM)	Rate (Δ OD/min)
0.10	0	0.1911
0.30	0	0.4943
0.50	0	0.7083
0.70	0	0.8667
1.10	0	1.0150
0.10	1.0	0.1628
0.30	1.0	0.3868
0.50	1.0	0.5649
0.70	1.0	0.6845
0.90	1.0	0.7579
1.10	1.0	0.8671
0.10	3.0	0.1243
0.30	3.0	0.2923
0.50	3.0	0.4463
0.70	3.0	0.5157
0.90	3.0	0.6134
1.10	3.0	0.6602
0.10	5.0	0.0894
0.30	5.0	0.2199
0.50	5.0	0.3285
0.70	5.0	0.4305
1.10	5.0	0.4961

Table 10. The kinetic parameters obtained from the substrate analog inhibition studies.			
Substrate/Inhibitor	K_{IS} (competitive)	K_{II} (uncompetitive)	K_{II} (noncompetitive)
malate/citrate	3.2×10^{-1}	5.89×10^{-1}	N/A
malate/malonate	1.67	3.72	N/A
OAA/malonate	4.37	9.82	$1.13 \times 10^{+1}$
OAA/ketomalonate	3.22	8.85	7.34
NAD^{+}/ATP	2.20	1.97	6.92

6. Allosteric Effects:

To determine if MDH exhibited allosteric behavior, an experiment was performed, in which malate was varied between 0.1 and 13.0 mM, NAD^{+} was fixed at 0.10 mM, NADH was fixed at 0.065 mM, and 100 mM KCl. The allosteric effect plot is presented in Figure 8.

Table 11. Allosteric effect data. Conditions: 50mM Taps pH 9.0, 100 mM KCl, Malate varied, NAD⁺ fixed at 7 mM, and NADH fixed at 0.065 mM, and 25°C.

[malate] (mM)	Control Rate (ΔOD/min)	[NADH] (mM)	Rate (ΔOD/min)
0.10	0.032	0.065	0.002
0.50	0.071	0.065	0.004
1.0	0.123	0.065	0.0089
2.0	0.176	0.065	0.044
3.0	N/A	0.065	0.053
5.0	0.236	0.065	0.078
7.0	N/A	0.065	0.094
10.0	0.291	0.065	N/A
13.0	N/A	0.065	0.114

7. Chemical Modification:

For the chemical modification with DEPC, there were 14.85 μM histidines in the assay mixture. Table 12 shows the concentration of histidine residues modified with 0.75 mM DEPC over a 25 min time interval.

Fig. 8. An allosteric effect was determined by plotting the rate as function of malate concentration with or without NADH. Control (○), 0.065 mM NADH (●).

Table 12. Results of Chemical Modification with 0.75 mM DEPC.		
Time (min)	Absorbance = 340 nm	[Histidine] (μ M)
0	0.03	9
5	0.039	12
10	0.0455	14
15	0.0475	15
20	0.0485	15.2
25	0.050	15.6

From Table 12, we see that after 25 min all of the histidine residues have been modified. When treated with 0.75 mM DEPC, eMDH shows a loss of catalytic activity (Figure 9).

Table 13. Inactivation of eMDH with 0.75 mM DEPC		
Time (min)	Control Rate (Δ OD/min)	Modified Rate (Δ OD/min)
0	0.2788	0.2505
5	0.2781	0.1464
10	0.2759	0.1227
15	0.2737	0.1149
20	0.2689	0.1021
25	0.2691	0.0997

The loss of catalytic activity (Figure 10) corresponds to the concentration of modified histidine residues.

Fig. 9. The inactivation of eMDH with 0.75 mM DEPC. A loss of catalytic activity is observed in the presence of 0.75 mM DEPC. eMDH (7.4 μ M Active sites) was incubated with 0.75 mM DEPC at 25°C and assayed for activity every 5 min.

Fig. 10. Loss of eMDH activity is dependent on the concentration of modified histidine residues.

The reactivation study shows the reaction is reversible and the catalytic activity can be returned. Figure 11, shows 74% of the catalytic activity of MDH was returned by removal of the histidine modifications with 1 M hydroxylamine.

Table 14. Reactivation of DEPC modified eMDH with 1 M hydroxylamine.		
Time (min)	Control Rate (Δ OD/min)	Reactivation Rate (Δ OD/min)
0	0.2701	0.1033
15	0.2687	0.1141
30	0.2659	0.1867
45	0.2674	0.2067
60	0.2639	0.1991

Activity assays were also performed in order to determine the effects of 1,2-cyclohexanedione on the catalytic activity of eMDH. The effects of the treatment of MDH with 16 mM 1,2cyclohexanedione, which is an 81% decrease in catalytic activity, is presented in Figure 12.

Fig. 11. The reactivation of DEPC modified eMDH with 1 M hydroxylamine. DEPC chemically modified eMDH shows an increase in enzyme activity when treated with 1 M hydroxylamine. eMDH (7.4 μ M active sites) was incubated with 1 M hydroxylamine, pH 7.0 at 25°C and was assayed for activity every 15 min.

Fig. 12. The inactivation of eMDH with 16 mM 1,2-cyclohexanedione. A loss of catalytic activity is observed in the presence of 16 mM 1,2-cyclohexanedione. eMDH ($7.4 \mu\text{M}$ Active sites) was incubated with 16 mM 1,2-cyclohexanedione at 25°C and assayed for activity every 5 min.

Table 15. Inactivation of eMDH with 16 mM 1,2-cyclohexanedione.		
Time (min)	Control Rate (Δ OD/min)	Modified Rate (Δ OD/min)
0	0.3793	0.3743
5	0.3753	0.3560
10	0.3714	0.2588
15	0.3454	N/A
20	0.3324	0.1877
25	0.3219	0.1662
30	0.3604	0.1115
35	0.3266	0.0855
40	0.3497	0.0874
45	0.3491	0.0555
50	0.3760	0.0562
55	0.3130	0.0591

The catalytic activity was also determined in order to determine the effects of iodoacetic acid on MDH. The enzyme showed a 52% decrease in activity upon treatment with 10 mM iodoacetic acid (Figure 13). In addition, Figure 14 shows that the loss of enzyme activity by modification of iodoacetic acid can be prevented if the modification is carried out in the presence of 7 mM NAD⁺.

Fig. 13. The inactivation of eMDH with 10 mM iodoacetic acid. A loss of catalytic activity is observed in the presence of 10 mM iodoacetic acid. eMDH (7.4 μ M Active sites) was incubated with 10 mM iodoacetic acid at 25°C and assayed for activity every 5 min.

Fig. 14. Protection of eMDH against 10 mM iodoacetic acid with 7 mM NAD⁺. eMDH when treated with 10 mM iodoacetic acid, in the presence of 7 mM NAD⁺ at 25°C, shows virtually no loss of catalytic activity.

Table 16. Inactivation of eMDH with 10 mM Iodoacetic acid and protection with 7 mM NAD ⁺ .			
Time (min)	Control Rate (Δ OD/min)	Modified Rate (Δ OD/min)	Protected Rate (Δ OD/min)
0	0.4864	0.4115	0.4707
5	0.4169	0.2475	N/A
10	0.4134	0.2640	0.4024
15	0.4184	N/A	N/A
20	0.3960	0.2349	0.3850
25	0.4170	0.2178	0.3980

Chemical modification with DEPC was performed at various pH values to further identify the pK of the histidine residue involved in catalysis. The initial rates were plotted vs. time for pH 8.0-6.5. A pK of approximately 6.8 was determined from the plot of the rate of inactivation vs. pH (Figure 15).

Fig. 15. Rate of inactivation of eMDH plotted as a function of pH. From the plot, a pK of approximately 7.0 was determined.

Table 17. Inactivation of eMDH with 1.0 mM DEPC at various pH values.			
pH	Time (min)	Control Rate (averaged) (Δ OD/min)	Modified Rate (Δ OD/min)
6.0	0	N/A	N/A
	5	0.37	0.32
	10	N/A	0.30
	15	0.37	0.26
	20	N/A	0.24
	25	0.36	0.23
	35	N/A	N/A
	45	0.37	0.20
6.5	0	N/A	N/A
	5	0.37	0.34
	10	N/A	0.27
	15	0.37	0.20
	20	N/A	0.19
	25	0.36	0.20
	35	N/A	0.19
	45	0.37	0.17
7.0	0	N/A	N/A
	5	0.37	0.27
	10	N/A	0.23
	15	0.37	0.20
	20	N/A	0.17
	25	0.36	0.13
	35	N/A	0.13
	45	0.37	0.12

Table 17. Continued.			
pH	Time (min)	Control Rate (averaged) (Δ OD/min)	Modified Rate (Δ OD/min)
7.5	0	N/A	N/A
	5	0.37	0.27
	10	N/A	0.17
	15	0.37	0.11
	20	N/A	0.082
	25	0.36	0.064
	35	N/A	0.061
	45	0.037	0.060
8.0	0	N/A	0.16
	10	N/A	0.061
	15	0.037	0.045
	20	N/A	0.042
	25	0.36	0.022
	35	N/A	0.026
	45	0.37	0.018

Table 18. Rate of inactivation of eMDH in the presence of 1.0 mM DEPC at various pH values.		
pH	Control Slope	DEPC Modified Slope
8.0	6.95×10^{-4}	-2.02×10^{-2}
7.5	9.40×10^{-5}	-1.55×10^{-2}
7.0	-4.85×10^{-4}	-8.96×10^{-3}
6.5	-3.98×10^{-4}	-6.15×10^{-3}
6.0	1.89×10^{-3}	-3.89×10^{-3}

Chapter IV

Discussion

The results of this body of research in combination with the work of Fang Jenny Zhao [34] indicate that eMDH has a histidine residue that is essential for catalytic activity. This residue most probably functions as a general acid/base in the catalytic mechanism (Scheme III). The results of these studies are similar to those conducted by Bernstein and Everse, on mMDH from chicken heart, which indicated that an ionizing group with a pK of 7.5 is involved in the binding of malate and the substrate analogue hydroxymalate [33].

There are many reasons why rates may vary with pH: (1) the substrate or a catalytic group on the enzyme might have to be in a specific state of protonation for the reaction to occur, or (2) protonation of groups remote from the active site may cause a conformational change [13]. In the case of eMDH, the catalytic group(s) on the enzyme must be in a given state of protonation for the reaction to occur. The residue must be protonated for the forward reaction and unprotonated for the reverse reaction, which is characteristic for groups involved in acid/base catalysis.

1. Identification of an Active Site Histidine Residue

In order to determine the pK of the active site amino acid residue involved in catalysis, pH studies were performed. Only one ionizing group with a pK_a

Scheme III. A proposed role for the active site histidine.

between 7.8 and 8.7 was determined regardless of the direction of which the reaction was carried out. It is our belief that these two pK_a values represent the ionization of a histidine residue located in the active site of the eMDH. Further support comes from a pK of 8.7 (± 0.1), which was calculated from $\log V/K_{NADH}$ vs. pH profile [34] and from a pK_a of approximately 7 from a plot of rate of inactivation vs. pH (Fig 15).

A role proposed for this active site histidine is presented in Scheme III. The microenvironment within the active site perturbs the pK_a of histidine from the normal pK_a of 6.0 to an elevated value between 7.8 and 8.5. An explanation for the increase in pK can be found in the crystal structure [9, 10]. Asp150 forms an ion pair with the histidine residue thus elevating the pK_a of the catalytic essential His177 (Scheme III).

The results of the DEP chemical modification studies further substantiate that a histidine residue is located in the active site. The loss of enzyme activity can be directly correlated with the concentration of modified histidine residues, as presented in Fig. 10. Treatment of modified eMDH with 1 M hydroxylamine restored 74% of the enzyme's activity, again suggesting a histidine residue is involved in catalysis. Furthermore, the rates of inactivation by DEPC were decreased by a factor of 8 and 4 fold when NADH or OAA were present, indicating that the histidine residue is located in the active site [34].

To further support the conclusion that a histidine was involved in catalysis, eMDH was modified with iodoacetic acid which is another histidine-modifying reagent. When eMDH was treated with iodoacetic acid, a loss of activity was

observed.

There are many research reports which propose the participation of a histidine residue in malate dehydrogenases, [2, 25-27, and 35]. The crystal structure of eMDH has been determined in the presence of citrate, and NAD^+ , and it clearly shows His177 at the active site within hydrogen bond distance of the bound citrate and NAD^+ [9, 10].

The log V profile of malate indicates malate binding requires that the enzyme must be in the correct protonated form (Fig. 5). This is somewhat different from mMDH from chicken heart in which both malate and OAA bind to the unprotonated form of the enzyme [33].

2. Kinetic Isotope Effects

A critical process in enzyme catalysis is the abstraction of hydrogen from carbon, and primary hydrogen isotope effects have been useful in the investigation of enzyme catalyzed proton abstraction reactions. Hydrogen abstraction reactions usually have kinetic isotope effects that range from $k_{\text{H}}/k_{\text{D}} = 2-10$ [36]. Our primary deuterium isotope effect studies indicate that proton abstraction is partially rate determining at non-saturating substrate levels at pH 9.0. A primary isotope experiment was performed with NADD to determine if proton abstraction is partially rate limiting in the forward direction. Ordered mechanisms, such as that observed by eMDH where NADH (NAD^+) bind first, followed by OAA (MAL) exhibit no isotope

effect on V because the release of the second product (NADH) limits V . The kinetic isotope effect value of 1.44 for V/K is in good agreement with a value of 1.5 obtained for LDH using 2- ^2H -lactate[37]. This enzyme shows a small isotope effect on the chemical conversion step because the cofactor is bound very tightly to the enzyme. Many dehydrogenases are characterized by small isotope effects on V/K and no isotope effect on V . Northrop has suggested that there should be no KIE on V when the release of the cofactor is the slowest step in catalysis [38]. Small isotope effects on V and V/K_{OAA} were observed when NADD was present at fixed concentrations (0.13 mM) and variable OAA at pH 7.2. This coincides with an ordered mechanism in which the cofactor binds first and release of cofactor is rate determining. [38, 39]. No KIE was observed on $V/K_{\text{NADD(H)}}$, since binding of labeled substrate is not likely to be an isotopically sensitive step. This is again consistent with an ordered mechanism.

3. Kinetic Mechanism

Malate dehydrogenases are proposed to follow an ordered sequential mechanism, in which the cofactor is the first to bind and the last to be released (Scheme II), and the release of the cofactor is rate determining [2, 4, 25, 27, and 35]. Work by James Rardin has shown that eMDH operates by a Bi Bi sequential ordered mechanism.

The results of the pH studies, chemical modification studies with both DEPC and iodoacetic acid, and the kinetic isotope effect studies indicate that eMDH is quite

similar to both cMDH and mMDH as well as LDH. The proposed mechanism for eMDH involves a single ionizing group with a pK_a in the range of 7.8 to 8.5, which is most likely a histidine (His177). This histidine acts as a general acid in the reduction of OAA and a general base in the oxidation of malate. The chemical conversion step is partially rate determining under non-saturating conditions, while under saturating conditions the release of the cofactor is most likely rate determining.

4. Substrate Inhibition

Several other kinetic studies and another chemical modification experiment were performed in order to further characterize the enzyme. It was found that OAA acts as a substrate inhibitor, most probably because: 1.) High concentrations of substrate alter the order of addition of the reactants or the reaction pathway. 2.) The substrate may bind at an allosteric site and result in either partial or total substrate inhibition [14].

From the $1/\text{Rate}$ vs. $1/[S]$ plot, it can be seen that NADH or OAA displays the characteristic upward curve at low $1/[S]$ values. However, the K_i is negative for NADH; a meaningless results. The difficulty of measuring the rate at moderate to high NADH concentrations stems from the extremely high absorbances at moderately high NADH concentrations. Thus, the data from these studies are suspect and requires more investigation.

5. Allosteric Effects

As mentioned above, the substrate may possibly bind at an allosteric site which results in substrate inhibition. Sandwal, determined that eMDH was allosterically regulated in the presence of low concentrations of NADH [7]. In this research, an allosteric effect has been observed in the reverse direction in the presence of a low concentration (0.065 mM) of NADH and 100 mM KCl (Fig. 6). This suggests that NADH is binding to a site other than the active site, thereby causing a conformational change in the enzyme. An allosteric effect in the forward direction has not been observed due to the fast initial velocities with OAA, which could possibly yield data that exhibits a sigmoidal curve, but in fact was not allosteric in nature [1, 7].

6. Inhibition Studies with Substrate Analogs

By using substrate analogs, we are able to probe the active site of eMDH. At this point, we are unsure of the mode of inhibition of many of the inhibitors used. From the inhibitor studies however, we find that MDH is very specific for its substrates due to the relatively high K_i values (Table 10).

7. Identification of Active Site Arginine Residues

Arginine residues have been implicated in the binding of OAA and malate in eMDH. To determine their importance, a chemical modification experiment was carried out using 1,2-cyclohexanedione, an arginine specific reagent. When eMDH is treated with 1,2- cyclohexanedione, a loss of enzymatic activity is observed. This indicates that arginine residues are important to the function of eMDH, but does not

give any information regarding the location of the residues.

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APPENDIX I
KINETIC FIT PARAMETERS

CONST.	VALUE	S.E.	Weight
K=	2.7364E-01	3.3170E-02	9.0887E+02
V=	5.3556E-01	1.6607E-02	3.6259E+03

V/K ISOTOPE EFFECT = 1.436 S.E. = 1.4398E-01

SIGMA = 1.500109E-02
 VARIANCE = 2.250328E-04

V/K ISOTOPE EFFECT FIT-MALATE

CONST.	VALUE	S.E.	Weight
K=	1.0620E-01	3.2778E-02	9.3077E+02
V=	3.4439E+00	5.8636E-01	2.9085E+00

V ISOTOPE EFFECT = 1.385 S.E. = 1.9587E-01

SIGMA = .1398673
 VARIANCE = 1.956286E-02

Vmax ISOTOPE EFFECT FIT-NADH

1

CONST.	VALUE	S.E.	Weight
K=	8.5195E-02	3.2769E-02	9.3125E+02
V=	2.9639E+00	5.4109E-01	3.4155E+00

V/K ISOTOPE EFFECT = 1.209 S.E. = 2.2530E-01

SIGMA = .1766875
 VARIANCE = 3.121847E-02

V/K ISOTOPE EFFECT FIT-NADH

CONST.	VALUE	S.E.
C	9.9161D-01	2.7906D-01
1/C	1.0085D+00	2.8380D-01
pKa	7.7978D+00	1.8788D-01

SIGMA = 5.5330D-01
VARIANCE = 3.0614D-01

The variable V/K increases as the pH increases

CONST.	VALUE	S.E.	Weight
V=	5.8700D+00	2.2911D+00	1.9051E-01
K=	2.2125D-01	1.5100D-01	4.3855E+01
K/V=	3.7692D-02	1.2714D-02	6.1861E+03
1/V=	1.7036D-01	6.6490D-02	2.2620E+02
KI=	8.0773D-01	5.4219D-01	3.4017E+00
1/KI=	1.2380E+00	8.3104D-01	1.4480E+00

SIGMA= 4.182503D-01
VARIANCE= 1.749333D-01

SUBSTRATE INHIBITION-OAA

CONST.	VALUE	S.E.	Weight
V=	2.4010D+05	2.0557D+08	2.3663E-17
K=	-3.1195D+02	1.3341D+05	5.6189E-11
K/V=	-1.2992D-03	5.5677D-01	3.2259E+00
1/V=	4.1649D-06	3.5659D-03	7.8644E+04
KI=	-4.3570D-04	1.8640D-01	2.8781E+01
1/KI=	-2.2952E+03	9.8192D+05	1.0372E-12

SIGMA= 1.080286D+02
VARIANCE= 1.167018D+04

SUBSTRATE INHIBITION-NADH

CONST.	VALUE	S.E.	Weight
V=	5.6065D-01	4.8372D-02	4.2738E+02
K=	2.7254D+00	3.5121D-01	8.1070E+00
K/V=	4.8612D+00	2.4496D-01	1.6665E+01
1/V=	1.7836D+00	1.5389D-01	4.2226E+01
KI=	6.5948D+01	2.7503D+01	1.3221E-03
1/KI=	1.5163E-02	6.3237D-03	2.5007E+04

SIGMA= 9.802532D-03
 VARIANCE= 9.608964D-05

SUBSTRATE INHIBITION-MALATE

CONST.	VALUE	S.E.	Weight
V=	2.6888D-02	5.3680D-03	3.4704E+04
K=	6.6815D-02	9.7891D-02	1.0436E+02
K/V=	2.4849D+00	3.2770D+00	9.3119E-02
1/V=	3.7192D+01	7.4251D+00	1.8138E-02
KI=	-2.0081D+00	8.7720D-02	1.2996E+02
1/KI=	-4.9798E-01	2.1753D-02	2.1133E+03

SIGMA= 1.365744D-02
 VARIANCE= 1.865256D-04

SUBSTRATE INHIBITION-NAD+

CONST.	VALUE	S.E.	Weight
K=	5.9928D-01	1.8414D-01	2.9492E+01
V=	1.1294D+00	1.1987D-01	6.9595E+01
KIS=	3.2070D-01	8.9496D-02	1.2485E+02
1/KIS=	3.1182D+00	2.7907D-01	1.2841E+01

SIGMA= 7.509734D-02
 VARIANCE= 5.639611D-03

COMPETITIVE INHIBITION-MALATE VS CITRATE

CONST.	VALUE	S.E.	Weight
K=	5.1035D-01	1.6798D-01	3.5437E+01
V=	1.0395D+00	1.1019D-01	8.2358E+01
KIS=	1.6690D+00	5.0928D-01	3.8555E+00
1/KIS=	5.9917D-01	3.0515D-01	1.0739E+01

SIGMA= 8.422872D-02
 VARIANCE= 7.094476D-03

COMPETITIVE INHIBITION-MALATE VS MALONATE

CONST.	VALUE	S.E.	Weight
K=	2.6373D-02	1.1306D-02	7.8226E+03
V=	3.1156D-01	4.1074D-02	5.9274E+02
KIS=	4.3789D+00	1.6647D+00	3.6085E-01
1/KIS=	2.2837D-01	3.8016D-01	6.9194E+00

SIGMA= 2.728030D-02
 VARIANCE= 7.442147D-04

COMPETITIVE INHIBITION-OAA VS MALONATE

CONST.	VALUE	S.E.	Weight
K=	1.3752D-02	5.6280D-03	3.1572E+04
V=	2.7387D-01	2.4449D-02	1.6729E+03
KIS=	3.2153D+00	1.4854D+00	4.5324E-01
1/KIS=	3.1101D-01	4.6197D-01	4.6856E+00

SIGMA= 2.697033D-02
 VARIANCE= 7.273987D-04

COMPETITIVE INHIBITION-OAA VS KETOMALONATE

CONST.	VALUE	S.E.	Weight
K=	5.9823D-01	6.1643D-02	2.6317E+02
V=	1.5487D+00	7.4449D-02	1.8042E+02
KIS=	2.1991D+00	1.7175D-01	3.3899E+01
1/KIS=	4.5474D-01	7.8102D-02	1.6394E+02

SIGMA= 2.422137D-02
 VARIANCE= 5.866747D-04

COMPETITIVE INHIBITION-NAD+ VS ATP

CONST.	VALUE	S.E.	Weight
K=	8.6851D-01	1.9956D-01	2.5110E+01
V=	1.3051D+00	1.3022D-01	5.8969E+01
KII=	5.8938D-01	1.0967D-01	8.3142E+01
1/KII=	1.6967D+00	1.8608D-01	0.0000E+00

SIGMA= 6.209870D-02
 VARIANCE= 3.856248D-03

UNCOMPETITIVE INHIBITION-MALATE VS CITRATE

CONST.	VALUE	S.E.	Weight
K=	6.7623D-01	2.0423D-01	2.3975E+01
V=	1.1424D+00	1.3129D-01	5.8014E+01
KII=	3.7244D+00	8.4283D-01	1.4077E+00
1/KII=	2.6850D-01	2.2630D-01	0.0000E+00

SIGMA= 8.288515D-02
 VARIANCE= 6.869947D-03

UNCOMPETITIVE INHIBITION-MALATE VS MALONATE

CONST.	VALUE	S.E.	Weight
K=	3.5128D-02	1.5771D-02	4.0207E+03
V=	3.4475D-01	5.7872D-02	2.9858E+02
KII=	9.8173D+00	3.1086D+00	1.0349E-01
1/KII=	1.0186D-01	3.1664D-01	0.0000E+00

SIGMA= 3.213704D-02
 VARIANCE= 1.032790D-03

UNCOMPETITIVE INHIBITION-OAA VS MALONATE

CONST.	VALUE	S.E.	Weight
K=	2.2209D-02	4.8405D-03	4.2680E+04
V=	3.2310D-01	2.3213D-02	1.8558E+03
KII=	8.8536D+00	1.5907D+00	3.9521E-01
1/KII=	1.1295D-01	1.7966D-01	0.0000E+00

SIGMA= 1.857592D-02
 VARIANCE= 3.450648D-04

UNCOMPETITIVE INHIBITION-OAA VS KETOMALONATE

CONST.	VALUE	S.E.	Weight
K=	1.1756D+00	2.2588D-01	1.9600E+01
V=	2.2039D+00	2.6686D-01	1.4042E+01
KII=	1.9709D+00	3.1003D-01	1.0404E+01
1/KII=	5.0738D-01	1.5731D-01	0.0000E+00

SIGMA= 4.184771D-02
 VARIANCE= 1.751231D-03

UNCOMPETITIVE INHIBITION-NAD+ VS ATP

CONST.	VALUE	S.E.	Weight
K=	3.2998D-02	1.3113D-02	5.8158E+03
V=	3.3767D-01	4.7337D-02	4.4627E+02
KIS=	4.6630D+01	1.3141D+02	5.7912E-05
1/KIS=	2.1446D-02	2.8181D+00	1.2592E-01
KII=	1.1286D+01	5.2122D+00	3.6809E-02

SIGMA= 2.468139D-02

VARIANCE= 6.091709D-04

NONCOMPETITIVE INHIBITION-OAA VS MALONATE

CONST.	VALUE	S.E.	Weight
K=	2.5553D-02	7.5555D-03	1.7518E+04
V=	3.3568D-01	3.2049D-02	9.7358E+02
KIS=	1.1917D+07	8.6024D+12	1.3513E-26
1/KIS=	8.3911D-08	7.2184D+05	1.9192E-12
KII=	7.3433D+00	2.1807D+00	2.1028E-01

SIGMA= 1.873733D-02

VARIANCE= 3.510873D-04

NONCOMPETITIVE INHIBITION-OAA VS KETOMALONATE

CONST.	VALUE	S.E.	Weight
K=	7.2066D-01	6.0979D-02	2.6893E+02
V=	1.6997D+00	7.3401D-02	1.8561E+02
KIS=	3.7329D+00	5.5681D-01	3.2254E+00
1/KIS=	2.6789D-01	1.4916D-01	4.4943E+01
KII=	6.9239D+00	1.6783D+00	3.5503E-01

SIGMA= 1.686188D-02

VARIANCE= 2.843229D-04

NONCOMPETITIVE INHIBITION-NAD+ VS ATP