Introduction

Kinetics and Regulation of Mammalian MDH

This document is intended to support faculty who are interested in MDH-related CUREs using mammalian MDH. Each reviewed publication includes a summary and limited commentary. Interpretation of collections of publications are discussed as a mini review on a particular sub-topic. This work is meant to help lower the barrier in implementing a mammalian/human MDH CURE project. A complete description of the Malate Dehydrogenase CURE Community (MCC) was published recently.

Introduction

The following review article is a good place to begin a study of kinetics and regulation of MDH:

Function, kinetic properties, crystallization, and regulation of microbial malate dehydrogenase, *Takahashi-Iniguez, et al*., *(Biomed. & Biotechnol.)* (2016) 17:247. [Although this review focuses on the properties of less-well-known bacterial malate dehydrogenases, eukaryotic enzymes are discussed as well.]

Kinetics

Remarkably the Michalis-Menten kinetic data for mammalian and certainly human MDH are highly diverse depending on the pH, concentration of substrate and source. While both mitochondrial and cytosolic porcine MDH are commercially available and used for many studies, the simple kinetics of these isoforms of MDH are not easily found in the literature. Bernstein, et al., (*J. Biol. Chem*. (1978) 253:8697) demonstrated that human mitochondrial MDH is inhibited by OAA due to the formation of an abortive binary complex consisting of the enzyme and the enol form of OAA. The K_I was measured to be 2 mM at pH 7.4; at this pH, the about 15% of the OAA is in the enol form, so the K_I would be expected to be 0.3 mM.

Inhibition is dependent on substrate concentration and possibly pH. The roles of citrate, glutamate and other small metabolites on enzyme activity is not completely clear.

Abtahi, et al., (*Biomedical Research* (2017) 28:4328) measured K_M and V_{max} values of cytosolic MDH in normal and cancerous breast tumor cells. In their assays, they did not see inhibition of MDH1 by OAA. Inhibition of MDH2, but not MDH1 by OAA has been observed by others.

Table 1: Ranges of K_M values for MDH1, MDH2 and microbial MDH enzymes with substrates for both the forward and the reverse reactions

	K_M (µM)			
	OAA	**NADH**	**Malate**	**NAD$^+$**
MDH1	8-300	17-200	160-770	43-62 µM
MDH2	110-350	15-72	145-1600	60-170
Microbial MDH	22-150 to 300	14-100	170-5,000 and greater	24-100 to 300 µM

Physiological conditions for mammalian MDH studies

As pH and substrate concentration impact mammalian MDH activity it is important to keep in mind the physiologically relevant conditions used in our studies. Cardiac tissue is often used as a guide for substrate concentrations, as it is one of the most metabolically active tissues (Opie, et al., *Biochem J*. (1975) 148: 403 and Geisbuhler, et al., *Circ. Res*. (1984) 54:536). Typical concentrations of metabolites in rat heart are shown in Table 2.

Table 2: Typical metabolite concentrations in rat heart cells, by cellular location

	Metabolite concentration (µM)			
	NADH	**NAD$^+$**	**OAA**	**malate**
Cytosol	10	99	5	500
Mitochondrial matrix	800	5,800	0.3	5,000

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Cytoplasmic pH typically ranges from 7.0-7.4 but in an actively respiring or hypoxic cell the intracellular pH can drop as low as 6.2. Tumor cells activate proton transporters and other pH buffering mechanisms and may have higher intracellular pH values from 7.1-7.6. Mitochondrial matrix pH ranges between 7.0-7.4.

The kinetic parameters reported in the literature can vary widely for both isoforms. Incomplete work, as well as inconsistencies in the concentration of each substrate, the pH, and presence of allosteric regulators make it difficult to compare kinetic constants with confidence. In Table 3 we present representative mammalian kinetic constants for each MDH isoform; these values are aggregated from several published sources. All values were determined without citrate or other regulators and at pH 7.2-7.4. A range of experimentally determined values found in the literature are described in the **Kinetics** section of this document.

Table 3: Kinetic constants for mammalian MDH isoforms

	K_M (μM)			
	NAD^+	**malate**	**NADH**	**OAA**
MDH1	42	770	17	51
MDH2	60	1450	44	17

Table 4: Specific literature and database values

	K_M				Specific activity (μmol/min/mg)		Turnover (s^{-1})		
	NADH	OAA	Malate	NAD^+	Malate → OAA	OAA → malate	Malate	OAA	Reference
Human liver MDH1	17	51	770	42	102	11.2			Crow,1983
Rat liver MDH2	72	110	1600	170	380	39			Wiseman, 1991
pig MDH1		20, 8	300		182				BRENDA
pig MDH2	15, 21	350					18	377	BRENDA
Rat	50	40							BRENDA
Chicken liver MDH2	44	17	145	60					Gelpi, 1992

Kinetic characterization of malate dehydrogenase in normal and malignant human breast tissues, *Abtahi, et al., Biomed. Res. (2017) 28:4329*.

In this study, the kinetic characteristics of cMDH were determined from human breast tissue collected directly from the operating room. A summary of their results is shown in Table 5. In the paper, the authors' Table 1 shows the reaction in the forward (OAA → malate) direction and Table 2 shows the results from the reaction carried out in the reverse (malate → OAA) direction. The K_M values for the reaction in the forward direction were not significantly different in normal and tumor cells. The V_{max} for both OAA and NADH were elevated in tumor cells.

In the malate → OAA direction, V_{max} values for both substrates did not change significantly, but the K_M for both malate and NAD^+ were higher in tumor cells. The team reasoned that the higher K_M (decreased affinity) of MDH1 for OAA could lead to an increase in the pool of OAA in the cytosol, which could be

converted to pyruvate via malic enzyme, and then to lactate via LDH, to regenerate NAD^+ for glycolysis. These results are consistent with the observation that ME and LDH activities are increased in tumor cells.

Table 5: Kinetic constants as reported by Abtahi, et al., 2017.

Table 1. Kinetic parameters of MDH in forward reaction from breast tumors) and normal tissues.

Kinetic parameters	Groups	
	Tumor	Normal
S 0.5 OAA (mM)	0.2 ± 0.009	0.23 ± 0.007
S 0.5 NADH (mM)	0.16 ± 0.007	0.2 ± 0.005
V_{max} OAA (mU/g)	4509.8 ± 88*	2456 ± 46
V_{max} NADH (mU/g)	4233.3 ± 111*	2549 ± 37

Table 2. Kinetic parameters of MDH in reverse reaction from breast tumors and normal tissues.

Kinetic parameters	Groups	
	Tumor	Normal
Km malate (mM)	3.13 ± 0.4*	1.6 ± 0.2
Km NAD+ (mM)	0.63 ± 0.05	0.43 ± 0.06
V_{max} malate (mU/g)	78 ± 2.13	75 ± 2.7
V_{max} NAD+ (mU/g)	110 ± 3.32	102 ± 4.4

Some molecular and kinetic properties of heart malic dehydrogenase, *Wolfe and Neilands*, *J. Biol. Chem.* (1956) 221:61.

An early published work on purification and kinetic characterization of MDH from pig heart. Note that the kinetic studies were performed on enzyme isolated from tissue acetone powder, followed by a series of extractions with various solvents. No fractionation was perfomed during the purification process, which means that the MDH activity measured is either a mix of MDH1 and MDH2, or is enriched in one isoform or the other. They reported a turnover number in the malate → OAA direction as 9700 mol of DPN/mol enzyme/min (DPN = NAD^+) K_M and V_{max} were influenced by pH. But the buffers may have influenced the regulation of the enzyme as well as cross contamination with both mitochondrial and cytosolic enzymes. At pH 7.4 they report a K_M (NADH) of 4.2 µmol and a V_{max} of 3.8×10^{-4} µmol substrate/mol protein/min.

Malic dehydrogenase. II. Kinetic studies of the reaction mechanism, Raval and Wolfe, Biochemistry (1962) 1:263.

Using a more purified version than their earlier studies, but still extracting the enzyme from an acetone powder, the kinetics of porcine heart MDH were reported at pH 8.0 as shown in Table 6.

Table 6: Kinetic constants reported by Raval and Wolfe, 1962

	Malate → OAA (forward)		OAA → Malate (reverse)	
	NAD^+	Malate	NADH	OAA
K_M (µM)	200	800	18	40
V_{max} (mol substrate/mol enzyme/min)	1×10^4		3.5×10^4	

Malic dehydrogenase. III. Kinetic studies of the reaction mechanism by product inhibition, *Raval and Wolfe*, *Biochemistry (1962) 6:1112.*

Using the same preparation of pig heart MDH, Wolfe's group provides evidence for the formation of a ternary complex of enzyme-coenzyme-substrate, in which the coenzyme binds to the enzyme first, followed by the substrate. Upon completion of the reaction, substrate is released first, followed by coenzyme. They use this model to explain observed decreases in initial rates in the presence of products. When NADH is present in the reaction medium, the initial rate of the reaction in the malate → OAA direction decreases. Similarly, when NAD^+ is present in the reaction medium, the reaction in the OAA → malate direction decreases. They propose that the product could compete with the substrate for binding to the active site, and that an inactive binary complex forms between the enzyme and the coenzyme. They do not observe this when malate or OAA are

added as product inhibitors but speculate that perhaps their malate and OAA concentrations were too low, and if the concentrations were higher, that an inactive ternary complex (enzyme-coenzyme-product) could form.

Malic dehydrogenase. V. Kinetic studies of substrate inhibition by OAA, *Raval and Wolfe, Biochemistry (1963) 2:220.*

Pig heart MDH was purified as described previously. In this set of experiments, the authors examined inhibition by OAA. Lineweaver-Burk plots for OAA concentrations below 200-250 mM show that Michaelis-Menten kinetics apply. At concentrations higher than 250 mM, inhibition by OAA is observed, and since there is a change in both the slope and the y-intercept, the authors label this a "mixed" inhibition. This is one of the first papers published which describes this phenomenon for mammalian MDH. The authors suggest that the inhibition is competitive (with OAA competing for binding to the enzyme with NADH) when an enzyme-OAA inactive binary complex forms, and uncompetitive when an inactive enzyme-NAD-OAA complex forms.

Beef heart malic dehydrogenases. V. A kinetic study of the reaction catalyzed by the supernatant enzyme, *Cassman and Englard, J. Biol. Chem. (1966) 421:793.*

This team, using cytosolic beef heart MDH, finds a different reaction mechanism for MDH. They propose that substrate binding is random, rather than the ordered binding reported by Raval and Wolfe. They suggest that the Wolfe group's enzyme preparation was enriched in mitochondrial MDH. Cassman and Englard's kinetic results support the formation of a binary, not a ternary complex. They identify three different binary complexes: E-OAA, E-NADH and E-NAD^+ and suggest that reaction pathway proceeds through an enzyme-coenzyme binary complex. This team used a significantly higher pH of 8.0 in their kinetic assays and a higher ionic strength than previous studies, which could impact the results. They demonstrate that inhibition of cMDH by OAA is pH dependent (at pH values < 7.8); K_M values were reported as 4 µM at pH 6.4 and 20 µM at pH 8.3. In 1972, Holbrook and Wolfe showed that both cMDH and mMDH bind one NADH per subunit (there had been some disagreement about this stoichiometry in the past) and that both isoforms bind NADH less tightly as pH increases from 6.3 to 9.3. In 1974, Vetterlein and Cassman provide evidence for a phosphorylated MDH with different kinetics and decreased sensitivity to allosteric regulators than the unphosphorylated form.

Malic dehydrogenase. VI. A kinetic study of hydroxymalonate inhibition, Harada and Wolfe, *J. Biol. Chem. (1968) 243:4123*.

Pig heart MDH (likely mixed isoforms, based on their isolation method) is inhibited by hydroxymalonate, a malate analog. α-Ketomalonate is an OAA analog, but this study found that MDH could use α-ketomalonate as an alternative substrate, and reduce it to hydroxymalonate, but the opposite reaction (oxidation of hydroxymalonate to α-ketomalonate) did not occur. Hydroxymalonate inhibits MDH uncompetitively with respect to malate and competitively with respect to OAA; these results are consistent with the explanation that hydroxymalonate binds to E-NADH but not to E-NAD^+.

Kinetic studies on the mechanism of the malate dehydrogenase reaction, *Heyde and Ainsworth, J. Biol. Chem. (1968) 243:2413*.

This research purified bovine heat mitochondrial MDH and investigated the mechanism of the enzyme at pH 8. Their rate constants show that the simplest "ordered bi-bi" mechanism does not apply; instead they argue for a more complex bi-bi model in which the enzyme-oxidized coenzyme complex, forms, then isomerizes. The kinetic constants reported by the team are shown in Table 7.

Table 7: Kinetic constants reported by Heyde and Ainsworth

Substrate	K_M (µM)
NADH	260
OAA	80
Malate	386
NAD^+	60

Malate dehydrogenase. XII. Initial rate kinetic studies of substrate activation of porcine mitochondrial enzyme by malate, *Telegdi, et al., J. Biol. Chem.* (1973) 248:6484*.*

This research team shows that mitochondrial pig heart MDH when the assay is carried out in the malate → OAA direction, is activated by the malate substrate (at concentrations at or above 30 mM). They note that this concentration is well above the K_M for malate (5.6 mM) and suggest that this is evidence for a second malate binding site, in which malate binds to the enzyme other than the active site, and in so doing promotes a conformational change in the enzyme that results in an increased affinity of NAD^+ for the enzyme. This is supported by their data which indicates a decrease in the K_M for NAD^+ binding by 10-fold.

Kinetic studies on pig heart cytoplasmic malate dehydrogenase*, Frieden and Fernandez-Sousa, J. Biol. Chem.* (1975) 250:2106.

In this kinetic study, the authors use cytoplasmic pig heart MDH to study the mechanism of the enzyme. The authors discuss their results in detail, in context with previous studies. They show that at low substrate concentration (the reaction is run in the OAA → malate direction), the reaction proceeds by an ordered sequential mechanism in which the coenzyme binds to the enzyme first, followed by the substrate. The substrate is converted to product and is released, and the coenzyme is released second. The authors used their kinetic data to identify the rate determining step of the reaction, which they concluded involves either (1) a conformational change of the E-NADH complex, followed by rapid dissociation of NAD^+, or (2) a slow dissociation of NAD^+. They report that the K_M for NADH is independent of pH (the K_M ranges from 4-6 µM over a pH range of 6.5-8.5) while for OAA, the K_M increases (indicating decreased binding affinity) when pH increases. They did not find that NADH allosterically activates the unphosphorylated form of MDH1 as reported by Cassman and Vetterlein, 1974. However at high saturating levels of NADH, a 2-fold activation of the enzyme by OAA was observed.

Malate dehydrogenase. Kinetic studies of substrate activation of supernatant enzyme by L-malate, *Mueggler and* Wolfe, *Biochemistry* (1978) 17:4615.

In this study of pig heart cytosolic MDH, the authors collected kinetic data that demonstrated activation of MDH by the substrate malate (the reactions were carried out in the unfavorable malate → OAA direction). Activation of the enzyme by malate was not observed at malate concentrations of 20-150 µM, but was observed at concentrations of malate from 150-500 µM. Above 10 mM malate, inhibition was observed. They suggest that an allosteric binding site for malate exists. They claim that their data support an ordered bi-bi mechanism. They further suggest a mechanism that they term “reciprocating compulsory order mechanism” involving negative cooperativity (see Fig. 1). The non-activated form of the enzyme is proposed to carry out catalysis on a single subunit. The activated form of the enzyme coordinates the activities of both subunits. The authors argue that the transition from the non-activated to activated form does not involve NAD^+. Instead, the conversion correlates with the concentration of malate. If the malate concentration is low, it binds to a high-affinity site on one subunit and catalysis occurs. But at high concentrations of malate, malate is able to bind both to the high affinity site and to a low affinity binding site on the second subunit. A 3-fold increase in binding affinity for NAD^+ is observed and conversion from “non-activating” or “half active” to “activating” or “fully active” occurs.

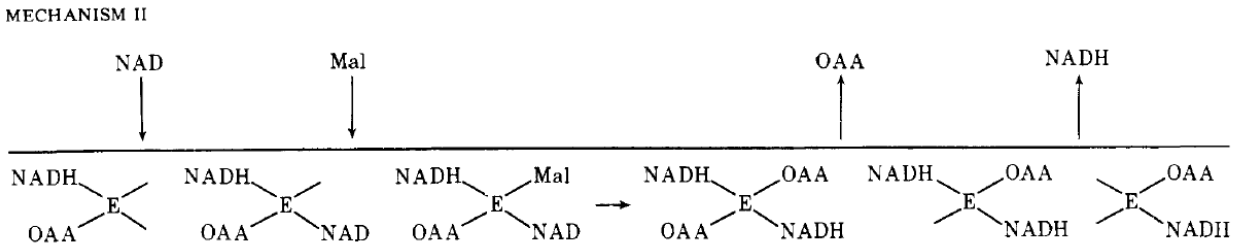

Figure 1: The reciprocating compulsory order mechanism proposed by Mueggler and Wolfe.

Introduction

Kinetics and Regulation of Mammalian MDH

Regulation

MDH activity is regulated by substrates and other small molecules including citrate, α-ketoglutarate, glutamate, and others. The inhibition or activation is both competitive and allosteric. Depending on the organism or isoform, select inhibitors may have an impact on either or both directions of the MDH reaction. OAA inhibits mitochondrial MDH but there is conflicting evidence if that is the case for cytosolic MDH. The pH of buffers used can decrease the affinity of substrates for MDH (a higher pH seems to result in a higher K_M), and that at some pHs and concentrations of substrates, OAA inhibition is not observed. At higher levels, malate increases MDH affinity for NAD^+. There is argument in the literature on how malate, OAA, citrate or other inhibitors impact MDH. Some research teams argue that their data support a model in which one subunit plays a regulatory role (binding citrate and perhaps other ligands) while allosterically acting on the second subunit, which is catalytically active. Others argue that binding sites for OAA and citrate exist and that MDH has two conformations similar to the tight / relaxed conformations of hemoglobin. In this model, both subunits are active. Further investigation is needed that reconciles the kinetic data with the structural data of mammalian MDH in various states bound to substrate. Also needed is additional data in which potential allosteric and regulatory sites differ between the mitochondrial and cytosolic forms of MDH. In the next section, we focus on classic key publications describing MDH kinetics and regulation.

Substrate inhibition of the mitochondrial and cytoplasmic malate dehydrogenases, *Bernstein, et al., J. Biol. Chem.* (1978) 253:8697.

Using commercial preparations of porcine heart MDH1 and MDH2 the authors describe the substrate inhibition of mitochondrial MDH. Maximal activity was observed at 300 µM OAA (140 µM NADH). At concentrations of OAA 400 µM and higher, a significant loss in activity was observed with a 60% loss in activity at 500 µM OAA. The authors measure the K_I values for OAA and report a K_I for MDH2 of 2.0 mM and a K_I for MDH1 of 4.5 mM (although they do note that physiological concentrations of OAA are on the order of 10 µM). They suggest that OAA acts as an inhibitor because of its ability to form an abortive ternary complex, E-NAD^+-OAA, in which OAA is in its enol form. Using Lineweaver-Burk analysis, the K_M for mitochondrial MDH with OAA was determined to be 40 µM, which was similar to the value of 38 µM obtained with chicken heart mitochondrial MDH. The K_M for cytosolic MDH is 30 µM.

Commentary: The results reported by Bernstein, et al. differ from those reported by Abtahi, et al. The latter team report that both K_M values for substrates malate and NAD^+ are 3-5 times higher in cancerous breast tissue and as evidenced by the graphs in the Abtahi publication, no OAA inhibition was observed. Abtahi et al. used 500 µM NADH when determining OAA kinetics at pH 8, whereas Bernstein et al. performed OAA kinetics at 140 µM NADH at pH 7.5. There is no indication that the higher NADH concentration was able to drive the difference in kinetic results, but it is possible. It should also be noted that the breast tissue was assayed in crude cytosol and not as a purified enzyme preparation. As described by Dasika et al. in the Mechanism section, the reaction in the forward rate for mitochondrial MDH is highly pH dependent with a reduction in activity when the pH is greater than 7.5 and a significant loss of activity at pH values greater than 8.0. NAD^+, when present at the start of the reaction, inhibits 20-60% of the activity of mitochondrial MDH at NAD^+ concentrations ranging from 12-16 mM NAD^+. NADH does not inhibit the reaction.

Regulation of mitochondrial malate dehydrogenase. Evidence for an allosteric citrate-binding site, *Mullinax et al., J. Biol. Chem.* (1982) 257:13233.

Porcine heart mitochondrial and cytosolic MDH were used for this work. When conducting the assay in the OAA → malate direction (the favorable direction, that normally occurs in the cytosol), the team used 200 µM NADH and 250 µM OAA. When the assay was conducted in the malate → OAA direction (the unfavorable direction that normally occurs in the mitochondrial matrix), they used 10 mM malate and 5 mM NAD^+. All assays were carried out at pH 8.1.

Measuring the MDH activity of both isoforms in each direction in the presence of 1-60 mM citrate, cytosolic MDH activity was inhibited by 15% in the malate → OAA direction and 60% in the OAA → malate at 25-50 mM citrate. Likewise, an even greater inhibition was observed for mitochondrial MDH in the OAA → malate direction at even lower citrate concentrations. However when mitochondrial MDH activity was measured in the malate → OAA direction, instead of inhibition, the MDH activity was increased by 170-180% of controls at 50-100 mM citrate. Essentially, citrate acts to encourage the reaction to proceed in one direction and not the other. The ability of one isoform to react in the presence of citrate indicates this is not a competitive inhibition but an allosteric regulation taking place at a site distal to the substrate binding site. Citrate binds to each monomer and reduces the affinity of each MDH monomer for NADH via some conformational shift to a conformation of MDH that favors NAD^+ binding. Additionally, citrate does not bind the OAA site, but at high concentrations of OAA (inhibitory for MDH) citrate binding to MDH is diminished. The authors argue that OAA and citrate do not share a binding site, with OAA having a low affinity (high K_M) for this binding site acting as a feedback inhibitor. A comparison of citrate and OAA structures may help inform this result. Malate at 20 mM or greater concentrations is an activator for MDH activity and may bind to two different sites, an active and a regulatory binding site. Both citrate and malate enhanced mMDH dimer formation at low pH values (less than 6.5) where the protein usually assumes the monomer form. The K_d for citrate binding is reported as 12.5 µM. The authors note that citrate concentrations in vivo range from 1.7 – 8.75 mM (and in a localized microenvironment may even be higher), so the citrate concentrations used in this vitro experiment could be relevant in vivo. They also revisit the results of Telegdi, et al., and suggest that instead of malate having two different binding sites, there may be a competition between malate and NADH for binding, not to the same site on the enzyme, but to different conformations of the enzyme. Malate at activating concentrations could bind to the citrate regulatory site, a site that exists only on the conformation of the enzyme that is able to bind to NAD^+; this would be conformation E in Figure 2.

Regulation of mitochondrial malate dehydrogenase. Kinetic modulation independent of subunit interaction, *McEvily, et al., Arch. Biochem. Biophys.* (1985) 238:229.

This study, also from the Mullinax group (see above) reports results with porcine mitochondrial MDH to further characterize citrate binding. They chemically modified histidines by iodoacetylation to inhibit the His in the active site, then they constructed heterodimers consisting of one active monomer and one chemically modified inactive monomer. Using Scatchard plot analysis, they found that citrate binds to the heterodimer with a K_d of 100 µM and to the native homodimer with a K_d of 75 µM. The heterodimer showed the same kinetics and the same activation/inhibition profile by citrate as the active homodimer even though in the heterodimer, only one catalytic site was active and only one site could bind citrate. K_M and V_{max} values for both dimers were essentially the same. Regulation by OAA and citrate were also similar for the homo- and hetereodimers. They "reinterpret" their work to show the binding of citrate is independent of interactions between MDH subunits. They also carry out experiments with TTFA, an NADH analog. They propose that TTFA at low concentrations binds to an allosteric site and activates the enzyme. At high concentrations of TTFA, the TTFA may bind to an allosteric site similar to the citrate binding site that is only available in the NAD^+-binding conformation of the enzyme (conformation E in Fig. 2), resulting in inhibition of the enzyme.

Commentary: Experiments involving heterodimers constructed with site-directed mutants of MDH would be interesting. One way to prepare the heterodimers would involve denaturing the monomers and then refolding them in a mixture that includes both wild-type and mutant monomers. Isolating the resulting heterodimers would be difficult; perhaps this could be done using different tags for each monomer?

Some kinetic characteristics of immobilized protomers and native dimers of mitochondrial malate dehydrogenase: An examination of the enzyme mechanism, *DuVal, et al., Biochemistry* (1985) 24:2067.

In this study, the research team immobilized pig heart mMDH and showed that the immobilized enzyme displayed the same kinetic behavior as the non-bound enzyme, in terms of K_M values and inhibition patterns. They report the following K_M values for the commercial prepared enzyme: 38 µM (NADH), 18 µM (OAA), 230 µM (NAD^+) and 370 µM (malate); similar values were reported for the immobilized enzyme. They observed

substrate inhibition by OAA, competitive inhibition by hydroxymalonate when α-ketomalonate was the substrate and uncompetitive inhibition with hydroxymalonate when malate was the substrate. They concluded that the model in which subunits interact in a reciprocating mechanism, as described by Harada and Wolfe, 1968, was not correct and that a mechanism in which there is an equilibrium state between two different conformations, as described by Mullinax et al. (see Fig. 2) is more consistent with their data as well as previously reported data.

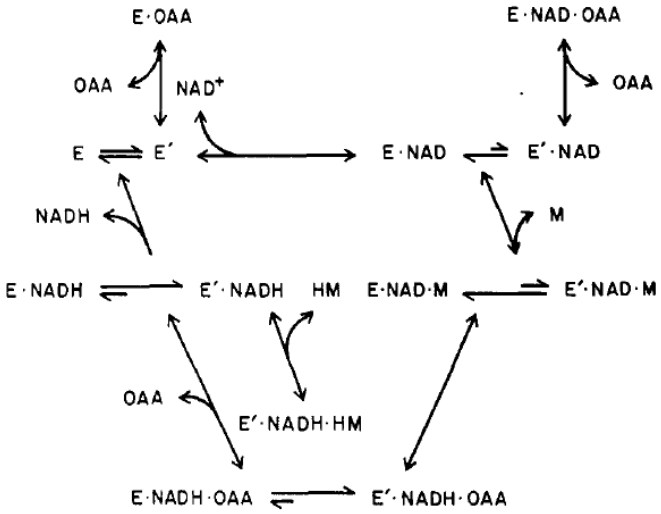

Figure 2: A catalytic mechanism proposed by DeVal, et al., in which MDH exists in two conformers, one which preferentially binds NAD^+ (E) and one which preferentially binds NADH (E'). (@ 1985, The American Chemical Society. All rights reserved.)

Regulation of malate dehydrogenase activity by glutamate, citrate, α-ketoglutarate and multienzyme interaction, *Fahien et al., J. Biol. Chem.* (1988) 263:10687.

The authors used purified rat liver mitochondrial enzyme and commercially purchased pig heart mitochondrial enzyme for this study. Their goal was to study MDH kinetics when MDH binds to AAT and α-ketoglutarate dehydrogenase as part of a ternary complex, and to investigate the effects of metabolites such as citrate and others on the activities of the individual enzymes that form the complex. The focus is on mMDH, which uses malate as a reactant and produces OAA as a product. The other enzymes in the ternary complex can either provide or compete with substrate, or can whisk away product as it is formed. For example, as the MDH-catalyzed reaction is unfavorable in the malate → OAA direction, it's important to keep OAA concentrations low so that the reaction isn't reversed. CS and AAT can assist in this regard, using OAA as a reactant immediately as it is formed. Kinetic data obtained by the team are shown in Table 8.

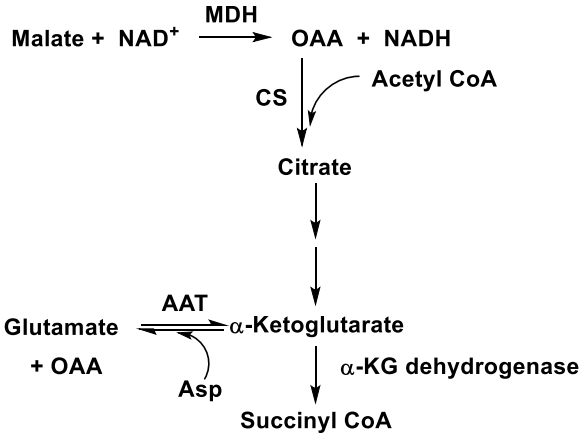

Figure 3: Three enzymes forming a ternary complex as studied by Fahien, et al.

Table 8: Kinetic constants for selected enzymes. K_i and K_i' values were determined in the presence of citrate.

Enzyme	Kinetic constant		
	K_i (mM)	K_i' (mM)	K_M (μM)
Citrate synthase	0.40	∞	7
MDH (malate → OAA)	0.84	1.8	420
Fumarase	1.0	∞	330
AAT	High	High	

A summary of the team's findings:

- Citrate competes with fumarate in the fumarase reaction and with OAA in the citrate synthase reaction, as shown in Table 8. Not shown are other metabolites, such as isocitrate, which produced < 20% inhibition.
- Citrate and α-ketoglutarate inhibit MDH, noncompetitively.
- High concentrations of malate activate MDH and abolish inhibition by citrate at pH 8. This supports the existence of an allosteric binding site shared by malate and citrate.
- At pH 7.0, binding of citrate to the allosteric site has a high affinity (citrate binds to MDH 6-fold greater at pH 7.0 than at 8.0), and decreases binding of both malate and NAD+ (the reactants in the MDH reaction). Differences observed at pH 7 and pH 8 could be explained by MDH assuming a different conformation at different pH values. [**Commentary:** As the pH of the mitochondrial matrix is 7.2-7.4, citrate may play a significant role in the regulation of MDH. If this occurs via a conformational change, protein-protein interactions may be impacted.]
- At pH 8.0 and at low (1.0 mM) levels of malate, glutamate (4.0 mM) is an activator and α-KG (4.0 mM) slightly inhibits MDH. These effects are reversed when the concentration of malate is increased to 10 mM. At pH 8.0, it is proposed that α-KG binds to a regulatory site. Glu activates MDH at pH 8.0 but acts as a competitive inhibitor (with malate) at pH 7.0. Glu could have a higher affinity for the regulatory site at pH 7.0. The authors argue that at pH 7, metabolic conditions exist in which Glu, citrate and α-KG could each be inhibitors of mMDH activity in the malate → OAA direction by 50-90%. [**Commentary:** The authors propose that binding sites on the enzyme bind various metabolites, under different conditions of pH and metabolite concentrations, but it is not clear whether the binding sites are the same as binding sites for malate and OAA, or different. Crystal structures of the enzyme with these metabolites could clarify this.]
- Adding CS did not alter MDH inhibition by citrate or α-KG, yet the presence of pyruvate dehydrogenase (PDH) or α-ketoglutarate dehydrogenase (α-KGDH) increased MDH activity even when inhibitory concentrations of glutamate were present.
- MDH associates with either the CS-α-KGDH complex, or the AAT-α-KGDH complex (but not both), and in each case, MDH activity increases. Cytosolic AAT is not bound to either dehydrogenase, indicating possibly that both dehydrogenases are part of the complex and only one of the MDH partners can bind at a time because of competition for a binding site?
- Citrate and α-KG both block MDH-CS interactions.
- Citrate blocks CS from binding to an MDH-PDH complex or an MDH-α-KGDH complex, but Glu does not.

Physiological significance:

Formation of an α-KGDH-MDH-AAT complex: The complex places MDH and AAT in close proximity which could facilitate direct transfer of OAA from MDH to AAT, which would decrease the local concentration of OAA and thus minimize OAA inhibition of MDH. This could also enable Glu to react with AAT and prevent Glu from inhibiting MDH. And α-KG from AAT could be transferred directly to α-KGDH and prevent α-KG from inhibiting MDH. In a ternary complex, MDH is activated. A ternary complex could also favor AAT activity over CS activity, because the product of the α-KGDH reaction is succinyl-CoA, which inhibits CS. Inhibition of CS results in low levels of citrate, which would then not be present to inhibit MDH and cause MDH to dissociate from the ternary complex.

Dissociating the α-KGDH-MDH-AAT complex: When citrate and α-KG levels are high and the α-KGDH complex is well supplied with substrate, the ternary complex is no required and dissociates and is inhibited.

Direct inhibition of MDH, CS and fumarase: Citrate inhibits all three of these enzymes (see Table 1) and α-KG inhibits MDH. Thus citrate and α-KG could inhibit MDH activity both by direct inhibition and by influencing whether or not MDH forms a complex with other mitochondrial matrix enzymes.

Commentary: The involvement of PDH or α-KGDH was not followed up in other later studies and might have been an anchoring point for aggregation or interaction or as a protein general buffer, as any time more than two proteins were involved there was a significant increase in MDH precipitation. BSA or other proteins should be used as a control in other experiments.

Introduction

Kinetics and Regulation of Mammalian MDH

Kinetic studies of the regulation of mitochondrial malate dehydrogenase by citrate, *Gelpi, et al., Biochem. J.* (1992) 283:298.

This team used both chicken liver and pig heart mMDH to conduct a rigorous examination of the impact of citrate on MDH kinetics at pH 8.1. They measured the activity of mMDH in the presence of increasing concentrations of citrate in both directions of the reaction, while varying the substrate concentration. Their results indicated that citrate both activates and inhibits mMDH, in both directions, depending on substrate concentration. Their results indicate that:

- mMDH follows an ordered bi-bi mechanism, in which the coenzyme binds first and is released second, when citrate is not present, according to Banaszak and Bradshaw.
- mMDH is activated by high concentrations of its substrate malate (Telegdi, et al and Mullinax et al.)
- Citrate inhibits the reaction in the OAA → malate direction independently of the concentration of NADH, with citrate acting as a competitive inhibitor of NADH and a mixed inhibitor with respect to OAA.
- Citrate activates the reaction in the malate → OAA direction at any concentration of malate, and when the NAD+ concentration (5 mM) is high; the activating effect at high concentrations of malate is abolished;
- Citrate inhibits the reaction in the malate → OAA direction when the concentration of NAD^+ is low (0.2 mM); no activation by malate is observed and citrate appears to act as a competitive inhibitor. Citrate appears to interfere with the ordered bi-bi mechanism.
- At an intermediate concentration of NAD^+ (1 mM), citrate inhibits mMDH at low malate concentrations and activates mMDH at high concentrations of malate. Citrate appears to interfere with the bi-bi mechanism.
- The enzyme is inhibited by excess OAA, independent of NADH concentration.

In order to explain their data as well as data collected by other, the authors proposed that there is a common regulatory site for OAA, citrate and malate, and that these metabolites are able to shift the conformation of MDH to either one of two confomers that they label E and F in Figure 3. Kinetic data for both conformations is shown in Table 9. There was about a 25-fold increase in the K_M values for NAD^+ and malate between conformations E and F whereas K_M values for NADH and OAA are unchanged in the presence of citrate.

Table 9: Kinetic data for conformations E (K_M) and F (K'_M) for mMDH for the metabolites listed, in the presence of citrate.

K_M and K'_M values (μM)			
Conformation E		**Conformation F**	
K_M (NAD^+)	60	K'_M (NAD^+)	1450
K_M (malate)	145	K'_M (malate)	4750
K_M (NADH)	44	K'_M (NADH)	38
K_M (OAA)	17	K'_M (OAA)	12

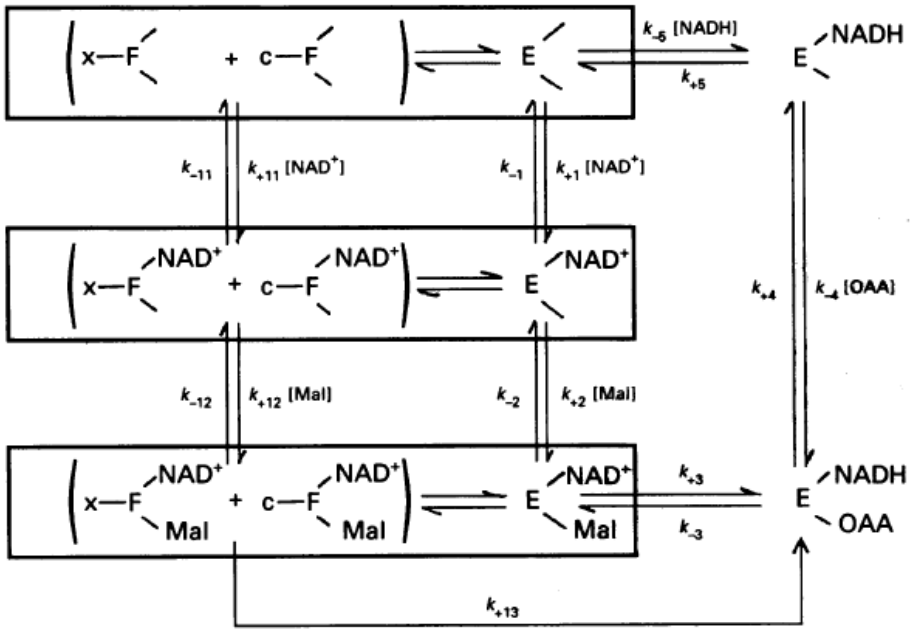

Figure 4: Kinetic mechanism for mMDH proposed by Gelpi, et al. Conformation E catalyzes the reaction in both directions. Conformation F has decreased substrate binding affinity but greater catalytic efficiency and catalyzes the reaction in the malate → OAA direction because the affinity of this conformation for NADH is too low. Conversion from the E to the F conformation occurs when either malate or OAA bind to allosteric sites. In the diagram, C = citrate and X = either malate or OAA.

Appendix

Table A1: Cellular enzymes important in the study of MDH1 and MDH2

Enzyme name	Abbreviation	Alternative names or abbreviations	Cellular location	UniProt ID	UniProt accession number
NADP dependent malic enzyme	ME1		Cytosol	MAOX_HUMAN	P48163
Glutamate oxaloacetate transaminase	GOT1	aspartate transaminase (cAST, AST1) aspartate aminotransferase (cAAT, AAT1)		AATC_HUMAN	P17174
ATP citrate lyase	ACL		Cytosol	ACLY_HUMAN	P53396
Phosphoenol-pyruvate carboxykinase	PCK1, cPEPCK		Cytosol	PCKGC_HUMAN	P35558
Citrate synthase	CS		Mitochondrion	CISY_HUMAN	O75390
Glutamate oxidase transaminase	GOT2	aspartate transaminase (mAST, AST2) aspartate aminotransferase (mAAT and AAT2)	Mitochondrion	AATM_HUMAN	P00505
NAD dependent malic enzyme	ME2		Mitochondrion	MAOM_HUMAN	P23368
NADP dependent malic enzyme	ME3		Mitochondrion	MAON_HUMAN	Q16798
Fumarase	FH		Mitochondrion	FUMH_HUMAN	P07954
Aconitase	ACO2		Mitochondrion	ACON_HUMAN	Q99798
Phosphoenol-pyruvate carboxykinase	PCK2, mPEPCK		Mitochondrion	PCKGM_HUMAN	Q16822