Malate Dehydrogenase CUREs Community – Mammalian Cytosolic MDH
Regulation, Interactions & Involvement Metabolism/Disease: Mini Review and Literature Summary

Purpose of this document: This document is intended for support faculty who are interested in MDH related CUREs using MDH. These include CUREs on kinetics, post translational modifications and protein-protein interactions with MDH. Each publication includes a summary and limited commentary-interpretation or groups of publications are discussed as a mini-review on a particular sub-topic. This work is meant to help reduce the barrier of starting a mammalian/human MDH CURE project. Protein kinetics, structure-function and cell biochemistry approaches are included as the role of MDH in mammalian cells is reviewed. This document focuses on mammalian/human MDH isoform structure, kinetics, protein-protein interaction, regulation and its role in various metabolic processes. Information on other enzymes such as ME1, PEPCK, GOT, IDH and fumarase is also included where appropriate. This document is not meant to take the place of a good literature review.

Abbreviations/Key Terms:

MDH1: Cytosolic Malate Dehydrogenase with splice variants 1, 2 and 3. UniPRotKB – P40925 (MDHC_HUMAN)
MDH2: Mitochondrial Malate Dehydrogenase UniPRotKB – P40926 (MDHM_HUMAN)

MDH splice variants: While MDH1 and MDH2 splice variants exist, only MDH1 splice variants are biochemically/physiologically relevant. Each differ at the N terminus. Variant 1 is considered the canonical version with less amino acids than splice variant 3. Splice variant 2 is the smallest variant and while expressed in a number of tissues, may not have enzymatic activity.

Cytosolic Enzymes:

ME1	Malic Enzyme NADP dependent UniProtKB - P48163 (MAOX_HUMAN)
GOT1	Glutamate oxidase transaminase, also known as (aspartate transaminase) cAST, AST1, and (aspartate aminotransferase) cAAT and AAT1. UniProtKB - P00505 (AATM_HUMAN)
ACL	ATP Citrate Lyase UniProtKB - P53396 (ACLY_HUMAN)
PCK1	(cPEPCK) Phosphoenolpyruvate carboxykinase, cytosolic [GTP] UniProtKB – P35558 (PCKGC_HUMAN)

Mitochondrial Enzymes:

CS	Citrate Synthase UniProtKB - O75390 (CISY_HUMAN)
GOT2	Glutamate oxidase transaminase, also known as (aspartate transaminase) mAST, AST2, and (aspartate aminotransferase) mAAT and AAT2. UniProtKB - P00505 (AATM_HUMAN)
ME2	NAD dependent Malic Enzyme UniProtKB - P23368 (MAOM_HUMAN)
ME3	NADP dependent Malic Enzyme NADP. UniProtKB - Q16798 (MAON_HUMAN)
FH	Fumarase UniProtKB - P07954 (FUMH_HUMAN)
ACO2	Aconitase UniProtKB - Q99798 (ACON_HUMAN)
PCK2	(mPEPCK) Phosphoenolpyruvate carboxykinase, mitochondria [GTP] UniProtKB – Q16822 (PCKGM_HUMAN)

Summary: Both mitochondrial MDH and cytosolic MDH are involved in more than the traditional TCA and asp-malate shuttle. Altering kinetics, regulation and interactions can shift cells to support various metabolic pathways including lipid synthesis and using glutamine as a primary carbon source for ATP production. A unique and very interesting metabolism is the production of L-2HG from alpha ketoglutarate. While MDH is not the biggest supplier of this compound, it is significant and beyond disease could be how evolution has taken place.Like watermelon and some other MDH isoforms, both human mitochondrial and cytosolic MDH are dimers. The kinetic parameters reported are widely varied for both isoforms. Concentration of each substrate, the pH and presence of allosteric regulators and a lack of complete work make it difficult to declare the clear kinetic constants. The most well examined studied is mitochondrial MDH. Representative Mammalian/Human Kinetic Constants (an aggregate of several publications)*:

- Mito: Km NAD^+ (60 μM), malate (1,450 μM), NADH (44 μM) and OAA (17 μM).
- Cyto: Km NAD^+ (42 μM), malate (770 μM), NADH (17 μM), and OAA (51 μM).

*All values were determined without citrate or other regulators and at pH 7.2-7.4. Experimentally determined values range and are found in the literature described in the kinetics section of this document.

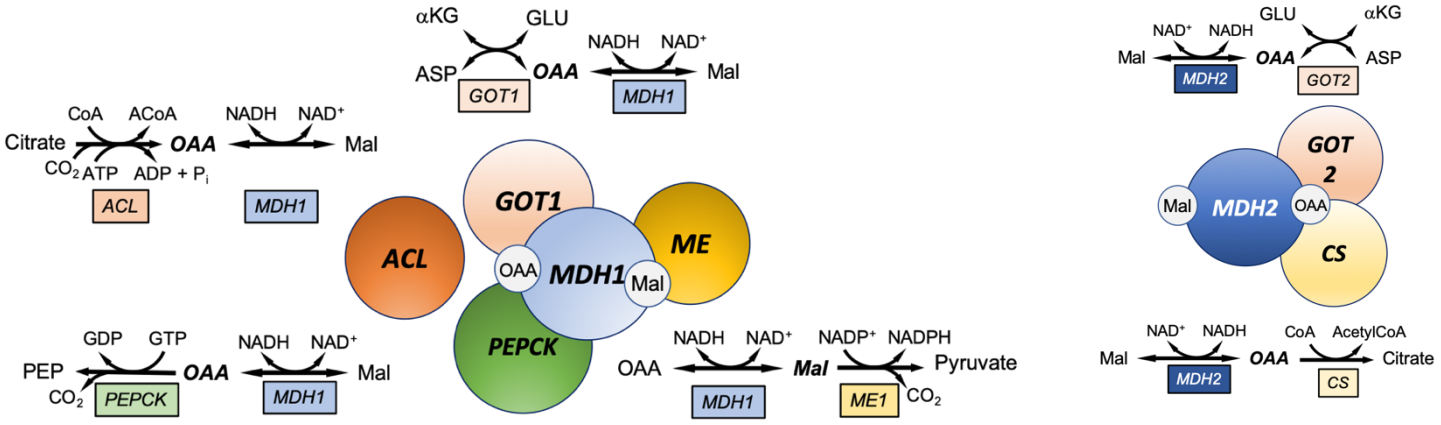
OAA inhibition is not universally observed for cytosolic MDH (mostly for mito MDH) but inhibition is due to substrate concentration and possibly pH. The role of citrate, glutamate and other small metabolites is not fully clear, although the last paper described here on this subject starts to but does not totally clarify such regulation.

MDH exists as multiple splice variants ; each variants taking place at the N terminus. Little is known of the impact of the N terminal differences but as this is where subunits of MDH interact and some degradation may take place at one of the extended

cytosolic MDH isoforms, lots of interesting work is possible here. There are several MDH expressed splice variants that are not likely to have activity. However as MDH binds as a dimer, this could be a way to alter the activity. MDH also binds to non-metabolic enzymes. It might be possible that these splice variants are involved in a unique form of regulation. While mitochondria also has splice variants, only one, the canonical form is highly expressed.

There are many sites of phosphorylation for both isoforms but very very little has ever been studied for mammalian MDH phosphorylation. One paper shows possible activation, while another may lead to degradation. The LDH paper on phosphorylation is very interesting and should be seriously looked at for analogous MDH studies. MDH methylation shows that either arginine 240 or 248 methylation inhibits MDH1 presumably by shifting the structure from the active dimer to a monomer form. Acetylation of lysine residues also impacts MDH activity. There are four mito MDH sites known to be acetylated (K185, 301, 307 and 314) and three cytosolic sites of acetylation (K118, 121, 298). While there is a little disagreement in the literature, acetylation of both (all 4 or 3 lysines or just one) this modification seems to activate MDH.

Interesting GOT, PEPCK and ME1 all enzymes that share substrates with MDH are also phosphorylated and methylated. The mitochondrial GOT-MDH and CS-MDH may very well be regulated not by phosphorylation but acetylation. There are, surprisingly very few publications of cytosolic MDH interactions with other proteins. My graduate work (not published) found GOT, PEPCK and ME1 all interacted with cytosolic MDH using liver purified enzymes. There is a lot of work on CS-MDH interactions. There is a complete and very detailed review on these interactions. While there is no mystery that these proteins interact, where they interact, how they interact and what regulates this interaction is a wide open area of CURE research. The reviews below comment on these interactions and the conditions to make these two proteins interact. The acetylation information is separate from the CS-MDH work but should be considered to fully understand the nature of these interactions.

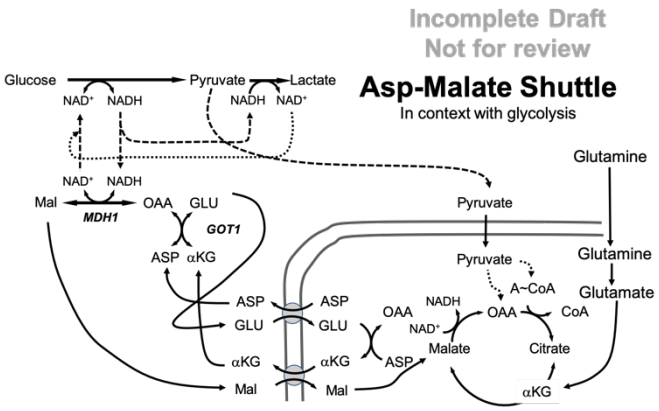


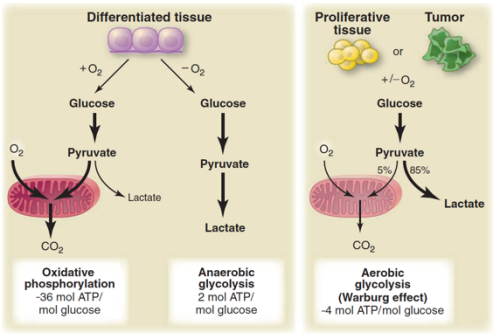
MDH Metabolism

For purposes of simplicity and because of the high homology between mammalian MDH we treat human, porcine and mouse enzymes as essentially identical. There are slight variations but that can be accounted for in a deeper, more detailed review. Yeast MDH is not included in this as there are some unique characteristics to that isoform and it deserves its own review. There are three isoforms of MDH in mammalians. Mitochondrial MDH (now more commonly labeled as MDH2), cytosolic MDH (MDH1) and peroxisomal MDH. While some organisms such as yeast, have a dedicated MDH peroxisomal gene, mammalian peroxisomal MDH is actually MDH1 whose stop codon has been read through resulting in an extended c-terminus (labeled MDH1x for extended) and are shuttled to the peroxisome (Hofhuis Open Biology 2016) where in general, MDH is thought to be involved in reducing equivalents for metabolism ($NADH/NAD^+$).

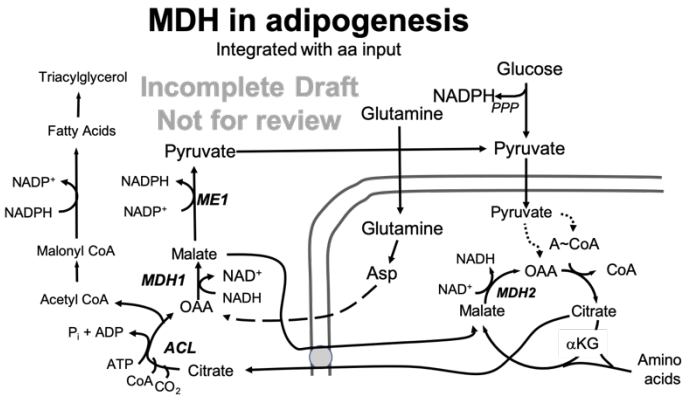
MDH REVIEWS - Malate dehydrogenase: a model for structure, evolution and catalysis. Goward and Nicholls. Protein Sci 3, 1883-1888, 1994. This is one of the key publications that along with the review from J Bell and E Bell will supply catalytic, evolution and structural information on MDH.

The traditional role for MDH is to 1) support NADH/NAD+ reducing equivalents in support of anaerobic metabolism via the aspartate-malate shuttle in the cytoplasm and 2) as a key component of the tricarboxylic acid (Krebs) cycle. Pretty standard stuff. Well over the past 4-7 years the involvement of MDH in unique and tissue specific metabolisms is increasingly clear. New evidence, discussed later in this document, highlight that regulation of the enzyme can shift carbon flux in support of amino acid utilization in cancer cells, high metabolic demand in kidney tissues, adipogenesis and other pathways. Additional complex and interesting fine-tuning of MDH takes place by phosphorylation, splice variants, acetylation, methylation and metabolite inhibitors/allosteric regulators can further adjust the carbon flux in support of cellular metabolic needs. However very little is known about many of these processes. In cytosol there are four different enzymes that share substrate/product with MDH1. These include malic enzyme (ME), glutamic-oxaloacetic transaminase (GOT), ATP citrate lyase (ACL) phosphoenolpyruvate carboxykinase (PEPCK). Malic enzyme (ME) also called NADP dependent malic enzyme is found in the cytosol (UniProtKB - P48163 (MAOX_HUMAN)) is involved in lipid and other macromolecular biosynthesis for the regeneration of NADPH. The enzyme with three names (glutamic-oxaloacetic transaminase (GOT) aka aspartate aminotransaminase (AAT) and aspartate transaminase (AST) Cytosolic UniProtKB - P17174 (AATC_HUMAN)), transfers the amino group and alpha keto acids in a reversible manner catalyzing Asp + α-ketoglutarate (αKG) ⇔Glu oxaloacetate (OAA). ATP citrate lyase (UniProtKB - P53396 (ACLY_HUMAN) is responsible for the conversion of Acetyl CoA, ADP and OAA to citrate, ATP and CoA involved in fatty acid synthesis. Finally, phosphoenolpyruvate carboxykinase (UniProtKB - P35558 (PCKGC_HUMAN) is one of the key enzymes of gluconeogenesis. ME, GOT and PEPCK also have mitochondrial isoforms which add to the complexity of possible research project for this or other CURE related projects.



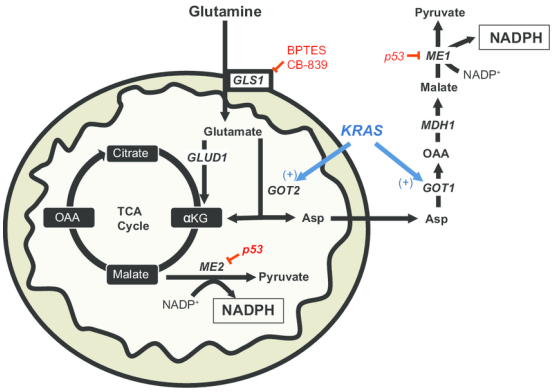


In glycolysis, the asp-malate shuttle is key to maintaining reducing equivalents in tissues without mitochondria or undergoing anaerobic supporting the NAD+ regeneration also conducted by lactate dehydrogenase. As tumor cells grow at a dysregulated pace, a high requirement for ATP is met by the Warburg effect. This is an adaptation by tumor cells where critical metabolic enzymes are expressed at higher levels and activated by a variety of post-translational modifications including phosphorylation and acetylation. This results in an aerobic-like metabolism to produce ATP even in the presence of oxygen and functional mitochondria/oxidative phosphorylation processes. As a result there is a significantly higher uptake of glucose producing lactate to continue the high glycolytic production of pyruvate. There are several reviews on this topic worth reading. While this is not as efficient as full oxidation of glucose to carbon dioxide and water, the rate of ATP production is higher in these cells. Depending on the tissue type, come of the glucose is shunted to pathways producing NADPH for biosynthesis of lipids and glucose carbons are converted to amino acids and nucleotides. Similar shifts in intermediate carbohydrate metabolism is seen in hypoxia, both in nascent tumors where diffusion of oxygen is insufficient to support cancer cell growth and in disease states including cardiac ischemia, brain injury.



MDH activity is critical in support of these dynamic metabolic processes as the NAD+ regenerated in the asp-malate shuttle is critical and in some cases are primary to support a proper NADH/ NAD+ ratio. Glucose alone is not enough to supply the glycolytic needs of active cells (proliferating, immune and cancer cells) and the increasing role of the amino acid glut amine to support his is becoming a therapeutic target for many researchers. MDH again plays a metabolic role in this metabolism. MDH is

expressed at higher levels and regulated in several cancer cells suggesting the enzyme is more than a household metabolic protein and understanding its role in directing reducing equivalents and carbohydrate metabolites.



Finally MDH plays a role in supporting fatty acid production as both mitochondrial and the cytosolic isoforms are involved and may even have pools of enzymes directing metabolic intermediates in divergent pathways. Thus for the purposes of this work, we will focus on the structure of MDH, its kinetics and regulation as well as how MDH interacts with proteins to support metabolism in normal and diseased states.

Physiologic 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 that might be used in our studies. Starting with substrate concentrations, cardiac tissue is often used as it is one of the most metabolically active tissues. Cytosol – NADH 10 μM, NAD^+ 99 μM, OAA 5 μM and malate 500 μM. Mitochondria - NADH 800 μM, NAD^+ 5,800 μM, OAA 0.3 μM and malate 5,000 μM (Opie Biochem J 1975 and Geisbuhler Circ Res 1984). Intracellular (cytoplasmic pH typically ranges from 7.0-7.4 but in an actively respiring or hypoxic cell the intracellular pH can get as low as 6.2. Tumor cells activate proton transporters and other pH buffering mechanisms and will have higher intracellular pH from 7.1-7.6. Mitochondrial (matrix) pH ranges between 7.0-7.4.

MDH, Metabolism and Roles in Disease:

This section will be a brief review of several papers that describe the role of either MDH1 or MDH1 in various pathways. The reason for including these is to show there is a more significant role for MDH in mammalian metabolism that we learn in a textbook and that our understanding of how MDH is regulated is growth area.

Characterization of the Role of the Malate Dehydrogenase to Lung Tumor Survival. Zhang et. al. Journal of Cancer, 8, 2088-2096, 2017.

Looking at non-small cell lung carcinoma (NSCLC) the most aggressive type of lung cancer, they reference several identified mutations of MDH1 in lung cancer whose impact has not been investigated. They used patient samples and found both MDH1 and MDH2 expression was increased and was associated with a poor patient survival prognosis. Using cell lines they found both higher expression and activity for both isoforms over control cultured cell lines. Interestingly while knocking down/out both MDH isoforms singly decreased cell survivability, MDH1 seemed to be much more important in cancer cell survival than the mitochondrial MDH2 isoform. In fact MDH1 activity was significantly higher in these cells, by 4-fold than MDH2 activity. *They did not show what control, non-transformed cell activity for MDH1/2 were or changes in the Km/Vmax/other characteristics. Could the difference be from expression alone? Based on the changes in expression, possibly not fully explained. Are there other regulations happening with MDH1 and MDH2? Not discussed here but fully in support of what we would like to do…*

Oxaloacetate induces apoptosis in HepG2 cells via inhibition of glycolysis. Kuang et.al. Cancer Medicine, 7, 1416-1429, 2018.

Using a liver cancer cell line (HepG2) and normal human cells (LO-2), they add OAA to cells and measure the impact on metabolism and cell behavior. OAA added to neuronal cells increases oxidative phosphorylation respiratory rates and they suggest that OAA can force Warburg glycolysis to mitochondrial respiration pushing cells away from metabolic support for cancer growth. Added OAA increased cell death in liver cancer cells and OAA also decreased ATP production and glucose consumption because cells are not as glycolytically active with the added OAA. This is supported as the mitochondrial function is increased in the OAA treated cells. In both nude mouse studies and in primary human tumor studies, OAA induced apoptosis and decreased tumor size. This was accompanied by a decrease in AKT and HIF signaling/activity. *This is an argument for MDH focused work as the obvious entry point for OAA metabolism.*

The fate of Glutamine in Human Metabolism. The Interplay with Glucose in Proliferating Cells. Mazat and Ransac. Metabolites, 9, 2019.

This paper is a deep dive into how glutamine can support the metabolic need of growing cells. They use modeling to identify the flux between glucose and glutamine in cells. Along with other papers, this really supports that glutamine is just as important as glucose in proliferating and cancerous cells.

Oxidative modification of proteins in the frontal cortex of Alzheimer's disease brain. Korolainen et.al. Neurobiol Aging, 27, 42-53, 2006.

This work supports that increased MDH activity in neurodegenerative disorders such as Alzheimer's Disease exhibit a significantly decreased degree of oxidation in diseased brains compared to controls. In ALS, MDH is also upregulated and may be interacting with SOD1 protein in these diseases. Interestingly, a patented filed in 2008 aimed to target MDH 217-239 (SWLKGEFITTVQQRGAAVIKARK) to block the SOD protein from MDH as a novel therapy.

Glutamate and malate dehydrogenase activities in Joseph disease and olivopontocerebellar atrophy. Grossman et.al. Neurology, 1987.

The activities of brain glutamate dehydrogenase and malate dehydrogenase were not statistically different in samples from patients with autosomal dominant olivopontocerebellar atrophy or Joseph disease compared with control subject samples. These two enzymes are thus not involved in the pathogenesis of these two separate dominantly inherited diseases. There are a lots of things wrong with Joseph, just not MDH!

NADH Shuttling Couples Cytosolic Reductive Carboxylation of Glutamine in Glycolysis in Cells with Mitochondrial Dysfunction. Gaude et. al. Molecular Cell 69, 581-593, 2018.

There are a number of papers presented in this document and others that demonstrate MDH as a key role in glutamine metabolism in addition to glucose. This work used a cell line with deficient mitochondrial function to investigate how glutamine metabolism to 2-oxoglutarate and then citrate where MDH links this metabolism to support glycolysis. i.e. citrate from glutamine in cytosol takes the place of lactate and mitochondria to regenerate NAD^+. In mitochondrial diminished cells, PEP, pyruvate and lactate increased while malate and citrate were decreased. Using a model similar to a publication already discussed, they predicted that under these conditions for glycolysis to continue, glutamine metabolism would have to be involved. This was supported by radiotracer experiments support this work suggesting that mitochondrial disfunction causes a switch to glycolysis and glutamine metabolism. Genetic knockdown experiments and radio-tracer work show that MDH1 ties the two pathways together (glutamine-citrate) and glycolysis. Cells with impaired mito function and lacking MDH proliferated and migrated much slower than controls. Furthermore they find that GAPDH and MDH interact in pull down experiments and that this is increased in the impaired cells indicating some factor enhances the binding. While surprising as GAPDH does not share substrate – it has been shown with LDH and MDH as well as MDH-GAPDH before (Ferreiro et. al.).

Mammalian MDH: Structure, Mechanism and Kinetics

MDH Structure – Both MDH1 and MDH2 are expressed as homodimers with expected native size of 72.8 kDa for MDH1v1, 77.2 for MDH1v3 and 71 kDa for MDH2. Many organisms express MDH as a dimer, yet there are several MDH microbiological and other isoforms that are found as monomeric, and others are tetrameric forms while some are described as both (Takahashi-Iniguez Biomed & Biotechnol 2016). While both mammalian enzymes catalyze the same reaction, the human isoforms possess only 19% identical positions (70 residues) and 35% sites that are similar (119 residues) as shown by clustal alignment for the canonical MDH1/2 variants. There are several highly conserved domains between mammalian MDH1/2 isoforms including the catalytic loop. These are highlighted in the alignment shown here and expanded in a separate document.

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MDHC_HUMAN          1   --------------------MSEPIRVLVTGAAGQIAYSLLYSIGNGSVFGKDQPIILVL       40
MDHM_HUMAN          1   MLSALARPASAALRRSFSTSAQNNAKVAVLGASGGIGQPLSLLLKNSPLVS-----RLTL       55
                                              .:  :* * **:* *.  *   : *. :..      *.*

MDHC_HUMAN         41   LDITPMMGVLDGVLMELQDC-ALPLLKDVIATDKEDVAFKDLDVAILVGSMPRREGMERK       99
MDHM_HUMAN         56   YDIAHTPGVAA----DLSHIETKAAVKGYLGPEQLPDCLKGCDVVVIPAGVPRKPGMTRD      111
                         **:   **      :*..  :   :*. :. ::   .:*. **.:: ..:**: ** *.

MDHC_HUMAN        100   DLLKANVKIFKSQGAALDKYAKKSVKVIVVGNPANTNCLTASK----SAPSIPKENFSCL      155
MDHM_HUMAN        112   DLFNTNATIVATLTAACAQHCPEAM-ICVIANPVNSTIPITAEVFKKHGVYNPNKIF-GV      169
                        **:::*..*. :  **  ::. ::: : *:.**.*:.   :::      .   *:: *  :

MDHC_HUMAN        156   TRLDHNRAKAQIALKLGVTANDVKNVIIWGNHSSTQYPDVNHAKVKL--QGKEVGVYEAL      213
MDHM_HUMAN        170   TTLDIVRANTFVAELKGLDPARVNVPVIGGHAGKTIIPLISQCTPKVDFPQDQL------      223
                        * **  **:: :*   *:    *:  :* *: ..*  * :.:.. *:    .::

MDHC_HUMAN        214   KDDSWLKGEFVTTVQQRGAAVIKARKLS------SAMSAAKAICDHVRDIWF---GTPEG      264
MDHM_HUMAN        224   -------TALTGRIQEAGTEVVKAKAGAGSATLSMAYAGARFVFSLV-DAMNGKEGVVEC      275
                              :.  :*: *: *:**:  :       * :.*: : . * *      *. *

MDHC_HUMAN        265   EFVSMGVISDGNSYGVPDDLLYSFPVVIKNKTWKFVEGLP-INDFSREKMDLTAKELTEE      323
MDHM_HUMAN        276   SFVKS---------QETECTYFSTPLLLGKKGIEKNLGIGKVSSFEEKMISDAIPELKAS      326
                        .**.            :   :* *::: :*  :   *:  :..*..: :. :  **. .

MDHC_HUMAN        324   KESAFEFLSSA-                                                    334
MDHM_HUMAN        327   IKKGEDFVKTLK                                                    338
                         :.. :*:.:
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MDH2 – The structure of human MDH2 has been published (apo) and with substrates and inhibitors bound (see Uniprot or PDB database). The table below displays the current available structures for mammalian MDH2 with various ligands and other information.

Mitochondrial MDH2 Structural Information

Species	Entry	Align	Ligands	Other Ligands	Chains	Res. (A)	Length
Homo sapiens	2DFD	/	NAD	HIS, MLT, ALA, CL	A/B/C/D	1.90	20-338
Homo sapiens	4WLE	/	CIT	/	A/B/C/D	1.90	20-338
Homo sapiens	4WLF	/	LMR	PO4	A/B/C/D	2.20	20-338
Homo sapiens	4WLN	/	APO	PO4	A/B/C/D	2.28	20-338
Homo sapiens	4WLO	/	OAA	NAI	A/B/C/D	2.50	20-338
Homo sapiens	4WLU	/	NAD	LMR	A/B/C/D	2.14	20-338
Homo sapiens	4WLV	/	NAD	PO4	A/B/C/D	2.40	20-338
Sus scrofa	1MLD	94.08%	CIT	/	A/B/C/D	1.83	25-338
S. cerevisiae	1HR9	51.60%	APO	EPE, ZN	O/P/Q/R	3.01	18-334

The differences between the two structures could be used to show tertiary and quaternary shifts in apo vs substrate bound structures when comparing interacting proteins, channeling and or important contact points vs post translational modification. Another missing and interesting project would be to overlay where citrate binds the mitochondrial isoform vs cytoplasmic, if they are the same, then a hybrid citrate/apo model could also be used to predict citrate's impact on protein interactions and channeling and/or might explain kinetic differences in citrate and other allosteric modifiers between the two isoforms.

Mechanism – A very comprehensive study (Daskika Biophysical J 2015) further established the mechanism and pH dependence of the activities of both cardiac cytosolic and mitochondrial MDH (see kinetics papers from Wolfe and others for the initial descriptions). The ordered mechanism for both isoforms is an ordered bi-bi where nucleotide (NADH or NAD+) binds followed by OAA or malate and after the proton transfer the active loop closes on the binding site. Under physiological conditions the mitochondrial MDH activity is predicted to be higher than cytosolic enzyme. Interestingly at low concentrations 5-150 μM malate both cytosolic and mitochondrial MDH have the same activity (mal->OAA), however at higher malate concentrations, the mitochondrial isoform is more active. While in the reverse direction (OAA->mal) at low NADH (~5 μM) cytosolic MDH activity is higher and at higher NADH mitochondrial activity is higher. When comparing these activities to the physiological concentrations cytosolic MDH activity is higher than mitochondrial in cytosolic conditions and the reciprocal is true in the mitochondria. pH dependency of both isoforms follows similar patterns. At mitochondrial physiological conditions at pH 6.5 both isoforms are relatively inactive, however as the pH raises to pH 7.5 and 8, both activities increase where mitochondrial MDH activity is 2-3 times greater than the cytosolic enzyme. While under cytosolic conditions, the cytosolic enzyme rate is greater at all pHs compared to mitochondrial – thus suggesting the ideal behavior for each isoform under physiological conditions. Interesting is that in the malate-OAA direction, in the presence of higher (mM) concentration of NAD+ and malate, increasing pH leads to increasing activity up to pH 9 for both enzymes. Yet at μM conditions, in the reverse direction (OAA->malate), activity is significantly higher at lower pH (6.5 – 7.5) and the activity continue to drop off significantly at pH8.5 and higher. Something to consider when conducting comparable enzyme kinetic studies!

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 is commercially available and used for many studies, the simple kinetics of these isoforms of MDH are not easily found in the literature. In general the human mitochondrial MDH is OAA inhibited at concentrations above 200-300 μM (Bernstein JBC 1978) while the cytoplasmic enzyme (Abtahi) does not display the significant inhibition. The lack of OAA inhibition is only shown once and not conclusively while it was reported in the Bernstein paper.

- The ranges of Km for cytosolic MDH are as follows: OAA 8-300 μM, for NADH 17-200 μM, malate 160-770 μM and NAD+ 43-62 μM. While the ranges of Km for mitochondrial MDH are as follows: OAA 110-350 μM, for NADH 15-72 μM, malate 145-1600 μM and NAD^+ 60-170 μM. Microbial MDH affinities ranges OAA 22-150/300 μM, for NADH 14-100 μM, malate 170-5,000 and greater μM and NAD^+ 24-100/300 μM (Takahashi-Iniguez).

Specific literature and database values are described here.

- Rat mitochondrial MDH gave the following results: Km NADH (72 μM), OAA (110 μM), malate (1,600 μM), and NAD^+ (170 μM) with a specific activity of 380 μmol/min/g of tissue (OAA->malate) and 39 μmol/min/g of tissue for malate->OAA. Porcine, chicken and Rat: Mitochondrial porcine turnover [1/S] for malate was 18 and OAA 377. Only one

specific activity for pig cytosolic MDH was found 182 μmol/min/mg protein. Chicken and Pig mitochondria Km (Gelpi Biochem J 1992) found Km for: OAA (17 μM) NADH (44 μM), Mal (145 μM) and NAD+ (60 μM).

Post Translational Modification: Phosphorylation – Direct and Indirect Evidence:

Identification of phosphorylation sites. There are only two reported mammalian MDH phosphorylation publications occurring in the current literature and a small handful of papers on MDH phosphorylated in other organisms. However a search of the web-based Phosphosite.net interface was used to find many LCMS identified phosphorylation sites of MDH. This website links to curated studies and databases of protein modifications (MDH1, MDH2; summer 2019) to identify protein phosphorylation. They also list what are labeled as "LTP - low throughput papers" which are published records of a phosphorylation not using LCMS. Like our search, their LTP show no sites for either isoform of MDH. The curated databases are large phospho-proteomic studies, none specific for MDH. In each case, tissues or cell lines were prepared and positive phosphorylated peptides hits made available. Some studies were in human tissues other in various mammalian tissues or cell lines. We scanned for all mammalian positive MDH phosphorylation results and cataloged them for each animal. Non-human positive hits (i.e. mouse, pig, rat…) were double checked against the human sequence and if the consensus sequence was an identical match, this was counted as a "known phosphorylation site" for human MDH. In no case did the studies indicate which kinase was responsible as it was a global assessment of protein phosphorylation. Depending on the tissue, metabolic / hormonal / disease state, it was not surprising to see each putative phosphorylation site show up in one or more study but not all studies. Thus one hit was enough to recognize the MDH Ser/Thr/Tyr as a "known phosphorylated residue". There are likely other databases and websites sites worth investigating to get a more complete study. It should be noted, that we have no idea what fraction of MDH was phosphorylated in any one study. The MS result could show a small percentage of the total MDH that are modified or that all of them are modified. Something to consider when analyzing the results. From this search we found the following: mitochondrial MDH was phosphorylated at 20 unique sites (10 serine, 15 threonine and 5 tyrosine residues) and cytosolic MDH is also phosphorylated at 20 sites (12 Ser, 4 Thr and 4 Tyr). In addition to these "known sites", there are additional putative phosphorylation sites predicted using three websites/databases (summer 2019: GPS5.0, NetPhos3.1, KinasePhos2.0 and DISPHOS) but at this time, no indication if these predicted sites are actually phosphorylated. Every "known" site identified by MS was also a predicted site. While we don't know which kinases phosphorylated mitochondrial MDH, each site can be predicted. It is likely that there will be several potential kinases predicted for each site depending on the sequence. For background review, protein kinases involved with mitochondria are described in this paper (Regulation of mitochondrial functions by protein phosphorylation and dephosphorylation Lim et. al. Cell Biosci, 6, 25, 2016). Each site is identified on their respective alignment maps and a report with both "known" and "predicted" sites.

Protein-Protein Interactions: Mitochondrial MDH

Mitochondrial MDH – CS (and others) A Chronological Publication Summary with Commentary:

An Immobilized Three-Enzyme System: A model for microenvironmental compartmentation in mitochondria. Srere, Mattiasson and Mosbach. Proc Nat Acad Sci. USA. 70, No 9, pp2534, 1973.

One of the initial papers indicating an interaction was advantageous. Limiting diffusion of MDH, CS and LDH by binding to sepharose or in a gel, indicate that when MDH was in close proximity to CS the net flux from one enzyme to the other was 4-5 times faster than alone or with controls.

Interaction between Citrate Synthase and Mitochondrial Malate Dehydrogenase in the presence of Polyethylene Glycol (PEG). Halper and Srere. ABB. 184, 529-534, 1977.

Start by describing Keq differences for MDH and CS, and that in the mitochondrial concentration of OAA (4 x 10^{-8}M) the Keq of CS would lead to only about 1% of CS possible activity. There are similar issues for coupling MDH with OAA in the TCA cycle for OAA production. Thus something else has to be going on… Shuttling of substrate. They mixed sigma pig CS and MDH (0.2 mg/ml) in PEG (14% in a 2 μM phosphate buffer). Looked at turbidity (@650 nm) to identify aggregated enzyme. CS only ppt with mito-MDH not BSA or cyto MDH. Increasing ionic strength in the presence of PEG decreased turbidity – interactions. Centrifuged down ppt and measured activity and saw mMDH and CS both in pellet vs low/none for cMDH or BSA and CS. PEG was 6,000-7,500 Mr. Acts as a crowding agent. That is, tying up bulk water, which increases the order of the water (more water is tied up H bonding to PEG) and limits free water to solvate surface of proteins. This helps to drive protein-protein interactions as there isn't enough water to solvate the surface of the protein. *Kind of a hydrophobic effect. May have some impact on slowing down free diffusion too – need to look that up more if one goes in molecular crowding direction. One of first papers to show CS-mMDH interactions and that cMDH does not interact with CS.*

Substrate Channeling of Oxalacetate in Solid-state Complexes of Malate Dehydrogenase and Citrate Synthase. Datta, Merz, and Spivey. JBC, 260, pp 15008-15012, 1985.

Pushed the concept that channeling of product-substrate of MDH-CS took place in PEG induced aggregates (the solid state). They do note that PEG alone can decrease MDH activity and needs to be accounted for when working with MDH activities. They also note that these enzymes are not stable in low ionic strength buffers without PEG and higher ionic strength buffers even with PEG defeat the MDH-CS interactions albeit slow. They ppt the proteins in PEG by adding both proteins at 2 mg/ml in 10 mM KPi buffer with 10% PEG for 1 hr and ppt centrifuged at 10,000 x g for 40 min. Addition of aspartate transaminase (aka AAT, GOT, AST) decreased activity of CS when OAA was supplied by MDH in solution but did not impact CS activity in solid state – indicating a scavenging effect of AAT is only seen in the absence of MDH-CS interaction induced substrate shuttling.

Polyethylene Glycol-Induced Heteroassociation of Malate Dehydrogenase and Citrate Synthase. Merz, Webster, Appleman, Manley, Yu, Datta, Ackerson and Spivey. ABB 258, pp132-142, 1987.

Used dynamic light scattering in PEG. At 6% PEG reasonable MDH-CS interactions were observed with 0.5 mg/ml protein. They did not see ppt aggregates with dextrans, ficoll or other crowding agents. They show mMDH-CS interactions in both solid phase and in solution – do not see with cMDH-CS or BSA-CS.

Quantitation of the Interaction between Citrate Synthase and Malate Dehydrogenase. Tompa, Batke, Ovadi, Welch and Srere. JBC 262, pp 6089-6092.

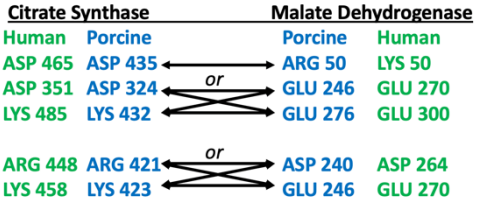
Used FITC labled CS or MDH and fluorescence anisotropy to study interaction between pig (sigma) MDH and CS. With mMDH – CS found dissociation constant = 1.0 x 10^{-6} M and 1.5 x 10^{-5} M (50 mM Tris pH 8.0). Aldolase, as a control showed no impact on anisotropy (binding). Each enzyme was 3.5 x 10^{-7}M (~0.023 mg/ml) when held steady. This is the first time a more sensitive assay was used to show interaction. OAA, citrate, ATP, malate and NAD+ had no impact on binding of mMDH-CS at 10^{-4}M. NADH increased Kd (decreased affinity of MDH-CS) by 5 fold and a-ketoglutarate decreased Kd (increased affinity) by 5 fold. CS kinetics were not impacted by either MDH – again in solution not PEG ppt as seen before. BUT mMDH (3.3 x 10^{-10} M) rates increased in presence of 1.4 x 10^{-6} M CS. Interesting points: cMDH did bind significantly more than control but an order of magnitude less than mMDH. No PEG or other agent was used to induce interactions. That some but not all tested metabolites impacted binding is interesting – we see this with MDH and IDH in an SPR study by others.

Preparation and Kinetic Characterization of a Fusion Protein of Yeast Mitochondrial Citrate Synthase and Malate Dehydrogenase. Lindbladh, Rault, Hagglund, Small, Mosbach, Bulow, Evans, and Srere. Biochemistry, 33, 11692-11698, 1994.

This is the first of several fusion MDH-CS studies. Yeast CS and MDH were fused N – CS – mMDH – C with a Gly-Ser-Gly linker. Obvious issues as both CS and MDH are dimers. MDH dimerizes and is regulated in part via the N term of the MDH dimer so there are limitations to meaning of some of the data. This was evident in the big decrease in MDH activity of fusion vs wild-type free protein. The Km was 3x lower and Vmax was 2X slower. CS kinetics were unchanged. Using the AAT/GOT/AST competitive or trapping assay, trapped free OAA from non-fusion mixtures but much less with the fusion (but still impacted the fusion protein) – so at least the proximity of MDH and CS allowed for some channeling. But this does match the PEG "solid state" results. This is also the first time kinetics of transient are measured to indicate either a shorter path or a preferred path of OAA from MDH to CS exists.

Evidence for Electrostatic Channeling in a Fusion Protein of Malate Dehydrogenase and Citrate Synthase. Elcock and McaCammon. Biochemistry 35, pp 12652-12658, 1996.

One of several computational – docking attempts to define the interaction between CS and MDH. Used a Brownian Dynamics to find an electrostatic channel. That is using surface charges of the proteins – to see if a "ditch" of positive charges could lead from MDH active site to CS active site. The idea – used by several other investigators hereafter – is that the negative charged OAA will stick to the positive charged "tunnel or channel" facilitating the transfer. What is missing in this, is how the other reactants and products enter and diffuse from the active site if there is some limited charged surface for OAA. It happens, not sure this is the ONLY way it can happen, but this is the favored idea that this author and others are looking for… the holy grail – find the interactions that creates a positively charged surface!!! They find a predicted fit of the two proteins where the active sites are close to each other (about 60 A), then change the surface charge and predict using modeling, how much of OAA would leave MDH to get to the active site of CS. Without charge, less than 1% of OAA is transferred. This means proximity alone is not enough. Including charge of protein surface or OAA gives a closer transfer rate to that observed experimentally by others.



Proposed ionic interactions based on docking model

Model of a Quinary Structure between Krebs TCA Cycle Enzymes: A Model for the Metabolon. Velot, Mixon, Teige, and Srere. Biochemistry 36, 14271-14276, 1997.

Extends fusion yeast CS-MDH to include aconitase with docking predictions. Used to identify charged channel to guide citrate between CS and Aconitase (Aco). Basically building on the idea of a large metabolon to show potential for hand-off (channeling) of product/substrate. Started with docking of all three enzymes, using the open non-substrate version of CS to dock to Aco. Then used the closed (with citrate) to identify potential residues involved in channel to Aco. As before alter the surface charge (electrostatic potential) and charge of citrate and then measured theoretical diffusion times to support a charged surface is key to direct product of CS / substrate of Aco. They produce a reasonable argument for docking vs the limiting orientations of a fused protein, and then use the fusion protein (vs free) to find evidence that supports the modeling. In this system they identified (using docked model) potential charged amino acids. They did identify possible charged, ionic binding partners between CS and MDH. They predict that most interactions between CS and MDH are due to global ionic charges. They identified (via modeling only) the potential binding pairs shown in the picture. They also note that while mMDH and cMDH (porcine) have only a 10% amino acid homology, the structure – based on a higher resolution porcine cMDH is high to both enzymes with the same global shape. WHICH helps our argument that there are similar and key unique residues on the surface responsible for the poor cMDH-CS binding and the stronger mMDH-CS binding we and others have identified. Via personal correspondence, both cMDH and mMDH have docked (using the older generation of software) similarly to CS. Something to consider. They point out that the mMDH residues which look to form a salt bridge with CS in this work (see figure above) are NOT conserved in cMDH. In later work, using lysing crosslinking, LYS 50 did not show up as bound to CS, but is adjacent to one of our predicted interaction domains. *GLU 270 is adjacent to a cross linked LYS for mMDH and cMDH has in place of GLU has a LYS without acidic aa upstream or downstream for several amino acids*. This is at the beginning of the C tail that may be part of the interactions between mMDH and CS (from Wu et. al. and Bulutoglu et. al.).

Interaction between Citrate Synthase and Malate Dehydrogenase. Substrate Channeling of Oxaloacetate. Morgunov and Srere. JBC 273, pp 29540-29544, 1998.

Used PEG to show interactions and the impact of ionic strength on CS-MDH interactions. Several important approaches all supporting CS-mMDH interactions. Frontal analysis SEC chromatography – saturate 0.7x300 cm (~50 ml) S200 column with 0.2 mM CS in 10% PEG and 10 mM KPi buffer pH 8.0. Then mMDH or cMDH is run through column and elution volume compared to chromatography without CS. Large shift in mMDH with CS in buffer than without indicating a complex is formed. The same experiment with cMDH did not show a change in elution buffer. Enzyme is ppt using 10% PEG for mMDH-CS and 25% PEG for cMDH-CS 2 mg/ml protein and the duration of the complex shown using turbidity (OD 650) in 0 or 30% PEG. Note that at higher conc of PEG cMDH will bind to CS, although we don't see how much bind at lower PEG… Assuming little thus the higher conc of PEG. BOTH MDH proteins complexed with CS with 200 mM KPi buffer for over 2 min with 30% PEG although slightly less than in lower ionic strength (10 mM) buffer was used. However, in even low ionic strength buffer, turbidity was lost without PEG in less than 20 seconds. WHAT we don't know yet, is the turbid issue is likely large aggregates of CS and MDH (see Merz 1987), and individual complexing of MDH-CS may still remain. The point is that PEG pushes the interaction even in higher salt conditions, and without, may not see the interaction. To show this, a competition/trapping assay using AAT with a ratio of 1:2:50 (CS-MDH-AAT) at 0.2, 0.4, and 10 units of enzyme respectively, in 0 or 30% PEG. They did the same assays with another OAA utilizing enzyme giving the same results as AAT. As before, AAT can trap free OAA produced by MDH if the substrate isn't shuttled directly to CS. Without PEG, neither mMDH or cMDH coupled well enough to prevent AAT from stealing OAA. In 30% PEG, mMDH-CS protected 60-70% of OAA from AAT, yet cMDH-CS did not shuttle (even though they form turbid complexes). So here we have some indication that CS-and both m/cMDH interact but not when using the AAT competition assay. The detection of interaction is low threshold but the assay is a nice functional approach. Not sure if it will be a subtle – fine-tunable assay to detect small changes, but as a measure of channeling it has become very important. ALSO note that the numbers of the assay are all normalized as 30% PEG inhibits MDH and AAT activity.

Substrate Channeling. Spivey and Ovadi. Methods 19, pp306-321, 1999.

This is a review of approaches to study channeling focusing on isotope dilution, transition time analysis, competing reaction and enzyme buffer methods. The authors provide a deep analysis of the approaches using a very quantitative look at each study. For competition assay, they re-evaluate Srere work and explain this is similar to what Ovadi calls the more quantitative model of Elcock. There is clearly a difference in interpretation between Srere and this group in looking at some of the results. If one plans to investigate transit state/fast kinetics to investigate protein-protein interactions this is a good place to start.

Electrostatic Channeling of Oxaloacetate in a Fusion Protein of Porcine Citrate Synthase and Porcine Mitochondrial Malate Dehydrogenase. Shatalin, Lebreton, Rault-Leonardon, Velot, and Srere. Biochemistry, 38, pp881-889, 1999.

Moved from yeast fusion MDH-CS-Aco to a mammalian (pig) fusion protein to show similar to yeast fusion protein that charged surface (channel) is responsible for guiding OAA between MDH and CS. As before – the limitations are orientation and impact of each being a monomer as both MDH and CS are functional homodimers. Both MDH as a fusion protein showed about the same Km for OAA and malate, slightly lower for NADH but a significant decrease in specific activity. BUT they do show that for the fusion protein, AST decreased MDH-CS channeling by up to 70% for free enzyme (mMDH & CS) but only 10-20% of fusion (mMDH-CS) activity was lost in presence of AST. This was performed without PEG as the fusion proteins were already "in proximity" as compared to the free enzyme. When cMDH-CS was assayed in presence of AST, no difference was observed between fusion and free enzyme. The majority (80%) of CS activity was lost for both cases. Again, arguing that cMDH does not bind CS nor will it channel CS (proximity was still a case here). For the mMDH-CS fusion protein, (no PEG) increasing ionic strength from 40-200 mM KPi buffer reduced channeling (AST competition assay) to same as free enzyme. Thus charge interferes with channeling as it isn't untethering the protein (proximity-docking vs channeling). Revisiting that cMDH and mMDH both dock to CS with the same orientation but the amino acids are very different, they looked at the surface (electroastatic potential) charge of the two proteins. The mMDH-CS surface charge interface (aka potential channel) was much more positively charged than cMDH-CS. Remembering that OAA is negative charged, the channel is more like a positive charged ditch that guides OAA between the enzymes via the positive surface charges. So this is more about channeling than docking. Subtle and interesting point… Also important is using the trapping/competition assay with 40-50 mM KPi buffer and no PEG found free enzyme interactions. May indicate more of a binary semi-qualitative result for this assay as compared to other protein-interaction techniques. OR that binding may be occurring but the environment for OAA channeling is not taking place…

Reversible Transdominant Inhibition of a Metabolic Pathway. In vivo evidence of interaction between two sequential tricarboxylic acid cycle enzymes in yeast. Velot and Srere. JBC 275, pp 12926-12933, 2000.

Use yeast genetics to develop an in vivo traditional acetate yeast-growth assay to show MDH-CS interactions. Lack of acetate growth is indicative of yeast TCA metabolism problems. There is a yeast CS mutation that retains enzyme activity but somehow blocks TCA production. They use this approach to find "assembly mutations" that is, mutations that block protein interactions but allow activity and result in a diminished TCA and thus yeast growth. The idea is that an uncoupling of protein interactions will slow metabolism and thus yeast growth – as long as the enzymes remain enzymatically active. Because they used an error prone polymerase when cloning initial fusion of peroxisomal and mitochondrial yeast CS, they found some recovery in CS deleted yeast cells. They then prepared the same fusion CS using a low - error polymerase without sequencing errors. The fusion wild-type restored full acetate growth but the mutant CS hybrid only partially reconstituted yeast acetate growth. Indicating that a some random mutation made as a mistake in cloning can't fully allow cells to use TCA cycle. The mutated hybrid CS did express and showed significant activity, near that found in wild-type cells. Sequence of the mutations found several differences: 1) one five aa mutation from three single bp mutations leaving the mutated protein missing one aa and with four different aa residues due to frame shifting all found in a solvent exposed loop of CS 2) A92T and 3) T166A. Using peptides fused to GFP as a carrier, they screened to see which peptide containing mutation was responsible for loss of TCA metabolism. The idea is that a peptide would compete for binding with whatever CS is binding. If a peptide mimicking one of the CS mutations had no impact on growth, then that wasn't the mutation of consequence. The first mutation of five amino acids (one deletion) was identified using the peptide studies on acetate growth. Expressing both mutant CS peptide and excess mMDH allowed growth, indicating that the extra MDH was able to "soak up" peptide and no longer block CS enzyme from binding to its partner at that site - MDH. Key points: it is possible to use yeast to find *in vivo* TCA alterations. How sensitive is not obvious but possible. Could a delta cMDH or delta mMDH strain be used to look at growth with replaced m/cMDH? This might be a more straight forward approach than the peptide inhibition approach. Also yeast CS Gly357 - Arg362 aligns to human CS Gly346 - Arg351. In the later binding papers based on Lys cross-links, no area of MDH was mapped to crosslink to this region of MDH. At least 10 or more residues more N terminal were the closest interactions. Thus there could be an unidentified interaction left to be discovered. Dr. Srere passed away before the publication of this paper in 1999.

Kinetics of the coupled reaction catalyzed by a fusion protein of yeast mitochondrial malate dehydrogenase and citrate synthase. Pettersson, Olsson, Bulow, and Petersson. Eur J. Biochem. 267, pp. 5041-5046 2000 .

This paper argues against channel diffusion and instead supports a free diffusion based on proximity using the same fusion protein described by the Srere group and the Ovadi/Lindbladh group. Assuming substrates are at a high enough concentration to allow for first order kinetic analysis, only the initial rates were measured. Using this approach, they only looked at generation of MDH products NADH and OAA to account for CoA and Citrate formation. Used same

concentrations in AAT trapping assay as Srere. They show no difference in initial rates without or with AAT in free enzymes. Furthermore in the fusion protein, no changes were observed as compared to the transient times of free diffusion from MDH to CS.

Florescence Characterization of Co-immobilization-Induced Multi-Enzyme Aggregation in a Polymer Matrix Using Forster Resonance Energy Transfer (FRET): Toward the Metabolon Biomimic. Wu and Minteer. Biomacromolecules, 14, pp 2739-2749, 2013.

This paper's focus is finding a polymer to slow down free diffusion and support assembly of protein complexes similar to found in cell. Using FRET they tested various compounds and used mMDH-CS and CS-Aco to identify material that increased protein-protein interaction via FRET. In several approaches, mMDH-CS were "FRET friendly". While not specific to this work, the FRET approach could be used in vivo with the right donor/acceptor pairs.

Complex formation between malate dehydrogenase and isocitrate dehydrogenase from Baccillus subtilis is regulated by tricarboxylic acid cycle metabolites. Bartholomae, Meyer, Commichau, Burkovski, Hillen, and Seidel. FEBS J. 281, pp. 1132-1143, 2013.

This is not an MDH-CS paper but investigates another interaction partner for mMDH, IDH, using sensitive SPR technique. No direct comparison of affinities between MDH-IDH or MDH-CS have been performed, so it is difficult to directly assess the affinity-binding strength of MDH with CS from this paper. However the direct binding without PEG of MDH-IDH was very weak and difficult to detect by SPR. But - as seen with MDH-CS, adding cofactors could significantly increase affinity which results in an SPR measurable trace. One approach they use is to create a kinetically dead IDH to allow for some of the metabolites at a concentration to show an effect without being consumed to products. For IDH, this happened to be a phosphorylatable serine near the active site which inhibits the enzyme when phosphorylated. There is a simple experiment where they show presence of MDH influences IDH activity two fold or more depending on MDH concentration. Using the Malate-OAA direction (J. Biochem Biophys Methods 68, 101-111, 2006) which uses XTT and NADH, and a catalytically dead IDH (NADPH interferes with the XTT reaction) slightly reduced MDH activity. However what this paper tells us, is that substrates can inhibit or activate interactions and interacting proteins can have impact on the partner protein activities.

Side note - Following the 1999/2000 papers from Srere lab and the Pettersson "rebuttal" no CS-MDH papers came out for nearly 15 years. Now the Minteer group and as will be discussed other groups looking at other organisms in large MS studies have brought CS-MDH back in focus. The Wu-Minteer group studies the interactions as part of a biofuels - metabolon assembly problem. Others identify MDH-CS interactions in plant and other organisms as big database studies. The addition of more specific and sensitive analytical techniques will give us more info on the MDH-CS interactions. Game on.

Krebs cycle metabolon formation: metabolite concentration gradient enhanced compartmentation of sequential enzymes. Wu, Felster, and Minteer. Chem Commun 51, pp 1244-1247, 2015.

Using a microfluidic device to measure florescent labled MDH - CS interactions in the presence of metabolites. This is the third paper that describes MDH related protein interactions are potentially influenced by metabolites. Diffusion of MDH and CS increased when malate (0-5 mM) was added in presence of NAD+ and ACoA. At 10 mM and above the diffusion was reduced. The authors indicate this might be due to increasing concentration of citrate which might be a feedback inhibitor for the interaction. Interestingly others have shown that citrate is an allosteric inhibitor (along with glutamate) of MDH. No mention of this was observed. When ATP, a competitive binding inhibitor of NAD+ was added, no diffusion of CS or MDH was seen even in the presence of 5 mM malate. So, both malate and NAD+ seem to be important for the increased diffusion - interaction? When only malate was added without NAD or ACoA, diffusion of both enzymes increased from 0-10 mM saturating at that point where neither 50 or 100 mM malate generated further diffusion-interaction. Key points - malate and NAD+ seem to be important for interaction - at least for diffusion. ATP can inhibit and presumably, while not tested, citrate can modulate the interaction. Stuff to test - not done anywhere yet.

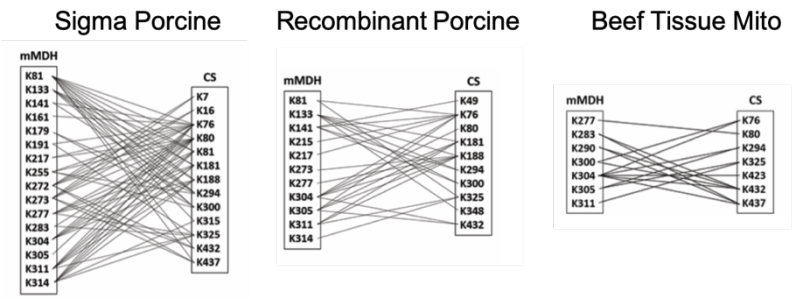
Krebs Cycle Metabolon: Structural Evidence of Substrate Channeling Revealed by Cross-linking and Mass Spectrometry. Wu and Minteer. Angew. Chem. Int. Ed. 54, pp 1851-1854, 2015 plus supplement.

One of two big papers that show where MDH and CS interact. They also looked at CS-Aco. Ignored here. Only focus on mMDH not cMDH. Beef heart mitochondria were treated with short chain, Lys-Lys cross-linker (disuccinimidyl glutarate - DSG) and separated by SDS-PAGE. Proteins were cleaved into peptides with trypsin and analyzed by LC MS/MS. Interesting, and telling of the interaction strength, only MDH-CS was found without cross-linking while other interactions were not strong enough to stay complexed through gel electrophoresis. Using the cross-linked lysines, global docking was predicted using ClusPro 2.0. Then lysines further apart than 25 A (comb of DSG and lysine distance and

involved backbone flexibility) were removed as false positive cross-links. Further restrains including solvent distances and others were set and local docking using Rosetta server was used to generate 10 models of interaction with lowest interface energy were screened by Xwalk to find the most observed cross-links. Unfortunately in this nor the next paper, the final structure of interaction was given. All cross-linked Lys were given in supplement materials and indicated on our mMDH alignment map. They found Lys300, Lys304 of mMDH were clearly linked after stringent removal using the described methods of docking. Two CS Lys were potentially linked to several mMDH sites. Their model showed one subunit each of mMDH and CS interacted, with mMDH residues Ile34-Pro38, Tyr56-Glu60, Gly77-Thr95, Lys215-Ala220 were interacting with CS. This model also indicated that both N and C term of a single CS subunit was near the mMDH-CS binding interface. Distance between active site is predicted to be 35A for one of the MDH-CS models. A positive charged surface between mMDH-CS active sites was identified in support of channeling requirements in the MCA1 model. Key points - this shows all lysines that cross-link to CS. Some (three)remain after stringent filtering. Three models remained, with one supporting a positive "ditch" channel from active site to active site. One MDH subunits binds one subunit of CS, with CS tails involved in the interaction. *While some residues in the MCA1 model are identified as close, there may be others. They do not describe the N or C terms of MDH in the docking. Don't limit to Lysines - the amino acids surrounding if close enough can interact. We need to develop a mature and well tested model ourselves and see if HINT lookup tables can identify other interaction residues.*

Direct Evidence for Metabolon Formation and Substrate Channeling in Recombinant TCA Cycle Enzymes. Bulutoglu, Garcia, Wu, Minteer and Banta. ACS Chem Bio, 11, pp. 2847-2853, 2016 and supplement.

They used crosslinking studies from beef mito (as in the Wu Minteer paper), Sigma purchased porcine mMDH and CS, and recombinant porcine mMDH and CS to perform the DSG Lysine crosslink experiments. CS-Aco interactions are ignored here.



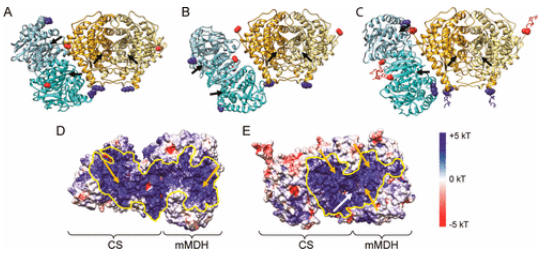


Figure 1. Structures of the mMDH–CS complex and the simulated electrostatic potential on the surface of the complex. (A) Native tissue mitochondrial metabolon. (B) *In vitro* complex formed by commercial enzymes. (C) *In vitro* complex formed by recombinant enzymes. N- and C-termini are represented by red and blue spheres, respectively. FLAG-tag and polyhistidine-tag are represented by red and blue sticks, respectively. mMDH and CS active sites are denoted by black arrows. (D) *In vitro* complex formed by commercial enzymes. (E) *In vitro* complex formed by recombinant enzymes. Surface regions of positive potential and negative potential are colored in blue and red, respectively. The electrostatic channeling path for OAA is highlighted by the yellow edge. Orange arrows indicate the active sites. The white arrow indicates the location of Arg65 and Arg67. The surface ESP was calculated with water molecules at pH 7.4.

The authors argue that tissue is most accurate as the proteins are naturally crowded leaving a more stable metabolon vs dilute free enzymes. But using pure proteins gives a clear, cleaner and strong signal to identify crosslinked lysines. Both recomb and tissue models have a similar orientation with a shorter distance between active sites (40A and 35A). The sigma model is flipped with a very long distance between active sites (73A). Most trusted Lysine beef tissue mMDH links are Lys191, Lys277, and Lys283. For recomb porcine Lys81, Lys217, Lys304, and Lys305. In both models, C&N term of CS lie in the interface of mMDH-CS binding plane. C term of mMDH for these models are close to mMDH-CS interface but N term of mMDH are away. The N terms interact with the other for MDH regulation making this model make more sense with established kinetic and regulatory data. The recombinant mMDH is 30 degrees shifted from CS as compared to tissue model. Thus no single model is correct. As described elsewhere the positive electrostatic charge between active sites creates a positively charged channel "ditch" for OAA to bind and more efficiently move to CS without free diffusion. Both tissue and recombinant models support this with shorter and positively charged surfaces. Interestingly, CS R65S mutant retained enzyme activity, but did not show up in cross-linking experiments. Nor in AAT trapping/competition assays did OAA channel well. None of the Lysines map to this region of CS, although K49 and K76 bracket R65. Both of these lysines do crosslink to MDH, BUT at several possible mMDH lysine residues. So the exact mMDH docking site for CS R65 is not known, but should be identified. Other competition assays with native, recombinant and commercial MDH and CS were only mildly trapped by ATT (30-10%) but the authors did not include PEG to induce interactions of free enzymes, something demonstrated in earlier experiments by others. Key points to this work are that we have two sets of Lys that crosslink and after a tight screen three or so remain to provide us with a couple of models of interaction. These models agree with the current idea of close active sites that have a positive surface to channel OAA to. Overlapped with human cMDH and watermelon MDH alignments (hMDH1 binds to CS at least half as strongly as hMD2 and wgMDH does not seem to bind at all; our and other work) gives a bigger picture of where the distinct and common MDH sites of CS

interaction remain. The CS sites that are responsible for binding to MDH can be looked at with a good pdb model, but we must create that ourselves as the authors did not provide the pdb file of any models.

Protein-protein interactions and metabolite channeling in the plant tricarboxylic acid cycle. Zhang, Beard, Swart, Bergmann, Krahnert, Nikoloski, Graf, Ratcliffe, Sweetlove, Fernie and Obata. Nature Comm. 8, 15212, 2017.

Advances the metabolon investigation into plants (*Arabidopsis thaliana*) using affinity MS, a luciferace assay and a yeast-two-hybrid approach. Combining these the positive results from all three were selected as positive hits. Both MDH1 and MDH2 (cyto & mito) were identified as interacting with CS although qualitatively in all three assays. Malic enzyme I and II also showed up as a positive interaction with MDH1 and 2. Both MDH isoform interactions with CS was confirmed using a complementation assay.

Brownian dynamic study of an enzyme metabolon in the TCA cycle: Substrate kinetics and channeling. Huang, Huber, Wang, Minteer, and McCammon. Protein Science, 27, 463-471, 2017.

A modeling paper looking at the channeling of OAA between the two active sites of MDH and CS. Using all three models from the Bulutoglu paper (even the sigma proteins as the active sites were flipped around), were used to identify the positively charged channel for OAA free vs guided diffusion. As a reminder, both the native tissue and recombinant MDH-CS had a positive charged surface for OAA to transfer. Using the previous calculated transit time, the Brownian diffusion model matched the predicted times of the native tissue and recombinant protein pairs. They end stating that these transient published times and the predicted times in this work are more efficient than the Elcock paper.

Interaction of enzymes of the tricarboxylic acid cycle in Bacillus subtilis and Escherichia coli: a comparative study. Jung and Mack. FEMS Microbiology Letters, 365, 2018.

Extending the IDH-MDH-CS in bacteria using purified proteins (tagged) added to a cell lysate with crosslinking. In this work, CS trapped IDH and MDH, IDH trapped CS and MDH and MDH trapped CS and IDH. To show that MDH interacted with CS, recombinant MDH-CS complex was mixed with excess mutant MDH that was catalytically inactive but able to bind to CS. The idea being that titrating in the dead MDH would stop OAA production for CS. BUT in these cases, CS activity increased not decreased, arguing against the channeling and instead was suggested that CS was allosterically being activated i.e. that MDH (and IDH) binding to CS would structurally alter CS resulting in a higher activity. Additional studies were conducted looking at the level of expression and where the gene for each enzyme was located supporting co-expression/interactions.

The role of dynamic enzyme assemblies and substrate channeling in metabolic regulation. Sweetlove and Fernie. Nature Comm. 9, 2136, 2018.

This is a review article that focuses on free vs diffusion calculations of substrates between several protein pairs. Worth reviewing for the kinetics / computational modeling alone and putting MDH-CS in perspective to other pairs.

Mito MDH – CS Interaction Summary: *mMDH and CS interact. As does CS and Aconitase and potentially MDH and Fumarase or GOT. The affinity and duration of the interaction can be defeated by ionic strength and dilution. Early studies using PEG helped drive the interactions in both solution and in crowded environments. PEG acts as a crowding agent by bonding to bulk water forcing proteins to interact and solvate their surfaces. There are several papers that use PEG to mimic the low bulk water environment of the cell and especially the mitochondria. But there are issues with PEG. For example, PEG alone decreases activity of MDH or CS. Thus we need to look at the interactions with and without crowding agents. The ionic strength arguments either block the interaction of the protein, or somehow negate the charged channel by which OAA is guided from MDH to CS. Interestingly, there are several papers that show that cMDH does indeed bind to CS, but usually when more sophisticated and more sensitive detection methods are used. When the cMDH-CS interactions are described, they seem to be an order of magnitude or more weaker than mMDH-CS. Very little investigation on the isoform specific domains between the two have been done. Thus a careful and consistent approach using the right buffer system with or without crowding agent should be used when conducting in vitro assays (i.e. pull down, thermal melts, fluorescent anisotropy, ect…).*

The trapping or competition assay using AAT (aka GOT and AST), is often used but a careful examination shows that the trapping is not efficient without crowding agent. Also important is the ionic strength and protein concentration for the trapping. It is a viable and necessary approach to study the interaction and when done right can support and demonstrate functional channeling of wild-type and mutants. We also see acetylation is key for GOT2-MDH2 interactions and the acetylation activates MDH2 activity. While CS is also acetylated, no studies on its function have happened. So what is going on with this regulation? In ME1, phosphorylation inhibits acetylation and the dimerization of that enzyme. Is there an analogous thing happening in mito for GOT-MDH-CS? Also – what is happening in the cytosol? There is evidence of GOT1-MDH1 interacting but little else has been looked at.

A lot of attention is paid and argued for and against transient time (lag between formation of OAA in MDH to use in CS) and the distance of active sites. Not sure if this is an area to get into without serious computational modeling power. However the last Srere paper and some of the others show an interesting opportunity to look for real interactions using traditional yeast genetic screening. For about 15 years the field was silent with the death of Srere while others moved to other projects. Recently Minteer and others have begun reinvestigating the MDH-CS interactions in part to examine ways to assembly macromolecular complexes (metabolons) for biofuel or other purposes. Yeast, bacteria and other species clearly show the interactions of MDH-CS. Some extend to the interaction of IDH-MDH-CS-Aco or MDH-CS-Aco. The most recent paper indicates that channeling may not be so important as an allosteric regulation activating CS after MDH or IDH interactions. The crosslinking and modeling done by the Minteer group is important but not totally conclusive-it isn't over yet people! First they have several cross-linked lysine that may be important to redefine the models that aren't used. They have two models that make sense if one biases for the electrostatic channel and proximity of active sites. Even then, why did all three proteins pair up differently in crosslinking and modeling.

They argue it is about dilute complexes (tissue vs purified protein) but don't explain why sigma MDH-CS linked and modeled differently than the recombinant proteins. Also the two "favored" models of interaction show different orientations to each other and the important N terminus of MDH. Thus this is a great start but bench work needs to be done to show what is going on.

Don't over-focus on the lysines. We know they are close enough to interact with CS but the crosslink is between two lys (from MDH and the other from CS) bridged by the linker. So Lys-Lys is not happening. But it tells us what parts of the two proteins are likely to be close enough together to make the cross link. How dynamic (floppy) a part of the protein is will impact the crosslinks - are they real or not? We need to refine our docking models but for now we are good. Also in diluted solutions, some of the crosslinks may not be more than a random and non-specific interaction. Missing is regulation of the interaction. Not only with MDH-CS but the other binders to MDH and CS. We need to look at both known Lys contacts and the sites of acetylation. A very interesting dynamic possibility here. Also phosphorylation…

There are many MS identified S/T/Y sites and a few more predicted phosphorylation sites of MDH. CS hasn't been analyzed yet. Some of these will play a role in regulation of dimerization of MDH and the active loop ect… but several may have an important role regulating the MDH-CS interaction. Finally, all interactions are focused on malate->OAA->citrate. BUT, during other metabolic needs, the interactions may need to be broken to allow pyruvate ->OAA->malate for shuttle out of the mitochondria. This may be different in liver, vs muscle or other mammalian tissue - who knows about other organisms and other pathways. At the end of this document are three papers that describe AST-MDH interactions and that acetylation supports this interaction. All three enzymes are acetylated, and while MDH acetylation (at least on the N term – where three sites happen and are predicted to interact with CS), acetylation of all four sites of MDH break the protein into less/inactive monomer. No information on CS and acetylation can be found. Thus what breaks the MDH-CS interaction to allow the reversal to take place? Phosphorylation, acetylation, both? Seems like an attractive hypothesis to develop.