Studies Of Phosphofructokinase In Human Erythrocytes

Lilian Miu Lee

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STUDIES OF PHOSPHOFRUCTOKINASE
IN
HUMAN ERYTHROCYTES

by

Lilian Miu Yee Lee
M.Sc.

Department of Pathological Chemistry

A thesis submitted in partial fulfilment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

FACULTY OF GRADUATE STUDIES
THE UNIVERSITY OF WESTERN ONTARIO
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<tr>
<td>PFK</td>
<td>phosphofructokinase</td>
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<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td></td>
<td>phosphate</td>
</tr>
<tr>
<td>F6P</td>
<td>fructose-6-phosphate</td>
</tr>
<tr>
<td>FDP</td>
<td>fructose-1,6-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
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<tr>
<td>Cyclic-3',5'-AMP</td>
<td>adenosine-3',5'-cyclic monophosphate</td>
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<tr>
<td>IMP</td>
<td>inosine-5'-monophosphate</td>
</tr>
<tr>
<td>IDP</td>
<td>inosine-5'-diphosphate</td>
</tr>
<tr>
<td>ITP</td>
<td>inosine-5'-triphosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine-5'-monophosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine-5'-monophosphate</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine-5'-diphosphate</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>diethyl amino ethyl cellulose</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose-6-phosphate</td>
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<tr>
<td>CTP</td>
<td>cytidine-5'-triphosphate</td>
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ABSTRACT

Phosphofructokinase (PFK) is recognized as one of the few rate-limiting glycolytic enzymes in the human erythrocyte. In the present study, a method involving DEAE-cellulose chromatography and ammonium sulphate fractionation is described for the preparation of relatively pure enzyme in reasonable quantities.

Optimal activity of purified PFK was observed between pH 8.2 and 8.5. In the analytical ultracentrifuge, the enzyme sedimented as a single boundary with $s_{20,w}$ values between 4.80 and 8.50 and exhibited a concentration dependence. The sedimentation coefficient extrapolated to zero concentration was approximately 2.2. On disc gel electrophoresis, two protein bands corresponding to enzyme activity were located.

The kinetic behavior of purified PFK was studied under different conditions. At pH 8.4, purified PFK exhibited Michaelis-Menten kinetics with respect to ATP, F6P and Mg$^{2+}$ when two of these substrates were present at saturating concentrations. Michaelis constants for these substrates were determined. The enzyme was inhibited by high concentrations of ATP and citrate. At this pH, the enzyme was totally insensitive to adenine nucleotide activation. In the presence of low (non-saturating) concentrations of substrates, 'apparent' negative homotropic interactions were observed.

At pH 6.8, the enzyme was very sensitive to ATP inhibition and exhibited 'apparent' negative cooperativity with respect to F6P, Cyclic-3',5'-AMP, AMP and, to a lesser extent, ADP were effective
activators of PFK between pH 6.8 and 7.0. The activating effects were competitive with respect to ATP suggesting that the enzyme has a regulatory (allosteric) binding site for ATP which is quite different from its substrate site.

Photooxidation of purified PFK in the presence of methylene blue and light at non-saturating concentrations of substrates shifted the 'apparent' negative cooperativity to what appeared to be first order kinetics at both pH 8.4 and 6.8. Analyses of the kinetic data by means of the Hill equation revealed that the apparent order of reactions with respect to substrates was less than unity.

Molecular weight determinations indicated that the enzyme exists in a tetramer-dimer-monomer equilibrium. Under normal conditions of isolation, the predominant form of the enzyme is a tetramer (M.W. approximately 80,000) which could dissociate into a dimer (M.W. approximately 40,000). Low temperature, saturating concentrations of ATP and photooxidation stabilized the tetrameric form of the enzyme while F6P stabilized the dimeric form. Large macromolecules (M.W. approximately 200,000) were obtained in the presence of inhibitory concentrations of ATP. Under slightly acidic conditions (pH 6.8), the dimer could be dissociated into two 'subactive' monomers (M.W. approximately 20,000). The equilibrium between the dimer and the 'subactive' monomer appeared to be a function of pH. Inactivation and reactivation studies indicated that this equilibrium was not completely dynamic. Urea and guanidine hydrochloride cleaved the active enzyme into inactive subunits with a molecular weight of approximately 20,000.
At pH 8.4, PFK appeared to be regulated by an association-dissociation equilibrium. At pH 6.8, the regulation of PFK kinetics seemed to be exerted through changes in the facility of binding of substrates and/or activators to the enzyme. When the regulatory site is occupied by ATP, the addition of cyclic-3',5'-AMP or AMP to the reaction mixture caused a competitive exclusion of ATP from the regulatory site. Photooxidation of the enzyme destroyed this allosteric site and the enzyme became insensitive to adenine nucleotide control. The present investigation suggests that the probable physiological significance of PFK regulation is to generate energy and valuable glycolytic intermediates for the mature human erythrocyte.
INTRODUCTION

1. Cellular energetics in mature erythrocytes

The mature mammalian erythrocyte depends on the metabolism of glucose by way of glycolysis for its source of energy. Since the cell has no nucleus, endoplasmic reticulum or mitochondria, it is incapable of de novo biosynthesis of protein (1-2), nucleotides (3), and lacks a tricarboxylic acid cycle (4) and electron transport system (5). The Embden-Meyerhof and pentose phosphate pathways for carbohydrate metabolism have been shown to be intact and functional in human normocytes (6-10).

It has been estimated that under physiological conditions, about 90% of the glucose consumed by the red cell is metabolized via the Embden-Meyerhof pathway to lactic acid, resulting in a net production of two molecules of ATP per molecule of glucose metabolized (11-13). The other 10% of the glucose is channelled through the pentose phosphate pathway (11, 14-15) which generates NADPH (11, 14-16). Although NADPH is a source of energy in most other tissues, the mature red cell (17) lacks the mechanism, e.g., oxidative phosphorylation, for utilizing this energy. NADPH has been shown to participate in the following reactions: the reduction of methemoglobin to hemoglobin catalyzed by NADPH-methemoglobin reductase (18), the reduction of oxidized glutathione catalyzed by glutathione reductase (19) and in the protection of the cell against various oxidants, most probably mediated through
the action of NADPH-methemoglobin reductase (18, 20). Thus, the mature red cell is entirely dependent on the Embden-Meyerhof pathway as a source of energy supply.

The rate of glycolysis in the mature human erythrocyte appears to be regulated by the action of a few rate-limiting enzymes. From the determination of glycolytic intermediates and nucleotides in circulating erythrocytes, and by thermodynamic calculations, Minakami et al. (21) and Minikami and Yoshikawa (22) have shown that the reactions catalyzed by the enzyme hexokinase, phosphofructokinase and pyruvic kinase are rate-limiting. There is some evidence that the reactions catalyzed by glyceraldehyde phosphate dehydrogenase (23) and phosphoglycerate kinase (24) may be rate-limiting also. Since the mature erythrocyte obtains energy solely from glycolysis, the regulation of this pathway is obviously of prime importance to cellular function and survival.

II. Regulatory (allosteric) enzymes

A large number of different regulatory (allosteric) enzymes show many structural and kinetic characteristics that are generally atypical of other enzymes. The term allosteric enzyme is usually used to describe an enzyme which (i) contains a site (allosteric site) which lacks catalytic activity and is topologically different from the active site, (ii) possesses multiple subunits, and (iii) occurs at a branch point in a metabolic pathway.
The homeostatic regulation of enzyme catalytic efficiency is mediated by changes in the concentration of substrates, coenzymes, activators or inhibitors. The activity of some key regulating enzyme is modulated by low molecular weight allostERIC effectorS (modifiers) which usually have little or no structural similarity to the substrates or coenzymes for the regulatory enzyme. According to Koshland (25), an allostERIC effeCt is defined as the effect of a modifier bound at a site which is topologically distinct from the active site at which reaction is occurring and which influences the binding or activity at this site by indirect means. AllostERIC effecTS resulting from interaction between identical ligands (i.e., a substrate binding to the enzyme at more than one site in the absence of other modifiers) have been termed homotropic effeCts which are almost always cooperative in nature. AllostERIC effecTS resulting from interactions between different ligands are termed heterotropic effeCts which may cause either activation or inhibition. AllostERIC effectors may influence enzyme activity by altering either the affinity of the activator site for substrate or the rate of conversion of substrate via the enzyme-substrate complex to products or by both of these mechanisms. Generally, heterotropic effeCts are partially competitive and may be relieved at higher substrate concentrations.

A. Classification of allostERIC enzymes

AllostERIC enzymes may be classified by their initial velocity response into three types (Fig. 1):
Fig. 1. Classification of allosteric enzymes.

Class 1. Positive cooperativity (sigmoid response)

Class 2. Negative cooperativity (substrate activation)

Class 3. Hyperbolic response
   (a) Substrate non-cooperativity
   (b) Effector (inhibitor or activator)-dependent substrate cooperativity

A: activator

I: inhibitor
1. **Positive cooperativity** (sigmoid response):

   The substrate concentration-velocity plot in this class of enzyme illustrates sigmoidal curves which normally indicate that at least two molecules of substrate interact with the enzyme and that the binding of one molecule in some manner facilitates the binding of the next, i.e., there is a cooperative effect in the binding of more than one substrate molecule to the enzyme. In the presence of **saturating** concentrations of enzyme modifiers (e.g., activator or inhibitor) the sigmoidal curves become hyperbolic. If the inhibitor is present in **less than saturating** concentrations, the substrate concentration-velocity curves become more sigmoid. It has been shown that the sigmoidal kinetics of PFK from sheep heart is converted to a rectangular hyperbola in the presence of non-saturating concentrations of F6P when one of its activators - cyclic-3',5'-AMP - is added (26). There are a few exceptions to this type of behavior, e.g., allosteric activators fail to convert the cooperative curve for substrates into a hyperbola in PFK isolated from *E. coli* (27).

2. **Negative cooperativity** (substrate activation) (28):

   Enzymes in this class exhibit two plateau regions in the substrate concentration-velocity curves and biphasic double reciprocal plots with two $K_m$ values. Negative cooperativity is characteristic of many regulatory enzymes with regulatory
sites, e.g., CTP synthetase (28), glutamic dehydrogenase (29), aspartyl transcarbamylase (30) or without, e.g., glyceraldehyde-3-phosphate dehydrogenase (31). In this class of enzyme, the binding of a modifier to the enzyme molecule decreases the affinity of the vacant sites of the neighboring subunits, causing cooperativity in a negative fashion. In most cases, negative cooperativity is explained by specific ligand-induced conformational changes on the enzyme molecule.

3. Hyperbolic response which may be divided into two sub-classes:
   a) Substrate non-cooperativity
      The enzymes in this class show no homotropic interactions of the substrate even in the presence of activator or inhibitor. Homotropic or cooperative interaction is seen with the inhibitor alone and during the course of enzyme catalysis only the $V_{max}$ is altered.
   b) Effector (inhibitor or activator)-dependent substrate cooperativity
      The substrate concentration-velocity plot is hyperbolic but in the presence of inhibitors or activators these curves become sigmoid. Unlike Class 3(a) enzymes, both activators and inhibitors show cooperativity for the substrate. Kinetic transitions alter the $K_m$ for substrate but the $V_{max}$ may or may not be affected.

B. Properties of allosteric enzymes

Some general properties of allosteric enzymes are:
1. **Desensitization**

   Treatment with mercurials, urea, proteolytic enzymes, high or low pH, etc. may produce loss of feedback regulatory control with retention of catalytic activity. Both homotropic and heterotropic interactions are lost usually as a consequence of alterations of protein structure.

2. **Heat stability**

   Most allosteric effectors, and in some cases, the substrates for the catalyzed reaction, confer enhanced resistance to heat denaturation of the allosteric enzyme. It is assumed that the protective effects are attributable to increased stability of the catalytic site as a consequence of conformational changes that accompany the binding of effectors at the allosteric site.

3. **Quaternary structure**

   All known allosteric enzymes are composed of more than one subunit, e.g., glycogen phosphorylase (32), pyruvate carboxylase (33) and acetyl CoA carboxylase (34). Some allosteric enzymes undergo reversible association-dissociation reactions of subunits, which may or may not be accompanied by a loss in catalytic activity or marked changes in susceptibility to allosteric effectors. The ability of various effector substances to induce changes in the state of aggregation has been speculated as a feasible mode for the regulation of enzyme activity.
Though not a general property, it is interesting to note that many regulatory enzymes undergo reversible inactivation (loss of catalytic activity) at 0-4°. In some cases this is accompanied by either dissociation or association of the enzyme which may be prevented by the presence of allosteric effectors. In other cases the enzyme becomes more sensitive to allosteric inhibition at low temperatures.

C. \textit{Models of allosterism}

Within the past decade, two useful models have been proposed for allosteric enzymes by relating in detail the subunit structure of the protein to its allosteric and cooperative effects. These are:

1. \textbf{The Monod-Changeux-Wyman model} (35):

   This model is based on the assumption that:
   
   a) All proteins are symmetrical.
   
   b) All allosteric enzymes are polymers of two or more identical subunits, each capable of existing with at least two conformational states in dynamic equilibrium with each other.
   
   c) All the subunits in a given protein exist in the same conformation at a given time.
d) Each subunit bears a catalytic (substrate) and an allosteric (inhibitor) site.

e) In each conformational state (the R or T states), the sites have equal affinities for their respective ligands (substrate or allosteric effector).

f) The transition from one state to another involves simultaneous changes in all identical subunits. The molecular symmetry is conserved during this transition.

In this model, it is assumed that the conformational change occurs through an isomerization between the R (relax) and T (tight) states of the protein. If one of these states, e.g., the R state binds substrate preferentially and is present in small amounts, addition of the substrate will tend to shift the equilibrium in the direction of the R state and the saturation curve will be sigmoid. The action of inhibitors and activators may be explained by assuming the former preferentially bind to the T state and the latter to the R state. Monod et al. pointed out that allosteric systems appear to be always positive or cooperative.

2. The Koshland, Némethy and Filmer model (36):

This model for allosteric proteins is based on the site interaction hypothesis of Adair (37) assuming the existence of at least two substrate-binding sites - one catalytic and the other regulatory. According to this model, both
substrate and allosteric activator binding at the regulatory site induce a conformational change favoring substrate binding at the catalytic site. On the other hand, allosteric inhibitors displace these activators, producing a conformational change favoring the dissociation of substrate at the catalytic site. This 'sequential' Koshland model postulates that:

a) Each subunit of the protein can exist in at least two conformations.

b) A change in conformation of one subunit may change the relative stability of conformations of neighboring subunits by subunit interactions.

c) The ligand binds preferentially to one of the conformations.

d) Interactions may be positive or negative, i.e., ligand binding at one site may increase or decrease the affinity of the remaining sites.

III. Properties of phosphofructokinase from other sources

Phosphofructokinase (ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) (PFK) catalyzes the relatively irreversible phosphorylation of fructose-6-phosphate by ATP to form fructose-1, 6-diphosphate and ADP. It is recognized as a key enzyme in the regulation of glycolysis (38-41). PFK isolated from heart (42), brain (43), muscle (44), liver (45), yeast (46), E. coli (27) and liver fluke (47) have been investigated and, in general, have similar characteristics.
All are inhibited by high concentrations of ATP (26, 41, 46-50) and the structurally unrelated compound, citrate (43, 46, 50-52). The inhibition of PFK by ATP and citrate is subject to the antagonistic actions of NH₄⁺, orthophosphate, F-6-P and 5'AMP (26, 43, 45, 50). There is strong evidence that each of these acts at a different site on the molecule since they are all synergistic in their actions and the dissociation constant of each is not changed or decreased by the other substances. ADP and cyclic-3',5'-AMP also activate PFK and it has been suggested that they probably act at the same site as 5'-AMP. In spite of an abundant literature on PFK, few attempts have been made to purify the enzyme due to its apparent instability and highly refractory properties. However, within the past five years, Ling et al. (44) purified PFK from rabbit skeletal muscle with a final specific activity of 130-150 (Specific activity expressed as μ moles of FDP formed per minute per mg protein) while Parmeggiani et al. (53) obtained crystals from the same source with a specific activity of 150. Crystals of PFK with specific activity of 100-120 were also obtained from sheep heart (54).

Recently, evidence has been provided that a control mechanism of PFK is represented by the presence of different molecular forms in several sources. The mechanisms of interconversion in vivo are still unresolved. Working with PFK from the liver fluke, Mansour and Mansour (47) were the first to report a shift in the equilibrium between the catalytically active and inactive forms of PFK with cyclic-3',5'-AMP mediating as an activator.
Further insight into the mechanism of PFK regulation was gained by the identification of two catalytically distinct forms of the enzyme from guinea pig heart (42). The inactive form regains its catalytic activity at slightly alkaline pH, in the presence of ADP, F6P or FDP. Sucrose gradient analysis in the ultracentrifuge indicated that inactive PFK has a lower sedimentation coefficient than the active form. With crystalline sheep heart PFK, Mansour et al. (54-55) confirmed that a reversible dissociation to subactive monomers was involved in enzyme regulation and provided evidence that this interconversion was not enzyme-catalyzed. The effects of several ligands were tested: ATP was found to favour the subactive, monomeric species (M.W. approximately 90,000 to 100,000). Two monomers could aggregate into a protomer (M.W. approximately 200,000) which, in turn, could self-aggregate into dimers (M.W. approximately 380,000 to 400,000). F6P or FDP shifted the equilibrium towards the dimer which could undergo further aggregation into large macromolecules. Each subactive monomer was capable of dissociating irreversibly into small subunits (M.W. approximately 32,000) in the presence of guanidine hydrochloride or urea. Removal of ATP and FDP gave rise to a molecular form of PFK which was more sensitive to ATP inhibition and showed a more cooperative saturation curve for substrate F6P. These experiments indicated that heart PFK underwent marked variations which were strictly dependent on ligands related to its catalytic activity.

Similarly, studies on the subunit structure of muscle PFK (56-58) indicated that the protomer (M.W. 93,200) consisted of four non-identical peptides of roughly equal molecular weight (24,000 to
25,000), i.e., more than one kind of subunit within the protomer and could be split into halves of identical molecular weight. Binding studies (59) indicated three sites for ATP per protomer, one of which was apparently also capable of binding AMP, cyclic-3',5'-AMP or ADP competitively. It was suggested that one of these ATP binding sites might be the catalytic site. Like PFK from sheep heart, the enzyme also exhibited cooperativity with regards to one of its substrates, F6P (60). This property was destroyed by modification of a single thiol group on the enzyme (61).

IV. Present investigation

Although recent detailed studies of PFK isolated from various sources have shown that it is a sensitive site of control of carbohydrate metabolism, few reports have been published concerning PFK from human erythrocytes. Preliminary kinetic data obtained from studies of a partially purified preparation have shown that inorganic phosphate activates the enzyme by relieving the inhibition of ATP (62-63). Following the discovery of a new inborn error of metabolism characterized by the deficiency of muscle and erythrocyte PFK in man (64-65), investigations of the physical and kinetic properties of the enzyme from these sources were undertaken. The studies of Layzer et al. (66) demonstrated that partially purified muscle and erythrocyte PFK, although immunologically related, were not identical. PFK from both sources was inhibited by ATP and citrate. It was suggested that the kinetics for both substrates, ATP and F6P, might follow a multi-substrate, multiproduct mechanism corresponding to that of a
'ping-pong' conversion sequence described by Cleland (67). Sucrose density gradient centrifugation of muscle PFK indicated a molecular weight in the range of 380,000 to 430,000. However, the molecular weight of erythrocyte PFK was not estimated because of the heterogeneity of the preparation (66).

In the light of recent detailed investigations of PFK from various mammalian sources, it was of interest to study the characteristics and properties of PFK from human erythrocytes. As a prerequisite to the biochemical and kinetic studies, it was desirable to obtain a relatively pure and stable preparation of the enzyme. The present work was conducted with the aim of elaborating the regulatory mechanism for this important rate-limiting glycolytic enzyme in the human erythrocyte.
EXPERIMENTAL

I. MATERIALS

Diethyl amino ethyl cellulose (DEAE-cellulose, anion exchanger; capacity: 0.9 mEq/gm; mesh: coarse); 2-mercapto-ethanol (2-hydroxy-ethylmercaptan; Type 1); D-fructose-6-phosphate (potassium salt: Grade VI); D-fructose-1,6-diphosphate (tetrasodium salt); dithiothreitol (Cleland's reagent; DTT); 5-diphosphopyridine nucleotide (5-DPN, 5-NAD, 5-nicotinamide adenine dinucleotide from yeast; Grade III); adenosine-5'-triphosphate (disodium salt from equine muscle; crystalline); adenosine-5'-diphosphate (sodium salt from equine muscle); adenosine-5'-monophosphoric acid (sodium salt from yeast; crystalline); adenosine-5'-5'-cyclic monophosphoric acid (crystalline); inosine-5'-monophosphate (disodium salt from yeast; crystalline); inosine-5'-diphosphate (sodium salt from muscle); inosine-5'-triphosphate (trisodium salt from equine muscle); guanosine-5'-monophosphate (sodium salt from yeast); guanosine-5'-diphosphate (trisodium salt from yeast); guanosine-5'-triphosphate (sodium salt from equine muscle); uridine-5'-phosphoric acid (disodium salt from yeast; crystalline) and uridine-5'-diphosphate (sodium salt from yeast) were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Aldolase and glyceraldehyde-3-phosphate dehydrogenase were obtained from Boehringer Mannheim Corporation, New York, U.S.A.

Sodium arsenate (anhydrous); magnesium chloride (crystals);
potassium chloride (white; granular, U.S.P.); cysteine hydrochloride monohydrate; boric acid (crystals, A.C.S.); sodium phosphate (dibasic, anhydrous powder); potassium phosphate (monobasic); potassium phosphate (dibasic, anhydrous); sodium citrate; sodium chloride (certified A.C.S.) and tris-(hydroxymethyl)-aminomethane (certified primary standard); sodium hydroxide; cupric sulphate (crystals) and sodium carbonate (anhydrous) were obtained from Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A.

Folin and Ciocalteu's phenol reagent (volumetric solution) was obtained from The British Drug Houses (Canada) Ltd., Toronto.

Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Company, Kankakee, Illinois, U.S.A.

Acrylamide (for electrophoresis); N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylethylendiamine were purchased from Eastman Organic Chemicals, Rochester, New York, U.S.A.

Bromophenol blue; glycine (reagent grade) and ammonium persulfate were obtained from Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A.

Buffalo blue black was obtained from Beckman Instruments, Inc., Fullerton, California, U.S.A.

Nitro blue tetrazolium (crystalline, Grade III); phenazine methosulfate and methylene blue were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.
II. METHODS

1. Isolation and purification of PFK from human erythrocytes

All operations were carried out at 0-4°C unless otherwise stated.

Fresh blood (about 100 ml) was collected in heparin and centrifuged at 2,000 g for 20 minutes. The plasma and buffy coat were discarded and the erythrocytes were washed three times by suspension and centrifugation at 2,000 g for 20 minutes with two volumes of cold 1.15% KCl. After the final wash, the cells were resuspended in one volume of water. The hemolysate was prepared by freezing and thawing the preparation three times. The stroma was separated from the hemolysate by centrifugation at 2,000 g for 20 minutes. A small aliquot of the stroma-free hemolysate was removed for PFK assay. The remainder was adsorbed onto a DEAE-cellulose suspension according to the procedure of Hennessey et al. (68) and then chromatographed. The eluate containing appreciable PFK activity was pooled and fractionated by (NH₄)₂SO₄.

2. Preparation of DEAE-cellulose for adsorption of hemolysate

The DEAE-cellulose was prepared according to the procedure of Hennessey et al. (68). Ten g of commercial DEAE-cellulose were suspended in 250 ml of 0.1 M K₂HPO₄, pH 9.1, and stirred mechanically for one hour. The fines were decanted and the DEAE-cellulose was then filtered with suction. This step was repeated several times until the filtrate was colorless. The DEAE-cellulose was washed
further with water until the pH of the filtrate was approximately 7.0. An aqueous suspension of the DEAE-cellulose was adjusted to pH 7.0 and stored at 4° prior to use.

3. Preparation of DEAE-cellulose for column chromatography

DEAE-cellulose for chromatography was prepared according to the procedure of Mansour et al. (54) by suspending the dry powder in 0.1N NaOH and then washed exhaustively with water. The fines were decanted and the slurry was then filtered through a Buchner funnel. The resultant DEAE-cellulose cake was suspended in M NaCl for approximately 20 minutes and again filtered. The DEAE-cellulose was washed and filtered several times with water until the pH of the filtrate was approximately 8.0. The final suspension of DEAE-cellulose was stored at 4°.

4. Measurement of PFK activity

Enzymatic activity was measured by a modification of the procedure of Blanchaer et al. (69) for PFK in hemolysates in which PFK was rate-limiting in a sequence of reactions leading to the reduction of NAD by glyceraldehyde-3-phosphate dehydrogenase. The resultant increase in optical density at 340 mu was followed for 10 min in a Gilford 2000-Beckman DU spectrophotometer equipped with a recording device. The temperature in the cells was maintained at 37° with the aid of 'thermospacers' fitted to the cell housing and connected to a constant temperature circulating water bath.
Initial reaction velocity was calculated as millimicro-
moles of FDP formed per minute per ml enzyme preparation
using a molar extinction coefficient of $6.22 \times 10^3 M^{-1}cm^{-1}$
for NADH (70). A unit of PFK activity is defined as the
amount of enzyme which catalyzes the formation of 1 µ mole
of FDP per min at 37°. Specific activity is expressed as
units per mg protein. Two reaction mixtures were used in
the kinetic studies:

i) Reaction mixture A: The following reagents were contained
in a final volume of 3.5 ml in the concentrations noted:
$57.0 \times 10^{-3} M$ Tris buffer, pH 9.0; $28.5 \times 10^{-3} M$ sodium
arsenate, pH 7.4; $28.5 \times 10^{-3} M$ phosphate buffer, pH 7.4;
$34.0 \times 10^{-3} M$ cysteine; $34.0 \times 10^{-3} M$ MgCl$_2$; $1.4 \times 10^{-3} M$ F6P;
$0.35 \times 10^{-3} M$ ATP; $0.16 \times 10^{-3} M$ NAD; 3.0 µg aldolase;
3.0 µg glyceraldehyde-3-phosphate dehydrogenase. The
final pH of this mixture was 8.4 and provided conditions
for maximal enzyme activity.

ii) Reaction mixture B: This mixture provided a medium for
studying the activation of PFK by cyclic-3',5'-AMP and
related adenyllic nucleotides. It is similar to reaction
mixture A with the exception that F6P and ATP were added
to final concentrations of $0.6 \times 10^{-6} M$ and $0.17 \times 10^{-3} M$
respectively. The pH of this mixture was adjusted to
6.8 before use.

5. Estimation of protein

The concentration of protein in PFK preparations was
estimated colorimetrically by the Lowry method (71). The
protein was precipitated with 10% trichloroacetic acid and then resuspended in water prior to determination. Crystalline bovine serum albumin was used as the standard.

6. **Disc electrophoresis on polyacrylamide gel**

Purified PFK was examined by the simplified method of Clarke (72). The enzyme (100–200 µg), made denser by mixing with a 5% sucrose solution, was pipetted directly onto the flat-top surface of the acrylamide separating gel. Electrophoresis was carried out at 4°C with Tris-glycine buffer, pH 8.4, containing 10⁻⁴M ATP, 10⁻⁵M FDP and 10⁻³M Cleland's reagent (150 volts, 5 mAmp per tube) for approximately 1 hour. At the conclusion of the run, the gel was stained for protein with a 0.075% (w/v) solution of Buffalo blue black containing 6% (w/v) HgCl₂ in 7/5% (v/v) glacial acetic acid. Unbound dye was removed by leaching the gels with 5% acetic acid. PFK activity was located by incubating the gels for 45 min at 37°C in 20 ml of reaction mixture containing 0.28M Tris buffer; 0.17M MgCl₂; 6.0 µg aldolase; 6.0 µg glyceraldehyde-3-phosphate dehydrogenase; 14.0 x 10⁻³M F6P; 3.5 x 10⁻³M ATP; 15.0 µg NAD; 1.0 mg phenazine methosulfate and 15.0 mg nitroblue tetrazolium.

7. **Analytical ultracentrifugation**

Sedimentation studies were carried out in single sector, capillary-type synthetic boundary cells at 20°C and 42,040 r.p.m. in a Spinco Model E analytical ultracentrifuge with the An-D rotor, absorption optics and photoelectric
scanner. Radial positions were measured by drawing a horizontal line on the chart halfway between the tracing for the solvent region and that of the plateau region of the cell. The point of intersection with the tracing of the boundary region was used as the centre of the boundary. The magnification factor was determined by measuring the distance between the reference edge images and dividing by the counterbalance dimension (nominally 1.60 cm). The observed sedimentation coefficient ($s_{obs}$) was calculated by using the equation according to Schachman (73):

$$s_{obs} = \frac{1}{\omega^2 r} \frac{dr}{dt}$$

where $\omega = \text{angular velocity (radians/sec)}$.
$r = \text{distance from axis of rotation (cm)}$
$t = \text{time in seconds}$

Sedimentation coefficients were corrected for standard conditions by using the equation:

$$s_{20,w} = s_{obs} \left( \frac{\eta_t}{\eta_{20}} \right) \left( \frac{\eta_{sol}}{\eta_w} \right) \left( \frac{1 - \bar{V} \rho}{1 - \bar{V} \rho_{t,sol}} \right)$$

where $s_{obs} = \text{observed sedimentation coefficient}$
$\eta_t = \text{viscosity of water at } t^\circ \text{ (temperature of centrifuge run)}$
$\eta_{20} = \text{viscosity of water at } 20^\circ$
$\eta_{sol} = \text{viscosity of sample solution at known temperature, } t'^\circ$
$\eta_w = \text{viscosity of water at } t'^\circ$
$\rho_{20,w} = \text{density of water at } 20^\circ$
\[ \rho_{t, \text{sol}} = \text{density of sample solution at } t \]  
(temperature of centrifuge run)

\[ \nabla = \text{partial specific volume} \]

Viscosity measurements were performed with an Ostwald viscometer and outflow times at 25.0 ± 0.005° were determined to ±0.05 seconds (73). Density measurements were obtained by pycnometry (73).

Molecular weight of PFK under various experimental conditions was determined by the equilibrium sedimentation method (74-78) in a Spinco Model E ultracentrifuge equipped with a monochromator and a photoelectric scanner. The sample, in a Kel F double sector 12 mm cell, was allowed to equilibrate for 18 to 24 hours at 5° at 15,220 r.p.m. in a titanium An-F rotor. The molecular weight was calculated by using the slope of the equilibrium curve in the expression:

\[ M = \frac{2RT}{(1 - \nabla \rho) \omega^2} \frac{d \ln c}{d (x^2)} \]  
(74-78)

where

\[ M = \text{molecular weight} \]

\[ R = \text{gas constant (8.313 x 10^7 ergs/degree mole)} \]

\[ T = \text{absolute temperature (degree Kelvin)} \]

\[ \nabla = \text{partial specific volume (ml/gm)} \]

\[ \rho = \text{density of solution (gm/ml)} \]

\[ \omega = \text{angular velocity (radians/sec)} \]

\[ c = \text{concentration (O.D. units)} \]

\[ x = \text{distance from the axis of rotation (cm)} \]
8. **Desensitization of PFK**

PFK was desensitized by photooxidation in the presence of methylene blue and light according to the method of Mansour and Ahlfors (79). 0.25 ml enzyme solution containing $2 \times 10^{-6}$M methylene blue was placed in a 12 ml centrifuge tube immersed in ice water. Photooxidation of the enzyme solution was allowed to take place for approximately 10-15 minutes by means of a 250 watt reflector bulb placed at a distance 20 cm above the top of the tube. The tube was shaken manually from time to time.
RESULTS

I. Isolation and purification of PFK: Summarized in Fig. 2 and consisted of the following steps:

1. Adsorption of hemolysate to DEAE-cellulose: Studies of individual enzymes in erythrocytes are hampered by the fact that lysates or partially purified systems contain vastly more hemoglobin than various enzymes. Hemoglobin may be removed efficiently by treatment of the hemolysate at pH 7.0 with DEAE-cellulose following the procedure of Hennessey et al. (68). At this pH, proteins having isoelectric points near neutrality are not adsorbed. In adsorbing the hemolysate, an equal volume of DEAE-cellulose suspension was added to the hemolysate and the mixture was allowed to stand at 4° for 20 minutes with manual stirring approximately every 4 minutes. The unadsorbed material in the suspension, principally hemoglobin, was separated by centrifugation at 2,000 g for 5 minutes. Residual hemoglobin on the cellulose was removed by washing the adsorbant with a total of about 500 ml of 0.003M phosphate buffer, pH 7.0. The nearly colorless supernatant of the final wash was discarded. The final DEAE-cellulose containing the hemoglobin-free protein fraction served as a starting material for the subsequent isolation and purification of PFK.
Fig. 2  Isolation and purification of PFK from human erythrocytes.
Fresh heparinized blood

Centrifuged

Erythrocytes

Plasma and buffy coat → Discard

Washed and centrifuged 3x

Washed erythrocytes

Supernatant → Discard

Equal vol water added
Freeze and thaw 3x
Centrifuged

Hemolyzed erythrocytes

Stroma → Discard

1) DEAE-cellulose adsorption
2) DEAE-cellulose column chromatography
   with a linear gradient of 0.15 M NaCl
   in 0.2M Tris-HCl buffer (pH 8.6)

PFK fractions

1) (NH₄)₂SO₄ fractionation
2) Centrifuged

Supernatant

Precipitate

Discard

Dissolved in 0.05M potassium phosphate buffer (pH 8.0)

Purified PFK preparation
2. **DEAE-cellulose column chromatography:** A column of DEAE-cellulose (2.2 x 25 cm) was prepared by allowing the first 5 cm of the slurry to settle by gravity. The remainder was then packed under pressure. The DEAE-cellulose column was equilibrated and washed with a 0.2M Tris-HCl buffer, pH 8.6, containing $10^{-4}$ M ATP, $10^{-5}$ M FDP and $10^{-3}$ M 2-mercaptoethanol. About 30 ml of this solution was added to the DEAE-cellulose containing the hemoglobin-free protein fraction [from step (1)] and this suspension was then applied to the top of the column. The wall of the column was washed with about 20 ml of the same buffer and then developed with a linear gradient of 0.15M NaCl in the same solution. The eluate was collected in 10 ml fractions with an LKB fraction collector. Protein was detected spectrophotometrically at 280 nm and PFK activity was assayed. The elution pattern as shown in Fig. 3 gave six peaks but only effluents between fractions 51 and 64 had PFK activity. Fractions 52 to 58 which contained most of the activity were pooled and transferred to an Erlenmeyer flask which had been pre-chilled on ice. 2-Mercaptoethanol (20 μl/10 ml eluate) was added to stabilize the enzyme.

3. **Ammonium sulphate precipitation:** Ammonium sulphate solution, saturated at room temperature, was added dropwise with continuous stirring to 0.4 saturation to the pooled enzyme fractions obtained from DEAE-cellulose chromatography. The resulting precipitate which had no measurable PFK activity
Fig. 3  The elution pattern of erythrocyte PFK from
the DEAE-cellulose column.

   O  O  , protein
   \^--\ , PFK

A PFK unit is expressed as μ moles FDP formed/min.
was sedimented at 20,000 g for 30 minutes and discarded. Ammonium sulphate was added to bring the solution to 0.6 saturation. After centrifugation at 20,000 g for 20 minutes, the precipitate was dissolved in a minimum volume (1 to 2 ml) of 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-4}$M ATP, $10^{-5}$M FDP and $10^{-3}$M Cleland's reagent (dithiothreitol) to give a faintly yellow solution containing 1 to 2 mg protein/ml with a specific activity between 20 and 40 PFK units. Purification from the crude hemolysate to the ammonium sulphate preparation was 700 to 1000 fold (Table 1). Re-chromatography on DEAE-cellulose failed to increase the specific activity. This preparation will be referred to as 'purified' PFK preparation.

'Purified' PFK isolated by ammonium sulphate fractionation inevitably contained some traces of ammonium sulphate. It was, therefore, necessary to remove the residual ammonium sulphate in the preparation. 'Purified' PFK was dialyzed against 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-4}$M ATP, $10^{-5}$M FDP and $10^{-3}$M Cleland's reagent at 4° for periods of $\frac{1}{2}$, 1, 2, 3 and 4 hours after which PFK activity was determined (Fig. 4). Activity was rapidly lost and after 3 hours none whatever remained. Ammonium ions may, therefore, be essential for stabilizing PFK after isolation. In the following studies, therefore, undialyzed preparations were used. 'Purified' PFK preparations were frozen in small volumes in tightly capped tubes.
Purification of phosphofructokinase from human erythrocyte.

Purified PFK was prepared from approximately 100 ml of fresh heparinized blood. The hemolysate was adsorbed and chromatographed on a DEAE-cellulose column with a linear gradient of 0.15M NaCl in 0.2M Tris-HCl buffer containing $10^{-5}M$ FDP, $10^{-4}M$ ATP and $10^{-3}M$ 2-mercaptoethanol. Ammonium sulphate fractionation in Step 3 was carried out on fractions containing appreciable PFK activity which was determined spectrophotometrically at 340 nm in reaction mixture A (pH 8.4) described in the text. A unit of PFK activity is defined as the amount of enzyme that catalyzes the formation of 1 μ mole of FDP/min and specific activity is expressed as units/mg protein.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification fold</th>
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<tr>
<td>Step 1 Homolysate</td>
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<td>0.045</td>
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<td>40.8</td>
<td>1.2</td>
<td>755.5</td>
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</tbody>
</table>
Fig. 4  Effect of dialysis on PFK activity
In view of recent reports that a form of PFK more sensitive to ATP inhibition could be isolated from sheep heart (55), attempts were made to isolate PFK from human erythrocyte by DEAE-cellulose column chromatography as described previously with a linear gradient of 0.15M NaCl in 0.2M Tris-HCl buffer, pH 8.6, in the absence of ATP and FDP. The elution pattern (Fig. 5) showed seven protein peaks, none of which, however, had PFK activity. The column was further developed with 500 ml of 0.2M Tris-HCl buffer, pH 8.0, containing 0.15M NaCl but no enzyme activity could be detected in the effluents. However, when the column was eluted with 100 ml of 0.5M KCl, weak PFK activity was detected in a few fractions. The results indicated that it was unlikely PFK could be isolated from human erythrocyte in the absence of ATP and FDP.

II. Properties of 'purified' PFK

1. Stability:

During the course of investigation, PFK isolated from human erythrocytes was stored in 0.05M potassium phosphate buffer, pH 8.0, containing \(10^{-4}\text{M}\) ATP, \(10^{-5}\text{M}\) FDP and \(10^{-3}\text{M}\) 2-mercaptoethanol. Unfortunately, PFK preparation frozen and stored this way lost all its activity within 5 days. Since Mansour et al. (54) and Layzer et al. (65-66) had used dithiothreitol (\(10^{-3}\text{M}\)) with no detrimental effect on their PFK preparations, it was of interest to investigate the
Fig. 5  The elution pattern of erythrocyte PFK from DEAE-cellulose column in the absence of ATP and FDP.

[Diagram: circles representing protein]

(PFK activity was not detectable in the eluate fractions.)
stability of 'purified PFK' isolated from human erythrocytes in the presence of this reducing agent.

Frozen preparations of 'purified' PFK (protein concentrations between 1 and 2 mg/ml) in 0.05M potassium phosphate buffer, pH 8.0, containing 10^{-4} M ATP, 10^{-5} M FDP and 10^{-3} M Cleland's reagent lost about 20% of its activity within 24 hours of storage (Fig. 6). After this initial period of rapid loss of enzyme activity, the enzyme was stable for at least two weeks, followed by a progressive loss of enzyme activity. After 3 to 4 weeks of storage, there was a complete loss of PFK activity. In the present work, 'purified' PFK preparations were used within 2 weeks after isolation.

The effect of different temperatures on PFK activity was studied at various time intervals. Enzyme preparations were incubated at 4°, 23° and 37° for 10, 25, 35, 45 minutes and 1\frac{1}{2}, 2, 3 and 24 hours at the end of which 20 \mu l (15 \mu g) were removed for enzyme assay. The results are shown in Fig. 7. At 4°, there was insignificant loss of enzyme activity in the first hour of incubation as compared to the 10% and 50% loss of enzyme activity in the preparations incubated at 23° and 37° respectively. Loss of enzyme activity in the preparation incubated at 37° was most pronounced during the first 5 minutes, accounting for 25% of its initial activity. In all three cases, there was a period during which the enzyme was relatively stable, i.e., between 1 and 2 hours. After 24 hours of incubation at 4°
Fig. 6  Stability of PFK during storage at -20°

(Conditions as described in the text.)
Fig. 7  Effect of temperature on PFK activity.

••• , 4°
○○ , 23°
•• , 37°
and 23°, 'purified' PFK preparations retained 80% and 50% of the initial activity respectively. However, PFK preparations incubated at 37° for 24 hours were completely inactive.

2. **Disc electrophoresis on polyacrylamide gel**

Purified PFK was examined by disc electrophoresis on polyacrylamide gel by the simplified method of Clarke (72) as described previously. It can be seen in Fig. 8 that there were two protein bands in the separating gel. When the gel was incubated for 45 min at 37° in a special reaction mixture containing phenazine methosulfate and nitro blue tetrazolium, two bands with enzyme activity were located. Since the separation of sharp bands in polyacrylamide gel is graded according to molecular size and charge density, it might be inferred that PFK isolated from human erythrocytes also exists in different molecular forms as demonstrated in other sources (42, 47, 54-58).

3. **Sedimentation behavior**

A typical sedimentation pattern of the enzyme in 0.05M potassium phosphate buffer containing 10^{-4}M ATP, 10^{-5}M FDP and 10^{-3}M dithiothreitol (Fig. 9) revealed a single boundary with \( s_{20,w} \) value of 7.56 (protein concentration 0.75 mg/ml).
Fig. 8  Disc electrophoresis of PFK on polyacrylamide gel with the simplified method of Clarke (72) at 4°.

Gel buffer: Tris-glycine buffer, pH 8.1

Chamber buffer: Tris-glycine buffer, pH 8.4

Electrophoresis was conducted at 150 volts, 5 mAmp per tube for 1 hour. Tube at left shows protein stained by a 0.075% solution of Buffalo blue black and tube at right shows PFK stained with phenazine methosulfate and nitro blue tetrazolium in a reaction mixture as described in the Methods section. 100 µg of protein were applied to the gel surface.
Fig. 9  Sedimentation pattern of phosphofructokinase in
0.05M potassium phosphate buffer, pH 8.0, containing
10^{-3}M dithiothreitol, 10^{-4}M ATP and 10^{-5}M fructose-
1,6-diphosphate. The direction of sedimentation is
to the right.

Enzyme concentration : 0.75 mg/ml
Speed : 42,040 r.p.m.
Temperature of run : 20°
(Note: The partial specific volume, $\bar{V}$, of proteins is generally between 0.70 and 0.75 ml/g and its accurate determination requires relatively large amounts of protein. In view of the small quantities of the 'purified' PFK available, the partial specific volume, $\bar{V}$, was not estimated. An assumed value of 0.72 ml/g was used in the calculation of $s_{20,w}$ values and molecular weights in the present investigations.). If a given sedimenting substance travels across the cell as a single boundary without too much broadening, one usually assumed a reasonable degree of homogeneity. Since sedimentation coefficients are functions of concentration in general, it was desirable to determine $s_{20,w}$ of PFK at a series of concentrations and to extrapolate the results to zero concentration to see if there is a concentration dependence in the sedimentation rate. As illustrated in Fig. 10, the sedimentation coefficient of PFK was indeed concentration-dependent and the value of $s_{20,w}$ at infinite dilution was approximately 2.2. Generally, in sedimentation velocity experiments, if sedimentation rates are found to increase with increasing concentration, then it can be assumed that solute molecules are interacting to form larger sedimenting species. The present data on PFK isolated from human erythrocytes is in keeping with this explanation.

It has been shown that in potassium phosphate buffer containing similar stabilizing agents, $s_{20,w}$ values for PFK from sheep heart were 8.21 and 41.0 (54) while that for rabbit skeletal muscle were 13.7, 20.9 and 31.0 (44). This suggests
Fig. 10 Concentration dependence of the sedimentation rate of human erythrocyte phosphofructokinase in 0.05M potassium phosphate buffer, pH 8.0, containing 10^{-3}M dithiothreitol, 10^{-4}M ATP and 10^{-5}M fructose-1, 6-diphosphate.
that PFK from these two sources is present in different polymeric forms. In the light of these findings, it appeared necessary to examine the effect of urea and guanidine hydrochloride on the sedimentation behavior of PFK from human erythrocytes to see if it, too, undergoes a dissociation-association phenomenon. 'Purified' PFK was incubated overnight with 8M urea or 5M guanidine hydrochloride at 4°. The sedimentation patterns in the presence of urea or guanidine hydrochloride (Figs. 11 and 12) showed a single boundary with s_{20,w} values of 5.70 and 5.65 respectively (protein concentrations 1.0 mg/ml).

It was mentioned earlier in the Results section that PFK from human erythrocytes was highly unstable when stored in the presence of 2-mercaptoethanol. It became interesting to investigate the effect of 2-mercaptoethanol on the sedimentation behavior of this enzyme. From Fig. 13 it was apparent that the enzyme dissociated into smaller molecular weight species in the presence of this reducing agent: the sedimentation coefficient (s_{20,w} = 2.90) was much lower compared to a parallel experiment with the same protein concentration in the presence of dithiothreitol (s_{20,w} = 4.80, see Fig. 10). The dissociation of PFK into smaller molecular weight species might be responsible for the apparent stability when the enzyme was frozen and stored in the presence of 2-mercaptoethanol.
The effect of 8M urea on the sedimentation pattern of phosphofructokinase. The ultracentrifugation of PFK in 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}$M dithiothreitol, $10^{-4}$M ATP and $10^{-5}$M fructose-1,6-diphosphate was carried out after incubating the enzyme overnight in the presence of 8M urea at 4°. The direction of sedimentation is to the right.

Enzyme concentration : 1.0 mg/ml
Speed : 42,040 r.p.m.
Temperature of run : 20°
Fig. 10  Concentration dependence of the sedimentation rate of human erythrocyte phosphofructokinase in 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}$M dithiothreitol, $10^{-4}$M ATP and $10^{-5}$M fructose-1, 6-diphosphate.
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The effect of 8M urea on the sedimentation pattern of phosphofructokinase. The ultracentrifugation of PFK in 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}$M dithiothreitol, $10^{-4}$M ATP and $10^{-5}$M fructose-1,6-diphosphate was carried out after incubating the enzyme overnight in the presence of 8M urea at 4°. The direction of sedimentation is to the right.

Enzyme concentration : 1.0 mg/ml
Speed : 42,040 r.p.m.
Temperature of run : 20°
Fig. 12  The effect of 5M guanidine HCl on the sedimentation pattern of phosphofructokinase. The ultracentrifugation of PFK in 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}$M dithiothreitol, $10^{-4}$M ATP and $10^{-5}$M fructose-1,6-diphosphate was carried out after incubating the enzyme overnight in the presence of 5M guanidine hydrochloride at 4º. The direction of sedimentation is to the right.

Enzyme concentration : 1.0 mg/ml
Speed : 42,040 r.p.m.
Temperature of run : 20º

(A higher O.D. reading in the base line was observed for some unknown reasons.)
Fig. 13  The effect of 2-mercaptoethanol on the sedimentation pattern of phosphofructokinase. The ultracentrifugation of PFK was carried out in a solution of 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}M$ 2-mercaptoethanol, $10^{-4}M$ ATP and $10^{-5}M$ fructose-1,6-diphosphate. (The 'purified' enzyme had been stored frozen in a medium containing $10^{-3}M$ 2-mercaptoethanol). The direction of sedimentation is to the right.

<table>
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<td>Speed</td>
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<td>Temperature of run</td>
<td>: 20°</td>
</tr>
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</table>
III. Kinetics at pH 8.4. In the present investigation, a typical kinetic experiment is presented on the basis of several analogous experiments. Assay conditions were as described under reaction mixture A in Methods unless otherwise stated.

1. Effect of pH: The activity of the enzyme was tested at different pH values at optimum substrate concentrations. Under these conditions, maximal enzyme activity was found to lie between pH 8.2 and 8.5 (Fig. 14). At pH 6.8, enzyme activity was reduced to 20% of the optimal activity.

2. Michaelis constants: Michaelis constants determined under assay conditions were $1.3 \times 10^{-9}$M for F6P (Fig. 15) and roughly $10^{-7}$M for ATP (Fig. 16). However, at concentrations greater than $4.3 \times 10^{-9}$M, ATP was a potent inhibitor of enzyme activity. When it was discovered, inhibition by ATP was tentatively attributed to a decrease in the concentration of free magnesium ions as a result of chelation with the nucleotides (48). The work of Ramaiah et al. (46) in yeast clearly indicated that ATP strongly inhibited the activity of PFK even when the total concentration of Mg$^{++}$ ions greatly exceeded that of ATP, suggesting that inhibition was not attributed to a lack of free Mg$^{++}$. Since it is not possible to overcome ATP inhibition by converting all of the nucleotide to its Mg$^{++}$ complex, both MgATP and ATP must be inhibitors. The use of a constant ratio of Mg$^{++}$ ions to ATP, e.g., 2:1, will cause a wide fluctuation in the MgATP:ATP ratio as total ATP concentration is changed. As can be seen in Fig. 16, when the MgCl$_2$:ATP
Fig. 14  Effect of pH on PFK activity.

○○ , Tris buffer

●● , Glycine buffer
Fig. 15  Plot of the reciprocal of initial reaction velocity versus the reciprocal of the molar concentration of F6P at pH 8.4. Initial reaction velocity was determined as a function of F6P which was varied in the range of $1.14 \times 10^{-4}$ M to $5.7 \times 10^{-3}$ M. Velocities are expressed as $\mu$ moles of FDP formed in the reaction mixture over a period of 10 minutes.
Fig. 16  Plot of the reciprocal of initial reaction velocity versus the reciprocal of the molar concentration of ATP at pH 8.4. Initial reaction velocity was determined as a function of ATP which was varied from $1.14 \times 10^{-4} \text{M}$ to $5.7 \times 10^{-3} \text{M}$. Velocities are expressed as $\mu$ moles of FDP formed in the reaction over a period of 10 minutes.

---

- The ratio of $\text{Mg}^{++}$ to ATP was held constant at 2:1.
- The ratio of $\text{Mg}^{++}$ to ATP used was as described under reaction mixture A in Methods section.
ratio was held constant at 2:1, strong inhibition by ATP was still observed but the Michaelis constant for ATP did not appear to be significantly altered. The Michaelis constant for MgCl₂ was roughly 0.6 x 10⁻⁴ M (Fig. 17).

3. **Effect of nucleotides on PFK activity:** It has been reported that certain nucleotides, in particular adenine nucleotides, function as activators of PFK at slightly acidic pH. It would be interesting to see if the same holds true at alkaline pH in human erythrocyte PFK. The nucleotides tested were IMP, IDP, ITP, GMP, GDP, GTP, UMP, UDP, AMP, ADP and cyclic-3',5'-AMP (10⁻⁴ M in all cases). None of these were effective in altering enzyme activity at pH 8.4.

4. **Effect of citrate:** One of the inhibitors for PFK isolated from other sources is citrate (43, 46, 50-52) which arises in vivo by the enzymic condensation of acetyl CoA with oxaloacetate in the citric acid cycle. All the reactions of the cycle take place in just one subcellular organelle - the mitochondria. Since the mature human erythrocyte lacks a functional oxidative phosphorylation pathway due to the absence of mitochondria, it was of interest to examine the effect of citrate on PFK isolated from human erythrocytes. Sodium citrate at concentrations between 5 x 10⁻¹ M and 10⁻⁵ M was investigated and the results are presented as percentage of the control reaction which was conducted in the absence of citrate. As shown in Fig. 18, there was
Fig. 17  Plot of the reciprocal of the initial velocity versus the reciprocal of the molar concentration of MgCl$_2$ at pH 8.4. Initial reaction velocity was determined as a function of MgCl$_2$ which was varied in the range of 0.6 x 10$^{-3}$M to 1.2 x 10$^{-5}$M. Velocities are expressed as μ moles of FDP formed in the reaction mixture over a period of 10 minutes.
Fig. 18  Effect of sodium citrate on PFK activity at pH 8.4.
a consistent loss of enzyme activity (about 20%) at concentrations between $10^{-5}$M and $10^{-3}$M. As concentration of citrate was increased, enzyme activity progressively decreased reaching a zero mark at 0.1M sodium citrate. The inhibition of PFK by citrate might be attributed to the chelation of Mg$^{++}$.

5. **Low concentrations of F6P:** Low concentrations of F6P from $1 \times 10^{-5}$M to $3 \times 10^{-4}$M were used to investigate the activity of the enzyme at fixed, non-inhibitory concentrations of $1 \times 10^{-5}$M, $4 \times 10^{-5}$M and $8 \times 10^{-5}$M ATP. As shown in Fig. 19(a), there are two plateau regions separated by a fairly sharp inflexion point on the substrate concentration-velocity plot. The double reciprocal plot of the same data [Fig. 19(b)] consisted of two linear regions separated by fairly sharp discontinuity; the slopes of the plot increased as the substrate concentration was increased. Such kinetic characteristics are often seen in enzymes which exhibit negative cooperativity toward their substrates or in associating-dissociating systems. When F6P concentrations were raised between $10^{-3}$M and $10^{-4}$M with ATP concentrations fixed at $1 \times 10^{-5}$M, $4 \times 10^{-5}$M and $8 \times 10^{-5}$M as in the previous experiment, PFK was found to exhibit Michaelis-Menten kinetics with respect to ATP (Fig. 20). The apparent $V_{\text{max}}$ for one substrate was increased by raising the concentration of the other substrate but the $K_m$ appeared to be unaltered.
Fig. 19(a)  Plot of the initial reaction velocity versus the molar concentration of F6P at fixed ATP concentrations at pH 8.4. F6P concentration was varied between $1 \times 10^{-5}$M and $3 \times 10^{-4}$M. Velocities are expressed as $\mu$ moles of FDP formed/10 min/ml enzyme.

- •• , $1 \times 10^{-5}$M ATP
- ○○ , $4 \times 10^{-5}$M ATP
- ▼▼ , $8 \times 10^{-5}$M ATP
Replots of Fig. 19(a) data by the Lineweaver-Burk method.

- , 1 x 10^{-5}M ATP
- , 4 x 10^{-5}M ATP
- , 8 x 10^{-5}M ATP
The graph shows a plot of $1/v \times 10^{-4}$ against $1/F6P \times 10^{-4}M$. The data points are represented by different symbols, and the trend lines indicate a linear relationship.
Fig. 20  Plot of the initial velocity versus the molar concentration of F6P at fixed ATP concentrations at pH 8.4. F6P was varied between $5 \times 10^{-5}$M and $1.2 \times 10^{-3}$M. Velocities are expressed as $\mu$moles of FDP formed/10 min/ml enzyme.

\[
\begin{align*}
\text{▼▼} & , \quad 1 \times 10^{-5}$M ATP \\
\text{●●} & , \quad 4 \times 10^{-5}$M ATP \\
\text{▼▼} & , \quad 8 \times 10^{-5}$M ATP
\end{align*}
\]
6. **Low concentrations of ATP:** The effect of low concentrations of ATP between $10^{-5}$M and $1.5 \times 10^{-4}$M was studied with respect to enzyme activity with F6P concentrations fixed at $1 \times 10^{-4}$M, $4 \times 10^{-4}$M, $8 \times 10^{-4}$M and $1.2 \times 10^{-3}$M. The results represented in Fig. 21 showed biphasic curves in the substrate concentration-velocity plot when F6P concentrations were fixed at $1 \times 10^{-4}$M, $4 \times 10^{-4}$M and $8 \times 10^{-4}$M. However, as F6P concentration was raised to $1.2 \times 10^{-3}$M a normal rectangular hyperbola was obtained but the $V_{\text{max}}$ was reduced. This might be the result of substrate inhibition since F6P concentrations greater than $1.2 \times 10^{-3}$M were found to cause marked loss of enzyme activity.

IV. **Kinetics at pH 6.8.** In the present investigation, a typical experiment is presented on the basis of several analogous experiments. Assay conditions were as described under reaction mixture B in Methods unless otherwise noted.

1. **Effect of ATP:** The effect of ATP on PFK activity at pH 6.8 was investigated. At concentrations greater than $2 \times 10^{-5}$M, ATP was a potent inhibitor of enzyme activity (Fig. 22) (Compare to $4.3 \times 10^{-4}$M at pH 8.4, Fig. 16). The $K_m$ for ATP at pH 6.8 was roughly $1.7 \times 10^{-6}$M.

2. **Reversible inactivation and reactivation:** Mild acidification (to pH 6.8) of the 'purified' PFK preparations resulted in a reduction of enzyme activity to about 16-20% of that observed at the optimal pH (Table 2). By increasing the pH to 8.1 - 8.4, approximately 40% of the enzyme activity lost during mild acidification was
Fig. 21

Effect of low ATP concentration on PFK activity at pH 8.4. ATP was varied between $1 \times 10^{-5}$ M and $1.4 \times 10^{-4}$ M. Velocities are expressed as μmol of FDP formed/10 min/ml enzyme.

- ○ ○ , $1 \times 10^{-4}$ M F6P
- • • , $4 \times 10^{-4}$ M F6P
- ▼ ▼ , $8 \times 10^{-4}$ M F6P
- ▼ ▼ , $1.2 \times 10^{-3}$ M F6P
Fig. 22  Effect of ATP on PFK activity at pH 6.8.

Concentration of ATP was varied between $5 \times 10^{-6}$M and $1.0 \times 10^{-4}$M. Velocities are expressed as $\mu$moles of FDP formed/10 min/ml enzyme. Other experimental conditions for measuring enzyme activity in reaction mixture B were used and are given in the text.
<table>
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<tr>
<td><strong>Inactivation and reactivation of PFK at 23°</strong></td>
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* PFK activity is expressed as μ moles of FDP formed/10 min/ml enzyme

** Experiments were done at 0-4°
<table>
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<th>pH of reaction mixture</th>
<th>Addition</th>
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recovered. The recovery of enzyme activity was dependent on the time of reactivation and was found to decrease as the time of inactivation was prolonged.

3. **Activation of PFK by adenine nucleotides:** At slightly acidic pH, adenine nucleotides have been reported as activators of PFK isolated from other sources. The effect of adenine nucleotides on PFK isolated from human erythrocytes was, therefore, studied. In keeping with these reports, AMP, cyclic-3',5'-AMP and, to a lesser extent, ADP were found to be effective activators at pH 6.8. The activation by different molar concentrations of these nucleotides is shown in Fig. 23. These agents, when used simultaneously, were not synergistic in action. The effect of cyclic-3',5'-AMP and AMP were best demonstrated between pH 6.8 and 7.0 (Fig. 24). As the pH of the reaction mixture was raised, the stimulatory effects of both nucleotides were diminished.

4. **Relationship between ATP inhibition and cyclic-3',5'-AMP activation:** The effect of cyclic-3',5'-AMP on enzyme activity was tested in the presence of different concentrations of ATP. It can be seen from Fig. 25 that the inhibitory effect of ATP was abolished in the presence of $10^{-4}$M cyclic-3',5'-AMP. The titration curve of the cyclic nucleotide (Fig. 26) showed that the inhibition was partially competitive. On account of this apparent competition and the close structural similarities of the two
Fig. 23

Activation of PFK by different molar concentrations of cyclic-3',5'-AMP (○○), AMP (●●) and ADP (▼▼) at pH 6.8. Assay conditions for reaction mixture B were used and are given in the text.
Fig. 24  Effect of AMP and cyclic-3',5'-AMP on PFK activity at different pH. Assay conditions for reaction mixture B were used and are given in the text.

 Control

 10^{-4}M AMP

 10^{-4}M cyclic-3',5'-AMP
Fig. 25  Effect of cyclic-3',5'-AMP on ATP inhibition
at pH 6.8. Assay conditions for reaction
mixture B were used and are given in the text.
Velocities are expressed as μ moles FDP formed/
10 min/ml enzyme.

● ● ● , Control
○ ○ ○ , 10^-4 M Cyclic-3',5'-AMP
Fig. 26  Plot of the initial reaction velocity versus the molar concentration of cyclic-3',5'-AMP at pH 6.8. Assay conditions were as described for reaction B in the text except as noted below. ATP concentrations were held constant at $3.5 \times 10^{-4} \text{M}$ (●●) and $1 \times 10^{-3} \text{M}$ (○○). Initial velocity was determined as a function of cyclic-3',5'-AMP concentration which was varied between $10^{-5} \text{M}$ and $5 \times 10^{-4} \text{M}$. Velocities are expressed as μ moles FDP formed/10 min/ml enzyme.
nucleotides, it might be possible that ATP and cyclic-3', 5'-'AMP share the same allosteric site on the enzyme.

5. **Relationship between F6P concentration and cyclic-3',5'-AMP effect:** The effect of cyclic-3',5'-AMP on PFK activity was tested in the presence of different concentrations of F6P at pH 6.8 and with inhibitory concentrations of ATP (0.17 x 10^{-5}M). It can be seen in Fig. 27 that in the absence of the cyclic nucleotide, there were two plateau regions on the rate-concentration plot. Similar results were observed at pH 8.4 at low concentrations of F6P and ATP (see Figs. 19 and 21). In the presence of the cyclic nucleotide at pH 6.8, the biphasic kinetics was converted to first order kinetics. The $K_m$ for F6P in the presence of the cyclic nucleotide was roughly 1.5 x 10^{-5}M. These results indicated that cyclic-3',5'-AMP increases the activity of the ATP-inhibited enzyme only when the substrate is present in relatively low concentrations.

6. **Time course of cyclic-3',5'-AMP effect:** In the previous experiments, the effect of cyclic-3',5'-AMP on PFK activity was studied by adding the nucleotide with the rest of the assay mixture. Under these conditions, the enzyme is exposed simultaneously to both an inhibitory concentration of ATP and cyclic-3',5'-AMP. Under such circumstance, it is possible that the cyclic nucleotide protects the enzyme from the inhibitory action of ATP. Experiments were
Fig. 27  Plot of the initial velocity versus F6P concentration at pH 6.8. Initial reaction velocities are expressed as μ moles FDP formed/10 min/ml enzyme. Assay conditions were as described for reaction B in the text except for the variation of F6P concentrations and the addition of cyclic-3',5'-AMP (10^-4M).

○○○○, Control with no cyclic-3',5'-AMP
○○○○, 10^-4M cyclic-3',5'-AMP
therefore designed to test the time course of enzyme activation by the cyclic nucleotide by adding it to the assay mixture after the reaction was started. As shown in Fig. 28, when cyclic-3',5'-AMP was added 4 minutes after starting the reaction, activation by the nucleotide was still observed. Whether the reaction was started with either substrates or enzyme did not alter the response to the cyclic nucleotide. It may be concluded that activation by the cyclic nucleotide can be demonstrated after ATP inhibition has occurred. AMP also exhibits similar activation kinetics. It may be pointed out that in both cases there was a lag of approximately 2-3 minutes prior to enzyme activation.

V. Desensitization of PFK: Previous work with heart PFK (26, 55) has shown that the enzyme exhibits cooperative kinetics at slightly acidic pH. Similar observations were reported for PFK from other sources (46-47, 51, 80-82). However, the foregoing results clearly indicate that PFK from human erythrocytes does not follow such a kinetic pattern, either at slightly acidic pH (6.8) or at low substrate concentrations at the optimal pH (8.4). In contrast, PFK from human erythrocytes exhibits an apparent negative cooperative homotropic interaction with its substrate. These kinetic properties may indicate that:

(i) The enzyme is regulated by a feedback allosteric mechanism
Fig. 28  Time course of cyclic-3',5'-AMP effect on ATP-inhibited PFK at pH 6.8. Assay conditions for reaction mixture B were used and are given in the text. Initial reaction velocities are expressed as μ mole FDP formed/10 min/ml enzyme. The addition of cyclic-3',5'-AMP (●, 10^{-4}M) to the test assay or an equivalent volume of water (〇〇) to the control is indicated by the arrow.

Protein concentrations were 80 μg (●) and 100 μg (〇) respectively.
possessing a site distinct from the catalytic site
where enzyme effectors or modifiers act, or
(ii) The enzyme is regulated by an association-dissociation
equilibrium of the enzyme molecule.

In many allosteric enzymes, the presence of modifier
sites can be easily recognized by desensitizing the enzyme to
its modifier without destroying the catalytic site (79, 83-
85). Since ATP is both a substrate as well as an inhibitor
for PFK, it is interesting to desensitize the enzyme to ATP
inhibition by photooxidation following the procedure of
Mansour and Ahlfors (79) and to study some of its properties
in order to determine the mechanism by which PFK is regulated.
In the following experiments, 'control' refers to the enzyme
preparations in the presence of methylene blue but not photo-
oxidized by light.

1. **Time course of photooxidation on PFK activity:** As a pre-
requisite to the kinetic studies of the photooxidized
enzyme it was necessary to investigate the enzyme
activity in relation to the time course of photooxidation
so that a period where there is not much loss of enzyme
activity could be selected. It can be seen from Table 3
that the enzyme lost over 80% of its initial activity
within one hour of photooxidation. It would appear that
a suitable period of photooxidation for the preparation
of desensitized PFK was between 10 and 15 minutes during
which 70-80% of the initial enzyme activity was still
intact.
TABLE 3

Time course of photooxidation on PFK activity

* PFK in the presence of $10^{-6}$M methylene blue but not exposed to light (see text).
<table>
<thead>
<tr>
<th>Time of photooxidation (mins)</th>
<th>PFK activity x 10^4 (µ moles FDP formed/10 min/ml)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control* Photooxidized PFK</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.55  2.55</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>2.55  2.20</td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>2.55  1.70</td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>2.55  1.43</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>2.55  0.84</td>
<td>33</td>
</tr>
<tr>
<td>60</td>
<td>2.20  0.034</td>
<td>15</td>
</tr>
</tbody>
</table>
2. **Effect of photooxidation on kinetics at pH 8.4**: Results on the effect of photooxidation on enzyme activity at pH 8.4 in the presence of low concentrations of F6P and ATP are summarized in Fig. 29(a), (b) and (c) and Fig. 30 (a), (b) and (c). These results showed that whereas there are two plateau regions on the rate-concentration plots [Fig. 29(a) and 30(a)] and two linear regions separated by distinct discontinuities on double reciprocal plots [Fig. 29(b) and 30(b)] in the control, the photooxidized enzyme appeared to follow first order kinetics with respect to both F6P and ATP.

When F6P concentration was varied between $10^{-4}$M and $10^{-5}$M, the $K_m$ values for the control were $1.2 \times 10^{-5}$M and $2.6 \times 10^{-5}$M while the $V_{max}$ were $1.8 \times 10^5$ μM moles FDP formed/10 min/ml enzyme and $2.7 \times 10^5$ μM moles FDP formed/10 min/ml enzyme, respectively. After photooxidation, there was only one $K_m$ ($1.8 \times 10^{-5}$M) and one $V_{max}$ ($2.0 \times 10^5$ μM moles FDP formed/10 min/ml enzyme) [Figs. 29(a) and (b)]. When plotted according to the Hill equation (86) the interaction coefficients (n) were 0.48 and 0.76 respectively for the control and photooxidized preparations [Fig. 29(c)].

When ATP concentration was varied between $10^{-4}$M and $10^{-5}$M, the $K_m$ values for the control were $2.7 \times 10^{-5}$M and $1.5 \times 10^{-4}$M while the $V_{max}$ values were $3.1 \times 10^5$ μM moles FDP formed/10 min/ml enzyme and $1.2 \times 10^5$ μM moles FDP formed/10 min/ml enzyme, respectively. After photooxidation, there was again
Fig. 29(a)  Effect of photooxidation on PFK in the presence of low concentrations of F6P at pH 8.4. Concentration of ATP was kept constant at 8 x 10^{-5}M. Photooxidation was carried out for 12 minutes in the presence of 10^{-6}M methylene blue and light.

\[\text{\textbullet \textbullet \textbullet}, 'control' \ PFK\]

\[\text{\textbullet \textbullet \textbullet}, 'photooxidized' \ PFK\]
Replots of Fig. 29 (a) data by the Lineweaver-Burk method.

 difficile, 'control' PFK

 difficile, 'photooxidized' PFK
Fig. 29(c) Plot of the Hill coefficient in 'control' and 'photooxidized' PFK in the presence of low concentrations of F6P at pH 8.4. Photooxidation was carried out for 12 minutes.

▼▼, 'control' PFK
●●●, 'photooxidized' PFK
Effect of photooxidation on PFK in the presence of low concentrations of ATP at pH 8.4. Concentration of F6P was fixed at $10^{-4}$ M. Initial reaction velocities are expressed as μ moles FDP formed/10 min/ml enzyme. Photooxidation was carried out for 12 minutes in the presence of $10^{-6}$ M methylene blue and light.

❖❖❖, Control

●●●, Photooxidized PFK
Fig. 30 (b) Replots of Fig. 30 (a) data by the Lineweaver-Burk method.

△ △, Control
● ●, Photooxidized PFK
one $K_m$ ($3.2 \times 10^{-5}$M) and one $V_{max}$ ($3.6 \times 10^5$ mµ moles FDP formed/10 min/ml enzyme) [Fig. 30(a) and (b)]. As in the case of low F6P concentrations seen previously, the Hill coefficients for the control and photooxidized enzymes were 0.38 and 0.51 respectively [Fig. 30(a)].

3. **Effect of photooxidation on kinetics at pH 6.8**: Titration on the control enzyme and the photooxidized enzyme with ATP showed typical inhibition curves at concentrations above $2 \times 10^{-5}$M (Fig. 31). Photooxidation did not alter the shape of the ATP inhibition curve. The $K_m$ and $V_{max}$ were not significantly different in both cases.

Titration curve for F6P at pH 6.8 with the control enzyme had inflexion points with two $K_m$ values [Fig. 32(a)]. On a double reciprocal plot [Fig. 32(b)] there was distinct discontinuity in the control enzyme giving two $K_m$ values of $0.4 \times 10^{-6}$M and $3.2 \times 10^{-5}$M respectively, while the $V_{max}$ values were $0.3 \times 10^5$ mµ FDP formed/10 min/ml enzyme and $0.9 \times 10^5$ mµ FDP formed/10 min/ml enzyme. After photooxidation, the kinetic curve for F6P was converted to a rectangular hyperbola with a $K_m$ of $0.7 \times 10^{-5}$M and a $V_{max}$ of $0.7 \times 10^5$ mµ FDP formed/10 min/ml enzyme. These results were plotted according to the Hill equation (86) [Fig. 32(c)] and the interaction coefficient in the case of the photooxidized enzyme was 0.73 while that of the control was 0.38. This indicated that the negative cooperative homotropic interaction shown by the control enzyme persists in the photooxidized enzyme but to a lesser degree.
Fig. 30(c)  Plot of the Hill coefficient in 'control'
and 'photooxidized' PFK in the presence of
low concentrations of ATP at pH 8.4 from data
in Fig. 30(a). Photooxidation was carried out
for 12 minutes in the presence of $10^{-6}$M
methylene blue and light.

\[\text{\textbullet \textbullet}, \text{ 'control' PFK}\]

\[\text{\textbullet \textbullet \textbullet}, \text{ 'photooxidized' PFK}\]
Fig. 31  
Effect of photooxidation on ATP inhibition at pH 6.8. Initial reaction velocities are expressed as μ moles FDP formed/10 min/ml enzyme. Photooxidation was carried out for 10 minutes in the presence of $10^{-6}$M methylene blue and light.

____, Control

_____ , Photooxidized PFK
Fig. 32(a)  Effect of photooxidation on PFK at low F6P concentrations at pH 6.8. Photooxidation was carried out for 10 minutes in the presence of $10^{-6}$M methylene blue and light.

▼▼, 'control' PFK
●●●, 'photooxidized' PFK
Replots of Fig. 32 (a) data by the Lineweaver-Burk method.

\[\text{\textbullet\textbullet\textbullet}, \text{ Control}\]

\[\text{\textbullet\textbullet\textbullet}, \text{ Photooxidized PFK}\]
Fig. 32(c)  Plot of the Hill coefficient in 'control' and 'photooxidized' PFK in the presence of low concentrations of F6P at pH 6.8 from data in Fig. 32(a). Photooxidation was carried out for 10 minutes in the presence of $10^{-6}$M methylene blue and light.

\[\begin{align*}
\text{\textbullet \textbullet}, \text{ 'control' PFK} \\
\text{\textbullet \textbullet}, \text{ 'photooxidized' PFK}
\end{align*}\]
Fig. 33  
Time course of cyclic-3',5'-AMP effect on the 'control' and 'photooxidized' PFK at pH 6.8. Assay conditions for reaction mixture B were used and are given in the text. Initial reaction velocities are expressed as µ moles FDP formed/10 min/ml enzyme. Photooxidation was carried out for 10 minutes in the presence of 10^{-6}M methylene blue and light. The addition of water or cyclic-3',5'-AMP is indicated by arrow.

- - 'Control' + water (protein conc. = 75 µg)
- - 'Control' + 10^{-4}M cyclic-3',5'-AMP (protein conc. = 75 µg)
- - 'Photooxidized' PFK + 10^{-4}M cyclic-3',5'-AMP (protein conc. = 100 µg)
The effect of cyclic-3',5'-AMP on the photooxidized enzyme at pH 6.8 was studied. The results (Fig. 33) showed that the stimulatory effect of the cyclic nucleotide was abolished by photooxidation.

VI. **Molecular weight:** In the present work, it has been found that PFK from human erythrocyte sedimented as a single sharp boundary with $s_{20,w}$ values between 4.80 and 8.50 (protein concentrations between 0.35 mg/ml and 0.90 mg/ml) in the sedimentation velocity experiments which employ high speed centrifugation. In recent years, the molecular weights of a number of substances have been obtained quite accurately from sedimentation equilibrium data using the absorption optics at relatively low rotor speeds for prolonged periods in the ultracentrifuge so that equilibrium is achieved. Since the rate at which a macromolecule sedimenting in an ultracentrifuge is dependent on its molecular weight, rate of diffusion, shape, concentration, tendency to polymerize and interaction with buffer, etc., the calculation of molecular weight from sedimentation coefficients would involve calibration of the diffusion constant. In a sedimentation equilibrium run, however, the movement of the solute to the bottom of the cell due to the centrifugal force is exactly balanced by the movement of the solute to the top of the cell by diffusion down the concentration gradient so that the molecular weight of a protein can be determined directly from the recorded data by the photoelectric scanner.

The sedimentation equilibrium method has certain advantages
over chromatography on sephadex for molecular weight determination:

1) It requires relatively low concentrations of protein. This presents advantages in cases when it is difficult to obtain a pure enzyme preparation in large quantities.

2) Only a small volume of protein solution is used in each double sector cell.

3) With multi-channel rotor heads, as much as three separate determinations can be performed simultaneously. This is particularly useful in studying alterations of molecular weights in the presence of added ligands.

In the present work, the molecular weights of purified PFK under various experimental conditions were determined by the sedimentation equilibrium method (74-78) in a Spinco Model E analytical ultracentrifuge. The optimal speed and the equilibrium time were determined by running a sample of the purified enzyme in the ultracentrifuge at an arbitrarily chosen speed suitable for a protein with \( s_{20,w} \) values between 4.0 and 8.0 over a period of 48 hours (78). Photoelectric scans of the enzyme were taken at various time intervals. This procedure was repeated with another rotor speed. Initial overspeeding and subsequent reduction of rotor speed would facilitate the attainment of equilibrium. In using the sedimentation equilibrium method for molecular weight determinations, the logarithms of optical densities are plotted as a function of square of radial position \( (X) \) at a series of points across the portion of the chart corresponding to the cell. In general, optical densities are linearly
proportional to concentrations so that conversions of O.D. into corresponding concentration values are not needed. The molecular weight is proportional to the slope of the log O.D. versus \( X^2 \) plot and can be obtained by substituting the numerical slope into the equation:

\[
M = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \times \frac{d \ln c}{d (X^2)}
\]

\[
= \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \times \text{slope} \times 2.303
\]

(Note: The partial specific volume, \( \bar{V} \), of most proteins is generally between 0.70 and 0.75 ml/g and its accurate determination requires relatively large amounts of protein. Due to the small quantities of 'purified' PFK available, \( \bar{V} \) was not estimated in the present work. An assumed value of 0.72 ml/g was used in the calculation of molecular weights.). The log O.D. versus \( X^2 \) plots for PFK in 0.05M potassium phosphate buffer containing \( 10^{-5}\text{M} \) fructose-1,6-diphosphate, \( 10^{-4}\text{M} \) ATP and \( 10^{-3}\text{M} \) dithiothreitol at 10,589 r.p.m., 13,410 r.p.m. and 15,220 r.p.m. within 48 hour intervals from the midpoint of the fluid column to the cell bottom are shown in Figs. 34, 35 and 36, respectively. Equilibrium was attained when the slope of the curve remained constant. Unless otherwise stated, molecular weight determinations of PFK in the present investigation were performed at 15,220 r.p.m. and 5° for 18 to 24 hours. The protein concentration of the enzyme was kept within 0.35 mg/ml to 0.45 mg/ml.
The change of concentration and concentration gradient with increasing time in a sedimentation equilibrium experiment for purified PFK from human erythrocytes in 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}$M Cleland's reagent, $10^{-4}$M ATP and $10^{-5}$M FDP at 10,589 r.p.m. and 5°.
Speed = 10,589 r.p.m.
Temp. = 5°C
Protein concentration = 0.40 mg/ml

10 hrs
26 hrs
34 hrs
46 hrs
Fig. 35  The change of concentration and concentration
gradient with increasing time in a sedimentation
equilibrium experiment for purified PFK from
human erythrocytes in 0.05M potassium phosphate
buffer, pH 8.0, containing 10^{-3}M Cleland's
reagent, 10^{-6}M ATP and 10^{-5}M FDP, at 13,410
r.p.m. and 5°.
Speed = 13,410 r.p.m.
Temp. = 5°C
Protein concentration = 0.40 mg/ml
The change of concentration and concentration gradient with increasing time in a sedimentation equilibrium experiment for purified PFK from human erythrocytes in 0.05M potassium phosphate buffer, pH 8.0, containing $10^{-3}$M Cleland's reagent, $10^{-4}$M ATP and $10^{-5}$M FDP at 15,220 r.p.m. and 5°.
Speed = 15,220 r.p.m.
Temp. = 5°C
Protein concentration = 0.40 mg/ml

- 12 hrs
- 17 hrs
- 23 hrs
- 29 hrs
A typical scan and the subsequent log O.D. versus $X^2$ plot throughout the entire double-sector cell for PFK from human erythrocytes in 0.05M potassium phosphate buffer containing $10^{-5}$M fructose-1, 6-diphosphate, $10^{-4}$M ATP and $10^{-3}$M dithiothreitol at 15,220 r.p.m. and 5° after 23 hours of ultracentrifugation are shown in Figs. 37 and 38. The slope of the log O.D. versus $X^2$ plot is directly proportional to the apparent molecular weight of the protein. According to Chervenka (78), the plot is a straight line if the solute is homogeneous and ideal. If the plot curves downward (i.e., molecular weight decreases with increasing concentration), this usually indicates that the solution is non-ideal. If the plot curves upward, then the general indication is that the solute is heterogeneous due to (i) a mixture of materials of differing molecular weights, (ii) the original sample having undergone degradation or (iii) the original material interacting to form aggregates of higher molecular weight.

It may be recalled that a concentration dependence of PFK from human erythrocytes was demonstrated in the sedimentation velocity studies and on polyacrylamide gel electrophoresis. In molecular weight determinations using the sedimentation equilibrium method, there is a concentration gradient from the top to the bottom of the cell. One would expect a curved log O.D. versus $X^2$ plot from a concentration-dependent interacting system such as PFK. When Figs. 34, 35, 36 and 38 are examined carefully, one would notice that all of the log O.D. versus $X^2$ plots concaved upwards toward the bottom of the double sector cell. This finding provides further evidence that PFK from human erythrocytes interacted to form aggregates of
Fig. 37  Photoelectric scan of 'purified' PFK from human erythrocytes in the determination of molecular weight by the equilibrium sedimentation method in the Spinco Model E analytical ultracentrifuge. The enzyme was in 0.05M potassium phosphate buffer, pH 8.0, containing 10^{-3}M dithiothreitol, 10^{-4}M fructose-1,6-diphosphate and 10^{-5}M ATP.

Scanning conditions:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Monochromator</td>
<td>280 μ</td>
</tr>
<tr>
<td>Rotor speed</td>
<td>15,220 r.p.m.</td>
</tr>
<tr>
<td>Chart speed</td>
<td>5 mm/sec</td>
</tr>
<tr>
<td>Temperature of run</td>
<td>5°C</td>
</tr>
<tr>
<td>Equilibration time</td>
<td>23 hours</td>
</tr>
</tbody>
</table>
Fig. 38  Plot of the log of the optical density (280 m\(\mu\)) versus distance \((X^2)\) from axis of rotation in the molecular weight determination of PFK from human erythrocyte by the equilibrium sedimentation method.
Speed = 15,220 r.p.m.
Temp. = 5°C
Protein concentration = 0.40 mg/ml
Time of run = 23 hrs

DISTANCE (X^2) in CM^2
higher molecular weights at higher concentration regions near the cell bottom. Experimentally, at equilibrium (15,220 r.p.m., 5° and 18-24 hours), a considerable portion of the log O.D. versus X² plot is linear (Fig. 38). In the present work, for the convenience of calculation, the slope of the log O.D. versus X² curve was plotted from the meniscus end of the scan to the midpoint of the fluid column in the cell.

The molecular weights of PFK isolated from human erythrocytes under various experimental conditions (Table 4) indicated that the enzyme, as in the case of PFK from other sources, existed in dissociated and associated forms. Previous experiments showed that PFK in potassium phosphate buffer, pH 8.0, containing 10⁻⁴M ATP, 10⁻⁸M FDP and 10⁻³M Cleland's reagent lost all of its activity in three hours when dialyzed free of stabilizers (see Fig. 4). However, during the course of investigation, it was found that the activity of the enzyme could be retained if the preparations were dialyzed in the same buffer containing 10⁻³M Cleland's reagent and 2% (NH₄)₂SO₄. Unfortunately, PFK preparations dialyzed this way were very unstable and could not be stored. Nevertheless, they provided conditions whereby the effects of ligand-binding on the enzyme could be studied adequately. It can be seen that the active 'native' enzyme (PFK dialyzed against 2% (NH₄)₂SO₄) has a molecular weight which is more or less identical to that of the inactive 'native' enzyme (PFK dialyzed without (NH₄)₂SO₄), i.e., approximately 40,000. It is quite likely that NH₄⁺ ions protect the 'native' enzyme against inactivation by preserving the integrity of the active
TABLE 4

Molecular forms of PFK from human erythrocyte in 0.05M potassium phosphate buffer

\( \nu = 0.72 \text{ ml/g} \) (an assumed value, see text).

* The sedimentation equilibrium runs were done at 8225 r.p.m. and 5° for 24 hours.
<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>Conditions</th>
<th>Ligand(s) Added</th>
<th>Calculated M.W.</th>
<th>Average M.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>1. Containing $10^{-4}$M ATP, $10^{-5}$M FDP and $10^{-3}$M Cleland's reagent</td>
<td>None</td>
<td>$72,600; 78,000; 84,000; 86,500$</td>
<td>$80,300$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}$M ATP*</td>
<td>$198,000; 205,000$</td>
<td>$201,500$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}$M F6P</td>
<td>$40,000; 44,000$</td>
<td>$42,000$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8M urea</td>
<td>$23,000; 24,000$</td>
<td>$23,500$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5M guanidine hydrochloride</td>
<td>$22,000; 27,000$</td>
<td>$24,500$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-6}$M methylene blue</td>
<td>$79,000; 83,000$</td>
<td>$81,000$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(photooxidized 10 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Same as (1) but dialyzed against 2% (NH₄)₂SO₄ at 4°</td>
<td>None</td>
<td>$40,600; 42,500$</td>
<td>$41,500$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}$M F6P</td>
<td>$41,000; 44,000$</td>
<td>$42,500$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}$M F6P and $10^{-4}$M ATP</td>
<td>$43,000; 38,000$</td>
<td>$40,500$</td>
</tr>
<tr>
<td></td>
<td>3. Same as (1) but dialyzed without (NH₄)₂SO₄</td>
<td>None</td>
<td>$42,700; 45,000$</td>
<td>$43,800$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}$M F6P</td>
<td>$40,500; 38,000$</td>
<td>$39,000$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-3}$M F6P and $10^{-4}$M ATP</td>
<td>$36,700; 40,300$</td>
<td>$38,500$</td>
</tr>
<tr>
<td></td>
<td>4. Same as (1) but totally inactive due to prolonged storage</td>
<td>None</td>
<td>$84,260; 82,000$</td>
<td>$83,100$</td>
</tr>
<tr>
<td>6.8</td>
<td>Containing $10^{-4}$M ATP, $10^{-5}$M FDP and $10^{-3}$M Cleland's reagent</td>
<td>None</td>
<td>$23,000; 18,800$</td>
<td>$20,900$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-4}$M cyclic-3',5'-AMP</td>
<td>$19,000; 24,500$</td>
<td>$21,700$</td>
</tr>
</tbody>
</table>
site. The addition of either ligand ATP or F6P to the active and inactive 'native' enzyme was not accompanied by a significant change in molecular weight. However, aggregation effect was observed in PFK preparations (active and inactive) stored in potassium phosphate buffer, pH 8.0, containing the added ligands FDP (10^{-5}M), ATP (10^{-4}M) and Cleland's reagent (10^{-3}M) as stabilizers and also in preparations photooxidized at this pH (M.W. approximately 80,000). The tendency for the enzyme to polymerize into still larger macromolecules was seen in the presence of inhibitory concentrations of ATP (10^{-3}M). On the other hand, agents such as 8M urea and 5M guanidine hydrochloride tend to dissociate the 'native' enzyme into inactive subunits (M.W. approximately 20,000). At pH 6.8, the enzyme also exists as a low molecular weight subunit (M.W. approximately 20,000) but it appeared to be partially active as shown in previous kinetic experiments at pH 6.8. It may be important to point out that the addition of cyclic-3',5'-AMP to PFK preparations at pH 6.8 was not accompanied by a significant alteration in molecular weight.
DISCUSSION

A unique kinetic property of PFK isolated from other sources is a sigmoidal dependence of the velocity on the F6P concentration when the other substrate, ATP, is present at inhibitory levels (26-27, 41, 45-46, 87). Activator effectors convert the sigmoidal curves into hyperbolic response by antagonizing the inhibitory effect of ATP probably by decreasing the binding of ATP at the allosteric site. Sigmoidal response is characteristic of a large number of regulatory enzymes and usually indicates some kind of cooperative interaction between the enzyme and more than one molecule of substrate. According to the allosteric transition theory of Monod et al. (35) which predicts only two states (R and T states) in dynamic equilibrium with each other, preferential binding of substrates or modifiers to one or the other state would lead to an apparent change in the equilibrium between these two states resulting in the expression of sigmoidal saturation kinetics.

In the present investigation, PFK isolated from human erythrocytes displayed a distinct departure from the classical sigmoidal kinetics exhibited by PFK from other sources. The enzyme exhibited Michaelis-Menten kinetics only when either one of its substrates, F6P or ATP, was present at saturating levels (Figs. 13, 14, 15 and 20). When either ATP or F6P was present in low (non-saturating) concentrations at pH 8.4, apparent negative cooperativity toward substrates prevailed. Similarly, apparent
negative homotropic interactions with respect to F6P were also observed at pH 6.8 when ATP was present at inhibitory concentrations [Figs. 27(a) and 27(b)]. A salient feature of all these substrate concentration versus initial velocity plots is the pronounced intermediary plateau regions, i.e., hyperbolic at low substrate concentrations and sigmoidal at higher substrate concentrations. Double reciprocal plots were also rather complex consisting of two portions with significantly different slopes (biphasic curves). Similar kinetics have been observed for glutamate dehydrogenase from several animal sources (29, 88-90), homoserine dehydrogenase (91), phosphoenolpyruvate carboxylase (92) and cytidine triphosphate synthetase (28). Such negative homotropic interactions cannot be accounted for by the symmetrical allosteric model of Monod et al. (35) which assumes very strong interactions between subunits which do not seem to be a necessary consequence of protein structure. On the other hand, the 'sequential' model of Koshland et al. (36), though more complex, is more flexible and can be used to explain a wide variety of kinetic observations. This model not only predicts positive and negative heterotropic interactions but also positive and negative homotropic interactions, making allowances that significant concentrations of molecular species containing hybrid conformational states can exist. In this model, it is assumed that the binding of a ligand induces a conformational change in neighboring subunits or neighboring parts of the same polypeptide chain which may be translated with varying degrees of efficiency to other subunits. A ligand which strengthens substrate-binding is an activator whereas a ligand which weakens substrate-binding is an
inhibitor. Binding of like ligands is said to be cooperative if the addition of the first substrate molecule increases the affinity of the second and this sequential change is transmitted to neighboring units. If the addition of the first substrate molecule decreases the binding of the second substrate molecule, a negative cooperative effect is observed. If the binding of the first ligand causes very little or no change in subunit interactions, then the kinetics will be a simple Michaelis-Menten type even though the subunits are held together in a polymeric form. In this case the subunits are very weakly coupled and consequently each one acts independently of others in a polymer. (Subunit interactions are considered basically the same as interactions within the polypeptide chain.). The homotropic interactions of like ligands are made completely analogous to the heterotropic interactions of unlike ligands and thus positive and negative allosteric effects are explained.

Levitski and Koshland (28) suggested several possibilities which may be responsible for the negative cooperativity observed in enzymic reactions:

1. Ligand-induced conformational changes which affect subunit interactions, i.e., ligand may decrease the affinities of unoccupied binding sites in the protein.

2. Electrostatic repulsion between ligands.

3. Non-identical peptide chains having active sites with different binding constants, i.e., in isozymes.

4. Two or more polymeric forms of the same enzymes.
5. Geometric arrangements of identical chains which produce non-
identical sites either because of static geometry or because
of subunit conformational changes during the association of
subunits, i.e., burying of some sites and the exposure of
others.

More recently, Teipel and Koshland (93) verified experimentally that saturation curves with intermediary plateau regions
could be obtained from:

1. Enzyme possessing more than two substrate-binding sites whose
catalytic or binding constants vary non-linearly with saturation,
e.g., glutamate dehydrogenase (29), cytidine triphosphate syn-
theticase (28) and glyceraldehyde-3-phosphate dehydrogenase (94)
are known to possess four or more subunits and exhibit negative
cooperativity.

2. Multisite enzymes whose catalytic or binding constants first de-
crease and then increase as the enzyme is saturated.

3. Single site enzymes when there is interaction between the sub-
strate and a modifier (95).

Enzyme model for human erythrocyte PFK

On the basis of molecular weight determinations (Table 4)
it is apparent that the enzyme is characterized by an association-
dissociation phenomenon. Association-dissociation equilibrium has also
been demonstrated in PFK obtained from other sources (44, 53-54, 56).
A number of enzymes which do not self-associate have been shown to be
capable of associating with other protein molecules, e.g., pyruvate or α-ketoglutarate dehydrogenase complexes (96). Protein-protein interaction has been speculated to play an important role in metabolic control (97). Frieden (98) divided protein-protein interactions observed in enzyme systems into two classes:

1. Those which involve interactions within a single enzyme species and are characterized by molecular weight changes between forms of the same enzyme which do not markedly differ in conformation. This class includes enzymes which undergo either reversible association-dissociation reaction or reversible inactivation associated with molecular weight changes.

2. Those which involve the interaction of a particular enzyme with a different enzyme or protein species. Enzymes represented in this class have a tendency to bind tightly to each other so that they persist even after disruption of the cell and subsequent dilution with a medium of quite different dielectric constants, e.g., pyruvate or α-ketoglutarate dehydrogenase and fatty acid synthetase (96).

A simple model (Fig. 39), based on the molecular weight determination of the enzyme under various experimental conditions (Table 4), may be postulated for the molecular transformation of PFK isolated from human erythrocytes. It is evident that protein-protein interactions will contribute to an alteration of the kinetic properties of the enzyme according to this model.

1. The enzyme exists in a tetramer-dimer-monomer equilibrium.
Fig. 39  Molecular model for human erythrocyte PFK
LARGE AGGREGATE ← High ATP

M·W: 200,000

Low temp:
ATP
F6P

M·W: 80,000

M·W: 40,000

OH⁻ → H⁺

M·W: 20,000

8M Urea
5M Guanidine hydrochloride

M·W: 20,000
2. The isolated enzyme stored in potassium phosphate buffer (pH 8.0) containing the added ligands FDP ($10^{-5}$M), ATP ($10^{-4}$M) and Cleland's reagent ($10^{-3}$M) as stabilizers exists predominantly as the tetramer (M.W. approximately 80,000) which is in equilibrium with the dimer (M.W. approximately 40,000). The equilibrium between the tetramer and dimer is regulated by some ligands which contribute to the observed peculiar kinetic properties of the enzyme. The tetramer dissociates readily into the dimer at higher concentrations of F6P. Due to the ease of dissociation, it may be postulated that the tetrameric form exists as a loosely-associated species and is unstable. On the other hand, low temperature and ATP favour the tetrameric form. It might be possible that under normal conditions of isolation PFK was obtained initially in the dimeric form but aggregated into the tetrameric form when the enzyme is stored in the cold. It has been found that one of the properties of allosteric enzymes is cold sensitivity. In most cases, it is accompanied by dissociation into smaller subunits resulting in a loss of enzyme activity but association has also been observed in allosteric enzymes which are labile at low temperature (33, 87-88). The initial loss of PFK activity (about 20%) observed during the first 24 hours of storage in the cold (Fig. 6) could therefore be a consequence of aggregation.

3. The dimer and tetramer are catalytically active and insensitive to nucleotide activation. If either one of these molecular forms predominates at pH 8.4 in the presence of optimal substrate
concentrations it is expected to yield Michaelis-Menten type kinetics. This prediction is satisfied when Figs. 15, 16, 17 and 20 are examined.

4. The tendency for the dimer or tetramer to aggregate into even larger macromolecules is promoted by inhibitory concentrations of ATP. This large aggregate, according to this model, is inactive.

5. The dimer may be dissociated reversibly into the 'subactive' monomer with a molecular weight of approximately 20,000. The equilibrium between the dimer and the 'subactive' monomer is a function of pH. Lowering the pH to 6.8 favors the dissociation of dimers into 'subactive' monomers while an increase in pH promotes association. This equilibrium is evident from the inactivation and reactivation studies of PFK at both acidic and alkaline pH (Table 2). Due to the poor recovery of enzyme activity on reactivation it would appear that this association-dissociation phenomenon is not a true dynamic equilibrium. Changes in pH may represent a great physiological flexibility in the regulation of the enzyme, particularly in conformational changes and alterations in electrostatic charges that may be involved in interprotomer bonds. Since the activating effect of adenine nucleotides was induced by pH alterations (Figs. 23 and 24), it may be logical to assume that the 'subactive' monomer is the predominant enzyme species under assay conditions at pH 6.8. (Data obtained in the present investigation does not exclude the possibility that the 'subactive' monomer can undergo aggregation during assay at pH 6.8.) The binding
of these activator effectors may be assumed to occur at the allostERIC site on the enzyme surface since there was no significant alteration in molecular weight as a result of ligand binding (Table 4).

6. Urea and guanidine hydrochloride cleaved the fully active enzyme irreversibly into totally inactive monomers (M.W. approximately 20,000).

7. Photooxidation of PFK in the presence of methylene blue and light stabilizes the tetramer at pH 8.4 (Table 4).

It should be pointed out that the experimental data could, of course, be fitted by a theoretical equation and values for the constants derived but this would be premature until sufficient equilibrium and detailed kinetic studies of the enzyme-coenzyme reactions are available to establish the number of binding sites and active centres per molecule and whether negative cooperativity is involved in these steps.

**Mechanism for the 'apparent' negative cooperativity at pH 8.4**

According to the enzyme model postulated for the molecular organization of PFK, it can be easily conceived that the 'apparent' negative cooperativity observed is largely attributed to the dissociation-association equilibrium between the tetramer and dimer. At low (non-saturating) concentrations of F6P, the predominant
enzyme species is the tetramer which can dissociate into the catalytically active dimeric form at higher F6P levels. On the other hand, at low (non-saturating) concentrations of ATP, the dimer is probably the predominant enzyme species which can aggregate either into the tetrameric form at higher ATP concentrations or into inactive large macromolecules at still higher ATP concentrations. When the system was saturated with either F6P or ATP, only one enzyme species was present and it is, therefore, not difficult to reconcile the observed Michaelis-Menten kinetics with this model.

**Mechanism for 'apparent' negative cooperativity at pH 6.8.**

At this pH, the enzyme attained its maximal activity over a narrow range of low ATP concentrations beyond which strong inhibition was observed (Fig. 22). Activators, e.g., cyclic-3',5'-AMP, AMP and, to a lesser extent, ADP relieved the ATP inhibition competitively (Figs. 23-26) and converted the typical inhibition curve into a hyperbola (Fig. 25) without an alteration in molecular weight (Table 4). In keeping with the fact that activation by the cyclic nucleotide (and AMP) occurred in the ATP-inhibited enzyme (Fig. 28), it is quite probable that the inhibition by ATP at pH 6.8 was due to the binding of ATP at a second regulatory site which is quite distinct from the substrate site. These activators therefore counteract the inhibitory effect of ATP either by directly competing with ATP for the regulatory site or indirectly as a result of interactions at other binding sites. Since it has been demonstrated that the predominant molecular species of PFK at pH 6.8
is a monomer with molecular weight of approximately 20,000
(Table 4), a mechanism may be postulated with the monomeric form
of the enzyme consisting of at least two ATP sites - a substrate
site and an inhibitor site. In this model, the substrate sites
for F6P and ATP are separate but adjacent to each other. For sim-
plicity, one ATP inhibitor site is placed at the opposite side of the
enzyme molecule in the diagram (Fig. 40). At low concentrations of
ATP, the enzyme is partially inhibited. In the presence of high con-
centrations of ATP, the inhibitor site is occupied by ATP and the
enzyme is severely inhibited. According to this scheme, attachment
of ATP occurring at the allosteric site on the enzyme induces a con-
formational change in the enzyme molecule so that the catalytic sites
for F6P and ATP are now far apart and enzyme catalysis becomes less
likely. In view of the fact that the adenine nucleotides - AMP, ADP
and cyclic-3',5'-AMP did not appreciably stimulate the activity of
PFK unless the enzyme was already inhibited by ATP, it may be assumed
that the binding of these nucleotides at the allosteric (regulatory)
site resulted in the competitive exclusion of ATP and restored the
enzyme to its original configuration so that catalysis could occur.
Present evidence does not eliminate a different regulatory site
specific for each of these nucleotides. The time lag observed for
a duration of approximately 2 to 3 minutes prior to enzyme
activation (Fig. 28) may be a reflection of the time required for
these conformational changes to occur.
Fig. 40  Enzyme model of human erythrocyte PFK at pH 6.8
Mechanism for the desensitization of PFK.

At pH 8.4, PFK photooxidized in the presence of methylene blue and light was shown to be a tetramer with M.W. of approximately 80,000 (Table 4) which retained its 'apparent' negative homotropic interactions toward both F6P and ATP when these substrates were present in low (non-saturating) concentrations [Figs. 29 (a), (b) and (c) and 30 (a), (b) and (c)]. The absence of both intermediary plateau regions on the substrate concentration-initial velocity plots and the distinct non-linearity on the double reciprocal plots may be rationalized as the inability of the tetramer to dissociate into the stable active dimer configuration, i.e., photooxidation conferred a stabilizing effect on the tetramer. The work of Ahlfors and Mansour (79) also showed that sheep heart PFK remains in a fully active, associated form after photooxidation in the presence of methylene blue.

At pH 6.8, the stable form of the enzyme, according to the proposed model, would be the monomer. Photooxidation did not alter the shape of the ATP inhibition curve (Fig. 31) but the enzyme was converted into a form which was totally insensitive to the activation by cyclic-3',5'-AMP (Fig. 33). These kinetic parameters are therefore consistent with the assumption that PFK has a regulatory site for ATP which is different from the catalytic site. It is quite probable that photooxidation of the enzyme might specifically affect those groups essential for binding ATP at the allosteric site and thus induce a different conformation in the protein resulting in different kinetic properties. The fact that maximum activity of the enzyme was
affected slightly suggests that both active and allosteric sites
might have some identical functional groups. It might also be
possible that both sites are altered by a conformational change
caused by alteration of one group. The work of Weil et al. (99-101)
on photooxidation of β-lactoglobulin and crystalline lysozyme in
the presence of methylene blue indicated that the amino acids
tyrosine, tryptophan, histidine, methionine and cysteine were highly
reactive but peptide bonds did not participate in the photo-
chemical reaction. Whether these amino acids are important con-
stituents in the reactive centre of PFK has yet to be determined
by amino acid composition analysis.

It is interesting to point out the more recent findings of
Setlow and Mansour (102) on sheep heart PFK desensitized to ATP in-
hibition by photooxidation of the enzyme in the presence of methylene
blue: of a total of 18.2 moles of histidine per 10^5g of enzyme an
average of 3 were lost after photooxidation. The total number of
cysteine residues was reduced from an average of 14.0 to 6.6 moles/
10^5g of the enzyme after such treatment. Methionine was thought to be
affected by photooxidation also. On the other hand, acylation of the
enzyme by ethoxyformic anhydride which reacts specifically with the
histidine residues in the enzyme showed that both the sensitivity to
ATP inhibition and the cooperative kinetics can be abolished without
affecting the sulfhydryl groups in the enzyme. On the basis of this
finding, these authors concluded that the loss of allosteric proper-
ties of the desensitized enzyme might be largely attributed to the
alteration of histidine residues.
Physiological significance of phosphofructokinase regulation.

The mature human erythrocyte is unique in that it lacks a functional tricarboxylic acid cycle (4) and thus presumably it is dependent on the Embden-Meyerhof pathway exclusively for ATP energy production. Although 10% of the glucose consumed by the cell may be shunted via the pentose phosphate pathway yielding NADPH (6-10), it is not evident that conversion of this energy to ATP occurs in these cells. ATP is known to be required for the preservation of the proper molecular configuration of the cellular membrane, the maintenance of ionic equilibrium, and the synthesis of coenzymes (103-104). In view of these considerations, it is reasonable to assume that the survival of the erythrocyte is fundamentally dependent on the functional state of the glycolytic pathway. Since the mature erythrocyte retains little capacity for de novo biosynthesis of protein (1-2) and nucleotides (3), the loss of a single most labile component of the glycolytic apparatus could effectively limit the life span of these cells.

Kinetic and molecular analyses of PFK in the present work strongly implied that the regulation of its activity in vitro seems to be exerted through an association-dissociation equilibrium which is characterized by the ubiquitous 'apparent' negative homotropic interactions both at alkaline and near-neutral pH values. Until more of its kinetic properties are examined, the validity of such a mechanism for cellular regulation in vivo remains speculative. Whereas the effect of positive interactions is to make an enzyme very sensitive to small changes of substrate or coenzyme concentrations
over a narrow range, the effect of negative interactions is to make
the reaction rate continuously responsive to changes in concentration
over a very wide range. The maximum rate decreases from the highest
to the lowest concentration range. Due to the rapid combination with
the first ligand on the enzyme molecule, a reasonably steady rate of
reaction is maintained with small ligand concentrations. At higher
ligand concentrations, the rate of reaction increases substantially.
It may be important that some enzymes on major pathways be of con-
stant activity despite fluctuations in metabolite concentrations.
Since the survival of the mature human erythrocyte is dependent on the
Embden-Meyerhof pathway for the generation of energy in the form of
ATP, it may be necessary that a 'pacemaker' glycolytic enzyme such
as PFK should be insulated against extreme fluctuations in metabolite
concentrations. Under such conditions, negative cooperativity
would certainly provide an advantageous mechanism for the cell.
Koshland and Conway (31) suggested that it would be desirable to have
sites of decreasing affinity so that under normal workday conditions
the turnover of substrate is rapid and efficient. They postulated
the existence of two kinds of binding sites with different affinity
for a single identical ligand in enzymes which exhibit negative
cooperativity.
1. The 'tight' sites which serve as 'reservoirs' to be used under
   more demanding conditions, and
2. The 'loose' sites for operation under normal metabolite levels.
Accordingly, 'loose' sites would have greater reactivity than the
'tight' sites. The kinetic data from the present investigation are
consistent with this hypothesis in that both the $K_m$ and $V_{max}$ values are less at lower substrate levels than at higher substrate concentrations (Figs. 19, 21 and 27). Sequential binding of ligand therefore appeared to create successive new sites of lower affinity but higher turnover. High substrate concentrations would then be required to saturate the weak affinity sites which, when saturated, would have a greater turnover than the 'tight' sites.

The activation and inhibition effects of adenine nucleotides could well serve as a fine regulatory valve for glycolytic sequences at the locus of PFK. The regulation of this rate-limiting enzyme by energy metabolites suggests that its physiological function is to generate energy and valuable glycolytic intermediates. According to the adenylate control hypothesis of Atkinson (46, 105), the cellular regulation of energy metabolism is dependent on the momentary cellular AMP:ADP:ATP concentration ratio. The energy state of the cell controls the level of hexose monophosphates, which, in turn, regulate the phosphorylation of glucose. Thus, accumulation of ATP in an energy-rich cell would be expected to increase the G-6-P concentration leading to a decrease of hexokinase activity. On the other hand, an increase in the rate of ATP consumption (and, hence, an increased AMP:ATP ratio) should:

1. Increase the rate of glycolysis by modifying PFK kinetics.
2. Increase the rate of hexose monophosphate supply both by relieving the hexokinase inhibition (as a result of reduced glucose-6-P concentration) and by activating phosphorylase (106).
3. Conserve substrates for energy metabolism by preventing gluconeogenesis (by inhibiting fructose diphosphate phosphatase due to the increased AMP concentration (107) as well as the decreased ATP level) and glycogen synthesis (because of the decreased glucose-6-P concentration).

Thus, the AMP:ADP:ATP ratio may regulate the intracellular metabolic direction toward energy release or energy storage. Since the mature red cell is not endowed with a mechanism for glycogenesis and glycogenolysis (108), it may be logical to assume that the regulatory role of adenylate in human erythrocyte is focused on the glycolytic pathway.

Cyclic-3', 5'-AMP was discovered by Sutherland and Rall (109-110) as a factor enhancing phosphorylase activity. The intracellular production of this nucleotide, in turn, modulates the kinetics of some enzymes by acting as an allosteric modifier in effecting a cascade of reactions usually involving a kinase activating a kinase which, in turn, activates phosphorylase (111). The phenomenon of cyclic-3', 5'-AMP regulation may be of evolutionary significance. It may be recalled that PFK isolated from mammalian sources is modulated by both AMP and cyclic-3', 5'-AMP (26, 45, 47) but the same enzyme from microorganisms does not respond to the cyclic nucleotide (27, 46). It appears that the regulation of enzyme activity by cyclic-3', 5'-AMP was probably evolved through evolutionary time but the physiological significance of this in the modulation of red cell metabolism has yet to be determined.
Since mitochondria are not present in the mature human erythrocytes, changes in citrate concentration would not be expected to play an important role in controlling PFK activity. The relative insensitivity of PFK towards citrate inhibition in the present study (Fig. 18) is in keeping with this prediction. However, it may be speculated that citrate could play a role in the regulation of carbohydrate metabolism in developing nucleated red cells and reticulocytes which possess an active tricarboxylic acid cycle.


