Understanding the Interface: Exploring Malate Dehydrogenase using Computational and Experimental Approaches

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Dimeric Malate Dehydrogenase exhibits properties attributed to subunit interactions. The dimer interface comprises 47 residues, clustered in four groupings in the sequence, 15 residues are conserved in eukaryata, with 7 more functionally conserved. Structures of watermelon glyoxysomal MDH with or without the allosteric ligand Citrate bound to one subunit, were examined to explore the nature of subunit contacts (HINT). In addition, to examine second sphere residues with potential roles in catalysis, and to establish differences in conserved crystallographic water molecules we used POOL and DRoP respectively. Intra- and inter-molecular HINT analysis with no ligands bound versus the dimer with Citrate bound to one subunit indicates that D87 forms multiple hydrogen bonds within the interfacial 266-270 loop region, some having increased intensity with Citrate bound, (mobile loop closed) as compared to no ligands bound, (mobile loop open). Further analysis suggests R196 and T268 lose favorable interactions with D87 on the opposite subunit, while E256 loses unfavorable interactions with D90 upon citrate binding which draws S266 further into the active site causing T268 to shift away from D87 and closer to Q58. This affects L269-Q58 interaction across the interface. S266A and L269A mutants show loss of citrate inhibition and binding, and diminished substrate inhibition. T268D and I88A show little impact on cofactor binding although I88A becomes monomeric as shown by SEC and cross-linking. Coupled with changes in S266, T268 and L269 interactions across the interface, it appears that subunit interactions are triggered by cofactor induced changes in L269-Q58 interactions between subunits.

The two MDH subunits dimerize in a flipped pairing such that the active sites are on the same side and a straight line can be drawn from one pocket to the other. The center of the interface is a non-polar core formed mainly between the two Helix 5's. The strongest of these helical interactions is L63-L63, M62-L63, L63-M62, and M62-M62. The upper portion of Helix 3 (267-9) also contributes to the hydrophobic core. Helix 6 of the adjacent subunit is tightly packed against the hydrophobic core through hydrogen bonds between D92 and Y273 & K277 on Helix 3, and V195 and N198 on Helix 2. Helix 6 and the loop that follows contains polar residues that influence the dynamics of the interface and subsequently the active site. Previous research has identified a variable active site loop, however a second loop between Helix 1 and Helix 3 is located near the active site and serves as a means of communication with the interface via interactions with D87 from the other subunit. Substrate binding causes this loop region to become re-ordered, which changes the backbone's configuration and the interactions with D87. Specifically, S266O-D87OD2 and A267CB-D87OD1 transition from attractive to repulsive interactions, however, these large switches in interaction type are not felt at the other active site. These repulsive interactions alter the placement of Helix 6, which is near the active site of the adjacent subunit. D87 is directed closer to L269 forming favorable interactions, however, the unbound subunit interaction becomes more attractive. D87 forms complex interactions with T268 that are also not propagated to the other subunit. For example, T268N-D87OD2 increases in interaction score by 111 with substrate binding, but D87OD2-T268N increases in attractiveness by 293. Further, there is a balance with the side chains of T268 that repels the D87 side chain. H90 is located further down Helix 6 and interacts with Helix 2, which is directly implicated in the active site via R196. Substrate binding causes V195CG-H90O to switch from attractive to repulsive forces, with the unbound subunit having stronger repulsive forces. The H90 side chain is able to form positive interactions with R196's side chain and may affect the chemistry present at the active site. The R196NH2-H90ND1 interaction becomes weaker and weak positive interactions between R196 and D87, H90, and A86 found in the AB interface disappear in CD. As a result of these arrangements, H90 is able to form a stronger hydrogen bond with E256. The G94CA-D208OD2 interaction switches from a repulsive interaction to an attractive interaction and may also play a role in the effects of D87&H90.

Figure 2. Initial rate kinetics of native and mutant wgMDH with OAA and NADH. (a) *OAA* Purified protein was used in an assay containing 100 µM NADH and varied OAA concentrations in 5 mM phosphate buffer (pH 8). (b) *NADH* Purified protein was used in an assay containing 200 µM OAA and varied NADH concentrations in 5 mM phosphate buffer (pH 8).

Figure 3. CD spectra and thermal melt curves of native and mutant wgMDH. (a) *CD spectra* Purified protein was dialyzed in 5 mM phosphate buffer (pH 8). An absorbance scan was performed to ensure protein samples had a minimum absorbance of 0.4 at 220 nm. The scans above are representative of 3 trials, each being an average of 3 accumulations. The scans were taken from 195 to 250 nm. (b) *Thermal melt curves* Purified protein was dialyzed in 5 mM phosphate buffer (pH 8). Thermal melts were conducted from 20°C to 70°C and monitored at 222 nm. Each graph is representative of 3 trials.

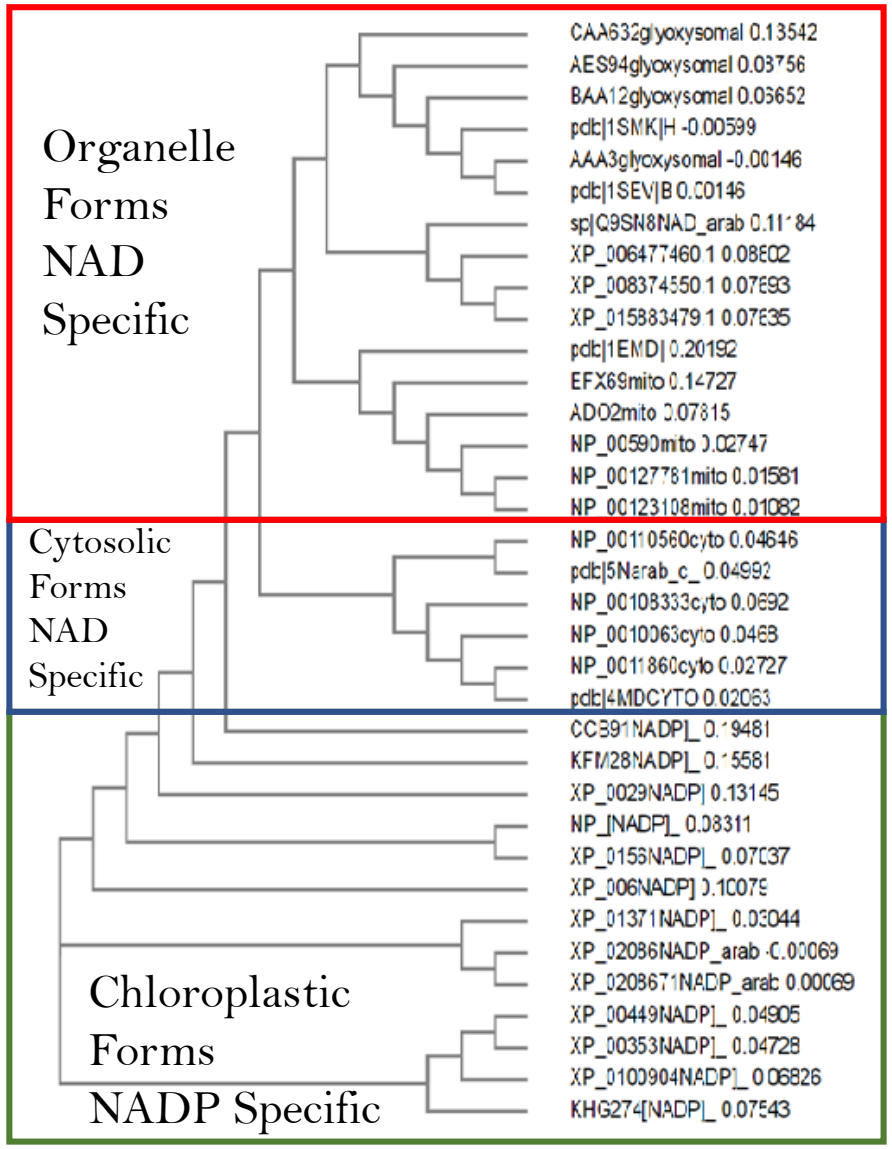
Figure 4. SEC and cross-linking with BS3 of native and mutant wgMDH. (a) *SEC* Purified protein was dialyzed in 25 mM phosphate buffer (pH 8), 150 mM NaCl, and 1 mM BME. The samples were run on an EnRICH650 column. (b) *Cross-linking with BS3* Purified protein was diluted to 50 µM and cross-linked with 100x BS3. Samples were run on a 4%-20% gradient SDS Page gel and stained using Coomasie Blue.

Figure 5: SYPRO-orange dye was used as a fluorescent probe of thermal denaturation via selective binding to exposed non-polar regions. An average midpoint of three replicates was determined by curve fitting to 4-parameter sigmoidal plot. A variety of ligand conditions [(a) Malate (b) OAA (c) NAD+ (d) NADH (e) Malate & NADH (f) Oxaloacetate & NAD+ (g) Citrate] were used to assess thermal stability.

*Fluorescence-based Thermal Shift Assay***:** A 96-well plate was used for high-throughput screening. Each plate consisted of thirty-two separate conditions including negative and positive controls. 20X SYPRO-orange and 4 uM WT (red [EE2205]), I88A (yellow [FFFC66]), L269A (green [61D836]), T255E (blue [0D8BFF]), S266A (purple [4E00FF]) gMDH in 5 mM pH 8.0 phosphate buffer were used. Ligand conditions ranged from 0-2.5 mM Malate, 0-2.2 mM OAA, 0-3.5 mM NAD+, 0-2.0 mM NADH, 0-200 mM Citrate, 1 mM NADH with 0-2.2 mM OAA, and 1 mM NAD+ with 0-2.2 mM Malate. The plate was sealed and centrifuged at 1000 rpm for 1 minute. A Bio-Rad CFX96 Real-Time system was programmed to measure fluorescence at 570 nm as the plate's temperature was first held at 15 ˚C for 30 minutes, increase by 0.5 ˚C until 95 ˚C, and was then cooled to 15 ˚C. Data was background corrected using the negative control and curve-fit using SigmaPlot to determine thermal melting point.

Clustal Omega Analysis of Major Classifications of Malate Dehydrogenase

Bell Lab

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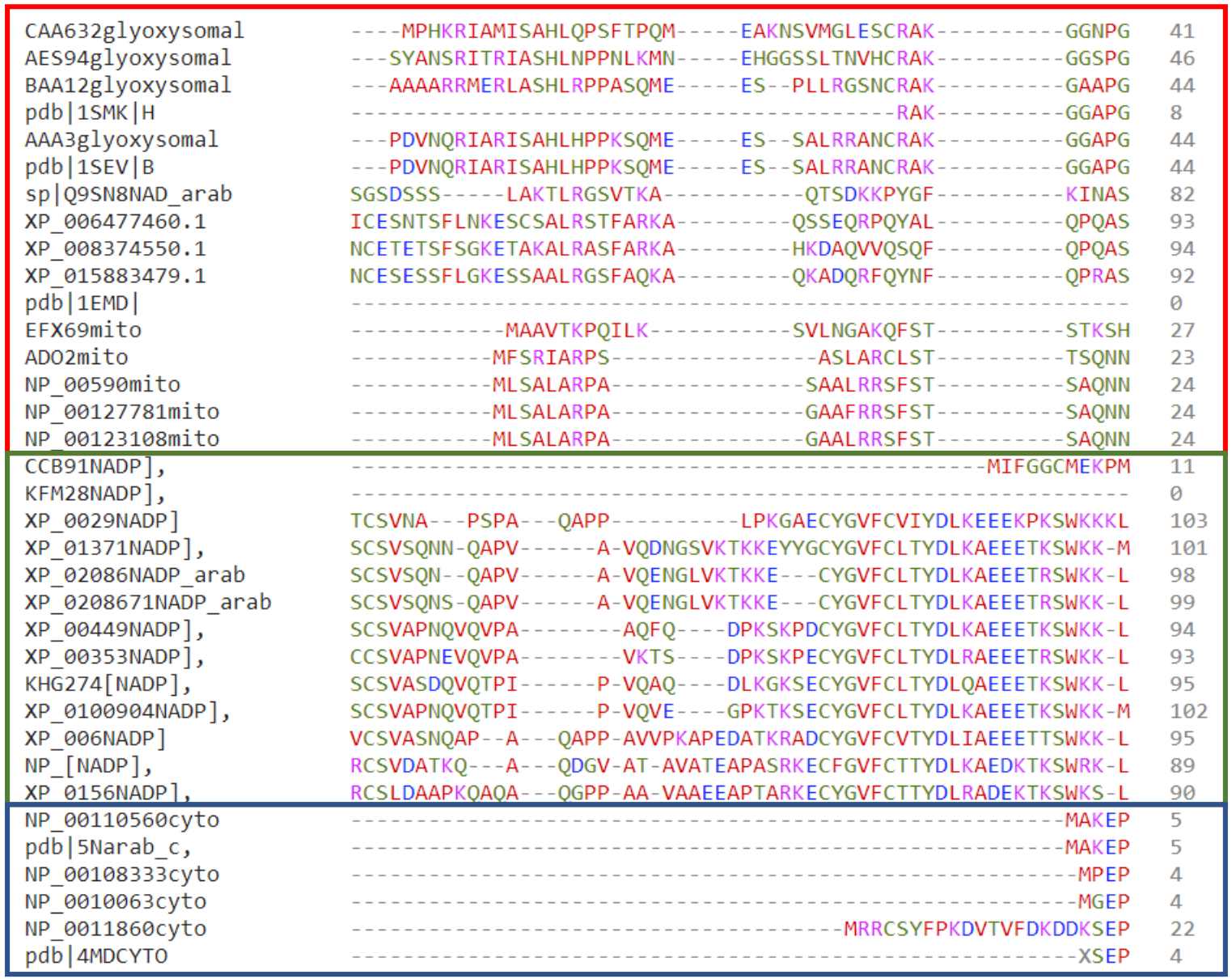
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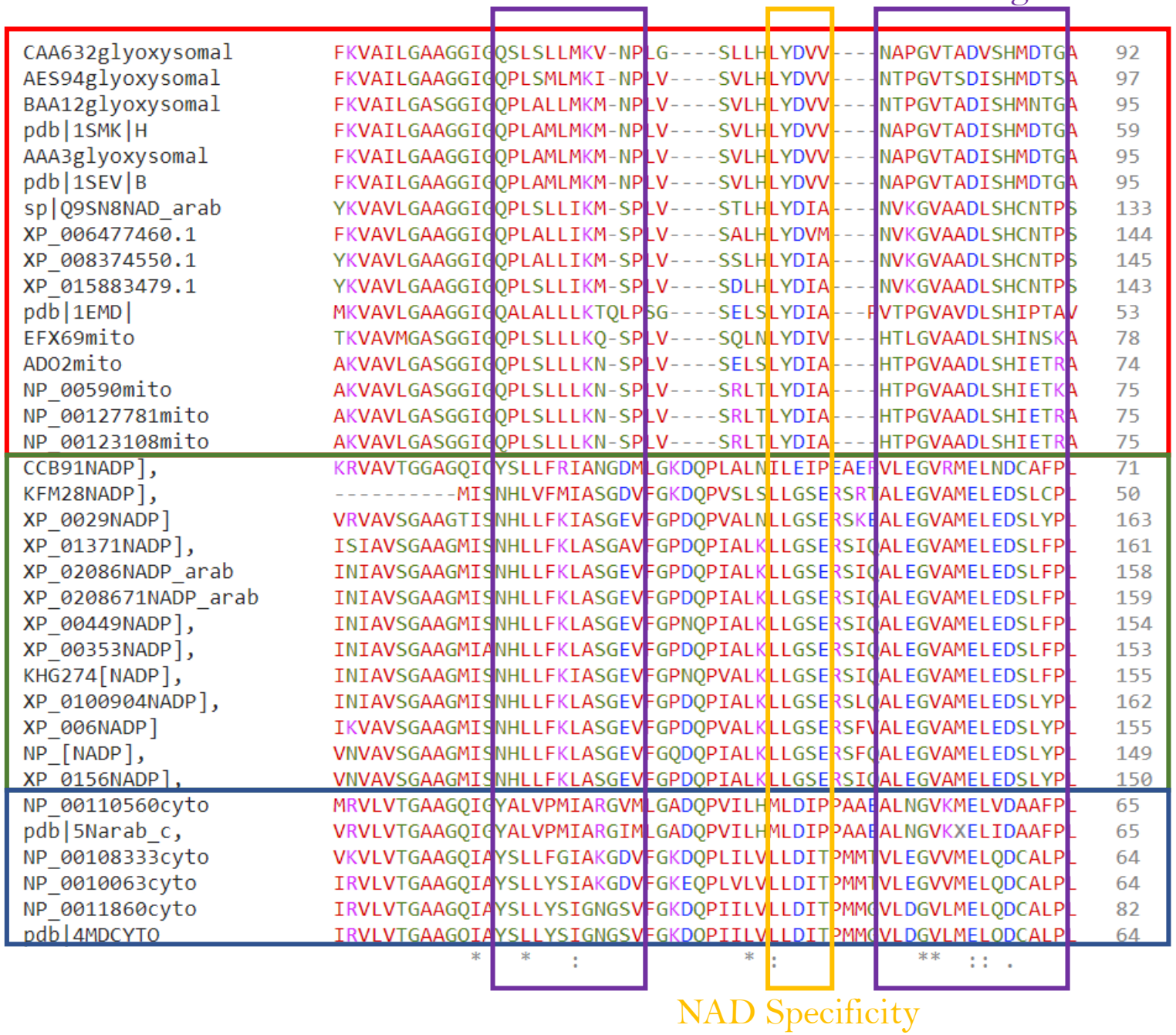
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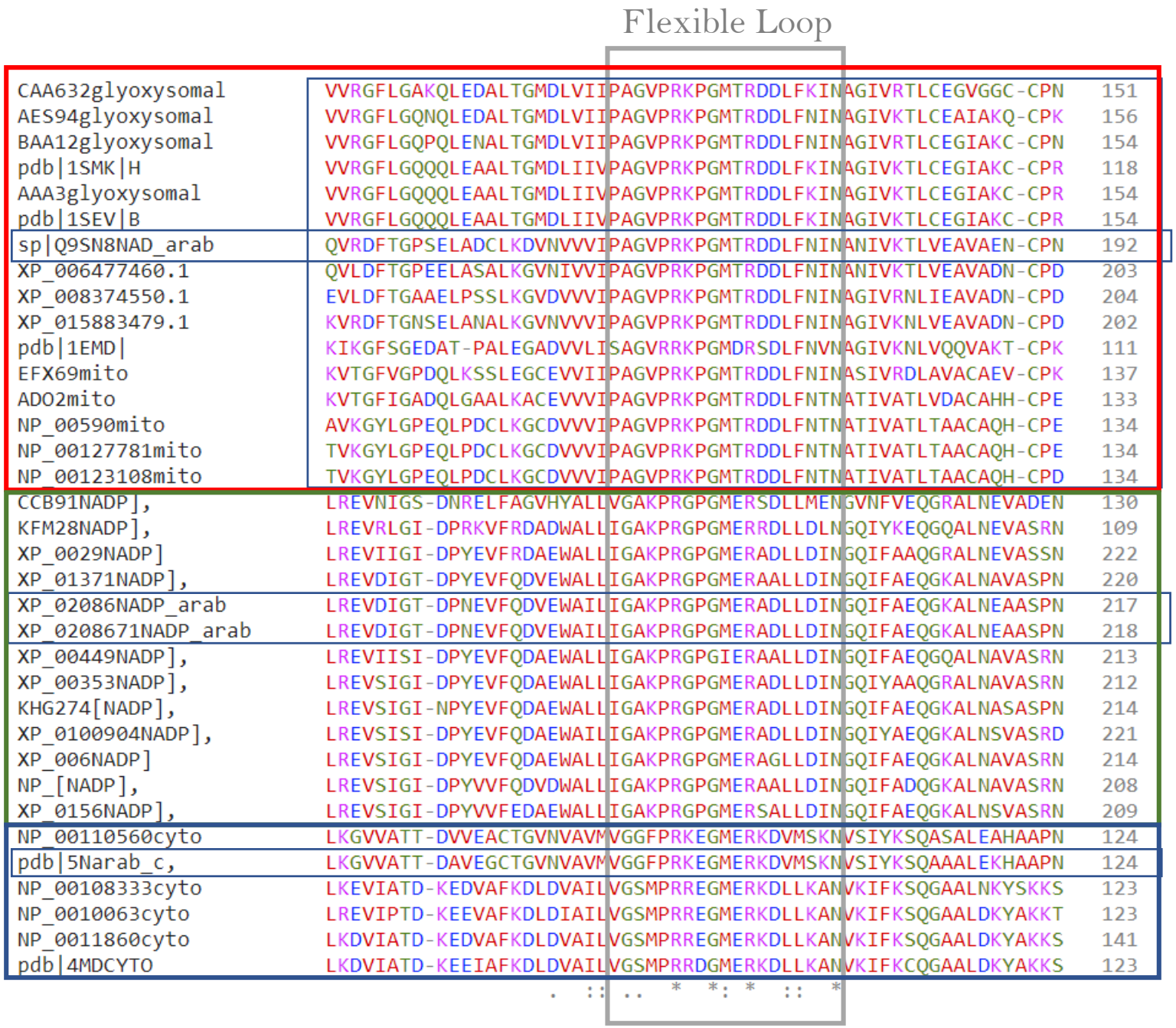
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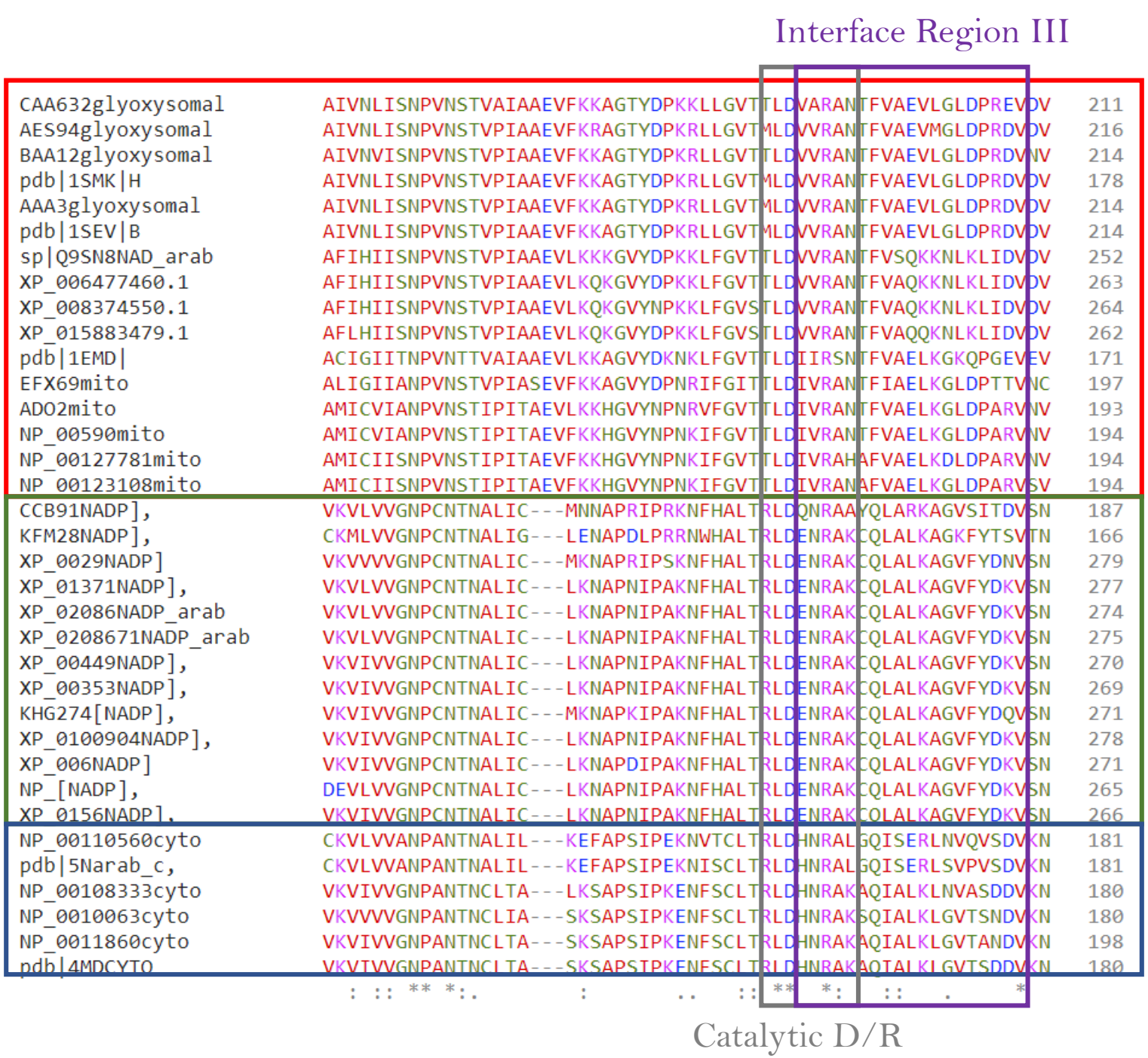
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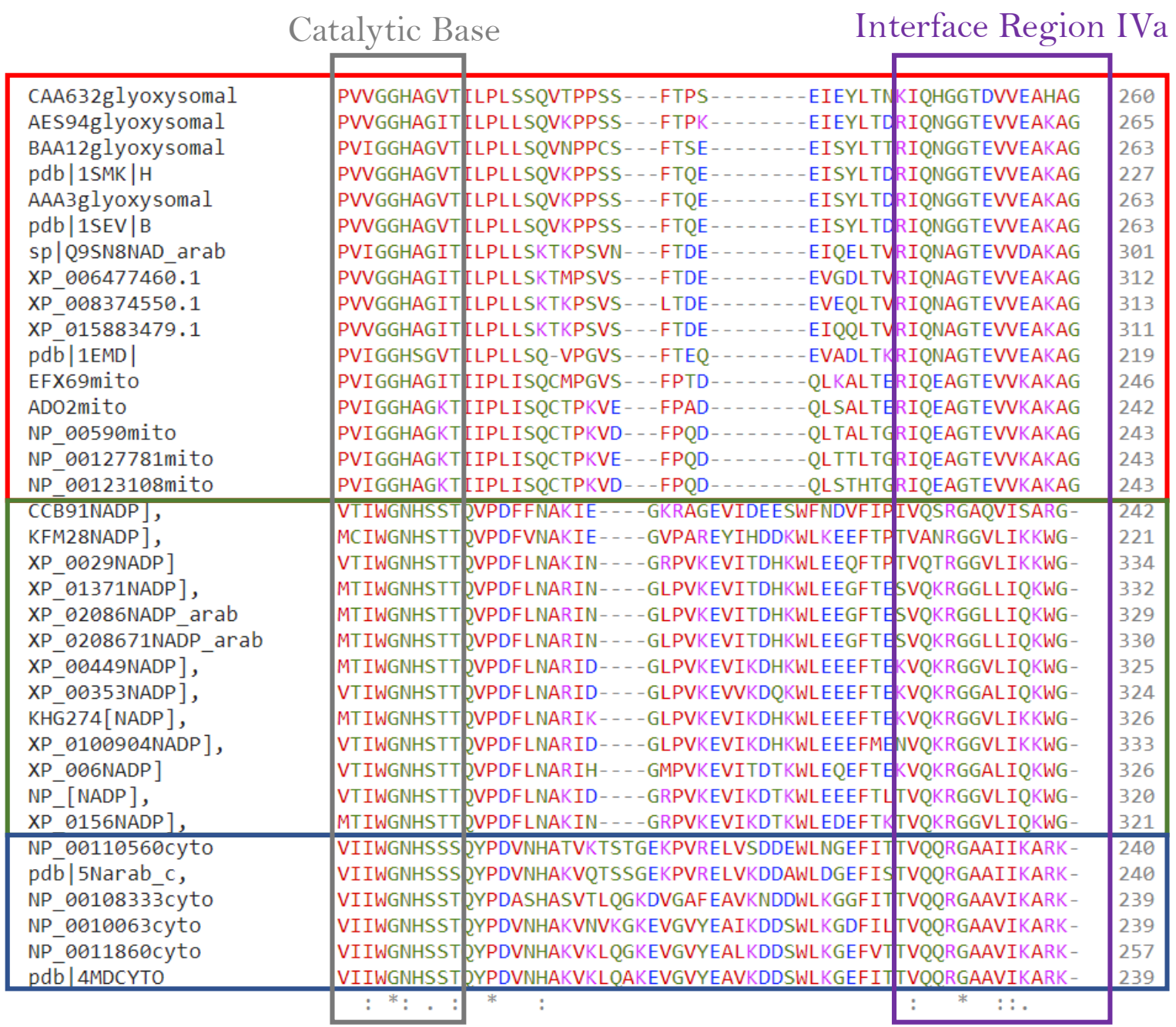
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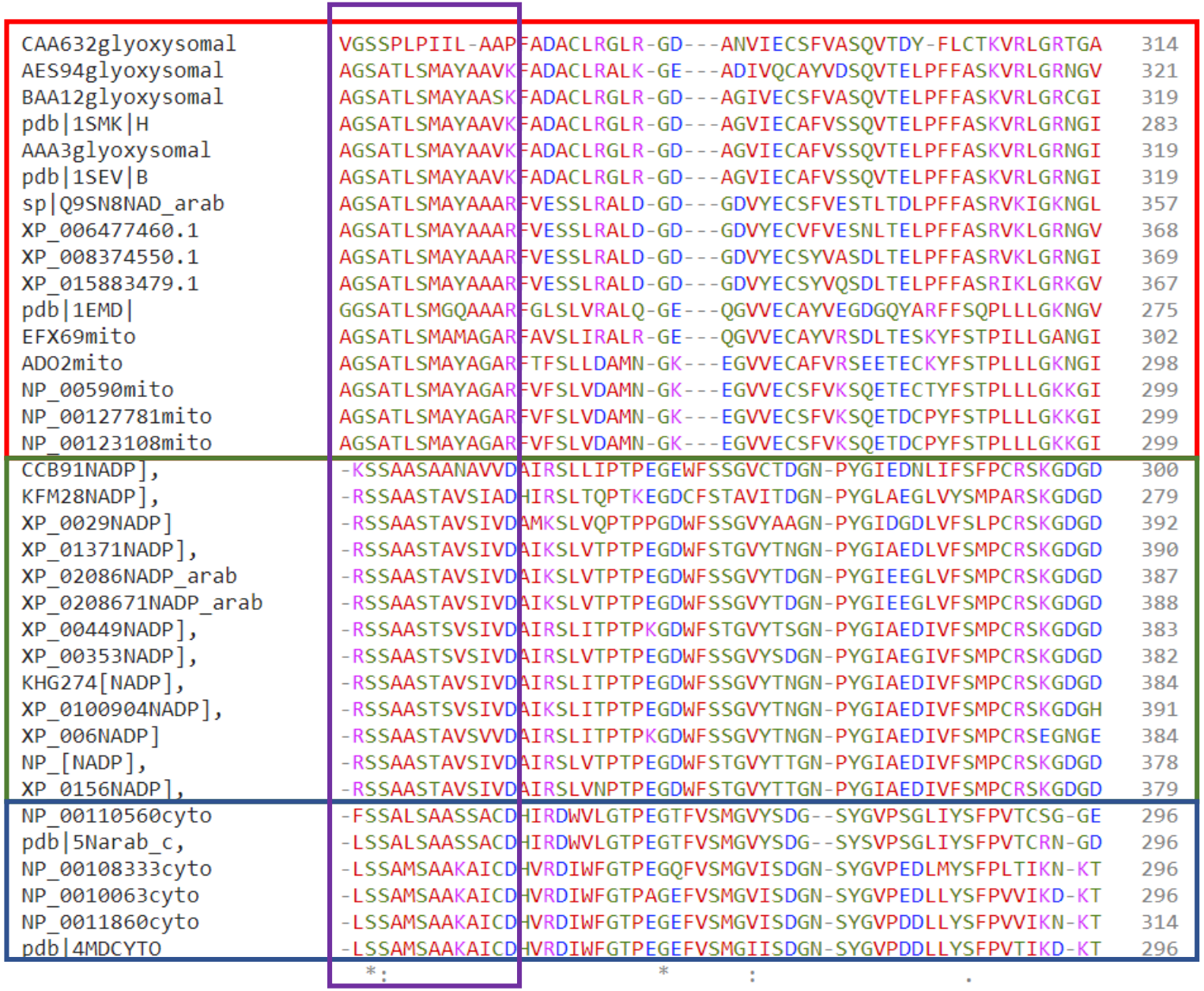
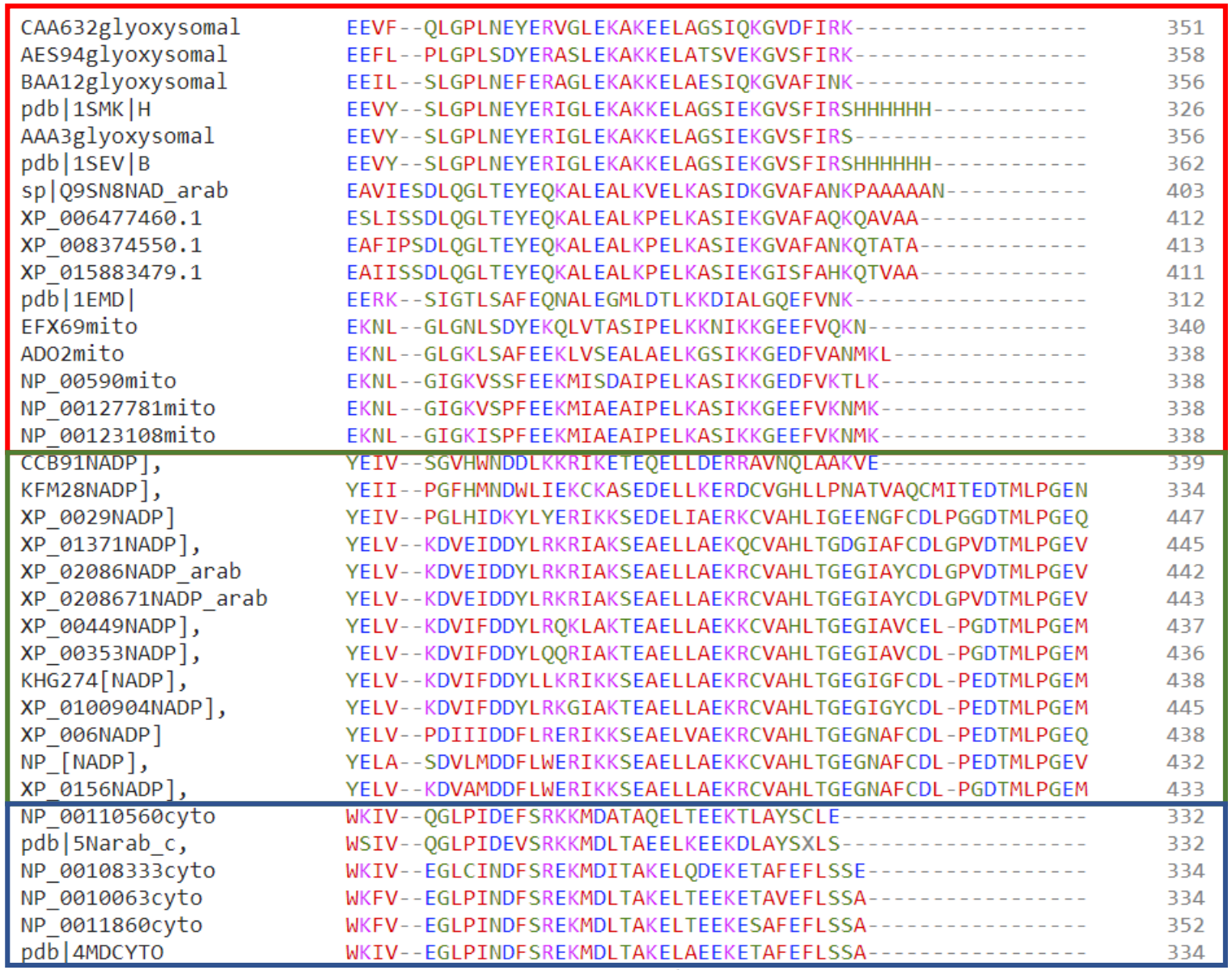
Interface Region IVb

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* .

A unique facet amongst Malate dehydrogenase is the conserved tertiary structure given variations in primary sequences. As a metabolism enzyme, ero critical to cell survival, malate dehydrogenases are under intense regulation and have evolved several isoforms. Obeying the theory of a symbiotic organelle relationship, the bacterial cytoplasmic form and ribosome/glyoxysome/chloroplast forms have higher sequence homology than cytosolic eukaryote forms. Five key residues are well-studied in terms of their necessity for catalytic function. A classic dehydrogenase his-asp pair participate in the hydride abstraction, while three arginines have been shown to be important for creating a binding pocket suitable for carboxylic rich substrates. Two of these arginines are located on a variable active site loop that cycles through an active/closed and inactive/open conformation, while the third is located in the protein interior. Several malate dehydrogenase crystal structures have been published from a variety of species and organelles. Our model system for experimentation utilized the glyoxysomal *C. lanatus* with PDB ID 1SMK structure as the main crystallographic model. In this model H220 and D193 are the catalytic pair with R124, R130, and R196 as the critical arginines. Given the high homology in malate dehydrogenase, the cofactor binding domain is presumed to be conserved. From the *e. Coli* 1IE3 structure, this translates to back bone interactions with G55, I56, G121, I160, N162, V189, and side chain interactions with H220, N137, and D77. Malate dehydrogenase require both substrate and cofactor binding for activity, and dimerization has also been demonstrated to be critical for function.

The asymmetric unit of the 1SMK model contains four homodimer complexes. Each monomeric unit contains eight alpha helices (α1:57-65, α2: 81-90, α3: 131-151, α4: 164-177, α5: 191-204, α6: 241-260, α7: 269-286, α8: 331-356), along with a 3/10 helix (106-110), nine beta sheets (β1: 46-50, β2: 73-76, β3: 96-100, β4: 115-118, β5: 156-159, β6: 186-188, β7: 293-298, β8: 308-313, β9: 320-322), and 17 loop/unstructured regions (L1: 51-56, L2: 66-72, L3: 77-80, L4: 91-95, L5: 101-105, L6: 111-114, L7: 119-130, L8: 152-155, L9: 160-163, L10: 178-185, L11: 189-190, L12: 205-240, L13: 261-268, L14: 287- 292, L15: 299-307, L16: 314-319, L17: 323-330).The back of the molecule (relative to the active site) contains a six strand parallel sheet consisting of a rossmann fold and 3/10 helix linking β3-β2-β1-β4-β5-β6. β7-9 participate in a hairpin-like structure that may also interact with the six strands. H220 is located on L12, while D193 and R196 are located on α5. A key difference between the AB and CD dimer is the placement of a single active site loop (L7), containing R124 and R130. In the A,B,&D subunit the active site loop is open, while the C subunit complex contains a tri-carboxylate substrate mimic (Citrate) and the closed loop conformation. Here we present data to support a hypothesis where modulation of a secondary active site loop (L13) by the subunit interface points to mechanism of subunit communication through the interface and implicates a mechanism that requires cofactor bind first to prime the active site for the dicarboxylic acid substrate.

The overall quaternary structure of malate dehydrogenases are the same, where the two homodimers come together in a reciprocal symmetry. The α1 helix is involved in non-polar interactions across the interface with α1 and α7 helices, while the α2 helix reaches into the active site pocket and interacts primarily with α5, α6, and α7. α2 serves as a mediator of intramolecular interactions between α5 and α7. The α7 helix serves as a critical junction between the highly charged active site and non-polar dimer core. Within these elements of secondary structure, key sequences are highly conserved amongst bacterial and organelle-specific malate dehydrogenases. For example, the reciprocal point of malate dehydrogenase dimer interface is the α1-α1 helix, which is a non-polar (M62/L18) residue in (1SMK/e. coli). Given the hydrophobic nature of the subunit interface, we turn to HINT to score the relative interactions between atoms at the

interface based on their calculated electrostatic potential. To compare how these forces change with the addition of substrate and cofactor molecules, and given the high homology of the MDH tertiary structure, we turn to several other *e. coli* models for comparison. The 1MLD structure contains two citrate molecules bound to the closed subunits and is sequentially more related to the 1SMK model. The 1IE3 structure contains two NAD molecules, a mono-carboxylic acid (pyruvate) bound to one subunit, and a R153C mutation. The 5KKA structure contains two NAD analog molecules bound, while 2PWZ has no ligands bound.

Our data from the fluorescence-based thermal shift (FTS) assay implicates cofactor as the first molecule to bind in the bimodal mechanism in order to prime the active site for substrate binding, however, citrate is able to overcome this necessary 'priming' and bind to the cofactor-less molecule. Malate/oxaloacetate are dicarboxylic acids with a large apparent negative charge and presumable bind readily to the solvent-exposed R124 and R130 loop residues, however, modulation of the relative baseicity of the third R196 residue plays a role in both substrate binding and creation of the catalytic environment needed for proton abstraction. Several conserved regions of the subunit interface also play a role in modulating this positive environment. Namely positions 87-90 in α2 on one subunit, along with positions 266-270 on the end of the active site secondary loop 13 residues on the adjacent subunit, which includes, branch out from the non-polar reciprocal point. While these regions are conserved, the regions immediately before and after (L4 interactions with α5 and α7) are different within the clad and may contribute to the different states of the primary active site loop and thus the ability to bind one or two substrate molecules.

In all malate dehydrogenases position 87 (D87; 1SMK), contributes a large negative charge at the subunit interface and modulates the 266-270 loop. In the unbound *e. coli* form, the side-chain carboxylic acid forms positive interactions with S266 backbone oxygen, while cofactor binding changes this to a repulsive force. This also has an effect of decreasing the repulsion of K261 with V84. Another force that appears with cofactor binding is the small attractive force between R196. Interestingly, the 1SMK unbound structure more closely relates to the cofactor bound *e. coli* structure, and thus the repulsive S266 backbone oxygen and positive guanidinium group of R196 interactions with D87 side chain are independent cofactor binding. Cofactor binding causes the A267 side chain to transition from attractive to D87 to repulsed by the carboxylic groups, while substrate binding changes it back to an attractive interaction. In contrast to pyruvate, citrate binding causes this interaction to remain negative, while dual citrate binding reduces some of the negative interactions; however, this could also be an affect of the R153C mutation. Substrate binding causes an induced fit that allows T268 to rotate bringing the side chain hydroxyl group closer and repelling the D87 side chain. Q58/Q14 is fully conserved and sits in between position 268 and 269 and adjusts in response to this rotation. This is different in the citrate form, where the T268 side chain is rotated to a midpoint between the substrate bound and unbound states. At this midpoint, the attractive forces are maximal, where the double citrate form is slightly less attractive due to weaker repulsions with the 269 position. These interactions with D87 intensify the non-polar interactions present at position 88 (I88; 1SMK).

In all malate dehydrogenases, position 88 is nonpolar and serves as a critical non-polar socket. The non-polar socket forms on α2 between the 84 and 88 positions and makes contact with the clad conserved non-polar 269 position to form a sort of joint. The local dielectric strength of the 269 location is greatly reduced by the surrounding D87 and Q58 residues, which increases the strength of the Van Der Waal interactions between position 84 and 88 on the adjacent subunit.

In all of the clade malate dehydrogenases position 90 is histidine that directly interacts with R196/R153 and E256/E212. In the *e. coli* form, cofactor binding brings the imizadole ring closer to the gunidinine group. This slight change also brings the 256 position closer to the histidine and introduces positive interactions. This is different in the 1SMK form, where H90 interacts with R196 independently of cofactor binding.

The large differences between the 1SMK and *e. coli* forms originates from two locations of low sequence homology on either side of conserved α2 positions, namely 86 and 92. The 86 side chain points in different directions between 1SMK and *e. coli* forms, where A86 (1SMK) points away from the α5, and V44 points towards it. This leads to a flip-flopped effect where strong forces occurring between the subunit interfaces are parallel, but on opposite sides. For example, D92 (which is a proline in *e. coli*) on L4 has strong interactions with α7 and α5, while in *e. coli* the strong attractive force occurs from the end of α5 to β3. These result is that different forces in either form stabilize the placement of H90 in the interface. For example, the T199/T156 position interacts with the H90 backbone in both structures. In 1SMK T199 is connected to D92 via positive interactions with N198 and N155, while T156 in *e. coli* is connected to position 54 (98 in 1SMK) and 96 (140 in 1SMK) via 160 (204 in 1SMK).

Several mutations were made. L269A and I88A mutants were made an affect the non-polar 'ball and socket' model. L269 serves as the ball to the 84/88 socket. In the L269A mutation, the side chain is shorter and thus unable to reach into the 84/88 socket. This likely has an effect of shifting the socket over to the α1 helix and maintaining dimerization. In the I88A mutation, the socket has been severely damaged and there is nothing for L269, or any non-polar residue to 'latch' into and explains why the I88A mutant is monomeric. The S266 side chain points into the active site and its position is modulated by D87 interactions with the backbone. In the S266A mutation the side chain is no longer a potential hydrogen bond donor to potential substrates, this removes some of the strain experienced by the loop and its interactions with D87 and directly affects the communication network.

Molecular Modeling.

Protein preparation: atomic coordinates from PDB entry 1SMK were used for molecular models of MDH. The A:B chains were used for apo inter-residue interaction scoring; the C:D chains were used for inter-residue interaction scoring for the citrate bound form. The A:B and C:D subunit dimers pairs were energy refined as separate entities. The 'protein preparation pizard' in Maestro version 2017-1 (Schrodinger LLC, New York, NY), was used to add hydrogens, assign bond orders, and cap termini. Following, the Discovery Module in Insight 2005 (Dassault Systèmes BIOVIA, San Diego, CA) was used to energy refine the models. The dielectric was set to 1.0*r and atom potentials were set using the cff91 forcefield. First, hydrogens only energy refinement (100 steps of steepest descents followed by conjugate gradients minimization until the the maximum derivative was < 0.001 kcal/Å) was performed. Next, with main chain heavy atoms frozen, side chains were energy refined (100 steps of steepest descents followed by conjugate gradients minimization until the maximum derivative was < 0.001 kcal/Å). In addition, for C:D subunit side chain refinement, a template force constant of 20 kcal/Å^2 was applied to the citrate molecule's heavy atoms. Final energy refinement for both the A:B and C:D subunit entities involved: a) all atoms free to move in coordinate space, but with a 20 kcal/Å^2 template force constant applied to backbone heavy atoms (100 steps of steepest descents followed by conjugate gradients until the maximum derivative was < 0.001 kcal/Å), and b) all atoms free to move in coordinate space using conjugate gradients minimization until the maximum derivative was < 0.001 kcal/Å.

Hint Scoring Algorithm.

The HINT (for "Hydropathic INTeractions") program (eduSoft LLC, Richmond, VA) scoring algorithm was used to calculate atom-atom (and hence residue:residue) inter-molecular interactions between the MDH A:B subunit interfaces and C:D subunit interfaces. The program has been described in detail previously (***Ref 1**: J.C., Burnett, C., Lim, B.D., Peyser, L.P., Samankumara, M., Kovaliov, R., Colombo, S.L., Bulfer, M.G., LaPorte, A.R., Hermone, C.F., McGrath, M.R., R., Gussio, D.M., Huryn, and P. Wipf, Org. Biomol. Chem., 2017, 15, 4096; **Ref 2**: Kellogg, G. E. & Abraham, D. J. KEY, LOCK, and LOCKSMITH: complementary hydropathic map predictions of drug structure from a known receptor-receptor structure from known drugs. J. Mol. Graph. 10, 212-217, 226 (1992)*). In brief, the algorithm scores a summation of the hydropathic interactions between all intermolecular atom-atom pairs based on six 6 classes. The "favorable" classes include hydrogen bonds, acid/base, and hydrophobic interactions, while acid/acid, base/base, and hydrophobic/polar interactions are "unfavorable" categories. Each atom potential type has a corresponding hydrophobic constant derived from the hydrophobic fragment constant approach of Hansch and Leo (***Ref 3**: Hansch, C. & Leo, A. Substituent Constants for Correlation Analysis in Chemistry. John Wiley & Sons: New York (1979)*). A summation of all of these constants for a given molecule results in the total molecular partition constant for that molecule. This methodology reduces the empirical information from bulk molecular solvent partitioning to discrete atom-atom interactions. Because of this approach, the hydrophobic constants derived include a linear free estimate of entropy, which is ignored in molecular mechanics models. Thus, a properly constructed set of comparisons using these hydrophobic parameters as its basis yields a much richer set of information when evaluating the interactions between two species than a method based solely on parameters derived from mechanics.

Protocol:

Protein Expression & Purification of wgMDH

Protein Expression

1. Make 1 L luria broth for each mutant and native gMDH by dissolving on capsule in 1 L deionized water. Autoclave this to sterilize – liquid cycle for 20 min at 121°C.
2. Prepare starter cultures (~5 pm):
    a. Use 12.5 g LB crystals and dissolve in 500 mL deionized water. Autoclave on liquid cycle for 20 min at 121°C.
    b. Aliquot 50 mL into sterilized 250 mL baffled Erlenmeyer flasks.
    c. Add 50 µl of 100 mg/mL carbenicillin or ampicillin into each flask + LB.
    d. Pick single colony from plate with sterile tip & eject tip into culture. OR Using glycerol stocks in -80°C, scratch stocks with sterile pipet tip and eject entire tip into flask.
    e. Incubate at 37°C overnight, shaking at 220 rpm. Should be turbid by next am (@ or before 8 am).
3. Add 1 mL of 100 mg/mL carbenicillin or ampicillin to each 1 L LB and 1-10 mL of each starter culture (depending on timing of induction).
4. Incubate the 1 L cultures at 37°C, shaking at 220 rpm.
5. Check OD at 600 nm using the "Simple Reads" program on the UV-Vis spec. Blank with autoclaved LB media. OD of culture should be at least 0.6, but not higher than 1 before inducing.
6. Induce with 2 mL of 1M IPTG.
7. Incubate overnight on shaker (220 rpm) at 22°C for 20 hrs.
8. Transfer 1 L cultures into centrifuge bottles. Spin down at 4K rpm for 15 min at 10°C.
9. Pour off supernatant and freeze pellet in -80°C, if not using right away.

Protein Purification

Buffers:

- Lysis buffer: 50 mM phosphate buffer (pH 8), 300 mM NaCl, 10 mM imidazole
- Wash buffer: 50 mM phosphate buffer (pH 8), 300 mM NaCl, 20 mM imidazole
- Elution buffer: 50 mM phosphate buffer (pH 8), 300 mM NaCl, 250 mM imidazole

1. Resuspend the frozen pellet in ~40 mL lysis buffer. Add 40 mg lysozyme (1mg of lysozyme/mL of lysis buffer) and protease inhibitor (1 cocktail pill (Roche Complete EDTA-free) dissolved in 1 mL lysis buffer). Mix well and incubate on ice for 1 hr.
2. Transfer solution into 50 mL falcon tubes. Sonicate 5 times for 30 seconds each at 50% power with 1 min rest between cycles – ON ICE in cold room.
3. Transfer to centrifuge tubes (50 mL) for centrifugation. Centrifuge for 30 min at 12k rpm (~14K xg) at 10°C.
4. While the centrifuge is running, equilibrate the Ni-NTA resin. NOTE: This can also be done in column format.
    a. 5 mL resin + 45 mL milliQ water in a 50 mL falcon tube. Centrifuge for 1 min at 1000 rpm using the bench top centrifuge.
    b. Discard water. Add 25 mL lysis buffer and centrifuge for 1 min at 1000 rpm using the bench top centrifuge.
    c. Discard almost all lysis buffer.
5. After centrifuging the cell lysate, keep the supernatant (protein is here) and discard the pellet.
6. Add the supernatant to the equilibrated resin. Rock in cold room for 1-2 hours. This can rock overnight, but remember proteases can degrade your protein.
7. Centrifuge 1 min, 1K rpm at 5°C using bench top centrifuge. Remove supernatant and keep as flow through (Can pour off or remove with plastic transfer pipette).
8. Add 40 mL lysis buffer. Rock in cold room for 10 min (or incubate on ice bucket and invert tubes every ~2 min).
9. Centrifuge 1 min at 1000 rpm at 5°C using bench top centrifuge. Discard supernatant.
10. Add 40 mL wash buffer. Rock in cold room for 10 min (or incubate on ice bucket and invert tubes every ~2 min).
11. Centrifuge 1 min at 1000 rpm at 5°C using bench top centrifuge. Discard supernatant.
12. Add 10 mL wash buffer. Transfer resin (where protein is bound) to 15 mL falcon tube. Rock in cold room for 10 min (or incubate on ice bucket and invert tubes every ~2 min). Centrifuge for 1 min at 1000 rpm at 5°C using bench top centrifuge. Remove supernatant.
13. Add 5 mL elution buffer. Rock in cold room for 10 min (or incubate on ice bucket and invert tubes every ~1 min). Centrifuge for 1 min at 1000 rpm at 5°C using bench top centrifuge.
14. Keep supernatant as Elution 1 in new, labeled falcon tube. Repeat Step 13 for 4 elutions for each mutant and the native. After 5 elution fractions, if protein is still eluting from resin, increase elution buffer to 10 mL/fraction.
15. NOTE: AFTER EVERY WASH, COMPLETE A BRADFORD. CONTINUE COLLECTING ELUTION FXNs UNTIL BRADFORD SAMPLE MATCHES BRADFORD BLANK.

a. Prepare Bradford dye (BioRad Assay Reagent). If your total volume per Bradford assay is 200 μL, recommend that your sample volume is 25 μL. Prepare dye: (40 μL dye per rxn (Dye is 5X; 40 μL is 1/5 of 200 μL) + 135 μL H2O)*(# of samples + 2). Place 175 μL of diluted dye/microcentrifuge tube for each sample to be tested. For control reaction tube, add 25 μL elution buffer to 175 μL diluted dye. Invert to mix. This is your blank/background. For each elution fraction, add 25 μL elution fraction to 175 μL diluted Bradford dye. Invert to mix. Compare to blank to determine if protein is present in fraction (presence of blue color=protein).

Partial Order Optimum Likelihood (POOL) is a computational program that employs machine learning to rank protein residues by the probability it is involved in the function of the protein. THEoretical Microscopic Anomalous TItration Curve Shapes (THEMATICS) and ConCavity are two algorithms that evaluate the protein structure and serve as input values into POOL. THEMATICS calculates a theoretical titration curve for each residue in the protein and ranks the residues by the deviation from a sigmoidal shape, which is evaluated as the fourth moment of the curve. ConCavity evaluates the shape and location of each residue on the ability for solvent molecules to bind; for example, concave and solvent exposed regions get higher scores. POOL analysis was applied to the CD dimer subunits in the 1SMK crystal structure. The CD dimer, C subunit, and D subunit were submitted to POOL separately for probability ranking; the top 15 predicted residues for each structure are shown as sticks. Residues within these three lists that appear in the dimer but not the monomer are colored blue, while residues that appear in either monomer and the dimer are shown in cyan. Residues within 5 angstroms of the citrate molecule (magenta, sticks) in either subunit are colored yellow and labeled.
Residues D193, R196, H220 in the C subunit are within this 5 angstrom range of citrate and occur in both POOL rankings, while residues I56 and I160 only appear in the C subunit of the dimer. Residues I160 and D193 in the D subunit are within this 5 angstrom range and occur in the POOL ranking for both rankings, while residues R196 and H220 are also within this range but only occur in the dimer.

Reference for POOL: Parasuram, Ramya, Timothy A. Coulther, Judith M. Hollander, Elise Keston-Smith, Mary Jo Ondrechen, and Penny J. Beuning. "Prediction of Active Site and Distal Residues in E. Coli DNA Polymerase III Alpha Polymerase Activity." Biochemistry 57.7 (2018): 1063-072. Web.

Here are also some analysis I had written in March:

DRoP of 1SMK
1SMK subunits were paired into the two populations: open & closed based on the bfactor values for the arginines 124 and 130 on the variable active site loop. The open population (chains BDGH) and the closed population (chain ACEF) are considered inactive and active states of the protein subunit respectively. The two sets were submitted to DRoP for analysis to condense the symmetry related water molecules and rank water molecules according to conservation in the prospective sets. The open set has only one water that is found in every subunit, while the closed set has three waters. The following contains a description of top/interesting waters.

Closed set:

- Water 1 in the closed set forms hydrogen bonds with the backbone oxygen of V184 and N-terminal of 192's at the beginning of a helix; water 1 is variably associated with the catalytic H220. In chain A H220NE2 is in hydrogen bonding distance from water 1, in chains E and F H220NE2 is rotated further away from the water molecule. Chain C there is a 1.8 Å shift in the position of H220NE2 from the rotation in chains E and F.
- Water 2 in the closed set forms hydrogen bonds with the side chain with the NE and NH1 of R248 and D289OD2.
- Water 3 has an RMDS of 0.635, almost double the RMSD value of water 2. In Chain A this water forms hydrogen bonds with P119O and G51O, however this water is located

near the phosphate oxygen of the NADH/NAD cofactor. In chain C this water is interacting with G57N and G51O. In chain E this water is interacting with G51O, I57N, and G57N, This water likely contributes to stabilizing the cofactor-substrate complex, as it is present in the 1IE3 structure where it bridges the phosphate of the cofactor and the backbone of the helix and loop region.

- Water 7 interacts with the N165ND2 and N165N (N-terminal of the helix) in the E chain this also interacts with H220O. This water is missing in from the C chain.

Open set:

- Water 1 is associated with H220O and the both D192OD1 and D192OD2 in all three chains. In the closed subunit this molecule maintains interactions with the D192 side chain, but not H220
- Water 5 is associated with the G51O, G57N, and P119O and water 7. It is missing from chain G.
- Water 7 is associated with G121O and P119O and is missing from the D chain.

Many of the conserved waters are located near the active site pocket suggesting a water molecule may interact with D192 to alter the basicity of H220. Many of the conserved waters in the closed subunit are interacting with a helix dipole, suggesting the closed conformation may be more stabilized by these interactions. As the water molecule (water 3 in closed set and waters 5&7 in open set) the cofactor binding pocket is missing in the closed set, this also suggests an asymmetric dimer may be more accessible to solvent molecules.

POOL Analysis

POOL is a machine learning program used to interpret the scores given by the Concavity and THEMATICS algorithms. Concavity scores residues by their location in the protein tertiary structure, where residues deep in the structure and exposed to solvent molecules are given high scores. THEMATICS calculates theoretical titration curves for protein residues and gives high scores to residues that deviate from the expected sigmoidal curve. POOL was used on the 1SMK CD dimer, C subunit, and D subunit. The results are summarized below:

H220 has experimentally shown to be involved in catalysis and is ranked number one and/or number two in the POOL analysis of both the *citrate bound* 1SMK subunit (Chain C) and the *citrate+unbound dimer* (Chain C+D), yet in the POOL analysis of the *unbound* (Chain D) monomer H220 is not even ranked in the top 20 residues. This suggests that interactions across the dimer interface affect either the theoretical pKa of the residue or the concavity of the active site. Residue C295 in the *citrate bound* and *unbound* subunits of the homodimer are ranked as 10 and 13, while separate analysis of either the *citrate bound* or the *unbound* subunit are ranked as six and three respectively. Although H220 has been shown to have a conformational change between the two subunits, C295 does not. A plausible explanation for this could be through a network of hydrogen bonds at the 'back' of the interface that links to C295 on either subunit. The change in this environment on either subunit likely affects the placement of the H220 in the active site via a network of hydrogen bonds from the interface. In this network the side chains of N198, K277, and Y273 interact with the side chain of D92 from the opposing subunit.

The *citrate bound* D92OD2- un*bound* K277NZ shifts from an interaction score of 671 in AB to 701 in CD and *citrate bound* D92OD1-K277NZ shifts from 533 in AB to 614 in CD, while the *citrate bound* K277NZ- un*bound* D92OD2 shifts from 703 in AB to 663 in CD and *citrate*

bound K277NZ-D92OD1 shifts from 654 in AB to 515 in CD. Y273 is also involved in a shift, where *citrate bound* Y273CE1- un*bound* D92OD1 shifts from -13 in AB to -58 in CD; this is coupled with the *citrate bound* Y273CE2- un*bound* D92OD1 shifts from -58 in AB to -13 in CD. However, the *citrate bound* D92OD1- un*bound* Y273OH changes from 702 in AB to 711 in CD, while *citrate bound* Y273OH- un*bound* D92OD1 shifts from 694 in AB to 704 in CD. *citrate bound* Y273OH- un*bound* D92OD2 interactions are much weaker and change from 77 in AB to 83 CD, while *citrate bound* D92OD2- un*bound* Y273OH shifts from 80 in AB to 82 in CD. The *citrate bound* D92OD2- un*bound* N198ND2 interactions shifts from 505 in AB to 480 in CD, while the *citrate bound* N198ND2- un*bound* D92OD2 shifts from 470 in AB to 509 in CD. *citrate bound* N198OD1- un*bound* V214N forms a hydrogen bond with a HINT score of 292 in the A subunit and *citrate unbound* N198OD1- un*bound* V214N forms a hydrogen bond of 283 in the B subunit. The *citrate bound* N198OD1-V214N interaction in the C subunit is 289, while the *citrate bound* N198OD1-V214N interaction in the D subunit is 275. These interactions likely change the position the V216O, which forms a hydrogen bond with C295SG with a HINT value of 92 in the C chain, 81 in the D chain, 86 in A chain, and 83 in B chain. Both V214 and V216 are located on the loop before H220 and likely affect the position of H220 in the active site to a more catalytically competent form.

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

Synopsis

The purification, characterization and quantitation of proteins depends upon the accurate determination of both the enzyme activity and the protein concentration. These are related in the term specific activity. Protein activity ranges from enzymatic activity to binding to structural roles in cells and can be assayed in a variety of ways including continuous or discontinuous (stop time) enzyme assays. ***If we are using an enzyme, like Malate Dehydrogenase, that either uses or creates NADH we can use absorbance measurements at 340nm to quantitate the reaction. The absorbance change will be +ve if we are creating NADH and –ve if we are using NADH- note. In either case, the rate (□A_{340nm}/minute) is positive!***

Introduction: What is "Specific Activity or Turn-Over Number"? In conjunction with the determination of the activity of an enzyme solution, you can use the protein concentration and activity measurement to determine a parameter known as the "specific activity" of an enzyme containing solution:

Specific Activity = Enzymatic Activity/Protein Concentration

What are the units of specific activity? The answer is complicated. If enzyme activity is measured in change of absorbance of the substrate as it is converted to product (often the case with Malate Dehydrogenase), the units of enzyme activity could be □Absorbance/minute. If the protein concentration in the cuvette is in mg/mL, then the units of specific activity would be

(ΔAbsorbance/min)/(mg/mL)

What is the Turn Over Number?

For example, if an activity of 63nmoles/min/mL is determined when one adds 7 pmoles/mL of enzyme to a standard assay, the specific activity of the enzyme would be:

63,000pmol/min/mL ÷ 7pmol/mL = 9000 min^{-1}

In such calculations, the units of the activity and the units of the protein concentration are arranged to cancel out and the final unit of the turn-over number is $time^{-1}$. A turn over number is a specific activity, but one requiring the concentration units to cancel out. The units of Turn Over Number are always $time^{-1}$.

Typical calculation for wild type watermelon glyoxysomal Malate Dehydrogenase

Starting Material: 1mg/mL wgMDH
Dilute 200 fold- add 5 μL to 1mL of 0.05M Phosphate Buffer, pH 8.

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

Assay 10µL of diluted enzyme in 3mL assay mix containing 100□M NADH and 200□M Oxaloacetate in 0.05M Phosphate pH 8.

Data:
ΔA340nm = 0.483/minute

Calculations:

Rate in Cuvette:
Use Beer-Lambert Law to convert absorbance to concentration where ε=6.22 mM^{-1} cm^{-1}, and pathlength is 1 cm.

0.483 ΔA340nm/min ÷ (6.22 $mM^{-1}cm^{-1}$)*(1cm pathlength) = 0.0776mM/min
= 77.6 µM/min

Protein concentration in cuvette:

$$1\frac{mg}{mL} * \frac{1}{200} * \left(\frac{0.01mL}{3.01mL}\right) = 1.66\ x\ 10^{-5}\frac{mg}{mL}$$

Convert to µM:
1.66 x 10^{-5} mg/mL is equivalent to 1.66 x 10^{-5} g/L

$$\frac{1.66x10^{-5}mg}{mL} * \frac{1}{29000MW} = 5.77x10^{-10}M$$

5.77x10^{-10} M is equivalent to 5.77 x 10^{-4} µM

Specific Activity:

(Rate in cuvette)/(protein concentration in cuvette), in same concentration units

ie:

77.6 µM min^{-1}/0.000577 µM = 134,000 min^{-1}

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

General Considerations: Saturating substrate concentrations in reaction mixtures are used to minimize experimental errors. It is also important that reaction rates be measured under conditions where a sufficiently small amount of substrate is utilized so that the rate does not change during the assay as a result of substrate depletion. Similarly, product buildup, which may lead to product inhibition, is to be avoided. In general, a convenient way to test that these factors do not become a problem is to measure activity at a series of protein concentrations: The rate should be directly proportional to the protein concentration, as in Figure 1. Deviations below the line indicate that substrate depletion or product accumulation may be occurring. Deviations from linearity can also result from protein aggregation or subunit dissociation affecting the rate of the catalyzed reaction.

For the study of enzyme kinetics, it is important that the rate that is measured is the "Initial" rate of the reaction. In addition to being linearly dependent of the amount of enzyme added, an important criterion of the initial rate is that whatever change is being measured to follow the activity extrapolate to zero change at the start of the reaction. This ensures that the measured rate is indeed the initial rate of the reaction and that some change in the rate of the reaction did not occur in whatever "deadtime" the physical measurement of the rate involves. For example, in the direct assays described below for dehydrogenases, the deadtime is the time interval between introducing the enzyme, mixing, and starting the actual absorbance measurements. This problem is illustrated in Figure 2. With an enzyme, such as Malate Dehydrogenase, which catalyzes a reaction that proceeds quickly to equilibrium, a small "deadtime" can lead to a large error in the estimated "initial" rate. However, such a situation is easily detected by the "must extrapolate to zero change at the start of the reaction" rule.

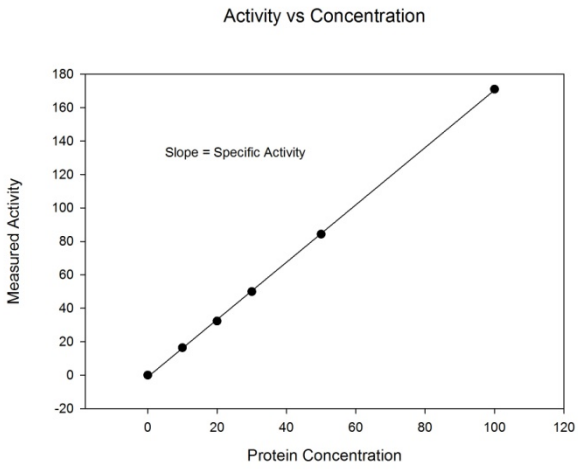


Figure 1: Activity is directly proportional to concentration.

How do you decide how much enzyme to use? When dealing with an enzyme where you do not know the specific activity, it is important to establish the correct amount of enzyme to use in assays. The trial and error approach is the only option you have. Try some amount (say

10µL of the solution you have) and measure the "rate." There are three possible outcomes of this experiment: Too much was added, too little was added, or approximately the right amount was added, as shown in Figure 2- curve d. If too much was added, you can make a best guess as to how much too much from the shape of the resultant curve. If, by the time you initiated the measurement, the reaction was already at, or close to equilibrium, you added much too much and probably need to dilute the enzyme 50-100 fold (Figure 2- curve a). If you added too little of the enzyme to get a reasonably measurable rate (curve b), you need to concentrate the enzyme or simply add more volume of the enzyme until you get a reasonably measurable rate. If you added approximately the right amount, the issue is whether or not it extrapolates back to the starting absorbance (usually about 0.6 in an MDH assay) at t = zero. In which case, it is fine to continue with your experiment (curve d), or whether the enzyme needs some dilution- curve c- (by either adding a smaller volume- this depends upon how small a volume you are comfortable being able to add accurately- or by diluting maybe 5-10 fold).

Notes on Volumes to Pipet: Pipetting small volumes is inherently less accurate (and reproducible) than pipetting larger volumes. It is recommended that you use volumes no smaller than 5µL and in general try to stay in the 10 µL or above range. Always use the appropriate pipettor and tip for the volume: eg if you are pipetting 5-20 µL volumes, use a p20 pipettor, if 20-200 µL, use a p200 pipettor, if 250-1000 µL, use a p1000 pipettor etc.

You then calculate the initial rate (dA/dt) from the linear region that extrapolates back to the correct absorbance at t = 0. Usually in an MDH assay, you can establish conditions where the plot is linear for about 30 seconds or more. Do not include any data from the curved region of the plot as this will distort the initial rate estimate.

Once you have established how much enzyme you need to add to give an accurately measurable initial rate, it is probably a good idea to test the highest and lowest combinations of substrates that you will use to ensure that you can make good measurements throughout the range you will use during a given experiment.

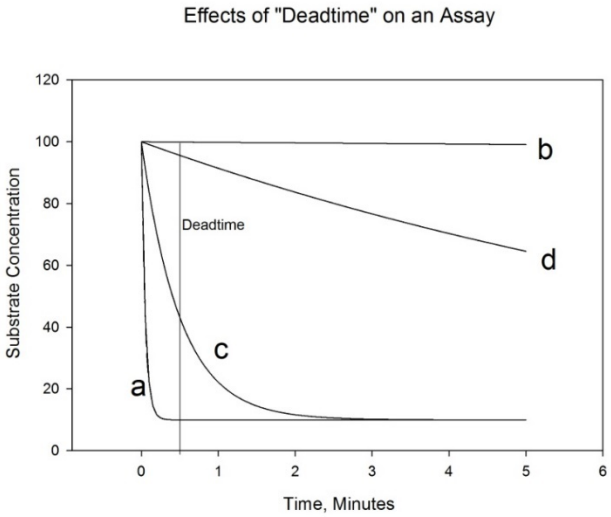


Figure 2. Effect of "deadtime" on accurate initial rate determination.

Continuous Assays: The activity of an enzyme can often be conveniently measured by following either the

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

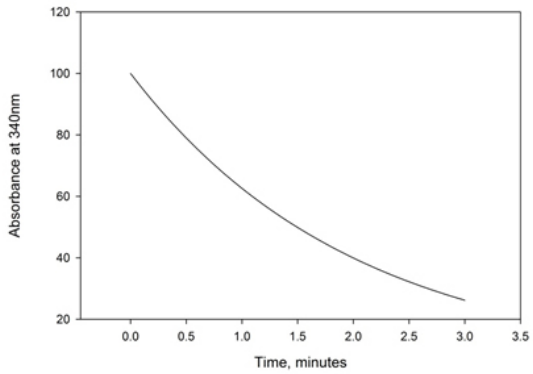


Figure 3: Time course of the reduction of oxaloacetate catalyzed by malate dehydrogenase using NADH as a coenzyme.

production of a product or the removal of a substrate. With certain classes of enzymes (e.g., dehydrogenases), the natural substrates are chromophoric and exhibit spectral changes that can be followed directly. For example, malate dehydrogenase catalyzes the reduction of oxaloacetate by the coenzyme NADH:

Oxaloacetate + NADH ←→ Malate + NAD^+

NADH has an absorption band centered at 340 nm with an extinction coefficient of 6.22 x 10^3 $cm^{-1}M^{-1}$, while NAD^+ has no absorbance at this wavelength. When malate dehydrogenase is added to a mixture of oxaloacetate and NADH, there is a time-dependent loss of absorbance at 340 nm.

Protocol For Initial Rate Measurements with Malate Dehydrogenase

Equipment: recording spectrophotometer capable of measuring absorbance at 340nm

Disposable supplies
4.5 mL plastic cuvettes that pass light at 340nm
Disposable plastic Pasteur pipettes with about 3mL volume: **Squishers!**

Reagents –
Make substrates in H2O, fresh and keep on ice (do not premix NADH and Oxaloacetate)
0.1M Sodium (or Potassium) Phosphate Buffer, pH 8.0
3mM NADH in H_2O. Make up more concentrated, check Absorbance at 340nm to calculate actual concentration and dilute as appropriate to 2mM
6mM Oxaloacetate in H_2O

Wet Lab Experiments

All experiments compare mutant with wildtype under same conditions (preferable performed on the same day etc)

Important experimental points:

1. Oxaloacetate and Citrate solutions should be made up fresh and in H_2O, immediately before the experiment- they do not seem to keep more that a few hours on ice.

2. Do not premix NADH and Oxaloacetate for more than a minute or so before using in an assay- typically we add buffer and NADH to cuvettes- allow to reach desired temperature. Then add OAA stock to give desired concentration and immediately add enzyme and record data.

Specific Activity under defined conditions

pH 8, 50mM Phosphate Buffer, 100µM NADH, 200µM Oxaloacetate

Recommended Stock solutions

NADH, 3mM in H2O

Oxaloacetate, 6mM in H2O

50mM Phosphate Buffer, pH 8.0

Keep both substrate stock solutions in an ice bucket- do not use the Oxaloacetate stock solution for more than 3-4 hours. Keep the phosphate buffer at the required temperature (eg. room temperature)

In a 3mL Cuvette, add 2.8mL 50mM Phosphate Buffer, pH 8, "Blank" the spectrophotometer at 340nm

Add 100µL stock NADH, mix, run a 'No enzyme" control for 30 seconds- this allows you to calculate the actual NADH concentration in the cuvette.

Add 100µL Stock Oxaloacetate solution and 10µL (to give a final concentration in the cuvette of about 0.005-0.01µg/mL enzyme, mix and start data collection for 0.5 minutes.

Typically we analyze the acquired data from 0.05 to 0.25 minutes since the first 0.05 minutes often reflects mixing etc.

Save the primary data as a plain text or csv file for later input into an excel file for data archiving.

Record the rate obtained over the 0.05-0.25 minute time frame

Repeat to give a total of three to five rate measurements to allow an average and standard deviation of the rate to be calculated.

If you wish you can scale down to use 1mL total volume in a 1.5mL cuvette.

Initial Rate Kinetics to determine Km and Vmax

Stock solutions

NADH, 6mM in H_2O

Oxaloacetate, i) 60mM in H_2O

ii) 6mM in H_2O

(Make up the 60mM and dilute some of it 10 fold with H_2O)

Keep all stock solutions on ice in an ice bucket

100mM Phosphate Buffer at pH 8: keep at room temperature or whatever temperature you wish if using some other temperature

H_2O, keep at room temperature or whatever temperature you wish if using some other temperature

NADH Varied: OAA fixed at 200 µM, NADH Varied 10 µM to 200 µM

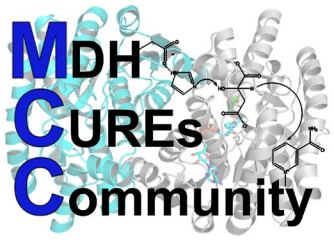
Oxaloacetate Varied: NADH fixed at 100 µM, OAA Varied from 20 µM to 2 mM

Recommended Approach

In 3mL cuvettes:

Add 1.5mL stock buffer

Add appropriate volume of H_2O so that the final volume in the cuvette after addition of NADH and Oxaloacetate is 3mL.



Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

Add appropriate volume of stock NADH, mix, run a 'No enzyme" control for 30 seconds- this allows you to calculate the actual NADH concentration in the cuvette.

Add appropriate volume of stock Oxaloacetate solution and 10µL (to give a final concentration in the cuvette of about 0.005-0.01µg/mL wildtype enzyme (for mutants you will have to establish the appropriate concentration to give linear kinetics), mix and start data collection for 0.5 minutes.

Typically we analyze the acquired data from 0.05 to 0.25 minutes since the first 0.05 minutes often reflects mixing etc.

Save the primary data as a plain text or csv file for later input into an excel file for data archiving.

Record the rate obtained over the 0.05-0.25 minute time frame

Repeat to give a total of three to five rate measurements to allow an average and standard deviation of the rate to be calculated.

Note on Mixing and initiating data collection

Mixing- pipetting the enzyme up and down a few times with your micropipet DOES NOT EFFECTIVELY MIX THE REACTION and you will get irreproducible data. With the cuvette in the spectrophotometer hold the Pasteur pipette, air expelled in one hand and the pipettor with the enzyme in the other. As soon as you pipet the enzyme into the reaction mix gently suck up the reaction mix into the Pasteur pipet and expel- Start recording A_{340nm} versus time. This effectively mixes the enzyme and reaction mix and you should get reproducible data. Because of the "deadtime" phenomenon discussed early you should develop a consistent pattern of these steps and not stop and talk before starting the recording of A_{340nm}.

When you have collected date calculate the rate over the linear region that extrapolates back to the starting absorbance. You can check that you have the initial rate by calculating the rate over two time periods within this range- if it is the initial rate you will get essentially the same rate from each segment.

Typically if you have a reasonable concentration of enzyme you will see an absorbance change of 0.03- 0.1/minute during the course of the assay- if you leave it to run longer it will eventually reach equilibrium and the absorbance will be about 0.02.

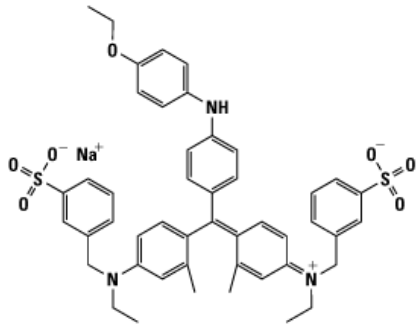
Suggested Set Ups for NADH or Oxaloacetate Varied Kinetics

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

Calculating the Rate of the Reaction

Since the mM extinction coefficient of NADH at 340nm is 6.22 you simply divide the ΔA_{340nm}/min by 6.22 and get the rate in mM NADH consumed per minute (remember mM is a concentration, not an amount)

Determining the Protein Concentration: Bradford Dye Binding Assay



The Bradford dye binding method for the determination of the concentration of a protein solution depends on the observation the dye Coomassie Brilliant Blue G-250undergoes a color transition, as shown in chemical structure, on going from a cationic species [with a wavelength of 470nm] to a neutral species [maximum at 650nm] to an anionic species, with a maximum at 595nm. The equilibria between these species are shifted when the dye binds to a protein due to the effects of the local environment on the protonation of the ionizable groups on the dye.

Protocol for Using the Bradford Dye Binding Assay

Equipment: Spectrophotometer capable of reading absorbance at 595nm

Disposable Supplies
1.5mL Plastic spectrophotometer cuvettes
Disposable plastic Pasteur pipettes

Reagents
0.1M Phosphate Buffer
1mg/mL BSA in 0.1M Phosphate Buffer pH 8
Bradford Reagent
Unknown Sample

The Basics:
In this assay, you will set up the standard curve using varied amounts of the "standard" protein BSA to a total volume of 800 microliters and then add 200 microliters of a Bradford Dye concentrate to give a final total volume of 1000 microliters. You must make sure that everything is thoroughly mixed before quantitating the results. The Unknown that you will use must be handled the same way.

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

To achieve the protein amounts that you will need for the standard curve for the Bradford Bye Binding Assay, you must first prepare a series of cuvettes containing different amounts of the protein standard, Bovine Serum Albumin (BSA) from a 1mg/mL stock BSA solution, using 0.05M phosphate buffer , pH 8, as the dilutant to give protein amounts from 100µg down to about 1µg per 800µL of added sample. In the protocol below, a BSA range of 0-10 micrograms is used which effectively keeps the "standard curve" in the linear range.

Set Up for Standard Curve and Unkowns

Tube Number	**Desired Amount of Protein, µg**	**Volume (µL) of 1mg/mL BSA Added**	**Volume (µL) of Buffer Added to give a total of 800µL**
1	0	0	800
2	2	2	798
3	4	4	796
4	6	6	794
5	8	8	792
6	10	10	790
Unknown	??	1-10 as appropriate	790-799 to make a total of 800

Add 0.2mL of Bradford reagent. Mix each tube, incubate for 5 minutes at room temperature, and determine the absorbance at 595nm, using a cuvette with buffer in place of the protein sample as the blank (tube 1 in above table).

For the unknowns, add sufficient volume of the sample to give an absorbance that lies on the standard curve

Typical Data for a standard curve under these conditions would be:

Tube Number	**Desired Amount of Protein, µg**	**Volume (µL) of 1mg/mL BSA Added**	**Absorbance at 595nm obtained by blanking on tube 1**
1	0	0	0
2	2	2	0.184
3	4	4	0.273
4	6	6	0.494
5	8	8	0.576
6	10	10	0.699

Protocol: Measuring the Specific Activity and Michaelis-Menten Kinetics of Malate Dehydrogenase

Unknown	? to be determined	10µL unknown added	0.317

Data Analysis

Plot the standard curves, using Absorbance at 595nm vs µg of BSA for the Bradford assay, similar to Figure 4.

Using linear regression, the slope of the standard line is 0.0699+/-0.0043 A595nm/microgram protein and the intercept is 0.022+/-0.026 (indistinguishable from zero as expected)

The line is described by the equation:

A595nm = 0.0699 x micrograms of protein

Note that the x axis of the graph is micrograms of protein. It didn't matter what volume we added, just the final number of micrograms.

From the standard curve determining the number of micrograms of protein in the unknown sample is quite simple. The unknown gave an absorbance of 0.317 and we simply solve the equation:

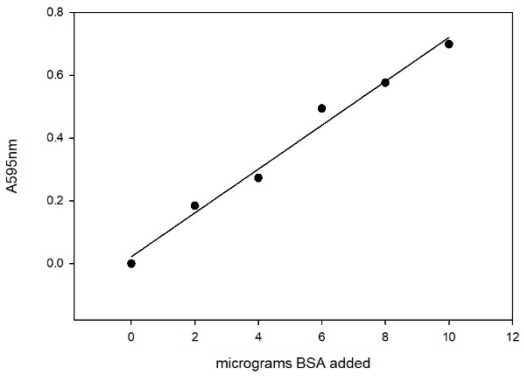


Figure 4. Typical standard curve for Bradford assay.

0.317 = 0.0699 x micrograms of protein to get the micrograms of protein in the unknown.

0.317/0.0699 = 4.54 micrograms of protein.
To express the results in mg/mL of the original stock solution of the unknown, we know that 10µL of the unknown (the amount we put in the assay) contained 4.54 micrograms and hence the sample contained

4.54/10 = 0.454 micrograms/microliter, which is the same as 0.454mg/mL.

Prepared by: Nina Marie Garcia
Spring 2018

Size-Exclusion Chromatography

Introduction: Size-exclusion chromatography allows us to separate proteins by size and can help us determine if the mutants retain their dimeric structure. Larger molecules will elute the column faster since they will not be able to interact as much with the porous beads. Conversely, smaller molecules will elute the column later.

Purpose: To determine if mutant gMDH retains dimeric structure.

Materials:

- SEC buffer: 25 mM phosphate buffer (pH 8), 150 mM NaCl, 1 mM BME
- Protein samples, dialyzed into SEC buffer.

Protocol:

1. De-gas buffer
    a. Pour buffer into giant vacuum filtration flask at an angle to minimize bubbles.
    b. Stir, attach vacuum, put black cap on top of flask. Turn on vacuum.
    c. Bubbles should form around stir bar.
    d. Stir for 30 min. Transfer to 500 mL glass bottle. Try not to get bubbles.
2. Prepare samples
    a. Dilute samples if needed. Usually want all samples at the same concentration. Final volume: 750 µL. Dilute in SEC buffer.
    b. Spin down on bench top centrifuge: 4*C, 14k rpm, 10 min.
    c. Add 60 glass tubes onto rack.
3. Load sample: there is a protocol created on the instrument: gMDH_Su17
    a. Isolate syringe. Detach syringe from system.
    b. Rinse 3x with DI water. Rinse 2x with buffer
    c. Reattach to system. Connect it to pup and inject 500 µl buffer to wash column.
    d. Isolate syringe. Detach syringe. Connect needle and take up 500 µl sample. MAKE SURE THERE ARE NO AIR BUBBLES.
    e. Remove needle and reattach to system. Connect to pump and inject sample. MAKE SURE THERE ARE NO AIR BUBBLES.
    f. Start run.
    g. Make sure the fractions are being collected.
4. Export data as CSV.

Prepared by: Nina Marie Garcia
Spring 2018

BS_3 Crosslinking Experiment Protocol

Purpose: To determine if the gMDH mutants maintain their dimeric structure. If they do, they should have the same band patterns as the native.

Materials:

- BS_3 cross-linker
- Gradient gel

Notes: We do not need to dialyze gMDH because our elution buffer does not contain any NH_3. NH_3 will be crosslinking by BS_3 if present and will skew results. If NH_3 is present, dialyze in PBS.

Protocol:

1. Determine what protein concentration you want in your x µl sample
    a. Let's say our final volume is 100 µL
    b. We want: 50 µM

	Initial Concentration (mg/ml)	Initial concentration (µM)	µl protein used for dilution	µl buffer for 100 µl total volume
WT Elution 1	6.8	194.7	25.7	74.3
S266A Elution 1	9.6	275	18.2	81.8
L269A Elution 4	2.35	67.3	74.3	25.7

2. Determine how much BS_3 you want to add in x µl final volume. And
    a. Want: 5 µL BS_3 in 100 µL final volume
    Dilution factor = 5 / 100 µl = 0.05
    [BS_3] = 5000 µM for 100x
    [BS_3] stock = (5000 / 0.05) / 1000 = 100 mM
    Add 35 µl milliQ water to 2 mg BS_3 aliquot.
3. Combine 5 µl BS_3 + 95 µl diluted protein in eppi tube. Incubate for 1 hr in the refrigerator.
4. Add 100 µl 0.5 M Tris-HCl. Incubate for 30 min at room temperature
5. Make gel samples
    a. 20 µl loading dye + 60 µl cross-linked protein sample
    b. Boil at 100 deg. C for 10 min.
    c. Spin down.
6. Load the gradient gel. Wells hold 30 µl

Lane	Sample
1	Standards
2	Blank
3	10 µl WT
4	30 µl WT
5	Blank
6	10 µl S266A
7	30 µl S266A
8	Blank
9	10 µl L269A
10	30 µl L269A

7. Run the gel at 200 V for ~30 min. Image gel.