A Structural Study of Fumarase C from *E. coli*

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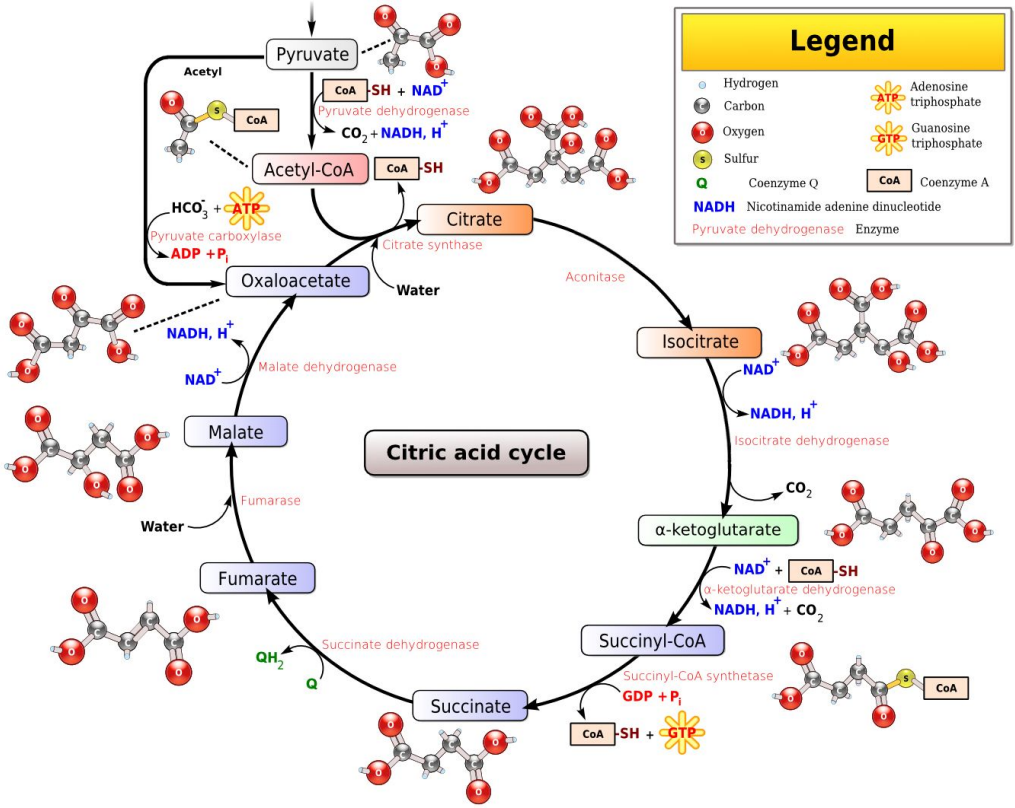
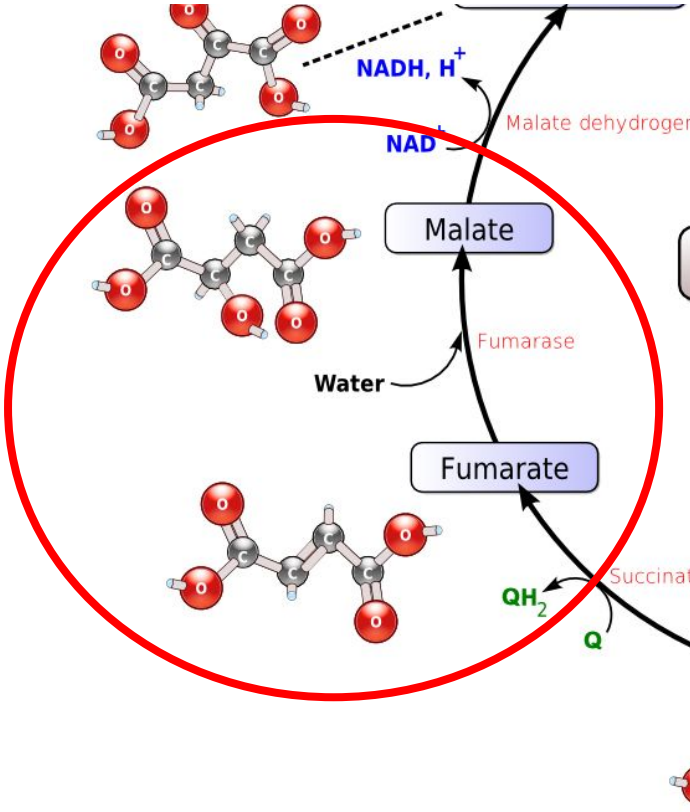
The Citric Acid Cycle

- Releases stored energy from acetyl-Coa into NADH, FADH, and GTP
- Eight different enzymes that catalyze the cycle
- Important for the breakdown of glucose and other small molecules into ATP



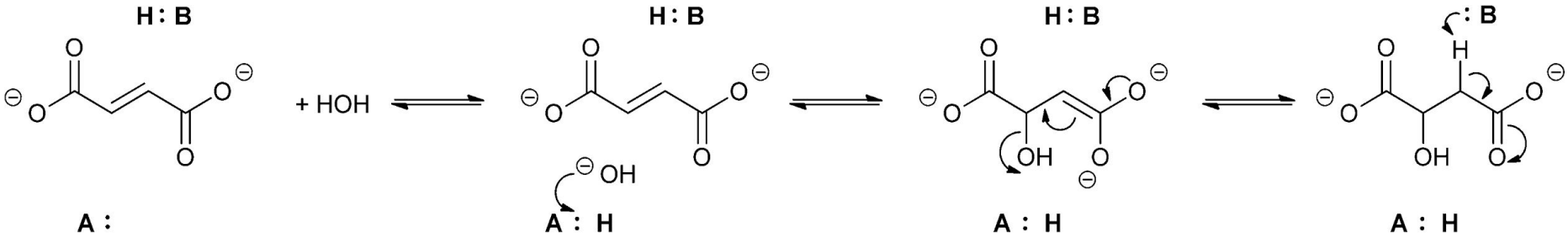
Fumarases in General

- Class I fumarase's
  - Heat liable, iron dependent, 4 Fe- 4 S clusters
  - Dimers of 120 kDa
  - Fumarase A and B
- Class II fumarase's
  - Thermostable, iron-independent proteins
  - Tetramic proteins around 200 kDa
  - Class II fumarase's can be identified via a GSSxMxKxnxxPxE specific sequence located between Gly317 and Glu331
  - FumC and fumarases found in *S .cerevisiae* and mammals



Reaction of Fumarase

- Fumarase catalyzes reversible hydration/dehydration of fumarate to L-malate.
- mechanism is an acid-base catalyzed elimination with an aci-carboxylate intermediate
- Two important catalytics bases A and B
- A is thought to be aspartate or glutamate
- B is thought to be a histidine



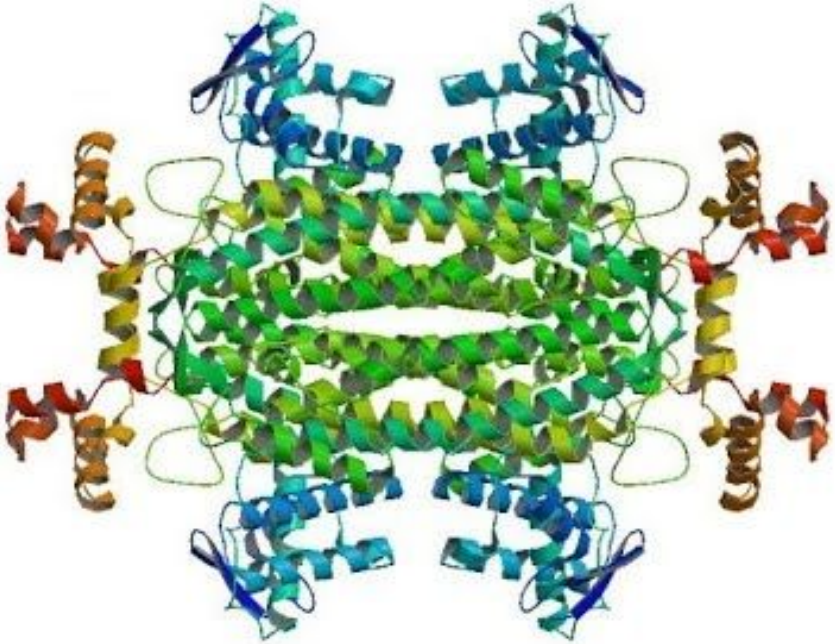
aci-carboxylate

An Interesting Case Regarding Fumarase

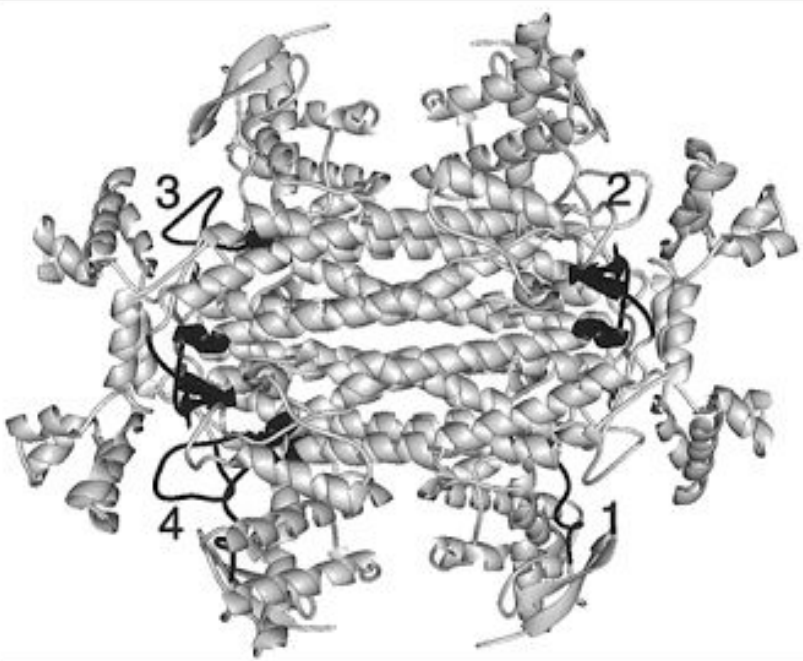
- Fumarase has been linked to a progressive metabolic encephalopathy.
- An inborn error of the gene, a missense mutation at position 955 causing a G to C transversion (glutamate to glutamine at protein level), results in an inactivation of the protein
- The inactive protein causes psychomotor retardation, microcephaly, and abnormal posture with hypotonia contrasting with hypertonia of limbs in humans.

Fumarase C from *E. coli*

Four Studies of fumarase have elucidated some of the key features of the enzyme



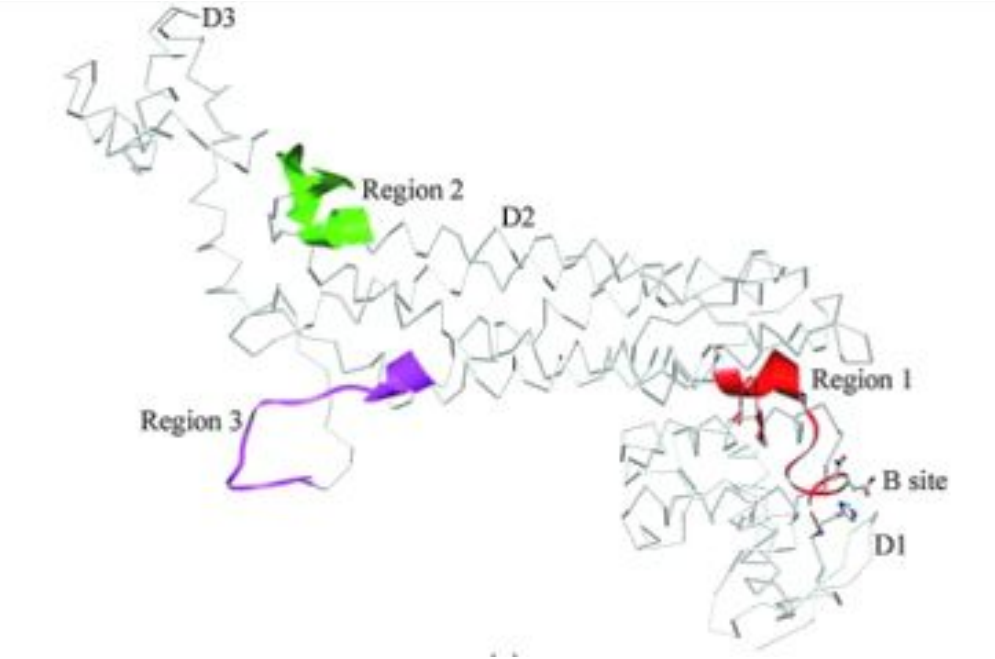
The First Important Study - the structure of citrate, a competitive inhibitor, bound in the active site of fumarase



- Fumarase is a tetramer in its active form
- The monomer is inactive
- More information from the monomer

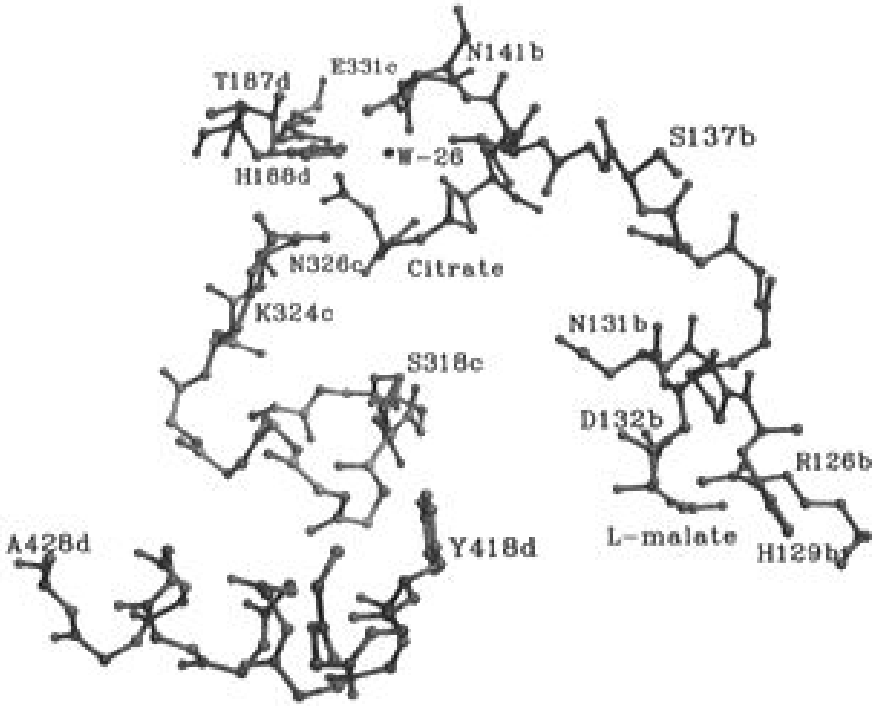
The First Important Study - the structure of citrate, a competitive inhibitor, bound in the active site of fumarase

- Three different domains: D1, D2, D3. D2 comprises a 5 helix bundle
- Three highly conserved regions across the superfamily of fumarases
- Two active sites: A-site and B-site
- A-site is formed by residues from regions 1-3 all coming from different subunits
- B-site is formed from one subunit



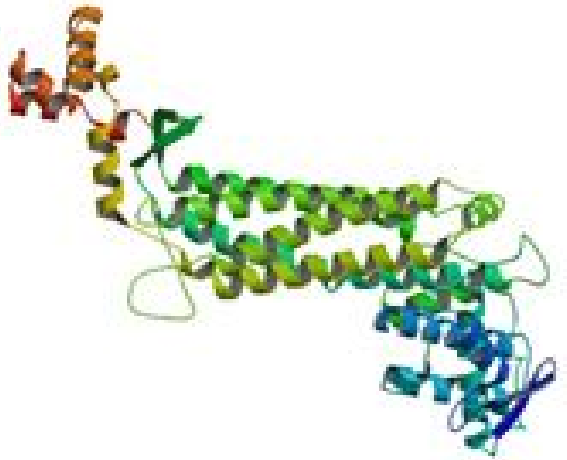
The First Important Study - the structure of citrate, a competitive inhibitor, bound in the active site of fumarase

- Key A-site residues
  - Thr187, H188, Lys324, and Asn326
  - Water 26
- Key B-site residues
  - Arg126, His129, Asn131, and Asp132



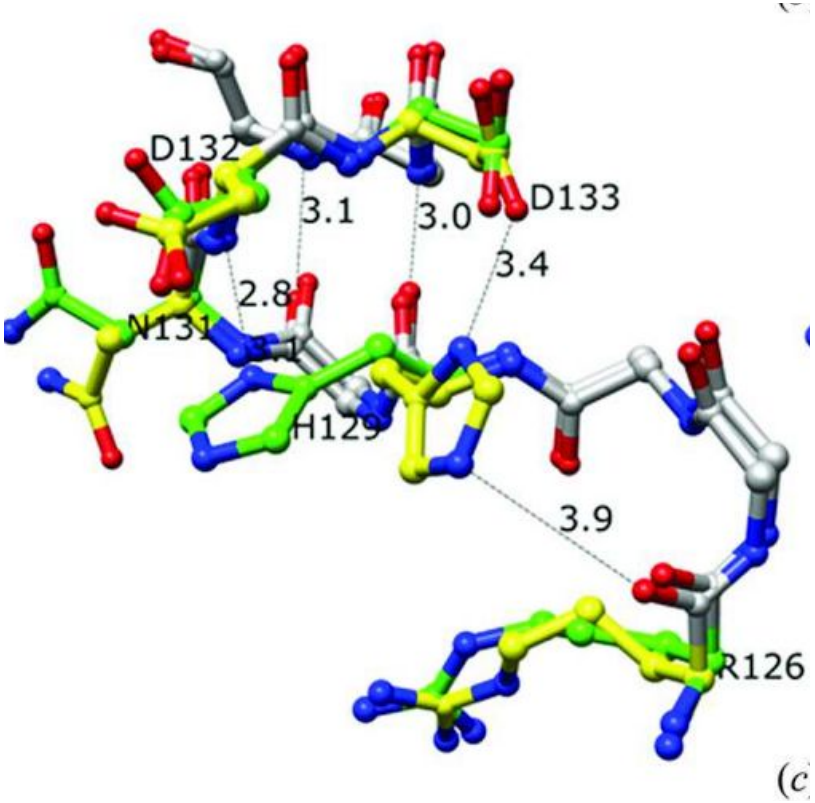
The Second Important Study - the active sites

- What active site is the catalytic site?
- Two mutagenesis experiments were performed
  - A-site mutation - H188N
  - B-site mutation - H129N
- H129N mutant had little or no effect on the catalytic activity of the enzyme
- H188N mutation showed a large decrease in catalytic activity
- With increased concentrations of malate present, increased in the rates of reaction.



The Third Important Study - the apo or free structure of fumarase

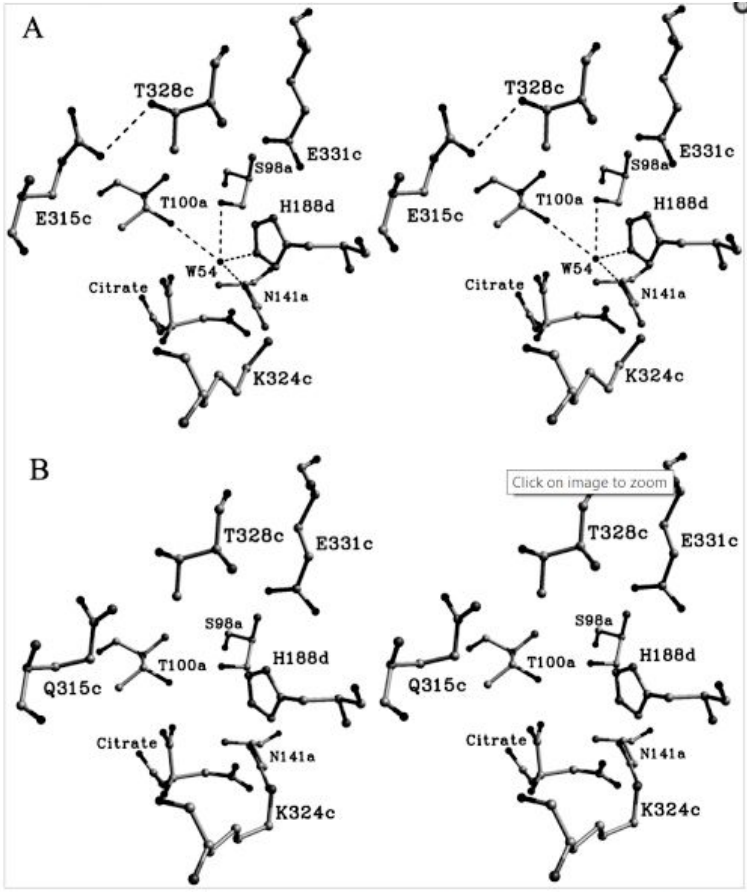
- A - site is conserved with minimal structural changes
- B - site has two main differences
  - Imidazole ring of His 129 rotated into the active site
  - Asn131 also had a notable rotation towards the active site



The Fourth Important Study - another mutagenesis experiment

- Mutated glutamate 315 to a glutamine (E315Q)
- Equivalent to a the same missense mutation in humans
- W - 26 was coordinated to 4 residues
  - Ser98, Thr100, Asn141, and His188
- k_{cat} decreased 10 fold in both directions of catalysis
- K_m did not change

Enzyme	Native	E315Q
S-malate → fumarate		
V_{max} (μmol substrate/min/mg enzyme)	178.6	16.11
K_m (mM)	0.857	0.885
k_{cat} (s^{-1})	595.2	55.32
k_{cat}/K_m (M^{-1} s^{-1})	6.95E5	6.25E4
Fumarate → *S*-malate		
V_{max} (μmol substrate/min/mg enzyme)	344.8	32.2
K_m (mM)	0.207	0.248
k_{cat} (s^{-1})	1149	107.1
k_{cat}/K_m (M^{-1} s^{-1})	5.56E6	4.32E5



Hypothesis

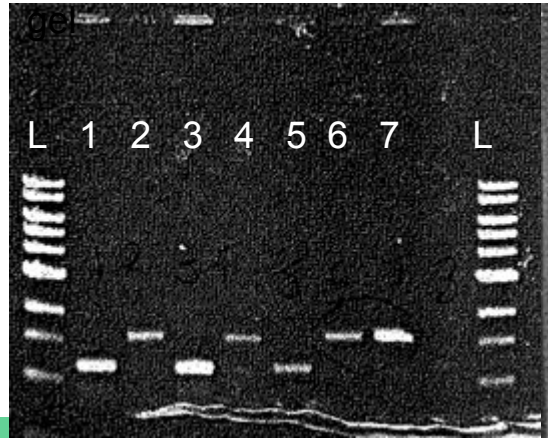
Co-crystallize fumarase C from *E. coli* with its substrate, L-malate, in order to elucidate the two catalytic bases in the mechanism, the catalytic importance of the A-site water, how the B-site plays a role in catalytic efficiency, and investigate the A-site's interactions with a substrate further

Results:
Gene to Protein - a couple gels

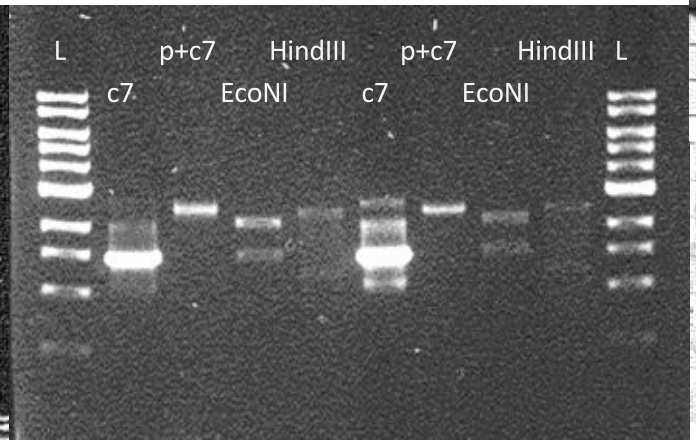
- Bottom left: colony PCR
- Bottom middle: restriction digests
- Right top-bottom: Purification of fumC C-ter

Protein purific. 12% SDS-phage gel's

Colony PCR 1% agarose gel



Restriction digest 1% agarose



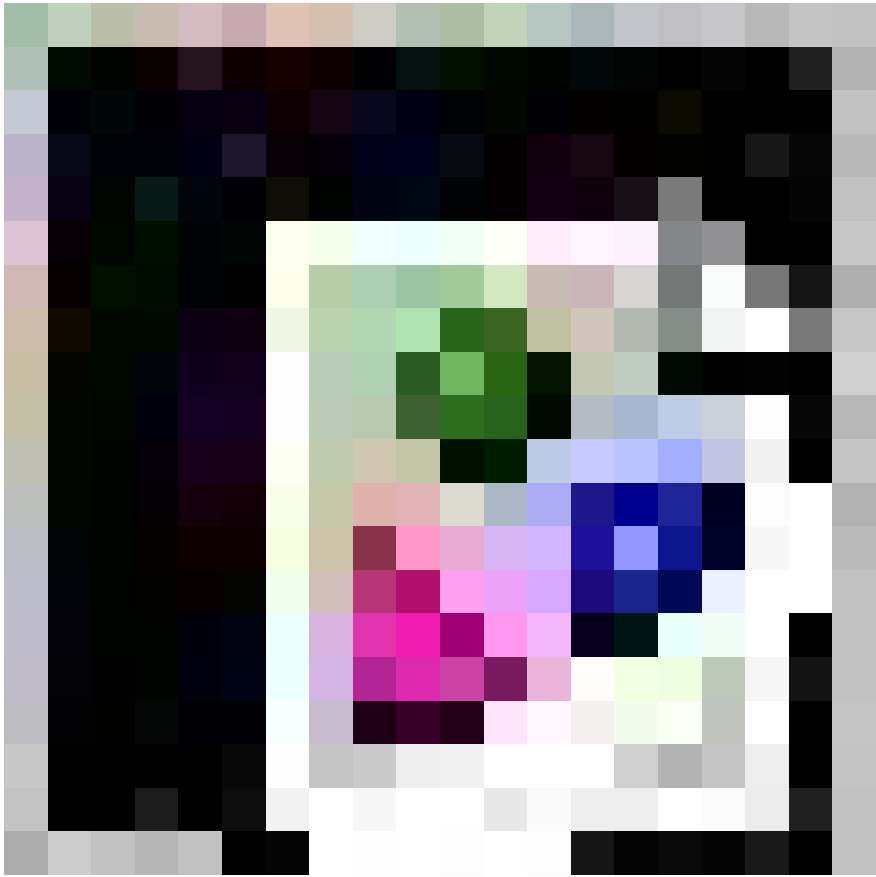
Crystallization - the tough part

- Two Sets of conditions
  - Previously published (MOPS, $LiSO_4$, PEG 4000) and new conditions (KCl, PEG 3350, BTP)
- Varied almost everything we could
  - Salts and their concentrations
  - PEG's and their concentrations
  - pH and concentrations of other buffers
  - Temperature of trays
  - Micro-seeding and filtering protein
  - Protein concentration
  - Droplet volume/mixtures
  - Concentrations of malate - in drop solution and post crystallization

Data set	fumC pre-refinement	fumC post-refinement
Space group, A (Å), B (Å), C (Å)	I222, 62.04, 121.14, 128.24	
Resolution (lower to higher limit) (Å)	13.9-2.2	
% Completeness	98.1 (95.8)	
I/σ(I)	5.7 (0.7)	
CC(1/2)	0.982 (0.197)	
R_{meas}	0.277 (2.11)	
R_{free}	0.2456	0.2450
R_{work}	0.2466	0.1847
R. m. s bond length (Å)	0.011	0.014
R. m. s bond angles (°)	1.454	1.325
Average mosaicity	0.67	
Mean B-factor ($Å^2$)		49.64
Ramachandran favored (%)		94.5

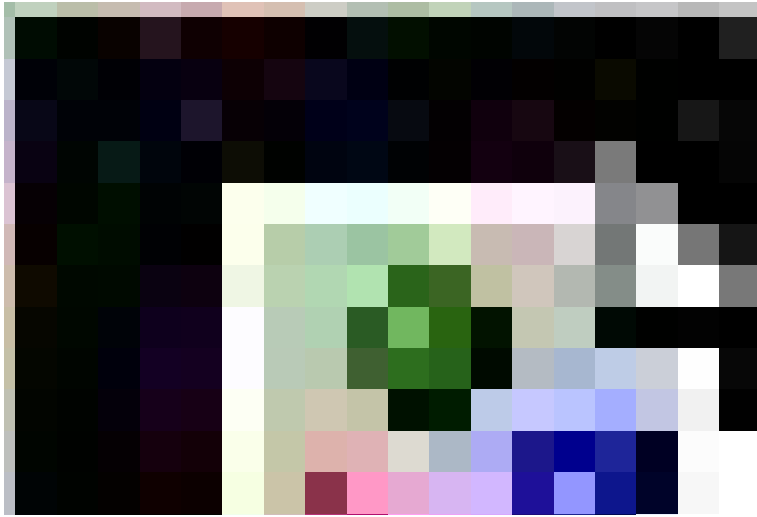
Omit Map of The A-site

- Shows electron density of active site
- Active site residues: Thr187, His188, Lys324, and Asn326
- No electron density in the active site cavity



The Omit Map of The B-site

- Active site residues: Arg126, His129, Asn131, and Asp132
- Top map shows the difference map with no electron density in the active site cavity
- Bottom shows our structure superimposed onto the deposited apo fumarase structure
- Note the position of His129



Discussion

- No electron density in the active cavity of the A-site
- No electron density in the active cavity of the B-site
- Nearly identical unit cell dimensions to apo fumC
- Previously published and PDB deposited apo fumarase C structure was 2.19 angstroms with similar R-factors

Conclusions

- Obtained an approximate 2.2 angstrom resolution structure of apo fumarase C in new crystallization conditions that contain malate

Acknowledgements

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Questions?