Reengineering Allosteric Regulation of Bacterial PFK-1

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Why is human PFK-1, but not *E. coli* PFK-1, allosterically regulated by citrate?

Can we reengineer E. coli PFK-1 to be allosterically inhibited by citrate?

Introduction

Phosphofructokinase (PFK-1) catalyzes a rate-limiting step of glycolysis and is allosterically regulated by ligands whose binding can elicit conformational changes ~40Å away in the protein's active sites. While the structure of PFK-1 is well conserved, eukaryotic orthologs are larger and bind more allosteric regulators than archaeal and bacterial PFK-1.[1] Sequence comparison and biochemical mutation studies suggest that mammalian PFK-1 evolved by a process of tandem gene duplication and fusion, ultimately resulting in additional allosteric binding sites derived from duplicated catalytic and regulatory sites of ancestral PFK-1.[2] One of these sites binds citrate, which allosterically inhibits PFK-1. However, bacterial PFK-1 is not allosterically regulated by citrate. **In order to elucidate the mechanisms behind the allosteric regulation of human PFK-1 by citrate, we sought to engineer its ancestral ortholog, bacterial PFK-1, to be allosterically inhibited by citrate binding using protein design methods in Rosetta.**

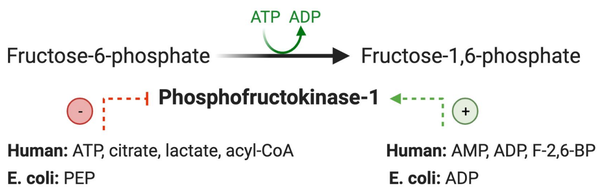


Fig 1. Enzymatic reaction catalyzed by PFK-1 showing inhibitors and activators in human and E. coli.

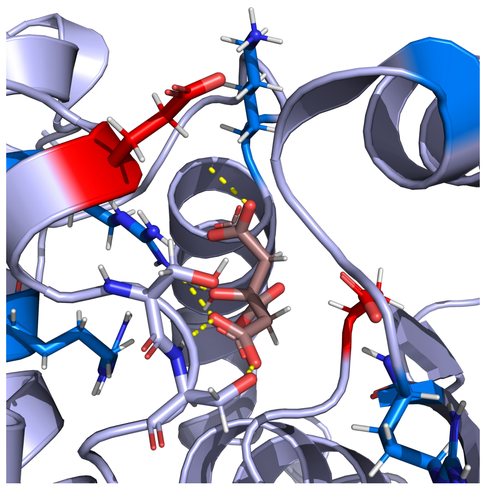


Fig 3. A citrate conformer docked in the hypothesized human PFK-1 binding site (PDB: 4XYJ). This site consists of many positively charged residues (positive shown in blue; negative shown in red; Predicted polar interactions between citrate and the binding pocket shown in yellow.)

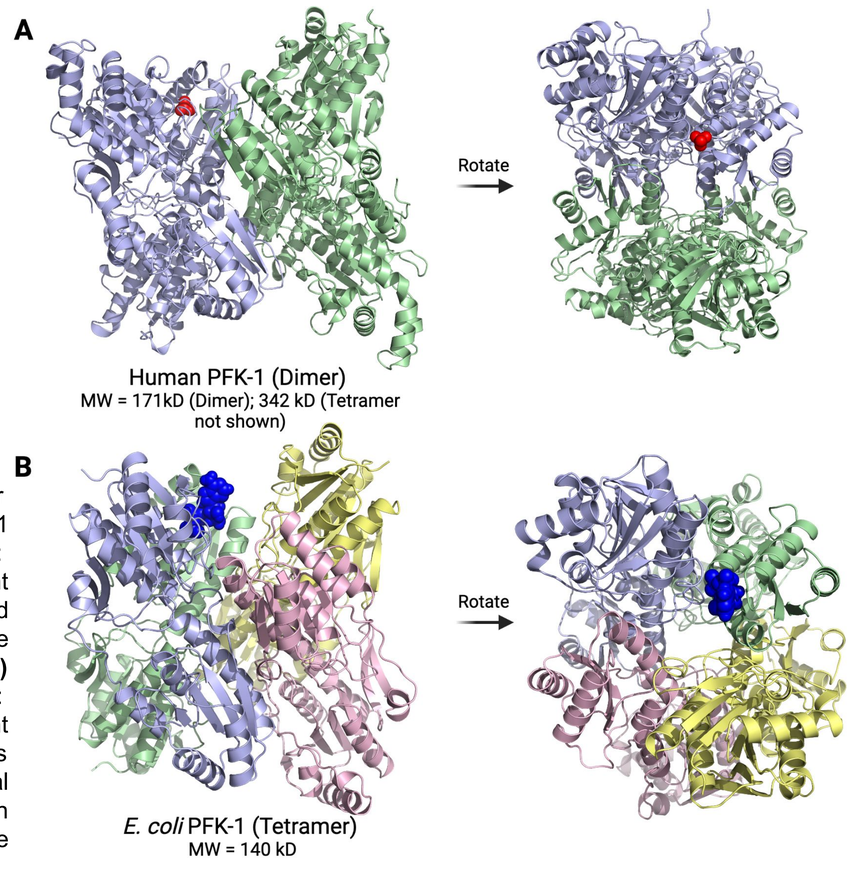


Fig 2. Bacterial PFK-1 tetramer corresponds to human PFK-1 dimer. **(A)** Human PFK-1 (PDB: 4XYJ). Colors show different subunits. Phosphate shown as red spheres is positioned in the prospective citrate binding site. **(B)** *E. coli* ADP-bound PFK-1 (PDB: 1PFK). Subunits shown as different colors. ADP shown as blue spheres is positioned in the bacterial allosteric effector site, which corresponds to the human citrate binding site.

Hypothesis: Allosteric regulation by citrate in *E. coli* can be engineered by introducing "humanizing" mutations to binding site residues

Computational Protein Design

No experimentally solved crystal structure of human PFK-1 with citrate bound is available. Therefore, citrate conformers were docked into the hypothesized citrate binding site in human platelet PFK-1 (PDB: 4XYJ, 4XYK). Because previous studies have hypothesized that the citrate binding site in higher eukaryotes evolved from the ADP/PEP effector site in bacterial PFK-1[3], citrate was docked into this site and Coupled Moves was used to design a binding site around the docked ligand.

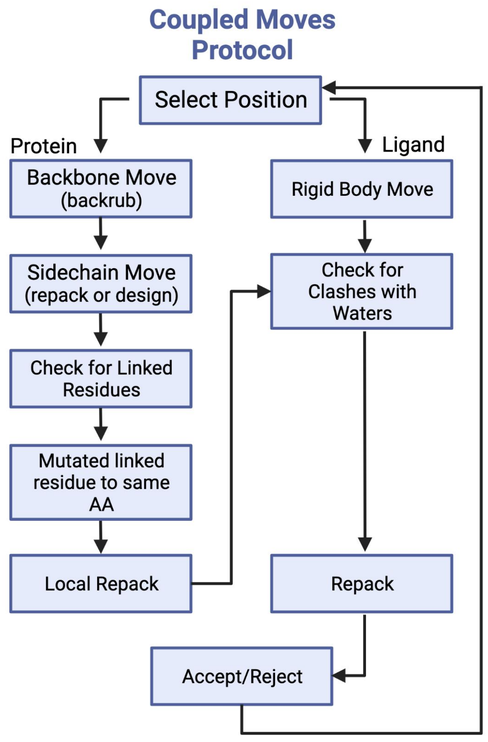
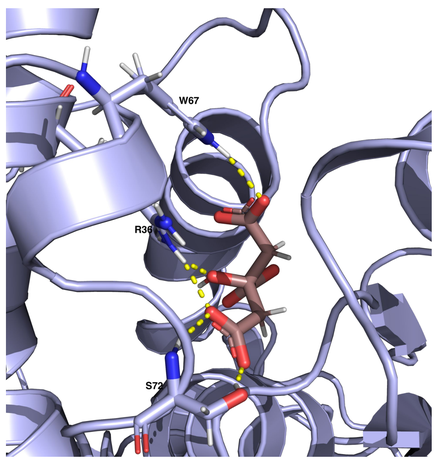
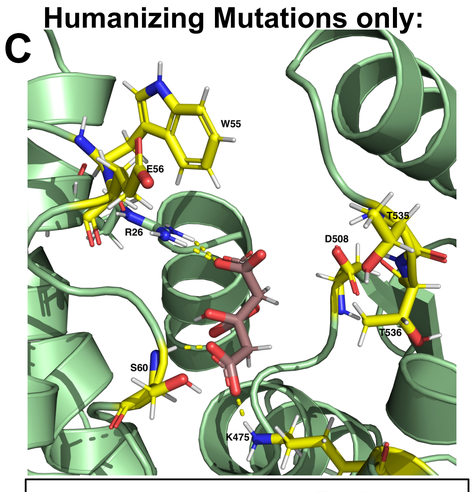


Fig 4. Non-Coupled protocol frameworks vs. Coupled Moves algorithm. The coupled algorithm integrates backbone flexibility with amino acid side chain movement, permitting subtle local arrangements

A

1PFK (*E. coli*)	4XYJ (Human)
22 Arg	32 Arg
26 Arg	36 Arg
55 Arg	**67 Trp**
56 Tyr	**68 Glu**
59 Ser	71 Ser
60 Asp	**72 Ser**
475 Arg	**555 Lys**
506 Gly	587 Gly
508 Glu	**589 Asp**
532 Lys	613 Lys
534 Lys	615 Lys
535 Lys	**616 Thr**
536 His	**617 Thr**



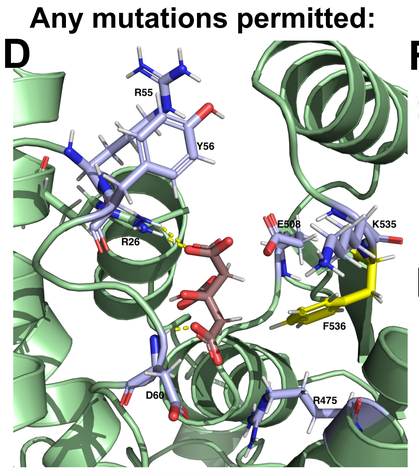


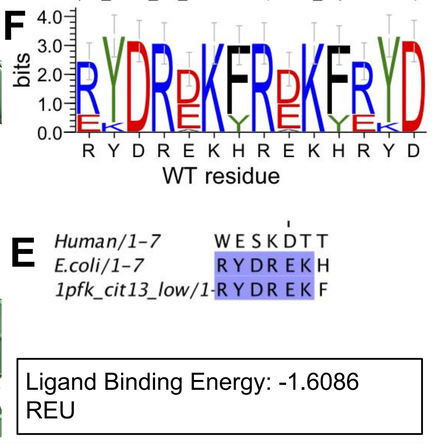
Ligand Binding Energy: -1.7295 REU

Figure 5. (A) List of important residues in E. coli ADP effector site.[4] Residues different in *E. coli* vs. human in red. **(B)** Human platelet PFK-1 with docked citrate conformer for comparison. **(C)** Lowest binding energy output structure after designing a "humanized" binding pocket with Coupled Moves (mutated residues in yellow)

Our most conservative computational design strategy models citrate in human PFK-1 and either grafts an identical citrate binding site in *E. coli* PFK-1. Our least conservative design strategy allows all binding site residues to mutate to build a new binding site from scratch.

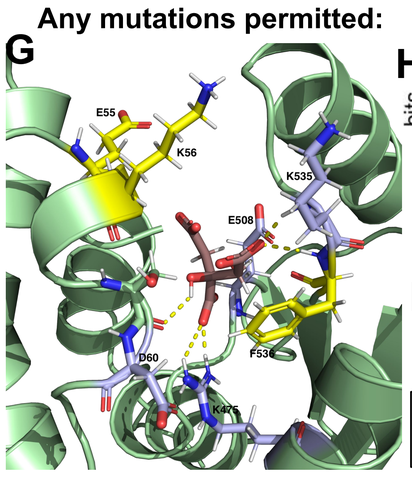
Any mutations permitted:

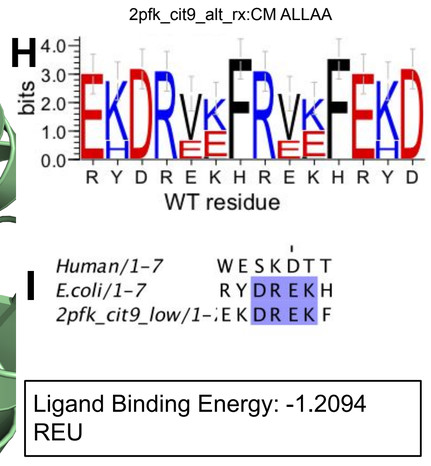




Ligand Binding Energy: -1.6086 REU

Any mutations permitted:





Ligand Binding Energy: -1.2094 REU

Coupled Moves generated designs exhibit high recovery of the native *E. coli* sequence, suggesting that citrate may bind the WT enzyme, or that only a few mutations are required for citrate binding.

(**D and G)** Lowest ligand binding energy output structure for a given PDB and citrate conformer (Designable residues as blue sticks; designed residues in yellow). **(E and I)** Designed sequence compared to human and *E. coli* WT sequences. **(F and H)** Sequence logo for 20 output designs. Ligand binding energy = Total Score Bound - Apo.

Ongoing and Future Work

1. Further analyze coupled moves designs to determine candidates for screening
2. Screen designs for binding and inhibition by citrate using activity assays and binding assays (Isothermal Titration Calorimetry (ITC) or Microscale Thermophoresis (MST))
3. Hydrogen-deuterium exchange (HDX) on human and *E. coli* PFK-1 to confirm hypothesized binding sites and to examine allosteric protein dynamics in WT and mutants based on differences in deuteration in the presence and absence of citrate.

Acknowledgements

Thank you to the Glasgow lab and the CMBS Program at Columbia University

References

[1] N. Sträter, et al., *FASEB J.* 25, 89–98 (2010).
[2] R. G. Kemp and D. Gunasekera, *Biochemistry*, 9426-9430 (2002).
[3] Usenik A, Legiša M (2010) Evolution of Allosteric Citrate Binding Sites on 6-phosphofructo-1-kinase. PLoS ONE 5(11): e15447. doi:10.1371/ journal.pone.0015447.
[4] Shirakihara, Y., and Evans, P.R. (1988) Crystal structure of the complex of phosphofructokinase from *Escherichia coli* with its reaction products. *J Mol Biol* 204: 973–994.