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Investigating the Allosteric Behavior of Malate Dehydrogenase from Escherichia coli

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BY

Eman M. Ghanem

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ABSTRACT

Regulatory mechanisms of malate dehydrogenase from *E.coli* (eMDH) involving NADH as an allosteric effector were investigated. The reaction was studied in both directions: malate oxidation and oxaloacetate reduction. When malate was the variable substrate, a plot of rate against substrate concentration was sigmoidal in the presence of 0.065 mM NADH, which indicates the presence of an allosteric site for NADH on the enzyme. Binding of NADH at the allosteric site causes conformational changes in the active site and, thereby, changes the catalytic activity of the enzyme. An increase in K_m value, from 1.3 to 3.9 mM malate was observed, which indicates a decrease in the enzyme affinity for the substrate.

When eMDH was chemically modified with 5'-p-flurosulfonyl-benzoyladenosine (FSBA) in the presence of 0.15 mM NADH as a protecting agent, the allosteric behavior was abolished, which suggests that FSBA is modifying specific amino acid residues in the allosteric site and, therefore, preventing NADH from binding. eMDH was inactivated by FSBA in the absence of NADH. The inactivation appears to result from covalent modification of His 177 in the active site, which is believed to be crucial for the catalytic mechanism of eMDH.

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LIST OF ABBREVIATIONS

ADP.....Adenosine diphosphate

AMP.....Adenosine monophosphate

Asp.....Aspartate

ATP.....Adenosine triphosphate

CoA.....Coenzyme A

DSS.....2,2-dimethylsilapentane-5-sulfonic acid

EDTA.....Disodium ethyldiaminetetraacetate

eMDH..... Malate dehydrogenase from *E.coli*

FSBA..... 5′-p-flurosulfonylbenzoyladenosine

His.....Histidine

KPi.....Potassium phosphate

Mal..... Malate

MDH.....Malate dehydrogenase

NAD^+.....Nicotinamide adenine dinucleotide

NADH.....Nicotinamide adenine dinucleotide, reduced form

OAA.....Oxaloacetate

TAPS.....N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid

TCA..... Tricarboxylic acid

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Chapter I

INTRODUCTION

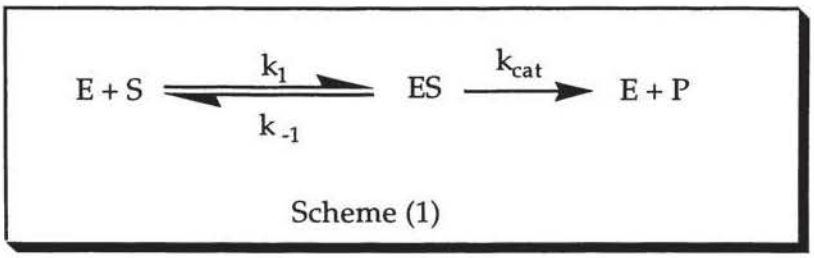
Enzymes are protein catalysts which catalyze the chemical reactions that take place in biological systems. Essentially, all biochemical reactions that are involved in metabolic pathways are enzyme-catalyzed. The rates of these reactions could be as much as 10^{12} times greater than the corresponding uncatalyzed reactions.

Enzymes can be distinguished from chemical catalysts by four characteristic features: (1) they have higher reaction rates, (2) they function under relatively mild conditions of temperature, pressure, and pH, (3) they are highly stereospecific with respect to both substrates and products, and (4) they can be regulated by a number of regulatory mechanisms.

Many enzymes catalyze reactions only in the presence of a specific non-protein organic molecule, the coenzyme or cofactor. Where the coenzyme is required, the complete system known as the holoenzyme, consists of the protein (the apoenzyme) plus the bound coenzyme. Most water soluble vitamins are coenzymes precursors. For example, the nicotinamide component of NAD^+ and NADH (coenzymes that are involved in redox reactions) is derived from niacin, one of the B complex vitamins.

In addition, enzymes display substrate saturation kinetics. In other words, at low substrate concentration, [S], the reaction shows a first-order dependence of the initial rates on [S]. However, instead of increasing indefinitely, at sufficiently high [S], the rate approaches a limiting value termed the maximum velocity, V_{max}, at which there is no dependency of the rate on [S] and, therefore, the reaction becomes zero-order with respect to [S].

The concept of enzyme kinetics was developed by Michaelis and Menten when they proposed the mechanism of an enzyme-catalyzed reaction [1 & 2]. Their mechanism supposes that the first step of the reaction is the binding of the substrate (S) to the enzyme (E) to form an enzyme-substrate intermediate (ES). The second step involves the conversion of the substrate to product (P), which is released, with the regeneration of the free enzyme. This mechanism is presented in Scheme (1):



Scheme (1)

where k_{cat} is the catalytic constant and is related to V_{max} and the total enzyme concentration, $[E]_0$, by equation (1):

$$k_{cat}\,[E]_0 = V_{max} \qquad (1)$$

The concentration of the total enzyme, $[E]_0$, is related to the concentration of the free enzyme, [E], by equation (2):

$$[E] = [E]_0 - [ES] \qquad (2)$$

By applying the steady state approximation to [ES]:

$$\frac{d\,[ES]}{d\,t} = k_1[E][S] - k_{cat}\,[ES] - k_{-1}\,[ES] = 0 \qquad (3)$$

Substituting $[E]_0 - [ES]$ for [E] in equation (3) and solving for [ES] gives equation (4):

$$[ES] = [E]_0\,[S] / \{[S] + (k_{cat} + k_{-1}) / k_1\} \qquad (4)$$

Since the initial rate, $v = k_{cat}\,[ES]$, it can be expressed by inserting the expression for [ES] from equation (4), which gives equation (5):

$$v = \frac{k_{cat}\,[E]_o\,[S]}{[S] + (k_{cat} + k_{-1})/k_1} \qquad (5)$$

where $(k_{cat}+k_{-1})/k_1 = K_m$ or the Michaelis constant, which is the substrate concentration that produces half the maximum velocity. It is also a measure of the affinity that an enzyme has for its substrate. The lower K_m, the greater the affinity between the enzyme and the substrate. Therefore, equation (5) can be rewritten as equation (6):

$$v = (V_{max}\,[S]) / (K_m + [S]) \qquad (6)$$

which is known as the Michaelis-Menten equation, which describes the saturation behavior that was previously discussed.

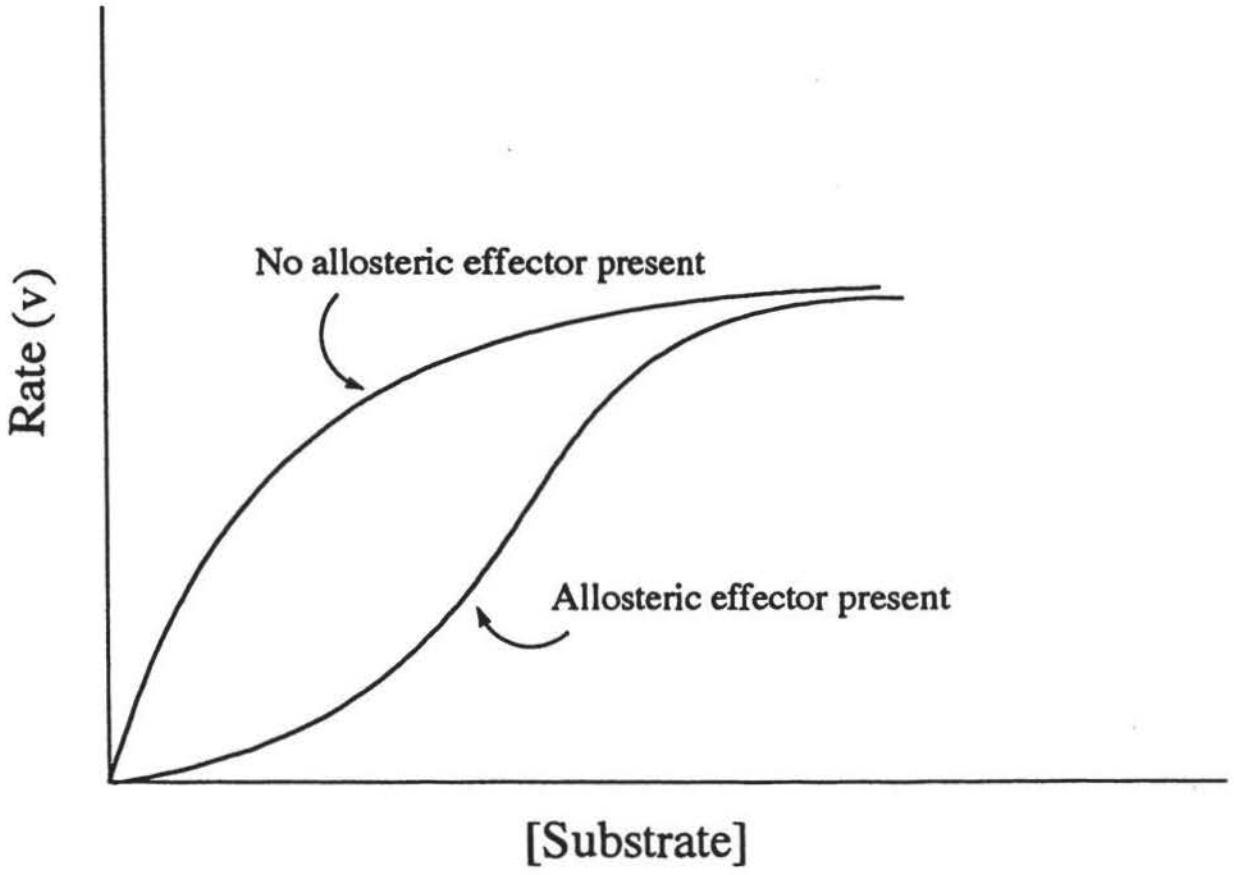
The regulation of enzyme activity sets them apart from non-enzyme catalysts. Enzyme activity can be regulated by altering the enzyme concentration, by the presence of inhibitors and/or activators, by substrate availability, and by chemical modification.

Allosterism is another regulatory mechanism that may control the catalytic activity of enzymes. Typically, an enzyme contains an active site where the substrate binds and initiates the chemical reaction. In addition to the active site, some enzymes contain another binding site, the allosteric site. Low molecular weight allosteric effectors (activator or inhibitor) control the catalytic activity of these enzymes. Binding of the allosteric molecule at the allosteric site causes conformational changes in the active site and therefore, changes the catalytic activity of the enzyme, making it higher or lower. In this case, the enzyme-catalyzed reaction does not follow Michaelis-Menten kinetics, which gives a hyperbolic plot of rate vs. [S]. Instead, a plot of rate vs. [S] is sigmoidal or has an "S" shape. Figure 1 shows the sigmoidal substrate saturation curve for an allosterically regulated enzyme in the presence of the allosteric effector.

The sigmoidal character of a plot of v against [S] in the presence of an allosteric inhibitor reflects the phenomenon of cooperativity. According to Figure 1, at low substrate concentration, the activity in the presence of the inhibitor is low relative to that in its absence. However, as [S] increases, the extent of inhibition becomes less severe. Therefore, allosterically regulated enzymes are more sensitive to the relative concentrations of their substrates. The kinetics are

Figure 1.

Allosteric Behavior: Kinetic Evidence



consistent with the presence of two or more interacting binding sites, where the presence of an effector molecule at the allosteric site facilitates or inhibits the binding of a substrate molecule at the active site.

Sigmoidal behavior can be described in several ways. The symmetry model of allosterism by Monod, Wyman, and Changeaux, which is termed MWC theory, is considered the most elegant model for describing cooperativity [2]. This model is defined by four rules:

- An allosteric enzyme is an oligomer.
- The protein can exist in two conformational states, T (tense) and R (relaxed). These states are in equilibrium whether or not a ligand (substrate) is bound to the protein.
- The T state has a lower affinity for the ligand and, therefore, is the less active form.
- The molecular symmetry of the protein is conserved during the conformational changes.

Allosteric control could be due to the change of the R/T ratio. An allosteric activator functions by binding to the R state and stabilizing it. An inhibitor functions by binding to the T state and causes the transition to the R state to be more difficult.

Allosterically regulated enzymes can be divided into two categories, K-series and V-series [3]. For the K-series allosteric enzymes, the allosteric inhibitor increases the K_m value and therefore, decreases the enzyme affinity for the

substrate without affecting V_{max}. For the V-series enzymes, the inhibitor lowers the catalytic efficiency of the enzyme by lowering V_{max} without affecting K_m.

As was mentioned earlier, Michaelis-Menten kinetics cannot be applied to the substrate saturation curve in the presence of the allosteric effector. Instead, the Hill equation:

$$log \frac{v}{V_{max} - v} = n\ log\ [S] - log K \qquad (7)$$

was derived to describe the allosteric behavior of proteins, where K is the dissociation constant of the ligand. The value of "n", the slope of the equation, is a measure of cooperativity. If n = 1, there is no cooperativity; if n > 1, there is positive cooperativity.

This project is concerned with the regulatory aspects of malate dehydrogenase from *E.coli* (eMDH). Dehydrogenases represent a class of enzymes that catalyze the oxidation of an alcohol to a keto-group. eMDH catalyzes the last step in the TCA cycle, the reversible oxidation of L-malate to oxaloacetate with the subsequent reduction of NAD^+ to NADH, a coenzyme utilized by most dehydrogenases (Figure 2). Although the equilibrium of this reaction is in favor of malate/NAD^+ by a factor of 10^5 (ΔG° = +29.7 KJ/mol), in a living cell, the net flux is toward the formation of oxaloacetate because it is continuously removed by the first reaction of the TCA cycle, the condensation of OAA with acetyl-CoA to form citrate.

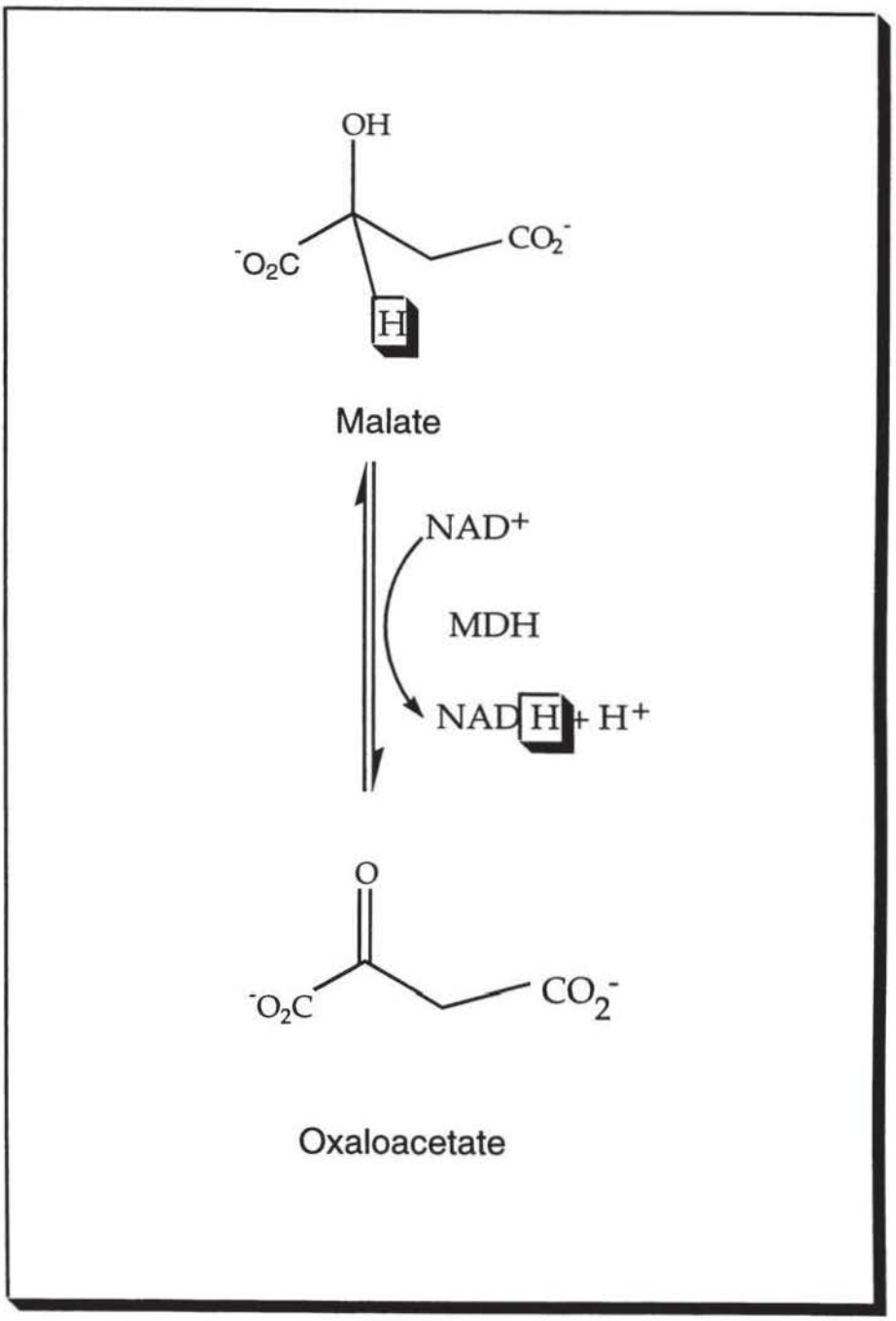


Figure 2. The Reaction Catalyzed by Malate Dehydrogenase (MDH)

In eukaryotic organisms, MDH exists in two forms, the cytosolic and the mitochondrial isozymes. Cytosolic MDH participates in the aspartate/malate shuttle by which the cytosolic reducing equivalents are transported across the mitochondrial membrane. Mitochondrial MDH participates on the other side of the aspartate/malate shuttle, in addition to catalyzing the formation of OAA.

eMDH was found to be closely homologous with mitochondrial MDH. It is a dimeric protein that consists of two identical subunits; each with a molecular weight of 35 kD [4]. Kinetic studies on eMDH have indicated that it, like other MDH's, follows an ordered Bi Bi kinetic mechanism, in which NAD^+ binds first followed by malate and OAA is released before NADH [5 & 6]. It was also indicated that eMDH has a single ionizing group in the active site with a pKa range between 7.8 and 8.5. This ionizing group is believed to be His 177, which acts as a general acid/base in the catalytic mechanism of the enzyme [5].

In 1966, research by Kuramitsu showed that AMP, ADP, and ATP activate the reaction catalyzed by eMDH in the direction of NAD^+ reduction [7]. According to this observation, it is unlikely for these nucleotides to bind at the active site. It was also observed that AMP decreases the K_m value for NAD^+ from 3.7 to 0.79 mM and therefore, increases the enzyme affinity for NAD^+ without altering V_{max} of the reaction. This work has led to the proposal of the presence of an allosteric site on eMDH where adenine nucleotides bind and alter the binding properties in the active site.

Three years later, in 1969, Sanwal presented evidence that eMDH may be regulated by NADH in an allosteric manner [8]. When OAA was the variable substrate, Sanwal showed eMDH to be allosterically regulated in the presence of 0.065 mM, 0.13 mM, and 0.32 mM NADH. However, the plots of rate vs. [OAA] under these conditions did not clearly present the sigmoidal curve, which characterizes the allosteric behavior. This observation, in addition to Kuramitsu's data, has led to the need of further investigations regarding the allosteric effect of eMDH.

The work presented here focuses on studying the allosteric behavior of eMDH in the presence of NADH as the allosteric effector. The main purpose is to determine whether or not NADH allosterically regulates the reaction catalyzed by eMDH. A kinetic tool was utilized which involved measuring the initial reaction rates under different concentrations of the substrate (MAL or OAA) and fixed concentrations of the cofactor (NAD^+ or NADH). The reaction was studied in both directions: NAD^+ reduction and NADH oxidation.

Another tool utilized to study enzyme kinetics involves chemical modification using reagents that can covalently bind to proteins. Binding of these modifying reagents to specific amino acid residues that play a key role in catalysis alters the catalytic activity of enzymes. In this project, eMDH was chemically modified in order to determine the effect of this modification on the allosteric behavior of the enzyme. The modifying agent used was 5'-p-flurosulfonylbenzoyladenosine (FSBA), shown in Figure 3.

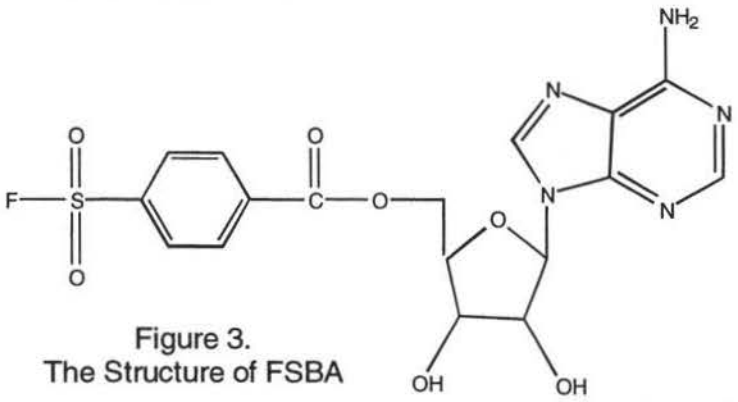


Figure 3.
The Structure of FSBA

FSBA was selected as the modifying agent since it has been found to be involved in specific modification of nucleotide binding sites of a wide variety of enzymes. Pyruvate kinase [9] and pig heart mitochondrial MDH [10] were shown to be chemically modified and, therefore, inactivated by FSBA. In addition, FSBA has the adenosine moiety, which makes it an appropriate analog of NAD^+ and NADH (Figure 4). Previous work has shown that this moiety is required for recognizing the active site of the enzyme being modified [9]. Different mechanisms that were proposed for the modification with FSBA are discussed in Chapter IV.

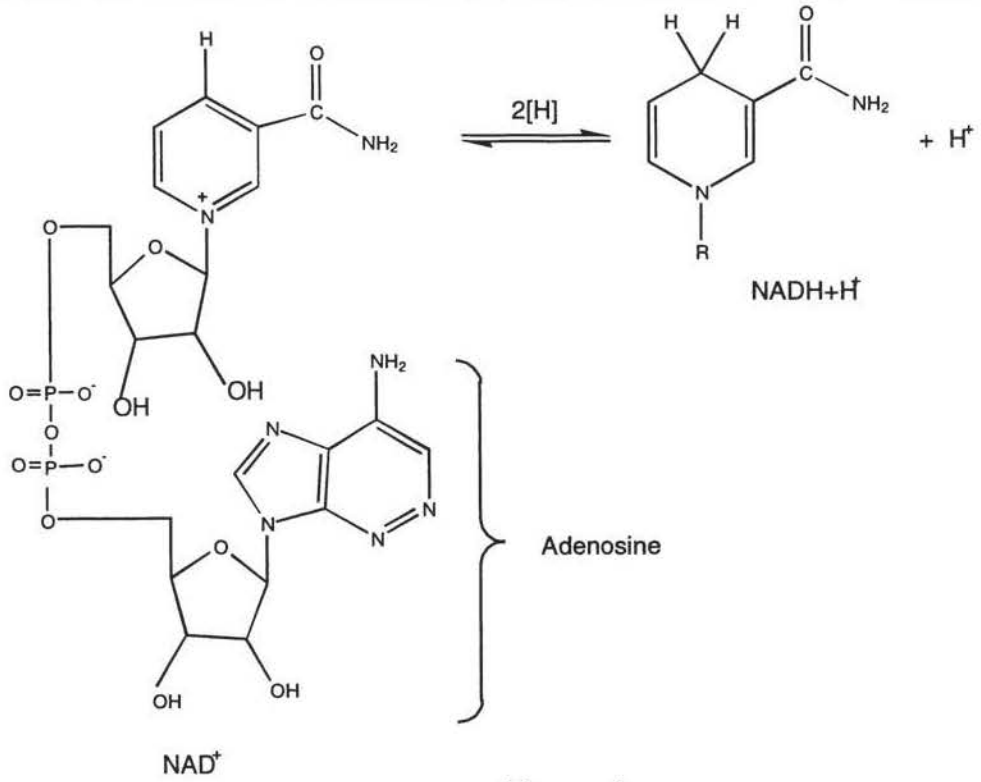


Figure 4.
The structure of NAD^+ and NADH

Another aspect of this project, unrelated to regulation, is concerned with strong hydrogen bonds, an interesting category of hydrogen bonds. Strong hydrogen bond energies are in excess of 12 kcal/mol [11], and sometimes equal those of covalent bonds. They have low-energy-barrier potential wells in which the position of the proton is variable and sensitive to the molecular environment. Therefore, they are referred to as low barrier hydrogen bonds (LBHB) or ionic hydrogen bonds. A LBHB is characterized by large infrared stretching frequency shifts and by relatively large downfield proton NMR chemical shifts (between 15 and 20 ppm).

Hydrogen bonds are known to be involved in enzyme catalysis through the formation of a structure called, the "charge-relay-system" between amino acid residues in the active site. This structure serves to conduct electrons through the hydrogen bonds [12 &13]. A LBHB was found to play an important role in the catalytic mechanism of chympotrypsin through the formation and stabilization of a tetrahedral intermediate between the enzyme and the substrate [14].

The crystal structure of eMDH in the presence of a substrate analog citrate has shown that His 177 at the active site is within a hydrogen bond distance of Asp 150, the bound citrate, and the cofactor [15]. NMR spectroscopy was utilized in an attempt to investigate the hydrogen bonds within the active site and their role in the catalytic mechanism of eMDH.

Chapter II

EXPERIMENTAL PROCEDURES

♦ Chemicals and Instruments:

1. Chemicals:

All reagents were purchased from Sigma Chemical Company. *E.coli* HB101 strain was obtained from American Type Culture Collection. Potassium phosphate dibasic was obtained from Aldrich Chemical Company Inc. The Bradford reagent and the Affi-Gel Blue agarose were obtained from Bio-Rad.

2. Instruments:

Centrifugation was performed on a Beckman Avanti J-30 I centrifuge at 4°C. The sonication of *E.coli* cell paste was performed on a Bronwill Scientific Biosonik III sonicator. The enzyme assays were performed using a Simadzu UV-3100 spectrophotometer with a thermostated microprocessor controlled cell holder. Stopped-flow measurements were performed using an Applied Photophysics Stopped-Flow Reaction Analyzer, SX-18 MV. K_m and V_{max} values were calculated using the software "Enzfitter" by Robin J. Leatherbarrow. The ^1H NMR spectroscopy was performed at 300 MHz using a General Electronics QE-300 Spectrometer.

- **Preparation of *E.coli* Culture:**

The *E.coli* HB101 strain colonies were used to inoculate 5 mL LB medium that was incubated at 37° C for 18 hours with shaking at low speed in the shaker room. When growth was observed, the cells were tested for the presence of pEM6, which carries the MDH gene. This was done by using 50 µL of the overnight culture to inoculate 3 test tubes, each one contained 5 mL LB medium in addition to 50 µg / mL ampicillin (63 µL of 4 mg/ml ampicillin). The cells were incubated at 37° C for 18 hours with shaking. The growth of *E.coli* colonies in the presence of ampicillin indicated the presence of the pEM6 plasmid. The contents of the test tubes were added to 3 one-liter flasks containing 1 liter of LB medium, in addition to 50mg ampicillin. The flasks were incubated at 37° C for 18 hours with shaking at low speed. The culture was centrifuged at 4,000 RPM for 20 min at 4° C. The supernatant was decanted and discarded. The cell paste was collected, weighted, and frozen until needed.

- **Enzyme Extraction and Purification:**

Approximately 0.5 g of the cell paste was thawed and mixed with 25 ml of 10 mM potassium pohosphate (KPi) buffer pH 7.2 on ice with a magnetic stir bar. The solution was then sonicated on ice at 65 % of the maximum power for 60 seconds. The solution was kept in ice for 60 seconds between sonications. This process was repeated 5 times. The protein extract was then centrifuged at 4,000

for 30 min at 4°C. The supernatant was assayed for activity and protein concentration.

Approximately 25 ml of Affi-Gel Blue was washed with 1L of 6 M urea solution followed by deionized water and was then equilibrated with 10 mM KPi buffer pH 7.2. The supernatant was mixed with blue agarose, and was allowed to stand for 15 min at 4°C. This allowed the binding of eMDH to the blue agarose. In the cold room, a column of the enzyme-bound blue agarose was built and washed overnight with 10 mM KPi buffer pH 7.2 to remove any unbound protein. The column was washed until the absorbance at 280 nm was less than 0.1. The enzyme was eluted from the column using 0 to1 M NaCl gradient in 10 mM KPi buffer pH 7.2. Fractions of 3 ml each were collected and assayed for enzyme activity. The fractions that showed high activity were combined and assayed for activity and protein concentration. The eMDH was concentrated by centrifugation at 2,000 RPM at 4°C using Centriplus concentrators. The centrifugation was continued until the total volume was 10% of the volume of the combined fractions. The concentrated protein was then assayed for total activity and protein concentration.

- **The Enzyme Assay in the Forward Direction (Malate Oxidation):**

Malate and NAD^+ were dissolved in 50 mM TAPS buffer pH 9.0 containing 100 mM KCl and 2 mM EDTA. The control assay was performed in the direction of NAD^+ reduction and consisted of 7 mM NAD^+, variable

concentrations of malate (0.15 to 12.0 mM), 50 mM TAPS buffer pH 9.0, 100 mM KCl and 2 mM EDTA. The reaction began with the addition of 6.7 nM active site MDH. The increase in the absorbance of NADH was recorded at 340 nm. In a separate experiment, the assay was performed in the presence of 0.18 mM NAD^+ instead of 7 mM as was stated in the literature (8).

- **The Enzyme Assay in the Reverse Direction (OAA Reduction):**

The routine assay for enzyme activity was performed in the direction of NADH oxidation. Oxaloacetate and NADH were dissolved in 50 mM KPi buffer pH 7.2 containing 100 mM KCl and 2 mM EDTA. The enzyme assay contained: 0.15 mM NADH, 0.10 mM OAA, 50 mM KPi buffer pH 7.2. The reaction was started by the addition of 0.01 mL of the diluted MDH. The assay was performed at 25°C. The decrease in NADH absorbance was recorded at 340 nm. The number of units was calculated according to the equation:

$$\text{MDH activity (u/mL)} = (\Delta\text{OD/min})\,(1/6.22)\,(0.01\text{ mL}) \qquad (8)$$

where 6.22 mM^{-1} is the extinction coefficient of NADH at 340 nm, and 0.01 mL is the volume of MDH.

- **The Allosteric Assay:**

The allosteric assay contained the same conditions as the control assay in the forward direction, in addition to the presence of 0.065 mM NADH as the allosteric effector. The assay was performed at 25°C and the increase in absorbance was recorded at 340 nm.

- **Protein Assay**

The protein concentration was determined using 1 mL of Bradford reagent, diluted 5-fold and 20 µL protein solution. The absorbance was recorded against reagent blank at 595 nm at 25°C and the concentration was calculated using the equation:

$$\text{Protein conc. (mg/mL)} = (\text{Abs} + 0.0166) / 1.1887 \qquad (9)$$

The specific activity was calculated as the number of units/mg protein.

Chemical Modification

1) Treatment with 5'-p-flurosulfonyl benzoyl adenosine (FSBA):

FSBA was dissolved in absolute ethanol. The concentration of the stock solution was 10 mM. The experiment was performed in the presence of NADH to protect the active site from binding to FSBA. Two eMDH samples were prepared: modified and control. The control sample consisted of: 300 nM MDH, 10% absolute ethanol, 0.15 mM NADH, 50 mM TAPS buffer, pH 9.0 and 100 mM KCl.

The modified sample contained the same concentrations except for the presence of 0.1 mL of 10 mM FSBA. NADH was added before the addition of FSBA. The two samples were incubated for 4 hours at room temperature. However, when the samples were assayed for enzyme activity, a significant drop of the rates was observed. As a result, the experiment was repeated with incubation time of one hour at room temperature. After the incubation period, the enzyme was washed using equal volume of 50 mM TAPS buffer pH 9.0 and centrifuged at 2000 RPM for 20 min, after being loaded in Centriplus concentrators, at 4°C in order to remove any unbound FSBA. The washing process was repeated 3 times.

In order to determine the effect of the modification, 10 µL aliquots from the control and the modified samples were assayed in the forward direction in the absence and presence of 0.065 mM NADH.

2) The Chemical Modification with FSBA without protecting the active site:

This experiment was performed in order to determine the role of NADH in protecting the active site from being modified by FSBA, which in turn might give an idea whether the FSBA is modifying the active site or the allosteric site.

In this experiment, two samples were prepared: protected and unprotected eMDH. The unprotected enzyme sample consisted of: 690 nM eMDH active sites, 0.1 mM FSBA, 50 mM TAPS buffer pH9.0. The protected sample contained the same concentrations in addition to the presence of 0.15 mM NADH. The two samples were incubated for 1 hour at room temperature and

washed as was described previously. The control assay in the forward direction was performed on both samples.

- **Allosteric Effect using the Stopped-Flow Spectrophotometer:**

The experiment was performed in the Department of Chemistry, University of Illinois, Urbana-Champaign using an Applied Photophysics stopped-flow spectrophotometer. The stopped-flow technique allowed us to record the exact initial rates of the reaction. The final volume of the assay was 100 µL. The control assay in the forward direction consisted of: variable concentrations of malate (from 0.043 to 8.5 mM), 0.18 mM NAD^+, 50 mM TAPS buffer pH 9.0 and 100 mM KCl. The increase in the NADH absorbance was recorded at 340 nm and 25°C. The rates were recorded as the change of the absorbance per second, which was converted to the change of the absorbance per minute. The allosteric assay was performed under the same conditions, in addition to the presence of 0.065 mM NADH as the allosteric effector.

- **The Allosteric Effect in the direction of NADH oxidation:**

The purpose of this experiment was to determine whether or not eMDH is allosterically regulated in the reverse direction, NADH oxidation. The experiment was performed using both the conventional and the stopped-flow spectrophotometers.

Both NADH and OAA were dissolved in 50 mM KPi buffer, pH 7.2. Three different concentrations of NADH were used: 0.017 mM, 0.15 mM, and 0.32 mM. The assay mixture consisted of variable concentrations of OAA (from 0.003 mM

to 0.16 mM), NADH concentration was fixed, 50 mM KPi buffer pH 7.2 containing 100 mM KCl, and 2 mM EDTA. The reaction was started by the addition of 0.8 nM eMDH (0.16 nM active sites). The decrease in absorbance was recorded at 340 nm and 25°C. Since the change in absorbance in the presence of 0.32 mM NADH was very small compared to the initial absorbance, the rates recorded in this assay were measured against a 0.15 mM NADH blank.

- **The Chemical Modification with FSBA at pH 7.2:**

The purpose of this experiment was to determine the effect of the chemical modification on the allosteric behavior of eMDH in the reverse direction. The enzyme was incubated with FSBA under the same conditions that were described above. However, 50 mM KPi buffer pH 7.2, containing 100 mM KCl, and 2 mM EDTA was used instead of the TAPS buffer. After the washing process, the enzyme was diluted and used in the enzyme assay in the presence of 0.32 mM NADH.

- **Protein NMR Studies:**

In order to run this experiment, high protein concentration was required. Therefore, two eMDH extracts were combined, concentrated and lyophilized.

Protein Lyophlization (Freeze Drying):

The protein solution needed to be lyophilized for two reasons: 1) to eliminate the water molecules, which will reduce the intensity of the water

signal, and 2) to achieve the highest possible protein concentration. The protein extract (approximately 1.9 mL) was frozen, in a 25 mL round bottom flask, using liquid nitrogen and was then dried under vacuum for 3 to 4 hours or until all the solution was converted to a white powder. The dry protein was then reconstituted in 0.6 mL D_2O. At this point, there was some protein left that could not be dissolved after swirling the solution on ice for approximately 15 min. As a result, the protein solution was centrifuged at 3,000 RPM for 20 min at 4°C. The supernatant was assayed for activity and protein concentration.

^{1}H NMR Experiment:

The NMR spectra were recorded for the lyophilized protein with DSS as an internal standard. The protein concentration was approximately 0.1 mM in 0.6 mL D_2O. The sweep width was 1000 ppm. In the first spectrum, the number of acquisitions was 496 and D5 (relaxation time) was 1.5 sec. In order to improve the signal-to-noise ratio, the second spectrum was obtained using 10512 acquisitions and D5 was 1.5 sec. All spectra were collected at 21°C.

Chapter III

RESULTS

- **Purification of Malate Dehydrogenase:**

Enzyme activity and protein concentration for the crude and purified MDH are summarized in Table 1.

Table 1: Results of MDH purification.

	Total Units	Protein Conc. (mg/ mL)	Specific Activity (unit/ mg)
After Extraction	5082.5	1.7	199.6
After Purification	1686.6	0.042	787.4
After Concentration	1656.5	0.54	808.5

- **The Allosteric Assay:**

In this experiment, malate concentration was varied from 0.15 to 12.0 mM, NAD^+ and NADH concentrations were held constant at 7 mM and 0.065 mM respectively. The buffer used was 50 mM TAPS pH 9.0 containing 100 mM KCl. The data from the control and the allosteric assays is summarized in Table 2, and the plot of the rate vs. malate concentration is presented in Figure 5.

Table 2. The allosteric effect of eMDH in the presence of 0.065 mM NADH
Conditions: 0.15 to 12.0 mM malate, NAD^+ was fixed at 7 mM, NADH was fixed at 0.065 mM, 50 mM TAPS pH 9.0 and 100 mM KCl

Malate conc. (mM)	Rate (ΔOD/min) Control	Rate (ΔOD/min) With 0.065 mM NADH
0.15	0.060	0.003
0.5	0.110	0.004
1.0	0.143	0.007
2.0	0.180	0.050
3.0	0.201	0.062
5.0	0.220	0.075
7.0	0.260	0.090
10.0	0.276	0.096
12.0	0.286	0.120

The sigmoidal curve in the presence of 0.065 mM NADH that is shown in Figure 5 supports the proposal of the presence of an allosteric site where NADH binds and changes the catalytic activity of eMDH.

- **The Chemical Modification with FSBA:**

The chemical modification experiment was performed to determine whether or not FSBA will covalently bind to the allosteric site and affect the binding of NADH at this site. The enzyme was incubated with 1 mM FSBA in the presence of 0.15 mM NADH to protect the active site from being modified. Both the control and the allosteric assays were performed on the control and the modified enzyme. Tables 3 & 4 list the results of the control and the allosteric

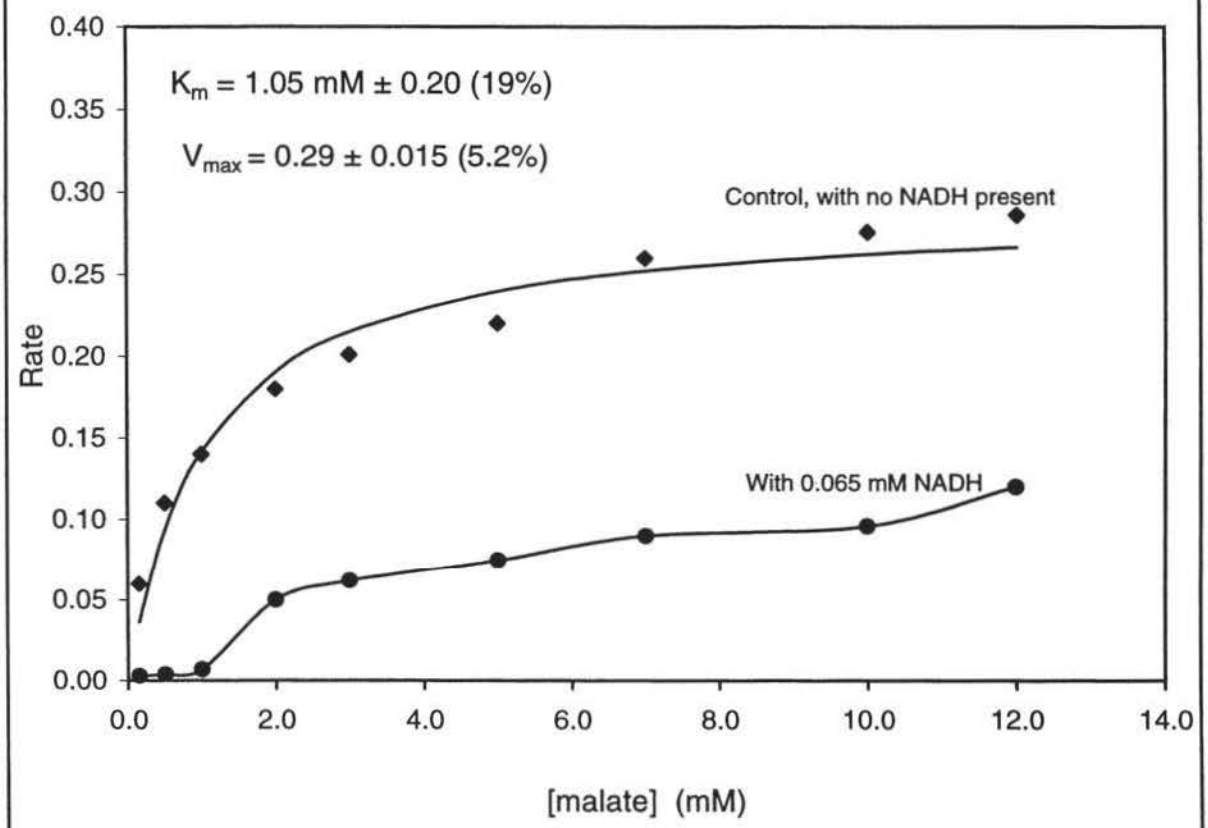


Figure 5.
A plot of the rate vs. [malate] in the absence and presence of 0.065 mM NADH showing the allosteric behavior of eMDH

assays for both the control and the modified eMDH respectively. The plots of this data are presented in Figures 6 & 7.

Table 3. Chemical Modification with FSBA
Control eMDH (not treated with FSBA)
Conditions: 7 mM NAD^+, 0.15 to 12.0 mM malate, 0.065 mM NADH, 50 mM TAPS pH 9.0 and 100 mM KCl

Malate conc. (mM)	Control Rate (ΔOD/min)	Rate (ΔOD/min) with 0.065 NADH
0.15	0.023	0.0005
0.5	0.030	0.0006
1.0	0.043	0.0017
2.0	0.054	0.0077
3.0	0.060	0.0129
5.0	0.073	0.0210
7.0	0.081	0.0226
10.0	0.080	0.0225
12.0	0.081	0.0225

Table 4. Chemical Modification with FSBA
Modified eMDH (treated with FSBA)
Conditions: 7 mM NAD^+, 0.15 to 12.0 mM malate, 0.065 mM NADH, 50 mM TAPS buffer pH 9.0 and 100 mM KCl

Malate conc. (mM)	Control Rate (ΔOD/min)	Rate (ΔOD/min) With 0.065 NADH
0.15	0.008	0.005
0.5	0.013	0.007
1.0	0.016	0.009
2.0	0.020	0.015
3.0	0.025	0.018
5.0	0.034	0.020
7.0	0.040	0.021
10.0	0.041	0.020
12.0	0.041	0.020

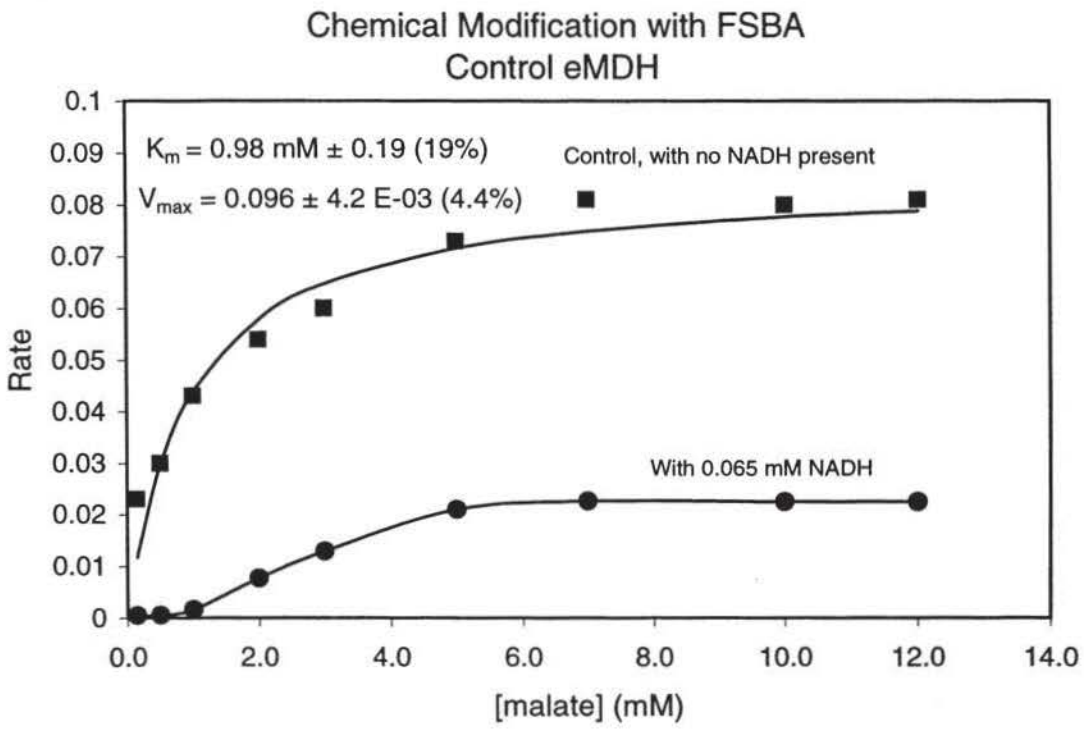


Figure 6.
Plot of rate vs. [MAL] in the absence and presence of NADH for the control eMDH that was not treated with FSBA
Conditions: 0.15 to 12.0 mM Malate, 7 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS buffer pH 9.0

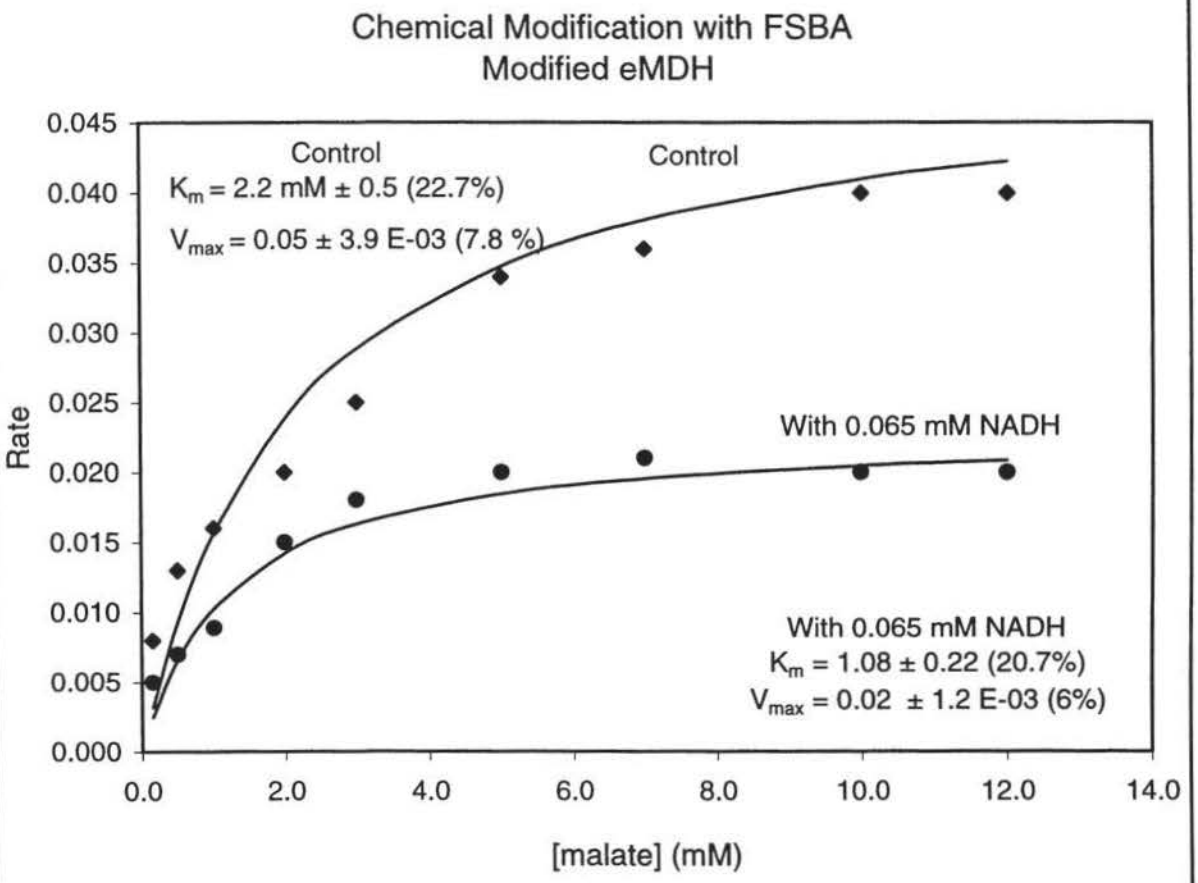


Figure 7.
Plot of rate vs. [MAL] in the absence and presence of NADH for the modified eMDH that was treated with FSBA
Conditions: 0.15 to 12.0 mM malate, 7 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS buffer pH 9.0

The eMDH sample that was treated with FSBA did not show the allosteric behavior in the presence of 0.065 mM NADH, which suggests that FSBA may have covalently modified a specific amino acid residue at the allosteric site and, therefore, prevented the binding of NADH.

However, the data obtained from the allosteric assay in the presence of NADH (Figure 5) was not reproducible when the assay was repeated several times in order to confirm this behavior.

In addition, the control and the allosteric assays were also performed in the presence of 0.18 mM NAD^+ as was stated in the literature instead of 7 mM [8]. Each data point of the assay in the presence of NADH was repeated three times and the average was considered as the actual rate. Tables 5 & 6 list the results of this assay. A plot of this data is presented in Figure 8.

Table 5. The control assay using 0.18 mM NAD^+

Malate conc. (mM)	Control Rate (ΔOD/min)
0.15	0.0030
0.5	0.0119
1.0	0.0235
2.0	0.0365
3.0	0.0501
5.0	0.0573
7.0	0.0721
10.0	0.0755
12.0	NA

Table 6. Results from the allosteric assay at 0.18 mM NAD^+ and 0.065 mM

[malate] (mM)	Rate (ΔOD/min) Trial # 1	Rate (ΔOD/min) Trial # 2	Rate (ΔOD/min) Trial # 3	Average rate
0.15	0.0004	NA	0.0004	0.0004
0.5	0.0051	0.0070	0.0037	0.0053
1.0	0.0067	0.0126	0.0069	0.0087
2.0	0.0145	0.0199	0.0177	0.0174
3.0	0.0186	0.0224	NA	0.0205
5.0	0.0289	0.0365	0.0335	0.0329
7.0	0.0346	0.0367	NA	0.0357
10.0	0.0394	NA	0.0361	0.0378
12.0	NA	NA	0.0420	0.0420

As Figure 8 shows, the allosteric effect could not be detected using 0.18 mM NAD^+. At this point, it was not clear whether or not eMDH is allostericaly regulated by NADH. The fact that this behavior was observed in the first set of data led to the idea of using a different technique to record the initial rates of the reaction.

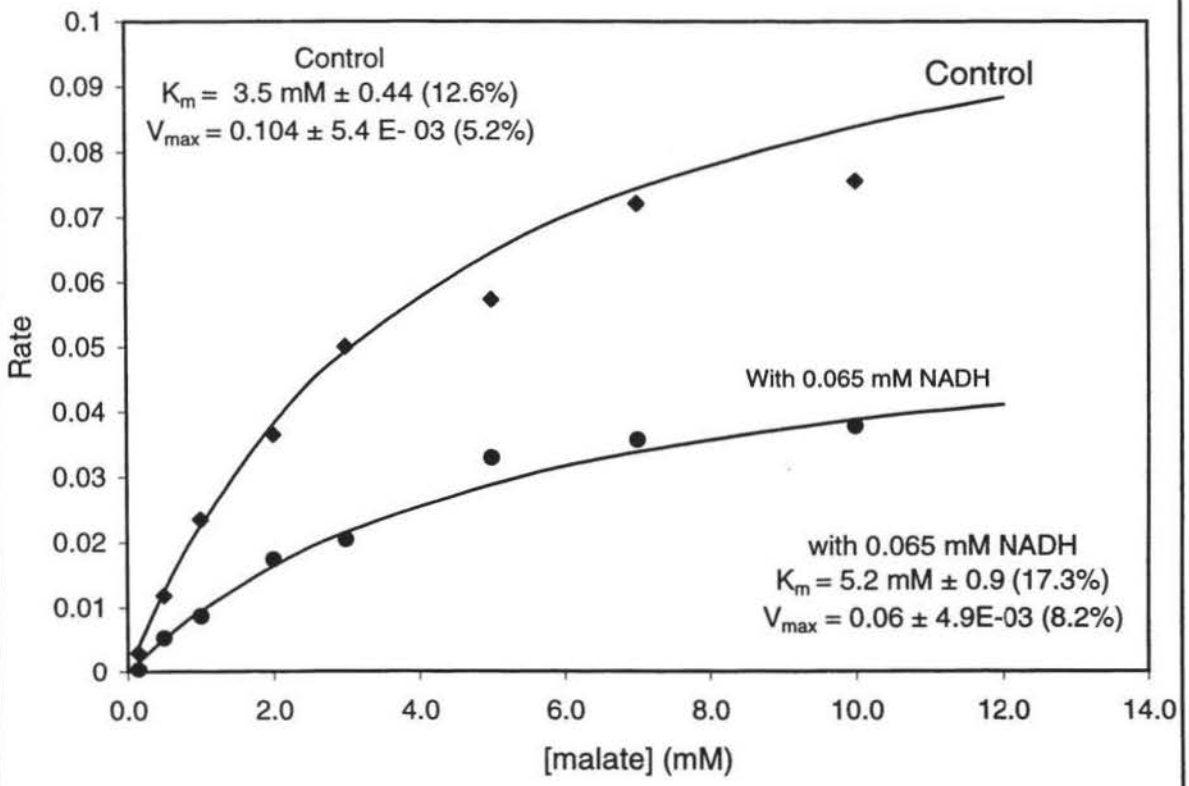


Figure 8.
Plot of rate vs. [MAL] using 0.18 mM NAD^+ in the absence and the presence of 0.065 mM NADH

- **The Enzyme Assay using the Stopped-Flow Spectrophotometer:**

The assay was performed using an Applied Photophysics stopped-flow spectrophotometer, which allowed us to record the exact initial rates of the reaction. In this experiment, lower concentrations of malate were used, varying from 0.04 to 8.5 mM. NAD^+ and NADH concentrations were held constant at 0.18 mM and 0.065 mM respectively. The rates were recorded as ΔOD/sec., which was converted to ΔOD/min. Table 7 shows the rates of the reaction from the control and the allosteric assays. A plot of v against [malate] is presented in Figure 9.

Table 7. The rates obtained from the control and the allosteric assays using the stopped-flow spectrophotometer.
Conditions: 0.0425 to 8.5 mM malate, 0.18 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS pH 9.0.

Malate conc. (mM)	Rate (ΔOD/min), Control	Rate (ΔOD/min), W/ 0.065 mM NADH
0.0425	0.0245	0.0047
0.085	0.0647	0.0101
0.213	0.1080	0.0219
0.425	0.1790	0.0476
0.850	0.4143	0.0795
1.70	0.800	0.1581
3.0	1.0448	0.3684
4.25	1.5190	0.6230
8.5	1.8280	0.6871

As Figure 9 shows, using lower concentrations of malate allowed the detection of the allosteric effect of eMDH at 0.18 mM NAD^+. The data obtained from this assay were fit to the Hill equation and "n" value of 1.2 was obtained. Figure 10 shows a plot of log v/V_{max}-v against log [S].

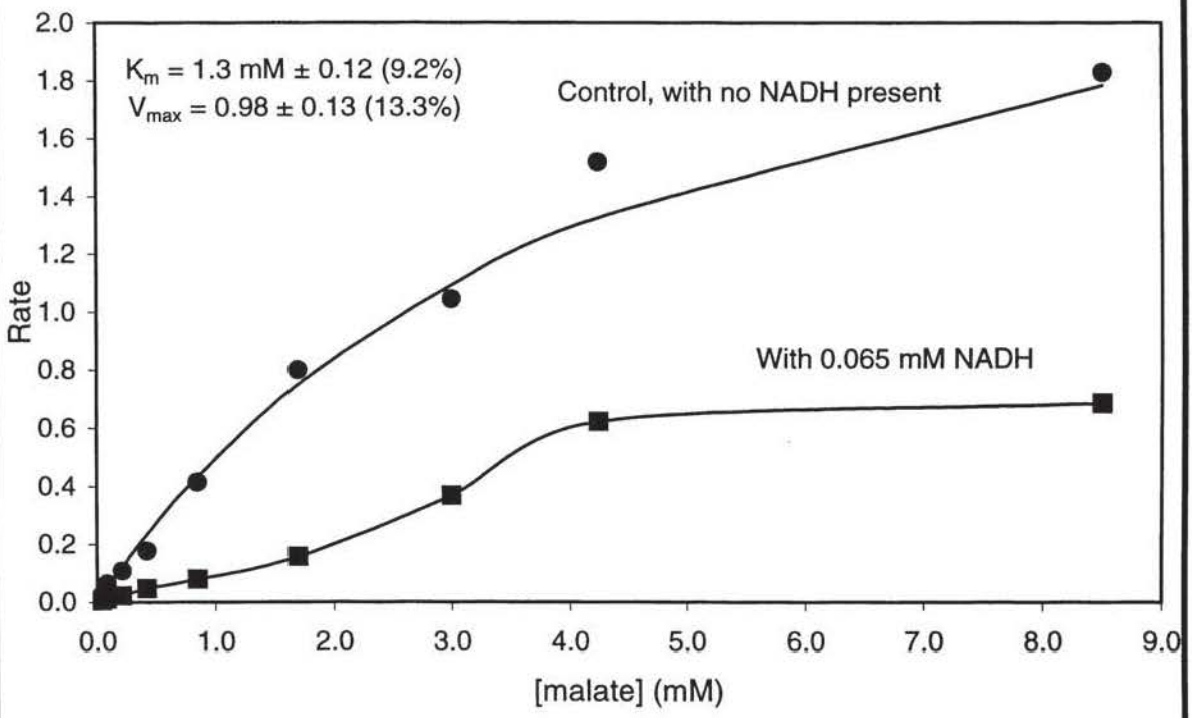


Figure 9.
Plot of rate vs. [MAL] using the stopped-flow spectrophotometer.
Conditions: 0.0425 to 8.5 mM malate, 0.18 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS buffer pH 9.0

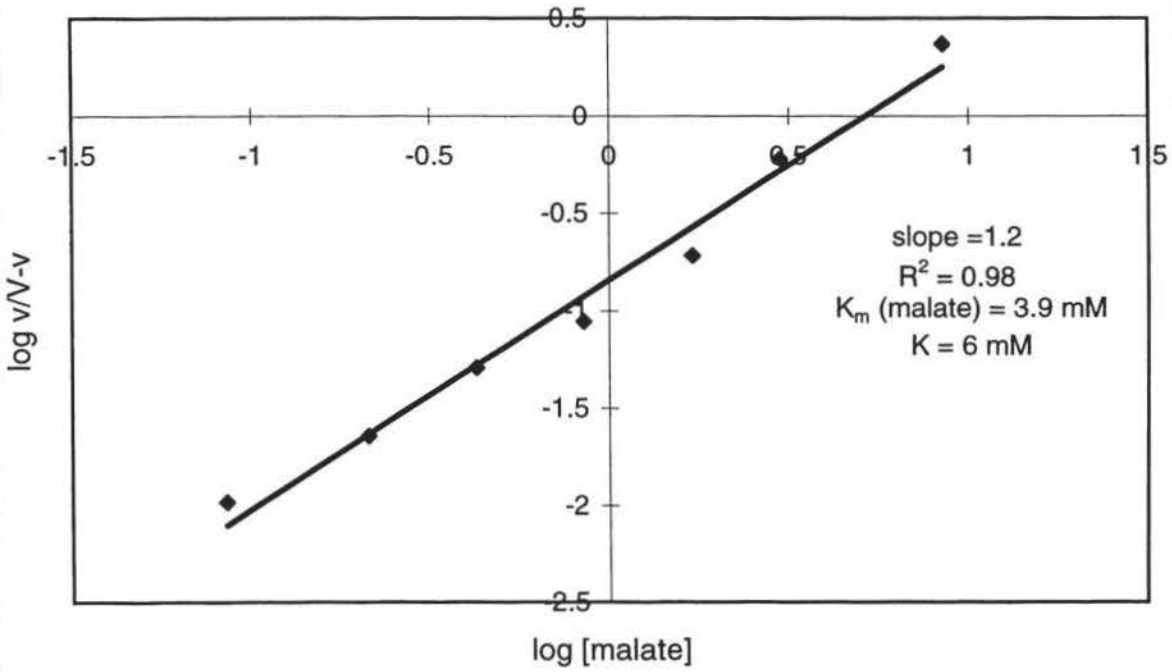


Figure 10.
The Hill plot for the data obtained using the stopped-flow spectrophotometer at 0.18 mM NAD^+
K_m was calculated as the substrate concentration when *log* $v/V_{max} - v = 0$

- **The Chemical Modification with FSBA, using 0.18 mM NAD^+ in the enzyme assay:**

The enzyme was incubated with FSBA under the same conditions that were described previously. Tables 8 & 9 list the rates that were obtained from the enzyme assays using the control and modified eMDH respectively. Figure 11 represents the control and the allosteric assays for the control eMDH (was not treated with FSBA) with the sigmoidal curve in the presence of 0.065 mM NADH. However, as Figure 12 shows, the FSBA did not completely block the allosteric site, since the plot of the enzyme assay in the presence of NADH does not follow Michaelis-Menten equation.

Since this behavior might be due to the incomplete substrate saturation of eMDH at low concentration of NAD^+, the enzyme assays were performed on the same samples of eMDH using 7 mM NAD^+. This data is presented in Tables 10 and 11. As figures 13 and 14 show, eMDH did not exhibit the allosteric effect after being modified with FSBA.

Table 8. Chemical Modification with FSBA Control eMDH that was not treated with FSBA **Conditions:** 0.05 to 10.0 mM malate, 0.18 mM NAD^+, 0.065 mM NADH and 50 mM TAPS pH 9.0		
Malate conc. (mM)	Rate (ΔOD/min) Control	Rate (ΔOD/min) With 0.065 mM NADH
0.05	0.005	0.002
0.10	0.009	0.003
0.25	0.019	0.004
0.5	0.032	0.004
1.0	0.048	0.013
2.0	0.086	0.023
5.0	NA	0.030
10.0	0.107	0.033

Table 9. Chemical Modification with FSBA Modified eMDH that was treated with FSBA **Conditions:** 0.05 to 10.0 mM malate, 0.18 mM NAD^+, 0.065 mM NADH and 50 mM TAPS pH 9.0		
Malate conc. (mM)	Rate (ΔOD/min) Control	Rate (ΔOD/min) With 0.065 mM NADH
0.05	0.003	0.0015
0.10	0.006	0.0035
0.25	0.011	0.0055
0.5	0.026	0.008
1.0	0.043	0.012
2.0	NA	0.026
5.0	0.084	0.039
10.0	0.092	0.039

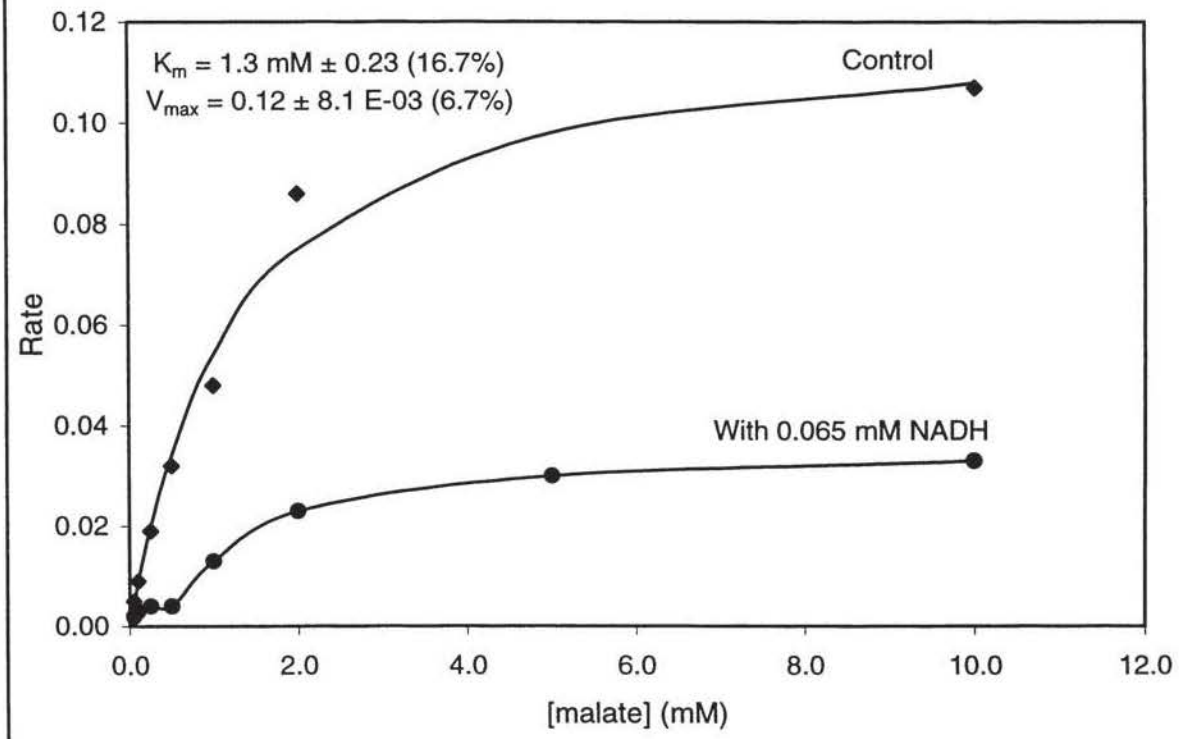


Figure 11.
Plot of rate vs. [malate] in the absence and presence of NADH for the control eMDH that was not treated with FSBA
Conditions: 0.05 to 10.0 mM malate, 0.18 mM NAD^+, 0.065 mM NADH and 50 mM TAPS pH 9.0

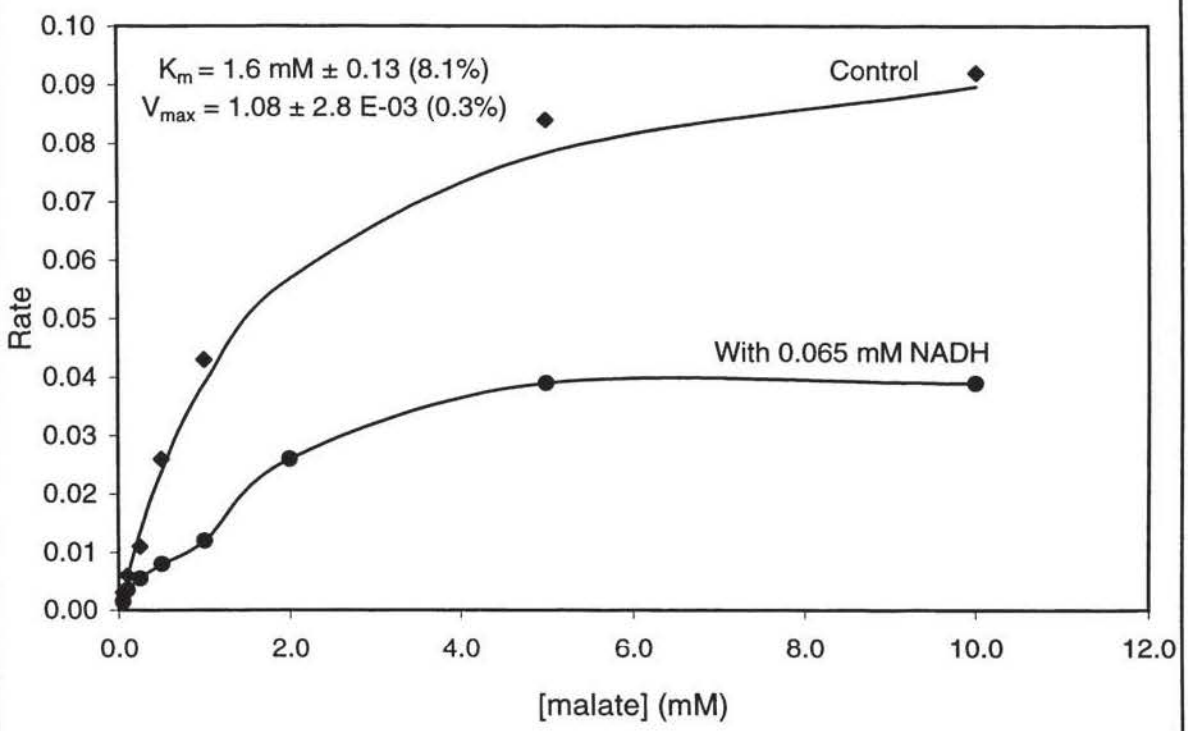


Figure 12.
Plot of rate vs. [malate] in the absence and presence of NADH for the modified sample that was treated with FSBA.
Conditions: 0.05 to 10.0 mM malate, 0.18 mM NAD^+, 0.065 mM NADH and 50 mM TAPS pH 9.0

Table 10. Chemical Modification with FSBA
Control eMDH that was not treated with FSBA
Conditions: 0.05 to 10.0 mM malate, 7 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS pH 9.0

Malate conc. (mM)	Rate (ΔOD/min) Control	Rate (ΔOD/min) With 0.065 mM NADH
0.05	0.018	0.001
0.10	0.051	0.0025
0.25	0.115	0.0038
0.50	0.147	0.009
1.00	0.200	0.042
2.00	0.211	0.058
5.00	0.222	0.101
10.00	NA	0.125

Table 11. Chemical Modification with FSBA
Modified eMDH that was treated with FSBA
Conditions: 0.05 to 10.0 mM malate, 7 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS pH 9.0

Malate conc. (mM)	Rate (ΔOD/min) Control	Rate (ΔOD/min) With 0.065 mM NADH
0.05	0.018	0.006
0.10	0.031	0.011
0.25	0.069	0.031
0.50	0.104	0.047
1.0	0.149	0.080
2.0	0.167	0.101
5.0	0.170	0.128
10.0	0.180	0.137

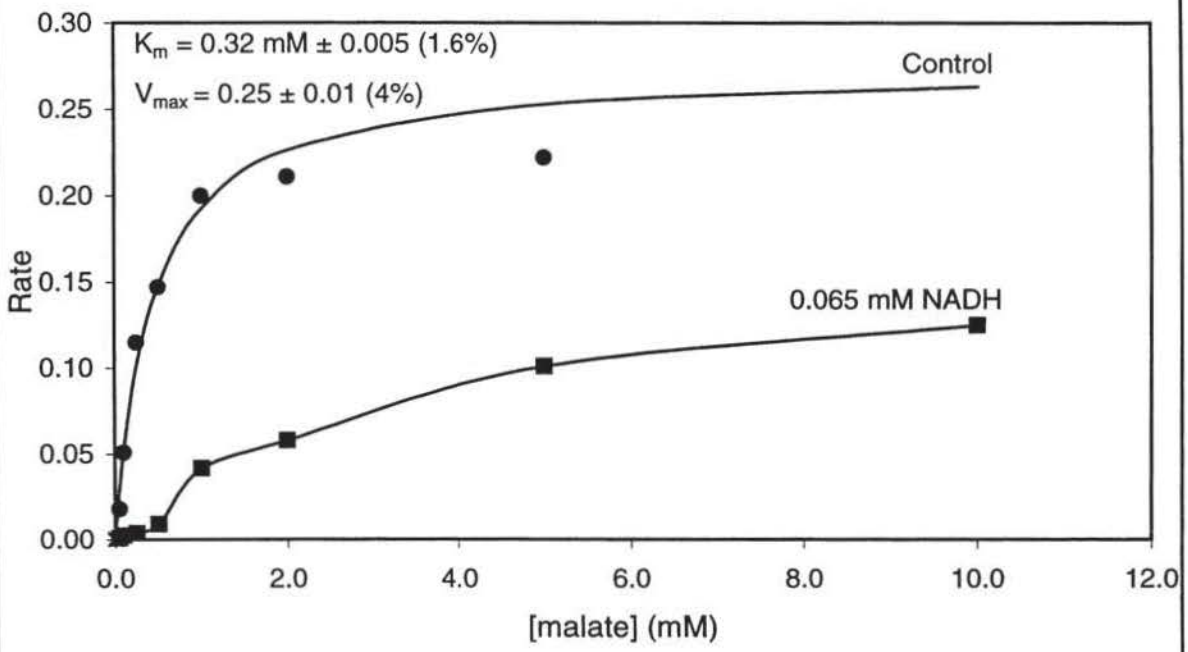


Figure 13.
Plot of rate vs. [malate] for the control eMDH that was not treated with FSBA
Conditions: 0.05 to 10.0 mM malate, 7 mM NAD^+, 0.065 mM NADH, and 50 mM TAPS buffer pH 9.0

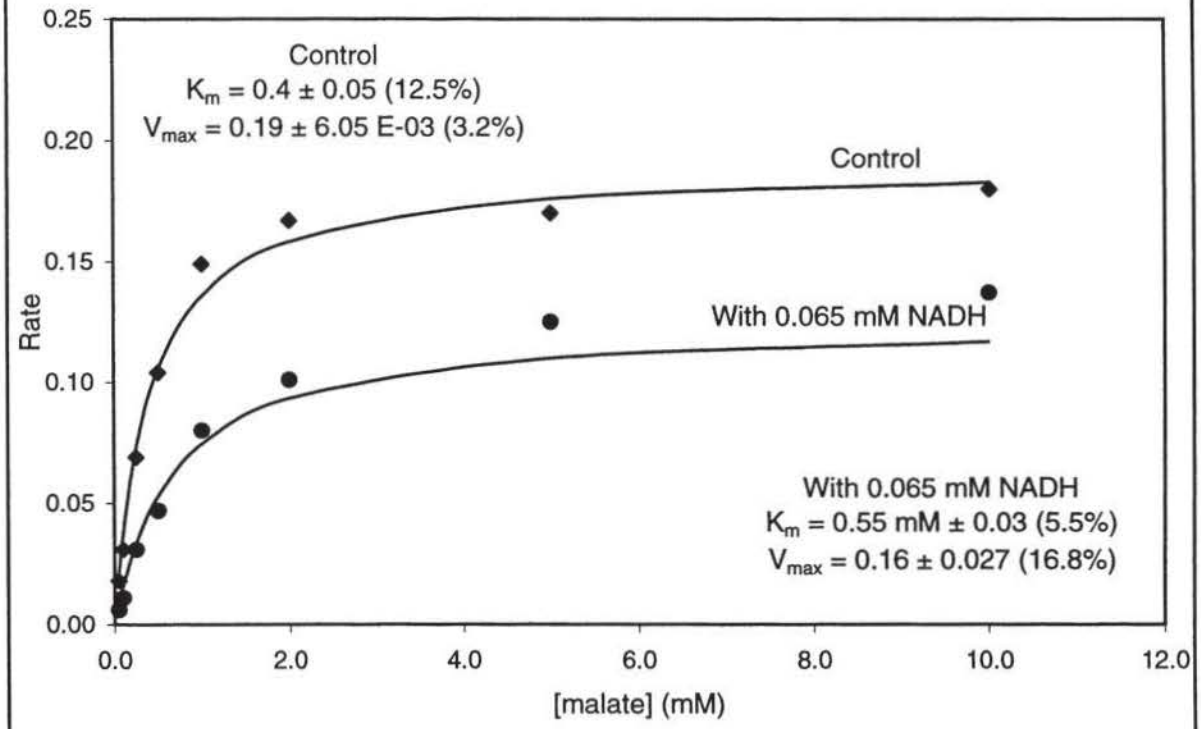


Figure 14.
Plot of rate vs. [malate] of the modified eMDH.
Condition: 0.05 to 10.0 mM malate, 7 mM NAD^+, 0.065 mM NADH and 50 mM TAPS buffer pH 9.0

- **Chemical Modification with FSBA without protecting the active site:**

This experiment was performed in order to determine the role that NADH plays in protecting the active site from being modified by FSBA. The unprotected MDH sample in this experiment consisted of: 1 mM FSBA in absolute ethanol, 690 nM eMDH, and 50 mM TAPS buffer pH 9.0. The protected enzyme sample contained the same components, in addition to the presence of 0.15mM NADH. The results of this experiment are summarized in Table 12, and a plot of rate vs. [malate] is presented in Figure 15.

Table 12. Rate data from the chemical modification with FSBA without protecting the active site with NADH
Conditions: 0.05 to 5.0 mM malate, 7 mM NAD^+, 50 mM TAPS pH 9.0 containing 100 mM KCl

Malate conc. (mM)	Rate (ΔOD/min) protected eMDH	Rate (ΔOD/min) unprotected eMDH
0.05	0.018	0.005
0.1	0.036	0.010
0.25	0.072	0.022
0.5	0.089	0.030
1.0	0.105	0.055
2.0	0.147	0.066
5.0	0.153	0.075

As the data and Figure 15 show, there was approximately 50-60 % drop in the catalytic activity when NADH was not added to the incubation mixture. This data suggests that, in the presence of NADH, FSBA might be modifying a specific amino acid residue in the allosteric site since the drop in rates was not observed in the chemical modification experiment.

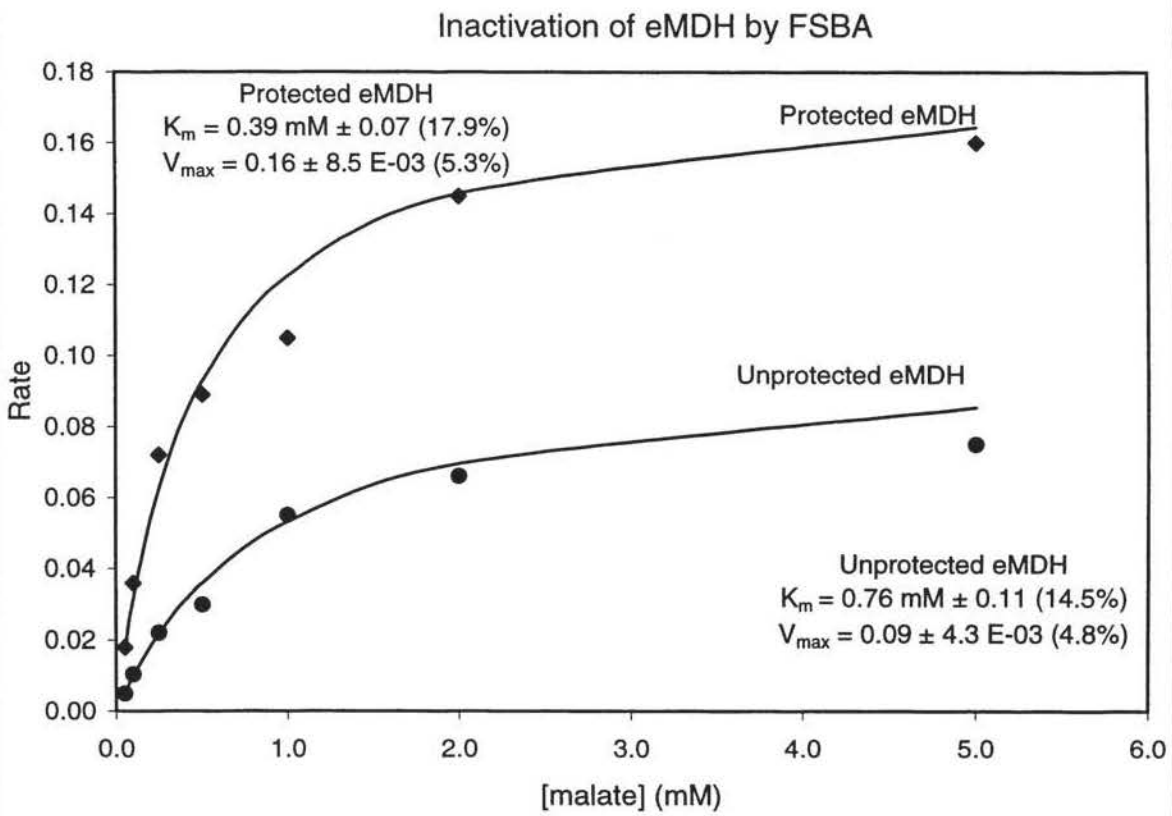


Figure15.
Plot of rate vs. [malate] of FSBA-modified eMDH with and without protecting the active site.
Conditions: 0.05 to 5.0 mM malate, 7 mM NAD^+, and 50 mM TAPS pH 9.0

- **The allosteric effect in the reverse direction:**

The allosteric assay was performed in the direction of NADH oxidation. Three different concentrations of NADH were used: 0.017 mM, 0.15 mM, and 0.32 mM. OAA concentration was varied from 0.003 to 0.16 mM. The enzyme assays in the presence of 0.017 and 0.15 mM NADH were performed using the stopped-flow spectrophotometer. The 0.32 mM NADH assay was performed on the Shimadzu UV-3100 spectrophotometer, using 0.15 mM NADH as blank. Table 13 shows the data obtained from the three enzyme assays. Figure 16 shows the plots of rate vs. [OAA] using 0.017 and 0.15 mM NADH. Double reciprocal plot of this data is presented in Figures 17a and 17b.

Table 13. Rate data in the reverse direction.
Conditions: OAA was varied from 0.003 to 0.16 mM, NADH was fixed at 0.017, 0.15 and 0.32 mM, 50 mM Kpi buffer pH 7.2

OAA conc. (mM)	Rate (ΔOD/min) 0.017 mM NADH	Rate (ΔOD/min) 0.15 mM NADH	Rate (ΔOD/min) 0.32 mM NADH
0.003	0.0139	NA	NA
0.005	0.0166	0.028	0.042
0.01	0.026	0.040	0.052
0.02	0.031	0.063	0.065
0.03	0.034	0.080	0.107
0.05	0.038	0.097	0.166
0.08	0.037	0. 121	0.198
0.16	NA	0.122	0.20

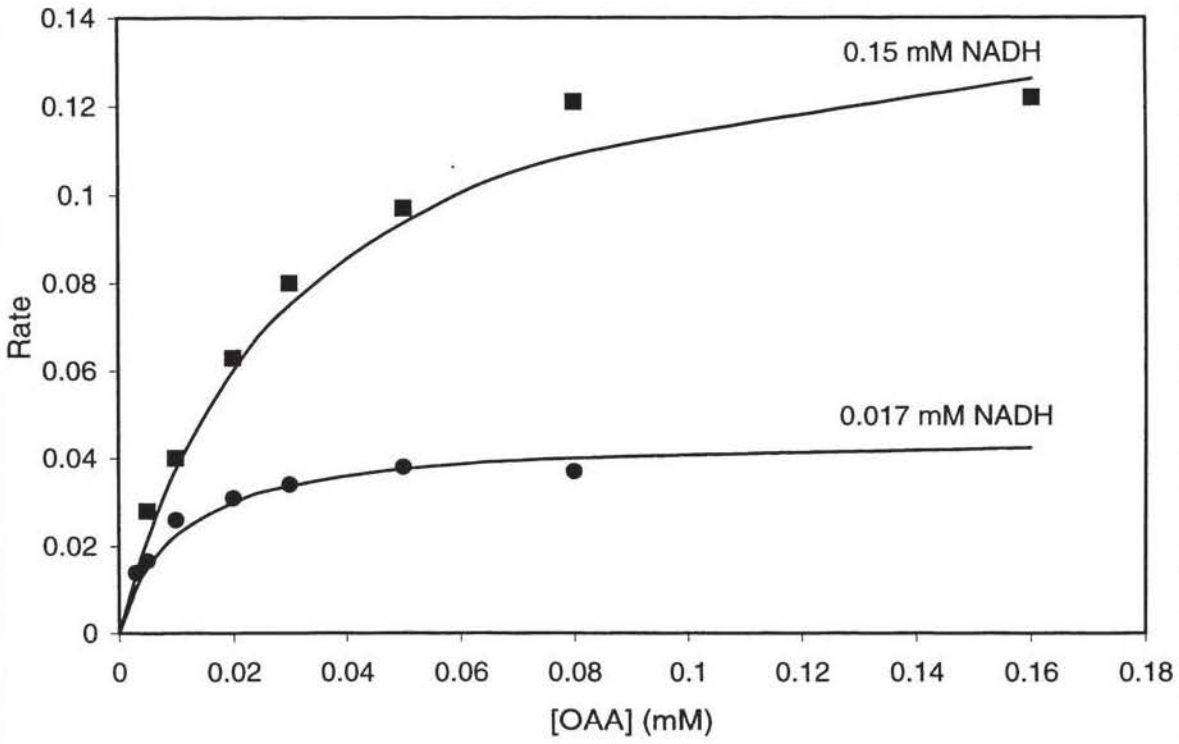
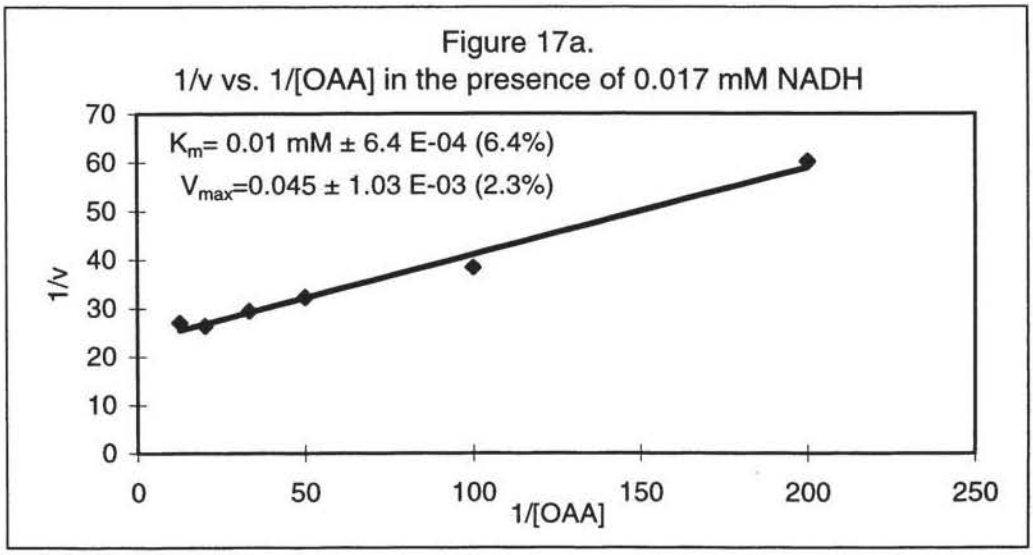


Figure 16.
Plot of rate vs. [OAA] in the reverse direction in the presence of 0.017 and 0.15 mM NADH

Double Reciprocal Plot of the Enzyme Assay in the Reverse Direction



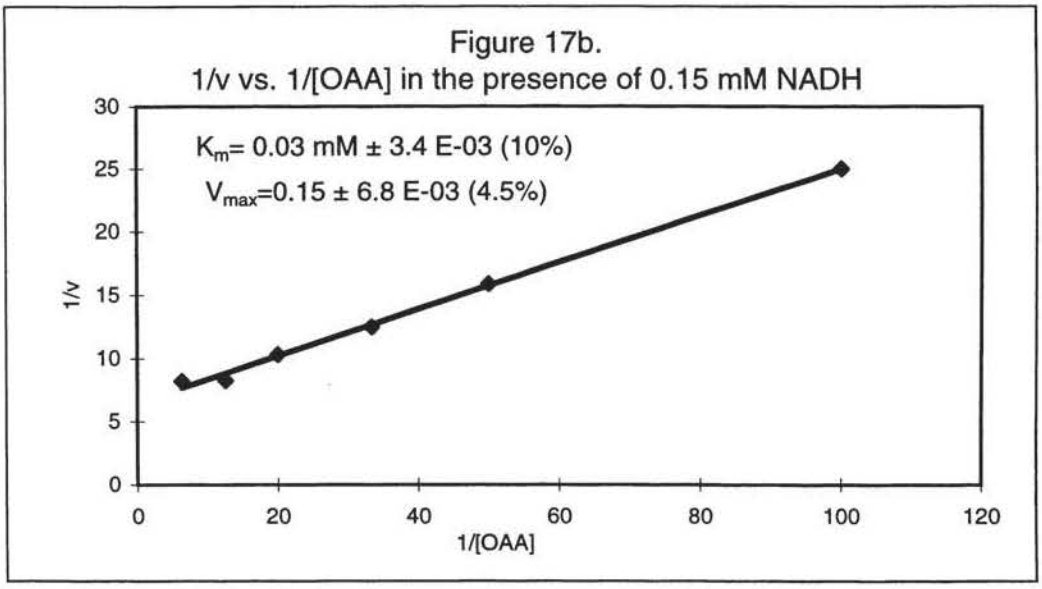


Figure 17.
A plot of 1/v vs. 1/[OAA] in the presence of a) 0.017 mM, and b) 0.15 mM NADH

As noted in Figure 16, at 0.017 mM and 0.15 mM NADH the plots of the rates against [OAA] are hyperbolic and follow Michaelis-Menten kinetics with K_m values of 0.01 mM and 0.03 mM OAA, respectively (Figures 17a and 17b). However, the hyperbolic curve becomes sigmoidal in the presence of 0.32 mM NADH (Figure 18), which suggests that at a higher concentration, NADH allosterically regulates the catalytic activity of eMDH in the direction of OAA reduction. The Hill constant in this direction is 1.6 and the K_m value is 0.025 mM OAA (Figure 19).

- **FSBA-modified enzyme activity in the reverse direction at pH 7.2:**

This experiment was performed to examine whether the FSBA-modified enzyme will exhibit the same behavior in the reverse direction as the forward direction. The rates recorded in this experiment are summarized in Table 14. A plot of this data is presented in Figures 20a and 20b.

Table 14. Rate data from the FSBA-modified eMDH at pH 7.2
Conditions: variable concentrations of OAA, 0.32 mM NADH, 50 mM KPi buffer pH 7.2 containg 100 mM KCl and 2 mM EDTA

OAA conc. (mM)	Rate (ΔOD/min) Control eMDH	Rate (ΔOD/min) Modified eMDH
0.005	0.032	0.026
0.01	0.043	0.035
0.02	0.055	0.053
0.03	0.095	0.093
0.05	0.103	0.097
0.08	NA	0.10
0.16	0.102	0.110

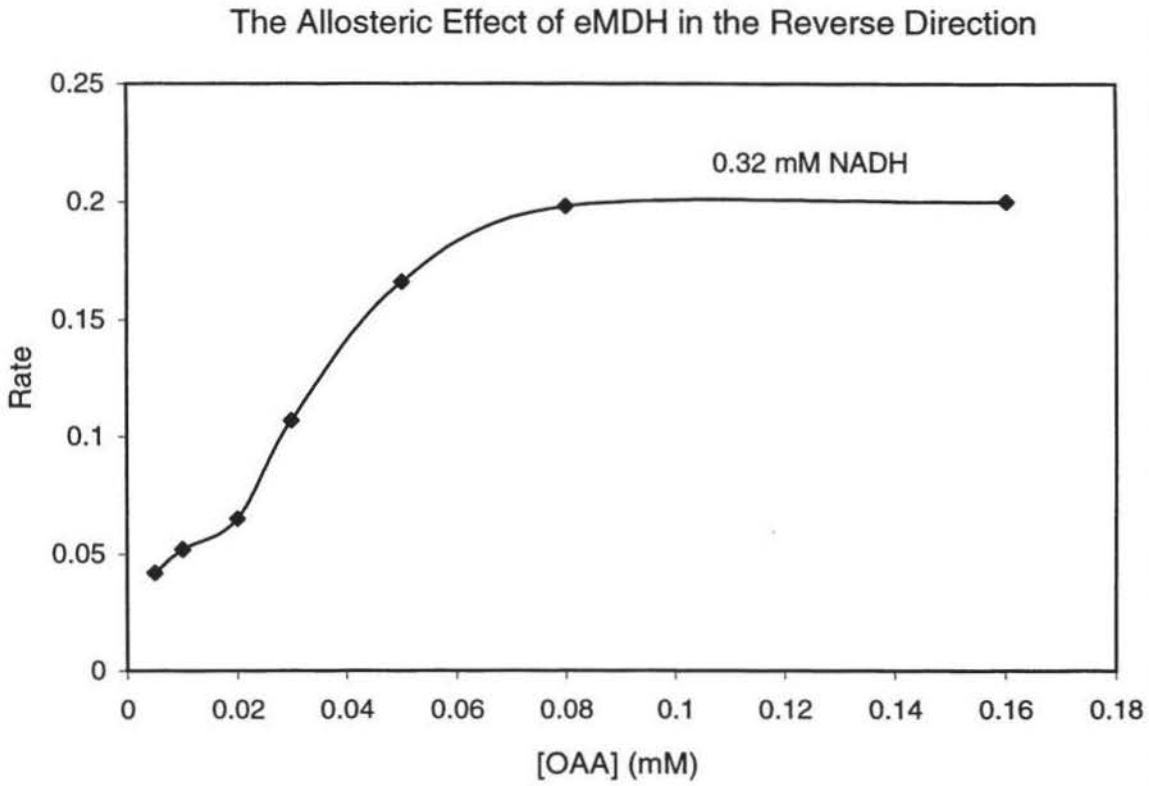


Figure 18.
Plot of rate vs. [OAA] in the reverse direction, in the presence of 0.32 mM NADH

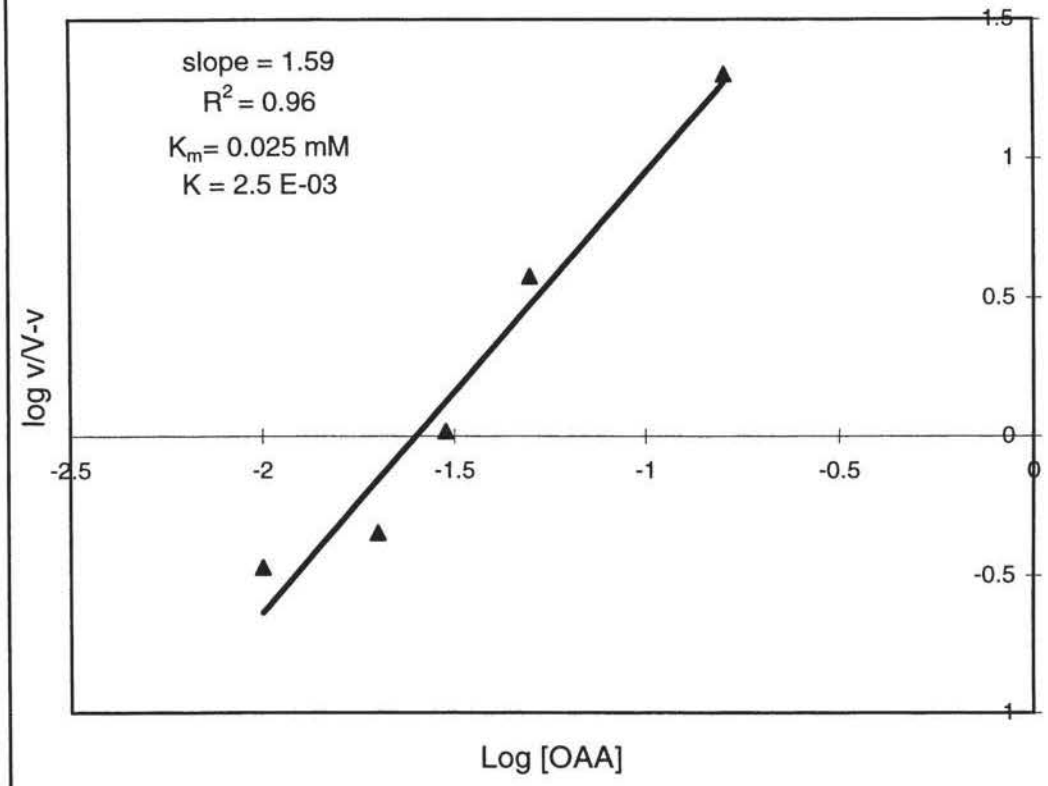
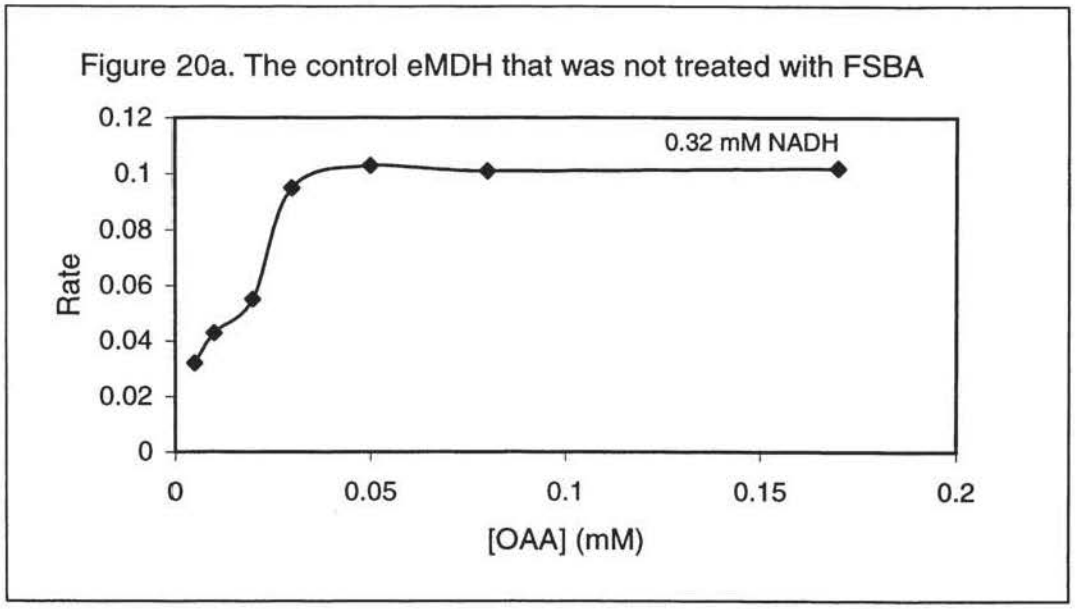


Figure19.
The Hill plot for eMDH in the reverse direction at 0.32 mM NADH

Chemical Modification with FSBA at pH 7.2



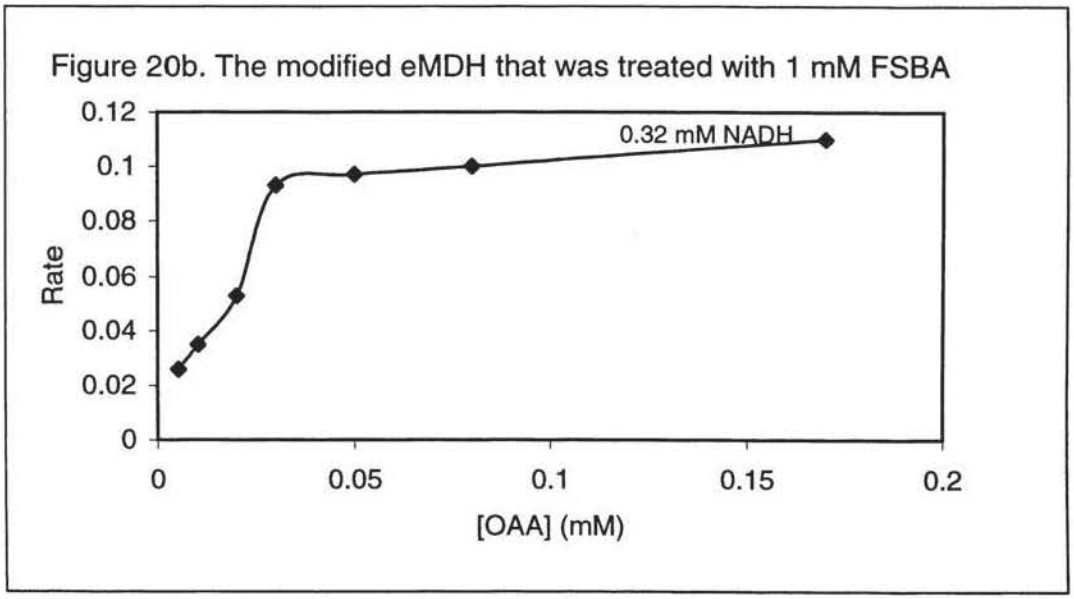


Figure 20.
Plot of rate vs. [OAA] of FSBA-modified eMDH at pH 7.2
Conditions: 0.005 to 0.16 mM OAA, 0.32 mM NADH, and 50 mM KPi buffer pH 7.2

As Figures 20a and 20b show, the allosteric effect of eMDH in the reverse direction seems to be unclear. The sigmoidal plot of rate vs. [OAA] of the control enzyme sample that was not treated with FSBA is not as distinct as the first assay (Figure 18). The fact that the reaction is much faster in this direction makes the detection of the initial rates using the UV-3100 Shimadzu spectrophotometer not reliable, particularly at low OAA concentrations. The enzyme assay at 0.32 mM NADH was performed using the stopped-flow spectrophotometer. However, the plots of absorbance vs. time were noisy since a blank of 0.15 mM NADH was not used and the change of absorbance was very small at low [OAA].

- **The results of protein lyophilization:**

The routine enzyme assay in the reverse direction was used to determine the effect of the lyophilization procedure on the specific activity of eMDH. The lyophilized protein was reconstituted in 0.6 mL D_2O. Table 15 shows a summary of this data.

Table 15. Effect of lyophilization on eMDH

	eMDH concentration (u/mL)	Protein concentration (mg/mL)	Specific Activity (u/mg)
Before lyophilization	3870	3.5	1105
First treatment	6917	6.9	1002 (90.1 %)
Second treatment	2714	4.1	662 (66.1%)

- **^{1}H NMR spectra:**

^{1}H NMR spectra were recorded using the lyophilized protein. The first spectrum that was recorded using 496 acquisitions at 21°C (Figure 21) showed a very intense water peak, which did not allow the detection of other peaks in the spectrum. Therefore, the protein was relyophilized and reconstituted in 0.6 mL fresh D_2O to minimize the exchange of deuterium with protons. The second spectrum was recorded using 10512 acquisitions to improve the signal-to-noise ratio. However, this did not reduce the intensity of the ^1H_2O peak (Figure 22). A water suppression technique in order to subtract the ^1H_2O signal needs to be used in this experiment, which will allow the observation of the other proton signals.

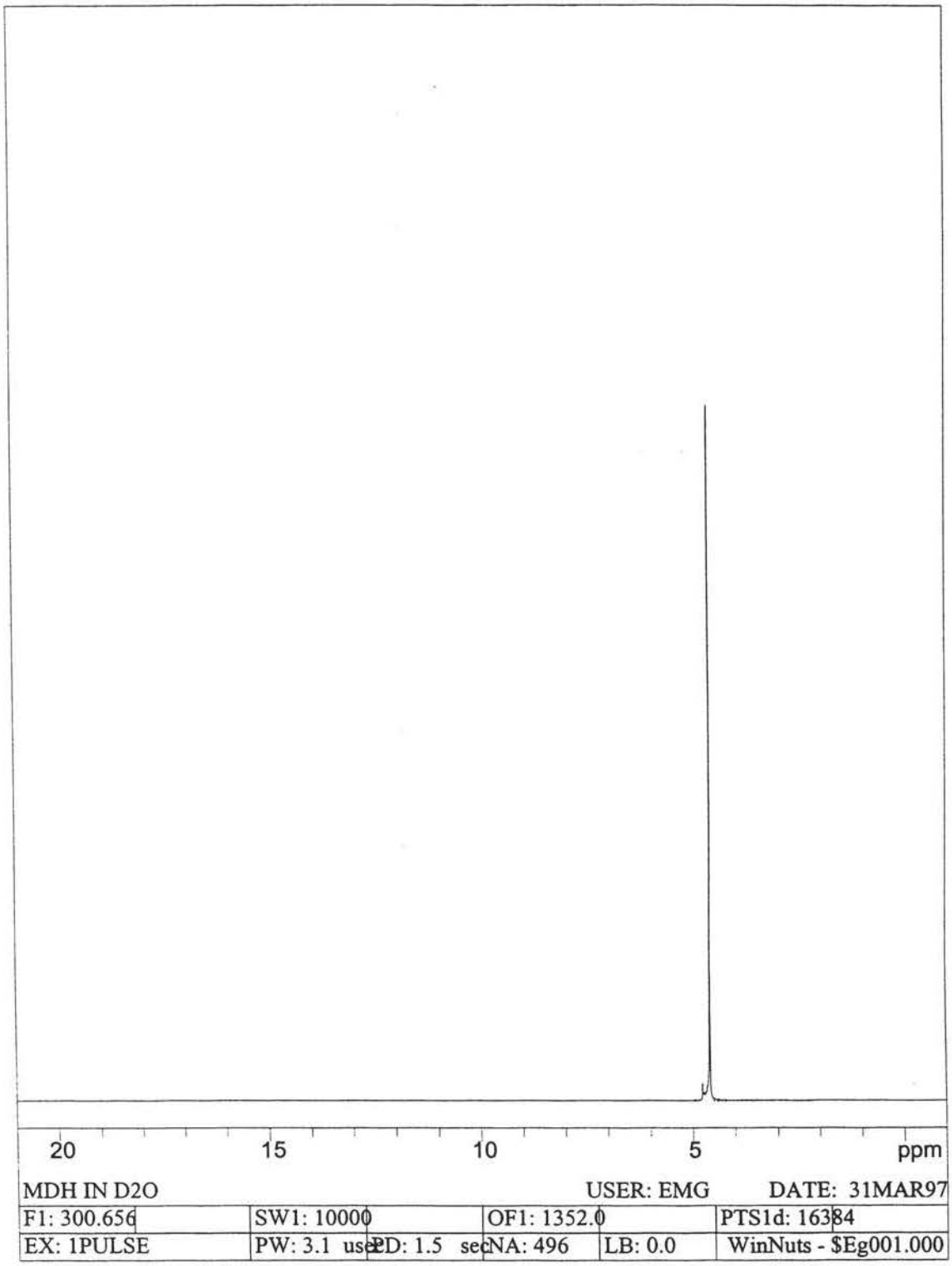


Figure 21

^1H NMR spectrum of eMDH in D_2O

The experiment was carried out using 496 acquisitions at 25°C

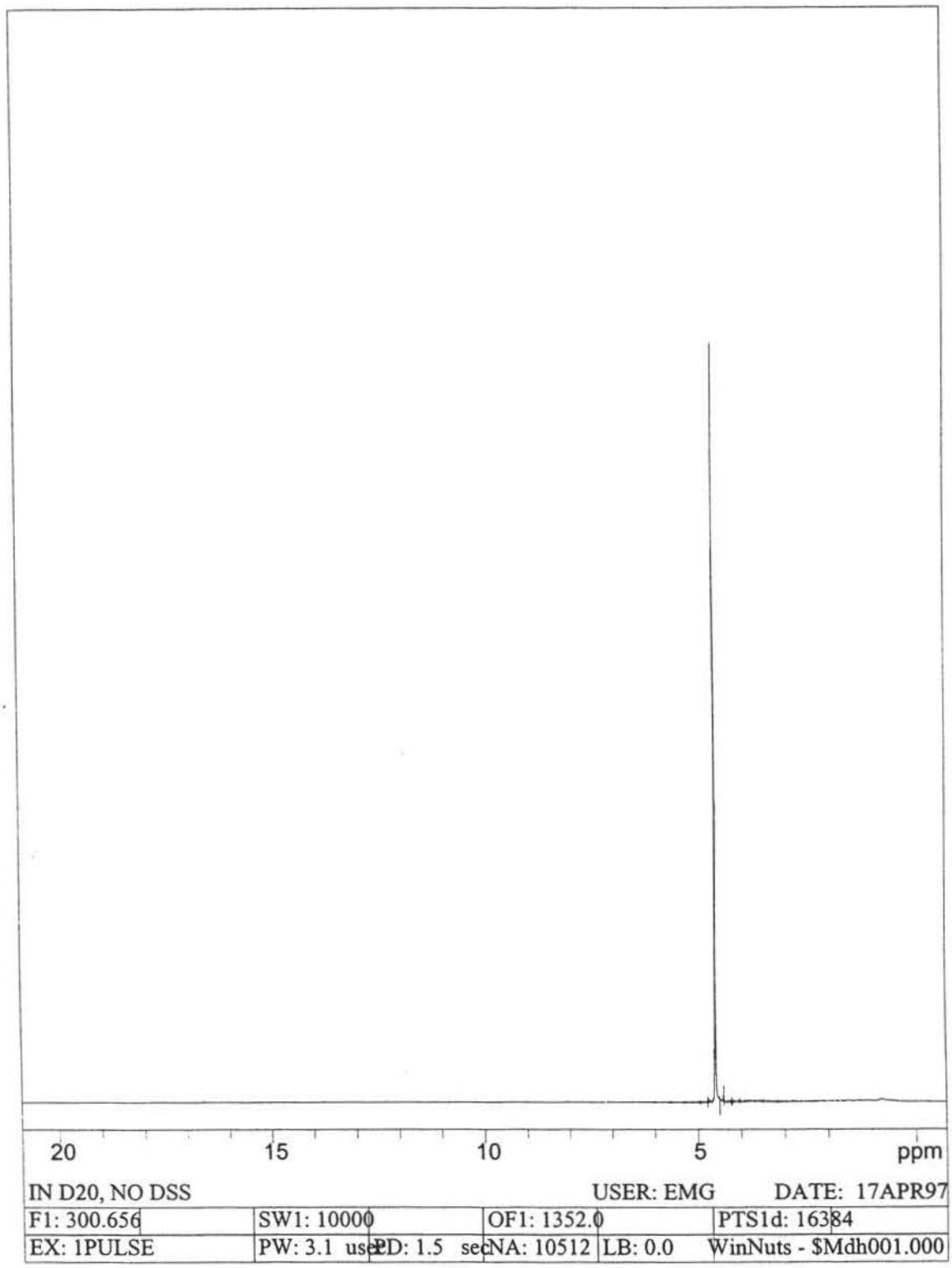


Figure 22

^1H spectrum of eMDH in D_2O

The experiment was performed using 10512 acquisitions at 25°C

Chapter IV
Discussion

The data presented in this research supports the proposal that eMDH is allosterically regulated by NADH in the direction of NAD^+ reduction. Other nucleotides including ATP, ADP, and AMP were found to bring about the same effect [7]. However, the high concentration required for this effect makes it unlikely that they are involved in the regulation of eMDH *in vivo*.

- Allosteric Effect in the forward direction:

When malate was the variable substrate in the presence of 0.065 mM NADH, a plot of v against [MAL] resulted in a sigmoidal curve, which reflects the allosteric behavior of eMDH (Figure 5). These results suggest that eMDH has an allosteric site, which is different from the active site, where NADH and possibly the other nucleotides bind and change the catalytic activity of the enzyme.

Since a plot of absorbance vs. time at low substrate concentration is linear for only few seconds, the rates recorded on the Shimadzu spectrophotometer might not be the actual initial rates. This might result in misinterpreting the data with respect to the allosteric effect since the recorded rates will be lower that the actual initial rates. Therefore, the experiment was repeated using the stopped-flow spectrophotometer, in addition to using lower concentrations of the

substrate (MAL). This data has further supported the proposal of the allosteric behavior of eMDH, since a sigmoidal curve was obtained in a plot of rate vs. [MAL] (Figure 9). Both the stopped-flow and the conventional techniques seem to be comparable, as far as the forward direction is concerned, since using lower concentrations of MAL resulted in a sigmoidal curve.

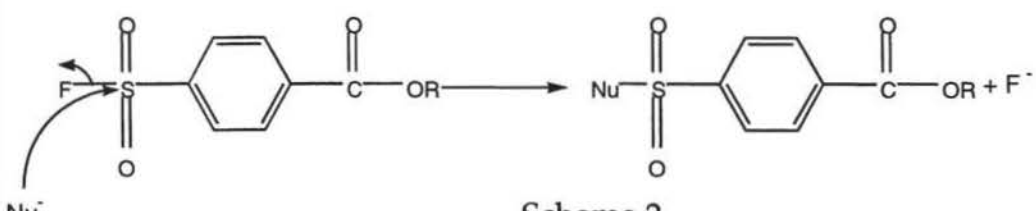
An interpretation of this data according to the MWC theory could be based on the following suggestions:

1. Binding of NADH at the allosteric site causes conformational changes in the subunits and, therefore, changes the catalytic properties of the active site. This assumption is based on the observed increase of K_m value (from 1.3 to 3.9 mM malate), which indicates a decrease in the enzyme affinity for the substrate.
2. Based on the MWC model, NADH seems to preferentially bind to the T state of eMDH and, thereby, increases the T/R ratio by inhibiting the transition to the R state.
3. Since NADH is a product of the reaction in this direction, product inhibition, in addition to the allosteric inhibition was observed. This explains the drop of V_{max} that accompanied the increase of K_m.
4. A Hill constant value of 1.2 was obtained when v/V_{max} -v was graphed against log [MAL], which suggests the presence of positive cooperativity between the allosteric and the active sites.

- The Chemical Modification at pH 9.0:

FSBA was utilized as an analog of NAD^+, NADH, ATP, and ADP in modifying the binding sites of these nucleotides in several enzymes. This compound is believed to chemically modify specific amino acid residues, including tyrosine, histidine, lysine, serine, and cysteine [16]. The sulfonyl fluoride is the reactive functional group, which acts as an electrophile that is attacked by these amino acids.

The modification process can take place by different mechanisms. Tyrosine and lysine covalently react with FSBA to yield stable derivatives [10]. When these derivatives were treated with 6 M HCl, the ester linkage between the benzoyl and the nucleoside moieties was cleaved, producing two acid-stable compounds, *N*-(4-carboxybenzenesulphonyl) lysine and *O*-(4-carboxybenzenesulphonyl) tyrosine. These two compounds are characterized by their hydophobicity and a net negative charge at neutral pH. A proposed mechanism for this type of modification is presented in Scheme 2 where Nu^- represents the amino acid residue that is being modified and R is the adenosine moiety.



Scheme 2.
A proposed mechanism for the covalent modification with FSBA

Another mechanism that does not involve covalent modification by FSBA involves the formation of a disulfide between two adjacent cysteine residues. This mechanism is believed to be involved in the FSBA-modified *S*-adenosylhomcysteinase since the inactivation was linearly related to the loss of sulfhydryl groups but none of the FSBA was covalently bound to the enzyme [17].

eMDH was treated with FSBA in order to determine whether this modification will affect the allosteric behavior of the enzyme. The results from these experiments suggest that, in the presence of 0.15 mM NADH as a protecting agent, FSBA may modify a specific amino acid at the allosteric site since the allosteric behavior was abolished (Figures 7 & 14). Since modification with FSBA is specific to tyrosine, histidine, lysine, cysteine, and/or serine, this data implies that one or more of these amino acids may be involved in binding of NADH at the allosteric site. When eMDH was treated with FSBA in the absence of NADH, approximately 50 % of the enzyme activity was lost (Figure15). This observation indicates that NADH protects the active site against the inactivation by FSBA.

Although the FSBA-modified eMDH did not exhibit the allosteric effect in the presence of NADH, it is not clear from this data whether FSBA is modifying the putative allosteric site or other amino acid residues that might interact with this site. Further experiments with respect to this modification may involve

analyzing the modified eMDH using an amino acid analysis technique to identify the residues that are modified by FSBA. In addition, incorporation of radioactive FSBA can be utilized in determining the stoichiometry of the reaction between the enzyme and the modifying agent.

- The Allosteric Effect in the reverse direction:

An allosteric effect in the direction of OAA reduction was observed at 0.32 mM NADH (Figure 16), with a Hill constant of 1.6 (Figure 17). In contrast to Sanwal's observation (the rates were measured using a Gilford 2000 recording spectrophotometer) [8], the rates recorded on the stopped-flow spectrophotometer at 0.15 mM NADH when OAA was the variable substrate were not sigmoidal, but instead followed Michaelis-Menten kinetics with K_m value of 0.03 mM OAA (Figure 15b).

- The Chemical modification at pH 7.2:

eMDH was incubated with FSBA at pH 7.2 in order to determine whether the modified enzyme will exhibit the same behavior in the reverse direction as in the forward direction. However, the sigmoidal curve in the presence of 0.32 mM NADH when OAA was the variable substrate (Figure 17a) was not as clear as data presented in Figure 16. Therefore, it could not be confirmed whether NADH allosterically regulates the reaction catalyzed by NADH in the reverse direction. Since the reaction rates are much faster in this direction, and in the presence of 0.32 mM NADH, the change in absorbance is very small particularly at low OAA

concentrations, obtaining the initial reaction rates using the routine spectrophotometers might not be reliable.

- Protein Lyophilization and ^1H NMR data:

The results obtained from the activity and protein assays of the lyophilized protein indicates that eMDH is stable under the conditions of lyophilization since only 10 % of the specific activity was lost. However, when the protein was relyophilized, the loss of the activity became more severe (34%).

The ^1H NMR spectra did not reveal any signals that might be significant because of the intensity of ^1H_2O signal. Applying a water suppression technique seems to be important in this experiment particularly at low protein concentration.

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