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**IDENTIFICATION OF NOVEL ALLOSTERIC REGULATORS OF HUMAN
ERYTHROCYTE PYRUVATE KINASE**

A dissertation submitted in partial fulfillment of the requirements for the
degree of Master of Science at Virginia Commonwealth University

by

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DEDICATION

To my family, friends and teachers for their love, support and guidance through the years.

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I sorely miss my father in this moment of happiness. His loving memory will always be with me. I would like to thank my friends and family members for always being there for me. I also thank my husband, Laxmikant for his patience and support during my graduate school.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cDNA	Complementary DNA
CoMFA	Comparative molecular field analyses
DEAE	Diethylaminoethyl
DEC	Dielectric constant
DNA	Deoxyribonucleic acid
DPG	2,3-diphosphoglycerate
EDTA	Ethylene diamine tetraacetic acid
EM	Emden-Meyerhoff
FBP	Fructose 1,6-bisphosphate
FDP	Fructose 2,6-bisphosphate
GOLD	Genetic Optimization for Ligand Docking
H ⁺	Hydrogen ion
Hb	Hemoglobin
HINT	Hydrophobic INTeraction
K ⁺	Potassium ion
KCl	Potassium chloride
K _M	Michaelis-Menten constant
K _{NF}	Koshland-Nemethy-Filmer
LB	Luria-Bertani
LDH	Lactate dehydrogenase
Log P _{o/w}	Partition coefficient for 1-octanol/water
M1-PK	Human muscle pyruvate kinase isozyme 1
M2-PK	Human muscle pyruvate kinase isozyme 2
Mg ²⁺	Manganese ion
MgCl ₂	Magnesium chloride
mM	Millimolar
Mn ²⁺	Magnesium ion
MnSO ₄	Manganese sulfate
MWC	Monod-Wyman-Changeux
NADH	Nicotinamide adenine dinucleotide
NCI	National Cancer Institute
n _H	Hill Coefficient
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid

OD	Optical density
PDB	Protein Data Bank
PEP	Phosphoenol pyruvate
PG	Phosphoglycolic acid
PK	Pyruvate kinase
PKD	Pyruvate kinase deficiency
PKLR	Pyruvate kinase liver red blood cell
PKM	Pyruvate kinase muscle
QSAR	Quantitative structure-activity relationship
QSAR	Quantitative structure activity relationship
RBC	Red blood cell
RNA	Ribonucleic acid
R-PK	Human erythrocyte pyruvate kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
V _{max}	Maximum velocity

ABSTRACT

IDENTIFICATION OF NOVEL ALLOSTERIC REGULATORS OF HUMAN ERYTHROCYTE PYRUVATE KINASE

Shilpa S. Kharalkar, M. Pharm.

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2006

Director: Donald J. Abraham, Ph.D.
Professor and Director ISBDD
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Erythrocyte pyruvate kinase (R-PK) is a key glycolytic enzyme catalyzing the transphosphorylation of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP respectively^{3,4}. The substrate PEP and product pyruvate of this reaction are involved in a number of energetic and biosynthetic pathways; hence a tight regulation of R-PK activity is crucial not only for glycolysis, but also for the entire cellular metabolism. Deficiency of R-PK is one of the most common enzymatic defects of RBC, and may be caused by mutations of the *PK-LR* (pyruvate kinase liver red blood cell) gene^{31,32}. Clinically, R-PK deficiency manifests itself as a chronic life-long hemolysis ranging from very mild or fully compensated anemia to life-threatening neonatal anemia and pronounced jaundice.

Current treatment options are limited to continuous blood transfusions and splenectomy. Thus, there is an urgent need for medications to counter R-PK deficiency without resorting to these complicated procedures. Our aim is to identify novel allosteric modifiers of R-PK using a combination of computational studies and enzyme activity assays. Such compounds could be of medical interest.

Human R-PK was expressed in DH-5 α cells and was purified by the procedure reported by Wang et al ⁵⁷. However, this method gave a very low yield of R-PK (5mg/L). In an attempt to increase the yield, we expressed R-PK in Rosetta strain cells. Further, addition of His-tag to the protein's N-terminus simplified purification to a one step Ni-NTA (Nickel- nitrilotriacetic acid) column resulting in a 6-fold increase in the yield.

Computational methods were applied to identify small molecules that bind to the allosteric activator fructose 1,6-bisphosphate (FBP) binding site of R-PK to identify compounds that could interact with the protein. The software UNITY, as present in the molecular modeling software Sybyl was used to perform 3D searches against the National Cancer Institute (NCI) chemical database. From these searches we obtained 29 hits that were subjected to further computational analysis. The small molecules were docked into the FBP binding site of R-PK with different docking methods including FlexX, GOLD and energy minimization. The energy scoring function of HINT was then applied to analyze the interactions between the docked molecules and R-PK. Compounds with highest HINT score were requested from NCI and were subjected to further kinetic analysis to identify possible allosteric effectors of R-PK.

In the kinetic analysis, we employed a lactate dehydrogenase (LDH) coupled spectrophotometric assay to determine the activity of R-PK in the presence of these compounds. The steady state kinetics of R-PK gave a typical S-shaped curve that fitted a sigmoidal function indicative of allosterism. All the kinetic parameters of our enzyme were in excellent agreement with native R-PK activity as previously reported^{5, 57}. R-PK activity in the presence of the analyzed compounds revealed both activators and inhibitors of R-PK. X-ray crystallographic analysis of R-PK in the presence of FBP and the identified small molecule effectors are currently in progress. These experiments were initiated to reveal the binding site of the compounds in R-PK, allowing for further optimization of the starting pharmacophores and syntheses of new molecular entities for enhanced allosteric activity.

In conclusion, we have developed a simple and efficient method for the expression and purification of R-PK. Using computational screening and HINT analysis we have also identified several compounds that interact with R-PK and kinetic analysis revealed both activators and inhibitors of the protein. Crystals of R-PK in the presence of effectors have been obtained and identification of the binding site on R-PK is under investigation. R-PK effectors discovered in this study could prove to be lead compounds for developing medications for the treatment of anemia and other disorders arising from R-PK malfunction.

INTRODUCTION

A. Pyruvate Kinase

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomitant production of a relatively small amount of ATP. Pyruvate kinase (PK: ATP: pyruvate 2-o-phosphotransferase, EC 2.7.1.40) is a key enzyme of this pathway that catalyzes the transphosphorylation of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP respectively ^{3,4}. This conversion takes place in two steps: initially, the phospho moiety of PEP is transferred to a Mg-ADP complex via an enolate intermediate; following, stereo-specific protonation and tautomerization yields the keto form of pyruvate. The enzyme requires divalent and monovalent ions, including Mn^{2+} , Mg^{2+} , H^{+} and K^{+} ions for activity ^{6,7}. The pyruvate kinase reaction is shown in Figure 1 and the glycolysis pathway is shown in Figure 2.

PK is an important regulatory enzyme and plays a dual role in glycolysis as it generates ATP in the cell, and is also involved in the regulation of glucose metabolism. As seen in Figure 1 and 2, the PK catalyzed reaction is bidirectional; however under physiological conditions the formation of energy rich ATP molecules is favored. The activation of PK increases the ATP concentration in cell whereas its down regulation allows for accumulation of phosphometabolite pools preceding the PK reaction that can

Figure 1. The pyruvate kinase reaction. The reaction mechanism favors the formation of pyruvate with the generation of ATP. The rate of this reaction can be regulated by the allosteric transition mechanism of pyruvate kinase enzyme.

Figure 2. Embden- Meyerhof pathway and the Rapaport Lubering shunt of the pathway in erythrocytes. The allosteric mechanism of pyruvate kinase (PK) plays a key role in regulation of lower half of the glycolysis pathway and maintenance of 2, 3-DGP and other metabolites in the red cell.

be directed into synthetic processes. The PK catalyzed reaction is also involved in the regulation of glucose consumption of the cell ⁸.

Among all PK enzymes sequenced to date, the amino acid residues directly involved in catalysis are nearly 100% conserved for all species ranging from bacteria to human ⁹⁻¹¹. In contrast, the allosteric sites of PK from different biological species display a much lower degree of sequence conservation ¹²⁻¹⁶. The PK enzyme is allosterically regulated by a variety of effectors, including fructose-1,6-bisphosphate (FBP), fructose-2,6-bisphosphate (FDP), L-alanine, and L-phenylalanine ¹²⁻¹⁶. In human, the enzyme is allosterically activated by FBP, while ATP, alanine, and phenylalanine act as inhibitors ¹⁷⁻¹⁹. This divergence appears to indicate that regulatory sites might be attractive drug targets for organism-specific and tissue-specific antagonists or deregulators ²⁰.

The three-dimensional structures are known for the PK enzymes from cat, rabbit muscle ^{21,22}, yeast ¹⁵, *Escherichia coli* ²³, *Leishmania mexicana* ²⁴ and human ^{5,25}. The structures show very similar architecture, with each subunit of the tetrameric protein arranged to form three main domains: A, B, and C ²⁶. For eukaryotes, there is an additional N-terminal domain ²⁷. The active or catalytic site is located at the interface of A and B domains, while the allosteric or activator-binding site is exclusively located within the regulatory C domain; approximately 36Å^o from the catalytic binding site. The multi-domain structure of PK is essential for the function and regulation of the activity of the enzyme. The subunits mainly interact through the A and C domains located along the molecular dyad. The crystal structure of human erythrocyte pyruvate kinase is shown in Figure 3.

Figure 3. Structure rendering of human R-PK. The enzyme is composed of four identical subunits. Each subunit contains a bound molecule of FBP and PG (space filled).

This structure shows the characteristic tetrameric structure of the PK enzymes with four subunits shown in different colors. The figure also shows the substrate PEP and allosteric effector FBP bound to the protein at separate locations.

Four PK isozymes have been identified in mammals²⁸. In humans, two separate genes; the *PKLR* (Pyruvate kinase liver, red blood cell) gene and the *PKM* (pyruvate kinase muscle) gene encode the PK enzyme isozymes. The regulatory behavior of each isozyme is different and designed to meet the metabolic requirements of the expressing tissues. The *PKLR* gene located on chromosome 1 uses alternate promoters for the expression of its two isozymes R and L. R-PK is exclusively expressed in erythrocytes whereas the L isoform is expressed in tissues involved in gluconeogenesis such as liver, renal cortex, and small intestine^{29,30}. Mutations in the R and L isoforms are associated with nonspherocytic hemolytic anemia and jaundice, respectively^{25,31,32}. The *PKM* gene located on chromosome 15 encode the M1 and M2 isozyme of PK by alternative splicing of the same RNA^{33,34}. M1-PK is expressed mostly in skeletal muscle, heart and brain tissues; and M2-PK, is expressed in kidney, lungs, fetal tissues, adipose tissues, platelets and spleen. The M2 isozyme is also expressed in proliferating cells such as embryonic cells, adult stem cells and tumor cells. With the exception of M1-PK, which displays hyperbolic kinetics, the other three isozymes display sigmoidal kinetics toward the substrate PEP^{21,22}. In human, the enzyme is activated by fructose 1,6-bisphosphate^{12-16,35}. These four PK isozymes also differ in their chemical, physical, kinetic, electrophoretic, and immunological properties^{36,37}.

B. Pyruvate kinase (R-PK): Its role in RBC

Red blood cells lack mitochondria and rely heavily on ATP production via the glycolytic cycle and hence PK. The rate of PK catalyzed reaction ensures the maintenance of pool of specific metabolites, biosynthetic precursors, and nucleotide triphosphates at appropriate levels for energy production in the cell. In addition, the metabolite pool is utilized to enhance the synthesis of nucleosides and amino acids in the cells³⁵. ATP maintains cation gradient in RBC and maintains the deformability and biconcavity of RBC. The ATP driven pump protects RBC from premature death. Hence, regulation of PK is an important key to control glycolysis in cells. In erythrocytes the Rapaport –Lubering shunt operates in close association with the glycolysis pathway (Figure 2). This shunt provides the RBCs with 2,3-diphosphoglycerate (DPG), an allosteric activator of hemoglobin (Hb).

C. R-PK crystal structure

The R-PK protein has been crystallized in the active R state and Vallentini et al. (2002)⁵ solved the 2.7Å^o resolution crystal structure of R-PK (Figure 3). This structure is that of a truncated form of the enzyme, lacking the first 49 amino acids in the protein. R-PK is a 240 kDa protein and is a homotetramer consisting of four identical subunits. The crystal structure shows similarities to other known PKs with the four subunits arranged to form D2 symmetry, resulting in a structure that has three, two fold symmetry axis intersecting each other at right angles.

Each R-PK subunit forms three main domains: A, B, and C. The A domain is the largest of the three domains with a $(\beta/\alpha)_8$ barrel topology. The B domain consists of

seven β -stranded β -barrel and a short α -helix and is inserted between the strand $\beta 3$ and helix $\alpha 3$ of the A domain whereas the C domain has α/β topology. In addition, R-PK shows a small N terminal domain. Inter-subunit interactions between these domains form two large contact areas; the A/A' interface that involves the A domains of subunits that are related by the vertical twofold axis whereas the C/C' interface involves the C domains of subunits interacting along the horizontal axis. This multi-domain structure of R-PK is essential for the function and regulation of the activity of this enzyme. R-PK is an allosteric enzyme and can exist in two different states. Transition between these states can be triggered by binding of one or more effectors and involves a combination of domain and subunit rotations that are coupled to conformational changes in the active site. Thus, the residues located at the domain and subunit interfaces of R-PK play a crucial role in the communication between the activator-binding site and the catalytic center.

R-PK binding sites

Two binding sites have been identified from R-PK crystal structure; the substrate-binding site where the substrate analog phosphoglycolic acid (PG) binds and, the allosteric activator-binding site where FBP bind. The active site binds the PG in a large cleft between the A and B domains and requires both Mg^{2+} and K^+ ions for its binding. The carboxylate moiety of PG engages in direct interactions with the protein residues Thr 371, Gly338, Asp339 and with the Mg^{2+} ion whereas the phosphate group interacts with the K^+ atom and with Arg116. These residues, all found in the A domain are completely conserved in *E.coli*, human, yeast, cat and rabbit PK.

The allosteric activator FBP binds in the allosteric site in the C domain approximately 36Å^o from the PEP binding site of R-PK. FBP molecule bound in the allosteric binding site is shown in Figure 4. The FBP binding site is located entirely within the C domains of each subunit, near the C-termini. FBP is sandwiched between loops 475-479 and 557-566 and interacts extensively with the protein. Each of the bound FBP molecules engages in identical non-covalent interaction with the surrounding polar residues. The 6'phosphate group of FBP engages in a direct salt bridge with Arg532 and in hydrogen bond interactions with residues Arg559 and Gly561, whereas the 1'phosphate group is involved in hydrogen bonding with the side chains of Thr475 and Ser480 as well as to the backbone nitrogen atoms of Thr476 and Thr477. Further, the negative charge of FBP is also compensated by the dipole of α -helix 480-486 that points towards the 1'phosphate. These interactions are very similar to those observed between yeast -PK and FBP. Mutation studies involving the residues of the allosteric binding site have identified Arg532 to play a major role in activator binding. The R532W mutation causes a complete loss of responsiveness to FBP and is associated with severe anemia. R479H mutation affects the N-terminus of helix 479-486, and prevents the compensation of the negative charge of FBP. This mutation is also associated with severe anemia.

D. Allosteric proteins

PK is an allosteric enzyme and control of its allosteric states plays a central role in the regulation of activity of this enzyme. Allostery or a different shape is defined as the coupling of conformational changes between two widely separated sites³⁸⁻⁴². Allosteric regulation refers to the regulation of an enzyme by binding of ligand at a site spatially

Figure 4. FBP binding site in human R-PK. The allosteric binding pocket shows interactions of FBP with the polar side chain residues lining the site. The primary binding determinants are an electrostatic bond between the 1'-phosphate and the side chain of Arg532 and a series of hydrogen bonds between 6'-phosphate and the side chains of Ser 480, Thr476, and Thr477.

different (or allosteric) from the enzyme active site. An allosteric protein is usually a multisubunit protein having multiple binding sites for the ligand, which can either be the native substrate (homotropic) or another effector molecule (heterotropic). Ligand binding induces a structural change in the protein. This results in alteration of the binding affinity for subsequent ligand molecules that could either activate or deactivate the enzyme. These change in the catalytic activity of the enzyme following this phenomenon results in cooperative kinetics; demonstrated by a sigmoidal curve obtained when enzyme velocity (or % saturation) is plotted against ligand concentration and can be differentiated from the hyperbolic curve obtained for enzymes following Michaelis-Menten kinetics. The sigmoidal curve better fit a modified Michaelis-Menten equation having the term for the constant of cooperativity, n_H , also known as the Hill coefficient. The value of n_H is less than unity for proteins exhibiting negative cooperativity whereas its value is greater than unity for proteins exhibiting positive cooperativity. Figure 5 shows the difference in kinetics of allosteric enzymes in contrast to enzymes following hyperbolic kinetics. Two models explain the behavior of allosteric protein; the concerted symmetry model, and the sequential interaction model:

Monod-Wyman-Changeux concerted model

The concerted –symmetry model by Monod, Wyman and Changeux (MWC) postulates that an allosteric protein is an oligomer of identical subunits termed protomers which, exist in equilibrium between a tense T- state having low affinity for substrate; and a relaxed R- state with high substrate affinity³⁸ (Figure 6). The conformations result from a change in the tertiary structure of the protomers, brought about by binding of various

Activity (or % saturation) vs. [Substrate]
Non-Co-operative enzymes

$$V = (V_{\max} \times [\text{Substrate}]) / K_m + [\text{Substrate}]$$

Activity (or % saturation) vs. [Substrate]
Co-operative enzymes

$$V = (V_{\max} \times [\text{Substrate}]^n) / K_m + [\text{Substrate}]^n$$

Hill Plot
Co-operative enzymes

$$\log(V / V_{\max} - V) = n_H \log[\text{Substrate}] - \log K$$

Figure 5. Graphical representation of protein/ enzyme binding and kinetic data, demonstrating the different behavior of non-cooperative and cooperative systems.

Figure 6. General model for the binding of ligands to a four-site allosteric enzyme or protein. Circles denote the R-state and the squares denote the T-state. The Monod-Wyman-Changeux (MWC) model encompasses the states enclosed by the vertical solid rectangles. The Koshland-Nemethy-Filmore (KNF) model is demarcated by the diagonal dotted rectangle. Both models are considered to be limiting cases of the general model allowing all possible conformational states and ligation patterns. Source: Seigel I. H {Seigel, 1975 #115}.

ligands to the protein. A key concept of the MWC model is that enzyme subunits are not independent acting but are connected in a manner that confers the conformational change in one subunit to all other subunits, thus maintaining the symmetry of the oligomer. Allosteric proteins such as R-PK thus exist in either the T-state or R-state with all subunits of the protein in the same conformation. The MWC model further assumes that the equilibrium favors the T-state of the protein in absence of the ligand. The binding of ligand to an all-T structure decreases the conformational equilibrium constant (termed k , equal to $[T]/[R]$), and increases the probability of the all-T to all-R structural transition. An allosteric activator binds preferentially to the R-state, whereas an allosteric inhibitor binds preferentially to the T-state. A major disadvantage of the MWC model is its incapability to address for the negative cooperativity, as ligand can only stabilize the all-R quaternary state. The MWC model is more widely used as it simplifies the construction of binding curves; only two substrate dissociation constants are needed, one for the all-T state, and other for the all-R state ⁴³.

Koshland Sequential model

The sequential interaction model by Koshland, Nemethy, Filmer (KNF) is also termed as an induced fit model. This model assumes sequential or progressive changes in the affinities of vacant sites as ligands occupy the protein sites (Figure 6). According to this model, individual subunits of proteins undergo tertiary structural changes from T-to R-states upon binding of ligand instead of a concerted all T to all R quaternary structure change. This structural change may be transmitted via the interfaces to neighboring subunits. Binding of each ligand molecule to the protein can either increase the binding

affinity of the subsequent ligand molecule (positive cooperativity) resulting in the characteristic S-shaped velocity curve showing a marked acceleration phase followed by the normal sloping off as the enzyme approaches saturation or result in decreased binding affinity (negative cooperativity). The binding curves for KNF model are more complex than those for MWC model and require a different dissociation constant for every intermediate state.

Basis for allosteric effector as potential therapeutic agents

The number of potential therapeutic agents among small molecule allosteric effectors of allosteric protein is growing rapidly. The elucidation of crystal structures of both high and low affinity states of allosteric proteins allow the design of state specific synthetic effectors. These drugs offer several important advantages over designing drugs that bind directly to the active site. First, these drugs are not limited by the need to generate molecules with receptor affinities several fold that of the natural substrate because allosteric binding sites offer a potential for greater specificity for target protein. Second, allosteric effectors can elicit an effect regardless of the concentration of endogenous substrate present since they bind to a location separate from the substrate-binding site. In addition, the activity of an allosteric protein can be modulated by degree, vs. all or none, allowing the possibility to tailor drug activity to the severity of the disease state ⁴⁴.

E. R-PK and allostereism

R-PK is an important glycolytic enzyme and manipulation of its regulatory behavior by allosteric modifiers is of interest for medical purposes. The FBP binding site of R-PK can be manipulated to identify effectors that bind the protein. These allosteric effectors could prove to be lead compounds for developing medication for the treatment of hemolytic anemia, sickle cell anemia, hypoxia-related diseases and other disorders arising from erythrocyte pyruvate kinase malfunction. An allosteric effector can either block the allosteric transition by stabilization of the region of the molecule across which this change takes place or it can stabilize either the T or R state appropriately. Such a drug would bind to some aspect of the conformation of either the R-or T state distinct for that state and displace the equilibrium between R and T toward the appropriate state. Figure 7 illustrates the allosteric transition mechanism of R-PK and its application for identifying drugs selective for either state.

R-PK and other enzymes use allosteric regulation to regulate biochemical reaction pathways and maintain an appropriate balance of intracellular substrate and product concentrations. This mechanism enables cells to coordinate the activity of a particular enzyme with other cellular reactions and signaling pathways¹⁵. The multidomain architecture of R-PK plays a central role in the regulation of activity of this enzyme. The exact mechanism of allosteric transition is not known, as R-PK or any other PK has not been crystallized in both T and R-state. Comparative studies of the crystal structure of allosteric type I PK from *E. coli*^{14,23} with the structures of cat and rabbit muscle enzymes^{21,22}, was used to study the mechanism of allosteric control of PK enzyme. These studies

Figure 7. Allosteric transition mechanism of R-PK. The transformation of the enzyme between the active R and inactive T state is brought about by binding of effectors. Stabilization of both states has potential therapeutic applications.

suggest that the $R \leftrightarrow T$ transition in PK involves modifications in the relative orientations of the domains and subunits coupled to conformational changes in the PEP-binding site and the allosteric effector binding sites. The subunit rotations observed in the T-state PK induce a loop shift in the enzyme, leading to a distortion of the PEP-binding site, which accounts for the low substrate affinity of the T-state enzyme. Site directed mutagenesis studies indicate that the intersubunit interactions at the A/A' and C/C' interfaces as well as the interdomain interactions at the A/B interface determine the allosteric responsiveness and the distribution of the conformations between active and inactive states. These studies along with crystallographic data indicate that the T and R forms correspond to ensembles of conformations characterized by the rotational flexibility of the B domain ^{5,45}.

R-PK enzyme behaves in similar manner and its crystal structure shows a flexible B domain that adopts different orientations in the crystallographically independent subunits. Site directed mutagenesis studies on R-PK indicate the A/A' interface of the protein to play a key role in the allosteric transition. D390N mutation, which targets a residue located in the core of the A/A' interface, produces an enzyme that retains a stable tetrameric state but almost entirely lacks enzymatic activity. On the other hand, the mutations targeting residues at the A/C interface do not alter the allosteric properties of R-PK and this domain interface appears to have little role in the transduction of the allosteric signal ⁵.

The allosteric transition of R-PK regulates the levels of important metabolic intermediates such as ATP and 2,3-diphosphoglycerate (DPG) in red blood cells ^{46,47}. The

allosteric transition mechanism of R-PK and its application is shown in Figure 7.

Allosteric effectors such as FBP and PEP that activate R-PK shift it towards the active R-state and increase the catalytic efficiency of the enzyme; consequently the energy production of RBC is increased. ATP maintains the deformability and biconcavity of erythrocyte through ATP driven pumps ⁴⁶. Activation of R-PK also causes a decrease in the levels of DPG ⁴⁸ that regulates oxygen delivery in response to stimulation by hypoxia, leading to an increase in the oxygen carrying capacity of the blood; potentially useful for the treatment of sickle cell anemia ⁴⁹⁻⁵¹.

On the other hand, inhibitors of R-PK, such as ATP, alanine and phenylalanine stabilize the T-state relative to the R-state ¹⁷⁻¹⁹. As a result the allosteric equilibrium is shifted toward the T-state leading to accumulation of DPG ⁴⁸. Under the conditions of hypoxia but no anemia, DPG level increases up to threefold and enhance oxygen delivery to the cardiovascular periphery by stabilizing the Hb deoxy state tissue ^{25,47,48,52}. Thus, an important aspect of R-PK inhibition involves identifying compounds that will effectively inhibit this enzyme and in turn will increase red cell DPG levels at least 2 to 3 fold. Development of R-PK allosteric inhibitors will make it feasible to significantly increase DPG concentrations in erythrocytes; potentially useful for the development of new treatment of a variety of ischemic diseased states, including Alzheimer's disease. This route of DPG modification for therapeutic application should be practical, as heterozygous individuals displaying 40-60 % normal PK activity display increased DPG levels, without adverse side effects ⁵³.

Thus, effectors of R-PK bind to multiple binding sites in the protein. PEP homotropically activates the enzyme by binding R-PK at the substrate-binding site, whereas the activator FBP binds at an allosteric binding site (Figure 3). Binding of ligand to the allosteric site alters the kinetics of enzyme that shows an accelerated binding curve indicating increased ligand affinity with binding of subsequent ligand molecules. Both PEP and FBP bind preferentially to the active R state causing a shift in R-PK equilibrium from an inactivated tense state “T” to an activated relaxed state “R”, thus increasing the catalytic efficiency of the enzyme and increasing the concentrations of ATP in the cell. The activity of R-PK decreases by binding of inhibitors such as ATP, alanine and phenylalanine, which shift the allosteric equilibrium towards the T state that slows the enzyme and causes a metabolic block in the glycolysis pathway. In addition to the regulation by effectors; the activity of R-PK can also be monitored by phosphorylation of N-terminal that favors the T-state⁵⁴. Partial activation of PK by proteolysis has also been reported⁵⁴ along with hormone-triggered dimerization for some forms of the enzyme.

The kinetic behavior of R-PK can be explained with the MWC model of allostery³⁸, which was proposed in the 1960's, and was subsequently corroborated by structural studies on Hb^{55,56}. According to this model, the multi-subunit structure of R-PK exists in equilibrium between an inactive tense state (T-state) that possesses low ligand/substrate affinity, and an active relaxed state (R-state) that possesses high affinity for the ligand/substrate⁵⁵. For R-PK, transition between these two equilibrium states can be triggered by binding of one or more effectors and involves a combination of domain and subunit rotations that are coupled to conformational changes in the active site²⁶. The

natural substrate, PEP and the allosteric effector, FBP bind and cause a shift in R-PK equilibrium from an inactivated T-state to activated R-state, thus increasing the catalytic efficiency of the enzyme. This results in increased concentrations of ATP in the RBCs; preventing their lyses^{5,46,57}. Therefore, R-PK activators that lead to stabilization of the R-state relative to the T-state and/or shift the allosteric equilibrium toward the R-state have potential application for the treatment of PKD causative hemolytic anemia. Activation of R-PK also causes a decrease in the levels of DPG⁴⁸ leading to an increase in the oxygen carrying capacity of the blood; potentially useful for the treatment of sickle cell anemia.⁴⁹⁻⁵¹ On the other hand, inhibitors of R-PK, such as ATP, alanine and phenylalanine stabilize the T-state relative to the R-state¹⁷⁻¹⁹. As a result the allosteric equilibrium is shifted toward the T-state leading to accumulation of DPG⁴⁸. Under the conditions of hypoxia but no anemia, DPG level increases up to threefold and enhance oxygen delivery to tissue^{25,47,48,52}. Therefore inhibitors of R-PK have potential application for the development of new treatments for hypoxic conditions, particularly Alzheimer and stroke.

F. Pyruvate kinase Deficiency

Pyruvate kinase deficiency (PKD) is one of the most common enzymatic defects of red blood cells (RBCs), and is caused by mutations in the *PK-LR* gene that code for R-PK⁵⁸. Mature RBCs lack mitochondria, and as such dependent on glycolysis for supply of ATP to function properly. Therefore, R-PK deficiency results in RBCs unable to maintain their cellular integrity and consequently undergo lyses causing hemolytic anemia⁴⁸. Severe PKD strongly alters the enzyme kinetics and affect the thermal stability

of the enzyme ⁵. It is also noted that, R-PK deficiency and/or transient inhibition of its activity creates a metabolic block in the glycolytic pathway and causes accumulation of proximal metabolic intermediates, such as 2,3-diphosphoglycerate in RBC ⁴⁸. DPG is an allosteric effector of hemoglobin (Hb) and regulates the affinity of Hb for oxygen. Increase in DPG levels facilitates oxygen delivery to tissues. During hypoxia, DPG levels increases up to threefold and the enhanced oxygen delivery to the tissues is useful to relieve hypoxia.

PKD is a hereditary genetic disorder and is transmitted as an autosomal recessive trait causing hemolytic anemia. More than 150 mutations have been identified worldwide in the gene coding R-PK among the R-PK deficient individuals, and new mutations are still being identified. These mutations mostly include missense mutations (69%), splicing and stop codon mutations (11% and 5%, respectively); whereas small insertions, deletions, and frame shift mutations are rare (12%). The treatment for PKD is mostly based on supportive measures such as blood transfusions or red cell transfusions to treat hemolytic anemia. In severe cases of PKD splenectomy is performed to increase the number of circulating erythrocytes (>40%) in the blood and to increase the hemoglobin levels to 2-3 g/100mL.

PKD has a world wide geographical distribution and its true prevalence is unknown. Individuals of northern European decent and certain other ethnic and racial groups have a higher prevalence of this disorder. The prevalence rate of heterozygote in Germany is estimated to be at 1% and in Hong Kong at 3%. In US, the occurrence of PK

deficiency is estimated to be 1:20000 in the general white population as assessed by gene frequency studies ⁵⁹.

G. Structure- based drug design

Structure- based drug design is one of the most logical approaches applied in drug discovery. It integrates information from several fields such as X-ray crystallography, NMR, molecular modeling, synthetic organic chemistry, quantitative structure-activity relationship (QSAR) studies, and biological evaluation to identify new lead molecules that can interact specifically with the target protein structure. This is a cyclic procedure that refines each stage of discovery ⁶⁰.

The first step in this process is identification of a biological target with known 3-d structure, the function of which can be modified to correct a pathophysiological state. This is followed by a detailed analysis of the binding site of the target protein, which is preferably complexed with a ligand. Different aspects responsible for the binding affinity and selectivity of the ligand for the target site are collected and used to optimize existing ligand or to develop new alternatives. This is followed by identification of lead molecules that can interact with this target. Computational methods and molecular modeling that perform elaborate computer searches of compound libraries provide new suggestions to generate new molecules. The new lead compounds must then be obtained (synthesized or purchased) and tested experimentally and their binding properties examined by spectroscopic, biochemical, and crystallographic methods. The process is repeated and the information obtained from these studies and the three-dimensional geometry of the new crystallographically characterized complexes is used to design improved ligand ⁶¹.

Computational studies

Computational methods play a crucial role in drug discovery programs, and are widely used for hit identification to lead molecule optimization and further; especially when the drug target is a protein. A variety of virtual screening techniques based on ligand or structure based screening are widely employed in many discovery programs for hit identification ⁶².

Computational techniques such as molecular modeling employ the known three-dimensional structural information about the therapeutic target to understand the interactions seen in receptor –ligand complexes and apply it to identify novel lead molecules ⁶³. The characteristics and unique conformations of the catalytic pockets of an individual enzyme are studied and exploited using a variety of approaches, both experimental and theoretical, aided by studies on the 3D structure of enzyme and its complex with ligand and supplemented by a variety of methods, including, molecular modeling and structure based drug design. A close inspection of the binding site and the possible interactions in it significantly improves the chance to find better/new ligands. Molecular modeling methods are then applied with the intent of designing more specific ligand. This information is used as a starting point of a 3D search in the NCI database ⁶⁴.

Virtual Screening and 3D database search

Virtual screening identifies new molecules that can interact with effector binding pocket of the protein. It is a fast and economical method to computationally screen compound databases for novel leads and is part of structure based drug design technique that includes database search, molecular modeling, docking and scoring the hits to

identify compounds that mimic portions of known active agent. This is a highly efficient process to reduce the vast number of molecules to be screened to a manageable number of compounds with a greater probability to become a successful drug candidate.

Chemical databases of small molecules employed in virtual screening contain records of hundreds of thousand of compounds in the form of explicit 3D atomic coordinates for each compound, obtained through direct biophysical analysis, or by automatic 3D structure generation.

A search can be performed by construction of search queries using combination of objects and constraints. Objects can be points, lines, or planes. Points are defined by an atomic nucleus, lone pair position, the spatial average of multiple points (for example, the centroid of a benzene ring), or an arbitrary point in space (“dummy” atom). Atomic centers can be defined as an explicit element type, or to maximize search results can be more general in notation, e.g., hydrogen bond donor/acceptor, hydrophobic centre, or positive/negative charge centre. Lines can be defined to encode 3D atom vectors. These have often been utilized as normal vectors in conjugation with a defined aromatic plane to spatially fix a ring orientation. Distance (two-point), angle (three-point), and torsional (four-point) constraints are set to define the geometric relationships between objects. If the structure of the receptor site is known, “exclusion spheres can be incorporated which represent steric “barriers “that a modeled ligand cannot overlap.

The HINT model

One computational tool currently in favor for analyzing protein- ligand interaction is the HINT (Hydropathic INteractions) program (eduSoft, LC). Unlike other simulations tools, HINT is a *natural* force field method that uses the empirically- derived octanol: water partition coefficient fragment rules of Hansch and Leo to measure the full range of electrostatic and hydrophobic interactions between protein and ligand. These interactions include the favorable interactions (hydrogen bond, acid/base, hydrophobic) and the unfavorable contacts (acid/acid, base/base, hydrophobic/polar). The total bimolecular interaction is determined by the following:

$$\sum_i \sum_j b_{ij} = \sum_i \sum_j (a_i S_i a_j S_j T_{ij} R_{ij} + r_{ij})$$

$$\sum a_i = \text{Log } P_{o/w}; \text{Log } P_{o/w} = -\Delta G/2.303 RT$$

$$\sum_i \sum_j b_{ij} = f(\Delta G)$$

where b_{ij} is the interaction score between atoms i and j , a the hydrophobic atom constant, S is the solvent accessible surface area, T a logic function that maintains the proper sign (+1 or -1) to signify attractive or repulsive interactions between atoms i and j , and R is an exponential function, and r is an adaptation of the Lennard-Jones function.

The factor $\log P_{o/w}$ (partition constant for 1-octanol/water) of a ligand or biological molecule is the sum of all the hydrophobic atomic constants for that molecule and, therefore HINT calculations encode more complete thermodynamic information than other molecular mechanics –based programs. HINT score therefore reveals information not only about enthalpy but also entropy of the bimolecular complex. It is particularly

valuable for evaluation of non-covalent molecular interactions, and pays particular attention to entropically driven hydrophobic effects.

A host of publications have shown HINT's applicability to predicting free energy of binding for protein–ligand systems ⁶⁵⁻⁶⁷, protein-protein ^{68,69}, and protein –DNA interactions ⁷⁰. In addition, HINT measurements have also been integrated into comparative molecular field analyses (CoMFA) and fitness functions for docking and virtual screening.

Protein crystallography

This structural information obtained from X-ray crystallography enables design of new molecules or ligands, and eventually drugs that interfere with the binding of natural ligands to the proteins and consequently alter their biological function ⁶¹. A major use of crystal structure is to identify new drug targets because the 3-D crystal structure of a protein reveals the architecture, surface properties, and ligand binding potential of a protein ⁷¹. The identification of potential binding sites and substrate geometries enables a targeted approach to screen potential lead molecules thereby reducing the number of compounds required for optimization of a potential lead compound. Further, additional crystal structures of alternate conformations of proteins identify alternate scaffolds or selectivity properties, which may be critical for ligand binding. Protein complexes with leads molecules (potential drugs) suggest synthetic variation strategies, depending on optimization needs: potency can be increased by optimizing interactions, pharmacokinetic properties may be optimized with targeted variation of moieties not

essential for binding, selectivity may be enhanced (or decreased) by comparing binding modes in related targets, and new binding modes may be discovered by targeted variation of “essential” binding moieties. The diversity of approaches requires a flexible, analytical methodology to drug design ⁶¹.

Protein crystallization is a trial and error process because several factors play an important role in the process: protein purity (>95%pure) and concentration (usually 10 mg/mL); the nature of precipitant (e.g., poly ethylene glycol and various salts) and its concentration; the nature, concentration and pH of buffer; the presence or absence of additives (e.g. metal ions, reducing agents, protease inhibitors, metal chelators, detergents) and effectors (e.g. ligands, cofactors, substrates, inhibitors); the rate of equilibrium between protein and the precipitant; the crystallization temperature; and so on. There are no general rules that correlate all these factors to the generation of crystals.

H. Central Hypothesis

Allosteric proteins (enzymes) are excellent therapeutic targets, as their activity can be manipulated without interfering with the active site. In contrast to the substrate binding sites, allosteric sites of the proteins vary widely among organisms and even among closely related isozymes within the same organism in response to the highly specialized biochemical requirements of different organisms or to the unique metabolic requirements of differentiated tissue types in complex multicellular organisms. Pyruvate kinase is a ubiquitous enzyme and highly conserved across all biological species. Several PK enzymes sequenced to date ranging from bacteria to humans indicate that the residues directly involved in catalysis are almost 100% conserved. In contrast, pyruvate kinase

enzymes are regulated by a wide variety of allosteric effectors including fructose-1,6-bisphosphate, fructose-2,6-bisphosphate, L-alanine, and L-phenylalanine or by covalent modification such as phosphorylation¹²⁻¹⁶. In addition, allosteric sites of PK from different biological species that are regulated by the same effector display a much lower degree of sequence conservation than do their corresponding active sites²². This divergence appears to indicate that regulatory sites might be attractive drug targets for organism-specific and tissue-specific antagonists or deregulators²⁰. So we set out to find allosteric modifies of R-PK at the allosteric FBP binding site. To achieve this goal, the study has been split into four specific aims:

Aim 1: Expression and purification of R-PK.

Aim 2: Computational studies of R-PK.

Aim 3: Identification of R-PK effectors.

Aim 4: Crystallization and structure determination.

This thesis describes identification of R-PK allosteric regulators, based on FBP binding site, through an integrated program that combined computational and kinetic studies along with X-ray crystallography. These effectors can regulate the activity of R-PK and thereby useful to control the levels of important substances in the erythrocytes. Enhancing the R-PK activity by stabilization of the R-state of R-PK increases glycolytic activity, resulting in ATP level increase in RBC. This allows the RBC's to maintain their cellular integrity and prevent their lyses. Also stabilization of the R-state of R-PK lowers the DPG levels in the red cells and prevents sickling of the RBC's associated with destabilization of Hb by DPG. Stabilization of the T-state of R-PK by binding of negative

allosteric effectors will decrease the metabolism of PEP to pyruvate and increases the levels of DPG in the RBC's allowing increase in oxygen delivery to the tissues, which has application in the development of treatment approaches for hypoxia, ischemia and trauma related blood loss. Thus these modifiers are of interest for medical purposes such as development of treatments for hemolytic anemia due to PKD, sickle cell anemia, and enhancing oxygen delivery to hypoxic tissues.

MATERIALS AND METHODS

A. Expression and Purification

The plasmid containing the cDNA coding for the truncated (lacking the first 49 amino acids residues from the N-terminal) form of R-PK (pLC3) was obtained from our Italian collaborators, Mattevi and colleagues, and transformed into competent *E.coli* DH5 α cells as previously reported^{5,57}. The transformed cells were grown in Luria – Bertani (LB) medium containing 100 μ g/ml ampicillin at 37°C. At OD_{600nm} of 0.5, the expression was induced by addition of 1.0 mM isopropyl- β -D thiogalactopyranoside for 12 hours at 30°C. The enzyme was purified by the procedure of Wang et al⁵⁷ using ammonium sulfate precipitations followed by DEAE sepharose and sephacryl S-200 column chromatography. Enzyme activity assay, as well as SDS 10% polyacrylamide gel electrophoresis was performed to confirm the purity and identity of the expressed protein. The protein yield was 5mg/L determined using Bradford assay. Due to poor expression, as well as time consuming and labor intensive purification procedure, an alternate method was developed (see below) for obtaining pure protein, in good yield.

Initially, we tried extracting the cDNA from DH5 α cells and expressing R-PK in the Rosetta cell line to improve the yield. Next, the plasmid containing the cDNA coding for the truncated form of R-PK (pLC3) and a pET 30a Novagen (Madison, Wisconsin)

vector carrying an N-terminal His-tag were digested with restriction enzymes EcoR1 and Nco1, and then run on an agarose gel. The R-PK fragment and the pET 30a vector were then extracted from the gel using a Qiagen (Valencia, California) DNA extraction kit and then ligated with T4 DNA ligase (Novagen). The ligated plasmid was then transformed into Rosetta (DE3) cells (Novagen), which were grown at 37°C in LB medium containing 40ug/ml kanamycin and 34ug/ml chloramphenicol, and after reaching an OD_{600nm} of 1.0, it was induced with 1.0 mM isopropyl- β -D thiogalactopyranoside. Incubation was carried out in a mechanical shaker at 37°C for 18 hours (overnight). Cells were harvested with centrifugation and broken with Avestin EmulsiFlex-C3 homogenizer. The solution was saturated with ammonium sulfate to 20% and the precipitate discarded. Second ammonium sulfate treatment (45% saturation) yielded a precipitate with 98% of the activity of the lysate. The precipitate was then dissolved in 50 mM potassium phosphate, 150 mM sodium chloride, pH 7.5 and dialyzed against the same buffer. The sample was loaded on a 2.5 x 7 cm Ni-NTA column, pre-equilibrated with the above buffer, and then washed with the buffer containing 50 mM imidazole. His-tagged R-PK was then eluted with 0.1 M EDTA in the buffer, and concentrated using amicon centricon cutoff 50K. The purity of the protein was determined to be greater than 95% using SDS gel electrophoresis.

B. Enzyme Activity Assay

The activity of R-PK was determined at 37°C by lactate dehydrogenase (LDH) coupled spectrophotometric assay as previously described^{5,57,72}. Slight modifications were

made in to accommodate screening of multiple samples simultaneously. This assay measures the change in absorbance at 340 nm due to oxidation of NADH by LDH. The standard assay conditions include the buffer (100 mM Tris pH, 8.0; 100 mM KCl; 10 mM MgCl_2 ; 0.5 mM EDTA), 1.5 mM ADP, 0.2 mM NADH, 10 $\mu\text{g/ml}$ LDH, and 0.5-1.0 $\mu\text{g/ml}$ R-PK in a final volume of 200 μl . The reaction was started by addition of varying concentration of PEP (0.25 mM- 3.0 mM) to the above mixture, and measuring the reaction velocity from the slope of the tangent to the reaction progress curve. The steady state kinetics of R-PK was determined by plotting the rate of reaction against PEP concentration. All the measurements were made in repeats of four (SpectraMax 190 spectrophotometer) and the results are shown as a function of PEP. All initial velocity data were fit to the Hill equations for the determination of apparent affinity for substrate ($S_{0.5}$), Hill coefficients (n_H), and maximal velocity (V_{max}) using the nonlinear regression program Sigma Plot with the Enzyme Kinetics module (SPSS Scientific).

C. Computational Studies

To discover R-PK allosteric regulators, computational methods were used to identify small molecules that could bind to the allosteric FBP site of R-PK. The crystallographic structure of R-PK in complex with FBP was obtained from the Protein Data Bank (PDB code 1LIU), and to simplify the computation, only subunit A of the tetrameric structure was used. Hydrogen atoms do not form part of the structure PDB files and are added to the protein using tools within the SYBYL biopolymer and Build/Edit menus. Further, the added hydrogen atoms are energy minimized to reduce

steric clashes with the protein residues to a gradient of 0.005 using Gasteiger Hückel charges, a dielectric constant (DEC) of 1.0, and a gradient of 0.005Kcal/molÅ[°] with 10,000 iterations. This ensures the ideal position and conformation for the added hydrogen atoms. Whereas, the coordinates of all non-hydrogen atoms were not altered during the minimization and were kept fixed⁶⁷. The individual software programs used for the studies are all incorporated in the molecular modeling and graphic program SYBYL 7.0 (Tripos Inc) software suite.

UNITY Search

First, the UNITY program was used to screen the National Cancer Institute (NCI) small molecule compound library for compounds with similar chemical features as FBP. UNITY is a pharmacophore-based chemical structure database-screening program which searches hit compounds against specific queries^{64,73}. Key molecular features that are responsible for favorable interaction between FBP and its binding site, including hydrophobic and hydrophilic interactions were identified and incorporated into a UNITY query^{61,64}. These include, hydrogen bond acceptors where the two phosphate groups are attached, and hydrophobic center at the central hydrophobic ring. Figure 8 illustrates the UNITY query employed in the database search. Using the defined query, various 3D flexible database searches were performed on the public release of the NCI compound library containing ~250,000 compounds, culminating in 4419 hits. The number of hits was reduced to 2115 by incorporating into the query, specific functional moieties, such as carboxylate, phosphate, hydroxide and sulfate groups. Excluding those that show incompatible size and/or shape with the FBP binding site reduced the number of

Figure 8. Unity Query. A UNITY generated hit (ball and stick) is shown superimposed over FBP molecule in the allosteric binding pocket. Features forming pharmacophore model include acceptor atom, distance constraint and hydrophobic center. Grey spheres: Spatial point constraint for acceptor atoms. Purple spheres: excluded volume constraint, and Yellow: Portions of the hit that match the query.

compounds further. This was done by imposing volume constraint that takes into account the size and shape of the FBP binding cavity. The volume constraint was added to the query by defining coordinates of protein atoms that line the binding site. These include Arg532 (guanidinium carbon), Ser480 (OH), Trp625 (ring nitrogen), Thr476 (carbonyl oxygen), and Arg559 (carbonyl oxygen). Subsequent Unity search reduced the number of hits to 327 compounds. A manual inspection of the compounds led to exclusion of more compounds, including those that were still too big to fit the FBP binding pocket, duplicate hits and linear chain molecules. Finally a list of 29 distinct hits was obtained.

Docking Studies

The 29 hit molecules were docked into the FBP binding site using three different procedures, followed by analyzing their interaction with the protein ⁷⁴. First, the automatic docking program, FlexX ⁷⁵ as incorporated in SYBYL software was used to dock and maximize the interactions between the 29 hit molecules and the FBP binding site of the protein ⁶². FlexX is a flexible docking program that uses an incremental construction algorithm to dock different conformations of a ligand into a receptor active site, when the active site radius and the sub pocket core radius are specified for the receptor. These ligand conformations are then ranked on the basis of estimation of the free binding energy, ΔG of the protein-ligand complex. An active site and sub-pocket core cutoff radii of 10.5Å and 2.5Å, respectively were used to define the active site during the computation. A FlexX run using default parameters and formal charges was conducted for 100 solutions for each ligand. FBP molecule from the crystal structure was used as a control for analysis.

Figure 9. Chemical structures of the 29 hits from NCI database identified in the UNITY search.

A second automatic docking program, GOLD (version 3.0) was also employed to dock the ligands at the FBP site. GOLD employs a genetic optimization algorithm to predict different binding conformations and orientations of ligand. The program then scores the different poses and generates a ranking list ⁷⁶. The active site for the docking experiment was specified using the coordinate of the FBP ring oxygen atom. A GOLD docking run was specified for 100 solutions of each ligand using default parameters of the program.

For the third docking procedure, the position and orientation of the ligands from the UNITY search were maintained, and then energy minimized with a subset of the binding site residues. The employment of a ligand-based search ensures that the UNITY generated hits possess arrangement of chemical groups such as charges, hydrophobic groups, and hydrogen bonding acceptor or donor atoms as stated by the query. A hot radius of protein-ligand subset of 6 Å, and surrounding radius of 12 Å were used for the computation. Other conditions specified for the minimization process include G. Hückel charges, dielectric constant of 1.0, a gradient of 0.005 and 10,000 iterations, whereas the coordinates of all other atoms outside the subset were kept fixed.

HINT Analysis

Following the docking of the hit compounds at the FBP binding site, the program HINT (eduSoft, LC) was used to analyze the interactions between the compounds and the protein. HINT uses a scoring function that employs empirically derived parameter $\log P_{o/w}$ (partition coefficient for 1-octanol/water) - utilizing fragment rules of Hansch and Leo ^{77,78}. The program calculates a score for each interaction between various atoms of

the protein and ligand, and the cumulative score is termed as HINT score. A positive HINT score value indicates hydrophatically favorable contacts (hydrogen bonds, acid/base, and hydrophobic/hydrophobic), while negative value indicates unfavorable contacts (acid /acid, base/base, and hydrophobic/polar). The factor $\log P_{o/w}$ of a ligand or biological molecule is the sum of all the hydrophobic atomic constants for that molecule, and directly correlated with free energy. HINT score therefore reveals information not only about enthalpy but also entropy of the bimolecular complex^{67,79,80}.

First, the protein complex is separated into different areas corresponding to its different components, namely protein, ligand and water molecules. Second, the partition method dictionary is used to calculate $\log P_{o/w}$ value for the protein, where HINT uses a lookup table of parameters based on residue type and solvent condition. The inferred option for the solvent condition automatically selects the state of each residue in the protein based on its atoms and $\log P$ calculated using the SASA +30 NH. The $\log P_{o/w}$ for the ligand is then determined using the calculate method, based on CLOG-P method of Leo. Polar residues line the FBP binding site of R-PK and HINT scores were calculated using the “essential” approach that treats only polar hydrogen atoms explicitly to account for the hydrogen atoms. The total HINT score takes into account all possible interactions between R-PK protein and the ligand. . Finally, rotation is performed using the HINT Score system menu that enables additional structure cleanup and optimizes the orientations of side chain hydrogen in His, Lys, Arg and other groups in the ligand. Improved HINT scores are obtained after rotation because groups such as OH, NH₂ on protein are positioned for maximum interaction with respect to ligand and vice versa⁸⁰.

D. Kinetic Screening of Compounds:

Seven compounds (Compounds 1-7; shown in Figure 9) identified through the computational studies were obtained from the NCI. These compounds, in addition to FBP, FDP, ATP and RBP were tested to determine their effect on R-PK activity. All compounds were soluble in aqueous buffer with the exception of compound 7, which was prepared as stock solutions in DMSO. Consequently, we employed two controls in the experiment, buffer as well as DMSO to ensure that these solvents do not affect the enzyme's activity. The compounds were screened at 37°C using conditions identical to the enzyme activity assay except for the presence of 1 mM effector in the assay mixture. Similar to the procedure outlined above, the reaction velocity was measured by addition of 10 mM PEP to the reaction mixture, and indirectly measuring the rate of conversion of PEP to pyruvate by following the disappearance of NADH. All measurements were made in triplicate (SpectraMax 190 spectrophotometer) and the results are shown as percent change in activity (activation or inhibition) of the enzyme in relation to the control reaction that has no effector compound. The percent change in activity was calculated by the following equation:

$$\% \text{ Activity change} = (V_{\text{effector}} \times 100 / V_{\text{control}}) - 100$$

V_{control} and V_{effector} are the reaction velocities in the absence and presence of effector respectively. The activity in the absence of effector is considered to be hundred percent. We used the following two criteria to judge the activity of compound in this experiment;

Figure 10. Chemical structures of compounds tested to determine their effect on R-PK activity.

if R-PK activity decreases in presence of compound, it was interpreted as inhibitor of protein; whereas if R-PK activity increases in presence of compound, it was interpreted as the compound acts as activator of the enzyme.

Kinetic Analyses of Selected Inhibitor and Activator:

Two of the seven-effector compounds 1 and 7 were further characterized kinetically. The enzymatic activity of R-PK was determined at 37°C by varying the concentrations of PEP (0.25 mM – 3 mM) at fixed effector concentration of 1.0 mM. In all cases the reaction was started with addition of PEP. All measurements were performed in repeats of four. The plot of Lineweaver-Burk^{plot} was used to determine apparent $V_{\max}$ and apparent K_m values and the Hill plot was used to determine the apparent $S_{0.5}$ and n_H values.

E. Crystallization and X-ray studies

R-PK was crystallized in the absence and presence of small molecule effectors to identify their binding site using x-ray analysis. Hanging drop vapor diffusion method (shown in Figure 10) was used to grow R-PK crystals. A droplet of purified protein, buffer and precipitant is allowed to equilibrate with a larger reservoir containing similar buffer and precipitant in higher concentration. Protein crystals form under favorable saturation conditions.

Crystal Condition Screening

Initial crystallization conditions for the His-tagged R-PK complex were determined from the Crystal screening kit I and II (Hampton Research) at 20°C. Several

Figure 11. Protein crystallization: Hanging drop vapor diffusion method. Reservoir solution contains buffer and precipitant. Protein solution contains the same compounds, but in lower concentrations. The protein solution may also contain trace metals or ions necessary for precipitation of particular proteins along with effector solution. Source: McRee {McRee, 1993 #116}.

crystallization conditions that resulted in R-PK micro-crystals were then optimized further by fine gridding around the initial conditions until well-diffracting crystals were obtained. Single crystals of R-PK suitable for X-ray analysis were obtained after optimization of two crystal screen conditions: screen II condition 48 (2% v/v Dioxane, 0.1 M Bicine pH 9.0 and 10% w/v Polyethylene glycol 20,000) and screen II condition 30 (0.1 M HEPES pH 7.5, 10% w/v Polyethylene glycol 6000, 5% v/v MPD). Numerous crystals of R-PK were screened by X-ray diffraction.

Crystallization with FBP

R-PK with the His-tag was crystallized in the presence of FBP under similar conditions as reported in literature for comparison with the previously published R-PK structure without His-tag⁵⁷. This structure also serves as a control for further analysis of other ligand-protein complex structures. The previously developed crystallization protocol⁵ was slightly modified, to yield co-crystals of R-PK with fructose -1,6-biphosphate (FBP) and phosphoglycolic acid (PG). Purified human R-PK with the attached 6x-His tag was used. The reservoir solution consisted of 3-17% PEG- 8K, 8 mM MnSO₄ and 50 mM MES/KOH at pH 6.4. The protein-ligand mixture complex was prepared by mixing 24 μ l of 10 mg mL⁻¹ protein solution (in buffer 20 mM KPi, pH 7.0, 50 mM KCl) with 1.2 μ l of FBP (100mM in ddH₂O) and 1.2 μ l of PG (100 mM in ddH₂O). The mixture was incubated for 1 hr at 4 °C prior to crystal setting. 4 μ l hanging drops were produced by mixing equal volumes of 2 μ l of protein containing FBP and PG

with 2 μ l of well solution and were equilibrated against 1mL reservoir solution. Micro-crystals were obtained for 6% PEG-8K concentration at 20°C after four days.

Crystallization with Compounds

Crystallization of R-PK with various active compounds was carried out using excess of the effector; molar ratio of R-PK: effector = 1: 5 to encourage complete binding. The active compounds were co-crystallized with the protein in absence of FBP using crystallization conditions similar to that of native crystals with FBP and PG. The following test compounds were used: 2, 6-FBP, compound 1, compound 2, and compound 7. The reservoir solution consisted of 5-15% PEG-8K, 6 mM MnSO_4 and 50 mM MES/KOH at pH 6.4. The crystallization drop was set up using R-PK His tagged protein solution which contains 6mM of compound and 6 mM of PG. Next, 2.72- 4.76 mM ADP was also added directly to the drop. The drops produced by mixing protein with the reservoir solution were equilibrated against the reservoir solution at 20 °C.

X-ray diffraction

X-ray diffraction studies were carried out on crystals, including co-crystals from compounds 1, 2, and 7 in addition to effectors FBP, and FDP. For data collection, the co-crystals were soaked for a short time in cryo-solution containing mother liquor and 25% PEG 400 (three steps of 35 seconds each) and then quickly transferred to a liquid N_2 stream maintained at 100° K. The crystals diffracted to resolution limits of 2.2-2.7Å°. Oscillation frames were measured using a rotating-anode X-ray generator (Rigaku

MicroMaxTM-007) operated at 20 mA and 40 kV and equipped with an OSMIC confocal mirror. An R-Axis IV++ imaging-plate system was used as a detector. A total of 180 one-degree rotation X-ray images were collected with 2-minute exposures at a crystal to detector distance of 200 mm.

F. Materials

All reagents were purchased from Sigma Chemicals Co. (St. Louis, Mo) unless otherwise noted.

RESULTS

Expression and Purification of R-PK

R-PK has been isolated previously from mature erythrocytes or by recombinant methods^{5,57}. In this study presented we used recombinant protein for the expression of R-PK. To make the recombinant protein, *E. coli* DH5 α cells transformed with pLC3 were used. The proteins were purified using ammonium sulfate precipitations, followed by DEAE sepharose and sephacryl S-200 column chromatography as previously described⁵⁷. Employing a 45% saturation of ammonium sulfate at which only R-PK is precipitated ensured the elimination of intrinsic *E. coli* PK from the protein mixture. The results of R-PK expression and purification are shown in Table 1. The yield of the protein using this cell line was 5mg/L of culture. The enzyme expressed was confirmed to be R-PK by sequencing the plasmid at the DNA core facility at VCU. The purified enzyme also showed activity in the subsequent R-PK assay. SDS PAGE gel of the purified enzyme (Figure 12) showed a band around 60 kDa corresponding to the molecular weight of R-PK monomer. Further purification of the major fraction that consists of the active protein (60 kDa) and inactive protein (57 kDa) by using hydroxyapatite and sephacryl S-200 columns was not successful. Thus, the quantities of pure R-PK obtained from the minor fraction at 60 kDa were limited.

To optimize the yield of R-PK, we used Rosetta cells to express the protein instead of DH5 α cells. The yield of the protein increased but the purification procedure was not convenient as a third hydroxyapatite column was added to the purification protocol. Next, His-tag was attached to R-PK's N-terminal to allow purification by a single step Ni-NTA column chromatography. Using this approach, the yield of pure R-PK improved significantly up to 6-folds, typically 30 mg/L. Figure 13 shows the SDS-PAGE gel of the purified enzyme. It shows a single band of the purified protein in contrast to the double band observed for the previous method employing DH5 α cells. Thus expression of the protein in Rosetta cells with the attached His-tag represents a new simple method providing improved yield for the expression and purification of R-PK.

Identification of R-PK Allosteric Regulators

FBP binding pocket is an allosteric site in human R-PK that regulates the conversion of the enzyme between the inactive T-state and the active R-state. Effectors that can interact with this site could act as potential regulators and thus control the enzyme activity. The program UNITY^{64,73}, as present in the molecular modeling software SYBYL 7.0 was used to perform 3D searches against the NCI database (~250,000 compounds) to identify potential R-PK regulators. Figure 8 illustrates an example of a UNITY 3D FlexX query generated from natural allosteric effector FBP in the crystallographic structure. It contains feature sets of the ligand that are considered essential for binding. The features of FBP such as acceptor atoms that denote sites for bond formation, hydrophobic center, distance constraints that constrain the features in a determined position and excluded volume constraints that help define the shape of the

active site and force the hits to occupy a certain volume were used. The initial UNITY search identified 4419 hits that matched the features defined by the ligand-based query. The large numbers of hits obtained from this general search were reduced further in a stepwise manner by increasing the constraints for the ligands. This process is summarized in Figure 14. The hits from initial search were sequentially narrowed from 4419 to 2115 by refining the query to include other structural features, such as carboxylate, phosphate, and hydroxide and sulfate functional groups. These groups can interact with the polar binding site in R-PK. Following; a new query with excluded volume constraint reduced the hits to 327 compounds. This query selected the molecules on the basis of their geometry in relation to the FBP binding site in R-PK. The results of each search were viewed using the “Hitlist Manager” in the SYBYL software package. Compounds too big to fit the FBP binding pocket, duplicate hits and linear chain like molecules were eliminated by visual inspection of the molecules. Twenty-nine compounds were selected finally as representative of the search results.

The 29 hit molecules were docked then into the FBP binding site of R-PK using automated docking programs of FlexX⁷⁵ and GOLD⁷⁶. In addition, the position and orientation of the ligand as obtained from the UNITY search were maintained, and then energy minimized with a subset of the binding site residues. This docking procedure was purely based on the screening technique that gave us molecules superimposed on FBP molecule in the crystal structure. The interaction score of protein ligand complexes using both the automated docking programs suggested that the compounds could bind and interact positively with the active site of protein. The energy scoring function of HINT

^{78,79} was then applied to analyze the interactions between the docked molecules and R-PK. The best ligand pose of each compound suggested by docking was employed to study HINT interaction with R-PK. The results of the comparative HINT scores are shown in Figure 15. The HINT scores were mostly consistent across the different docking methods thus validating the three different docking processes. The natural allosteric activator FBP gave a good HINT score with R-PK, indicating very favorable interaction with the protein. The positive HINT scores for the other tested compounds also strongly suggest that the ligands interact favorably with R-PK. The only exceptions were compounds 1 and compound 18 which were found to have a negative HINT score indicating unfavorable interaction with R-PK. A list of twenty best compounds was prepared that scored high in the HINT calculation and requested from NCI for further experimental analysis. Out of the requested compounds, seven were obtained from NCI (Figure 10).

Enzymatic Activity

The activity of R-PK was determined using steady state kinetics as a function of the substrate PEP (Figure 16). Parameters derived from this kinetic study are summarized in Table 2. The activity of R-PK increased with increasing concentrations of PEP. The data fitted a sigmoidal function indicative of allosterism. Thus, as shown in Figure 16, R-PK demonstrated a typical homotropic response to PEP with a Hill coefficient of 1.69 ± 0.11 . This value is greater than unity and indicates positive cooperativity for the binding of PEP. As indicated in Table 2, the kinetic profile of the truncated His-tagged R-PK was

similar to those reported for R-PK in the literature. Thus, attachment of His-tag and modification of the purification procedure does not perturb the substrate-binding site.

R-PK kinetics in the presence of compounds

Seven compounds, 1-7 (Figure 10) identified in the computational studies were obtained from NCI and examined as possible new leads for R-PK regulation. The percent change in R-PK activity in the presence of these compounds was determined and compared against activity of the native protein. For comparison, percent change in activity of R-PK in presence of FBP and ATP were also carried out. FBP is a natural allosteric activator of R-PK and showed a maximum stimulation of R-PK activity, whereas ATP, a natural inhibitor, showed a decrease in the activity of R-PK. All the examined compounds at 1.0 mM concentration altered R-PK activity, ranging from inhibition to activation (Figure 17). Of the seven molecules tested, five compounds were found to be moderate activators of R-PK activity with compounds 2 and 3 being the most potent activators ($32.39 \pm 5.17\%$ and $32.02 \pm 1.79\%$ activation, respectively at 1 mM). FDP, a natural allosteric activator of *Leishmania mexicana* and *Chlamydia trachomatis* PKs^{24,81,82} caused only a minor activation of R-PK. Also, RBP caused no significant change in the activity of R-PK despite being suggested to be a potent effector in the computational studies. Compounds 6 and 7 decreased R-PK activity (11% and 22% inhibition, respectively at 1 mM) compared to the natural inhibitors ATP (8% at 1 mM) and therefore were identified as potential inhibitors. Based on the quantities available to us, compounds 1 and 7 were analyzed further as representatives of an allosteric activator and inhibitor.

R-PK kinetics in the presence of an activator, compound 1

Kinetic analysis of R-PK activation was carried out in the presence of saturating concentration of compound 1 (1 mM). The results are shown in Figures 18 and 19. Under the assay conditions, the $S_{0.5}$ for PEP decreased from 1.05 ± 0.05 mM to 0.6 ± 0.06 mM and the sigmoidal curve of R-PK shifted towards the left, suggesting that compound 1 was an allosteric activator of R-PK. The efficiency of R-PK ($k_{cat}/S_{0.5}$) in the presence of compound 1 in converting PEP to product was also tested. This ratio was found to be $615 \text{ s}^{-1}/\text{mM}$; almost a third compared to that of the natural allosteric activator, FBP ($1972 \text{ s}^{-1}/\text{mM}$). Hill coefficient of R-PK alone was 1.69 ± 0.11 . This R-PK Hill coefficient in presence of compound 1 was decreased to 1.49 ± 0.15 indicating a slight decrease in cooperativity of R-PK.

R-PK kinetics in the presence of an inhibitor, compound 7

The steady state kinetics of R-PK was also studied in the presence of compound 7. The results are shown in Figures 20 and 21. A rightward shift in the sigmoidal R-PK steady state curve was observed in the presence of compound 7 (1 mM), indicating that it acts as an allosteric inhibitor of the protein. Compound 7 also significantly decreased the efficiency of the enzyme ($232 \text{ s}^{-1}/\text{mM}$) in concerting PEP to its products. The Hill coefficient of R-PK in the presence of this compound 7 was 1.70 ± 0.11 which was not significantly different from R-PK alone (1.69 ± 0.11).

Computational versus Kinetic

There is a good structure-function relationship between the results from the kinetic studies and those from the HINT computational analysis. This is shown in Figure 22. However, there are significant exceptions. For example, compound RBP although showed a very good HINT score indicating strong interaction with the protein; it does not significantly affect the activity of R-PK. Most likely, the compound does not perturb the binding site protein residues that directly affect allosteric properties even though the compound may strongly bind to the allosteric site. Similar observation are noted with binding of effectors in the central water cavity of Hb, and exhibit varying degree of allosteric potencies^{83,84}. A note of caution is that, the computational analysis is based on a model and does not always represent the actual interactions between the protein and ligand. Therefore, we cannot say with certainty the specific interactions between the ligands and the protein, as well as which interactions are important for allostereism until crystallographic analyses of protein-compound complexes are obtained. Interestingly, the compounds that acted as activators have similar structural scaffold as FBP, while the two inhibitors also have similar structural scaffold but differs from that of the activators. The two different scaffolds are potential lead pharmacophores for the development of inhibitors and activators of R-PK. We also note that FDP, an allosteric activator of *Leishmania mexicana* PK^{24,81,82} has lower HINT score, as well as less potent than FBP, consistent with the fact that the allosteric sites of R-PK from various sources may be ligand-specific.

Crystallization studies

R-PK was crystallized in the presence of small molecule effectors to identify their binding site. We reproduced crystals of R-PK in presence of FBP. These crystals were isomorphous to the one reported in the literature thus verifying that the attached His-tag does not affect the crystallization of the protein. These crystals could further serve as a reference for other ligand-protein complexes. We also obtained R-PK crystals in the presence of compounds 1, 2, and 7 and effector fructose 2,6-bisphosphate. These crystals are shown in Figure 23. The conditions used to obtain crystals are shown in Table 3. X-ray data for these crystals were collected at 2.2- 2.7 Å resolution. Some of these crystals were tetragonal with P422 space group and cell dimensions $a=78.8$, $b=78.8$, $c=204.1$, 90.0, 90.0, 90.0. Others crystals were monoclinic with space group $P2_1$ and cell dimensions $a=74.4$, $b=172.1$, $c=85.5$, 90.0, 92.5, 90.0 for the R-PK crystal in presence of FBP. Table 4 summarizes the crystallographic parameters of these crystals. Further modifications of the crystallization conditions are being carried out for other crystal forms as the crystals obtained are twinned and proving difficult to work with. We have also obtained crystals of R-PK in absence of effectors under new crystallization conditions (crystal screen 2 No. 30 and 48). X-ray analysis of these crystals is still in progress. Since no effector or substrate aided the crystallization of protein we are hopeful that new alternate crystal form would be revealed.

Table 1. Results of R-PK expression and purification.

Method	Yield (mg/L)
DH-5 α cells	5
Rosetta cells	10
Rosetta cells + His-tag	30

Figure 12. 10% SDS-PAGE gel of recombinant human R-PK protein purified using the procedure recommended by Wang et al. Three fractions of protein were run in parallel with molecular mass standards. Lanes (1) Protein standard (Bio-Rad Precision Plus), (2) minor protein fraction one, (3) major protein fraction two showing twin band and (4) minor protein fraction.

Figure 13. 10% SDS-PAGE analysis of recombinant human R-PK protein purification. Lane 1, Protein standard (Bio-Rad Precision Plus). Lanes 2, 3 and 4 show varying concentrations of R-PK with attached His tag.

NCI: ~250,000 compounds

Kinetic analysis

Figure 14. Unity screening of the NCI database and sequential hit reduction.

Figure 15. Comparative HINT scores of compounds using different docking methods (●) Energy minimized UNITY search position and orientation; (○) FlexX docked ligand; and (▼) GOLD docked ligand. For clarity, only selected compounds (19 out of the 29) are shown in this figure.

Figure 16. Kinetic activity of R-PK as a function of the natural substrate, PEP.

Table: 2 Kinetic studies of R-PK in the presence and absence of allosteric regulators^a

Protein	K_{cat}^c (s ⁻¹)	$S_{0.5}^d$ (mM)	$K_{cat}/S_{0.5}^e$ (s ⁻¹ /mM)	n_H^f
R-PK ^b	355 ± 12	1.10 ± 0.04	323	1.60 ± 0.16
R-PK (His-tag)	347 ± 22	1.05 ± 0.05	332	1.69 ± 0.11
R-PK+FBP ^b	355 ± 11	0.18 ± 0.02	1972	1.05 ± 0.07
R-PK + compound 1	369 ± 16	0.6 ± 0.06	615	1.49 ± 0.15
R-PK + compound 7	325 ± 18	1.4 ± 0.04	232	1.70 ± 0.11

^aAll the measurements were made in repeats of four; ^bValentini et al. ⁵, ^c K_{cat} is the number of catalytic events per second per active site; ^d $S_{0.5}$ is the apparent affinity, a parameter used in sigmoidal kinetics and is defined as K_m or the substrate concentration at which the reaction velocity is half the maximal; ^e $K_{cat}/S_{0.5}$ is a measure of how efficiently an enzyme converts substrate to product at low substrate concentration; ^f n_H (Hill coefficient) is an empirical parameter related to the degree of cooperativity for binding of substrate. Values of Hill coefficient above unity indicate positive cooperativity among ligand binding.

Figure 17. Kinetic activity of R-PK in the presence of 1 mM allosteric regulators.

Figure 18. Steady state kinetic activity of R-PK in the absence and presence of compound 1. (●) R-PK; (○) R-PK in presence of 1mM compound 1. Data were fit to the Michaelis –Menten or Hill equations, and the resulting kinetic parameters are summarized in Table 1.

Figure 19. R-PK kinetics in presence and absence of compound 1. ($\bullet$) R-PK; ($\circ$) R-PK in presence of 1mM compound 1. Data was fit to the Hill equations, and the resulting kinetic parameters are summarized in Table 1.

Figure 20. Steady state kinetic activity of R-PK in the absence and presence of compound 7. (●) R-PK; (○) R-PK in presence of 1mM compound 7. Data were fit to the Hill equations, and the resulting kinetic parameters are summarized in Table 1.

Figure 21. R-PK kinetics in presence and absence of compound 7. (●) R-PK; (○) R-PK in presence of 1mM compound 7. Data was fit to the Hill equation, and the resulting kinetic parameters are summarized in Table 1.

Figure 22. Comparative results from the kinetic studies and HINT computational analysis. HINT scores are shown as line graph (●) Energy minimized UNITY search position and orientation; (○) FlexX docked ligand; and (▼) GOLD docked ligand. The bar graph shows the percent change in activity of R-PK in the presence of 1 mM compound

Table 3. R-PK crystallization studies.

Compound	Co-crystal Conditions
FBP	6 mM 1,6-FBP and 6 mM PG
FDP	9.5 mM 2,6-FBP, 9.5 mM PG, 7 mM ADP
Compound 1	8 mM compound 1, 9.5 mM PG, 7 mM ADP
Compound 2	7 mM compound 2, 9.5 mM PG, 7 mM ADP
Compound 7	8 mM compound 7, 9.5 mM PG, 7 mM ADP

R-PK co-crystals are obtained in presence of several effectors. The table shows the crystallization conditions for specific effectors. Addition of ADP produces new crystal form not yet reported.

Figure 23. R-PK co-crystals in presence of effectors. Photos of R-PK co-crystals grown by hanging drop method. Crystals in presence of FBP are monoclinic whereas other crystals are tetragonal.

Table 4. Crystallographic parameters of R-PK co- crystals.

Effectors	Resolution (Å°)	Space group/ cell parameters	Remarks
1,6 FBP	2.7	P2 ₁ / 74.4, 172.1, 85.5, 90.0, 92.5, 90.0	Isomorphous to known R-PK crystal
Others effectors	2.7-3.0	P422 78.8, 78.8, 204.1, 90.0, 90.0, 90.0	Crystals twinned

X-ray diffraction was carried out on some of the crystals, including co-crystals from compounds 1, 2, and 7 in addition to effectors FBP and FDP. The crystals diffracted to resolution limits of 2.2-2.7Å°.

DISCUSSION

Several classes of Hb allosteric effectors have been reported that shift the Hb oxygen equilibrium curve to the left (low-affinity or T state) or to the right (high-affinity or R state)^{83,84}. Interestingly, the compounds in each of these classes have been found to bind to the same novel allosteric sites of Hb, but produce very different magnitudes of OEC shift – i.e. these compounds express a wide range of activity^{83,84}. Based on these results, a general hypothesis of allosteric effectors is that the effectors ability to shift the allosteric equilibrium (i.e. the effectors potency) is due not only to where the molecule binds, but also depends on how it interacts with the protein at the binding site^{83,84}. This general concept can be applied to R-PK, as we observe not only varying allosteric potency among the tested compounds but also opposite effects. The allosteric transition mechanism of R-PK is finely regulated to meet the metabolic requirements of the red blood cell (RBC). It regulates the levels of important intermediates such as ATP and DPG in the red cells^{46,47}. The Embden-Meyerhof (EM) pathway produces ATP that is used for maintenance of deformability and biconcavity through ATP driven pumps whereas the Rapoport-Luebering shunt of the EM pathway provide the RBC with DPG when stimulated by hypoxia⁴⁶. Inhibition of FBP binding in R-PK prevents conversion of the enzyme from T-state to R-state resulting in increased levels of DPG - the primary in vivo allosteric effector of Hb in humans that enhances the delivery of oxygen to the

cardiovascular periphery by stabilizing the deoxy state. Thus, an important aspect of R-PK inhibition involves identifying compounds that will effectively inhibit this enzyme to increase red cell DPG levels at least 2 to 3 fold. Such compounds are potentially useful for the development of new treatment of a variety of ischemic diseased states, including Alzheimer's disease. This route of DPG modification for therapeutic application should be practical, as heterozygous individuals displaying 40-60 % normal PK activity display increased DPG levels, without adverse side effects ⁵³.

R-PK deficiency is primarily inherited as an autosomal disorder, and is the most common enzymopathy of glycolysis ^{53,85-88}. Clinical symptoms associated with R-PK deficiency are most often confined to homozygotes or compound heterozygotes ⁵², and in severe cases result in ATP depletion and RBCs unable to maintain their cellular integrity and consequently undergo lyses causing non-spherocytic hemolytic anemia. There is no specific therapy for this disease, which varies from mild to life-threatening neonatal anemia and jaundice, necessitating blood transfusions and splenectomy. Binding of FBP to R-PK activates and shifts the equilibrium of the protein toward the R-state with a concomitant increase in ATP production. It is also recognized that activation of R-PK, which leads to increase levels of ATP in the RBCs prevents their lyses ⁸⁹. Therefore, compounds that are more potent than FBP could potentially be useful to treat R-PK deficiency related diseases.

Sickle cell anemia arises from a single point mutation of β Glu6 in normal Hb to β Val6 in sickle Hb. Deoxygenation of the sickle Hb *in vivo* results in the polymerization of the protein that deforms the red cell membrane, with a number of the cells resembling a

sickle. The rigid sickled RBCs eventually block capillaries resulting in both local tissue/organ damage and severe pain. Interestingly, the mutant oxygenated Hb does not polymerize like the deoxygenated protein, therefore activation of R-PK with its concomitant decrease in DPG levels and increase in the oxygen carrying capacity of blood could potentially be useful for the treatment of sickle cell anemia. Thus R-PK activators designed for treatment of PK deficiency hemolytic anemia can also be used to treat sickle cell anemia. This study combined computational and kinetic studies along with X-ray crystallography to identify novel R-PK allosteric regulators that could potentially regulate R-PK activity in erythrocytes by binding to the FBP site.

Expression and purification of R-PK

R-PK is a tetrameric protein consisting of four subunits of 62 kDa each encoded by the *RP-LP* gene. However, R-PK isolated from mature erythrocytes^{54,90} or obtained as a recombinant form consists of two subunits of 62 kDa and another two subunits of 58 kDa; the latter due to proteolytic cleavage of the N-terminus⁵⁷. We developed a simple and efficient method for the expression and purification of R-PK. Human R-PK was expressed in DH5 α and Rosetta cells transformed with the R-PK cDNA and purified by the methods described in the experimental procedures. Attempts to obtain pure R-PK in substantial amounts using the Wang et al.⁵⁷ method proved difficult. There are two reasons for the sub-optimal yield. First, human R-PK is a eukaryotic protein and does not express optimally in DH5 α cells. Second, the protocol followed for the purification of R-PK was less efficient, resulting in degradation of the protein as evidenced by double

bands around 60kDa and 57kDa on the SDS gel, indicating proteolytic cleavage. DEAE sepharose column separates the protein into three fractions, which are seen on the SDS gel as a band around 60 kDa (minor fraction), a twin band at 60kDa and 57 kDa (major fraction), and a third band at 57 kDa (minor fraction). The minor fraction at 57kDa shows no activity, while that at the 60 kDa shows R-PK activity. Hence, alternate methods to increase the protein yield were attempted.

In an attempt to improve the purification of R-PK from the protein mixture a His-tag was attached to R-PK cDNA followed by a one-step Ni-NTA column for purification. Rosetta (DE3) cells were employed to express the ligated plasmid to improve R-PK expression. Rosetta strains are specially designed cells that increase the expression of eukaryotic proteins in *E. coli* in contrast to DH5 α cells. R-PK cDNA has thirty codons requiring the rare t-RNA's present in the Rosetta cell line. Expressing R-PK using this modified procedure greatly enhanced its yield up to 6-times compared to the traditional method. Thus, the expression of the protein in Rosetta cells with the attached His-tag represents a new streamlined method for purification of recombinant R-PK.

Computational studies

NCI structure database is a free database of small molecule structures that can be screened using computer-modeling software such as UNITY. Small molecules identified as effectors by searching the NCI Chemical database can be requested from the Developmental Therapeutics Program of the National Cancer Institute. 3D search of the NCI database has the obvious advantage of identifying existing molecules that are either easily available or that could be synthesized with a known protocol. Besides, the NCI

structure library being “virtual” allows for a computational screen using 3D or flexible search greatly improving the chances of obtaining “hits” that can match defined search criteria. This is a highly efficient method that eliminates the need for synthesizing huge compound libraries for initial screening. The search procedure takes place in two steps: first, the software identifies compounds that have the atom types and functional groups required by the pharmacophore (as defined by a query), and then it checks whether the spatial arrangement of these elements matches the query⁹¹. We employed UNITY program available in SYBYL software to virtually screen the NCI database. A gradual stepwise approach using structural determinants allowed us to come up with a list of 29 compounds that were most likely to interact with FBP binding site of R-PK and there by act as allosteric regulators.

The 29 UNITY generated molecules were next docked in the FBP binding pocket of R-PK using different docking programs to analyze their interaction with the protein. Docking is a computational technique that generates various conformations of small molecules, dock them in protein binding sites and predict the structure of the resulting complex. The ligand conformation that best compliments the protein binding site is then assessed by a scoring function Thus, docking process involves prediction of the ligand conformation and orientation within the protein active site and is used to determine accurate structural modeling and predict the activity of the ligand. The three different docking programs employed in our analysis of the ligands; FlexX, GOLD, and SYBYL suggested that the identified compounds could bind and interact positively with the active site of protein.

HINT analysis was then carried out on the best-docked conformations of the compounds from different docking programs. The results of the comparative HINT scores for the three docking procedures were mostly consistent with very few exceptions thus validating the three docking processes. The high HINT score values for compounds suggest energetically favorable interaction of the molecules with R-PK. The few anomalies can easily be explained by the limitations of the programs used. First, compound 18 using the latter docking procedure shows negative interaction with the protein. On closer examination, this compound was found to have an orientation in the binding pocket that resulted in unfavorable interaction with the protein. In contrast, the compound orientations obtained from GOLD and FlexX were optimal for favorable interactions with the protein. Second, compound 1 shows negative HINT interaction with R-PK when docked with FlexX, due to the fact that the compound resides outside the binding pocket. There is no steric reason for the expulsion of this compound from the active site. However, it should be noted that docking program have certain limitations. For instance, they do not consider the flexibility of the protein binding site when docking which may cause unfavorable interactions between the binding pocket residues and the molecule and help explain the resulting expulsion of the molecule from the binding pocket. In contrast to the other two docking methods, favorable interactions are noted for all compounds using GOLD. This program uses three genetic operators; crossover, mutation and migration to select and rank the best ligand pose on basis of their interaction with the binding site. Further, it has also been observed that both FlexX and GOLD consistently identified better ligand conformations compared to third docking

process. However, it is difficult to single out any single method as the best docking method because of inherent imperfections in the docking programs. Hence employing a combination of different docking methods ensures a consensus and improves the probability of identifying true ligands.

To summarize, the computational studies identified several compounds that were previously unknown or uncharacterized as promising candidates to act as allosteric effectors of R-PK. Also the computational screening and HINT analysis^{78,79} identified several compounds that could interact with R-PK, most likely at the FBP binding site. FBP stabilizes the active R-state of the enzyme by regulates the conversion of R-PK between the inactive T-state and an active R-state. Effectors that can interact with this site can thus act as regulators and control the activity of the enzyme. These effectors could either mimic or antagonize the physiological effector. In addition, the possible effect of such compounds on substrate affinity, on maximal velocity, and on enzyme cooperativity might be separable and distinct.

Kinetic studies

It has been reported that approximately 65% of the listed compounds in NCI are actually available, some on a commercial basis whereas some are provided complimentary. For the compounds we requested the percentage is substantially lower, around 35%. Of the final list of twenty best compounds obtained from the computational search that scored highest in the HINT calculation and were identified as promising leads by the docking methods, seven compounds were obtained from NCI for further experimental analysis. Nevertheless, once we identify new lead molecules our future

database searches will focus on commercial chemical databases featuring more tailored compounds based on the ligands identified in this study. Biological testing is necessary for the compounds tested virtually because although a compound may match a pharmacophore, thus showing receptor compatibility, its activity is not guaranteed. The free energy scoring function of HINT was used to evaluate the interaction between R-PK and the molecules found with the pharmacophore generation/3D database search tools. The molecules predicted to bind well according to the hydrophobic forcefield, were experimentally tested. The seven compounds obtained from NCI were subjected to kinetic analysis.

R-PK activity was measured by a continuous assay coupled to LDH as described in the experimental section. R-PK is an allosteric enzyme and shows a characteristic S-shape curve in presence of substrate PEP. R-PK responds to increasing concentration of substrate PEP by showing a typical homotropic effect and allosterically activates the enzyme. The kinetic parameters of the enzyme especially the Hill coefficient of R-PK indicate that the subunits in R-PK act cooperatively to alter its kinetics. Comparative analysis of R-PK kinetics reported in literature is similar to the one observed for the His-tagged enzyme. This helped establish the identity of the enzyme as R-PK and also showed that His-tag and additional amino acid residues attached to the N-terminal does not perturb the kinetics of R-PK. This was our concern since we did not remove the His-tag chain attached to the protein's N-terminal after purification.

Kinetic analysis of R-PK with the compounds revealed both activators and inhibitors of the protein, suggesting that an allosteric effector can bind to the same site

but produce opposite allosteric properties. This apparent contradiction could be explained by taking into consideration the fact that study of the molecules is based on a “model” of ligand –receptor complex. Given this limitation, we can predict but cannot state for sure where these molecules bind in the protein. Further, solving the structure of co-crystals of R-PK in presence of these molecules currently in progress in our laboratory will address the issue by identifying the binding site of these molecules.

The compounds identified in our computational studies were active; however, none of them were as potent as the natural allosteric activator, FBP. All tested compounds were active, but only at mM concentrations, which would make them clinically insignificant; however, we consider these results to be very encouraging because the molecules can prove as lead compounds to facilitate discovery of better effectors of R-PK in future. Compounds 2 and 3 are the most potent activators ($32.39 \pm 5.17\%$ and $32.02 \pm 1.79\%$ activation, respectively at 1 mM). In contrast, compounds 6 and 7 were found to be inhibitors and decreased the activity of R-PK ($11.26 \pm 0.82\%$ and $20 \pm 2.71\%$ inhibition, respectively at 1 mM). Compared to the natural inhibitors ATP (8% at 1 mM) and the previously reported L-alanine (30% at 70 mM) and L-phenylalanine (72% at 30 mM)¹²⁻¹⁶ these compounds inhibit R-PK at lower concentration. Our results also showed that FDP, a natural allosteric activator of *Leishmania mexicana* and *Chlamydia trachomatis* PKs^{24,81,82} caused only a minor activation of R-PK. Also, RBP caused no significant change in the activity of R-PK despite being suggested to be a potent effector in the computational studies. These results are consistent with the varying degree of sequence conservation observed in the allosteric sites of PKs from different

biological species. Manipulation of structural variation in the FBP binding site present excellent opportunity to distinguish the different PK isoforms and prove valuable in the identification of small molecules effectors that can selectively bind PK to regulate or stabilize its activity for targeted therapies of several diseases in which PK actively participates. For instance, manipulation of R-PK activity in either direction is desirable to find new treatment options for hypoxia, PKD or sickle cell anemia. Similarly, identification of the binding sites of thyroid hormone analogues known to inhibit human M2 PK would prove important to find new potent inhibitors. Based on the quantities available to us we further explored the kinetics of compounds 1 and 7 to assess their behavior.

Computational analysis and initial screening process identified compound 1 as a moderate activator of R-PK. Detailed kinetic analysis of R-PK activation was conducted in presence of 1 mM concentration of compound 1, since we were able to obtain co-crystals of R-PK in presence of this compound. The dose response curve of R-PK in presence of compound 1 is more hyperbolic, which is also evidenced by the decrease in Hill coefficient of R-PK from 1.69 ± 0.11 to 1.49 ± 0.15 (Figure 19, table 2). An allosteric activator mimics the substrate PEP and effector FBP by binding preferentially to R-form of the protein. As a result, the velocity curve becomes less sigmoidal, i.e. more hyperbolic at saturating activator and $S_{0.5}$ decreases. The $S_{0.5}$ for PEP decreased from 1.05 ± 0.05 mM to 0.6 ± 0.06 mM and the sigmoidal curve of R-PK is shifted towards the left, suggesting that compound 1 is an activator of R-PK and increases the conversion of PEP to pyruvate. However compared to FBP, the activation property of compound is

moderate, the efficiency of R-PK ($k_{cat}/S_{0.5}$) in the presence of compound 1 is $615 \text{ s}^{-1}/\text{mM}$; almost a third of that of the natural allosteric activator ($1972 \text{ s}^{-1}/\text{mM}$).

Similar steady state kinetics studies of R-PK were also conducted in the presence 1 mM of compound 7 that was identified as potential inhibitor in computational studies. In presence of this compound the allosteric behavior of R-PK is more pronounced and the sigmoidal curve of R-PK shifts right, indicating that it acts as an allosteric inhibitor of the protein. The kinetic parameters of R-PK in the presence of compound 7 indicates that it binds preferentially to the T-form of R-PK and causes further increase in the sigmoidal behavior of the protein with a higher $S_{0.5}$ indicating stabilization of inactivated T-state over active R-state. The stabilization of inactivated T-state over active R-state has special significance as it causes a metabolic block in the glycolysis pathway at this point and increases the cellular concentrations of metabolites previous to the R-PK catalyzed step. One of the most important metabolite is DPG that regulate oxygen affinity of Hb. Therefore; allosteric inhibitors of R-PK can be used to increase oxygen delivery to tissues and to relieve hypoxia. Compound 7 was found to be a potent inhibitor of R-PK and it significantly decreases the efficiency of the enzyme ($232 \text{ s}^{-1}/\text{mM}$) in converting PEP to products. However, more efficient inhibitors are necessary that can act in micro molar concentration.

Crystallization studies

X-ray crystallographic analysis of R-PK in the presence of the identified small molecule effectors is currently in progress to reveal the binding site(s) of the compounds. It would help to precisely describe the interaction formed in the allosteric site and their similarity or differences from the modeled compounds. The structural analysis of the enzyme complex with these compounds would also allow optimization of the starting pharmacophores and syntheses of new molecular entities for enhanced allosteric activity at much lower concentrations. Since we obtained both allosteric activator and inhibitor in our studies, we cannot rule out the identification of new binding sites in R-PK. Previous computational docking studies with yeast PK aimed at structure-based discovery of novel R-PK allosteric regulators yielded three activators²⁰. Furthermore, structure-based drug design for novel Hb regulators has led to the discovery of compounds that bind to or react at the allosteric site of Hb, which have the potential for the treatment of sickle cell disease, brain tumors, hypoxia, ischemia, and trauma-related blood loss⁹². One such drug is currently undergoing Phase III clinical test as a radio-sensitizing agent for brain tumor treatment⁹³. Similar structure-based design studies on human R-PK may one day lead to allosteric drugs for the treatment of hypoxia and nonspherocytic hemolytic anemia²⁵. Determination of the structure of these crystals will allow knowledge of the residues of R-PK involved in ligand binding and involved in allosteric transition.

To summarize, most of the compounds identified in our computational studies act as activators, however, none of them were as potent as the natural allosteric activator, FBP. Like the inhibitors, these activators will serve as leads for structure-based

modification to identify more potent and R-PK selective regulators. Our simple and efficient method for the expression and purification of R-PK is much better compared to the procedure reported by Wang et al.⁵⁷ and results in a 6-fold increase in the yield. Using computational screening and HINT analysis, we have identified several compounds that interact with R-PK, most likely at the FBP site. Kinetic analysis of R-PK with the compounds revealed both activators and inhibitors of the protein, suggesting that an allosteric effector can bind to the same site but produce opposite allosteric properties. All tested compounds were active, but only at mM concentrations, which would make them clinically insignificant; however, we consider these results to be very encouraging. X-ray crystallographic analysis of R-PK in the presence of the identified small molecule effectors is currently in progress to reveal the binding site(s) of the compounds. This would allow optimization of the starting pharmacophores and syntheses of new molecular entities for enhanced allosteric activity at much lower concentrations. We postulate that structure-based design studies on human R-PK may lead to new treatment alternatives for hypoxia, nonspherocytic hemolytic anemia and other disorders.

SUMMARY AND CONCLUSIONS

Allosteric proteins make excellent targets for structure based drug discovery. Erythrocyte pyruvate kinase is an important glycolytic enzyme and manipulation of its regulatory behavior by allosteric modifiers is of interest for medicinal purposes. Deficiency of this enzyme causes PKD in which energy-deprived erythrocytes fail to maintain structural integrity resulting in hemolytic anemia. Our laboratory became interested in studying R-PK protein because we have a long-standing interest in increasing oxygen levels to hypoxic tissues. Hypoxia results in ischemic insults, stroke, and trauma (blood loss). One method to treat chronic hypoxia would be to increase the concentration of the natural allosteric effector of Hb, DPG. The much lower preponderance of red cell pyruvate kinase to Hb levels makes this an interesting target for treating chronic hypoxia. New allosteric effectors of human R-PK will help understand how they mechanistically shift the allosteric equilibrium to increase DPG or ATP in the red cells. These effectors will also be useful to identify new binding sites in the protein. In comparison with Hb we postulate that R-PK bind several allosteric effectors at different sites in the protein. Discovery of these sites and the effectors would further accentuate drug discovery.

We applied the structure based drug design approach to identify new allosteric effectors of R-PK. Human erythrocyte pyruvate kinase was expressed in Rosetta cells and

purified using Ni-NTA column. Next we used the human R-PK structure in “structure-based” computational studies. A search of the National Cancer Institute small molecule compound database using the UNITY software led to the identification of twenty nine compounds with similar pharmacophore as fructose-1,6-bisphosphate, the natural allosteric activator of the human kinase. These small molecules were docked into the fructose-1,6-bisphosphate binding site using the programs FlexX and GOLD; and their mode of interactions with the protein analyzed with the energy scoring function of HINT. The compounds with the highest HINT scores were requested from the National Cancer Institute and subjected to kinetic analysis, which revealed both activators and inhibitors of the kinase. The allosteric effectors discovered in this study could prove to be lead compounds for developing medications for the treatment of hemolytic anemia, sickle cell anemia, hypoxia-related diseases and other disorders arising from erythrocyte pyruvate kinase malfunction.

In conclusion, we have developed a simple and efficient method for the expression and purification of R-PK. Human R-PK was expressed in DH-5 α cells and was purified by the procedure reported by Wang et al. However, this method gave a very low yield of R-PK (5mg/L). In an attempt to increase the yield, we expressed R-PK in Rosetta strain cells. Further, addition of His-tag to the protein’s N-terminus simplified purification to a one step Ni-NTA column resulting in a 6-fold increase in the yield. Using computational screening and HINT analysis, we have identified several compounds that interact with R-PK, most likely at the FBP site. Kinetic analysis of R-PK with the compounds revealed both activators and inhibitors of the protein, suggesting that

an allosteric effector can bind to the same site but produce opposite allosteric properties. All tested compounds were active, but only at mM concentrations, which would make them clinically insignificant; however, we consider these results to be very encouraging. X-ray crystallographic analyses of R-PK in the presence of the identified effectors are currently in progress to reveal the binding site(s) of the compounds. This would allow for further optimization of the starting pharmacophores and syntheses of new molecular entities for enhanced allosteric activity at much lower concentrations.

The findings in this dissertation may represent an important new direction for unraveling the allosteric mechanism of human R-PK thereby enabling the discovery of new effective treatment options for diseases arising from R-PK malfunction. It also provides a start for the drug discovery process by identifying new ligands that alter R-PK behavior. Future advancement of the project will follow the identification of R-PK crystal in the T-state. Since no PK enzyme has been crystallized in both the T-and R-state this would immensely improve the understanding of allosteric transition mechanism in the protein. Besides that, the identified molecules can be subjected to structure activity relationship studies to find compound with increased potency. In addition, screening of commercially available databases for active compounds is also a viable option.

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A list of Ms. Kharalkar's publications and abstracts is provided below:

Kharalkar S.S, Safo M.K, Joshi G.S, Musayev F.N, Fornabaio M and Abraham D.J. Identification of novel allosteric regulators of human erythrocyte pyruvate kinase. (*In Preparation*) .

Micaela Fornabaio, Fraydoon Rastinejad, **Shilpa Kharalkar**, Martin Safo, Donald J. Abraham, and Glen E. Kellogg. Virtual Screening Approach: Application of a Hydropathic Forcefield to 3D Database Searches. *The 229th ACS National Meeting, March 13-17'2005, San Diego, CA (COMP-171).*

Micaela Fornabaio, Fraydoon Rastinejad, Li-Zhi Mi, **Shilpa Kharalkar**, Martin Safo, Matteo Porotto, Anne Moscona, Donald J. Abraham, and Glen E. Kellogg. Hydropathic Forcefield in 3D Database Searches and Computational Titration Applications *Gordon Research Conference, 2005.*