DESENSITIZED PHOSPHOFRUCTOKINASE FROM Ascaris suum:

A STUDY IN NONCOOPERATIVE ALLOSTERY

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Marvin A. Payne, A.A., B.S., B.S., M.S.T.
Denton, Texas
May, 1993
DESENSITIZED PHOSPHOFRUCTOKINASE FROM *Ascaris suum*:
A STUDY IN NONCOOPERATIVE ALLOSTERY

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Marvin A. Payne, A.A., B.S., B.S., M.S.T.
Denton, Texas
May, 1993
Payne, Marvin A., *Desensitized Phosphofructokinase from Ascaris suum: A Study in Noncooperative Allostery*. Doctor of Philosophy (Biochemistry), May, 1993, 90pp., 1 table, 18 figures, 1 scheme, 1 reaction, 3 mechanisms, references, 50 titles.

Kinetic data have been collected suggesting that heterotropic activation by fructose 2,6-bisphosphate and AMP is a result not only of the relief of allosteric inhibition by ATP but is also the result of an increase in the affinity of phosphofructokinase for fructose 6-phosphate. Modification of the *Ascaris suum* phosphofructokinase at the ATP inhibitory site produces a form of the enzyme that no longer has hysteretic time courses or homotropic positive cooperativity (fructose 6-phosphate) and allosteric inhibition by ATP (Rao, G.S.J., Wariso, B.A., Cook, P.F., Hofer, H.W., and Harris, B.G. (1987) *J. Biol. Chem.* 262, 14068-14073). This Michaelis-Menton behaving form of phosphofructokinase is still activated by fructose 2,6-bisphosphate, AMP, and is phosphorylated by the catalytic subunit of cyclic AMP dependent protein kinase. Fructose 2,6-bisphosphate also decreases $K_{F-6-P}$ by about 15 fold and has an activation constant of 92 nM, while AMP decreases $K_{F-6-P}$ about 6-fold and has an activation constant of 93 μM. Double activation experiments suggest that
fructose 2,6-bisphosphate and AMP are synergistic in their activation. The desensitized form of the enzyme is also activated by phosphorylation which increases the affinity of free enzyme for fructose 6-phosphate such that the order of addition of reactants changes from that with MgATP adding first for the non-phosphorylated enzyme to addition of fructose 6-phosphate first. The phosphorylated form of the enzyme is still activated by fructose 2,6-bisphosphate and AMP.

The activation of d-PFK by fructose-2,6-bisphosphate has been studied at varied pH. In the absence of effector the maximum velocity is constant over the pH range of 6 to 9 while the $V/K_{F-6-p}$ decreases at high and low pH giving pK values of $6.2 \pm 0.3$, $6.8 \pm 0.3$, and $8.6 \pm 0.2$. The measured pKs are not perturbed in the presence of saturating fructose-2,6-bisphosphate, that is the maximum activation remains constant across the pH range studied. A titration of $K_{act}$ for fructose-2,6-bisphosphate yields a pK of $7.4 \pm 0.1$. 
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.  INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Regulatory Properties of PFK</td>
<td>3</td>
</tr>
<tr>
<td>Desensitization of Ascaris PFK</td>
<td>4</td>
</tr>
<tr>
<td>Activation studies using initial rate steady state kinetics</td>
<td>5</td>
</tr>
<tr>
<td>Other studies</td>
<td>6</td>
</tr>
<tr>
<td>II. METHODS</td>
<td>8</td>
</tr>
<tr>
<td>Enzyme purification</td>
<td>8</td>
</tr>
<tr>
<td>Desensitization</td>
<td>9</td>
</tr>
<tr>
<td>Assays of PFK</td>
<td>9</td>
</tr>
<tr>
<td>Phosphorylation of d-PFK</td>
<td>9</td>
</tr>
<tr>
<td>Circular dichroism studies</td>
<td>10</td>
</tr>
<tr>
<td>pH studies</td>
<td>11</td>
</tr>
<tr>
<td>Data Processing</td>
<td>13</td>
</tr>
<tr>
<td>III. ACTIVATION STUDIES WITH F-2,6-P$_2$ AND AMP</td>
<td>16</td>
</tr>
<tr>
<td>Activation of d-PFK by F-2,6-P$_2$ and AMP</td>
<td>16</td>
</tr>
<tr>
<td>Circular dichroism</td>
<td>32</td>
</tr>
<tr>
<td>Double activation</td>
<td>32</td>
</tr>
<tr>
<td>Phosphorylated d-PFK</td>
<td>34</td>
</tr>
<tr>
<td>IV. DERIVATION OF THE RATE EQUATION FOR DOUBLE COMPETITIVE ACTIVATION</td>
<td>36</td>
</tr>
<tr>
<td>V. ACTIVATION BY F-2,6-P$_2$ WITH VARIED PH</td>
<td>45</td>
</tr>
<tr>
<td>Effects of pH changes on d-PFK</td>
<td>45</td>
</tr>
<tr>
<td>VI. DISCUSSION</td>
<td>70</td>
</tr>
<tr>
<td>Effects of F-2,6-P$_2$ and AMP</td>
<td>70</td>
</tr>
<tr>
<td>Effects of Phosphorylation</td>
<td>73</td>
</tr>
<tr>
<td>Effects of Varied pH</td>
<td>73</td>
</tr>
<tr>
<td>Allosteric Models</td>
<td>80</td>
</tr>
<tr>
<td>VII. REFERENCES</td>
<td>88</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

Reaction I. The PFK Reaction..........................2
Figure 1. F-2,6-P$_2$ Activation of d-PFK.................17
Figure 2. K$_{act}$ for F-2,6-P$_2$..........................19
Figure 3. K$_{act}$ for AMP.................................21
Figure 4. Circular Dichroism Spectra of d-PFK............23
Figure 5. Double Activation Experiment....................25
Scheme I. Double Activation Model.......................27
Figure 6. Initial Velocity Pattern with p$_d$-PFK...........29
Table I. Summary of Activation Constants..................31
Figure 7. Theoretical Plot of appKa vs. [I]................37
Figure 8. Theoretical Plot of % Activation vs. [I]........39
Figure 9. Initial Velocity Pattern with d-PFK, pH 5.95..49
Figure 10. Initial Velocity Pattern with d-PFK, pH 8.9...51
Figure 11. Variation of V with pH........................53
Figure 12. Variation of V/K$_F$ with pH....................55
Figure 13. The maximum activation by F-2,6-P$_2$ plotted against pH..........................57
Figure 14. The Variation of pK$_{P-6-P}$ with pH in the absence of effector..........................59
Figure 15. The Variation of pK$_{P-6-P}$ with pH in the presence of saturating effector.................61
Figure 16. The Variation of pK$_{ID}$ with pH...............63
Figure 17. The Variation of pK$_{IN}$ with pH...............65
Figure 18. The Ratio K$_{IN}$/K$_{ID}$ plotted against pH........67
Mechanism I. Proposed Acid-Base Mechanism for 
Ascaris PFK.................................76

Mechanism II. Proposed F-2,6-P₂ Binding Mechanism for 
Ascaris PFK.................................79

Mechanism III. A Mechanical Linkage Model for Ascaris 
PFK Allosteric Regulation...............83
LIST OF ABBREVIATIONS

cAPK  catalytic subunit of cyclic AMP dependent protein kinase
BME  β-mercaptoethanol
DEPC diethylpyrocarbonate
F-6-P fructose 6-phosphate
F-2,6-P$_2$ fructose 2,6-bisphosphate
FBP fructose 1,6-bisphosphate
GDP guanosine diphosphate
PFK phosphofructokinase with the leading consonants n, d and pd indicating the native, desensitized and phosphorylated-desensitized forms, respectively
CHAPTER I

INTRODUCTION

Phosphofructokinase (PFK) catalyzes the phosphorylation of fructose-6-phosphate at the C$_1$ hydroxyl to form fructose-1,6-bisphosphate with MgATP serving as the phosphoryl donor (Reaction I). The activity of PFK is ubiquitous, being involved in the glycolytic pathway. Most PFK's are regulated by a number of glycolytic and/or citric acid cycle intermediates depending on the source of the enzyme. Some of the higher forms of PFK are also phosphorylated at specific consensus sequences (with varied effects) and are subject to regulation by the potent activator F-2,6-P$_2$. The bifunctional enzyme regulating the concentration of F-2,6-P$_2$ is also regulated by cAMP dependent phosphorylation and other effectors. The role of Fru-2,6-P$_2$ is discussed in detail in a compilation of reviews edited by Pilkis (1990). The above considerations taken together with accounting for the physiological concentrations of Fru-6-P, ATP, and Fru-1,6-P$_2$ can be interpreted to mean that the reaction catalyzed by PFK is a key regulatory point in the glycolytic pathway. The latter has been indicated for Ascaris PFK (Barrett and Beis, 1973) and for PFK from mammalian systems see introduction to Starling et al., 1982).
REACTION I.

The PFK Reaction.

\[
\begin{align*}
&\text{Prenyl diphosphate} + \text{Fructose-6-P} \\
&\xrightarrow{\text{PFK}} \text{Fructose-1,6-bisphosphate} + \text{ADP}
\end{align*}
\]
The physiological significance of several PFKs have been reviewed by Dunaway, (1983).

Regulatory properties of PFK. The most potent activators of *Ascaris suum* phosphofructokinase (PFK) are F-2,6-P$_2$ and AMP, while alkaline pH and some small ions also have a stimulatory effect (Hofer et al., 1982a). In contrast to mammalian PFK (Kemp and Foe, 1983), phosphorylation of *Ascaris* PFK by cAPK (Hofer et al., 1982b) causes marked stimulation of activity. This has also been observed with PFK from *Fasciola hepatica* (Kamemoto and Mansour, 1986, Kamemoto et al., 1987) and from *Dirofilaria immitis* (Srinivasan et al., 1988). The sequence of the peptide phosphorylated by the protein kinase in the *Ascaris* PFK has been reported by Kulkarni et al. (1987).

Several kinetic mechanisms for various forms of PFK are extant. Merry and Britton (1985) determined that the mechanism for rabbit muscle PFK at pH 8 is random with 72% of the reaction proceeding with MgATP binding first at low substrate concentration. Other PFK's exhibiting random mechanisms include the enzyme utilizing MgPP$_i$ from *Propionibacterium freudenreichii* or *Entamoeba histolytica* (Bertagnolli and Cook, 1984) and *Phaseolus aureus* (Bertagnolli et al., 1986). An ordered mechanism with sugar substrates binding first has been reported for the enzyme from *Lactobacillus plantarum* (Simon and Hofer, 1978).

The application of steady state kinetic analysis to the
study of the regulatory mechanism of phosphofructokinase from organisms such as *Ascaris* and rabbit muscle has been complicated by the allosteric properties of the enzyme. In particular, *Ascaris* PFK in its native form (n-PFK) exhibits strong ATP inhibition, hysteresis in the time courses, and homotropic cooperativity of F6P binding (Cook et al., 1987, Rao et al., 1987a).

**Desensitization of Ascaris PFK.** A form of *Ascaris* PFK has been prepared by reaction with diethylpyrocarbonate that effectively lacks ATP inhibition, exhibits non-cooperative binding of F6P, and gives linear time courses (Rao et al., 1987a). This form of PFK has been designated d-PFK. During the course of reaction with DEPC, the PFK is inactivated concomitant with the derivatization of about 5-6 histidines per subunit. The substrate F-6-P largely protects against inactivation and prevents derivatization of about 2 histidines per subunit. By examining the variation of the first order inactivation rate constant with pH, Rao et al. (1987a) obtained a pKₐ of 6.4 ± 0.1 representing titration of a single proton - most likely on the essential histidine being derivatized by DEPC.

The kinetic mechanism of d-PFK (Rao et al., 1987b) is predominantly steady state ordered with MgATP binding first. The alternate pathway with F-6-P binding first, although allowed, is blocked due to the very high Kᵢ for F-6-P which is estimated to be about 50 mM from F-6-P protection against
inactivation by DEPC. Previous studies have suggested that d-PFK is not activated by F-2,6-P₂ and AMP (Rao et al. 1987a), but these studies were carried out at saturating concentrations of F-6-P and AMP. It has recently been observed that d-PFK can be phosphorylated by cAPK and that both F-2,6-P₂ and AMP stimulate the d-PFK reaction at non-saturating F-6-P.

Activation studies using initial rate steady state kinetics. The theory of initial rate steady state kinetics must be extended somewhat to design experiments such as those described in this dissertation. Although the theory for particular experiments is explained in the relevant sections, its basis has been described by Cook (1982). Basically, an effector can be treated as a pseudo-reactant provided that its binding does not disallow the steady state. It is assumed that the allosteric transitions induced by the binding of an effector are rapid with respect to the establishment of the steady state. Slow allosteric transitions can give rise to hysteresis in the time courses. Hysteresis that is very sensitive to the order of addition of reactants, effectors, and enzyme to the assay mix, is especially pronounced with the native form of Ascaris PFK (Cook et al., 1987, Payne, M. A. unpublished observations). The time course for the d-PFK reaction, on the other hand, is insensitive to the order of addition of reactants, effectors, and enzyme.
The studies described in this dissertation examine the effects of F-2,6-P$_2$ and AMP or phosphorylation on the kinetic mechanism of d-PFK. The effect of varied pH on the activation by F-2,6-P$_2$ is also described. The Activation studies at pH 6.8 and the derivation of the double activation rate equation have been published (Payne, et al., 1991, Payne and Cook, 1991). The pH studies are to be published.

Other studies. As partial fulfillment of my research requirement, I assisted with the development of a computer-assisted sulfide ion selective electrode assay for O-acetylserine sulfhydrylase from Salmonella typhimurium. The work has been published along with a rapid purification procedure for O-acetylserine sulfhydrylase (Hara et al., 1990). An abstract follows.


An improved method for purifying O-acetylserine sulfhydrylase from Salmonella typhymurium is described as well as a new computer-controlled assay making use of the sulfide ion selective electrode. The purification method uses gradient elution from Q-Sepharose Fast Flow and phenyl-Sepharose columns to give 75 mg (50% yield) of the enzyme starting from 300 g of starting material in 3 days. The sulfide electrode assay makes use of sulfide and calomel electrodes attached to a signal buffer which serves as an impedance match. The output of the signal buffer is linked in parallel to a strip chart recorder and a Keithley Model 575 data acquisition and control system. The system 575 is interfaced to a Packard-Bell AT computer. In
addition, two BASIC computer programs have been written to convert potential measured by the electrode to sulfide concentration and to convert the time course data to rates.
Enzyme Purification. Phosphofructokinase was purified from 200 g of *Ascaris suum* according to Starling et al. (1982) except for the addition of a cocktail of protease inhibitors containing aprotinin, trypsin inhibitor, and phenylmethylsulfonfyl fluoride to the crude extract and DEAE sepharose eluate. After purification, the n-PFK was dialyzed into pH 7.0 storage buffer containing 50 mM potassium phosphate, 20 mM BME, and 2 % (v/v) glycerol, placed in 1 ml aliquots and stored at -20 °C. The d-PFK prepared for the pH studies was treated as above but was dialyzed after purification into pH 7.4 storage buffer containing 50 mM potassium phosphate, 3 mM dithiothreitol, and 15 % (v/v) glycerol, placed in 1 ml aliquots and stored at -20 °C. The d-PFK stored thus is more stable to repeated freeze-thaw cycles than the previous method. The enzyme was diluted before use into the same buffer but with 2 % (v/v) glycerol. Dilutions varied from 1:8 to 1:12. The catalytic subunit of cyclic AMP dependent protein kinase was purified according to Sugden et al. (1976) to a specific activity of 36 units/mg (using Leu-Arg-Arg-Ala-Ser-Leu-Gly as the substrate) and stored at 4 °C. The cAPK was provided by
Nuggehalli Srinivasn.

**Desensitization.** The desensitized form of PFK (d-PFK) was prepared by the method of Rao et al. (1987a).

**Assays of PFK.** All assays were carried out in a volume of 1.00 mL at pH 6.8 in 50 mM imidazole-Cl containing 8 mM MgCl₂, 2% glycerol (v/v), 0.2 mM NADH, 2.1 units aldolase, 65 units triosephosphate isomerase, 25 units α-glycerolphosphate dehydrogenase, and variable concentrations of ATP, F-6-P, effector(s), and d-PFK. Assays with F-2,6-P₂ had the effector added to each cuvette just prior to addition of enzyme to minimize degradation of F-2,6-P₂ at pH 6.8. All reactions were started with F-6-P. Typically, 8 milliunits was used for the activation experiments with n-PFK and 10 milliunits of d-PFK was used for all other assays. The rate of disappearance of NADH was monitored at 340 nm with either a Gilford 250 or a Gilford 260 UV/VIS spectrophotometer attached to a strip-chart recorder. Temperature was maintained at 30 °C with a circulating water bath with the capacity to heat and cool the thermospacers of the cell compartment.

**Phosphorylation.** Phosphorylation of d-PFK with cAPK was accomplished under conditions similar to those used by Kulkarni et al. (1987). The incorporation of Γ⁻³²P from [Γ⁻³²P]ATP was monitored by TCA precipitation of the phosphorylated protein and scintillation counting. In addition, d-PFK subjected to the same conditions in the
absence and presence of cAPK in phosphorylation assays was chromatographed isocratically on a TSK-400 gel filtration column at 1.0 mL/min. The buffer contained 50 mM sodium phosphate and 20 mM sodium sulfate at pH 6.8. A Beckman System Gold integrated high performance liquid chromatography system was used for the experiments including both a Beckman 161 UV detector and a Beckman 171 radioisotope detector to monitor the absorbance at 280 nm and radioactivity respectively. All the eluent was passed through the radioisotope detector equipped with a 1 mL liquid flow cell and Cherenkov radiation was detected and counted.

Phosphorylation by cAPK was carried out during the d-PFK assay. First, assays were initiated at pH 6.8, 50 mM imidazole-Cl, 8 mM Mg\textsuperscript{2+}, 2% glycerol (v/v), 100 μM ATP and 70 μM F-6-P. A linear time course was obtained, after which the amount of cAPK added was varied to determine the amount needed to increase the rate to obtain a second rate without a noticeable lag. As a result of these preliminary studies, 29 milliunits of cAPK was added to all subsequent assays. The assay mix for pd-PFK contained the following: buffer, NADH, coupling enzymes, 100 μM ATP, and 10 milliunits of d-PFK. The cAPK was added and the mixture allowed to stand for 1 min. Effectors were added if required and the PFK reaction was started by the addition of F-6-P.

Circular dichroism studies. The circular dichroism
studies were carried out using the procedure described by Rao et al., (1991).

pH studies. The d-PFK assay was modified somewhat for purposes of performing F-2,6-P$_2$ activation studies as a function of pH. All assays were carried out in a volume of 1.0 mL in 200 mM bis-tris-propane-CL at the appropriate pH, 8 mM MgCl$_2$, 0.2 mM NADH, 2.1 units aldolase, 34 units triose-phosphate isomerase, 3.3 units α-glycerolphosphate dehydrogenase, and variable concentrations of ATP, F-6-P, effector(s), and d-PFK. 10 μL/assay of aldolase was used which had been previously extensively dialyzed from an ammonium sulfate suspension against 50 mM imidazole-CL pH 6.8, 3 mM DTT, 10 % (v/v) glycerol. An aliquot of 3 μL/assay triosephosphate isomerase and α-glycerolphosphate dehydrogenase were added directly from an ammonium sulfate suspension (Sigma chemical company) to circumvent problems associated with stability of the enzymes upon dialysis. The above amounts of coupling enzymes were sufficient to support the highest rate observed without lags except at the pH extremes, and then about a minute was required to establish the steady state. Assays with the highest rates were periodically checked for linearity with d-PFK concentration. Assays with F-2,6-P$_2$ had the effector added to each cuvette immediately prior to addition of enzyme to minimize degradation of F-2,6-P$_2$. All reactions were started with d-PFK. Typically, 10 to 15 milliunits of d-PFK were used for
the experiments. The rate of disappearance of NADH was monitored at 340 nm with a Beckman Kintrac monochromator upgraded with a Gilford light source, sample compartment, and detector, the output of which was connected to a strip chart recorder. The temperature was regulated at 30 °C using a circulating water bath to heat and cool the thermospacers of the cell compartment, and a cuvette heating block to maintain the temperature of the assay mixes prior to the assays.

Typically, enough assay mix was prepared for 10 assays. 2.0 mL of concentrated assay buffer (500 mM bis-tris-propane, 40 mM MgCl$_2$ pre adjusted to the appropriate pH) were diluted with an amount of water necessary to make 10 mL assay mix after all additions were accounted for. This gave a final concentration of 200 mM bis-tris-propane, 8 mM MgCl$_2$. Appropriate amounts of the reagents were added except for effector, F-6-P, and d-PFK. 1/10 of the above mix was placed in several cuvettes which were allowed to come to temperature in the heating block for at least 8 - 9 minutes. The F-6-P and effector solutions were added followed by addition of enzyme to start the PFK reaction. The pH of each assay was checked immediately following the assay with a Radiometer PHM83 pH meter equipped with a small bore glass Ag/AgCl with calomel reference electrode.

The determination of protein concentration for the pH studies was done spectrophotometrically using the $E_{1%}^{280}$ at 280
nm of 6.5 reported by Starling et al. (1982). 0.25 ml of 50% Trichloroacetic acid (TCA) was added to 1.00 ml d-PFK in storage buffer to make a final concentration of 10% TCA. After a few minutes, two portions of 20 μL of the 50% TCA were added to insure complete precipitation. The precipitate was centrifuged and the pellet washed three times with 1.0 ml of 10% TCA solution, followed by three washes with 1 ml diethyl ether. The pellet was dissolved in 88% formic acid and the absorbance was measured at 280 nm. By this method, the concentration of d-PFK in the stock solution was determined to be 0.55 mg/ml assuming all protein was d-PFK.

Data processing. All data were fitted using the appropriate rate equation and with the exception of double activation data using computer programs developed by Cleland (1979) which use a non-linear least squares fitting algorithm. Substrate saturation curves were fitted using equation 1. Hyperbolic functions in which a finite value of Y was observed at zero effector concentration were fitted using equation 2. In the pH studies, values of Y obtained at 200 μM F-2,6-P$_2$ were weighted by a factor of 10. Curves exhibiting sigmoidicity were fitted using equation 3. The $K_{F-6-P}$ obtained from double activation experiments in which F-6-P was varied at different fixed levels of F-2,6-P$_2$ and AMP were fitted with equations 4 and 5 using the Marquardt-Levinthal algorithm supplied with Sigmaplot 4.0
from Jandel, Inc.. Equations 6 and 7 were used for fitting data from experiments in which the pH was varied. Equation 6 involves the titration of a single acidic proton, while Equation 7 involves the titration of two acidic and one basic protons. Equation 8 was used for fitting mechanism data in which both substrates were varied.

\[ v = \frac{V A}{(K_m + A)} \]  
\[ Y = A \left( 1 + \frac{X}{K_{IN}} \right) \left( 1 + \frac{X}{K_{ID}} \right) \]  
\[ v = \log \left( \frac{V X^2}{A + 2B X + X^2} \right) \]  

\[ \text{app} K = K_m \left( 1 + I/K_I + J/K_J + IJ/\alpha K_I K_J \right) / \left( 1 + I/\beta K_I + J/\gamma K_J + IJ/\delta K_I K_J \right) \]  
\[ \text{app} K = K_m \left( 1 + I/K_I + J/K_J \right) / \left( 1 + I/\beta K_I + J/\gamma K_J \right) \]  
\[ \log Y = \log \left( \frac{C}{1 + H/K_1} \right) \]  
\[ \log Y = \log \left( \frac{C}{1 + H/K_1 + K_2/H + (H^2)/K_0} \right) \]  
\[ v = \frac{V A B}{(K_a K_b + K_a B + K_b A + A B)} \]  

In equation 1, \( v \) is the initial velocity, \( V \) is the maximum velocity, \( A \) is the reactant concentration and \( K_m \) is the Michaelis constant for \( A \). In eq. 2, \( Y \) is the observed value of \( K_{F-6-P} \) at any effector concentration, \( A \) is the
value of \( K_{F-6-P} \) at zero effector, \( X \) is the effector concentration, \( K_{ID} \) is the dissociation constant for effector, \( K_{IN} \) is a ratio of rate constants that causes \( Y \) to level off at a finite value, and \( A(K_{IN}/K_{ID}) \) is the value of \( K_{F-6-P} \) at infinite effector concentration. In eq. 3, \( X \) represents the concentration of F-6-P, \( A \) and \( B \) are constants and all other terms are defined for equation 1. In equations 4 and 5, \( \text{app} K \) is the \( K_{F-6-P} \) obtained at any effector concentrations, \( K_m \) is the true \( K_{F-6-P} \), \( I \) and \( J \) are F-2,6-P\(_2\) and AMP, and the remaining constants are defined in Chapter IV. In equation 6, \( Y \) is the V/K or pK, \( C \) is the pH independent value of \( Y \), \( H \) is the concentration of hydronium ion, and \( K_1 \) is the acid dissociation constant for an ionizable group. The parameters for equation 7 are the same as for equation 6 with the following additions: \( K_2 \) is the base dissociation constant for the group titrating at alkaline pH, and \( K_0 \) is the product of the dissociation constants for the two groups titrating at acidic pH. In equation 8, \( v \) is the velocity, \( V \) is the maximum velocity, \( A \) and \( B \) are the concentrations of the substrates, \( K_{ia} \) is the dissociation constant for A from free enzyme, \( K_a \) and \( K_b \) are the Michaelis constants for A and B respectively.
CHAPTER III

ACTIVATION STUDIES WITH F-2,6-P₂ AND AMP

Activation of d-PFK by F-2,6-P₂ and AMP. It has been shown that the mechanism of action of allosteric effectors can be examined using initial velocity studies where the effector is treated as a pseudoreactant (Cook, 1982). Initial velocity patterns are obtained in the absence of effectors and in the presence of varied concentrations of effectors. The changes in the kinetic parameters V and V/K yield activation constants that are thermodynamic dissociation constants for given enzyme-effector complexes.

In this study, initial velocity patterns were obtained by varying MgATP and F-6-P at various fixed concentrations of AMP and F-2,6-P₂ (data not shown). It was found that the effect of AMP and F-2,6-P₂ is to lower K₆-P with no effect observed on any of the other kinetic parameters. It was thus possible to simplify the system by fixing the concentration of MgATP at 100 μM (10 times Kₐₗ₃₃₄₅₆₇₈₉) and treating the effector as a pseudoreactant. This was accomplished by measuring successive F-6-P saturation curves at various fixed concentrations of the effectors. A double reciprocal plot of one such study is shown in Figure 1.
Figure 1. F-2,6-P$_2$ Activation of d-PFK.

F-6-P saturation curves obtained at different fixed F-2,6-P$_2$ concentrations are presented in double reciprocal form. The line with the steepest slope is at zero F-2,6-P$_2$. The line with the least slope is at saturating F-2,6-P$_2$. The concentrations of F-2,6-P$_2$ used are given in Figure 2. For illustrative purposes, unweighted linear regression lines were used.
Figure 2. $K_{act}$ for F-2,6-P$_2$.

The $V/K$-6-P obtained from a fit of each line in Figure 1 using Equation 1 is plotted against the concentration of F-2,6-P$_2$. Data known were fitted using Equation 2 to obtain $V/K^0$ and $V/K^\infty$, the $V/K$ values for F-6-P at zero and infinite concentration of F-2,6-P$_2$, respectively. As presented, the data give the fractional activation as a function of F-2,6-P$_2$ concentration. On this scale, 1.0 is equal to 15.2 fold activation. The $K_{F-2,6-P_2}$ is 92 ± 7 nM.
Figure 3. $K_{act}$ for AMP.

The data in this figure were collected and treated in the same manner as in Figures 1 and 2 except $K_{p-6-p}$ was used directly instead of $V/K$ as explained chapter III. On this scale, 1.0 is equal to 5.5 fold activation. The $K_{AMP}$ is 93 ± 12 μM.
Figure 4. Circular Dichroism Spectra of d-PFK.

Circular dichroism spectra of d-PFK are shown for d-PFK (solid), d-PFK plus 100 μM AMP (short dash), d-PFK with 10μM F-2,6-P$_2$ (long dash). The inset gives the difference spectra between d-PFK without effectors and d-PFK with added AMP (short dash), and with added F-2,6-P$_2$ (long dash). The spectra were provided by Jaganatha Rao.
Figure 5. Double Activation Experiment.

Data were collected as for Figure 1, but with the experiment being repeated at varying AMP concentrations. The % activations were obtained as in Figure 2 except that $K^\infty$ was an estimate rather than a fitted value. The concentrations of AMP used are as follows: open circles, zero AMP, triangles, 80 µM AMP, squares, 200 µM AMP, and diamonds, 1000 µM AMP.
Scheme I. Double Activation Model.

Double activation model for double competitive activation by two K-type effectors as described in chapters III and IV.
Figure 6. Initial Velocity Pattern with pd-PFK.

Initial velocities were obtained as described in chapter II in the presence of cAPK, varying the concentration of MgATP at different fixed concentrations of F-6-P including from top to bottom, 70, 100, 150, and 1500 μM. A fit of the data using the equation for rapid equilibrium ordered gave the following kinetic parameters: $V_m = 0.0091 \pm 0.0006$ units, $K_{\text{MgATP}} = 6.5 \pm 1.2 \ \mu\text{M}$, $K_{i\text{F-6-P}} = 143 \pm 25 \ \mu\text{M}$. 
## Table I

*Summary of activation constants*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AMP</th>
<th>F-2,6-P&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{AMP}$</td>
<td>activity</td>
</tr>
<tr>
<td>n-PFK</td>
<td>13 ± 2</td>
<td>24.4</td>
</tr>
<tr>
<td>d-PFK</td>
<td>93 ± 12</td>
<td>5.5</td>
</tr>
<tr>
<td>pd-PFK</td>
<td>177 ± 22</td>
<td>13</td>
</tr>
</tbody>
</table>
As the concentration of F-2,6-P$_2$ is increased, the slope (K/V) decreases. Intersection of the lines on the ordinate indicates that V is not affected and that the effector is of the K-type. A replot of the fractional activation versus F-2,6-P$_2$ concentrations from the data in Figure 1 is shown in Figure 2 giving a K$_{F-2,6-P_2}$ of 92 ± 7 nM and a maximum stimulation of 15.2 fold. Results of a similar experiment with AMP shown in Figure 3 gives a K$_{AMP}$ of 93 ± 12 µM and a maximum stimulation of 5.5 fold. It is not possible to use V/K directly to obtain the activation constant since AMP causes some inhibition at very high concentrations as a result of competition with MgATP at the active site. The addition of higher MgATP overcomes the inhibition (data not shown). As a result, K$_{F-6-P}$ is a better indicator in the present case.

Circular Dichroism. AMP and F-2,6-P$_2$ cause similar changes in the CD spectrum of d-PFK. Figure 4 shows superimposed spectra of d-PFK obtained in the absence of effectors and in the presence of AMP and F-2,6-P$_2$ respectively. The inset shows the difference spectra between d-PFK without effector and d-PFK with added AMP and F-2,6-P$_2$.

Double Activation. As shown above, AMP and F-2,6-P$_2$ both decrease K$_{F-6-P}$. It is of interest to determine whether the effectors act independently or whether some interaction between the effectors is allowed. A double
activation experiment was carried out in which F-6-P was varied at several fixed concentrations of AMP and this was then repeated at several different concentrations of F-2,6-P$_2$. Figure 5 shows a plot of percent activation against F-2,6-P$_2$ concentration at several different fixed levels of AMP. For analysis of this type of data, a double activation model was developed that can distinguish between independence and synergism/antagonism. The model is presented as Scheme I and the derivation of the rate equation based on Scheme I is presented in chapter IV.

Similar double activation protocols were used by Uyeda et al. (1981) and Van Schaftingen et al. (1981) to study phosphofructokinase from rat liver and muscle, but only a qualitative treatment of the data was presented.

The interaction constants for the formation of E'I·J and E'A·I·J are $\alpha$ and $\alpha\delta$, respectively. These parameters are defined at intermediate to high concentrations of both effectors. Under these conditions, the change in the dependent variable ($K_{F-6-P}$) is small making it statistically difficult to define $\alpha$ and $\alpha\delta$. Data presented in Figure 5 and that from two similar experiments did not allow us to define $\alpha$ and $\alpha\delta$ when fitted using equation 4. The qualitative determination that synergism exists between the effectors was made from comparisons of line by line fits of the data of Figure 5 using equation 2. For example, the $K_{\text{AMP}}$ at zero F-2,6-P$_2$ is $104 \pm 36 \mu$M, in good agreement with
the value determined independently (see above), and at 50 nM F-2,6-P$_2$ the $K_{AMP}$ is 43 ± 19 μM. Similarly, the $K_{F-2,6-P2}$ decreases from 97 ± 47 nM at zero AMP to 47 ± 14 nM at 80 μM AMP.

**Phosphorylated d-PFK.** d-PFK is phosphorylated by cAPK to the same extent as is n-PFK in parallel experiments (data not shown). In this study, the amount of $^{32}$P incorporated was 0.6-0.8 mol phosphate/mol subunit. To avoid problems of additional dialysis, dilution of the enzyme, and the loss of activity associated with isolation of the phosphorylated enzyme, the method of in situ phosphorylation was employed for the kinetic studies of pd-PFK. This method also ensures that PFK is maximally phosphorylated for a given set of experiments, avoiding problems associated with lability of the phosphate on the enzyme. A potential problem in these experiments is the inherent ATPase activity of cAPK. Although this activity is very low, (1/1200)th the $V_{max}$ of protein or peptide phosphorylation, (Yoon and Cook, 1987), the activity was not measured in the milieu represented by the present reaction mixture. A control with PFK in the absence of F-6-P was carried out coupling the production of ADP to the pyruvate kinase and lactate dehydrogenase reactions. The preparation of cAPK used in these studies showed insignificant ATPase activity under these conditions.

The phosphorylated form of d-PFK, pd-PFK, is essentially a separate form of the enzyme and was treated as such in
kinetic analysis. The initial velocity pattern obtained with pd-PFK varying the concentration of MgATP at different fixed levels of F-6-P is shown in Figure 6. The double reciprocal plot exhibits a pattern intersecting on the ordinate characteristic of a rapid equilibrium ordered mechanism with F-6-P binding first. When a fit using the general equation for a sequential mechanism \( v = \frac{V_{AB}}{K_{iA}K_{B} + K_{BA} + K_{AB} + AB} \) was attempted, the fit did not converge. Parameter values are provided in the legend to Fig. 6. When subjected to activation studies similar to d-PFK, it was found that the pd-PFK is also activated by F-2,6-P₂ and AMP in a manner qualitatively identical. The results of these studies are summarized in Table I along with studies carried out for d-PFK and native PFK (n-PFK). In the case of n-PFK, the \( K_{\text{act}} \) for both effectors was determined from the variation in \( S_{0.5} \) values obtained at various effector concentrations.
CHAPTER IV

DERIVATION OF THE RATE EQUATION FOR DOUBLE COMPETITIVE ACTIVATION

A rate equation is derived for the case in which two effectors decrease the $K_m$ for a given substrate and thus exhibit competitive activation. In Scheme I, E actually represents E·MgATP, A represents F-6-P, and I and J are F-2,6-P$_2$ and AMP, respectively. As with a terreactant mechanism, three binary complexes, three ternary complexes, and one quaternary complex are potentially present. It is assumed that the binding of A, I, and J are in rapid equilibrium and the rate of catalysis ($k_3$) is the same regardless of which complex is turning over. The latter must be true for PFK since V is not affected by the presence of F-2,6-P$_2$ or AMP. If this were not the case, however, different rate constants would have to be assigned for turnover of each of the complexes. Changes in the derivation as a result of different rate constants for turnover will be pointed out below.

The above assumptions allow the reaction pathway to be described by a series of equilibrium dissociation constants except for the catalytic step (Cha, 1968). The rate equation is then derived based on the method of King and Altman (1956).
Figure 7. Theoretical Plot of $a_{pp}^{K_A}$ vs. I.
Figure 8. Theoretical Plot of % activation vs. I.
All steps except those represented by $k_3$ are contained in the rapid equilibrium segment $X$. The initial velocity is simply the sum of the products of $k_3$ and the fractional concentration of each complex that turns over. In the case where different rate constants are required for turnover, each of the $f$ values would be multiplied by a different constant giving a more complex rate equation.

$$v = k_3(f_{EA} + f_{EAI} + f_{EAJ} + f_{EAIJ}) \quad (9)$$

In equation 9, $f_i = [E_i]/[E_X]$ where $E_X$ is the sum of the concentrations of all enzyme forms in the rapid equilibrium segment $X$. In this case, $E_X$ is $E_t$ where

$$E_t = [E] + [EA] + [EI] + [EJ] + [EAI] + [EAJ] + [EIJ] + [EAIJ] \quad (10)$$

Substituting into equation 9 yields:

$$v/[E_t] = k_3([EA]+[EAI]+[EAJ]+[EAIJ])/([E]+[EA]+[EI]+[EJ]+[EAI]+[EAJ]+[EIJ]+[EAIJ]) \quad (11)$$

The concentration of each of the complexes listed can be expressed in terms of the dissociation constants of each of the complexes. Thus, $EA$, $EI$, $EJ$, $EAI$, $EAJ$, and $EIJ$ are equal to $[E][A]/K_A$, $[E][I]/K_I$, $[E][J]/K_J$, $[E][A][I]/\beta K_A K_I$, $[E][A][J]/\Gamma K_A K_J$, and $[E][I][J]/\alpha K_I K_J$, respectively. In the
expression for EAI, EAJ and EIJ, $\alpha, \beta$ and $\Gamma$ are the interaction coefficients between $A$ and $I$, $A$ and $J$, and $I$ and $J$.

Dissociation of the quaternary complex can proceed via several pathways giving three possible expressions for the concentration of $E\cdot A\cdot I\cdot J$. Each can be used in the derivation of the equation, yielding a final form with different constants in the cross terms for effector interactions. The equations can be interconverted by recognizing that the product $\alpha\delta = \sigma\Gamma$ is a condition of this model. For example, $[EAIJ]$ is equal to $[E][A][I][J]/\alpha\delta K_A K_I K_J$.

Substituting all of the above into equation 11 and factoring out $[E]$ gives:


(12)

Simplifying gives equation 13.
\[
\frac{v}{[E_t]} = \frac{k_3[A]}{\{(A)+K_a*(1+[I]/K_I+[J]/K_J+[I][J]/\alpha K_I K_J)/\}
\]
\[
(1+[I]/\beta K_I+[J]/\Gamma K_J+[I][J]/\alpha \delta K_I K_J})
\] (13)

The rate equation at zero effector concentration is the Michaelis-Menton equation. The apparent \(K_m\) at any effector concentration is given by equation 14.

\[
appK_A = K_A*(1+[I]/K_I+[J]/K_J+[I][J]/\alpha K_I K_J)/
\]
\[
(1+[I]/\beta K_I+[J]/\Gamma K_J+[I][J]/\alpha \delta K_I K_J)
\] (14)

A plot of \(appK_A\) versus \(I\) at different concentrations of \(J\) is hyperbolic beginning at zero \(I\) and asymptotically approaching a minimum at Infinite \(I\). Repeating this at different \(J\) values gives a series of hyperbolae. These data can then be fit using the Marquardt algorithm. An example of data for a case in which \(K_A\) is 1, \(K_I\) and \(K_J\) are 0.01, \(\alpha\), \(\beta\), and \(\Gamma\) are 0.5, and \(\delta\) is 0.48 is given in Fig. 7.

Another possible way of presenting the data is as a percent of total activation. In this case, one simply calculates \((K_A^0 - K_A)/(K_A^0 - K_A^\infty)\) where \(K_A^0\) and \(K_A^\infty\) are values of \(K_A\) at zero and infinite concentrations of the varied effector and \(K_A\) is the value observed at any effector concentration. This is done at each of the fixed \(J\) concentrations. In each case a hyperbola is obtained that
gives a value of 1 at infinite I concentration. Such a plot is shown in Figure 8 using the same values for parameters as Figure 7. These data can then be fitted as a hyperbola or in double reciprocal form.
CHAPTER V

ACTIVATION BY F-2,6-P₂ WITH VARIED PH

Effects of pH changes on d-PFK. All known PFK enzymes are pH sensitive. Since varying the pH from neutral to alkaline causes similar kinetic changes in PFK as is observed by the addition of effector(s), it is of interest to systematically study the action of effector(s) with varied pH. The kinetic theory applied to effector studies at varied pH is the same as described in chapter III provided that the mechanism does not change in the range of pH studied. Such mechanistic changes have recently been observed (Qamar and Cook, 1993) and must be accounted for if present. The relevant parameters obtained in such a study with respect to the effector will be the $K_{\text{act}}$ and the maximum activation obtained as a function of pH. To obtain those parameters, an entire activation study as shown in Figures 1 and 2 (chapter III) must be performed at each pH studied. Tertiary replots of $pK_{\text{act}}$ and fold activation versus pH can be used to obtain pKs for ionizable groups responsible for binding of the effector, the conformational changes brought about by binding of the effector, or changes in the pH dependence of events at the active site. The latter is observed in the course of the study since pH
profiles are obtained in the absence of effector and in the presence of saturating effector.

In the present study, initial velocity patterns in the absence of F-2,6-P$_2$ were obtained at the pH extremes of the study. The pattern obtained at pH 5.95 is shown in Figure 9. The lines intersect to the left of the ordinate diagnostic of a sequential mechanism as observed at pH 6.8 (Rao et al., 1987b). A fit of the equation for a steady-state ordered bireactant model (Equation 8) to these data gives the following parameters: $V = 0.023 \pm 0.003$ units (based on a 1:8 dilution of d-PFK from stock), $K_{P-6-P} = 7.9 \pm 1.6$ mM, $K_{ATP} = 36 \pm 8$ $\mu$M, $K_{IF-6-P} = 0.8 \pm 0.7$ mM, $K_{iATP} = 4 \pm 3$ $\mu$M. Figure 10 exhibits a similar pattern obtained at pH 8.85. A fit to these data as above gives the following parameters: $V = 0.0232 \pm 0.0017$ units (based on a 1:8 dilution of d-PFK from stock), $K_{P-6-P} = 0.597 \pm 0.097$ mM, $K_{ATP} = 41.7 \pm 6.9$ $\mu$M, $K_{IF-6-P}$ and $K_{iATP}$ were undefined. In both patterns, the lines are nearly parallel due to the use of reactant concentrations far greater than the $K_i$s. In fact, the pattern obtained at pH 8.85 fits the equation without the constant term ($K_iK_b$) equally well. Results similar to the above obtained with rabbit muscle PFK at pH 8 led Uyeda to conclude that the PFK reaction proceeded with a covalently bound phosphoryl-enzyme intermediate (Uyeda, 1970). No evidence for such an intermediate was ever found, however. In any case, the parameters $V$ and $K_{ATP}$ are not affected by changes in the pH.
Taken together with observations that $V$ and $K_{ATP}$ are not affected by the presence of saturating F-2,6-P$_2$ at pH 6.8, it was possible to simplify the experiments by fixing the concentration of MgATP at close to saturation and examining the variation of $K_{F-6-P}$ at varied pH and with varied concentrations of F-2,6-P$_2$. Data were collected and treated as previously described for pH 6.8 (see Figures 1 and 2, chapter III) at 0.5 pH unit intervals from approximately pH 6 to 9. The relevant parameters were then plotted against pH to obtain pKs for essential ionizable groups.

Figure 11 shows superimposed plots of log $V$ versus pH in the absence of F-2,6-P$_2$ and with 200 $\mu$M F-2,6-P$_2$ (known to be saturating across the range of pH studied). $V$ is independent of pH and the presence of F-2,6-P$_2$. Figure 12 shows pH profiles obtained in the absence and presence of 200 $\mu$M F-2,6-P$_2$. pH profiles were obtained for $V/K_{F-6-P}$.

Figure 13 is a treatment of the data in Figure 12 for which the ratio $K^0_{F-6-P}/K^\infty_{F-6-P}$ was calculated at each pH to give the fold activation as a function of pH. The above ratio is also the allosteric parameter (or interaction coefficient) $Q_{AX}$ as defined from thermodynamic linkage analysis (Symcox and Reinhart, 1992, Reinhart, 1983) where A represents F-6-P and X represents F-2,6-P$_2$. As shown in Figure 13, the fold activation is constant with varied pH. $Q_{AX}$ is related to the free energy of binding ($\Delta G$) of the effector which is the amount of energy released upon binding of F-2,6-P$_2$ provided
that it binds in rapid equilibrium (Symcox and Reinhart, 1992). The two are related as follows.

$$\Delta G_{AX} = -RT \ln Q_{AX}$$ (15)

Calculations based on the above, estimate the free energy of binding of F-2,6-P₂ to d-PFK to be 1.7 Kcal/mole at 30 °C in this system. Figure 14 shows \(pK_{F-6-p}\) in the absence of effector from the same data set plotted against pH. Figure 15 is an analogous plot in the presence of saturating effector. Since \(V\) is independent of pH and varies independently of \(K_{F-6-p}\), the pKs obtained will have the same interpretation as from a plot of \(\log(V/K)_{F-6-p}\) versus pH — namely that the pKs are the true pKs for the groups involved and that all groups must be in the correct protonation states for binding to occur (see discussion). Data from Figure 15 were used to obtain fits using pH profile equations for a single acidic proton, one acidic and one basic proton, and two acidic protons and one basic proton. For both the \(K^0_{F-6-p}\) data and \(K^\infty_{F-6-p}\) data, the best fit was obtained with the equation for titration of two acidic protons and one basic proton. The pKs thus obtained are given in the legends to Figures 14 and 15.

As a check of the validity of using determined values of \(K_{F-6-p}\) for pH profiles, the fitted parameters \(K^0_{F-6-p}\) and \(K^\infty_{F-6-p}\) obtained by fitting the activation data at each pH using Equation 2 and its rearranged form, Equation 16, to solve for \(K^\infty_{F-6-p}\).
Figure 9. Initial Velocity Pattern, d-PFK, pH 5.95.

The data were collected by varying the concentration of F-6-P at various fixed concentrations of MgATP. The data are presented in double reciprocal form. The concentrations of MgATP used were (circles) 10 μM, (inverted triangles) 20 μM, and (squares) 100 μM. The lines were calculated from a fit of the equation for a sequential mechanism (Equation 8) to the entire data set. The fitted parameter values are given in the text.
\[
\frac{[E_i]}{v} \text{ sec}
\]

\[
\frac{1}{[F-6-P]} \text{ mM}^{-1}
\]

\[\text{MgATP}\]
Figure 10. Initial Velocity Pattern, d-PFK, pH 8.9.

The data were collected by varying the concentration of F-6-P at various fixed concentrations of MgATP. The data are presented in double reciprocal form. The concentrations of MgATP used were (circles) 10 µM, (inverted triangles) 20 µM, (squares) 50 µM, and (triangles) 100 µM. The lines were calculated from a fit of the equation for a sequential mechanism (Equation 8) to the entire data set. The fitted parameter values are given in the text.
$V_{1/[F-6-P]} \text{ mM}$
Figure 11. Variation of V with pH.

The values of V used in this figure were obtained from fits of Equation 1 to F-6-P saturation experiments at each pH. The log of the maximum velocity is plotted against pH. Open circles are values obtained with 200 µM F-2,6-P₂. Inverted triangles are values obtained in the absence of effector. The lines are from linear regressions to the data.
Figure 12. Variation of $V/K_{F-6-P}$ with pH.

The values of $V/K_{F-6-P}$ used in this figure were obtained from fits of Equation 1 to F-6-P saturation experiments at each pH. Open circles are values determined with 200 µM F-2,6-P$_2$. Inverted triangles are values obtained in the absence of effector.
Figure 13. The maximum activation by F-2,6-P_2 plotted against pH.

The log(fold activation) was obtained from the data in Figure 12 by taking the logarithm of the ratio of the V/K_{F-6-p} in the presence of 200 µM F-2,6-P_2 to the V/K_{F-6-p} with F-2,6-P_2 absent. The line is from a linear regression to the data.
Figure 14. The variation of $pK_{F-6-P}$ with pH in the absence of effector.

The values of $K_{F-6-P}$ used in this figure were obtained from fits of Equation 1 to F-6-P saturation experiments at each pH. The parameters are from the same primary data used in Figures 11 and 12. The line is from a fit of Equation 7 to the data. The pKs obtained from the fit are: $pK_1 = 6.2 \pm 0.3$, $pK_2 = 6.8 \pm 0.3$, and $pK_3 = 8.6 \pm 0.2$. 
Figure 15. The variation of $pK_{F-6-P}$ with pH in the presence of saturating effector.

The values of $K_{F-6-P}$ used in this figure were obtained from fits of Equation 1 to F-6-P saturation experiments at each pH. The parameters are from the same primary data used in Figures 11 and 12. The line is from a fit of Equation 7 to the data. The pKs obtained from the fit are: $pK_1 = 6.4 \pm 0.6$, $pK_2 = 6.4 \pm 0.6$, and $pK_3 = 8.9 \pm 0.4$
Figure 16. The variation of $pK_{ID}$ with $pH$.

These data are a tertiary replot of data from activation studies performed as in Figures 1 and 2 and repeated at each $pH$ shown. The values for $K_{ID}$ were obtained from a fit of Equation 2 to values of $a_{PP}K_{F-6-P}$ from individual saturations done at various intermediate concentrations of $F-2,6-P_2$. The line is from a fit of equation 6 to the $K_{ID}$ values. The $pK$ obtained was $7.4 \pm 0.1$. The $pH$ independent value of $K_{ID}$ is $0.20 \pm 0.18$ $\mu$M.
Figure 17. The variation of $pK_{IN}$ with pH.

These data are a tertiary replot of data from activation studies performed as in Figures 1 and 2 and repeated at each pH shown. The values for $K_{IN}$ were obtained from a fit of Equation 2 to values of $a_{PP}K_{F-6-P}$ from individual saturations done at various intermediate concentrations of $F-2,6-P_2$. The line is from a fit of equation 6 to the $K_{IN}$ values. The $pK$ obtained was $7.5 \pm 0.2$. The pH independent value of $K_{IN}$ is $2.9 \pm 0.3 \mu M$. 
Figure 18. The ratio $K_{IN}/K_{ID}$ plotted against pH.

Data from Figures 16 and 17 were used to take the ratio $K_{IN}/K_{ID}$ at each pH. This ratio is $\beta$ - the interaction constant between F-6-P and F-2,6-P$_2$. The line is a regression line fitted to the data.
Equation 16 is obtained by taking the limit of Equation 2 as I goes to infinity and substituting back into equation 2. A comparison of the fitted and determined values are in close agreement. Other parameters obtained in the above fits are $K_{ID}$ ($K_{act}$) and $K_{IN}$ (a parameter which causes the $appK_{F-6-P}$ to level off at a finite value). Figures 16 and 17 show the variation of $K_{ID}$ and $K_{IN}$ with pH. In Figure 18, the ratio of $p(K_{IN}/K_{ID})$ versus pH is shown. That the above ratio is constant with varied pH is consistent with the finding that the maximum fold activation is also constant, and is another indicator that the models used accurately describe the determined values.
CHAPTER VI

DISCUSSION

Effects of F-2,6-P$_2$ and AMP. The d-PFK, devoid of allosteric inhibition by ATP, is still activated by F-2,6-P$_2$ and AMP. This suggests a duality of function for the effectors, i.e. the relief of ATP inhibition, and an increase in the affinity for F-6-P. With the native form of PFK, it is difficult to distinguish between the two roles since relief of ATP inhibition accompanies a dramatic decrease in the $S_{0.5}$ for F-6-P and the Hill number (Uyeda, 1979, Hofer et al. 1982a, Srinivasan et al., 1990). As a result, the effects of AMP and F-2,6-P$_2$ have been generally interpreted in relation to the antagonism of ATP allosteric effects (Kemp and Foe, 1983, Uyeda, 1979). F-2,6-P$_2$ and AMP act upon the same kinetic parameter, $K_{F-6-P}$. That they appear to do so synergistically suggests that they have separate binding sites. The synergism could either be the combination of a direct effect of the binding of one effector on the binding of the second and a link through the F-6-P portion of the active site or the latter alone. That there is a link through the active site comes from thermodynamic considerations. If an allosteric effector increases the affinity of enzyme for substrate, the
substrate must also to the same extent affect the binding of the allosteric effectors. Thus, when two effectors increase the affinity of enzyme for substrate, it follows that, through the active site modulation, the allosteric sites are linked unless there are two separate and quite different active forms of the enzyme obtained as a result of the binding of the two effectors. The latter is unlikely in the case of the Ascaris PFK since very similar changes in the circular dichroism spectra are observed upon binding of F-2,6-P$_2$ or AMP to d-PFK. This is supported by CD studies of n-PFK in which larger changes are observed than those observed for d-PFK (Rao et al., 1991). The binding of AMP and F-2,6-P$_2$ to n-PFK also cause very similar changes in the fluorescence spectra of n-PFK.

Based on the above considerations, it follows that the E'A'I'J and E'I'J complexes (see chapter IV) are allowed and the E'A'I'J complex is kinetically competent. The difference in affinity for F-6-P between the E'I or E'J complex and the E'I'J complex is small relative to the difference between E and E'I or E'J, and, as a result, is difficult to see kinetically as stated in chapter III. Most of the activation is realized by the independent effect of F-2,6-P$_2$ or AMP when either is present at a saturating concentration. Thus, the proportion of complexes that have high affinity for F-6-P will be determined mainly by the sum of the contributions of each effector. In this experimental
system, the interpretation is complicated somewhat by the fact that AMP is capable of decreasing $K_{\text{F-6-P}}$ 5.5 fold while F-2,6-P$_2$ decreases $K_{\text{F-6-P}}$ by about 15 fold. When non-saturating concentrations of one of the effectors is present, addition of the other will result in a further decrease in $K_{\text{F-6-P}}$. However, when 10 $\mu$M F-2,6-P$_2$ is present, the addition of AMP has no effect (data not shown). Apparently, saturating F-2,6-P$_2$ provides maximum activation. These data suggest that heterotropic activation of d-PFK does not adhere to a single R/T model as suggested by Monod et al. (1965). If that were the case, saturating concentrations of either of the effectors should drive the enzyme completely to the R state - an effect not seen with AMP. A similar phenomenon has been observed with the native form of the closely related PFK from *Dirofilaria immitis* (Srinivasan et al., 1990). At zero effector concentration, the $S_{0.5}$ for F-6-P is 17.4 mM. At 1 mM MgATP, 10 $\mu$M F-2,6-P$_2$ lowers the $S_{0.5}$ for F-6-P to 98 $\mu$M while 1 mM AMP lowers $S_{0.5}$ for F-6-P to 360 $\mu$M. The $S_{0.5}$ for F-6-P with 1 mM AMP and 10 $\mu$M F-2,6-P$_2$ is 30 $\mu$M implying that synergism between the effectors also occurs with the *D. immitis* PFK.

Product inhibition patterns of FBP vs. MgATP obtained for d-PFK in the presence of saturating F-2,6-P$_2$ were uncompetitive (data not shown). This pattern is diagnostic of an ordered mechanism with MgATP binding first and further suggests that FBP has very low affinity for E·MgADP (Rao et
al., 1987b). Thus, the influences of the effectors alone are insufficient to cause a change in mechanism, although they increase the affinity of E'MgATP (and likely also E) for F-6-P. Most likely, the mode of action of the effectors is to decrease the off-rate for F-6-P and perhaps the first product released.

Effects of phosphorylation. Phosphorylation of d-PFK by the catalytic subunit of cyclic AMP dependent protein kinase greatly lowers $K_{IF-6-P}$ (where $K_{IF-6-P}$ represents the dissociation constant for E*F-6-P) to about 150 $\mu$M from a value of 50 mM estimated for the non-phosphorylated form of d-PFK (Rao et al., 1987b). As a result, the alternate pathway with F-6-P binding first becomes preferred. The above behavior represents an apparent change in mechanism from the predominantly steady state ordered mechanism with MgATP binding first reported for d-PFK (Rao et al., 1987b).

When pd-PFK is assayed at 100 $\mu$M MgATP (10 times $K_{MgATP}$), it is activated by F-2,6-P$_2$ and AMP as is the d-PFK. Therefore, phosphorylation of d-PFK is not sufficient to drive the enzyme into a fully activated state in the presence of saturating MgATP. These results also argue against a simple R/T equilibrium.

Effects of varied pH. The experiments described in chapter V yield information about both the acid-base chemistry at the active site and the optimum protonation state for binding of F-6-P at the active site and F-2,6-P$_2$
at the allosteric site. The observation that \( V \) is invariant with varied \( \text{pH} \) indicates that all essential ionizable groups on enzyme and substrates must be in their correct protonation states for binding and subsequent catalysis to occur. The variation in \( pK_{F-6-P} \) with \( \text{pH} \) yields two acidic \( pK_s \), \( pK_1 = 6.2 \), and \( pK_2 = 6.8 \), and one basic \( pK \), \( pK_3 = 8.6 \). The acidic groups must be deprotonated, while the basic group must be protonated for binding and/or catalysis to occur. Based on the geometry of the active site in the crystal structure of E. coli PFK in the presence of its reaction products (Shirakihara and Evans, 1988), and the observation that phosphoryl transfer in most phosphotransferases (Cullis, P. M., 1987), including PFK from Bacillus stearothermophilus and rabbit skeletal-muscle (Jarvest et al., 1981), occurs with inversion of configuration of the transferred phosphoryl group, a mechanism that is consistent with the observations is shown in Mechanism I.

In Mechanism I, the reactants are shown immediately prior to reaction and the products are shown immediately after reaction. At least two groups on enzyme are required to be in specific protonation states. One is the general base which must be unprotonated in order to accept the proton from the \( C_1 \) hydroxyl of F-6-P (polarizing the O-H bond) to facilitate nucleophilic attack on the \( \Gamma \)-phosphorous of ATP. An electrophilic group on the enzyme is also needed
to decrease the electron density around the γ-phosphorous of ATP to make the group more susceptible to the nucleophilic attack. The latter group, or one in close association would need to be protonated to fulfill its function. The pK₃ of 8.6 is assigned to this function.

The lower pKs are more difficult to assign. pK₂ probably belongs to the essential histidine observed by Rao et al. (1987) in inactivation experiments using DEPC. The residue showed a pK of 6.4 in the free enzyme under the reaction conditions. The phosphates of ATP should have pKs < 5.5 while the 6-phosphate of F-6-P should have a pK slightly above 6. Based on the above, pK₂ is assigned to the general base, and pK₁ is assigned to the 6-phosphate of F-6-P.

The pH dependence of the activation by F-2,6-P₂ reveals important information about the action of the effector. First, the observation that the maximum activation (which is related to the free energy of binding) is constant pH implies that the allosteric transition induced by the effector is pH insensitive. That the pKs observed for the binding of F-6-P are not perturbed by the presence of the effector further suggests that the allosteric transition acts in a way only to facilitate access to the active site for F-6-P. The allosteric transition must be carefully positioned so as not to affect the binding of MgATP.

Circular dichroism spectra have been obtained for n-PFK
MECHANISM I

Proposed Acid-Base Mechanism for Ascaris PFK.
at pH 6 and pH 8 in the absence and presence of saturating F-2,6-P$_2$ (Rao, G. S. J., unpublished observations). It was found that raising the pH from 6 to 8 caused an opposite shift in ellipticity to that observed after addition of the effector at pH 6. The change upon addition of effector at pH 8 was present, but reduced in magnitude. In contrast, spectra of d-PFK were the same at pH 6 and 8 and were very similar to the spectra of n-PFK at pH 8. Apparently d-PFK is locked in a pH activated conformation. Taken with the activation studies presented in this dissertation, it is evident that changes in pH and the effectors have different modes of action toward PFK activation, and consequently induce (or stabilize) different conformations of the enzyme. It would be interesting to see the nature of the effector induced conformational change which subtly perturbs the secondary structure yet releases a reasonable amount of free energy and is pH insensitive. A possible explanation is that the conformational change induced by the binding of F-2,6-P$_2$ is transmitted almost entirely through the interior of the protein, shielded from solvent.

The pH dependence of F-2,6-P$_2$ activation can be explained by the titration of a group with a pK of about 7 at the F-2,6-P$_2$ site responsible only for binding of the effector and not the allosteric transition. This group must be unprotonated for binding to occur. This is likely to be a group on the enzyme, as the pKs of the F-2,6-P$_2$ phosphates
should be much lower. This group probably hydrogen bonds to one of the free hydroxyls of F-2,6-P$_2$. The information on the acid-base chemistry of effector binding is summarized in Mechanism II.

The PFK from *Escherichia coli* has also been studied with respect to the pH dependence of its kinetic properties (Deville-Bonne et al., 1991, Auzat and Garel, 1992). The pH dependence of the forward reaction kinetic parameters show notable differences compared to the *Ascaris* enzyme. First, the apparent affinity for F-6-P as measured by $S_{0.5}$ is invariant across the pH range 6 to 9 in the absence and the presence of the activator, GDP, although the affinity of enzyme for F-6-P was about 4 fold higher in the presence of GDP. GDP also drives the Hill coefficient ($n_H$) to a value close to 1 across the entire pH range. In the absence of GDP, $n_H$ increases from about 2.5 at pH 6 to about 5.5 at pH 9. Also, the catalytic rate constant ($k_{cat}$) increased over the pH range 6 to 9 with the titration of one group with a pK of 7.1 ± 0.1. The presence of GDP shifted the pK to 6.6 ± 0.1 and increased $k_{cat}$ by 15-20%. The presence of the inhibitor PEP reduced $k_{cat}$ and gave a pK of 7.0 ± 0.2. The activation of $k_{cat}$ by GDP was accounted for by the increase in the relative proportions of active enzyme at lower pH brought about by the shift in a critical pK. The pK of the group was found to be independent of the concentration of F-6-P suggesting that it was not involved in the binding
MECHANISM II

Proposed F-2,6-P2 Binding Mechanism for Ascaris PFK.
of F-6-P.

Allosteric models. The PFK from *Escherichia coli* has been demonstrated through crystal structure studies to have at least two separate conformational changes occurring: one change restricted to the active site area caused by the binding of MgATP ("open" or "closed" conformations) (Shirakihara and Evans, 1988), and another subunit-subunit twist caused by binding at the effector site (Schirmer and Evans, 1990). Rypniewski and Evans (1989) have also isolated an unliganded form of *E. coli* PFK which appears to be in an active conformation. This species should have been a very minor component of the total number of conformations present in solution and should not have crystallized. However, several possible explanations were put forward by Rypniewski and Evans which are consistent with the R/T model. Early conformational studies of rabbit muscle PFK (Gottschalk and Kemp, 1981) indicated that occupancy of an effector site did not necessarily cause a conformational change as monitored by thiol reactivity. Those results were interpreted to mean that an induced change could be caused by the binding of an effector instead of the perturbation of an R/T equilibrium (Kemp and Foe, 1983). Kemp and Foe also pointed out that sugar bisphosphates competing for the same effector site activate the rabbit muscle enzyme to different extents - similar to the differences in AMP and F-2,6-P$_2$ activation presented here. In principle, the presence of
multiple effector sites and the discrete effector domains in the larger PFKs (Poorman et al., 1984) allows for numerous active conformations with differing affinities for substrates and differing catalytic activity. A model for PFK activation by allosteric effectors should not be limited by the constraints imposed by an R/T model. To be accurate, a model must be able to account for factors such as differing maximal activation by effectors and activation distinct from the relief of ATP inhibition.

This study and others have revealed a complex set of allosteric linkages and multiple active forms of the Ascaris PFK. In addition to n-PFK, there are at least two chemically modified forms of the enzyme, d-PFK (locked in an active form) and o-PFK (locked in a less active form (Rao et al., 1991a)) which mimic possible natural conformations of the enzyme. Also, the effects of F-2,6-P_2, AMP, ATP, and pH have been studied in detail. A linkage model incorporating at least this subset of interactions is presented as Mechanism III.

The basis for this representation is the concept that thermodynamic links can be represented by a series of mechanical links attached to levers of varied lengths to illustrate the relative strengths of the effects. The binding of a ligand can be thought of as applying a "force" to its binding site. The force is transmitted to the levers through displacement of the mechanical linkages.
Conceptually, it is acceptable to think in terms of a static force applied by a ligand to its binding site. For calculation purposes, it must be realized that the net force applied to a binding site actually depends on the relative on and off rates, along with the concentration, of the ligand.

This method of describing allosteric linkages in terms of mechanics has potential for assisting with the interpretation of molecular movements within crystals which are beginning to be described in terms of torque applied to various bonds (Liang, 1992). The torque is interpreted as being relieved by the displacement of atoms in a transition to a new conformation. For example, F-2,6-P$_2$ binding to its site would cause displacement of the center lever at the active site, thus allowing F-6-P better access to the active site. This would be expressed as increased affinity for F-6-P. The F-6-P would then be able to exert more force on other levers affecting its affinity. Among other things, F-6-P would exert a counter force on the lever linked to the ATP inhibitory site. This counter force would be transmitted through the cooperativity link to increase the affinity of another active site for F-6-P. This model reflects the results of this study by showing the effects of AMP and F-2,6-P$_2$ as separate from the ATP inhibition. The effects are linked indirectly since any affect on the affinity of the enzyme for F-6-P will be transmitted via the
MECHANISM III

A Mechanical Linkage Model for *Ascaris* PFK Allosteric Regulation.
extra force exerted by F-6-P upon binding to the active site. If F-2,6-P$_2$ activates to the same extent in n-PFK through the primary link (about 16 fold), it should require about a 16 fold higher concentration of ATP to inhibit in the presence of the effector at a given pH. Without the existence of d-PFK, the nature of the interaction between the positive effectors and the ATP inhibition would be very difficult to discern.

It is evident from the model that the cooperativity of F-6-P binding is also only indirectly related to the positive effectors. That is consistent with the observation that d-PFK is noncooperative with respect to F-6-P binding. Since n-PFK is also largely noncooperative at pH 8, it appears that the essential group at or near the ATP inhibitory site (probably a histidine) is not only responsible for allowing ATP inhibition, but also establishes cooperativity. Protonation of this group establishes an inhibited conformation of the enzyme which is stabilized in the presence of ATP. In mechanical terms, the ATP simply increases the strength of the holding force applied to the link already displaced by H$^+$. 

It has been pointed out on several occasions by myself and others, that the data collected for PFK appears to be inconsistent with the MWC concerted transition model originally proposed as a mechanism explaining the allosteric properties of PFK (Blangy, et al., 1968). The statements
have generally been made without properly examining the
postulates of that model and other alternatives such as the
KNF model. Wyman and Gill (1990) point out that both the
MWC and KNF models for allosteric enzymes are actually
restricted versions of the general allosteric model (based
on concepts introduced by Adair). Essentially, the MWC
model allows for only two states of the enzyme: T (inactive)
and R (active). A ligand binds preferentially to one or the
other of the states, possibly affecting the R/T equilibrium
and the subsequent binding of other ligands. A hallmark of
this model is that both the R and T forms are present in the
absence of ligand. In a multimeric protein, the transition
from R to T occurs simultaneously for all subunits, a
"concerted transition". In the KNF model, the binding of a
ligand allows the formation of a previously inaccessible
conformational state of the enzyme which may, via an
"induced fit", have altered affinity for the next ligand.

It is clear that while the presence of multiple active
enzyme forms as seen with Ascaris PFK, and the existence of
the noncooperative, allosterically regulated d-PFK,
disallows the simple MWC model, the general allosteric model
can account for such a situation. However, the general
allosteric model, applied without restrictions, has so many
parameters that many may not have physical meaning. Any
reasonable allosteric model of this sort is dependent on
physically and/or kinetically definable "states" of the
enzyme. For PFKs from rabbit muscle, extensions of the simple MWC model have been formulated (Frieden et al., 1976, Pettigrew and Frieden, 1979a, 1979b, Goldhammer and Hammes, 1978). Although these models are reasonable, they all are constrained by the assumption of a pre-existing equilibrium of conformers with a fully active R reference state and an inactive T state.

An alternative approach to studying allosteric behavior has been developed by Reinhart (1983) which combines ideas from thermodynamic linkage analysis with those of initial rate steady state kinetics. Reinhart's approach has the advantage of examining ligand-ligand interactions directly in terms of free energy, independent of the pathway by which the energy is released. Thus, no a priori assumptions are made about the existence of any particular conformations of the enzyme or their relative activities. This approach has been used to describe the allosteric behavior of rat liver PFK in detail (Reinhart, 1985, Reinhart and Hartleip, 1986, 1992).

The approach used in this study is very similar to that used by Reinhart but is more straightforward. Treating an effector as a pseudoreactant allows the interpretation of results with only limited extension of existing kinetic theory. A limitation of this approach is that the system should be noncooperative for ease of interpretation of the parameters, although general comparisons to parameters
obtained with a cooperative system can be made (see Table I). This approach, applied to d-PFK, supplemented by physical studies and the kinetics of n-PFK, has allowed the development of Mechanism III. This represents the most complete description of Ascaris PFK regulation available to date.
REFERENCES


