From One Function to Another: The Physical Strategies of Protein Evolution

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Introduction: Cellular chemistry is carried out by enzymes that have evolved to specifically and efficiently catalyze the reactions that are necessary for life. A physical and energetic accounting of the mechanisms through which natural selection acts on enzymes to achieve this specificity and catalytic ability would advance our basic understanding of how current biological systems came to be, and could further aid in efforts to design proteins for use in the areas of alternative energy, environmental remediation, health, and others. Rather than designing enzymes *de novo*, equipped with a physical understanding of the strategies used by evolution to convert an enzyme with one function into another, extant enzymes could be altered to catalyze different reactions. Yet it is difficult to reconstruct the past. Much evolutionary research has focused on sequence data, applying phylogenetic approaches. Structural data has also been used, both in comparing many related structures and in the reconstruction of ancestral enzymes.[1] Directed evolution studies have tried to recreate and characterize molecular evolution experimentally. I propose to take a distinct and complementary approach, systematically and thoroughly characterizing the physical differences between two enzymes from the same superfamily that utilize similar catalytic mechanisms and apparati, but that have evolved to catalyze different reactions.

The two proteins I will compare are alkaline phosphatase (AP) and cofactor-independent phosphoglycerate mutase (iPGM). AP is arguably one of the best-studied enzymes, and its mechanism of phosphate hydrolysis using two Zn^{2+} ions and proceeding through a phosphoserine intermediate has been thoroughly characterized.[2] iPGM catalyzes the interconversion of 2- and 3-phosphoglycerates (2PGA, 3PGA) and is structurally homologous to AP, containing a nearly identical active site—the main difference being the incorporation of two Mn^{2+} ions rather than two Zn^{2+} ions. In the mutase reaction, phosphate is removed from glycerate and transferred to the active site serine. Glycerate then repositions in the active site, and phosphate is transferred back to a different glycerate hydroxyl group. Crystal structures show that iPGM is composed of two domains that are closely associated when substrate is bound, but swing over 10 Å apart when no substrate is present. One domain contains the conserved active site, while the other contains at least one catalytic residue and other residues thought to be involved in glycerate positioning.[3]

Hypothesis: <u>The following adaptations that distinguish iPGM from AP are proposed to allow iPGM to specifically catalyze the mutase reaction, instead of phosphate hydrolysis: 1) binding interactions with glycerate, 2) the substitution of Zn^{2+} for Mn^{2+}, and 3) the relegation of catalytic residues from the active site to a second domain that must open and close on the same timescale as catalysis</u>. To investigate this hypothesis, I will monitor the fate of the phosphoenzyme intermediate (**Fig. 1**), and thus iPGM's ability to promote glycerate phosphorylation and the mutase reaction over phosphate hydrolysis and an AP-like phosphatase reaction. I will then both mutate iPGM and exchange Zn^{2+} for Mn^{2+} and observe how these changes affect the ratio of hydrolysis to PGA formation, as well as their influence on the rate of enzyme opening and closing to determine what role, if any, this mechanism plays in promoting the mutase reaction.

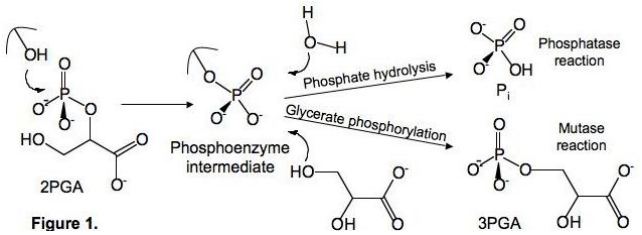

Figure 1.

Aim 1: ***Compare rates of phosphate hydrolysis and glycerate phosphorylation***

I will first establish a kinetic assay to monitor the fate of the phosphorylated iPGM intermediate. (A) The below outlines a strategy for generating a stable form of this intermediate. iPGM will be

exposed to phosphorylation agents such as *para*-nitrophenylphosphate that contain radioactive phosphate (^{32}P) and the fraction of enzyme that is radioactively labeled will be determined. By varying the temperature, buffer, and pH of the reaction and comparing the fraction of protein that is phosphorylated in every condition, I will define reaction conditions that optimally stabilize the phosphoenzyme intermediate. (B) The ratio of phosphate hydrolysis to glycerate phosphorylation will be measured using a pulse-chase experiment. iPGM will be pre-incubated with ^{32}P labeled phosphorylation agent, and then excess glycerate and non-radioactive phosphorylation agent will be added. The ratio of hydrolysis to PGA formation will be determined by monitoring the fate of ^{32}P—either as inorganic phosphate or phosphoglycerate—using thin layer chromatography.

Aim 2: ***Measure the rates of conversion and the equilibrium between open and closed iPGM***

I will use two different biophysical techniques to measure the equilibrium between open and closed iPGM. (A) Donor and acceptor fluorophores will be attached to both domains of iPGM, and FRET traces from individual proteins will be monitored using fluorescence microscopy. The FRET signal is correlated to the proximity of the two fluorophores, allowing the determination of opening and closing rates for individual molecules, as well as the equilibrium between the two states and thus the ΔG for closing. These same measurements will be repeated in the presence of phosphorylation agent and glycerate to determine the effects these molecules have on closure. (B) Small-angle X-ray scattering (SAXS) can provide information about the conformational behavior of a protein. I will collect SAXS diffraction data for iPGM in solution in the absence and presence of phosphorylation agent and glycerate. This data will be used to reconstruct low-resolution shapes of the different conformations of iPGM, which, through further analysis, can provide information about the relative numbers of molecules that adopt each conformation.[4]

Aim 3: ***Characterize functional implications of amino acid and metal substitutions***

Once the above schemes are developed, I will be able to probe the contributions individual residues and metal ligands make to the mutase reaction. I will make mutations in iPGM as well as exchange Zn^{2+} for Mn^{2+}, and measure the functional effects. The results will be interpreted in comparison to AP and in the context of evolution. Residues involved in catalysis and glycerate binding will be identified, as well as more complicated situations, such as the following two examples. (A) Mutation of a residue reduces PGA formation drastically and phosphate hydrolysis slightly, and shifts the equilibrium toward the open state. One model is that this mutation causes the enzyme to close less frequently, and that closure is not only essential for glycerate binding, but also involved in the chemical step since the rate of hydrolysis is also reduced. (B) Substitution of Zn^{2+} for Mn^{2+} increases hydrolysis, decreases PGA formation, and does not change the $\Delta G_{closing}$. These results would suggest a model in which Zn^{2+} enhances the hydrolysis reaction to the point where the phosphoenzyme intermediate is so short-lived that glycerate is simply not phosphorylated. In this model, the incorporation of Mn^{2+} in iPGM would be an important adaptation for slowing down the hydrolysis reaction and promoting PGA formation.

Conclusion: The scheme outlined above presents a novel approach to studying the physical mechanisms of protein evolution. In comparing related enzymes that catalyze distinct reactions, I hope to elucidate the specific adaptations in iPGM that have allowed it to promote the mutase reaction. This information will not only deepen our basic understanding of how proteins evolve, but it also has the potential to impact the field of enzyme design.

Statement of Originality: I assert that this proposal is my own work.

References: 1. Ortlund, E.A. et al. *Science,* 317: 1544-1548 (2007) **2.** Coleman, J.E. *Annu. Rev. Biophys. Biomol. Struct.*, 21: 441-483 (1992) **3.** Nukui, M. et al. *Biophys J.*, 92: 977-988 (2007) **4.** Svergun, D.I. *Biophys J.*, 76: 2879-2886 (1999)