The binding of 1,N⁶-ethenoadenosine-triphosphate (εATP) to rabbit muscle phosphofructokinase has been studied by fluorescence and circular dichroism, and compared to that of its counterpart, ATP. Muscle phosphofructokinase binds 11.3 ± 1.2 moles of εATP per tetramer with an average dissociation constant of 60 μM. This is in agreement with the report of 3 ATP binding sites per phosphofructokinase protomer (Kemp and Krebs, 1967). The binding of εATP is relatively homogeneous in comparison to the biphasic binding of ATP. Saturating concentrations of ATP, GTP, and ADP displace about 80% of the bound εATP from the enzyme whereas FDP and AMP displace only 27%. Citrate, on the other hand, enhances the affinity of phosphofructokinase for εATP. The effects of the binding of ATP and εATP on the conformation of the enzyme have also been compared. Binding of ATP results in increases in both the local rigidity and the ellipticity of the tryptophanyl side chains whereas binding of εATP causes a slight decrease in the local rigidity and has
virtually no effect on the ellipticity.

The interaction of rabbit muscle phosphofructokinase with F-actin has also been studied in vitro by quantitative SDS-polyacrylamide gel electrophoresis. Adsorption of the enzyme to F-actin produces a flocculent precipitate. The data indicate an extensive association with essentially stoichiometric binding of one phosphofructokinase tetramer per actin monomer and additional weaker binding at higher ratios. Precipitation of the enzyme by F-actin is complete at pH values below 7.3 and minimal at pH values greater than 8. Very low concentrations of ATP and ADP efficiently solubilize the complex. Activity measurements on the adsorbed phosphofructokinase show major alterations in catalytic activity, both with F-actin and the reconstituted thin filament. The interaction reverses the ATP inhibition and appears to enhance the binding of fructose-6-phosphate to the enzyme.
Binding Studies of Rabbit Muscle Phosphofructokinase:  
I. Binding of ATP and of 1,N\(^6\)-Ethenoadenosine-triphosphate  
to Phosphofructokinase. II. Binding and Activation of  
Phosphofructokinase by F-Actin.  

by  

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ABBREVIATIONS

εATP: 1, N^6-ethenoadenosine-5'-triphosphate
F6P: fructose-6-phosphate
FDP: fructose-1,6-diphosphate
PFK: phosphofructokinase
SDS: sodium dodecyl sulfate
I. INTRODUCTION

General Background: Regulation of Phosphofructokinase

Phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) has been a subject of considerable interest and extensive study. This enzyme has been purified from a number of sources and is believed to play a key role in the regulation of glycolysis (Mansour, 1972; Bloxham and Lardy, 1973; Hofmann, 1976).

Phosphofructokinase (PFK) catalyzes the transfer of the terminal phosphate of ATP to the C-1 hydroxyl of fructose-6-phosphate (F6P). The products are fructose-1,6-diphosphate (FDP) and ADP. A divalent cation (such as Mg\(^{2+}\) or Mn\(^{2+}\)) is required for activity. The large negative value of the free energy change of the reaction (Hanson et al., 1973) renders the reversal of the reaction very unfavorable in living organisms, and in gluconeogenesis the conversion of FDP to F6P is mediated through a hydrolytic reaction catalyzed by the enzyme fructose diphosphatase.

Phosphofructokinases from a variety of biological origin have complex molecular properties. A common feature of the active phosphofructokinases is the tetrameric structure. The molecular weights of the subunits differ widely, however. Whereas the subunit molecular weight of bacterial phosphofructokinase so far investigated is about
35,000 (Hengartner and Harris, 1975; Babul, 1978), it is 100,000 for yeast (Kopperschlager et al., 1977) and about 85,000 in mammals (Hofmann, 1976). In mammals a number of phosphofructokinase isoenzymes have been identified (Hofmann, 1976). Their active forms are the tetramer or its multiple forms.

Another common feature of phosphofructokinases from different sources is the complex kinetic properties. AT pH 8.2, which is the optimal pH for enzyme activity, phosphofructokinase exhibits Michaelis-Menten kinetics. At neutral or slightly acidic pH, typical allosteric kinetics are observed. The enzyme exhibits sigmoidal kinetics with respect to the substrate fructose-6-phosphate, inhibition by high concentration of ATP and citrate, and activation by AMP and ADP (Mansour, 1972).

Because of the molecular and kinetic complexities of this enzyme, its susceptibility towards various effectors, its strategic location in the metabolic process, and the quasi-irreversibility of the reaction, phosphofructokinase is highly adapted to be rate-controlling for the substrate flux through the glycolytic pathway (Heinrich et al., 1977) and to be the source of glycolytic oscillations (Hess, 1977). The unique properties of the enzyme and the important role of the enzyme in the regulation of glycolysis have rendered phosphofructokinase an intriguing subject in both biochemical and physiological studies. In the study of the complex nature of the regulatory behavior of the enzyme, substantial evidence shows that the state of aggregation of the enzyme as determined by several factors including allosteric
effectors are closely related to the activity of the enzyme. The mechanisms determining the regulatory behavior of phosphofructokinase are just beginning to unfold.

Association-Dissociation of Phosphofructokinase

The phosphofructokinases from a number of mammalian tissues have been demonstrated to be self-associating enzymes; they can reversibly associate to form aggregates higher than the tetramer (Aaronson and Frieden, 1972; Leonard and Walker, 1972; Wenzel et al., 1976) and dissociate into molecular forms smaller than the tetramer (Pavelich and Hammes, 1973). The state of aggregation of the enzyme is determined by several factors including pH, concentration, and various allosteric effectors. In contrast to mammalian phosphofructokinase, the yeast enzyme does not show an association-dissociation behavior over a wide range of protein concentration (Kopperschlager et al., 1978).

The polymerization of phosphofructokinase to high aggregates is largely dependent on enzyme concentration (Aaronson and Frieden, 1972). It appears to be of minor importance in the regulation of the enzyme, since the polymerization rarely occurs with change in enzyme activity.

Dissociation into the dimers is more complex. Phosphofructokinases from heart and skeletal muscle has been shown to dissociate into inactive dimers and reassociate to active tetramer by lowering the pH to about 6 and then raising the pH back to neutral (Mansour, 1965; Paetkau and Lardy, 1967; Mansour and Ahlfors, 1968). Quantitative measurements showed that activity loss and dissociation of enzyme are
a highly cooperative function of pH (Aarenson and Frieden, 1972; Pavelich and Hammes, 1973). Combination of pH and low temperature produces even more profound effect on the dissociation-inactivation of the enzyme (Bock and Frieden, 1974). The ionization of specific groups, presumably histidine residues, is considered responsible for the pH-dependent loss of activity (Bock et al, 1975).

Concentration also plays a role in triggering the dissociation of phosphofructokinase. Dilution of the enzyme at a given pH causes the active phosphofructokinase to dissociate into inactive dimers (Pavelich and Hammes, 1973). The influence of allosteric effectors is much more intriguing. Allosteric effectors appear to determine the state of aggregation of phosphofructokinase (Lad et al, 1973; Wenzel et al, 1976). For instance, at an enzyme concentration of 0.15 mg/ml and pH 7.0, strong activators such as fructose-1,6-diphosphate stabilize fully active tetramers, whereas citrate, a potent inhibitor of the enzyme, stabilizes the inactive dimers (Lad et al, 1973).

To summarize these observations, Bock and Frieden (1976a) postulated a mechanism to elucidate the effects of pH, temperature, and concentration on the dissociation-inactivation of phosphofructokinase. The mechanism assumes four species, two tetrameric and two dimeric, each of which can exist in a protonated and unprotonated form. Inactivation or reactivation induced by changes in pH or temperature reflect the kinetic establishment of new steady state between these forms. Thus, for example, lowering of pH causes the active tetramer to become protonated, presumably through histidine, which, followed
by isomerization, then dissociates into the inactive dimers. The reactivation may proceed through the kinetically different pathway of the same mechanism. The effects of ligands, including substrates and allosteric effectors, on the inactivation and reactivation of the enzyme are through their preferential binding to either protonated or unprotonated forms of the enzyme (Bock and Frieden, 1976b). It has been shown that the sigmoidal dependence of the reaction rate on the fructose-6-phosphate concentration and the inhibitory action of ATP are due to their preferential binding to two different forms of the tetrameric enzyme; fructose-6-phosphate binds to an unprotonated form and ATP to a protonated form of the tetramer (Frieden et al, 1976).

The mechanism, therefore, reasonably well accounts for the pH and ligand-dependent inactivation as well as the pH-dependent regulatory kinetic properties of phosphofructokinase. Kinetic data, however, pointed out that regulatory behavior is inherent in the tetrameric (active) form of the enzyme (Frieden et al, 1976). The dissociation process is too slow to be involved in, but is rather a consequence of, the formation of the enzyme conformation which controls the regulatory
properties. In that sense, the regulatory kinetic behavior can then be explained in a manner analogous to the allosteric model proposed by Monod et al (1965). In that model, sigmoidal kinetic behavior arises as a consequence of preferential binding of substrate to one of two conformational forms which are in rapid equilibrium.

**Binding of Substrates and Effectors**

A more profound insight into the regulatory properties of phosphofructokinase can be provided by quantitative studies of the binding of substrates and effectors to the enzyme. The binding studies have been carried out extensively on phosphofructokinases from rabbit skeletal muscle (Kemp and Krebs, 1967; Kemp, 1969; Colombo et al, 1975), sheep heart (Lorenson and Mansour, 1969; Setlow and Mansour, 1972), and recently, yeast (Nissler et al, 1976; Nissler et al, 1977).

Skeletal muscle phosphofructokinase binds one molecule of fructose-6-phosphate, AMP, ADP, cyclic 3',5'-AMP, and three molecules of ATP per protomer of 90,000 daltons (Kemp and Krebs, 1967). It also binds one molecule of citrate per enzyme protomer (Colombo et al, 1975). ATP and citrate bind to the enzyme at distinctive sites. Both inhibitors diminish the affinity of the enzyme for fructose-6-phosphate, whereas AMP increases it. Citrate increases the affinity for ATP and decreases it for AMP and fructose-6-phosphate. The affinity of the enzyme for MgATP is reduced by AMP, as well as by cyclic 3',5'-AMP and inorganic phosphate (Kemp and Krebs, 1967; Colombo et al, 1975). One of the three ATP binding sites could be the inhibitory site. Its affinity for MgATP is strongly influenced by fructose-6-
phosphate (Kemp, 1969). In a more recent report, Wolfman et al (1978) showed that rabbit muscle phosphofructokinase binds two moles of adenylyl imidodiphosphate, an ATP analog, per protomer. The "tight" binding site has been attributed to the catalytic site, and the "loose" one, regulatory site.

For heart phosphofructokinase, 3.6 moles of ATP, two moles of citrate, and 1.75 moles of fructose-6-phosphate are bound per protomer (Lorenson and Mansour, 1969). Fructose-6-phosphate is bound to two different classes of sites, one exhibiting low affinity and the other high affinity to this substrate. Both classes of sites change their affinity for fructose-6-phosphate in response to allosteric inhibitors and activators (Setlow and Mansour, 1972). When the enzyme is photooxidized and becomes insensitive to ATP and citrate inhibition, it binds two moles of ATP, two moles of fructose-6-phosphate and no citrate. Hence, heart phosphofructokinase seems to have more than one ATP inhibitory site (Lorenson and Mansour, 1969).

One protomer of yeast phosphofructokinase binds one molecule of fructose-6-phosphate (Nissler et al, 1977) and two molecules of ATP at two different classes of sites (Nissler et al, 1976).

In spite of these observations, analysis of the binding data and attempts to correlate them with the regulatory kinetics of phosphofructokinase have been impeded by the pH and ligand-dependent self-association of the mammalian enzyme. It has been shown that the more dissociated forms of the enzyme have a lower affinity for fructose-6-phosphate than more associated forms (Hofer and Radda, 1974).
Recently, Hill and Hammes (1975) demonstrated that homotropic interactions between fructose-6-phosphate binding sites may exhibit positive, negative, or no cooperativity depending on pH, allosteric effectors, and the association state of the enzyme. They showed that at pH 7.0 and 8.0, the binding isotherms of fructose-6-phosphate and fructose-1,6-diphosphate exhibit negative cooperativity. The results fit a model in which the dimers bind the respective phosphate with extreme negative cooperativity, the tetramers with less cooperativity, and larger aggregates with little or no cooperativity. In presence of an ATP analogue, the isotherm for fructose-6-phosphate binding is sigmoidal at pH 7.0, but hyperbolic at pH 8.0. Therefore, the characteristic sigmoidal fructose-6-phosphate velocity curve in initial kinetics at pH 7.0 is due to heterototropic interactions between ATP and fructose-6-phosphate binding sites.

With the equilibrium binding data available (Kemp and Krebs, 1967; Kemp, 1969; Hill and Hammes, 1975; Wolfman et al, 1978) in conjunction with steady state kinetic data measured under the conditions where the enzyme is tetrameric, Goldhammer and Hammes (1978) have recently interpreted the regulatory properties of phosphofructokinase in terms of the simple Monod-Wyman-Changeux model (Monod et al, 1965). According to these authors, each enzyme protomer contains a catalytic site which binds MgATP and F6P and a regulatory site which binds MgATP and cAMP. The ligand binding equilibria are adjusted rapidly relative to the rate-determining step. When binding to the catalytic site, MgATP shows no preference between the R (active) and T (inactive) states,
but it binds only to the regulatory site of the T state thereby inhibiting enzyme activity. Fructose-6-phosphate binds preferentially to the catalytic site of the R state at pH 7.0, but shows no preference between the R and T states at pH 7.43. The cAMP binds only to the regulatory site of the R state and is, therefore, a strong activator.

Adsorption of Phosphofructokinase to Cellular Components

In addition to the aggregation state of the enzyme and the presence of small ligands, the allosteric properties of phosphofructokinase seem to be affected by adsorption to the cellular components, for example, the inner surface of the erythrocyte membrane (Karadsheh and Uyeda, 1977). The membrane-bound phosphofructokinase loses its sensitivity to inhibition by ATP and 2,3-diphosphoglycerate. In addition, the dependence of its catalytic activity on fructose-6-phosphate concentration becomes non-sigmoidal.

The adsorption of phosphofructokinase to the inner surface of the erythrocyte membrane is reminiscent of the adsorption of glycolytic enzymes to various membrane or other cellular components in a variety of cells (Clarke and Masters, 1976). For instance, the association of entire glycolytic enzymes with the erythrocyte membrane is well documented (Green et al, 1965), and indeed the affinity of glyceraldehyde-3-phosphate dehydrogenase in this system is so high that it has been described as one of the structural proteins of the erythrocyte membrane (Steck, 1974). Other glycolytic enzymes, such as hexokinase (Tuttle and Wilson, 1970; Wilson, 1972), aldolase (Clarke and Masters, 1972;
1975b), pyruvate kinase (Tamir et al, 1972) and lactate dehydrogenase (De Domench et al, 1970; Wins and Schoffeniels, 1969) have been reported individually to associate with mitochondrial membrane or other cellular components. For phosphofructokinase, the enzyme activity of nervous tissue has been found to associate with mitochondrial membrane of brain cells (Craven and Basford, 1974). In most cases the adsorbed enzymes exhibit modified catalytic properties.

More detailed information on the interaction between glycolytic enzymes and the cellular components has been provided by the studies using skeletal muscle (Arnold and Pette, 1968; Melnick and Hultin, 1973). Histochemical experiments have shown that the glycolytic enzymes are located within the isotropic band of the muscle fibre, corresponding to the site of thin filaments in the relaxed myofibril (Sigel and Pette, 1969). Experiments with reconstituted thin filaments and with press juice extracts have demonstrated in vitro adsorption of several glycolytic enzymes, with phosphofructokinase being the most strongly adsorbed (Clarke and Masters, 1975a). Actin is believed to be the major site of association. Studies using purified enzymes such as aldolase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase and others not only demonstrate that glycolytic enzymes adsorb strongly to F-actin but also showed that the adsorption is reversible and dependent upon pH, ionic strength and specific metabolites (Arnold and Pette, 1970; Arnold et al, 1971). Moreover, it has been shown that the binding of aldolase to F-actin (Arnold and Pette, 1970) and to reconstituted thin filaments (Walsh
et al, 1977) results in changes in its kinetic properties. The regulatory implications of these observations, namely, the control of certain glycolytic enzymes may be accomplished by regulating the soluble-particulate equilibrium, are beginning to attract some attention (Masters, 1977; 1978).

**Purpose and Scope of This Work**

This work is concerned with two aspects: first, the use of fluorescence polarization and circular dichroism to study the binding to rabbit muscle phosphofructokinase of ATP and its analogue $1,N^6$-etheno-adenosine triphosphate (cATP); second, to study the interaction between phosphofructokinase and F-actin by the use of quantitative SDS-polyacrylamide gel electrophoresis, and to investigate the influence of the interaction with actin on the kinetic properties of phosphofructokinase. Both studies are under the same goal of seeking better understanding of the regulatory properties of phosphofructokinase.

**Binding of ATP and cATP to Phosphofructokinase**

The binding of ATP to phosphofructokinase has attracted interest because this nucleotide serves as both substrate and allosteric inhibitor for the enzyme. Of particular interest is the stoichiometry of the binding. Binding experiments using gel filtration technique have indicated three moles of ATP bound per protomer (90,000) for rabbit muscle phosphofructokinase (Kemp and Krebes, 1967). One of the three
binding sites has been attributed to the inhibitory site (Kemp, 1969). In the case of sheep heart phosphofructokinase 3.6 molecules of ATP are bound per 100,000 daltons of enzyme, and the enzyme protomer appears to have more than one inhibitory site (Lorenson and Mansour, 1969).

*eATP* is a fluorescent analog of ATP. Its characteristic fluorescence properties include a high quantum yield (about 50%), moderately long lifetime (20 nsec), and broad emission band whose shape and position (415 nm maximum) are relatively insensitive to changes in solvent. The use of *eATP* as an ATP analog in a number of enzyme systems is well documented (Leonard and Tolman, 1975). In rabbit muscle phosphofructokinase, Secrist *et al* (1972) have reported that *eATP* can replace ATP in its function as both substrate and allosteric inhibitor of the enzyme.

It was believed that a better determination of ATP binding could be obtained using fluorescence polarization measurements on *eATP*. The first part of the work, therefore, is devoted to the fluorescence polarization and circular dichroism studies of the system. The study shows that the enzyme binds three moles of *eATP* per protomer. In addition to the binding stoichiometry, the influence of various allosteric effectors on the binding of *eATP* to the enzyme is investigated. The effects of ATP and *eATP* on the conformation of the enzyme have also been studied.

**Interaction of Phosphofructokinase with F-Actin**

The adsorption of glycolytic enzymes to muscle proteins has been
studied extensively (Clarke and Masters, 1976; Masters, 1978). It has been established that the adsorption of the enzyme is reversible and dependent upon pH, ionic strength, and specific metabolites (Arnold and Pette, 1968; 1970). Actin has been identified as the major site of adsorption. Individual enzymes of the glycolytic pathway show markedly different affinity for actin (Arnold et al, 1971; Calarke and Masters, 1975a). Moreover, modified kinetic properties have been reported for actin-bound aldolase (Arnold and Pette, 1970; Walsh et al, 1977). These observations suggest that adsorption to subcellular structures such as F-actin may offer a means to regulate the intracellular distribution and activities of glycolytic enzymes (Masters, 1977; 1978).

Contractile proteins are known to occur in a variety of tissues and life forms in addition to skeletal muscle (Pollard and Weihing, 1974). The presence of actin on the inner surface of the membrane is a well-known example (Marchesi et al, 1976). Accumulating evidence shows that actin is very nearly a universal protein, comprising almost 10 - 20% of the total cell protein (Korn, 1978). Besides participating in muscle contraction, actin forms the microfilaments found in all eucaryotic cells (Hitchcock, 1977). It follows that interactions such as those observed for skeletal muscle may also take place in many other cells and tissues. The prevalent observation of the association of glycolytic enzymes to the actin-containing membranous structure may suggest such interactions (Masters, 1977). It is also interesting to note that the subcellular distribution of aldolase and pyruvate kinase
in brain parallels that of brain actin (Clarke and Masters, 1973; Blitz and Fine, 1974). The adsorption of glycolytic enzymes, therefore, may be a widespread phenomenon, occurring in both muscle and non-muscle cells.

In order to provide additional experimental data, the second part of the work is devoted to the study of the interaction between F-actin and phosphofructokinase. The adsorption of phosphofructokinase has been suggested by the studies using purified enzymes (Arnold et al, 1971) as well as press juice extracts of muscle (Clarke and Masters, 1975a). However, unequivocal demonstration of the adsorption of purified phosphofructokinase to F-actin has been impeded because of its extreme instability (Arnold et al, 1971). Therefore, the work was directed to seek optimal conditions to demonstrate the interaction of the enzyme with F-actin. The experiments were designed to provide information on the stoichiometry, to delineate the influence of various effectors on the interaction, and more importantly, to answer the question whether the interaction modifies the kinetic properties of the enzyme. The results show that adsorption to reconstituted thin filaments and particularly to isolated F-actin produces major changes in the catalytic properties of phosphofructokinase. The interaction with F-actin reverses the ATP inhibition and appears to enhance the binding of fructose-6-phosphate to the enzyme.
II. MATERIALS AND METHODS

Materials

Aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase, the auxiliary enzymes for phosphofructokinase activity assay, were obtained from P-L Biochemicals, Inc. P-L Biochemicals was also the source for ADP, AMP, and GTP. ATP, fructose-6-phosphate, fructose-1,6-diphosphate, dithiothreitol, creatine, and phosphocreatine were purchased from Sigma Chemical Co. Glycylglycine, α-glycerophosphate and β-mercaptoethanol were from Calbiochem. Acrylamide, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylenediamine, and ammonium persulfate, were from Canalco, Inc.; sodium dodecyl sulfate, from Bio-Rad Laboratories; Coomassie brilliant blue G-250, from Eastman Kodak; and pyronin Y, from Matheson Coleman and Bell. These materials were used without further purification. All solutions were prepared using deionized glass distilled water. Other chemicals were standard reagent grade.

Dialysis tubing, purchased from Union Carbide Corporation, was prepared for use by the method of McPhie (1971). The treated dialysis tubing was rinsed and stored at 4° under water with trace of sodium azide to inhibit the growth of microorganisms.

1,N6-Ethenoadenosine 5'-triphosphate (εATP) was prepared according to the method of Secrist et al (1972). The product, which contained a small amount of contaminating εADP after prolonged storage, was further purified by passing through a DEAE-cellulase (DE-52) column
(1.0 x 5.0 cm) which had been equilibrated with 0.09 M sodium acetate buffer, pH 4.4. The column was first washed with the same buffer. After the contaminating materials had been eluted, the column was then washed with 0.02 N HCl. The εATP fractions were pooled and neutralized immediately with 1 N NaOH. It was then lyophilized to dryness and stored at 0°. For experiments, it was redissolved in 25 mM glycylglycine, pH 7.0. The concentration of εATP was measured using the extinction coefficient of 5.6 x 10^3 mol^-1 cm^-1 at 275 nm (Secrist et al, 1972).

Phosphofructokinase

Phosphofructokinase from the skeletal muscle of New Zealand white rabbits, obtained from the Laboratory Animal Resources Center, Oregon State University, was prepared by the method of Ling et al (1966). The final ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate, pH 8.0, containing 1 mM EDTA and 1 mM dithiothreitol and dialyzed against the same buffer to give a stock enzyme solution of 10 to 15 mg/ml. Specific activities were usually in the range 100 to 120 units per mg. The enzyme was stored in this buffer in the cold. It was usually discarded after one month.

Before experiments the enzyme was dialyzed overnight against the buffer consisting of 25 mM glycylglycine, 25 mM glycerophosphate, 1 mM EDTA and 5 mM β-mercaptoethanol, pH 7.0. This buffer provides a degree of stability to the enzyme without interfering with the various kinetic parameters (Kemp and Krebs, 1967; Colombo et al, 1975). The
dialysate was then treated with about one mg per ml of Norit A for 30 minutes to remove excess nucleotides (Aaronson and Frieden, 1972). In the studies of the interaction of phosphofructokinase with F-actin this treatment was omitted. The enzyme was clarified by centrifuge at 12,000 rpm for 10 min, using SS-34 roter.

Phosphofructokinase concentrations were determined by using $E_{283}^{1%} = 10.9$ in 0.1 N NaOH (Paetkau et al, 1968). Molar concentrations of phosphofructokinase were based on a protomer molecular weight of 90,000 (Colombo et al, 1975).

Assays of Phosphofructokinase Activity

During purification of the enzyme, the activity of phosphofructokinase was assayed spectrophotometrically by coupling of fructose-1,6-diphosphate formation to the oxidation of NADH with the use of aldolase triosephosphate isomerase, and α-glycerophosphate dehydrogenase (Racter, 1947). The assay solution consists of 50 mM glycylglycine, pH 8.2, 1 mM EDTA, 0.01% bovine serum albumin, 2.5 mM dithiothreitol, 5 mM ammonium sulfate, 0.16 mM NADH, 5 mM MgCl$_2$, 1 mM ATP, 1 mM fructose-6-phosphate, aldolase (0.4 unit), triosephosphate isomerase (2 units), and α-glycerophosphate dehydrogenase (0.4 unit) in a total volume of 1 ml. The reaction was initiated by the addition of phosphofructokinase, and the absorbance at 340 nm of the reaction mixture was measured continuously with Varian 635D recording spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole of fructose-1,6-diphosphate per min
under these conditions.

In the studies of the interaction between F-actin and phosphofructokinase, a pH-stat assay, method by Dyson and Noltmann (1965), was adopted for the determination of the catalytic properties of the free and actin-bound phosphofructokinases. The standard reaction mixture contained final concentrations of 1 mM ATP, 1 mM fructose-6-phosphate, 5 mM MgCl$_2$, and 10 mM KCl, adjusted to pH 7.00 in a total volume of 2 ml. Two tenth mg per ml of either F-actin or reconstituted thin filaments was added when the bound phosphofructokinase was assayed. After waiting a short period for the instrument to attain a steady pH reading, the reaction was initiated by the addition of a 10 - 25 µl aliquot of phosphofructokinase diluted to give approximately 0.1 to 0.2 unit per aliquot. The phosphofructokinase was diluted with the buffer containing 12.5 mM glycylglycine, 12.5 mM glycerophosphate, 0.5 mM EDTA, adjusted to pH 7.00, and preincubated at room temperature for 5 min to ensure maximal activity. The enzyme was stable in this buffer for at least 4 hours at room temperature without losing activity.

Base consumption as a function of time was controlled and recorded with Radiometer titrator (Type SBR2/SBU1/TTA3). NaOH (2 mM) was used as titrant and was standardized against potassium hydrogen phthalate at the beginning of the experiment. The unit of phosphofructokinase activity is defined as that amount of enzyme which causes a reaction rate equal to the consumption of 1 µ equiv. of NaOH per min under the stated assay conditions.
**Actin**

An acetone powder of rabbit back and leg muscle was prepared by the Straub procedure as modified by Szent-Gyorgyi (1947). The powder was dried at room temperature and stored in a tightly closed container at -10° C.

Actin was prepared according to the method of Spudich and Watt (1971). After the second polymerization, F-actin was centrifuged at 80,000 x g for 3 hours and the sediment was resuspended in a solution of 2 mM Tris-HCl, pH 8.0, containing 50 mM KCl. For resuspension, a 7-ml Dunce tissue grinder was used. The concentration of the resuspended F-actin was adjusted to 10 mg/ml. This preparation of F-actin was stored in the cold, but was usually used within 10 days.

The actin concentration was measured using the extinction coefficient of 0.63 mg⁻¹ ml cm⁻¹ at 290 nm (Lehrer and Kerwar, 1972).

**Tropomyosin**

Bailey's (1948) method as modified by Mueller (1966) was followed for the preparation of tropomyosin. The tropomyosin was collected between 40 and 55% ammonium sulfate saturation. It was then dissolved in 25 mM imidizole, pH 7.0, 60 mM KCl and 2 mM MgCl₂ and dialyzed overnight against the same solution. The concentration of tropomyosin was measured spectrophotometrically using the extinction coefficient of 0.33 mg⁻¹ ml cm⁻¹ at 277 nm in a 1.1 ionic strength buffer at pH 7.0 (Woods, 1967).
Troponin

Troponin, prepared by the method of Greaser and Gergely (1973), was a generous gift of Dr. D. Malencik.

F-Actin-tropomyosin-troponin Complex

The reconstituted thin filament, the complex consisting of F-actin, tropomyosin, and troponin, was prepared according to the method of Ishiwata (1973) by mixing these three proteins in this order in the ratio 10:2:1 (by weight).

Fluorescence Measurement

Fluorescence Titration

The procedure for the titration was adapted from that described by Anderson and Weber (1965). A solution of phosphofructokinase (2 ml) was placed in a 1-cm cuvet. The εATP solution was added to the cuvet from a Hamilton microsyringe. The contents were mixed gently with a polyethylene rod and the fluorescence anisotropy of εATP was measured after each addition. The total dilution never exceeded 10% and was usually less than 5%.

Fluorescence Anisotropy Measurements

Fluorescence anisotropy measurements were made using the Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer equipped with polarization accessory. In the studies of the effect of metabolites on
the fluorescence anisotropy of εATP-phosphofructokinase complex as well as the intrinsic protein fluorescence, the measurements were made using a computer interfaced polarization spectrometer constructed in Dr. Isenberg's laboratory (Evett and Isenberg, 1969; Evett et al, 1970; Ayres et al, 1974).

Excitation and emission wavelengths were set at 310 nm and 440 nm, respectively for εATP fluorescence. Constant temperature was maintained by circulating water from a Forma constant temperature bath.

Emission anisotropy is defined by the equation (Jablonski, 1960)

\[ r = \frac{I_\| - I_\perp}{I_\| + 2I_\perp} \]

where \( I_\| \) is the intensity of the component vibrating in the direction of propagation of the exciting light and \( I_\perp \), the intensity of the component vibrating normally to the plane defined by the direction of excitation observation.

The anisotropy values were used to estimate the fraction of εATP bound to phosphofructokinase from the relation

\[ f_b = \frac{r_\varepsilon - r_2}{r_1 - r_2} \]

where \( r_\varepsilon \) is the observed anisotropy of the equilibrium mixture; \( r_1 \) and \( r_2 \), both constants, are the respective anisotropies of bound and free εATP. This equation is obtained directly from the principle of additivity of anisotropies (Weber, 1952).

The anisotropy of bound ligand is usually obtained by extrapolation
to infinite protein concentration. Since the binding of εATP to phosphofructokinase is relatively weak, a second approach based on the fact that the anisotropy of εATP dissolved in glycerol at infinite viscosity should be the same as that of εATP rigidly bound to a high molecular weight protein was adopted. The anisotropy extrapolated to infinite protein concentration was 0.12 ± 0.02 while that extrapolated to infinite viscosity in glycerol was 0.128 ± 0.004. Because of its greater precision, the value of $r_1$ obtained from measurements on the glycerol solution was used in the calculations. The error in $r_c$ and $r_2$ was estimated to be 2%. The use of this equation assumes that there are two fluorescence species, free and bound εATP, with identical extinction coefficients and quantum yields. Absorption and intensity measurements vindicated the latter two assumptions. The observed anisotropies were corrected for the small contribution of protein 'blank' by use of the addition law (Weber, 1952).

Whether the anisotropy of the complex is independent of the number of ligand molecules bound is difficult to verify in this case. Several independent binding measurements using the ultracentrifuge equipped with scanner (Schachman, 1963) were carried out. General agreement between the results obtained by ultracentrifugation and fluorescence anisotropy supports the applicability of the equation $f_b = (r_c - r_2)/(r_1 - r_2)$ to the binding of εATP to phosphofructokinase.  

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1 The binding was also examined in the ultracentrifuge equipped with scanner (Schachman, 1963). A solution containing 240 μM εATP and 2.13 mg/ml of phosphofructokinase was sedimented at 36,000 rpm at 22°C until the boundary was well separated from the meniscus. The absor-
Measurement of the Intrinsic Protein Fluorescence

In the measurement of the intrinsic fluorescence of phosphofructokinase, the excitation and emission wavelengths were set at 298 and 340 nm, respectively. Contribution of $\varepsilon$ATP to the fluorescence anisotropy observed in the mixture containing phosphofructokinase and $\varepsilon$ATP was always subtracted according to the addition law.

The apparent decrease in protein fluorescence intensity, due to the competition of $\varepsilon$ATP and the aromatic amino acid residues for the exciting light, was corrected using a method similar to that used in the studies on heme proteins (Seery and Muller-Eberhard, 1973). The fluorescence intensity of a tryptophan solution, with optical density at 298 nm equivalent to that of the enzyme, was measured in the presence of varying concentrations of $\varepsilon$ATP. The small linear decrease in tryptophan fluorescence (23% 'screening' was observed at 100 $\mu$M $\varepsilon$ATP) was used to correct corresponding points in the titration of phosphofructokinase. This method assumes that $\varepsilon$ATP and tryptophan do not interact directly in this concentration range.

Circular Dichroism

Circular dichroism spectra were measured on a Jasco J-41A.

(Continued)

Uptake of the supernate liquid and of the plateau region measured at 305 nm yielded the proportion of free and bound $\varepsilon$ATP. The value of $\bar{\Pi}$ obtained under this condition was 5.3. Similar experiments carried out in the presence of 0.47 mM and 0.94 mM citrate gave values of 5.8 and 6.2, respectively. These values were similar to an average value of 5.5 ± 0.4 calculated from the fluorescence measurement under similar conditions.
spectropolarimeter under nitrogen flush at 22°. The absorbancy of all samples was kept near 1.5. Cuvets with light paths of 1 mm to 10 mm were used.

**F-actin-Phosphofructokinase Binding**

The binding experiments were performed in 15-ml Corex centrifuge tubes. Solutions of F-actin and dialyzed phosphofructokinase were mixed thoroughly. The incubation solution contained, in addition to phosphofructokinase and F-actin, final concentrations of 5 mM glycylglycine, 5 mM glycerophosphate, 1 mM MgCl₂, and 5 mM KCl, pH 7.0, in a total volume of 1 ml. The mixture was incubated for 30 min at room temperature and then centrifuged at 12,000 rpm for 10 min in SS-34 rotor. The clear supernatant was decanted and saved for measurement of soluble phosphofructokinase. For washing, the sediment was dispersed and stirred in 2 ml of the same buffer used for binding. The mixture was then centrifuged for 10 min at 12,000 rpm, and the amount of phosphofructokinase in the sediment was determined with quantitative SDS-polyacrylamide electrophoresis described in the following section. The amount of phosphofructokinase in the sediment thus determined is defined as the quantity of enzyme adsorbed to F-actin under the conditions of the experiment. In most of the experiments the quantities of adsorbed and soluble enzyme were checked with the total amount of the enzyme present in the reaction mixture.
Quantitation of Protein by SDS-Polyacrylamide Gel Electrophoresis

The procedure of Weber et al (1972) with minor modifications suggested by Malencik (1972) was adapted for the quantitative determination of phosphofructokinase in F-actin-phosphofructokinase complex.

Samples for electrophoresis were prepared by dissolving the washed precipitate described previously in 0.2 ml of 0.01 M Tris-PO₄ buffer, pH 6.8, containing 1% SDS, 1% β-mercaptoethanol, 20% glycerol, 10⁻⁴ M EDTA, and 0.01% pyronin Y (tracking dye). After the precipitates were thoroughly dissolved, the clear sample solutions were heated in a boiling water bath for 2 min. The volume of each sample solution was measured with Hamilton microsyringe after they were cooled to room temperature. They were then diluted with the same buffer solution to suitable concentrations, usually in the range of 0.1 - 0.5 mg/ml in phosphofructokinase, for electrophoresis.

Electrophoresis was carried out in gel tubes 7.5 cm in length, I.D. 0.49 cm, using 7.5% polyacrylamide gel. The preparation of the concentrated stock solutions and the SDS-polyacrylamide gels has been described (Malencik, 1972). Electrophoresis was carried out with a Buchler Polyanalyst electrophoresis chamber. A maximum of 18 gel columns can be run each time. Each sample was taken up and discharged onto the top of the gel with Hamilton microsyringe. Between each operation of sample application the syringe was flushed with distilled water for at least 1 min and dried with vacuum suction. The quantity of sample applied was such that the phosphofructokinase content in
each gel was no more than 5 μg. Within this range the color density of phosphofructokinase band was easily monitored and linearly proportional to the concentration of the enzyme. For each quantitative determination of phosphofructokinase a calibration standard of the enzyme in a range between 1 and 5 μg was also established simultaneously. A Bio-Rad Model 400 power supply was employed, operated in the constant current mode, generally at 4 ma per tube. The running time was about 2 hours.

For staining, the gels were placed in small tubes and first fixed with 12.5% trichloroacetic acid for 10 min, then stained for 2 hours with a fresh solution prepared by dissolving 0.75 g of Coomassie blue G-250 in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid. Destaining was accomplished in 7.5% acetic acid using the Canalco quick gel destainer.

To quantitate phosphofructokinase, the gels were scanned at 595 nm with the Beckman DU Model 2400 Spectrometer equipped with a Gilford gel scanner (Model 2520). Weight integration was employed to measure the surface area of phosphofructokinase peaks. Each peak was cut and then weighed with the Mettler model H20 balance. The area was calculated by dividing the weight of the PFK peak by the weight of the unit area of the recording paper. The overall error of this method was estimated to be ±5%. 
III. RESULTS

A. Binding of ATP and of εATP to Phosphofructokinase

Binding of εATP

Titration of rabbit muscle phosphofructokinase with εATP, both in the presence (6 mM) and in the absence of MgCl₂, are shown as plots of \( \frac{1}{n} \) versus \( \frac{1}{[x]} \) in Figure 1. The values of \( \bar{n} \), the ratio of the concentration of bound εATP to enzyme concentration, were calculated in terms of the enzyme tetramer, assuming a protomer molecular weight of 90,000 (Colombo et al., 1975); \([x]\) is the free εATP concentration.

These results, covering a 200-fold range of εATP concentration, show that saturation was never reached. Further increases in εATP concentration (to more than 150 μM) result in a large increase in the proportion of free ligand. Correspondingly, the error in the determination of the fraction of bound ligand becomes large as the observed anisotropy approaches that of free εATP.

In order to estimate stoichiometry, double reciprocal plots of \( \frac{1}{n} \) versus \( \frac{1}{[x]} \) based on the following equation for simple, homogeneous binding (Klotz, 1946) were prepared.

\[
\frac{1}{n} = \frac{1}{N K} \cdot \frac{1}{[x]} + \frac{1}{N}
\]

In this equation \( K \) is the association constant and \( N \) is the number of binding sites occupied by the ligand when it is present at saturating concentrations. Least squares analysis of the nearly linear
Figure 1. Double reciprocal plot for binding of εATP by phosphofructokinase. Π is the average number of moles of εATP bound per tetramer of protein. The phosphofructokinase concentrations were 1.52 mg/ml (Δ), no MgCl₂ added; 1.52 mg/ml (○) and 1.0 mg/ml (●) with 6 mM MgCl₂ added. The buffer contained 25 mM glycylglycine, 25 mM glycerophosphate, 1 mM EDTA and 5 mM β-mercaptoethanol, pH 7.0. The temperature was 20°. The portion of the curve in the lower left-hand corner is enlarged in the insert.
plots obtained in the presence of magnesium gave values for $N$ and $K_d$ of $11.30 \pm 1.20$ and $6.0 \pm 2.2 \times 10^{-5}$ M, respectively. Since this method of graphical treatment does not consider possible occupation of low affinity sites at much higher ligand concentrations, the stoichiometry obtained must be regarded as a minimum value. Hill plots, based on $N = 12$, give a Hill coefficient of $0.93 \pm 0.06$. The very incomplete data obtained in the absence of magnesium ion indicate that the average affinity of the enzyme for $\varepsilon$ATP is an order of magnitude lower.

Influence of Magnesium ion on the binding of $\varepsilon$ATP

Figure 2 shows the effect of increasing magnesium concentration on the fluorescence anisotropy of a mixture containing phosphofructokinase and $\varepsilon$ATP. The experiment was performed at a low molar ratio of $\varepsilon$ATP to phosphofructokinase, where a significant enhancement of anisotropy is obtained. The fractional change in anisotropy is directly related to the fractional change in the amount of $\varepsilon$ATP bound. Differentiation of the equation $f_b = (r_\varepsilon - r_2)/(r_1 - r_2)$ (see page 21) gives $df_b = dr_\varepsilon/(r_1 - r_2)$. It follows that

$$\frac{f_b'' - f_b'}{f_b'} = \frac{r_\varepsilon'' - r_\varepsilon'}{r_\varepsilon' - r_2}$$

where the primes designate corresponding values of $f_b$ and $r_\varepsilon$. Since $r_2$ is small (0.0032), the relative change in the amount of $\varepsilon$ATP bound approximates the fractional change in anisotropy. The data in Figure
Figure 2. Influence of magnesium ion on the fluorescence anisotropy of a solution containing εATP and phosphofructokinase. A mixture of 0.75 mg/ml of rabbit muscle phosphofructokinase and 1.78 x 10⁻⁶ M εATP was titrated with increments of 0.15 M MgCl₂. Excitation was at 310 nm and emission, at 440 nm. Slit width for both excitation and emission was 2 mm. Refer to Figure 1 for other conditions.
2 show that saturating magnesium ion concentrations produce a 3.3 fold increase in the amount of εATP bound. Half-maximum binding of εATP to the enzyme occurs at a magnesium concentration of 1 mM.

**Influence of Substrate, Products, and Other Effectors on the Binding of εATP**

Figures 3 and 4 show the influence of substrate (ATP), products (ADP, FDP), and other effectors (AMP, GTP, citrate) of the enzyme on the fluorescence anisotropy of εATP-phosphofructokinase mixtures. Except for citrate, all of these compounds cause a decrease in the amount of εATP bound to phosphofructokinase. Saturating concentrations of ADP, GTP, and ATP displace about 80% of the bound εATP. The concentrations required to produce a half-maximal decrease in anisotropy are 5, 15, and 20 μM for ADP, GTP, and ATP, respectively. This concentration of ATP is in accord with the Michaelis constant (20 μM) reported for muscle phosphofructokinase (Hanson, 1970; Hanson et al, 1973). ADP, on the other hand, is more effective in displacing bound εATP than it is in reversing the ATP inhibition of catalysis, which often requires concentrations greater than 100 μM (Passoneau and Lardy, 1962).

The action of ATP, however, differs from that of ADP or GTP in that there is an enhancement occurring at 4.5 μM.\(^2\) This observation

\(^2\) An increase in the anisotropy of the bound εATP due to increased rigidity of the binding site could also lead to this effect. However, the fact that most (72%) of the εATP is free would require the anisotropy to exceed the limiting anisotropy of εATP (0.156 versus 0.128).
Figure 3. Influence of substrate, ATP (●); products, ADP (◇), FDP (△); and other effectors, GTP (●), AMP (◇), on the fluorescence anisotropy of a solution containing phosphofructokinase (0.73 mg/ml) and εATP (1.6 x 10^-6 M) at 22°C. Excitation and emission wavelengths were 310 nm and 440 nm, respectively. Slit width for both excitation and emission was 3 mm.
Figure 4. Influence of citrate on the fluorescence anisotropy of a solution containing phosphofructokinase and eATP. Refer to Figure 3.
was repeatable; the maximum enhancement ranged between 10 and 20% in several measurements. The precise reason for this enhancement is unknown. It could be the result of a conformational change in the enzyme accompanying the binding of ATP. Since the molar ratio of εATP to phosphofructokinase is very low, about 1, most of the binding sites are vacant. The initial binding of ATP at low concentrations does not necessarily involve competition for the sites already occupied by εATP. A conformation change induced by ATP binding may facilitate the binding of εATP to remaining unoccupied binding sites.

Saturating levels of AMP and FDP, both activators of the enzyme, displace about 27% of the bound εATP. The fact that these two effectors show similar concentration dependence agrees with kinetic data on the activation of ATP-inhibited phosphofructokinase (Mansour, 1972). Citrate causes a dramatic enhancement in the anisotropy, corresponding to 2.6-fold increase in the amount of εATP bound. Citrate has been shown to enhance the affinity of the enzyme for ATP (Kemp and Krebs, 1967).

Influence of ATP and εATP on the Intrinsic Protein Fluorescence of Phosphofructokinase

Polarization spectra, obtained by measuring the polarization of fluorescence produced by excitation with light of varying wavelengths, have been used to detect conformational changes affecting the local freedom of rotation of the tryptophanyl and tyrosyl side chains of proteins (Anderson and Weber, 1966). Since these aromatic amino acids have short fluorescence lifetimes, the polarization spectra of pro-
teins as large as phosphofructokinase are not affected by the rotational diffusion of the macromolecule as a whole.

Figure 5 shows the fluorescence polarization spectra of 2.13 μM rabbit muscle phosphofructokinase and its complexes with 140 μM ATP and 104 μM εATP recorded at a fixed emission wavelength of 340 nm. The binding of ATP results in a uniform increase in anisotropy over the entire range of wavelengths. This effect is usually explained by increased local rigidity of the tryptophanyl side chains (Anderson and Weber, 1966). An alternate explanation, that the average anisotropy is increased by the selective enhancement of the quantum yield of a single rigidly bound residue, is less likely since the observed changes in fluorescence intensity are small and the anisotropy is initially close to the limiting value. The binding of εATP causes a small decrease in anisotropy. Since εATP itself emits at 340 nm, the spectrum was corrected for a small contribution (7 - 12%) from εATP by using the addition law (Weber, 1952).

Titrations of the enzyme with either ATP or εATP were carried out measuring both the anisotropy and the total emission intensity obtained at fixed excitation and emission wavelengths of 298 and 340 nm, respectively. The results in Figure 6 show that the anisotropy increases monotonically with increasing concentrations of ATP. The intensity, on the other hand, undergoes a biphasic change with a maximum effect occurring in the same ATP concentration range producing enhancement of εATP binding (Figure 3).

There is little or no energy transfer from the tryptophanyl side
Figure 5. Polarization spectra of the intrinsic protein fluorescence of native phosphofructokinase (0.81 mg/ml) (●) and its complexes with ATP (1.40 x 10^{-4} M) (○), and εATP (1.04 x 10^{-4} M) (△) at 22°. Emission at 340 nm, respectively. The direct contribution of εATP to the spectrum was subtracted according to Weber's addition law.
Figure 6. Influence of ATP and cATP on the intrinsic protein fluorescence of phosphofructokinase.
A) Influence of ATP on the anisotropy and B) on the intensity.
C) Influence of cATP on the anisotropy and D) on the intensity.
The excitation and emission wavelengths were fixed at 298 nm and 340 nm, respectively. Enzyme concentration: 0.8 mg/ml, 22°.
Other conditions are listed under Figure 1.
chains to the bound εATP, evidenced by the fact that the protein fluorescence intensity is virtually unaffected by the binding. The spectral overlap integral (Forster, 1947) indicates that the characteristic energy transfer distance, at which the probability of transfer between optimally oriented molecules is 50%, is about 19 Å for tryptophan and εATP. The absence of transfer in phosphofructokinase suggests that the distances and/or orientations between donor and acceptor are unfavorable.

Circular Dichroism Studies

Figures 7 and 8 show the circular dichroism spectra of muscle phosphofructokinase and its complexes with ATP and εATP. Both nucleotides cause barely detectable changes in ellipticity in the wavelength range 205 - 240 nm, suggesting that the binding of either nucleotide causes little change in the secondary structure of the protein.

ATP and εATP are remarkably dissimilar in their effects on the ellipticities recorded between 250 and 310 nm. The dramatic effect observed on the binding of ATP to the enzyme is emphasized by the circular dichroism difference spectrum shown in Figure 9 (C). This difference spectrum comprises a strong positive band corresponding to the 260-nm absorption maximum of ATP and a negative band in the wavelength region corresponding almost exclusively to absorption by tryptophan. The strong positive CD band centered at 260 nm is due to the induced asymmetry of the adenine ring, reflecting the asymmetric environment of the binding site. The circular dichroism spectra of both
Figure 7. The circular dichroism spectra of solutions containing 0.21 mg/ml native phosphofructokinase and A) no addition, B) $1.1 \times 10^{-4}$ M ATP, C) $1.04 \times 10^{-4}$ M εATP. The spectra were expressed in terms of mean residue ellipticity. Path length : 1 mm. Refer to Figure 1 for condition.
Figure 8. The circular dichroism spectra of solutions containing 1.4 mg/ml native phosphofructokinase and A) no additions, B) $8.2 \times 10^{-5}$ M ATP, C) $9.6 \times 10^{-5}$ M εATP. The spectra were expressed in terms of observed ellipticity. Refer to Figure 1 for other conditions.
Figure 9. The circular dichroism difference spectrum of solutions containing native phosphofructokinase and its complexes with ATP (curve C). This difference spectrum was obtained by subtracting the CD spectrum of native phosphofructokinase (curve A, Figure 7) from the CD spectrum of the ATP-phosphofructokinase complex (curve B, Figure 7), and is expressed in terms of ATP molecular ellipticity. Also shown are the circular dichroism spectra of free εATP (curve A) and free ATP (curve B). Conditions: 22°, pathlength 10 mm.
free ATP (B) and εATP (A) are included here for comparative purposes. Titration of the enzyme with these two nucleotides show that the ellipticity of the protein at 288 nm undergoes a maximum change of 50% upon the addition of ATP and virtually no change when εATP is added (Figure 10). This striking observation is not simply due to a difference in the amount of the coenzyme bound. Calculations show that at a ligand concentration of 100 μM, more than 6.6 mol of ATP (Kemp and Krebs, 1967) or 5.7 mol of εATP will bind per mol of enzyme.
Figure 10. Influence of ATP (○) and εATP (●) on the circular dichroism at 288 nm. Phosphofructokinase concentration was 1.54 mg/ml. Either ligand alone gave negligible circular dichroism at this wavelength. The values of circular dichroism are given in millidegrees. Path length was 10 mm.
B. Binding and Activation of Phosphofructokinase by F-Actin

The Adsorption of Phosphofructokinase to F-Actin

Adsorption to the structural proteins of muscle, particularly F-actin, has been demonstrated with several purified glycolytic enzymes including aldolase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase and others (Arnold et al, 1971). The interaction of these enzymes with F-actin is characterized by reversible formation of a precipitable complex. Under the conditions described in the 'Materials and Methods' section, the adsorption of phosphofructokinase to F-actin results in a visible increase in the turbidity of the solution apparent within a few seconds after the addition of the enzyme. Quantitative SDS-polyacrylamide gel electrophoresis of the precipitate (which was collected by low speed centrifugation and washed) reveals the presence of the two protein components: phosphofructokinase and actin (Figure 11). Similar experiments utilizing bovine serum albumin in place of phosphofructokinase gave no detectable precipitation. This suggests that the formation of the precipitable complex is specific for phosphofructokinase, as it has been for other glycolytic enzymes.

The adsorption of increasing concentrations of phosphofructokinase to F-actin is shown in Figure 12. The results, covering a range of phosphofructokinase to actin ratios up to ten, indicate that saturation could be reached only at much higher phosphofructokinase concentrations. Direct measurement of the molar ratio of phosphofructokinase to actin
Figure 11. SDS-Polyacrylamide gel electrophoresis of actin-phosphofructokinase complex in 7.5% gel at pH 6.8. 
a) Phosphofructokinase, b) actin-phosphofructokinase complex, c) actin.
Figure 12. Binding of phosphofructokinase to F-actin. Solutions of F-actin (0.2 mg) were mixed with various amounts of phosphofructokinase in a buffer containing a final concentration of 5 mM glycylglycine, 5 mM glycerophosphate, 1 mM MgCl$_2$, and 5 mM KCl, pH 7.0. The total volume of the reaction mixture was 1 ml. After 30 min incubation at room temperature, the precipitate was collected and washed two times with the same buffer. The soluble and bound enzymes were determined with quantitative SDS-polyacrylamide gel electrophoresis as described under 'Materials and Methods'. The insert shows a plot of the ratio of phosphofructokinase and actin (in terms of protomer) in the actin-phosphofructokinase complex versus the ratio of these two proteins in the reaction mixtures.
PHOSPHOFRUCTOKINASE PRECIPITATED (mg)

PHOSPHOFRUCTOKINASE ADDED (mg)
in the precipitated complex (Figure 12, insert) shows the adsorption of one phosphofructokinase tetramer per actin protomer. Additional weaker adsorption takes at higher phosphofructokinase to actin ratios.

**Influence of Ionic Strength and pH on the Precipitation of Phosphofructokinase by F-actin**

Figure 13 shows the influence of increasing KCl and NaCl concentrations on the precipitation of phosphofructokinase by F-actin. The experiments were performed with phosphofructokinase and F-actin present in equal molarity of protein protomer (4.4 μM). Under such condition more than 85% of the added phosphofructokinase was precipitated by F-actin. With increasing concentrations of either KCl or NaCl, the amount of enzyme precipitated by F-actin decreases. NaCl is less effective than KCl in solubilizing the precipitate. The concentrations required to dissolve 50% of the precipitated enzyme are 20 mM and 70 mM for KCl and NaCl, respectively.

The pH of the reaction mixture has a profound influence on the precipitation of phosphofructokinase by F-actin. Figure 14 shows that precipitation of the enzyme by F-actin is essentially complete at pH values between 6.0 and 7.2 and minimal at pH values above 8.2, where only 10% precipitation occurs. In contrast, the aggregation of the enzyme alone occurs at pH values near 8 and above (Aaronson and Frieden, 1972).
Figure 13. Influence of potassium chloride (○), and sodium chloride (●) on the precipitation of phosphofructokinase by F-actin. 0.4 mg of phosphofructokinase were mixed with 0.2 mg of F-actin in a solution containing final concentrations of 5 mM glycylglycine, 5 mM glycerophosphate, and 1 mM MgCl$_2$, pH 7.0. The effects of these two salts were examined at increasing concentrations. The curves represent plots of the amount of the enzyme precipitated by F-actin at the respective salt concentration relative to the amount in which no salt is present.
Figure 14. Influence of pH on the precipitation of phosphofructokinase by F-actin. 0.4 mg of phosphofructokinase were incubated together with 0.99 mg of F-actin. The buffer was the same one used in Figure 12. The pH was adjusted to the indicated value by the addition of either NaOH or HCl (0.1 N).
RELATIVE PRECIPITATION OF PHOSPHOFRUCTOKINASE (%) vs pH

RELATIVE PRECIPITATION OF PHOSPHOFRUCTOKINASE (%)

pH

0 10 20 30 40 50 60 70 80 90 100
6.0 6.5 7.0 7.5 8.0 8.5
Figure 15 shows the effect of increasing magnesium and calcium ion concentrations on the precipitation of phosphofructokinase by F-actin. At low concentrations, both ions enhance the amount of enzyme precipitated by F-actin. Maximum enhancement occurs at approximately 1 mM, corresponding to a 1.6 fold increase in the amount of phosphofructokinase precipitated. At concentrations exceeding 1 mM, however, the effects of the two ions diverge. Whereas higher magnesium ion concentrations up to 10 mM have no further effect, the amount of enzyme precipitated by F-actin declines at calcium concentrations above 1 mM. However, the amount of the enzyme precipitated by F-actin remains higher than the control even in the presence of 10 mM calcium ion. Still higher magnesium or calcium ion concentrations, near 100 mM, suppress the precipitation of phosphofructokinase to 17%. This effect is similar to that exerted by high levels of KCl or NaCl (Figure 13), and may be due largely to electrostatic effects.

Clarke and Masters (1975a) found that low levels of magnesium and calcium enhance the adsorption of both phosphofructokinase and several other glycolytic enzymes to F-actin in myogen preparations. The biphasic effect of these two ions on the adsorption of purified phosphofructokinase, however, is contrary to what had been reported for the adsorption of purified aldolase to F-actin (Arnold and Pette, 1970). In that study, magnesium and calcium ions at low levels (less than 1 mM) did not appear to enhance the adsorption of aldolase to F-actin.
Figure 15. Influence of magnesium chloride (○) and calcium chloride (●) on the precipitation of phosphofructokinase by F-actin. Refer to Figure 12 for experimental conditions.
Influence of Metabolites on the Precipitation of Phosphofructokinase by F-Actin

Figure 16 shows the influence of various substrates (ATP, F6P), products (ADP, FDP), and allosteric effectors (AMP, citrate) of phosphofructokinase on the precipitation of the enzyme by F-actin. Except for AMP, the amount of precipitate formed is diminished in the presence of these ligands. ATP and ADP are the most effective of the compounds tested. At ATP or ADP concentrations as low as 0.1 mM, precipitation is completely suppressed. The concentrations required for 50% solubilization are 25 μM for ATP and 35 μM for ADP. F6P and FDP are less effective in preventing precipitation, and the concentrations required for 50% solubilization are 2 mM and 3.8 mM, respectively. Citrate exhibits an effect comparable to the hexose phosphates, with 50% solubilization at 5.3 mM. AMP, on the other hand, shows little or no detectable effect on the interaction of the enzyme with F-actin in the concentration range tested. Creatine and phosphocreatine, which occur in muscle tissue at rather high levels, also produce little effect at concentrations up to 10 mM.

Catalytic Properties of Phosphofructokinase Bound to F-Actin

Phosphofructokinase is a key regulating enzyme in glycolysis. The results presented so far raise the question as to whether the kinetic properties of the enzyme are modified by the interaction with actin. To answer this, the pH-stat assay of Dyson and Noltman (1965), in which the H⁺ produced by the enzymatic reaction is titrated with standard
Figure 16. Effect of various metabolites on the precipitation of phosphofructokinase by F-actin. Refer to Figure 12 for experimental conditions. Various ligands used are indicated by the corresponding curve.
NaOH was used. This method avoids complication due to the auxiliary

\[
F-6-P^{2-} + ATP^{4-} \rightarrow F-1,6-P_2^{4-} + ADP^{3-} + H^+
\]

enzymes used in the standard coupled assay. Aldolase, one of the auxiliary enzymes, has altered catalytic properties when adsorbed to either F-actin or reconstituted thin filaments (Arnold and Pette, 1970; Walsh et al., 1977).

The sigmoidal plot (Figure 17) of the activity of free phosphofructokinase as a function of fructose-6-phosphate concentration conforms to the well known allosteric behavior of the enzyme at pH 7. In the presence of 0.2 mg/ml of F-actin, the apparent affinity of the enzyme for the hexose phosphate increases, but the plateau activity \(V_{max}\) essentially remains the same. The reconstituted thin filaments exhibit a similar but less pronounced effect. The addition of 0.2 mg/ml of bovine serum albumin to the assay mixture resulted in no change in phosphofructokinase activity. The value for the \(K_m\) are 0.55 mM, 0.35 mM, and 0.15 mM for free, thin filament-bound, and F-actin-bound phosphofructokinase, respectively. It is noteworthy, however, that the Hill coefficient (2.02) does not appear to be changed.

Similar experiments using ATP as the variable substrate show that the presence of either F-actin or the reconstituted thin filament tends to reverse the ATP inhibition observed at high concentrations. As shown in Figure 18, the concentration of ATP required to produce 50% inhibition of enzyme activity increases from 2.5 mM for free phosphofructokinase to 3.7 mM and 4.2 mM for the enzyme bound to the recons-
Figure 17. Plot of initial reaction velocity with respect to fructose-6-phosphate concentration. The free phosphofructokinase (○), F-actin-bound (△) and reconstituted thin filament-bound (●) phosphofructokinases were assayed at pH 7.00 in the presence of 1 mM ATP, 10 mM KCl, 5 mM MgCl₂, and the indicated concentration of F6P. In the assay of the bound enzymes, 0.2 mg/ml of either F-actin or reconstituted thin filament were also added in the assay mixture.
Figure 18. Plot of initial reaction velocity with respect to ATP concentration. The enzymes were assayed at the indicated concentration of ATP and 1 mM F6P. Other conditions were same as in Figure 17. (o), free; (Δ), F-actin-bound; (●), reconstituted thin filament-bound phosphofructokinase.
tituted thin filaments and F-actin, respectively. The maximal activity of the enzyme at moderately high ATP concentration (1 mM), however, remains unaffected by either F-actin or the reconstituted thin filaments. Similar observations have been reported for the enzyme adsorbed to erythrocyte membranes (Karadsheh and Uyeda, 1977).

The tendency of F-actin and the reconstituted thin filaments to reverse the inhibition of phosphofructokinase by ATP is reminiscent of AMP activation. The results in Figure 19 illustrate the effect of AMP on the ATP-inhibited phosphofructokinase. In the presence an ATP concentrations reducing the free enzyme activity to one-half its maximum value, AMP activates the enzyme with maximal effect occurring at 0.1 mM AMP. The plateau activity remains the same even in the presence of 0.5 mM AMP, never reaching the maximal activity of the enzyme under conditions in which ATP is not inhibitory. Phosphofructokinase bound to either F-actin or the reconstituted thin filaments exhibit higher activities in the absence of AMP, but still shows significant response to AMP activation, with maximal activation also occurring at 0.1 mM AMP. It is interesting to note that the maximum activation obtained using combinations of either F-actin or the reconstituted thin filaments with AMP is about the same as the maximum activation found with AMP alone. In the presence of an ATP concentration (4 mM) that inhibits the free enzyme to less than 20% maximal activity, varying amounts of F-actin show progressive effects in reversing the ATP inhibition (Figure 20). A saturating level of AMP, however, activates the enzyme activity to a plateau value which is nearly independent of F-actin concentration,
Figure 19. Sensitivity of free phosphofructokinase (○), and of phosphofructokinase adsorbed to F-actin (△), or reconstituted thin filaments (●), to activation by AMP. The enzyme was assayed in the solution containing a final concentration of 1 mM F6P, 2.5 mM ATP, 5 mM MgCl2, and 10 mM KC1, pH 7.00 at room temperature with varying concentrations of AMP. 0.2 mg/ml of either F-actin or the reconstituted thin filaments were also added in the assay mixture during the assay of bound phosphofructokinases.
Figure 20. Sensitivity of phosphofructokinase to activation by AMP in the presence of various amount of F-actin. (○) no F-actin, (●) 0.05 mg/ml, (▲) 0.2 mg/ml, and (◇) 0.4 mg/ml of F-actin. Assay solution contains a final concentration of 1 mM F6P, 4 mM ATP, 5 mM MgCl₂, and 10 mM KCl, pH 7.00.
PHOSPHOFRUCTIKINASE ACTIVITY (UNIT x 100)

AMP (mM)
approaching the maximum activation obtained with AMP alone. In no case is the maximum activity of the enzyme restored. These results suggest that F-actin and AMP are not synergistic in activating the enzyme and may act on phosphofructokinase through a common mechanism.

Citrate is known to inhibit phosphofructokinase at pH 7.0 (Bloxham and Lardy, 1973). The effect of citrate on both free and bound phosphofructokinases was examined under conditions where the enzyme exhibits maximum activity. The results in Figure 21 show that in the presence of 1 mM ATP, citrate markedly inhibits the free phosphofructokinase. F-actin and the reconstituted thin filaments tend to decrease the inhibition by citrate. The concentration of citrate required to produce 50% inhibition increases from 50 μM for free phosphofructokinase to 55 μM and 80 μM, respectively, when the reconstituted thin filaments or F-actin is present.
Figure 21. Sensitivity of phosphofructokinase to inhibition by citrate. The enzyme was assayed in the presence of 1 mM F6P, and 1 mM ATP with varying concentrations of citrate. Other conditions were same as Figure 19. Citrate titration curves were done either for the soluble phosphofructokinase (o), or for the enzyme adsorbed to F-actin (●), and the reconstituted thin filaments (▲).
IV. DISCUSSION

Binding of ATP and of εATP to Phosphofructokinase

The use of εATP as an ATP analogue is well documented (Leonard and Tolman, 1975; McCubbin et al, 1973; Kaplan and Coleman, 1978). In the case of rabbit muscle phosphofructokinase, it has been shown that εATP can replace ATP in its function as both substrate and allosteric inhibitor (Secrist et al, 1972). The results presented on the stoichiometry of εATP binding to rabbit muscle phosphofructokinase show that the enzyme binds 11.3 ± 1.2 moles of εATP per mole of active enzyme. This value corresponds to approximately 3 moles of εATP bound per enzyme protomer, and is in agreement with the earlier work of Kemp and Krebs (1967) on ATP binding by the same enzyme. Recently, Wolfman et al (1978) published experiments showing that rabbit muscle phosphofructokinase binds two moles of adenylyl imidodiphosphate, another ATP analog, per protomer. There are two possible reasons for this apparent discrepancy. The first lies in the methods of measurement. Wolfman et al used an equilibrium method giving direct measurement of the free and bound ligand concentrations. Calculations from fluorescence polarization measurements in the present work are based on application of the principle of additivity of anisotropies to a two component system comprising free and bound εATP. If the anisotropy of the bound ligand is different for the two kinds of binding site, the calculations would be oversimplified and in error. However, the calculations were substantiated with direct binding mea-
surements using the ultracentrifuge equipped with scanner.

The other explanation lies in the different binding equilibria exhibited by the nucleotides with phosphofructokinase. The binding of either ATP or adenylyl imidodiphosphate shows pronounced negative cooperativity while the binding of εATP is simple. The difference in stoichiometry may reflect the difficulty in obtaining saturation in an anti-cooperative system. A stoichiometry of two seemed reasonable in view of the two known functions of ATP; one for the catalytic binding site and the other, the inhibitory binding site. However, ATP may have a third function, such as phosphorylation catalyzed by a specific protein kinase (Mendicino et al, 1978), involving the third binding site.

ATP and εATP differ in some subtle effects of the conformation of the enzyme. The binding of ATP to phosphofructokinase has been shown to cause a change in the conformation of the enzyme leading to a decrease in the local mobility (Jones et al, 1972; and 1973) as well as in the reactivity (Kemp, 1969; Mathias and Kemp, 1972) of the 'class I' thiol group. The results on the fluorescence polarization and circular dichroism spectra of the ATP-phosphofructokinase complex add to the complexity of the nature of ATP binding by showing that the binding of ATP also leads to increases in both the local rigidity and the ellipticity of the trytophanyl side chains of the enzyme. The

3 Designation of the 'class I' thiol group of phosphofructokinase is taken from the classification of Kemp and Forest (1968).
binding of εATP, on the other hand, causes a slight increase in the local freedom of rotation of tryptophanyl side chains and has virtually no effect on the ellipticity. These differences may be related to the distinctive binding equilibria obtained with the two nucleotides. One may ask whether these pronounced local effects are linked to extensive alterations in the overall three-dimensional folding of phosphofructokinase. Circular dichroism measurements show that there is little effect on the long-range secondary structure. Sedimentation velocity studies show that the tetramer predominates under the conditions of experiments. Thus changes in aggregation, such as the dissociation obtained at very high (10 mM) ATP concentration (Uyeda, 1970), are not involved.

Nonetheless, the two nucleotides exhibit many similarities in their binding to phosphofructokinase. The binding of εATP is highly dependent on the magnesium ion concentration. In fact, the effect of magnesium on εATP binding is cooperative. The depletion of magnesium ion results in a rather incomplete binding, and the affinity of the enzyme for εATP diminishes about 8 fold. A similar observation has also been noted on the binding of ATP to spin-labeled muscle phosphofructokinase (Jones et al, 1973). In that instance, MgATP binds sigmoidally to the enzyme labeled with 4-(2-iodoacetamide)-2,2,6,6-tetramethylpiperidinoxoyl at the class I thiol group, with a half-saturation point of 400 μM at pH 7.5. The binding of free ATP is hyperbolic and considerably weaker (K_s = 1.3 mM). Moreover, saturating concentrations of ATP displace most of the bound εATP from the enzyme, with
the half-saturation point comparable to the Michaelis constant. This suggests that εATP is bound to phosphofructokinase at sites very similar, if not identical, to those for ATP. The observation that activators such as ADP, AMP, and fructose-1,6-diphosphate are also able to displace the bound εATP, with ADP being most effective, is of interest. MgATP has been shown to bind to phosphofructokinase at specific inhibitory sites (Kemp, 1969; Mathias and Kemp, 1972). Because of the similarities between the binding of εATP and ATP to muscle phosphofructokinase, both in stoichiometry and sites, it is very likely that εATP binds to the inhibitory sites under the conditions of the experiments. The demonstration that ADP, AMP, and fructose-1,6-diphosphate partially displace the bound εATP from the enzyme is consistent with this interpretation.

It is also worthy to note the effect of citrate on the binding of εATP. Kemp and his coworkers have suggested that citrates inhibits phosphofructokinase by decreasing the affinity of the enzyme for fructose-6-phosphate and increasing the affinity for ATP (Kemp and Krebs, 1967; Kemp, 1969; Mathias and Kemp, 1972). This enhancement in ATP affinity is achieved by the binding of citrate to the enzyme at sites distinct from the ATP binding sites (Colombo et al, 1975). In a study of the binding of ATP to sheep heart phosphofructokinase, Lorenson and Mansour (1969) observed that citrate did not enhance the binding of ATP; instead they found that high concentrations (1 mM) of citrate inhibit ATP binding. The observations on the effect of citrate on εATP binding indicate that citrate enhances the affinity of the enzyme
for εATP. This suggests that citrate may as well enhance the affinity of phosphofructokinase for ATP, and thus lends further support to the observation of Kemp and his coworkers.

**Binding and Activation of Phosphofructokinase by F-Actin**

In recent years much consideration has been directed towards the question of an association between glycolytic enzymes and the particulate components of cells and tissues (Friedreich, 1974; Clarke and Masters, 1976). In the case of skeletal muscle, some of the specific interactions have been studied in detail. For instance, several glycolytic enzymes have been shown to adsorb to structural components such as F-actin (Arnold and Pette, 1970; Arnold et al, 1971). Although the adsorption of phosphofructokinase to F-actin has been suggested in studies using myogen preparations (Arnold and Pette, 1968; Clarke and Masters, 1975a), unequivocal demonstration of the interaction has been hampered because of the strong tendency of the enzyme to aggregate under low ionic strength conditions (Arnold et al, 1971).

In the present study it is demonstrated that under suitable conditions, including the use of glycerophosphate to stabilize the enzyme (Colombo et al, 1975), phosphofructokinase can be precipitated by F-actin. That phosphofructokinase is adsorbed to F-actin is supported by the following observations. (1) Both protein components are present in the washed precipitate obtained by low speed centrifugation under conditions where neither F-actin nor phosphofructokinase alone sediments. (2) The flocculent precipitate is solubilized by several
factors including alkaline pH, high level of cations such as K⁺ or Na⁺, and a variety of metabolic effectors of the enzyme. (3) The catalytic properties of the enzyme are markedly altered by F-actin.

By varying the proportions of the enzyme and F-actin, it is shown that the degree of adsorption can be extensive with essentially stoichiometric binding of one phosphofructokinase tetramer per actin monomer and additional weaker binding at higher ratios. It must be recognized, of course, that such extensive association is not likely to occur in vivo. Hasselbach and Schneider (1951) have shown that actin represent 15% of the total muscle protein, that is, 1 g of muscle contains 25 to 30 mg of actin. On the other hand, the physiological concentration of phosphofructokinase amounts to 0.33 mg per gram of skeletal muscle (De Duve, 1972). Thus, the average physiological ratio is only 1 mg of phosphofructokinase per 75 to 90 mg of actin. Furthermore, the ionic strength of the in vitro system employed in the present study is below the physiological value. Nevertheless, the data help us to visualize the interaction between phosphofructokinase and F-actin. Since phosphofructokinase molecules are much larger than the globular actin molecules, a possible scheme of interaction satisfying the observed stoichiometry is as follows. Each phosphofructokinase molecule may bind to a specific site on the actin filament, encompassing several actin molecules. The actin-bound enzyme may then associate either with free phosphofructokinase or with other phosphofructokinase molecules bound to actin, producing a cross-linked network. The resulting aggregate may be so large that it precipitates. The
aggregation of phosphofructokinase molecules may occur in such a way that it fulfills the observed stoichiometry. Evidence of this kind has been provided to elucidate the interaction between F-actin and aldolase (Masters, 1978).

The analytical procedures used in the study are selective for the extensive, three-dimensional association envisioned above. The references to the complex apply specifically to the protein which was collected by low speed centrifugation. In examining the solubility of this complex under various conditions, one must keep in mind that the formation of low molecular weight complexes, possibly even containing G-actin, would not be distinguished from complete dissociation. Further studies to determine the extent of association in the soluble systems could be interesting.

Since the precipitation of phosphofructokinase by F-actin is suppressed by increasing concentrations of KCl or NaCl, one might argue that such association could not occur in vivo, where the ionic strength is believed to be equivalent to 0.1 - 0.12 M KCl (Mannherz and Goody, 1976). Careful examinations of the results, however, shows that an appreciable amount of the enzyme is associated with F-actin even in the presence of 0.12 M KCl. The precipitation of the enzyme is even higher when the same level of NaCl is present. Indeed, Clarke and Masters (1975a) have pointed out that interaction of the glycolytic enzymes with structural proteins of muscle does occur under conditions of physiological ionic strength provided that the protein concentrations also approach the physiological levels.
Intracellular variations of pH appear to have no direct role in the association. The results show that the adsorption of the enzyme to F-actin occurs within the physiological pH range. Other effects, such as variations of specific metabolites or of magnesium and calcium ions, may have a significant role. Most of the metabolites tested increase the solubility of the complex. ADP and ATP are remarkable for their effectiveness at very low concentrations, F6P and FDP being less effective. In comparison with other metabolites, such as creatine and phosphocreatinine, these ligands appear to have specific effects. ATP and FDP have also been shown to have pronounced effects on the adsorption of aldolase and triosephosphate dehydrogenase to F-actin in the purified in vitro system (Arnold and Pette, 1970) and in the myogen preparation (Clarke and Masters, 1975a). There is no obvious explanation at the present for the effects of ATP and FDP on the adsorption of glycolytic enzymes. Low levels of magnesium and calcium ions cause increased precipitation of phosphofructokinase. This effect may be noteworthy in view of the importance of these ions in the kinetic activity of the enzyme and in the control of muscle contraction, respectively. The present observations, taken with previous work on the subject (Clarke and Masters, 1976), demonstrate that specific metabolites and effectors may offer a means of regulating the intracellular distribution of important glycolytic enzymes.

The most striking observation in the present study is the effect on the catalytic properties of phosphofructokinase exerted by F-actin. At pH 7.0, where the enzyme exhibits allosteric kinetics, adsorption
to F-actin or to reconstituted thin filament produces major changes in the kinetic properties of the enzyme. The interaction with F-actin decreases the sensitivity of the enzyme to inhibition by ATP. Changes in the fructose-6-phosphate kinetics are characterized by a decrease in the concentration of the hexose phosphate necessary for half-maximal activity. Cooperativity of the adsorbed enzyme with respect to fructose-6-phosphate does not appear to be changed. The effects of F-actin on the activity of phosphofructokinase are therefore similar to those of the allosteric activator AMP in at least two features: the lowering of the apparent $K_m$ for fructose-6-phosphate and the decrease in sensitivity to ATP inhibition. Activation by AMP, however, usually occur with the abolishment, or at least diminution, of the cooperativity among the fructose-6-phosphate sites of the enzyme. The constancy of the Hill coefficient suggests that interaction with F-actin leads phosphofructokinase to a conformation that is neither 'relaxed' nor 'taut' but rather in between. This is similar to some of the properties of affinity labeled phosphofructokinase (Mansour and Martenson, 1978). It is also worthy to note that similar activation results on adsorption of the enzyme to erythrocyte membranes (Karadsheh and Uyeda, 1977). This may be due to the well known presence of actin on the inner surface of the membrane (Marchesi et al, 1976). The influence of F-actin on the enzyme activities at pH 8.2 where Michaelis-Menton kinetics is observed, was not monitored because of the apparently weak interaction between these two proteins.

In conclusion, the data demonstrate that the key regulating enzyme
in glycolysis, phosphofructokinase, associates with F-actin as do the majority of glycolytic enzymes. The influence of several specific effectors on the adsorption and the changes in catalytic properties of the enzyme suggest that the interaction may help to regulate both the intracellular distribution and activity of the enzyme.
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