The consistent application of phosphatase inhibitors and a novel final purification step using a connected series of DE-51, DE-52, and DE-53 anion exchange chromatography columns facilitate the preparation of electrophoretically homogeneous sub-pupulations of rabbit muscle phosphofructokinase which differ in their catalytic properties and endogenous covalent phosphate contents. A band of "high" phosphate enzyme (fraction II) flanked by regions of "low" phosphate enzyme (I and III) is an unusual feature of the final purification profile. Fractions I (in this case 0.42 mol P/84,000 g enzyme) and II (1.26 mol P/84,000) exhibit the most pronounced functional differences of the fractions. Both are activated by the addition of rabbit skeletal muscle F-actin. Under the assay condition, $K_m$ of phosphofructokinase activity occurs at 15.4 nM actin monomer for fraction I and 9.7 nM for fraction II. The "low" phosphate enzyme is synergistically activated in the presence of 0.12 μM actin plus 3.0 μM F 2,6-P$_2$, with a marked increase in $V_{max}$, while the "high" phosphate enzyme is not. Neither
fraction is activated appreciably by the addition of G-actin or the chymotrypsin-resistant actin "core". The covalently cross-linked trimer of actin stimulates the activity of both the "low" and "high" phosphate enzyme fractions. However, the previously mentioned synergistic activation characteristic of fraction I fails to occur in solutions containing the trimer plus F\textsubscript{2,6-P\textsubscript{2}}.

In vitro phosphorylation of fraction I catalyzed by the cAMP-dependent protein kinase causes its properties to become more like, but not the same as those of fraction II. The total amount of covalent phosphate present approaches 2 mol P/84,000 g for both fractions. Alkaline phosphatase and calcineurin catalyze partial (30-45\%) dephosphorylation of the enzyme. Pilot studies just initiated with the cGMP-dependent protein kinase show that it catalyzes the incorporation of 1.03 mol P/mol protomer at a rate 1.7 fold greater than obtained with the cAMP-dependent protein kinase.

Two actin binding proteins, filamin and \(\alpha\)-actinin, influence only the activity of "low" phosphate PFK-with resulting activation and inhibition, respectively--in the absence of actin. The addition of excess actin to the solutions largely reverses these effects.
Factors Affecting the Activation of Rabbit Muscle Phosphofructokinase by Actin

by

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ABBREVIATIONS

ATP  Adenosine 5'-triphosphate
ADP  Adenosine 5'-diphosphate
AMP  Adenosine 5'-monophosphate
cAMP  Adenosine 3',5'-cyclic monophosphate
CTP  Cytidine 5'-triphosphate
EDTA  (Ethylenedinitrilo)tetraacetic acid
EGTA  Ethyleneglycol-bis-(β-Aminoethyl ether)N,N'-tetraacetic acid
GTP  Guanosine 5'-triphosphate
cGMP  Guanosine 3',5'-cyclic monophosphate
F 6-P  Fructose 6-phosphate
F 1,6-P2  Fructose 1,6-bisphosphate
F 2,6-P2  Fructose 2,6-bisphosphate
ITP  Inosine 5'-triphosphate
MOPS  Morpholinopropanesulfonic acid
NAD+  Nicotinamide adenine dinucleotide (oxidized form)
NADH  Nicotinamide adenine dinucleotide (reduced form)
PFK  Phosphofructokinase
SDS  Sodium dodecyl sulfate
TEMED  N,N,N',N'-tetramethylenediamine
FACTORS AFFECTING THE ACTIVATION OF RABBIT MUSCLE
PHOSPHOFRUCTOKINASE BY ACTIN

INTRODUCTION

General background on phosphofructokinase

Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) is an indispensable enzyme in the Embden-Meyerhof-Warburg pathway. It catalyzes the transfer of the γ-phosphoryl group of ATP to the first carbon atom of D-fructose 6-phosphate, producing D-fructose 1,6-bisphosphate and ADP.

Fructose 6-phosphate + ATP $\xrightarrow{PFK}^{Mg^{++}}$ Fructose 1,6-bisphosphate + ADP + H⁺.

The reaction was discovered in red blood cells by Dische (1935) and in muscle by Ostern et al. (1936). Shortly thereafter, phosphofructokinase was isolated from yeast by Negelein (1936). Magnesium is required for the formation of the MgATP complex, the essential substrate of the enzyme (Paetkau and Lardy, 1967). Other divalent metals, including Mn⁺⁺ and Co⁺⁺ are also active with brain and muscle phosphofructokinase (Cottam and Uyeda, 1973), substituting for magnesium. Phosphofructokinase is considered to be the rate-limiting enzyme in the glycolytic pathway of yeast and mammals. The regulatory significance of phosphofructokinase in ascites tumor cells has been demonstrated by Lonberg-Holm (1959), Hess (1963) and Schulz (1968).
A number of sugar phosphates including D-tagatose 6-phosphate, D-fructose 1-phosphate, D-glucose 1-phosphate, D-sedoheptulose 1-phosphate, and D-fructose 6-sulphate can be used as substrates at rates slower than those encountered with fructose 6-phosphate. ATP may be replaced by other nucleoside triphosphates such as ITP, GTP, UTP and CTP. (cf reviews by Uyeda, 1979; Goldhammer & Paradies, 1979).

In general, phosphofructokinase is regulated by a multitude of metabolites including the two substrates, ATP and fructose 6-phosphate, and a series of negative and positive effectors that reinforce the action of the substrates. Citrate, H⁺, phosphoenolpyruvate, creatine phosphate, long chain fatty acids and glycerol 3-phosphate (Claus et al., 1982) are negative effectors which shift the sigmoidal fructose 6-phosphate velocity curve to the right, decreasing the apparent affinity of the enzyme for the substrate. Glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, ADP, AMP (Torheim and Lowenstein, 1976), inorganic phosphate (Tejwani & Mousa, 1981), OH⁻, 6-phosphogluconate (Sommercorn & Freedland, 1982), and fructose 2,6-bisphosphate (Uyeda et al., 1981a, 1981b; Hers & Van Schaftingen, 1982) are positive effectors which increase the apparent affinity of the enzyme for fructose 6-phosphate and relieve ATP inhibition. Phosphofructokinase is also strongly activated by low concentrations of NH₄⁺ or K⁺ (Otto, et al., 1976). All of these effectors may be physiologically significant. However, not all sources of phosphofructokinase are equally sensitive to them. The kinetic effects of such a variety of compounds point to the existence of a number of effector binding sites on the enzyme which may be
distinguished from the substrate or catalytic sites.

Fructose 2,6-bisphosphate is the most potent activator of phosphofructokinase. It was first isolated from rat liver (Furuya & Uyeda, 1980; Pilkis et al., 1981) and later synthesized chemically from fructose 1,6-bisphosphate (Pilkis et al., 1982). The formation of fructose 2,6-bisphosphate from fructose 6-phosphate and ATP is catalyzed by fructose 6-phosphate, 2-kinase (phosphofructokinase 2), which is different from fructose 6-phosphate, 1-kinase (phosphofructokinase 1). (As is customary in the current literature, I will continue to designate phosphofructokinase 1 as simply phosphofructokinase.)

\[
\text{Fructose 6-phosphate} + \text{ATP} \xrightarrow{\text{PFK}_2, \text{Mg}^{++}} \text{fructose 2,6-bisphosphate} + \text{ADP} + \text{H}^+.
\]

Phosphofructokinase 2 has been partially purified from hepatocytes of rats (Furuya & Uyeda, 1981; El-Maghrabi et al., 1981; Van Schaftingen & Hers, 1981; Hue et al., 1981). Physiological concentrations of inorganic phosphate (Pi) and AMP stimulate the activity of phosphofructokinase 2, whereas both phosphoenolpyruvate and citrate are inhibitory in either the presence or absence of Pi (Van Schaftingen & Hers, 1981; Laloux et al., 1985). The degradation of fructose 2,6-bisphosphate in vivo is catalyzed by fructose 2,6-bisphosphatase (Van Schaftingen et al., 1982; cf review by Uyeda et al., 1982). Pilkis et al. (1984) have reported that the fructose 6-phosphate 2-kinase and fructose 2,6-bisphosphatase activities are located within a single polypeptide chain with different reaction sites. The tissue distribution of fructose 2,6-bisphosphate and
fructose 6-phosphate 2-kinase in rat was determined by Kawajima and Uyeda (1982). Liver, containing 20 n moles/g, has the highest concentration of fructose 2,6-bisphosphate followed in decreasing order by brain, heart muscle, kidney, testis and skeletal muscle, which has only 2.6 nmoles/g. The activity of fructose 6-phosphate 2-kinase is also the highest in liver and the lowest in skeletal muscle. Fructose 2,6-bisphosphate and fructose 6-phosphate 2-kinase are not detectable in red blood cells of rats.

The biosynthesis and biodegradation of fructose 2,6-bisphosphate are controlled by hormones and metabolites. For instance, the levels of both fructose 2,6-bisphosphate and phosphofructokinase 2 in hepatocytes of rats increase when large concentration of glucose are administered. The concentrations of both rapidly decrease upon the addition of glucagon to isolated hepatocytes from fasted rats. Fructose 2,6-bisphosphate and phosphofructokinase 2 concentrations in perfused muscle are increased by insulin and epinephrine but decreased after electrical stimulation (van Schaftingen et al., 1982; Richards et al., 1981; Rue et al., 1981; Kawajima et al., 1982). It has been reported that the hormonal control of phosphofructokinase 2 and fructose 2,6-bisphosphatase are mediated by certain specific protein kinases and protein phosphatases that phosphorylate and dephosphorylate the enzyme, resulting in inactivation and activation phosphofructokinase 2, respectively. In comparison to phosphofructokinase 2, the effects of phosphorylation and dephosphorylation on fructose 2,6-bisphosphatase are reversed. The cAMP-dependent protein kinase is known to phosphorylate phosphofructokinase 2 in vitro, with resulting inactivation. The
enzyme can also be phosphorylated by phosphorylase kinase in the
presence of calcium, resulting in an 80% decrease in catalytic
activity (Uyeda et al., 1982).

Different biological sources of phosphofructokinase exhibit widely
varying kinetic properties. Allosteric properties have been
demonstrated in purified preparations of phosphofructokinase from
various animals, plants, yeast, and certain other microorganisms.
In general, the enzyme from these sources is characterized by the
sigmoidal dependence of the rate on the concentration of fructose
6-phosphate and by the dual role of ATP, which acts as both substrate
and inhibitor. Strong kinetic interactions between the two substrates
are readily demonstrated. The shape and position of the fructose
6-phosphate isotherm depends on the ATP concentration and the
inhibition by ATP is a function of the fructose 6-phosphate concen-
tration (Freyer et al., 1976). The sigmoid shape of the fructose
6-phosphate isotherm is not necessarily the result of the inhibitory
action of ATP. Yeast phosphofructokinase shows cooperativity with
respect to fructose 6-phosphate when noninhibiting phosphoryl donors
are employed as the second substrate (Kopperschläger et al., 1968).
Phosphofructokinase from several microorganisms, including E. coli,
exhibits a sigmoid rate dependence on fructose 6-phosphate
concentration but is not inhibited by ATP (Blangy et al., 1968).

The allosteric behavior of phosphofructokinase is also affected by
the pH of the reaction solution (Freyer & Hofmann, 1967; Lindell &
Stellwagen, 1968). Animal and yeast phosphofructokinases are
oppositely influenced by changes in pH. With the animal enzyme, an
increase in pH from 6 to 8 causes a shift of the sigmoidal fructose
6-phosphate velocity curve to the left with reversal of the inhibitory action of ATP. With the yeast enzyme, the reverse is found (Hill and Hames, 1975). The effect of temperature on the allosteric properties of phosphofructokinase is strongly dependent on the source of enzyme as well. Phosphofructokinase from ascites tumor cells shows a decrease in the apparent affinity for fructose 6-phosphate and an increase in the inhibition by ATP as the temperature increases (Freyer & Hofmann, 1967). On the other hand, phosphofructokinase from either rabbit muscle or chicken liver exhibits cold lability (Kono and Uyeda, 1973; Bock & Frieden, 1974). The accompanying loss of activity is reversible and becomes more extensive at lower temperatures, lower pH values, and lower enzyme concentrations. Bock and Frieden (1976) demonstrated that the inactivation involves protonation of specific residues in the tetrameter, presumably histidine, followed by isomerization to an inactive form which dissociates to the dimer. The dissociation-association reaction is a characteristic feature of phosphofructokinase which is strongly dependent on protein concentration, pH and the presence of effectors. Fructose 6-phosphate (Lad et al., 1974) and fructose 2,6-bisphosphate (Reinhart, 1983) stabilize the tetrameter while negative effectors, such as citrate and ATP, stabilize the dimer and possibly the protomer (Kono & Uyeda, 1973). Crosslinking experiments using dimethyl suberimidate and electron micrographs of the large helical aggregates of phosphofructokinase both suggest that the dimer is the fundamental unit of polymerization (Lad & Hammers, 1974; Telford et al., 1975). The tetrameter, possessing a molecular weight of 320,000 to 380,000, is thought to be the smallest active form of phosphofructokinase from
animal tissue. It has a strong tendency to self associate, leading to polymers with molecular weights of several million. Depending on the biological source and the method of determination, the protomer molecular weights range from 75,000 to 95,000 daltons (Coffee et al., 1973; Kono et al., 1973; Brennan et al., 1974).

Animal tissues contain multiple phosphofructokinase isoenzymes, which can be distinguished by their immunologic, chromatographic and electrophoretic properties (Meienhofer et al., 1972; Kirby and Taylor, 1974). Four isozymes of human and rat tissue phosphofructokinase have been separated and immunologically characterized. In the case of the rabbit, at least three different kinds of subunit have been demonstrated. A4 occurs in skeletal muscle and heart while B4 occurs in liver and erythrocytes. C4, which does not react with antisera to muscle or liver phosphofructokinase, is found in the brain which also contains A-B-C hybrid isozymes (Foe & Kemp, 1984). These isozymes are the product of independent genetic loci (Kirby & Taylor, 1975). Other rabbit tissues--such as lung, adipose tissue and stomach--contain a complete set of five A-B hybrids (A4, A3B, A2B2, AB3 and B4) (Tasi & Kemp, 1972, 1973, 1974).

Phosphofructokinase, like several other glycolytic or glycogenolytic enzymes, belongs to a group of proteins which undergo reversible covalent modification by side chain phosphorylation (cf review by Soling and Brand, 1981). The enzyme can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase either in vivo or in vitro (Kagimoto & Uyeda, 1979). Both native and in vitro phosphorylated phosphofructokinase can be partially dephosphorylated in vitro by alkaline phosphatase from bovine intestine mucosa (Foe &
Presumably the enzyme also can be dephosphorylated in vivo by as yet unidentified phosphoprotein phosphatases.

The rate of phosphorylation of phosphofructokinase by the catalytic subunit of cAMP-dependent protein kinase is strongly accelerated by positive enzyme effectors such as fructose 2,6-bisphosphate without changing the total amount of phosphate incorporated (Kemp et al., 1981; Foe et al., 1983). In the presence of 1 mM AMP and in the presence of 0.1 mM fructose 1,6-bisphosphate, the rate of phosphorylation was increased by 34% and 91%, respectively. However, the inclusion of AMP in assay mixtures containing 22 μM fructose 2,6-bisphosphate has no further effect on the initial phosphorylation rate (Kemp et al., 1981). Foe et al. (1983) reported that the addition of 22 μM fructose 2,6-bisphosphate or 100 μM fructose 1,6-bisphosphate or 560 μM glucose 1,6-bisphosphate into the reaction mixture increased the initial phosphorylation rate of phosphofructokinase by the catalytic subunit of cAMP-dependent protein kinase to 277%, 270% and 260% of the control rate, respectively. On the other hand, the phosphorylation rate was decreased to 65% of the control value in the presence of 2 mM citrate. The effects of citrate and fructose 1,6-bisphosphate or citrate and AMP were additive. Citrate counteracts the influence of fructose 1,6-bisphosphate and glucose 1,6-bisphosphate but not of fructose 2,6-bisphosphate on the initial phosphorylation rate (Foe et al., 1983).

Accumulating evidence shows that the extent of phosphorylation of muscle phosphofructokinase is strongly dependent on both the physiological state and the methods of tissue extraction used. Varying phosphate contents of ca. from 0.08 to 1.17 mol phosphate per
protomer of phosphofructokinase isolated from resting muscle have been obtained by different research groups (Hussey et al., 1977; Riquelme et al., 1978; Uyeda et al., 1978; Hofer & Sorensen-Ziganke, 1979; Soling & Brand, 1981). Muscle contraction increases the average phosphate content of phosphofructokinase significantly. The phosphate content of phosphofructokinase isolated from electrically stimulated muscle increased from 1.0 to 2.0 mol phosphate per protomer as originally reported by Hofer & Sorensen-Ziganke (1979) and from 0.54 to 0.96 mol phosphate per protomer as later reported by Hofer (1985). Only one phosphorylation site, a serine residue in the sixth position from the carboxyl terminus of rabbit muscle phosphofructokinase, has been specifically identified (Kemp et al., 1981). Hussey et al. (1977) reported that differential phosphorylation of phosphofructokinase results in different degrees of self-aggregation. They found that their preparation of rabbit skeletal muscle phosphofructokinase could be fractionated into three distinct species with sedimentation coefficients of 30S, 18S, and 13S by chromatography on agarose gels, hydroxyapatite or DE-cellulose. Measurements of alkali-labile phosphate showed that the fractions consisting almost exclusively of 30S species and the fractions consisting predominantly of 18S and 13S species contain approximately 0.15 and 0.29 mole of phosphate per phosphofructokinase protomer, respectively. Uyeda et al. (1978), on the other hand, separated rabbit muscle phosphofructokinase into two fractions by DEAE-cellulose chromatography. The fraction which was eluted with 0.1 M Tris-phosphate buffer contained 0.08 mol phosphate per protomer while the fraction eluted with 0.3 M Tris-phosphate buffer contained 0.2 mol
phosphate per protomer. The two fractions of phosphofructokinase also differed from each other in aggregation state. Sedimentation velocity patterns of the enzyme fraction containing 0.08 mol phosphate showed two major peaks with sedimentation constants of 12 S and 18 S and a minor 24 S peak whereas the fraction containing 0.20 mol phosphate consisted mainly of 30 S material with minor 24 S, 18 S and 12 S peaks. Clearly, the fractions of "phosphorylated" phosphofructokinase isolated by the two groups differ and the total phosphate in all cases is much below the ideal minimum stoichiometry of one mol phosphate/mol protomer. No differences in the allosteric properties of the various fractions was observed by either group.

The difference of allosteric kinetic properties between "low" (<0.5 mol p/protomer) and "high" (>0.8 mol p/protomer) phosphate form of phosphofructokinase from rat liver (Sakakibara & Uyeda, 1983) and rabbit skeletal muscle (Foe & Kemp, 1982; Kitajima et al., 1983) have been reported recently. (The low phosphate fractions demonstrated in these papers were either the native form or treated by subtlisin or alkali phosphatase. The high phosphate fractions corresponded to the enzyme phosphorylated by cAMP-dependent protein kinase in vitro.) The reports showed that the phosphorylated enzymes were more sensitive to the inhibition by ATP and citrate and less sensitive to the activation by AMP and fructose bisphosphates including fructose 1,6-bisphosphate, fructose 2,6-bisphosphate and glucose 1,6-bisphosphate. The difference between the differentially phosphorylated species is larger in the liver enzyme than in the skeletal muscle enzyme. The pH-dependence of ligand binding and the cold inactivation phenomena are also affected by the degree of phosphorylation (Kitajima et al.,
1983). Foe and Kemp (1982) reported no differences in the sedimentation properties under a variety of conditions or in the stability of phosphorylated and dephosphorylated phosphofructokinase as shown by sucrose density gradient centrifugation and low pH inactivation. On the other hand, the influence of the degree of phosphorylation on the pH-dependence of ligand binding and the cold inactivation phenomena were reported by Kitajima et al. (1983). Their report shows that the binding of ATP to the inhibitory site of the phosphorylated enzyme is characteristically enhanced at lower pH. A pH-dependent cold inactivation study has shown that the phosphorylation of the enzyme causes an increase in the pK value for the inactivation, and the extent of the pK shift depends upon the degree of phosphorylation. However, the reported difference between the allosteric properties of the "high" and "low" phosphate forms of phosphofructokinase are relatively small and the physiological role of this covalent modification remains uncertain.

The degree of phosphorylation of either liver or heart muscle phosphofructokinase is affected by both metabolic state and glucagon levels (Clark and Patten, 1984; Brand and Soling, 1982). Glucose increases the incorporation of phosphate into phosphofructokinase to almost maximal levels in isolated hepatocytes from fed rats, whereas L-alanine abolishes the glucose induced phosphorylation in both fasted and fed animals (Brand & Soling, 1982). Glucagon stimulates the phosphorylation of liver phosphofructokinase approximately 3 to 5 fold while increasing the cAMP levels 5 fold and the blood glucose levels 2-fold over the values obtained for control rats (Kagimoto and Uyeda, 1979; Castano et al., 1979).
Macromolecular associations of phosphofructokinase

Histochemical techniques are widely used in studies of the cellular localization of glycolytic and glycogenolytic enzymes. Longitudinal sections of frog and rabbit skeletal muscle, rat heart muscle and locust flight muscle display a striated distribution of all glycolytic enzymes except hexokinase with the heavily staining region corresponding to the site of the I-band in the relaxed myofibril. The glycogenolytic enzyme phosphorylase also associates with the I-band in some muscle (cf review by Ottaway & Mowbray, 1977). The mean concentration of phosphofructokinase in the I-band region is 3.7 mg/ml \(10^{-5}\) M) in the case of white muscle and 0.35 mg/ml \(10^{-6}\) M) in red muscle (Hofer, 1971).

Figure 1 shows the structure of the sarcomere, the repeating structural unit of striated muscle fibers. The Z-lines, composed largely of the actin-binding protein \(\alpha\)-actinin, are the attachment sites for the thin filaments. The thin filaments are polar structures comprising a backbone of filamentous actin (F-actin) and the associated proteins tropomyosin and troponin. The thick filaments are bipolar structures produced by the linear polymerization of myosin. In the sliding filament theory of muscle contraction, the thick and thin filaments slide over each other to shorten the distance between the Z-lines. The center of the sarcomere is occupied by the M-line proteins. The I-bands, seen as light regions in electron micrographs, are centered around the Z-lines. They comprise those sections of the thin filaments which are not interdigitated with the thick filaments. (Cohen, 1974).
Figure 1. Myofibril. Figure here shows the interpenetrating arrays of thick and thin filaments.
Actin, a highly conserved protein, occurs in all eukaryotic cells. In addition to its function in the thin filament of muscle, it is also a major component of the microfilaments—which are among a variety of filaments occurring in the cytoskeleton (Osborn & Weber, 1979; Payne & Rudnick, 1984). The temporal and spatial regulation of actin polymerization is central to the structural and motile activities of nonmuscle cells (Korn, 1982). The polymerization mechanism, which involves nucleation and elongation steps (Frieden, 1985), is very similar for both muscle and nonmuscle actins. However, under conditions where essentially 100% of muscle actin would be polymerized either in vivo or in vitro, at least 50% of the actin of nonmuscle cells may be nonpolymerized (Korn, 1978). It is now evident that the polymerization and depolymerization of actin in nonmuscle cells is controlled by a variety of actin-binding proteins (Stossel et al., 1985). Some of them, such as α-actinin and filamin, have been found both in muscle and the microfilaments (Osborn & Weber, 1979).

α-Actinin is a structural component of skeletal muscle myofibrils (Ebashi & Ebashi, 1965; Suzuki et al., 1976), where it is localized in the Z-lines of the sarcomere (Maruyama, 1976; Gomer & Lazarides, 1983). The protein has been found in a variety of biological sources including cardiac muscle (Robson & Zeece, 1973), smooth muscle (Feramisco & Burridge, 1980) and non-muscle cells (Lazarides & Burridge, 1975; Nunnally et al., 1980). In general, α-actinin is a homodimer of identical polypeptides of about 100,000 daltons, each having the ability to cross-link actin filaments (Blikstad et al., 1980; Craig & Pollard, 1982; Kobayashi et al., 1984). Filamin, a 500,000-dalton actin cross-linking protein, contains two identical
polypeptide chains (Shizuta et al., 1976). Although, it was first isolated from chicken smooth muscle (Wang & Singer, 1977), filamin has been found in a different variety of cells (Osborn & Weber, 1979; Tyler et al., 1980; Nunnally et al., 1980; Weihing & Franklin, 1983), and muscle types (Bechtel, 1979; Gomer & Lazarides, 1983). Investigation of striated muscle has revealed filamin at the Z-lines of both skeletal and cardiac muscle (Bechtel, 1979; Gomer & Lazarides, 1983). Gomer and Lazarides (1981) observed that filamin is located at the rim of the Z-lines, surrounding a central region containing α-actinin.

The interactions of glycolytic enzymes with components of the cytosol and with subcellular structures has been studied using both sedimentation and chromatographic methods. The adsorption of the glycolytic enzymes is generally reversible and dependent on pH, ionic strength and metabolite levels (Clarke and Masters, 1975a,b, 1976; Choate et al., 1985). Cravan and Basford (1974) found that the phosphofructokinase activity of nerve tissue is associated with the mitochondria. Clarke and Masters (1975b, 1976) examined a pressed juice extract prepared from minced muscle which contained enhanced levels of enzymes (e.g. aspartate aminotransferase, isocitrate dehydrogenase) that are either absent or present in very low amounts in the normal myogen preparation, which is obtained by extraction of the minced muscle with buffer containing potassium phosphate (0.025 M), dithiothreitol (0.002 M), pH 7.5. The phosphofructokinase present in these preparation fails to display its usual tendency to aggregate. Their experiments also showed that phosphofructokinase is the most completely adsorbed enzyme of those bound to the thin filament.

The experiments with pressed juice extracts show that the
adsorption of the glycolytic enzymes to the thin filament is affected by the levels of calcium and magnesium. The adsorption of aldolase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase increases on the addition of 2 mM MgCl₂ to the binding assay system, while that of the other enzymes is largely unaffected. When 0.2 mM CaCl₂ is present in the assay system, the adsorption of aldolase, pyruvate kinase and lactate dehydrogenase is increased while that of the other glycolytic enzymes remains the same. Approximately ninety per cent of the phosphofructokinase of rabbit skeletal muscle was bound to the thin filament, with eighty per cent bound to the F-actin backbone (Clarke & Masters, 1975b). Experiments with cardiac myofibrils and purified glycolytic enzymes gave similar results, with the association of the enzyme dependent on pH and the presence of enzyme effectors (Clark et al., 1984; Choate et al., 1985). The lower the pH, the stronger the binding of phosphofructokinase to the cardiac myofibril. Bronstein and Knull (1981) reported that enzyme-enzyme interaction also affects the association of glycolytic enzymes with the reconstituted thin filament. They found that, when examined individually, the enzymes triosephosphate isomerase, glucose-6-phosphate isomerase and phosphoglucomutase do not bind to the thin filament. However, in a complex mixture containing all the other glycolytic enzymes, significant binding of the three enzymes does occur.

The interaction of phosphofructokinase with other subcellular proteins in vitro has also been observed. Starr and Offer (1982) reported the copurification of rabbit skeletal muscle phosphofructokinase with myosin, the major component of the thick
filament in skeletal muscle. Mayr and Heilmeyer (1983) discovered that skeletal muscle phosphofructokinase can be isolated by using a calmodulin-Sepharose affinity column. However, work in this laboratory shows that the association of phosphofructokinase with calmodulin is at least two orders of magnitude less effective than that of recognized calmodulin-dependent enzymes (Malencik and Anderson, unpublished results).

The catalytic properties of glycolytic enzymes may change upon adsorption to membrane or subcellular components. Arnold and Pette (1970) reported modification of the kinetic properties of aldolase when it is bound to F-actin. Similarly, the kinetic properties of glyceraldehyde 3-phosphate dehydrogenase (Dagher & Hultin, 1975) and lactate dehydrogenase A₄ (Ehmann and Hultin, 1973) are also altered upon adsorption to muscle subcellular particulate structure. In the case of human erythrocyte and rabbit skeletal muscle phosphofructokinases, the membrane bound enzyme—unlike free phosphofructokinase—is not inhibited by ATP or 2,3-diphosphoglycerate. The fructose 6-phosphate saturation curve of the bound enzyme is also nonsigmoidal (Karadsheh and Uyeda, 1977).

That the activity of rabbit skeletal muscle phosphofructokinase increases upon association with either F-actin or the thin filaments was first reported by Liou and Anderson (1980). They found that F-actin, either separately or in conjunction with AMP, promotes a decrease in the $K_m$ of the enzyme for fructose 6-phosphate and a decrease in its sensitivity to inhibition by either citrate or high concentrations of ATP. The cooperative properties of the enzyme are retained upon adsorption and the effects of the thin filament are
slightly less pronounced than those of actin.

Goals and Significance of This Project

The first part of this project entails the isolation of differentially phosphorylated species of rabbit skeletal muscle phosphofructokinase. Two fractions with substantially different endogenous phosphate contents are characterized in detail with respect to their specific activities and sensitivities to activation by F-actin and fructose 2,6-bisphosphate. The effects of protein kinases, especially cAMP-dependent protein kinase, and preliminary experiments of phosphoprotein phosphatases on the fractions are also examined. Pilot experiments were performed to determine how phosphofructokinase obtained from fasted rabbits (4 days on water) compares to the enzyme routinely prepared from well-fed rabbits.

The second aspect deals with the effects of chemical modification of actin on its ability to activate phosphofructokinase and with the differences between the effects obtained with F-actin and G-actin, the globular actin monomer. Since actin is a nearly universal protein, comprising 10-20% of the cell protein in all eukaryotic cells (Korn, 1978), its binding of phosphofructokinase and other glycolytic enzymes may be a widespread phenomenon. Accordingly, the additional effects of several actin binding proteins known to be involved in both cytoskeletal organization and smooth muscle structure—α-actinin and filamin—are described in the final stage of this work.
MATERIALS AND METHODS

MATERIALS

Fructose 6-phosphate, fructose 2,6-bisphosphate, fructose 1,6-bisphosphate, ATP, glycine, phenylmethylsulfonyl fluoride, glycylglycine, N,N'-p-phenylenebismaleimide, ammonium sulphate, dithiothreitol, tetrapotassium pyrophosphate, phosphoserine, 2-mercaptoethanol, bromochlorophenol blue, benzamidine, tris(hydroxymethyl)aminomethane (Tris) and sodium dodecylsulphate were purchased from Sigma company. The reagents were used without further purification. Aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase and carbonic anhydrase as well as alkaline phosphatase (bovine intestinal mucose or rabbit skeletal muscle) were obtained from the same company.

Sephacryl S-200 and S-300 superfine were purchased from Pharmacia; DE-51, DE-52 and DE-53 from Whatman; 4-phenylbutylamine sepharose was from Pierce Co.; P-2 and P-60 gels were from BioRad laboratories. N,N'-dimethylformamide and thymol crystal were purchased from Mallinckrodt Inc. and J.T. Baker Chemical Co., respectively. Coomassie brilliant G-250 was obtained from Eastman Kodak; coomassie brilliant R-250, acrylamide, N,N'-methylene bisacrylamide, N,N'-diallyltartardiamide and N,N,N',N'-tetramethylenediamine were obtained from Bio Rad; sterile millipore filters were from Amicon company; Norite was from Matheson Coleman & Bell Co.; bovine serum albumin was from Armour Pharmaceutical Company; Permablend III was from Packard Instrument Company, Inc. The catalytic subunit of type II
cAMP-dependent protein kinase, cGMP-dependent protein kinase, rabbit skeletal muscle myosin light chain kinase, turkey gizzard myosin light chain kinase, phosphorylase kinase, cardiac troponin I and C, calcineurin and bovine pancrease deoxyribonuclease I were kindly provided by Drs. Sonia R. Anderson and Dean A. Malencik. The rabbits were purchased from Animal Resource Center of Oregon State University.

METHODS

Phosphofructokinase purification

Rabbit skeletal muscle phosphofructokinase was purified from fresh skeletal muscle according to the method of Kemp (1975) with modifications. A large female New Zealand rabbit, weighing about 4 kilograms, was killed by injection of approximately 1.5 ml beuthanasia-D. The hind legs and back muscle were quickly removed and chilled in an ice bath immediately. The muscle was then ground in an electric meat grinder with a 1/8 inch plate. About 800 grams of ground muscle was normally obtained. All subsequent steps were performed using buffers at 4°C. After weighing, the muscle was extracted by stirring in three volumes of 30 mM KF, 4 mM EDTA, 15 mM 2-mercaptoethanol pH 7.5 for 25 minutes. The clear supernatant fraction was collected by centrifugation at 2000 x g in a Beckman model J-6B centrifuge for 10 minutes and brought to 5 mM tetrapotassium pyrophosphate. The pH of the supernatant was adjusted to 6.8 by the addition of 1 M glacial acetic acid. Then, the supernatant fraction was immersed in a constant temperature dry
ice-acetone bath to cool the extract to 4°C and the dropwise addition of one fifth volume of ice cold isopropanol began while continuously stirring. The temperature was held at 4°C for 20 min. The pellet was collected by centrifugation at 3000 x g for 20 minutes, dissolved in 0.1 M Tris-phosphate, 5 mM PPi, 0.2 mM EDTA, 0.2 mM fructose 1,6-bisphosphate, 1 mM 2-mercaptoethanol, pH 8.0 and dialyzed against the solution for several hours to get rid of the isopropanol. The dialysate was then brought to 57-59°C and held at this temperature for three minutes. After this heat treatment, the solution was immediately immersed in an ice bath to cool to 4°C and centrifuged at the same temperature. The yellowish supernatant was collected and the pellet was washed once with the same buffer. The supernatants were combined and ammonium sulfate (213 g/l) was added. After the ammonium sulphate was totally dissolved, the solution was stirred at 4°C for another thirty minutes. The supernatant was collected and brought to 55% ammonium sulphate (101 g/l) and stirred for at least 2 hours. The precipitate was collected and taken in 1/30 volume (referenced to the first supernatant) of 50 mM Tris-phosphate, 0.2 mM EDTA, 1.0 mM 2-mercaptoethanol, 5 mM pyrophosphate, pH 8.0 (solution A) and dialyzed against it in preparation for anion exchange column chromatography. The DE-51, DE-52 and DE-53 were packed into three columns (2.5 x 10 cm), connected in series and equilibrated with solution A. The dialysate was collected after overnight dialysis and applied to the anion exchanging cellulose columns. Immediately after the sample was applied, the columns were washed with the equilibration buffer and a linear gradient of 0.1 M to 0.3 M Tris-phosphate buffer, pH 8.0, containing 0.2 mM EDTA, 1.0 mM 2-mercaptoethanol and 5 mM
pyrophosphate. The total volume was 1200 ml. The phosphofructokinase was eluted with a Tris-phosphate concentration of approximately 0.14 M. The enzyme fractions were saved individually or pooled according to the peaks separated. For storage the enzyme was concentrated by ultrafiltration. A small amount of enzyme approximate 1 mg total were taken and precipitated with 10% trichloroacetic acid and saved in refrigerator for phosphate determination. Then, the concentrated enzyme samples were dialyzed against 0.1 M potassium phosphate pH 8.0, containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM pyrophosphate and 50% glycerol and saved in a -80°C deep freezer.

The average activity of this phosphofructokinase was about 200 units/mg when assayed at optimal condition in normal well fed rabbits.

**Acetone Powder Preparation**

Acetone powder for actin preparation was prepared according to Pardee and Spudich (1982). A large female New Zealand rabbit was sacrificed as described previously. After grinding in an electric grinder, the muscle was weighed and extracted with three volumes of 0.1 M KCl, 0.15 M potassium phosphate buffer pH 6.5 for ten minutes. The pellet was collected by centrifugation in Beckman J-6B centrifuge at 2,000 x g for 5 minutes and then extracted with 6 volumes of 0.05 M NaHCO₃ for 10 minutes. The pellet was collected as before and extracted with another three volumes of 1 mM EDTA, pH 7.0. The sediment was washed twice with 6 volumes of water for 5 minutes. Then, the pellet was extracted five times with 2 volumes of cold acetone (around 20°C). The acetone washed sediment was collected by passing through
several layers of cheesecloth and dried under the hood for several hours. The dried acetone powder was saved in a -20°C freezer and used for actin preparation.

**Actin Preparation**

Actin was prepared following the general procedure Pardee and Spudich (1982). The acetone powder was extracted with 30 volumes (by weight) of 2 mM Tris-Cl, 0.2 mM ATP (fresh prepared and titrated with NaOH), 1 mM 2-mercaptoethanol, 0.2 mM calcium acetate, pH 8.0 (depolymerization buffer) at 4°C for 30 minutes. The subsequent procedures were performed at 4°C. The solution was then passed through four layers cheesecloth, the filtrate was brought to 50 mM KCl, 2 mM MgCl₂, 1 mM ATP and allowed to set at 4°C for 2 hours. After that, the concentration of KCl was brought to 0.6 M and continuously stirred for 30 minutes. The polymerized actin was centrifuged down in 60 ml tubes at 80,000 x g for 3 hours at 4°C. The pellet of F-actin was resuspended by homogenization in 3 ml of cold depolymerization buffer per gram of acetone powder and dialyzed against 1 liter of prechilled depolymerization buffer with two changes over a 3 days period. The dialysate was centrifuged at 80,000 x g for 3 hours to remove undepolymerized actin. The supernatant of pure actin was passed through 0.45 μm sterile millipore (Grazi and Magri, 1981) to remove the trace amount of F-actin and saved in the atmosphere of thymol, or they can be brought to 0.05 N KCl, 1 mM MgCl₂, then set at 4° for one hour for complete polymerization. The polymerized actin was recollected by centrifugation at 80,000 x g 3
hours and saved in the atmosphere of thymol at 4°.

**Actin Core Preparation**

Actin core was prepared according to the method of Jacobson and Rosenbusch (1976) with major modifications. The actin had to be freshly prepared as described previously. Instead of preparing the final F-actin solution, the G-actin was saved. The protein concentration was adjusted to 2 mg/ml by the addition of more 2 mM Tris-Cl, 0.2 mM ATP, 0.2 mM calcium acetate, 1 mM 2-mercaptoethanol, pH 8.0 (buffer A). The G-actin solution (2 mg/ml) (total volume 20 ml) was incubated with 10% chymotrypsin (wt/wt) for 20 minutes at 25°C. The solution was brought to 3 mM phenylmethylsulfonylfluoride (dissolved in DMSO, 0.8 M in stock) and filtered immediately by passing through Whatman No. 1 filter paper. The mixture was then applied to a small affinity column of 4-phenylbutylamine sepharose (1.0 x 3.0 cm) which was equilibrated with buffer A to remove the last traces of chymotrypsin (Stevenson & Landman, 1970). (This step should be finished within one hour.) Approximately eighty per cent of the protein containing the actin core will be tightly bound to the column and can be eluted out by 1 mM of ATP, pH 7.0. The peak of actin core was pooled and dialyzed against buffer A immediately for several hours and concentrated by Amicon ultrafiltration. The concentrated core solution (10 ml) was then passed over a Sephacryl S-200 column (1.5 x 90 cm) that had been equilibrated with 2 mM Tris-Cl, 0.2 mM calcium acetate, 1 mM 2-mercaptoethanol and 0.3 M NaCl, pH 8.0 (Fig. 2). The peak of actin core was identified by sodium dodecylsulphate gel
Figure 2. Sephacryl S-200 column profile of rabbit muscle skeletal actin core preparation. The actin core containing solution (eluate of 4-phenylbutylamine sepharose) was applied to a 1.8 x 90 cm Sephacryl S-200 column which was washed with 2 mM Tris-Cl, 0.5 mM 2-mercaptoethanol, 0.2 mM ATP, 0.2 CaCl$_2$ and 0.3 N NaCl, pH 8.0 buffer. The actin core was eluted at flow rate 16 ml/hr. Peak I was actin core and peak II was actin fragments.
Figure 2.
electrophoresis and quickly dialyzed against buffer A to remove salt and concentrated by Amicon ultrafiltration. The actin core solution was passed through 0.45 μm sterile millipore and saved in a thymol atmosphere at 4°C. The yield of actin core is about 50%. The actin core solution was used within 2 days.

Actin Trimer Preparation

The actin trimer preparation was followed by the method of Gilbert and Frieden (1983). Freshly prepared G-actin solution was dialyzed against 4 mM imidazole-Cl, 2 mM MgCl₂, pH 7.5 for overnight. The protein concentration of actin solution was adjusted to 7 mg/ml then mixed with an equal portion of 5 mM sodium borate, 0.2 M KCl, 0.4 mM ATP, 0.2 mM CaCl₂, pH 9.3. A portion of stock N',N'-phenylenebismaleimide (2 mM dissolved in N,N'-dimethylformamide) was added to bring the concentration to 0.126 mM. The reaction mixture was set at 25°C for ten minutes and a small amount of 2-mercaptoethanol (final concentration 40 mM) was added to stop the reaction. The cross-linked actin was collected by centrifugation 100,000 x g for 3 hours at 4°C and immediately dissolved in 5 mM Tris-Cl, 0.2 mM ATP, 0.1 mM CaCl₂, 0.01% sodium azide pH 7.0 (depolymerization buffer). The suspension was dialyzed against the depolymerization buffer for 3 days; the dialyzing buffer was changed twice within this period. The unpolymerized actin was spun down by centrifugation at 100,000 x g for 90 minutes at 4°C. The supernatant (2 mg/ml) was applied to a Sephacryl S-300 column (1.8 x 90 cm) which was equilibrated with depolymerization buffer. The
trimer containing fractions were identified by sodium dodecylsulfate gel electrophoresis and pooled, and the protein was concentrated to ca. 1 mg/ml. After the MgCl₂ concentration was brought to 1 mM, the solution was set at 25°C for 5 minutes. The pellet containing the actin trimer was collected by centrifugation at 100,000 x g for 2 hours and dissolved in depolymerization buffer. The suspended solution was treated as previously described and the gel filtration procedure repeated. The actin trimer was pooled on the basis of sodium dodecylsulfate gel electrophoresis. The final profile of gel filtration chromatography is shown in Figure 3. The yield is about 3%. The trimer containing solution was saved in the atmosphere of thymol at 4°C and used within two weeks.

Preparation of Smooth Muscle α-actinin

The preparation of turkey gizzard α-actinin was based on the method of Craig et al. (1982). Turkey gizzards that had been trimmed were stored in 100% glycerol at -20°C for 2 weeks. Before use, the gizzard was washed in distilled water to remove the glycerol, then passed through an electric meat grinder. About 200 grams of ground tissue was collected. The ground tissue was suspended in two volumes (wt./v) of the extraction buffer (1 mM NaHCO₃ and 0.5 ml/l of 10% benzamidine). The slurry was placed into several large dialysis tubes and dialyzed against the same extraction buffer for 2 days. The dialysis buffer was changed at least twice over this period. The slurry was centrifuged at 2,000 x g, 20 min. in a Beckman model J-68 centrifuge and the supernatant was saved. The sample was brought to
Figure 3. Preparation of actin trimer. The final profile of actin trimer purification on a Sephacryl S-300 column is shown. The actin trimer containing solution was applied on a 1.8 x 80 cm Sephacryl S-300 column which was washed with 5 mM Tris-Cl, pH 8.0 buffer containing 0.1 mM CaCl₂, 0.2 mM ATP and 1% sodium azide. The protein was eluted with column washing buffer at the flow rate 16 ml/hr. Peak I contains actin oligomer and trace amounts of actin trimer; Peak II contains actin trimer; Peak III contains actin monomer.
Figure 3.
17% ammonium sulphate (94.5 g/l) with stirring and maintained at pH 7.0. After all the salt was dissolved, the sample was kept in an ice bath for another hour. The solution was clarified by centrifugation and brought to 30% ammonium sulphate (81.5 g/l). The pellet was collected, dissolved in 10 mM Tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 2 mM MgCl$_2$ and 0.5 ml 10% benzamidine per liter pH 7.4 at 24°C (buffer A) and dialyzed against it overnight. The sample was clarified by centrifugation and applied on a DE-52 column (4.0 x 8.0 cm) equilibrated with buffer A. The column was washed with buffer A and then by a salt gradient ranging from 0.1 N to 0.3 N NaCl (Fig. 4). The α-actinin containing peak was pooled, based on the sodium dodecylsulphate gel electrophoresis. The sample was then concentrated and passed over a large Sephacryl S-300 column (5.0 x 90 cm) which had been washed with 10 mM Tris-Cl, 0.6 N NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 2 mM MgCl$_2$, 0.02% NaN$_3$, pH 7.4 at 24°C. The result is shown in Figure 5. The pure alpha-actinin was identified by sodium dodecylsulphate gel electrophoresis, and its distinctive properties of crosslinking and gelling actin filaments. After concentration by Amicon ultrafiltration and dialysis against 50% glycerol, 10 mM Tris-Cl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 2 mM MgCl$_2$, 0.02% sodium azide, pH 7.4, the α-actinin solution was saved -80°C.

Preparation of Smooth Muscle Filamin

The turkey gizzard filamin was purified according to the method of Davies (1982) with several modifications by Dr. Dean A. Malencik. The pellet of 40% ammonium sulphate precipitation of turkey gizzard myosin
Figure 4. DE-52 column profile for purification of turkey gizzard α-actinin. The proteins were eluted with 10 mM Tris-Cl, 1 mM EDTA, 2 mM MgCl₂, 5 mM 2-mercaptoethanol and 0.5 ml benzamidine/1, pH 7.4 buffer with NaCl gradient, concentration ranging from 0 to 0.3 M. Peak I contains a protein with a molecular weight of 130000 daltons; peaks II and III contain the major protein-filamin; Peak IV contains α-actinin and trace amount of filamin.
Figure 4.
Figure 5. Purification of turkey gizzard α-actinin: profile of a Sephacryl S-300 column. The α-actinin containing solution eluted out from a DE-52 column was passed over a Sephacryl S-300 column (5 x 90 cm) which was washed with 10 mM Tris-Cl, pH 7.4 buffer containing 5 mM 2-mercaptoethanol, 1 mM EDTA, 2 mM MgCl$_2$ and 0.02% sodium azide and 0.6 N NaCl. The protein was eluted out with the same buffer at flow rate, 30 ml/hr. Peak I contains α-actinin and filamin: peak II contains α-actinin.
Figure 5.
light chain kinase preparation (Adelstein and Klee, 1981) was dissolved in 20 mM Tris-Cl, 5 mM EDTA, 15 mM 2-mercaptoethanol, 0.5 N NaCl pH 7.5 at 4°C and dialyzed against this solution in preparation for passage over a Sephacryl S-300 column (5 x 90 cm) which had been previously equilibrated. The filamin containing fractions were pooled on the basis of sodium dodecylsulphate gel electrophoresis. A conductivity meter was used to adjust the NaCl concentration to 150 mM by the slow addition of cold distilled water. The DEAE-cellulose (Whatman DE-52) was slowly added to the sample and continuously stirred for twenty minutes. The DEAE-cellulose was then removed by centrifugation. The DEAE-cellulose free supernatant was saved for the next step. Most of the contaminating actin was removed in this step. The sample was dialyzed against 20 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.5, at 4°C and applied to a DEAE-cellulose (Whatman DE-52) column which was equilibrated with the same buffer. The filamin was eluted by application of a linear gradient of NaCl concentration ranging from 50 mM to 500 mM in a 20 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, and 15 mM 2-mercaptoethanol. The filamin containing fractions were identified by sodium dodecylsulphate gel electrophoresis and were pooled. After this step the sample showed only a small amount (less than 10%) of actin contamination.

The filamin containing sample was then passed over a Sephacryl S-300 (5 x 90 cm) column. The protein was eluted with 50 mM potassium phosphate, pH 7.5 buffer with 1 mM EDTA, 15 mM 2-mercaptoethanol and 100 mM NaCl at a flow rate of 30 ml/hr (Fig. 6). The purity of the individual fractions were identified by sodium dodecylsulphate gel
Figure 6. Purification of turkey gizzard filamin: Profile of a Sephacryl S-300 column. A 5 x 90 cm Sephacryl S-300 column was used. The filamin was eluted with 50 mM potassium phosphate, pH 7.5 buffer containing 1 mM EDTA, 15 mM 2-mercaptoethanol and 100 mM NaCl with a flow rate of 30 ml/hr. Peak I contains filamin.
Figure 6.
electrophoresis and its actin cross-linking characteristics. The pure filamin containing fractions were pooled and dialyzed against 20 mM potassium phosphate pH 7.5, 15 mM 2-mercaptoethanol and 50% glycerol and saved at -80°C.

**Phosphorylase a Preparation**

Rabbit skeletal muscle phosphorylase b was purified according to the method of Krebs et al. (1964). The purified phosphorylase b was dialyzed against 50 mM glycerophosphate, 15 mM 2-mercaptoethanol, pH 7.0. The solution was clarified by centrifugation and protein concentration was checked by measuring the absorbance at 280 nm (A₀.₁% = 1.3). The sample was then mixed with a small amount of active charcoal (Norite) and re-clarified by centrifugation. The pH of the Norite treated supernatant was checked and adjusted to 8.2 with the slow addition of 2 M Tris. For the conversion of the phosphorylase b to a, 1/100 volume of 0.1 M ATP, 1/100 volume of 1.0 M Mg(OAc)₂ and 1/400 volume of phosphorylase kinase (depending on the activity) were added. The conversion was carried out at 30°C for 40 min. The reaction was stopped by adjusting the pH to 7.0 with 1 M acetic acid. Then, the mixture was set in ice for crystallization. The pellet was collected by centrifugation at 15,000 rpm for 10 min. at 0°C. The pellet was suspended in 50 mM glycerolphosphate, 15 mM 2-mercaptoethanol, pH 7.0 buffer and a 5 M NaCl solution was slowly added until the pellet was totally solubilized. The final concentration of NaCl was about 0.45 M. The sample was then treated with Norite to remove unreacted ATP. After the Norite treatment, the
sample was crystallized by dialysis against cold 50 mM glycerophosphate, 15 mM 2-mercaptoethanol, pH 7.0 buffer with two changes of buffer. The recrystallized phosphorylase a was stored in a -20° freezer.

Protein Bound Phosphate Determination

Protein bound phosphate of phosphofructokinase was determined according to the method of Hasegawa et al. (1982). Purified phosphorylase a (Fisher & Krebs, 1966) and/or phosphoserine and/or phosphorylated myosin light chain (prepared by Dr. Anderson) were used as standards. F-actin was used as a control to show that non-covalently bound nucleotides were removed in the washing procedure. Depending on how much protein may be on hand, approximately 1.0 mg to 0.5 mg of total protein was used each time to do the determination. Phosphofructokinase was dialyzed against 0.05 M Tris-phosphate, pH 8.0 buffer containing 5 mM pyrophosphate, 1 mM 2-mercaptoethanol, 0.2 mM EDTA to remove the glycerol, if there was any. Usually, the enzyme which was in 0.15 M to 0.18 M Tris-phosphate pH 8.0 buffer containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM pyrophosphate (eluate of DE-51, DE-52 and DE-53 connected columns) was immediately precipitated by 10% trichloroacetic acid and saved, refrigerated for phosphate determination. The samples were set in an ice bath for at least one hour, if the phosphate determination was performed immediately after the protein was precipitated by 10% trichloroacetic acid. The protein was collected by centrifugation and the supernatant was carefully removed. The pellet was washed twice
with 2.5 ml of distilled water and dissolved in 15% formic acid (370 μl of distilled water and 80 μl of concentrated formic acid) in a boiling water bath for 20 minutes. The samples were cooled and reprecipitated by 10% trichloroacetic acid and set in an ice bath for at least one hour. Then, the sample was washed again as previously mentioned. The precipitation and washing procedure was repeated another time before doing the phosphate determination. The protein concentration was determined by Bradford reagent. Typically, 90% of the protein was recovered after the washing procedures. Phosphorylase a, myosin light chain, bovine serum albumin and F-actin were washed following the same procedure.

Samples were divided into suitable aliquots and placed in individual 10 x 75 mm culture tubes. Phosphoserine standards, dissolved in distilled water, were treated in a similar manner. The sample volume of each tube was adjusted to less than 300 μl. Samples were dried by heating in an aluminum block by increasing the temperature slowly to 120°C. After drying, the tubes were cooled to room temperature. (The subsequent steps were performed in a well-ventilated fume hood.) Fifteen microliters of 50% sulfuric acid and 15 μl of 50% perchloric acid were added to each sample. After thorough mixing, the tubes were placed in the aluminum block, covered by an aluminum foil wrapped rack and the temperature was raised to 140°C (setting 6 on the heat block until the temperature was within 10°C and then turned to setting of 1), maintained at that temperature for 10 min., and then the temperature was increased to 190°C (same setting as above) and held there for 20 min. The samples were cooled to room temperature and 80 μl of distilled water was added to each
tube.

To determine the protein bound phosphate, 25 μl of freshly prepared ascorbic acid solution (10%) was added to each sample. The solution was mixed well, and then 150 μl of ammonium molybdate solution (0.42%) was added and the solution was mixed again. The samples were incubated at 65°C for 20 min, cooled on ice, and the absorbance at 820 nm was measured. Figure 7 illustrates the standard curves.

The unknown samples were treated by the same way and protein bound phosphate is extrapolated from the standard curves.

**Sodium Dodecylsulphate Gel Electrophoresis**

Sodium dodecyl sulphate slab gels were prepared and run according to Pollard (1982). For the preparation of two 0.8 mm x 5.6 cm x 8.7 cm slab gels with 10% acrylamide, 3.3 ml of 3 M Tris-HCl (pH 8.8), 3.8 ml of resolving gel acrylamide (28 g of acrylamide and 1.09 g of diallyltartardiamide with water to 100 ml), 0.13 ml of 10% SDS, and 2.74 ml of distilled water were mixed. Then 0.33 ml of ammonium persulfate (0.016 g in 1 ml of water) and 3 μl of TEMED were added and the mixture was gently poured between the plates. After overlaying with 0.1% SDS and the acrylamide polymerized for one hour at room temperature. After the gel is polymerized, the stacking gel was prepared by mixing 2 ml of 1.0 M Tris-HCl, pH 6.8 buffer, 1.6 ml of stacking gel acrylamide (21.5 g of acrylamide and 3.75 g of diallytartardiamide with water to 100 ml.), 80 μl of SDS, 4.26 ml of distilled water, 0.4 ml of ammonium persulfate and 2 μl of TEMED.
Figure 7. (a) Standard curve of protein bound phosphate determination. Phosphoserine, ○ − ○; phosphorylase a, □ − □; phosphorylase b, ■ − ■; in vitro phosphorylated myosin light chain, △ − △; myosin light chain, ▲ − ▲ and bovine serine albumin, ◊ − ◊, were chosen as standard.

(b) Standard curve of inorganic phosphate.
Figure 7.
After pouring the stacking gel, the sample well comb was inserted and then left to polymerize for 30 min. The protein samples were dissolved in 2 x concentrated sample buffer (10 ml of 20% SDS, 5 ml glycerol, 10 ml 1 M Tris-Cl, pH 6.8, 5 mg bromophenolblue, 19 ml water and 0.1 mM EDTA) and boiled for 10 sec. The samples were applied in the sample wells underneath the electrode buffer (0.05 M Tris, 0.38 M glycine and 0.1% SDS, pH 8.3 or 60 mM boric acid, 50 mM Tris, 1 mM EDTA and 0.1% SDS, pH 8.4) and run at 200 V at 4°C. The tracking dye will reach the bottom of the gel in about 1.5 hr. Gels were stained in a dye solution containing 0.15% coomassie blue G250 or R250, 45% methanol and 9.2% glacial acetic acid for 30 min. The gel was then destained in 20% methanol and 10% acetic acid. Myosin heavy chain, phosphorylase, bovine serine albumin, actin, carbonic anhydrase, troponin c and parvalbumin were chosen as molecular standards and were prepared in this laboratory.

Protein Concentration Determination

The protein concentration was determined using the dye binding method (Bradford, 1976) with bovine serum albumin as a standard. The coomassie blue dye reagent was made from 100 mg coomassie blue G-250, 48 ml concentrated phosphoric acid, 50 ml 90% ethanol and 902 ml of distilled water. Aliquots (5 to 100 µl) of stock bovine serum albumin solution (1 mg/ml) were placed in individual test tubes. Different volumes (0 to 95 µl) of distilled water were added in each tube to make 100 µl of total sample volume. Five milliliters of dye reagent was added to each tube and mixed well, then the absorbance of each
sample at 595 nm was measured. The unknown sample was measured in the same manner. Protein concentration can be extrapolated from the standard curve or calculated from the following equation.

$$\text{Protein (µg/ml)} = \frac{(A_{595\text{nm sample}} - A_{595\text{nm blank}})(\text{vol. sample ml})}{0.06 \times (\text{vol. unknown solution ml})}$$

The protein concentration can be also calculated from the absorbance at different wavelength, if the extinction coefficient at that particular wavelength was known. For phosphofructokinase, the absorbance value of $A_{280\text{ nm}}$ is 1.07 for 0.1% enzyme solution (Hesterberg and Lee, 1980); for actin $A_{290}^{0.1\%} = 0.63$ (Leher and Kerwar, 1972); for filamin $A_{280}^{0.1\%} = 0.74$ (Davies et al., 1982); for phosphorylase $A_{280}^{0.1\%} = 1.3$.

**Gel Filtration Experiment**

The gel filtration experiment was performed on a Sephacryl S-200 column (1.8 x 90 cm) which was equilibrated with 100 mM Tris-phosphate pH 8.0 or pH 7.0 buffers containing 0.2 mM EDTA and 1 mM 2-mercaptoethanol. Phosphofructokinase, G-actin, actin core, troponin C and bovine serum albumin were used as standards. Phosphofructokinase which was saved in 0.1 M potassium phosphate, pH 8.0 buffer containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 50% glycerol was dialyzed against equilibration buffer to remove the potassium ions. G-actin and actin core which were in 2 mM Tris-Cl, pH 8.0 buffer containing 0.2 mM calcium acetate, 0.2 mM ATP and 1 mM 2-mercaptoethanol were used directly. A molar ratio of 1:1 (protomer:
phosphofructokinase-84,000 daltons; actin-42,000 daltons; actin core-33,500 daltons) of phosphofructokinase (2.3 mg/ml) and G-actin (1.15 mg/ml) or phosphofructokinase (2.3 mg/ml) and actin core (0.92 mg/ml) with total sample volume of 10 ml were incubated at 25°C for 10 minutes or at 4°C for 1 hr. then quickly applied to the S-200 column. The protein samples were eluted out by washing with the previous buffer at a flow rate 16 ml/hr and 2 ml per tube was collected. Twelve per cent sodium dodecylsulphate polyacrylamide gels were used to identify protein in each fraction.

**Assay of Phosphofructokinase**

The phosphofructokinase was assayed spectrophotometrically by coupling fructose 1,6-bisphosphate formation to the oxidation of NADH through the use of the coupling enzymes aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase. One unit of enzyme activity is defined as the amount of enzyme which catalyzed the formation of 1 umole of fructose 1,6-bisphosphate per minute. It equals half the amount of NADH concentration decrease. The concentration of NADH oxidized was calculated using the extinction coefficient of 6200 1/mol. cm for NADH.

The optimal activity of phosphofructokinase is generated in 50 mM glycyglycine, pH 8.2 containing 1 mM EDTA, 2.5 mM dithiothreital, 5 mM ammonium sulfate, 0.16 mM NADH, 5 mM MgCl₂, 1 mM ATP, 1 mM fructose 6-phosphate, 0.1 mg/ml bovine serum albumin, aldolase (0.4 units), triosephosphate isomerase (2.0 units) and α-glycerolphosphate dehydrogenase (0.4 units) in a total volume of 1 ml (Racker, 1947).
The reaction was initiated by the addition of 5 μl phosphofructokinase (0.1 mg/ml) at room temperature and the absorbance at 340 nm of the reaction mixture was measured continuously with a Varian 635D recording spectrophotometer or LKB spectrophotometer interfaced with an Apple IIe microcomputer.

For studying the allosteric properties of phosphofructokinase, a pH 7.0 buffer was chosen because it enhanced these effects. This assay mixture contained 50 mM MOPS/KOH, pH 7.0, 2.5 mM dithiothreitol, 0.5 mM ammonium sulphate, 3.0 mM MgCl₂, 1 mM ATP, 0.16 mM NADH, variable amount of fructose 6-phosphate, 0.4 units aldolase, 0.4 units-glycerolphosphate dehydrogenase, 2.4 units triosephosphate isomerase and 0.5 μg phosphofructokinase in 1 ml total volume. After addition of the indicated fructose 6-phosphate and all other components except ATP, the reaction mixture which has ± fructose 2,6-bisphosphate, ± F-actin, ± G-actin, ± α-actinin, ± filamin, ± actin trimer and ± actin core was incubated at room temperature for one minute and the reaction was initiated with ATP.

Phosphofructokinase, α-actinin, filamin and F-actin were dialyzed against the assay buffer, 50 mM MOPS/KOH pH 7.0 containing 2.5 mM dithiothreitol, 3.0 mM MgCl₂ and 0.5 mM ammonium sulphate, several hours before use. G-actin and actin trimer in depolymerization buffer were passed through a 0.45 μm sterile millipore filter before use. The stock fructose 2,6-bisphosphate was made in 1 mM NaOH solution and diluted to 0.2 mM with assay buffer before use. Before addition to the assay mixture, the coupling enzymes were passed over a small Bio-Rad P-2 column to remove ammonium sulfate.
Deoxyribonuclease I Activity Assay

Bovine pancreatic deoxyribonuclease I was assayed spectrophotometrically at 260 nm according to the method of Hitchcock et al. (1977). The phosphofructokinase, saved in 0.1 M potassium phosphate buffer, pH 8.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol was dialyzed against 10 mM Tris-Cl pH 7.5 containing 0.1 mM Ca\(^{+2}\), 1 mM Mg\(^{+2}\) several hour before the assay was performed. This step is necessary since even trace amounts of potassium (4.5 x 10\(^{-7}\) M) inhibit DNAase I activity by 40%. Calf thymus DNA was cut into fine pieces with a scissors and dissolved in 10 mM Tris-Cl, pH 7.5 buffer with 1 mM Mg\(^{+2}\), 0.1 mM Ca\(^{+2}\). The final concentration was adjusted to 1 mg/ml (OD\(_{260}\) = 18/cm). G-actin and actin core which were saved in 2 mM Tris-Cl, pH 8.0 buffer containing 0.2 mM Ca\(^{++}\), 0.2 mM ATP and 1.0 mM 2-mercaptoethanol were diluted with assay buffer to 0.1 mg/ml and used directly. The reaction mixture contained 10 mM Tris-Cl, pH 7.5, 1 mM Mg\(^{++}\), 0.1 mM Ca\(^{++}\), 30 µl/ml DNA, 6.45 nM DNase I, 5 nM G-actin or 5 nM actin core and variable amount of phosphofructokinase. The reaction was initiated by the addition of DNA solution. The absorbance at 260 nm of the reaction mixture was measured continuously with Varian 635D recording spectrophotometer.

Phosphorylation of Phosphofructokinase

Cyclic AMP-dependent protein kinase, phosphorylase kinase and myosin light chain kinase from rabbit skeletal muscle or turkey gizzard were used to phosphorylate phosphofructokinase *in vitro*. 
Phosphorylation reactions, employing the catalytic subunit of the cAMP-dependent protein kinase were performed in 50 mM MOPS/KOH pH 7.0 buffer containing 0.5 mM EGTA, 5 mM MgCl₂, 0.5 mM ATP, 2.5 mM dithiothreitol and 1 mg/ml of phosphofructokinase which was dialyzed against MOPS/KOH pH 7.0 (containing EGTA, MgCl₂ and dithiothreitol) before use. The reaction was initiated by the addition of 5 µg/ml catalytic subunit of cAMP-dependent protein kinase and the reaction mixture was incubated at 30°C in a heat block. An aliquot of the phosphorylated phosphofructokinase was precipitated by 10% trichloroacetic acid and immediately saved in freezer for direct measurement of phosphate content. The remaining enzyme was saved for doing the coupling assay with other effectors including fructose 2,6-bisphosphate and F-actin.

Time course experiments, employing the catalytic subunit of the cAMP-dependent protein kinase from bovine heart and the cGMP-dependent protein kinase from porcine, were performed in 50 mM MOPS/KOH, pH 7.0 buffer containing 0.5 mM EGTA, 3 mM MgCl₂, 2.5 mM DTT and 0.1 mM γ-32P ATP (specific activity 0.03 Ci/m mole). The reactions were initiated by the addition of protein kinase. Seventy microliter samples were taken out at each time interval and put into a 0.5 ml microcentrifuge tube containing 7 µl 0.15% sodium deoxycholate. Then, 55 µl of aliquot was taken out immediately and spotted on a 2.5 cm diameter glass filter paper. The filter paper was then mounted on a small stainless steel cage which was set in a 1 liter beaker containing chilled 10% trichloroacetic acid and 2% sodium pyrophosphate with stirring. The filter papers were taken out after 15 minutes and put on a Buchner funnel with a 5 mm filter paper lying at the bottom.
connected with a vacuum suction and washed with 5 ml 15% trichloroacetic acid per disc followed by 95% ethanol and ethyl ether. The glass filter papers disc were then air dried and put into scintillation vials with 3 ml scintillation fluid containing 0.3% permablend III, 23.75% Triton X-100 and toluene. The incorporated phosphate was measured by Beckman LS 6800 scintillation counter.

Phosphorylation reactions employing turkey gizzard or rabbit skeletal muscle myosin light chain kinase were performed in 25 mM Tris-Cl, pH 7.5 buffer containing 4 mM MgCl₂, 0.1 mM CaCl₂, 60 mM KCl, 10 mM calmodulin, 0.2 mM ATP and 0.5 mg phosphofructokinase. The reaction was initiated by the addition of 16.6 µg myosin light chain kinase then the mixture was incubated at 30°C for 60 minutes. The reaction were stopped by the addition of 10% trichloroacetic acid. Aliquots were taken out for phosphate determination.

For the reaction utilizing phosphorylase kinase as the protein kinase, the phosphorylation of phosphofructokinase was performed in 25 mM Tris-25 mM glycerophosphate/HCl pH 8.2 buffer containing 70 µM calcium, 10 mM magnesium acetate, 0.2 mM ATP and 1.0 mg phosphofructokinase in a total volume of 1 ml. The reaction was started by the addition of 0.14 mg phosphorylase kinase and set at room temperature for two hours. The reaction was stopped by adding 100% trichloroacetic acid to bring the final concentration to 10% and the incorporated phosphate was measured directly. (See protein bound phosphate determination.)
Dephosphorylation of Phosphofructokinase

Dephosphorylation of phosphofructokinase was done utilizing bovine intestinal mucosa alkaline phosphatase in 50 mM Tris-sulphate buffer pH 8.0 containing 50 mM 2-mercaptoethanol and 1 mg phosphofructokinase with total volume of 1 ml at 30°C. The reaction was initiated by the addition of 0.16 μg phosphatase. Aliquots were taken out for phosphate determination and for the phosphofructokinase assay at each time interval. The maximal reaction time was two hours.

The calcineurin dephosphorylation reaction was in 50 mM Tris-Cl, pH 8.0 containing 0.5 mM dithiothreitol, 4 μM CaCl₂, 0.03 μM calmodulin and 1 mg phosphofructokinase with total volume 1 ml at 25°C for 2 hr. The reaction was initiated by the addition of 1 μg calcineurin (0.1%) and stopped by the addition of 100% trichloroacetic acid to a final concentration of 10%. Phosphate content of phosphofructokinase was measured directly.
RESULTS

Problems in the Purification of Phosphofructokinase

The initial preparations of rabbit skeletal muscle phosphofructokinase in this laboratory followed the procedure of Ling et al. (1966). Assay of the fractions obtained from diethylaminoethyl-cellulose (DE-52) chromatography revealed that only 70% of the enzyme is bound by the column and that the other 30% typically appears in the breakthrough (unbound) fractions. Ionic strength gradient elution of the bound enzyme yields a pool of electrophoretically homogeneous phosphofructokinase. The unbound fractions contain a prominent protein of apparent molecular weight 42,000 daltons, present in approximate equimolar ratio with the enzyme. No additional adsorption occurs when the pool of unbound protein is reapplied to an equilibrated DE-52 column. The contaminant has tentatively been identified as actin in this laboratory, based on the facts that its molecular weight is close to that of the actin monomer and that it inhibits the catalytic activity of DNase I (Kuo et al., 1986). DNase I forms a high affinity complex with G-actin resulting in depolymerization of F-actin and 95% inhibition of DNase I activity (Lindbergh, 1967; Lazarides & Lindbergh, 1974).

Since a major part of this project concerns the effect of actin on phosphofructokinase activity, the isolation of actin-free enzyme was given high priority. Several attempts to separate actin and phosphofructokinase chromatographically using hydroxyapatite (ion exchange), Sephacryl S-200 and S-300 (gel filtration), or Bio-Rad blue
dextran (affinity) columns were without success.

Adoption and modification of the purification procedure of Kemp (1975) allowed us to obtain actin-free phosphofructokinase. (Table I contains the outlines comparing the enzyme purification methods of Ling et al., and of Kemp with my final modification of the Kemp procedure.) The first modification I made was direct application of the dialyzed protein, obtained after the 55% ammonium sulfate precipitation, to the DE-52 column (Fig. 8). (This protein fraction contained only a trace of actin.) The phosphofructokinase which was eluted upon application 0.3 M Tris-phosphate, pH 8.0, containing 0.2 mM EDTA and 1 mM 2-mercaptoethanol was electrophoretically homogenous (Fig. 8). However, protein bound phosphate determinations on individual fractions revealed varying phosphorous contents which decreased rather quickly even during storage at -80° (Table II:A). Since the instability probably results from endogenous phosphatase activity present as a trace contaminant, 1 mM KF—a known inhibitor of phosphoprotein phosphatases (Li et al., 1978) was added to the solutions used in every step of the purification procedure. The elution buffer for the DE-52 column also was changed to a linear gradient in which the Tris-phosphate concentration ranged from 0.1 M to 0.3 M (pH 8.0). This buffer also contained the indicated fixed concentration of KF (1 mM), 2-mercaptoethanol (1 mM) and EDTA (0.2 mM) (second modification of the Kemp procedure). The resulting elution profile (Figure 33 in the last section) revealed overlapping peaks of electrophoretically homogenous phosphofructokinase eluting at Tris-phosphate concentrations 0.16 M, 0.17 M and 0.19 M, respectively. Although the protein-bound phosphate content was more stable than that
Table I. PFK Purification Procedures of Ling et al. (1966), Kemp (1972) and Kuo.

Ground Rabbit Muscle

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Ling's</th>
<th>Kemp's</th>
<th>Kuo's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenization</td>
<td>Homogenized in 30 mM KF, 1 mM EDTA</td>
<td>Homogenized in 30 mM KF, 4 mM EDTA, 1 mM DTT, pH 7.5</td>
<td>Stirred in 30 mM KF, 4 mM EDTA</td>
</tr>
<tr>
<td>Supernatant</td>
<td>pH=6.6</td>
<td>pH=6.6</td>
<td>15 mM 2-mercaptoethanol, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>Heat to 40°C, hold at that temp. and</td>
<td>Supernatant pH=6.6</td>
<td>5 mM PPi added to supernatant extract,</td>
</tr>
<tr>
<td></td>
<td>followed by isopropanol precipitation</td>
<td>Adjusted to 6.8 with 1 M Tris</td>
<td>supernatant pH=7.0</td>
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<tr>
<td></td>
<td>Pellet dissolved in 0.1 M Tris-PO4, pH 8.0</td>
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<td>Adjusted to 6.8 with 1 M Acetic acid</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE-52 cellulose</td>
<td>DE-52 cellulose chromatography</td>
<td>Heat treatment 57-59°C</td>
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</tr>
<tr>
<td>Crystallization</td>
<td>Crystallization at least 2 weeks</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Precipitation</td>
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<td>38% to 55% (NH₄)₂SO₄ 38% to 55% (NH₄)₂SO₄</td>
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<td>Crystallization</td>
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<td>PFK</td>
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Figure 8. The purification profile of rabbit skeletal muscle phosphofructokinase obtained with the first modification of the Kemp procedure.

The first arrow indicates the application of 0.1 M Tris-phosphate, pH 8.0, containing 0.2 mM EDTA and 1.0 mM 2-mercaptoethanol. The second arrow shows the application of an equivalent 0.3 M Tris-phosphate buffer, which promotes the elution of phosphofructokinase (peak II). Peak I contains one major protein having a molecular weight of 96,000. The proteins are shown in a 12% SDS polyacrylamide gel at the upper left corner. Columns 1. Protein (top to bottom) standards: myosin heavy chain (200,000), debranching enzyme (130,000), phosphorylase (96,000), bovine serum albumin (68,000), actin (45,000), carbonic anhydrase (29,000), troponin C (18,000) and parvalbumin (10,400); 2. Sample applied to the DE-52 column; 3 to 8: Different fractions of peak II of figure 8. The gel was stained with comassie blue G-250. See Materials and Methods for other details.
Figure 8.

Fraction No. (4.5 ml/tube)
Table II. Protein-bound phosphate content of rabbit skeletal muscle phosphofructokinase in different storage condition and time.

<table>
<thead>
<tr>
<th>Storage Buffer and Preparation Method</th>
<th>&quot;zero&quot; time</th>
<th>1 week</th>
<th>2 week</th>
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<th>6 months</th>
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<tr>
<td>A</td>
<td>0.23</td>
<td>0.10</td>
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<tr>
<td></td>
<td>0.41</td>
<td>--</td>
<td>0.40</td>
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<td>0.53</td>
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<td>1.22</td>
<td>0.44</td>
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<tr>
<td></td>
<td>2.60</td>
<td>0.47</td>
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</tr>
<tr>
<td>B</td>
<td>0.67</td>
<td>--</td>
<td>--</td>
<td>0.60</td>
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</tr>
<tr>
<td></td>
<td>1.04</td>
<td>--</td>
<td>--</td>
<td>0.54</td>
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<td>1.17</td>
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<td>C</td>
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<td></td>
<td>1.29</td>
<td>--</td>
<td>1.27</td>
<td>--</td>
<td>1.19</td>
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</table>

A: Enzyme was purified by the first modification of the Kemp (1972) method. Enzyme was saved in 0.2 M Tris-PO₄ pH 8.0 buffer containing 0.2 mM EDTA, 1 mM 2-mercaptoethanol and 50% glycerol. The phosphate contents shown correspond to those of individual fractions.

B: Enzyme was purified by the second modification of the Kemp (1972) method and was saved in 0.1 M potassium phosphate pH 8.0 buffer containing 0.2 mM EDTA, 1 mM 2-mercaptoethanol, 1.0 mM KF, and 50% glycerol. The phosphate contents correspond to individual enzyme fractions.

C: Enzyme was purified by the third modification of the Kemp (1972) method and was saved in 0.1 M potassium phosphate pH 8.0 buffer containing 1.0 mM EDTA, 1.0 mM 2-mercaptoethanol, 5 mM K₄P₂O₇ and 50% glycerol. The phosphate contents shown correspond to the pooled enzyme fractions of Figure 10.
found with the previous enzyme preparation (Table II:B), the phosphate
distribution in the fractions was more or less random.

In order to obtain both stability and better resolution of the
differentially phosphorylated fractions of enzyme, we added 5 mM
tetrapotassium pyrophosphate (Khandelwal, 1978; Khandelwal & Kamari,
1980)—another protein phosphatase inhibitor—to all except one of the
solutions used in the purification of the enzyme. The addition of
pyrophosphate to the tissue extraction buffer (30 mM KF, 4 mM EDTA, 15
mM 2-mercaptoethanol, pH 7.5) was avoided since it results in
increased contamination of the enzyme with myosin (Fig. 9). Starr &
Offer (1982) previously noted that myosin copurifies with
phosphofructokinase. Accordingly, pyrophosphate was added to the
supernatant extract of rabbit muscle and to the solutions used in all
subsequent fractionations. As a final modification, I used a
connected series of DE-51, DE-52, and DE-53 columns in place of the
original single DE-52 column. This procedure resulted in consistent
resolution of phosphofructokinase into fractions exhibiting varying
degrees of phosphorylation (Fig. 10, Table II:C). Figure 10 shows a
representative enzyme fractionation profile obtained with the
connected series of ion exchange columns. Table III shows the yields
and specific activities corresponding to the individual steps in the
final purification scheme. The overall yield of phosphofructokinase
is 51%. The gel in Figure 9 shows the proteins present after each
purification step. Determinations of total phosphate showed that the
most heavily phosphorylated enzyme typically occurs in fractions
89-93. The average composition for this particular pool is 1.29 mole
phosphate per enzyme protomer. Charcoal and/or gel filtration
Figure 9. SDS polyacrylamide gel electrophoresis of fractions from the phosphofructokinase purification procedures preceding ion exchange chromatography. 1: the protein standards (listed in Figure 8); 2: supernatant from the first buffer extraction; 3: supernatant from the isopropanol precipitation; 4: pellet from the isopropanol precipitation; 5: supernatant from the heat treatment procedure; 6: pellet from heat treatment procedure; 7: supernatant from 38% ammonium sulphate precipitation step; 8: pellet from 38% ammonium sulphate precipitation step; 9: pellet from 55% ammonium sulphate precipitation step; 10: protein standards; 11: sample applied to DE-51, DE-52 and DE-53 connected column (duplicate).
Figure 10. Fractionation of rabbit muscle phosphofructokinase on a connected series of DE-51, DE-52 and DE-53 columns. The enzyme was eluted with a gradient ranging from 0.1 M to 0.3 M Tris-phosphate in 5 mM sodium pyrophosphate, 0.2 mM EDTA and 1 mM 2-mercaptoethanol, pH 8.0. The fractions contained 6.5 ml each. The Roman numerals designate pools containing 0.42 mol P/84,000 g enzyme(I); 1.3 mol P/84,000 g(II); 0.18 mol P/84,000(III) and 0.24 mol P/84,000(IV). The figures above the peak show the corresponding specific activities determined under the standard assay conditions. The inset shows photos of polyacrylamide NaDodSO₄ electrophoresis gels representing the four major fractions. From left to right, the channels correspond to the standards and to fractions IV, IV, III, III, II, II, I and I.
Figure 10.
Table III. Purification of rabbit muscle phosphofructokinase according to the modified Kemp procedure.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total units</th>
<th>Protein (mg/ml)</th>
<th>Units/mg*</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>2400</td>
<td>68,112</td>
<td>12.9</td>
<td>2.2</td>
<td>--</td>
</tr>
<tr>
<td>Isopropanol Precipitation</td>
<td>180</td>
<td>55,872</td>
<td>19.4</td>
<td>16.0</td>
<td>82</td>
</tr>
<tr>
<td>Heat Treatment</td>
<td>300</td>
<td>55,080</td>
<td>2.7</td>
<td>67.0</td>
<td>81</td>
</tr>
<tr>
<td>55% Ammonium Sulfate Precipitation</td>
<td>76</td>
<td>43,502</td>
<td>3.6</td>
<td>159.0</td>
<td>64</td>
</tr>
<tr>
<td>DE-51-52-53 Chromatography</td>
<td>42</td>
<td>34,675</td>
<td>3.2</td>
<td>258.0a</td>
<td>50.9</td>
</tr>
</tbody>
</table>

* Specific activity was determined under the optimal condition at pH 8.0. (See material and method for details.)

a The average value for fractions 71 to 125.
(Bio-Rad P-2) treatments of the enzyme have no effects on its phosphate content. Most of the experiments in the following section were performed with fractions I and II from several different preparations. They are obtained by the final purification method, designated, respectively, as "low" phosphate-< 0.5 mol P/mol PFK protomer- and "high" phosphate - > 0.5 mol P/mol PFK protomer-phosphofructokinase. Independent preparations of enzyme from well fed rabbits gave "high" phosphate fractions containing 0.91 to 1.30 mol P/mol protomer and "low" phosphate fractions containing 0.04 to 0.42 mol P/mol protomer.

Association of Phosphofructokinase with Actin

The association of phosphofructokinase with actin has been observed in this laboratory on several levels. The addition of phosphofructokinase to solutions containing 0.2 mg/ml F-actin in 5 mM glycylglycine, 5 mM glycerophosphate, 5 mM KCl, 1 mM MgCl$_2$, pH 7.0 at 25° results in precipitation. An average of four moles of actin protomer bind tightly to each mole of phosphofructokinase tetramer, according to the centrifugation experiments described by Liou (1979). (Further incomplete association of phosphofructokinase continues to occur in solutions containing higher ratios of enzyme to actin.) The solubility of the F-actin-phosphofructokinase complex is enhanced by low concentrations of ADP (34 μM) and ATP (25 μM) and by high concentrations of fructose 6-phosphate (2 mM), fructose 1,6-bisphosphate (4 mM) and citrate (5.6 mM). AMP, an allosteric activator, has been tested at concentration up to 0.01 M with no
effect on the solubility of the complex. Liou and Anderson (1980) demonstrated the distinctive effects of F-actin on the catalytic activity of phosphofructokinase using a pH stat to monitor the reaction. The results obtained when excess concentration of ATP or citrate were present indicated that the interaction between the two proteins still occurs after solubilization. Although additions of NaCl or KCl also increase the solubility, appreciable precipitation of the enzyme by actin still occurs in the presence of 0.1 M salt. The enzyme to actin ratio (one phosphofructokinase tetramer per 640 actin monomer units) used in their activity measurements approximated that found in rabbit skeletal muscle.

One of the major effects of F-actin on phosphofructokinase is reflected in a decrease in its $K_m$ for the substrate, fructose 6-phosphate (Liou & Anderson, 1980). Accordingly, most of my experiments were performed at a fixed non-inhibitory level of ATP (1 mM) and varied concentrations of fructose 6-phosphate. In view of the tedious nature of the pH-stat assay used in the experiments performed by Liou and Anderson, the assay method of Racker (1947) which couples the production of fructose 1,6-bisphosphate to the reaction catalyzed by aldolase, triosephosphate isomerase and $\alpha$-glycerolphosphate dehydrogenase was adopted in the present study.

$$\text{PFK, MgCl}_2$$ $\text{Fructose 6-P + ATP} \xrightarrow{\text{Fructose 6-P + ATP}} \text{Fructose 1,6-P}_2 + \text{ADP} + H^+$

aldolase
$\text{Fructose 1,6-P}_2 \xrightarrow{\text{aldolase}} \text{Dihydroxyacetone phosphate +}$

$\text{Glyceraldehyde 3-phosphate}$
This method also was used by Karadsheh and Uyeda et al. (1977) to determine the effects of the inner erythrocyte membrane on phosphofructokinase activity.

Figure 11 shows the increases in phosphofructokinase activity measured at a non-saturating level of fructose 6-phosphate (0.1 mM) when varying proportions of F-actin are added to solutions containing 1.5 nM enzyme tetramer. Added F-actin has a larger activating effect on the "low" phosphate enzyme (0.42 mole p/mole protomer) than on the "high" phosphate enzyme (1.29 mole p/mole protomer). Even though the binding equilibrium is likely to be complex, the concentration dependence can be described simply. In the case of the "low" phosphate enzyme, apparent $K_d = 15.4 \pm 1.7$ nM actin protomer, the Hill coefficient ($n$) = 1.3, and the maximum activation = 3.3 fold. With the "high" phosphate enzyme, $K_d = 9.7 \pm 1.9$ nM actin protomer, $n = 1.3$ and the maximum activation = 2.3 fold. Since these actin concentrations are smaller than the critical level necessary for polymerization (about 0.04 mg/ml) (Rouayrenc and Travers, 1981), a certain amount of dissociation of F-actin to G-actin may occur in this experiment. Rapid dissociation of actin filaments is known to take place on dilution (Walsh et al., 1982). However, the distribution of
Figure 11. Activation of rabbit muscle phosphofructokinase as a function of the concentration of added F-actin. Results are shown for two phosphofructokinase fractions containing 0.42 mole phosphate/mole enzyme protomer (○) and 1.29 mole phosphate/mole enzyme protomer (□). The assay mixture contains 0.5 μg/ml phosphofructokinase, varying amount of added F-actin (given in terms of protomer concentration), 50 mM MOPS/KOH, 0.1 mM fructose 6-phosphate, 1.0 mM ATP, 3.0 mM MgCl₂, 0.5 mM (NH₄)SO₄ and 2.5 mM dithiothreitol, pH 7.0 at 25.
Figure 11.
small oligomers and monomers in the disassembled state remains undetermined (Maruyama & Tsukagashi, 1984). Differences in the effects of F- and G-actin are considered in the next section. The values for $K_d$ obtained here correspond to upper limits for the true values. Association of actin with the aldolase used in the coupled reaction and partial depolymerization to G-actin are both likely to result in overestimation of the dissociation constant. Later experiments were performed at an excess actin concentration, 0.12 $\mu$M or 70 mol actin/mol enzyme, giving nearly maximal enhancement of activity.

There is no sign of the precipitation which takes place when higher concentrations of the enzyme and F-actin are mixed. The apparent avidity of enzyme for actin suggests that highly efficient binding of the enzyme could take place under physiological conditions.

**Factors Influencing the Activation of Phosphofructokinase by Actin**

The activity of phosphofructokinase is affected by a number of metabolites and other effectors (cf reviews Hers & Hue, 1983). In addition the polymerization of actin is influenced both by solution conditions and by several proteins including G-actin stabilizing proteins, end-blocking proteins, cross-linking proteins and side-binding proteins (cf. reviews Korn, 1982; Mooseker, 1983; Payne & Rudnick, 1984; Stossel, 1985). The exploration of the role of all known effectors on the interaction of the two proteins is clearly a multi-dimensional problem. The work described here concentrates on the effects of the state of actin polymerization, on the roles of the
variously phosphorylated states of the enzyme, on the action of fructose 2,6-bisphosphate, and on the effects of two actin cross-linking proteins: α-actinin and filamin.

(a). Effects of fructose 2,6-bisphosphate

Fructose 2,6-bisphosphate is a very strong activator of phosphofructokinase from many different biological sources (cf. reviews by Pilkis et al., 1982; Hers et al., 1982). Its effects have been more completely studied in the case of the liver enzyme than in the case of skeletal muscle phosphofructokinase (cf. reviews by Furuya & Uyeda, 1980; Uyeda et al., 1982; Foe et al., 1983; Bartons et al., 1984; Claus et al., 1984). The effects of fructose 2,6-bisphosphate and fructose 1,6-bisphosphate, which is both a product and an activator of the enzyme, on the activity of a rabbit muscle phosphofructokinase sample purified according to my first modification of the Kemp method (phosphate content = 0.96 mol P/protomer) are shown in Figure 12. Fructose 2,6-bisphosphate is more effective at low concentrations than fructose 1,6-bisphosphate. Fifty percent activation occurs at a concentration of 0.277 μM with the former and at 2.17 μM with the latter. At concentration above 3 μM, which corresponds to the concentration of fructose 2,6-bisphosphate (2.6 nmol/g) (Kuwajima & Uyeda, 1982) and 1/22nd of the concentration of fructose 1,6-bisphosphate (65 nmol/g) in skeletal muscle (Veech et al., 1969), both fructose bisphosphates have similar effects. In the following experiments, the concentration of fructose 2,6-bisphosphate was fixed at 3 μM. Note that since fructose 1,6-bisphosphate is the
substrate of aldolase, one of the coupling enzymes in the reaction mixture, the effect of fructose 1,6-bisphosphate may be underestimated at low concentrations and overestimated at high concentrations. The aldolase-catalyzed reaction removes fructose 1,6-bisphosphate—thus necessitating the addition of extra fructose 1,6-bisphosphate in order to activate phosphofructokinase. But as the concentration of added fructose 1,6-bisphosphate is increased, it also makes a larger direct concentration to the reaction rate. These two effects would result in the apparent cooperativity (Fig. 12). The rather large apparent Hill coefficient (3.22) for fructose 1,6-bisphosphate suggests the influence of these two factors on the observed reaction rates. The influence of a fructose bisphosphate analogue—fructose 1,2-cyclic 6-bisphosphate, the intermediate in the chemical synthesis of fructose 2,6-bisphosphate (Pilkis et al., 1982), on phosphofructokinase was also tested. No effects on activity were found at fructose 1,2-cyclic 6-bisphosphate concentrations up to 10 mM. Possible interaction of fructose 2,6-bisphosphate with aldolase was investigated in a control experiment. No effects of the phosphofructokinase activator on aldolase activity were detected at concentration up to 50 μM.

Figure 13 shows the results obtained with the "low" (0.42 mol phosphate/mol of PFK protomer, fraction I) and "high" (1.29 mol phosphate/mol of PFK protomer, fraction II) phosphate phosphofructokinase samples when either F-actin or G-actin was added to the assay in conjunction with fructose 2,6-bisphosphate. The activity measurements were obtained as a function of the fructose 6-phosphate concentration, ranging from 0 to 1.0 mM, using a
Figure 12. Influence of fructose bisphosphates on the catalytic activity of phosphofructokinase. Enzyme was prepared according to the first modification of the Kemp procedure. The relative catalytic activity of phosphofructokinase is recorded as a function of varied concentrations of fructose 2,6-bisphosphate (●) or of fructose 1,6-bisphosphate (○). The reaction mixture contained enzyme (0.5 μg/ml), ATP (1 mM), fructose 6-phosphate (1 mM). Condition: 50 mM MOPS/KOH, 3 mM MgCl₂, 2.5 mM DTT, and 0.5 mM (NH₄)₂SO₄, pH 7.0.
The non-inhibitory concentration of ATP (1 mM) which gives nearly optimal reaction rates. The two fractions show pronounced cooperativity with respect to the substrate in the absence of added effectors. They have similar Hill coefficients (n = 3.0) and Kₘ's for fructose 6-phosphate (Table IV). The presence of 0.12 μM F-actin stimulates the activity of both forms of enzyme: the apparent Kₘ's and Hill coefficients for fructose 6-phosphate decrease approximately 60% and 40%, respectively, while Vₘₐₓ increases by ca. 15% for both enzyme fractions (Table IV). G-actin (0.12 μM), in contrast, has very little effect on either.

The largest difference between the "low" and "high" phosphate enzymes appears when F-actin and fructose 2,6-bisphosphate are studied in combination. Figure 13 demonstrates the marked increase in Vₘₐₓ obtained with the "low" phosphate enzyme and F-actin plus fructose 2,6-bisphosphate but not with the "high" phosphate enzyme.

Observations on five different enzyme preparation have confirmed this substantial difference in the properties of "low" and "high" phosphate phosphofructokinase. The synergistic activation of the "low" phosphate enzyme obtained with F-actin and fructose 2,6-bisphosphate apparently involves polymerized species of actin since solutions containing G-actin behave quite differently. In fact, the activity of either enzyme in the presence of G-actin and fructose 2,6-bisphosphate is greater than that obtained with G-actin but significantly less than determined with the fructose 2,6-bisphosphate alone (Fig. 13).

Fraction III, containing 0.18 mol phosphate/mol PFK protomer, was characterized in less detail (Fig. 10). It is generally similar to fractions I and II. However, its sensitivity to the combined effects
Figure 13. Influence of F-actin, G-actin and fructose 2,6-bisphosphate on the catalytic activity of the two major fractions of rabbit muscle phosphofructokinase. Reaction rates were determined using varied concentrations of fructose 6-phosphate, and fixed concentrations of ATP (1 mM) and enzyme (0.5 μg/ml). The effects of F-actin, 0.12 μM (□); G-actin, 0.12 μM (△); fructose 2,6-bisphosphate, 3.0 μM (●); F-actin plus fructose 2,6-bisphosphate, 0.12 μM plus 3 μM (■); G-actin plus fructose 2,6-bisphosphate, 0.12 μM plus 3 μM (▲), and of no additions (○), are shown. Panel A contains observations on the low phosphate fraction (0.42 mole phosphate/PFK protomer; fraction I) and panel B, on the high phosphate fraction (1.29 mole phosphate/PFK protomer; fraction II). Conditions: 50 mM MOPS/KOH, 3 mM MgCl₂, 2.5 mM dithiothreitol, 0.5 mM (NH₄)₂SO₄, pH 7.0.
Figure 13.
Table IV. Summary of Catalytic Properties of Fractions I and II Phosphofructokinase when Fructose 6-Phosphate is the Variable Substrate.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$K_m$ app (mM) I</th>
<th>$V_{max}$ (units/mg) I</th>
<th>$n_H$ I</th>
<th>$K_m$ app (mM) II</th>
<th>$V_{max}$ (units/mg) II</th>
<th>$n_H$ II</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.26</td>
<td>23.8</td>
<td>3.0</td>
<td>0.27</td>
<td>21.5</td>
<td>3.0</td>
</tr>
<tr>
<td>F2,6-P2</td>
<td>0.081</td>
<td>29.6</td>
<td>1.7</td>
<td>0.105</td>
<td>24.9</td>
<td>1.6</td>
</tr>
<tr>
<td>F-actin</td>
<td>0.15</td>
<td>27.7</td>
<td>2.0</td>
<td>0.22</td>
<td>24.6</td>
<td>2.5</td>
</tr>
<tr>
<td>F-actin + F2,6-P2</td>
<td>0.086</td>
<td>53.8</td>
<td>1.4</td>
<td>0.093</td>
<td>25.1</td>
<td>1.6</td>
</tr>
<tr>
<td>G-actin</td>
<td>0.25</td>
<td>24.9</td>
<td>2.1</td>
<td>0.26</td>
<td>23.1</td>
<td>1.8</td>
</tr>
<tr>
<td>G-actin + F2,6-P2</td>
<td>0.13</td>
<td>27.2</td>
<td>2.2</td>
<td>0.19</td>
<td>25.8</td>
<td>1.5</td>
</tr>
<tr>
<td>actin trimer</td>
<td>0.15</td>
<td>24.0</td>
<td>3.0</td>
<td>0.13</td>
<td>21.5</td>
<td>3.1</td>
</tr>
<tr>
<td>trimer + F2,6-P2</td>
<td>0.060</td>
<td>26.3</td>
<td>1.8</td>
<td>0.096</td>
<td>25.0</td>
<td>1.5</td>
</tr>
<tr>
<td>actin core</td>
<td>0.30</td>
<td>22.5</td>
<td>3.2</td>
<td>0.32</td>
<td>21.2</td>
<td>3.3</td>
</tr>
<tr>
<td>actin core + F2,6-P2</td>
<td>0.12</td>
<td>25.4</td>
<td>1.8</td>
<td>0.078</td>
<td>28.8</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The error of individual experiments was ± 9% for $K_m$, ± 2.6% for $V_{max}$, and ± 12% for $n_H$.

Conditions: 0.50 μg/mL enzyme, 1.0 mM ATP, 3.0 mM MgCl$_2$, 0.5 mM (NH$_4$)$_2$SO$_4$, 2.5 mM dithiothreitol, and 50 mM MOPS/KOH (pH 7.0). When present, the concentrations of F-actin, G-actin, and trimer were 0.12 μM; actin core, 50 μg/mL; and fructose 2,6-bisphosphate, 3.0 μM.
of F-actin and fructose 2,6-bisphosphate is not as pronounced as that of fraction I. The value of $V_{\text{max}}$ is increased by 70% in the case of fraction I; 2% with fraction II; and 29% with fraction III when F-actin and fructose 2,6-bisphosphate are both present.

(b). Effects of actin modification

The actin core, which is obtained by removal of the first 67 or so amino acid residues from the N-terminus of actin by chymotrypsin digestion, retains full capacity to bind ATP but is unable to polymerize, activate myosin, or bind calcium ions (Jacobson & Rosenbushch, 1976; Mornet & Ue, 1984). Covalently cross-linked actin trimers, prepared using the reagent phenylenebismaleimide, retain a conformation very similar to that of F-actin and unlike that of G-actin (Knight & Offer, 1978; Mockrin & Korn, 1981; Lal et al., 1984).

The actin core, tested at 1.5 $\mu$M, has minimal effect on the activities of both the "low" and "high" phosphate fractions of phosphofructokinase (Fig. 14). The combination of actin core and fructose 2,6-bisphosphate gives the same results obtained with fructose 2,6-bisphosphate alone with either of the enzyme fractions. The core also fails to inhibit DNase I at concentration where G-actin (5 nM) gives 44% inhibition (Fig. 15). Under some conditions, the actin core is even slightly inhibitory to phosphofructokinase.

The purified trimer, tested at 5 $\mu$g/ml, activates both fractions of phosphofructokinase efficiently (Fig. 14). The major difference between the trimer and F-actin is obtained with the "low" phosphate
Figure 14. Effects of actin modification: the cross-linked trimer and the chymotrypsin-resistant actin core. Reaction rates were determined as a function of varied concentrations of fructose 6-phosphate and fixed concentrations of ATP (1 mM) and enzyme (0.5 µg/ml). Results are shown for trimer, 5 µg/ml (▽); fructose 2,6-bisphosphate, 3.0 µM (●); trimer + fructose 2,6-bisphosphate, 5 µg/ml + 3 µM (▼); actin core, 50 µg/ml (◇); actin core + fructose 2,6-bisphosphate, 50 µg/ml + 3 µM (◆); and no additions (○). See Figure 13 for assay condition.
Figure 14.
enzyme and fructose 2,6-bisphosphate. No synergistic effect was detected with presence the actin trimer and fructose 2,6-bisphosphate (Fig. 14). The trimer also differs from F-actin in its failure to reduce the Hill coefficients. However, the actin trimer apparently approaches a minimal unit capable of activating both myosin ATPase activity (Knight & Offer, 1978) and phosphofructokinase phosphotransferase activity. Polymerization of the trimer to F-actin under these experimental conditions is unlikely, unless the phosphofructokinase molecule can serve as a nucleus for the association.

(c). Effect of phosphofructokinase on the association of actin and DNase I

In contrast to F-actin, G-actin has minimal effects on the catalytic activity of phosphofructokinase (Fig. 13). Nonetheless, phosphofructokinase and G-actin may interact to some extent. To investigate this possibility, I determined the effect of phosphofructokinase on the association of actin with DNase I. DNase I, an endonuclease which is highly specific for DNA, forms a stable 1:1 complex with G-actin which is readily dissociated only under conditions that denature the proteins (Lindberg, 1967; Lazarides and Lindberg, 1974). If the binding of DNase I and phosphofructokinase by G-actin are mutually exclusive, the addition of excess concentrations of phosphofructokinase to the assay media should protect DNase I from inhibition by G-actin. The results of DNase I assays shown in Figure 15 strongly suggest an interaction between phosphofructokinase and
G-actin. (The phosphofructokinase used purified by my first modification of the Kemp method. The phosphate content was undetermined.) Possibly through competition, the addition of phosphofructokinase to the DNase I assay effectively blocks the inhibitory effect of G-actin. With 10 nM phosphofructokinase tetramer, the inhibition obtained with 5 nM G-actin is only 2%. In contrast, 44% inhibition occurs in the absence of phosphofructokinase. The latter value is consistent with that predicted (48%) when the dissociation constant (2 nM) (Mannherz et al., 1980) of the DNase I-actin complex and the protein concentrations are taken into account. The small inhibition (2%) obtained in the presence of phosphofructokinase suggests that the free G-actin concentration has been decreased to 0.04 nM or less. If there is a 1:1 interaction between G-actin and the phosphofructokinase protomer, the dissociation constant of the complex would have to be on the order of 0.04 nM. The actin core is unable to inhibit DNase I, either in the presence or in the absence of phosphofructokinase (Fig. 15). Phosphofructokinase alone has no effect on DNase I activity. The results with the actin core suggest that the N-terminus (amino acid residues 1 to 67) of the actin monomer is involved, directly or indirectly, in complex formation with both DNase and phosphofructokinase. Additional experimental evidence for an interaction between phosphofructokinase and monomeric actin is given in the following section.

(d). Chromatographic evidence for the association between phosphofructokinase and G-actin
Figure 15. Effect of phosphofructokinase on the inhibition of DNAase I by G-actin and the actin core. The assay solution contains 10 mM Tris-Cl, pH 7.5, 1 mM MgCl₂, 0.1 mM CaCl₂, 30 µg/ml DNA, 6.5 nM DNase I, 5 nM G-actin or actin core and varying amount of phosphofructokinase (in term of protomer). The protein mixtures were incubated at room temperature for 2 minutes before the reaction was initiated by the addition of DNA. ○—○, G-actin; ●—●, actin core.
Figure 15.
Gel filtration chromatography is a useful technique to study the molecular weights of proteins (Belew et al., 1978), to determine the equilibrium constants of slow reactions (Killander, 1964), to determine the molecular weight distribution of polymers (Ackers, 1968) and to separate cells and particles (Alonso et al., 1978). To further study the interaction between G-actin and phosphofructokinase, a Sephacryl S-200 superfine chromatography column, which has a fractionation range from 5,000 to 250,000, was used. Figure 16 shows the gel filtration profiles of protein standards—phosphofructokinase, bovine serum albumin, G-actin and actin core—with molecular weights, in term of protomer, of 84,000, 68,000, 45,000 and 33,500, respectively. The proteins elute in different fractions (phosphofructokinase, 90.2 ml at the peak; bovine serum albumin, 103.4 ml; G-actin, 107.8 ml and actin core, 112.2 ml) according to their molecular weights. There is no obvious interaction between the proteins and the Sephacryl matrix (Fig. 16). Furthermore, little polymerization is detected in the actin sample under these conditions.

Figure 17 shows the elution profile of a Sephacryl S-200 column to which 10 ml of pH 8.0 solution containing phosphofructokinase (2.32 mg/ml) and G-actin (1.6 mg/ml), corresponding to a molar ratio of phosphofructokinase to G-actin of 1:1.3, had been applied. The 12% polyacrylamide SDS electrophoresis gel shows the distribution of the proteins in the different fractions obtained (Fig. 17). The profile was very similar to the calculated profile (Fig. 18) obtained by a combination of the separate phosphofructokinase and G-actin elution profiles. The results indicate little interaction of the enzyme and G-actin under these experimental conditions. Fraction no. 41 (90.2
Figure 16. Standard profiles of protein elution on a Sephacryl S-200 gel filtration column. A: phosphofructokinase (18 mg/ml); B: bovine serum albumin (2 mg/ml); C: G-actin (2.4 mg/ml); and D: actin core (0.5 mg/ml). Column size was 1.8 x 90 cm; elution rate was 16 ml/hr. The experiments were performed at 4° in 100 mM Tris phosphate pH 8.0 buffer containing 0.2 mM EDTA and 1 mM 2-mercaptoethanol.
Figure 16.
Figure 17. Gel filtration profile of a mixture of phosphofructokinase and G-actin at pH 8.0. Phosphofructokinase (2.32 mg/ml) and G-actin (1.6 mg/ml) in a total volume of 10 ml of solution containing 100 mM Tris-phosphate buffer, pH 8.0 containing 0.2 mM EDTA and 1 mM 2-mercaptoethanol was incubated at 4 for 1 hr. and applied to a Sephacryl S-200 column (1.8 x 90 cm). The protein was eluted with the preceding buffer. SDS polyacrylamide 12% gel electrophoresis of the different fractions was shown at upper left corner. From column 1 to 8 are protein standards, fraction no 38, 41, 43, 45, 47, 51 and 55. Each column (except column 1) had total protein about 2 to 3 μg.
Figure 17.
Figure 18. The calculated gel filtration profile as combination of individual proteins—phosphofructokinase (2.3 mg/ml) and G-actin (1.6 mg/ml).
Figure 18.
ml) contains no detectable actin. Since the binding of F-actin by phosphofructokinase is enhanced at pH values below 8 (Liou, 1979), the experiment was repeated using a pH 7.0 buffer. The results are shown in Figure 19. Both proteins now appear in the effluent starting at fraction number 27 (59 ml). (The Sephacryl S-200 profile of G-actin or phosphofructokinase was no different when experiments were performed either in pH 7.0 or pH 8.0 buffer.) The SDS polyacrylamide electrophoresis gel of the different fractions under the peak reveal the presence of both phosphofructokinase and actin in every fraction. (Compare with the results in Figure 17.) The results indicate some degree of interaction between phosphofructokinase and G-actin. In fact, the early appearance of both components—in fractions where the standard enzyme did not occur, even though its initial concentration was 10 mg/ml—suggests that some higher degree of polymerization is involved. The trailing edge of the peak may result from an interaction between phosphofructokinase-actin complex and the Sephacryl matrix. This characteristic elution profile was obtained in three different experiments. A binding experiment with the actin core and phosphofructokinase was also performed at pH 7.0. No interaction between the proteins was detected under these conditions (Fig. 20).

(e). Effect of actin binding proteins—α-actinin and filamin

The results in this section deal with the effects of two actin binding proteins—α-actinin and filamin—on the catalytic activity of phosphofructokinase. Perhaps unexpectedly, these proteins interact
Figure 19. Gel filtration profile of a mixture of phosphofructokinase and G-actin monomer at pH 7.0. The sample contained phosphofructokinase (2.03 mg/ml) and G-actin (1.08 mg/ml) in an initial volume 6 ml. The conditions of the experiment were otherwise the same as those used at pH 8.0. (See legend to Figure 16.) SDS polyacrylamide 12% gel electrophoresis of different fractions was shown at upper right corner. From left to right are the protein standards and the fraction numbers 33, 37, 43, 47, 55, 65, and 71. The protein (except protein standards) applied in each column was approximately 1.5 µg to 3 µg.
Figure 19.

[Graph showing OD at 280 nm against Fraction No. (2.2 ml/tube)]
Figure 20. Gel filtration profile of a mixture of phosphofructokinase and actin core at pH 7.0. The applied sample contained phosphofructokinase (2.32 mg/ml) and actin core (0.9 mg/ml) in total volume 10 ml. The experimental conditions were otherwise the same as those in Figures 17 and 16. SDS polyacrylamide 12% gel electrophoresis of the different fractions was shown at up left corner. From left to right are protein standards (in duplicate), the actin core, and fraction no 41, 45, 48, 49, 53.
with phosphofructokinase even in the absence of actin. Figure 21 shows determinations of phosphofructokinase activity on two fractions—"low" phosphate enzyme (0.24 mol P/protomer) which can be synergistically activated by actin filaments and fructose 2,6-bisphosphate (Fig. 22) and "high" phosphate enzyme (0.91 mol P/protomer) as a function of fructose 6-phosphate concentration, ranging from 0-1.0 mM. The effects of α-actinin (45 nM) on the reaction ratios were examined in the presence of F-actin (1.2 μM) and/or fructose 2,6-bisphosphate (3.0 μM). No precipitation was seen under the assay condition. The ratio of α-actinin to actin used (1:26.6) approximates that found in skeletal muscle. (Rabbit muscle contains 26 mg actin/g (Hanson & Lowy, 1963; Weber et al., 1969) and 0.32 mg α-actinin/g (Arakawa et al., 1970).) In the case of the "low" phosphate enzyme, about 83% inhibition occurs in the presence of α-actinin and saturating concentrations of fructose 6-phosphate. The inhibition is readily reversed by the addition of F-actin but not of fructose 2,6-bisphosphate. The "high" phosphate enzyme is much less sensitive to α-actinin, with only 25% inhibition occurring. Although there may be some additional changes in activity with the various combinations examined, none approaches the magnitude of that obtained with the enzyme and α-actinin alone. Assuming that both phosphofructokinase fractions have the same binding sites, the presence of α-actinin may affect the polymerization state of the enzyme. Possibly α-actinin stabilizes the free monomer of phosphofructokinases—which is considered to be inactive (Lad & Hammers, 1974; Telford et al., 1975). The two enzyme fractions could differ in their ease of dissociation.
Figure 21. Effects of α-actinin. Reaction rates of A: "low" (0.24 mol P/mole PFK protomer and B: "high" (0.91 mol P/mole PFK protomer phosphate phosphofructokinase were determined as a function of varied concentration of fructose 6-phosphate and fixed concentration of ATP (1 mM), 5 μg/ml of α-actinin and 0.5 μg/ml of enzyme. Results show for α-actinin, 5 μg/ml (△); F-actin, 50 μg/ml + α-actinin (□); fructose 2,6-bisphosphate, 3 μM + α-actinin (▲); F-actin + fructose 2,6-bisphosphate + α-actinin (■); and no addition (○). Condition: 50 mM MOPS/KOH, 3 mM MgCl₂, 2.5 mM DTT, and 0.5 mM (NH₄)₂SO₄, pH 7.0. See Figure 22 for the corresponding control experiments performed in the absence of α-actin.
Figure 21.
Figure 22. Influence of effectors on activity of the phosphofructokinase. The reaction rates of the "low", 0.24 mol P/protomer, (A) and the "high", 0.91 mol P/protomer, (B) phosphate enzymes were determined as a function of fructose 6-phosphate concentration. Other conditions are described under Figure 21. Results are shown for no additions (○); F-actin, 50 μg/ml (□); fructose 2,6-bisphosphate, 3 μM (●); and F-actin + fructose 2,6-bisphosphate (■) at the preceding concentration. Control experiment corresponding to Figure 21.
Figure 22.
Figure 23 shows assays of both "low" (Fig. 23A) and "high" (Fig. 23B) phosphate phosphofructokinase (5.9 nM) in the presence of variable amounts of alpha-actinin (0 to 12 µg/ml) and a non-saturating concentration of fructose 6-phosphate (0.2 mM). The F-actin and fructose 2,6-bisphosphate concentration tested were 50 µg/ml (1.2 µM of monomer) and 3 µM, respectively. The results show that the activity of the "low" phosphate enzyme decreases to 36% as the alpha-actinin/phosphofructokinase ratio (in term of protomer) increases from 0 to 20, with no significant changes obtained in the "high" phosphate enzyme (Fig. 24). With addition of 1.2 µM of F-actin, the maximum inhibition of "low" phosphate enzyme by alpha-actinin is less than 10%. The presence of either fructose 2,6-bisphosphate or fructose 2,6-bisphosphate and F-actin has no large effect on either enzyme fraction. This is partly due to the fact that three of the curves in Fig. 21 are close together when the fructose 6-phosphate concentration is 0.2 mM.

Parallel experiments were conducted using varying amounts of phosphofructokinase (from 0.05 mg/ml to 0.5 mg/ml) plus alpha-actinin (0.06 mg/ml) which had been incubated at room temperature in the assay buffer (50 mM MOPS (K+), 3 mM MgCl₂, 0.5 mM (NH₄)₂SO₄, and 2.5 mM DTT, pH 7.0), for 15 min. (The visible viscosity of the solution containing as 1.2 mg/ml of alpha-actinin and 2 mg/ml of "low" phosphate phosphofructokinase increased markedly during the incubation.) The resulting pellet was spun down with a table top microfuge at 12000 rpm for 3 min. The pellet was then washed twice with two volumes of the incubation buffer. Protein concentrations were then determined using the Bradford reagent. The results showed that 38% to 60% of the total
Figure 23. Effects of α-actinin on the activity of phosphofructokinase. Reaction rates of the "low" (A) and the "high" (B) phosphate phosphofructokinase fractions (see Figure 22 for phosphate contents) were determined as a function of the molar ratio of α-actinin to phosphofructokinase (in term of protomer) at a nonsaturating concentration of fructose 6-phosphate (0.2 mM) and fixed concentration of phosphofructokinase (0.5 μg/ml) and ATP (1 mM). The assay conditions are the same as in Figure 21. Results are shown for no additions (○); F-actin, 50 μg/ml (□); fructose 2,6-bisphosphate, 3 μM (●); and F-actin + fructose 2,6-bisphosphate (■) at the preceding concentration. Figure 24 uses an expanded vertical scale to show the effects of α-actinin in the absence of other effectors.
Figure 23.

[Graph showing PFK activity against the ratio of [α-actinin]/[PFK] (mol/mol).]
Figure 24. Effects of α-actinin on the activity of phosphofructokinase in the absence of other effectors. The reaction rates of the "low" (○) and the "high" (●) phosphate enzyme were determined as a function of molar ratio of α-actinin to phosphofructokinase. These data were also shown in Figure 23.
Figure 24.
protein was precipitated with increasing initial concentrations of "low" phosphate phosphofructokinase ranging from 0.05 mg/ml to 0.5 mg/ml. A 10% SDS polyacrylamide gel revealed the presence of both α-actinin and phosphofructokinase in the pellet and in the supernatant. Precipitation of the "high" phosphate enzyme fraction occurred only when 0.1 mg/ml of F-actin was also present in the mixture. Although this evidence is indirect, it suggests that α-actinin binds to the two enzyme fractions in differing degrees.

Figure 25 contains the results of activity measurement obtained as a function of the molar ratio of filamin to phosphofructokinase. The phosphofructokinase preparation used here is the same as that used in experiments shown in experiments shown in Figures 21, 22 and 23. Once again, both enzyme fractions behave differently. The influence of filamin alone on the "low" phosphate enzyme fraction is much larger than that on the "high" phosphate fraction. Low concentrations of filamin (> 10 nM) activate the low phosphate fraction to about the same extent as F-actin does. However, unlike actin, filamin does not synergistically activate the low phosphate enzyme when fructose 2,6-bisphosphate is present. Filamin has little effect when F-actin is initially present. Filamin partially reverses the synergistic activation obtained with 1.2 μM F-actin and 3 μM fructose 2,6-bisphosphate. The initial decline in enzyme activity accompanying the addition of 0 to 1.1 μg/ml filamin in the presence of F-actin is hard to explain. However, this phenomenon disappeared when the concentration of F-actin was decreased to 5 μg/ml or replaced by 5 μg/ml G-actin (Fig. 26). Filamin has little stimulatory effect on the "high" phosphate enzyme fraction in the absence of other effectors.
Figure 25. Effects of filamin on the activity of phosphofructokinase.

The reaction rates of the "low" (A) and the "high" (B) phosphate enzymes (same fractions used in Figure 22) were recorded as a function of molar ratio of filamin to enzyme at nonsaturating concentration of fructose 6-phosphate (0.2 mM), 1 mM ATP and 0.5 μg/ml enzyme. The assay condition was given under Figure 22. Results are shown for no additions (○); F-actin, 50 μg/ml (□); fructose 2,6-bisphosphate, 3 μM (●) and fructose 2,6-bisphosphate + F-actin (■) at preceding concentrations.
Figure 25.
Figure 26. Effects of filamin on the activity of phosphofructokinase in the presence of either F-actin or G-actin. The assay condition was given under Figure 22. Results are shown for no addition (○); F-actin, 5 μg/ml (□); G-actin, 5 μg/ml (△).
Figure 26.
However, a moderate activation--up to 30% or so--does occur when F-actin and/or fructose 2,6-bisphosphate are present. Precipitation experiments involving the same conditions employed with α-actinin used 0.15 mg/ml of filamin and varying phosphofructokinase concentrations ranging from 0.05–0.5 mg/ml. Precipitation, affecting 10% to 32% of the total protein, occurred only with the "low" phosphate enzyme fractions.

**Relationship Between the Low Phosphate and High Phosphate Phosphofructokinase Fractions**

(a). *In vitro* phosphorylation of phosphofructokinase

The attempted *in vitro* phosphorylation of phosphofructokinase by several different protein kinases including phosphorylase kinase from rabbit skeletal muscle, myosin light chain kinases from both rabbit skeletal muscle and turkey gizzard, c-AMP dependent protein kinase from bovine heart, and c-GMP-dependent protein kinase from porcine lung is described in this section. The results are shown in Table V. Phosphorylase kinase and myosin light chain kinase, either from rabbit muscle or turkey gizzard, did not phosphorylate phosphofructokinase under the condition applied. Both cyclic AMP-and cyclic GMP-dependent protein kinases, however, showed ability to phosphorylate either "low" or "high" phosphate fractions. The amount of phosphate incorporable is limited by the extent of endogenous phosphorylation. The total phosphate present after incubation with the purified catalytic subunit of the cAMP-dependent protein kinase (15 μg/ml) at room temperature
Table V. Phosphorylation of rabbit skeletal muscle phosphofructokinase, *in vitro.*

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Source</th>
<th>Phosphate Content (mol P/protomer)</th>
<th>Endogenous* incorporation</th>
<th>32p incorporated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase Kinase</td>
<td>Rabbit muscle</td>
<td>0.585 0.065 <strong>--</strong></td>
<td></td>
<td>0.65</td>
<td>1.26</td>
</tr>
<tr>
<td>Myosin Light Kinase</td>
<td>Turkey Gizzard</td>
<td>0.42 <strong>--</strong></td>
<td>0.03</td>
<td>0.45</td>
<td>1.47</td>
</tr>
<tr>
<td>Myosin Light Kinase</td>
<td>Rabbit Muscle</td>
<td>0.46 0.03 <strong>--</strong></td>
<td></td>
<td>0.49</td>
<td>0.52</td>
</tr>
<tr>
<td>cAMP-dependent Protein Kinase</td>
<td>Bovine Heart</td>
<td>0.42 1.63 <strong>--</strong></td>
<td></td>
<td>2.05</td>
<td>1.98</td>
</tr>
<tr>
<td>cGMP-dependent Protein Kinase</td>
<td>Porcine Lung</td>
<td>1.05 <strong>--</strong></td>
<td>0.92</td>
<td>1.97</td>
<td>2.27</td>
</tr>
</tbody>
</table>

* Phosphate determination is according to Hasegawa et al. (1982).

a Measurements of 32P-ATP incorporation which values were based on the count per minute of 32P incorporated into the enzyme.
for 2 hrs. approaches two mol phosphate/mol enzyme protomer with both the "low" and the "high" phosphate enzyme forms. Incubation of the phosphofructokinase fractions with the cGMP-dependent protein kinase (30 μg/ml) and cGMP (10 μM) at room temperature for 2 hrs. resulted in approximately one mol phosphate/mol enzyme protein in both enzyme fractions.

Accumulating evidence suggests that the cAMP-dependent protein kinase is the enzyme most likely to be responsible for the in vivo phosphorylation of phosphofructokinase (Uyeda et al., 1978; Kemp et al., 1981; cf reviews by Soling and Brand, 1981). Consequently, my characterizations concentrate on fractions which had been phosphorylated in vitro by the cAMP-dependent protein kinase. Figure 27 shows the effects of varying concentrations of fructose 2,6-bisphosphate on the native "low" and "high" phosphate enzyme fractions and on a sample (1.96 mol P/mol protomer) that had been phosphorylated in vitro. In this demonstration, the enzyme that had been phosphorylated in vitro closely resembles the native "high" phosphate fraction. Figure 28 shows the initial catalytic properties of the enzyme fraction (containing 0.5 mol endogenous P/mol protomer) used in the subsequent in vitro phosphorylation. The in vitro reaction resulted in a product containing a total of 2.3 mol P/mol protomer. However, only 1.3 mol P/mol protomer remained 3 hours later after passage of the sample over a BioRad p 60 column. At this time, the activity measurements in Figure 26B were obtained. They show a decline in V_max from 31.2 units/mg to 14.5 units/mg and an increase in the K_m for fructose 6-phosphate from 0.124 mM to 0.312 mM. The in vitro phosphorylated sample has lost much of its sensitivity to
Figure 27. Effect of *in vitro* phosphorylation, catalyzed by the cAMP-dependent protein kinase, on the sensitivity of phosphofructokinase fractions to fructose 2,6-bisphosphate. Results are shown for native "low" phosphate enzyme (0.42 mol P/mol protomer) (○), for native "high" phosphate (1.29 mol P/mol protomer) (□) enzyme fraction and for *in vitro* phosphorylated enzyme (1.96 mol P/mol protomer) (■). Conditions: 0.5 μg/ml enzyme; 50 mM MOPS/KOH; 0.1 mM fructose 6-phosphate; 1.0 mM ATP; 3.0 mM MgCl₂; 0.5 mM (NH₄)₂SO₄; and 2.5 mM dithiothreitol (pH 7.0 at 25°C).
Figure 27.
Figure 28: Effect of *in vitro* phosphorylation by cAMP-dependent protein kinase on the catalytic activity of phosphofructokinase. The reaction rate of native (0.5 mol P/mol protomer) (A) and *in vitro* phosphorylated (1.29 mol P/mol protomer) (B) enzyme were recorded as a function of fructose 6-phosphate concentration using fixed concentrations ATP (1 mM) and phosphofructokinase (1.5 μg/ml). Assay conditions: 50 mM MOPS/KOH, 3 mM MgCl₂, 2.5 mM DTT and 0.5 mM (NH₄)SO₄, pH 7.0. Results are shown for no additions (○); F-actin, 50 μg/ml (□); fructose 2,6-bisphosphate, 3 μM (●); and the combination of F-actin and fructose 2,6-bisphosphate (■).
Figure 28.
F-actin, both in the presence of fructose 2,6-bisphosphate and, markedly so, in its absence. The \textit{in vitro} phosphorylated enzyme is, in these respects, rather different from the "high" phosphate enzyme isolated from \textit{in vivo}. This result was obtained with two different preparations of the native "low" phosphate enzyme.

The initial phosphorylation rates of one phosphofructokinase fraction with an endogenous phosphate content 0.04 mol P/protomer, by either 0.38 \( \mu \text{M} \) of cAMP- or 0.38 \( \mu \text{M} \) of cGMP-dependent protein kinase were 0.14 mol P/protomer/min and 0.24 mol P/protomer/min, respectively (Fig. 29). Fifty percent of phosphate incorporation could be achieved at 6.08 ± 1.73 min for cAMP-dependent protein kinase phosphorylation and at 1.71 ± 0.40 min for cGMP-dependent protein kinase phosphorylation. The extrapolated maximal phosphate incorporations for cAMP- and cGMP-dependent protein kinase were 1.9 ± 0.12 mol P/protomer and 1.45 ± 0.10 mol P/protomer, respectively. The actual amounts of phosphate incorporation obtained with cAMP-dependent protein kinase was 1.85 mol P/protomer and with cGMP-dependent protein kinase, 1.03 mol P/protomer after 2 hrs of reaction. Fifty percent of the maximum phosphate incorporation was achieved at 1.40 ± 0.33 min when phosphofructokinase was phosphorylated by the combination of cAMP- and cGMP-dependent protein kinase at preceding concentrations (Fig. 30). After two hours reaction, 2.39 mol P/protomer could be obtained in the present of both protein kinases. Extrapolation to infinite time suggested a maximum of 2.5 ± 0.1 mol P/mol protomer.

The phosphorylation rates of phosphofructokinase by either cAMP-dependent protein kinase or cGMP-dependent protein kinase are slower than the phosphorylation of cardiac troponin I by either
Figure 29. The phosphorylation of phosphofructokinase by cAMP- or cGMP-dependent protein kinase. Assay conditions: 50 mM MOPS/KOH, 2.5 mM dithiothreital, 3.0 mM MgCl₂, 0.5 mM EGTA, 0.1 mM ³²p-ATP (0.03 Ci/mmol), 0.5 mg/ml phosphofructokinase and 0.38 μM purified catalytic subunit of cAMP-dependent protein kinase (○) or 0.38 μM cGMP-dependent protein kinase plus 10 μM cGMP (●). The reactions were initiated by the addition of protein kinase.
Figure 29.
Figure 30. The phosphorylation of phosphofructokinase by a mixture of cAMP- and cGMP-dependent protein kinases. Assay conditions: 50 mM MOPS/KOH, 2.5 mM dithiothreital, 3.0 mM MgCl₂, 0.5 mM EGTA, 0.1 mM ³²P-ATP (0.03 Ci/m mol), 0.5 mg/ml phosphofructokinase, 0.38 μM purified catalytic subunit cAMP-dependent protein kinase and 0.38 μM cGMP-dependent protein kinase plus 10 μM cGMP. The reactions were initiated by the addition of the mixture of protein kinases.
Figure 30.
Figure 31. The phosphorylation of cardiac troponin I by cAMP- or cGMP-dependent protein kinase. Assay conditions: 50 mM MOPS/KOH, 2.5 mM dithiothreital, 3.0 mM MgCl₂, 0.5 mM EGTA, 0.1 mM ^32P-ATP (0.03 Ci/m mol), 0.25 mg/ml cardiac troponin I, 8.4 nM purified catalytic subunit cAMP-dependent protein kinase and 8.3 nM cGMP-dependent protein kinase plus 10 µM CGMP. The reactions were initiated by the addition of protein kinases. (O) phosphorylation by cAMP-dependent protein kinase; (●) phosphorylation by cGMP-dependent protein kinase.
Figure 31.
protein kinase. The initial phosphorylation rates of cardiac troponin I by 8.4 nM of cAMP-dependent protein kinase is 0.11 mol P/mol/min; by 8.3 nM of cGMP-dependent protein kinase, the rate is 0.020 mol P/mol/min (Fig. 31).

(b). In vitro dephosphorylation of phosphofructokinase

The dephosphorylation of phosphofructokinase in vitro was attempted using several phosphatases, including alkaline phosphatase from either bovine intestinal mucosa or rabbit skeletal muscle and calcineurin from bovine brain. (The assay conditions were described in the Material and Methods section.) The results are shown in Table VI. No dephosphorylation of phosphofructokinase was obtained with alkaline phosphatase from rabbit skeletal muscle. The amount of phosphate removed by alkaline phosphatase from bovine intestinal mucosa and by calcineurin from bovine brain was 30-50% and 45% of the total, respectively. Preliminary experiments also showed that the synergistic activation characteristic of native low phosphate phosphofructokinase by fructose 2,6-bisphosphate and F-actin could not be detected in the partially dephosphorylated enzyme sample (Fig. 32).

The $K_m$ value for fructose 6-phosphate of native enzyme (1.6 mol P/protomer) purified by the second modification method of the Kemp procedure shifted from 0.24 mM to 0.21 mM after dephosphorylation by bovine intestine mucosa alkaline phosphatase with 45% phosphate removal. The $V_{max}$ values, 23.0 units/mg for native enzyme and 22.4 units/mg for dephosphorylated enzyme showed no difference. With the combination of F-actin (1.2 μM) and fructose 2,6-bisphosphate (3 μM),
Table VI. Dephosphorylation of phosphofructokinase in vitro.

<table>
<thead>
<tr>
<th>Phosphatase</th>
<th>Source</th>
<th>phosphorous content (mol P/mol protomer)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>original</td>
<td>dephosphorylated</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Bovine</td>
<td>0.16±0.03</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td></td>
<td>intestinal mucosa</td>
<td>1.08±0.16</td>
<td>0.7±0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.29±0.2</td>
<td>0.49±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6±0.1</td>
<td>0.89±0.09</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Rabbit skeletal</td>
<td>1.08±0.16</td>
<td>1.06±0.2</td>
</tr>
<tr>
<td></td>
<td>muscle</td>
<td>1.6±0.1</td>
<td>1.62±0.2</td>
</tr>
<tr>
<td>Calcineurin</td>
<td>Bovine brain</td>
<td>0.7±0.07</td>
<td>0.41±0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.86±0.05</td>
<td>0.47±0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.06±0.03</td>
<td>0.5±0.03</td>
</tr>
</tbody>
</table>

*a Phosphate determination is according to Hasegawa et al. (1982).*
Figure 32. Activity assays of native "high" phosphate and dephosphorylated phosphofructokinase. The assay conditions: 50 mM MOPS/KOH, 3 mM MgCl$_2$, 2.5 mM DTT, 0.5 mM (NH$_4$)$_2$SO$_4$, 1.0 mM ATP, 0.5 µg/ml phosphofructokinase, at pH 7.0. (A): native high phosphate enzyme with phosphate content of 1.6 mol P/protomer; (B): dephosphorylated enzyme with phosphate content of 0.89 mol P/protomer. Results are shown for F-actin, 50 µg/ml (□); F-actin + fructose 2,6-bisphosphate, 50 µg/ml + 3 µM (■); fructose 2,6-bisphosphate, 3 µM (●); and no addition (○).
Figure 32.
the $K_m$ shifted to 0.13 mM for the native enzyme and to 0.11 mM for dephosphorylated enzyme whereas the $V_{max}$ was 25.70 units/mg for native enzyme and 31.90 units/mg for dephosphorylated enzyme.

**Pilot Comparison of Skeletal Muscle Phosphofructokinase from Normal Well Fed and Long Term Fasted Rabbits**

The hormone levels and metabolic state have been known to be able to control the phosphorylation of phosphofructokinase in tissues (Brand & Soling, 1982; Dunaway, 1983, 1984; Frucht et al., 1984). Glucagon (0.1 μM) stimulated the phosphorylation of phosphofructokinase to the maximal value (1.2 mol P/mole enzyme) when the rat hepatocytes were preincubated with 20 mM glucose. Glucose (20 mM) addition to isolated hepatocytes from fed rats resulted in an increase in the incorporation of phosphate content from 0.64 mol P/mol enzyme to 0.86 mol P/mole enzyme for phosphofructokinase (Brand & Soling, 1982). Insulin (Dunaway, 1978) and epinephrine (Clark et al., 1982; Patten et al., 1982; Patten & Clark, 1983) were also reported to be able to influence the activity and phosphorylation degree of phosphofructokinase from liver and heart, respectively. Bradykinin, a nonapeptide hormone, rapidly produced in damaged tissues, is reported to significantly increase the level of cyclic GMP in muscle (Frucht et al., 1984), which is reported to decrease the activity of muscle phosphofructokinase to 47% of control activity at concentration 10 μM. To reveal possible effects of metabolic state on skeletal muscle phosphofructokinase, the enzyme was isolated from both normal well fed and short term fasted (4 days on water) rabbits.
Preliminary experiments were then performed to compare the influence of the various effectors on the two enzyme preparation. Two well fed and two fasted rabbits were tested.

Figures 33 and 34 show the representative DE-52 elution profiles of phosphofructokinase purified from both a well fed and a fasted rabbits. (My second modification of the Kemp procedure was used in the purification.) In both cases, the enzyme started to elute at a Tris-phosphate concentration around 0.16 M. The highest phosphate content fractions were eluted at Tris phosphate concentration around 0.166 M for well fed rabbits and around 0.18 M for fasted rabbits. No differences in the electrophoretic distribution of proteins could be seen in the individual purification steps used with the two preparations (Figure 35). The 12% polyacrylamide SDS gels showed that phosphofructokinase fractions purified from either the well fed or the fasted rabbits have same subunit molecular weights. However, the enzyme purified from fasted rabbits contains, on the average, more phosphate (Table VII) and is less active than the enzyme purified from the well fed rabbit. The "low" phosphate fraction, 0.56 mol P/protomer, from the fasted rabbit is less sensitive to activation by F-actin, by fructose 2,6-bisphosphate, and by the combination of F-actin and fructose 2,6-bisphosphate (Fig. 36A) than similar fractions from the well fed rabbit. The "high" phosphate enzyme from the fasted rabbit, containing 4.45 mol P/mol protomer, is completely insensitive to these effectors (Fig. 36B). The phosphate contents indicated here were determined immediately after the enzymes were purified.
Figure 33. The DE-52 column profile of phosphofructokinase purification from a well fed rabbit. The phosphofructokinase containing solution having a protein concentration 7.4 mg/ml (41 ml) was passed over a 4x11 cm DE-52 column. The enzyme was eluted with 0.1 M to 0.4 M Tris-phosphate buffer, pH 8.0 containing 0.2 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM KF. Phosphofructokinase was started to be eluted out at Tris-phosphate concentration around 0.15 M. The rabbit weighed 4.4 kilograms. See Table VII for the range of phosphate contents.
Figure 33.
Figure 34. The DE-52 column profile of phosphofructokinase purified from a fasted rabbit. The phosphofructokinase containing solution having a protein concentration 7.1 mg/ml (41 ml) was passed over a 4x11 cm DE-column. The experimental conditions were same as Figure 33. Phosphofructokinase was started to be eluted out at Tris-phosphate concentration 0.16 M. The rabbit weighed 3.7 kilograms. See Table VII for the range of phosphate contents.
Figure 34.
Figure 35. Comparison of the results of the individual steps in the fractionation of phosphofructokinase from a well fed and from a fasted rabbit. Column A: Protein standards from top to bottom are myosin heavy chain (200,000), debranching enzyme (130,000), phosphorylase (96,000), bovine serum albumin (68,000), actin (45,000), carbonic anhydrase (29,000), troponin C (18,000) and parvalbumin (104,000); B: actin; C and D: supernatant of first extract from fasting (C) and well fed (D) rabbits; E and F: supernatant of isopropanol precipitation with fasting (E) and well fed (F) rabbits; G: protein standards; H: Isopropanol pellet from well fed rabbit; I and J: supernatant of heat treated extract from fasting (I) and well fed (J) rabbits; K and L: pellet of heat treatment from fasting (K) and well fed (L) rabbits; M and N: 33% ammonium sulphate precipitation pellet from fasting (M) and well fed (N) rabbit; O and P: 55% ammonium sulphate precipitation pellet (sample for DE-cellulose chromatography) from fasting (O) and well fed (P) rabbits.
Table VII. Comparison of the phosphate contents of phosphofructokinase isolated from normal well fed and fasted rabbits. Phosphofructokinase was purified by the second modification of the Kemp (1972) procedure.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average</th>
<th>Fraction No.*</th>
<th>Highest Phosphate Content (mol P/mol protomer)</th>
<th>Fraction No.*</th>
<th>Lowest Phosphate Content (mol P/mol protomer)</th>
<th>Fraction No.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well fed</td>
<td>0.54</td>
<td>39-60(a)</td>
<td>1.3</td>
<td>43</td>
<td>0.25</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>39-60</td>
<td>2.65</td>
<td>46</td>
<td>0.2</td>
<td>39</td>
</tr>
<tr>
<td>Fasted</td>
<td>1.33</td>
<td>31-51(a)</td>
<td>3.0</td>
<td>37</td>
<td>0.05</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2.23</td>
<td>24-35</td>
<td>4.45</td>
<td>28</td>
<td>0.56</td>
<td>26</td>
</tr>
</tbody>
</table>

\(a\) The DE-52 profiles were shown in Figure 31 and 32, respectively.

* Fraction no. Each purification involved different fraction volumes. From top to bottom, the volumes of each individual tube were 4.5 ml, 5 ml, 5.6 ml and 7 ml, respectively.
Figure 36. Catalytic properties of the "low" and the "high" phosphate phosphofructokinase fractions purified from a fasted rabbit. The enzyme contained 0.56 mol P/mol protomer (A) and 4.45 mol P/mol protomer (B), respectively. Reaction conditions: phosphofructokinase, 3.0 µg/ml; 50 mM MOPS/KOH, 3.0 mM MgCl₂, 2.5 mM DTT, 0.5 mM (NH₄)₂SO₄, pH 7.0. Results are shown for no additions (○); F-actin, 50 µg/ml (□); fructose 2,6-bisphosphate, 3 µM (●); the combination of F-actin and fructose 2,6-bisphosphate (■) at preceding concentrations.
Figure 36.
DISCUSSION

The major accomplishments of this project can be summarized as follows.

1. Development of an Improved Purification Procedure for Rabbit Muscle Phosphofructokinase

Modification of the purification procedure of Kemp (1975) has allowed me to obtain preparations of the enzyme that are stable in terms of specific activity, subunit molecular weight, and endogenous phosphate content for periods of time up to 6 months at -80°C. The addition of tetrapotassium pyrophosphate (5 mM)—an effective phosphoprotein phosphatase inhibitor (Khandelwal, 1977; Khandelwal & Kaman, 1978)—to all but one of the standard solutions used, including the storage buffer, accounts for both the reproducibility and stability of the phosphate determinations. The preparation is also free of actin, which tends to copurify with a fraction of phosphofructokinase when the procedure of Ling et al. (1966) is applied.

An ionic strength gradient elution using a connected series of DE-51, DE-52, and DE-53 columns facilitates the partial resolution of the enzyme into electrophoretically homogenous (SDS polyacrylamide gel electrophoresis) subpopulations that differ in endogenous phosphate levels and in catalytic activity. The two fractions showing the greatest functional differences—"low" and "high" phosphate phosphofructokinase—were selected for a detailed study of the
influence of various known effectors of the enzyme.

2. Demonstration of the Functionally Disperse Properties of Phosphofructokinase

Characterization of the sub-populations of phosphofructokinase emphasized the influence of two effectors—fructose 2,6-bisphosphate (Hers & Van Schaftingen, 1982; Uyeda et al., 1982) and F-actin (Liou & Anderson, 1980). "Low" phosphate enzyme, containing less than 0.5 mol P/mol protomer, and "high" phosphate enzyme, containing more than 0.5 mol P/mol protomer, were similar in terms of their fructose 6-phosphate saturation curves and responded to addition of either F-actin or fructose 2,6-bisphosphate alone. Their $V_{\text{max}}$ values varied moderately. Unexpectedly, the largest difference between the two fractions was obtained with the combination of fructose 2,6-bisphosphate and F-actin. In the case of the "low" phosphate enzyme, the amount of activation obtained with these two effectors is much larger than that expected from addition of the separate effects. This pronounced synergism is absent in the case of the "high" phosphate enzyme. Previous studies on native (0.13 mol P/mol protomer), in vitro dephosphorylated (0.02 mol P/mol protomer), and in vitro phosphorylated (0.75 mol P/mol protomer) muscle phosphofructokinases demonstrated generally smaller differences—with the phosphorylated enzyme being somewhat more sensitive to inhibition by citrate or high concentrations of ATP and less sensitive to activation by AMP, glucose 1,6-bisphosphate, and inorganic phosphate than untreated or dephosphorylated enzyme (Poe & Kemp, 1982). The
effects of F-actin and/or fructose 2,6-bisphosphate were not considered in the latter study.

The observations on the influence of fructose 2,6-bisphosphate on native "low" and "high" and in vitro cAMP-dependent protein kinase phosphorylated phosphofructokinase are consistent with the known role of cAMP in the regulation of fructose 2,6-bisphosphatase and fructose 6-phosphate 2-kinase. Acting through the cAMP-dependent protein kinase, cAMP both prevents the formation and favors the destruction of fructose 2,6-bisphosphate (Richards et al., 1982; Van Schaftingen et al., 1982). When fructose 2,6-bisphosphate levels are high, the phosphorylation level of phosphofructokinase is expected to be low and its activation by F-actin maximal. However, additional experiments suggested that although the state of phosphorylation of phosphofructokinase is involved in the distinctive catalytic properties observed---there may be other differences within the enzyme population.

Calcineurin and alkaline phosphatase digestions of phosphofructokinase preparations phosphorylated in vivo ("high" phosphate enzyme) resulted in incomplete (30-50%) removal of covalent phosphate. Activity measurements showed incomplete recovery of the catalytic properties characteristic of the native "low" phosphate enzyme. (The properties of the digested samples remained closer to those of the native "high" phosphate enzyme.) In vitro phosphorylation, catalyzed by the cAMP-dependent protein kinase, caused the properties of phosphofructokinase to become more like--but not the same as--those of the "high" phosphate enzyme obtained from DE51-DE52-DE53 chromatography. The response of the in vitro phosphorylated enzyme to fructose 2,6-bisphosphate alone is the same
as that of the native "high" phosphate fraction. On the other hand, the in vitro phosphorylated enzyme retains a greater sensitivity to the combined effects of F-actin and fructose 2,6-bisphosphate than the native "high" phosphate enzyme.

3. Demonstration of Two Phosphorylation Sites in Phosphofructokinase

The total amount of covalently bound phosphate approaches 2 mol P/mol protomer when either "low" or "high" phosphate phosphofructokinase was phosphorylated in vitro in a reaction catalyzed by the cAMP-dependent protein kinase. Although previous attempts at in vitro phosphorylation usually resulted in the incorporation of less than one mole/mol protomer, Sorensen-Ziganke and Hofer (1979) identified two different phosphopeptides in digest of phosphofructokinase isolated from electrically stimulated muscle. Ascaris muscle phosphofructokinase readily incorporates up to two mol P/mol protomer (Hofer et al., 1982). The sequence of phosphofructokinase contains a second possible phosphorylation site far removed from the serine at position 348 identified by Kemp et al. (1981). Observations by Huang et al. (1979) on other physiologically significant protein kinase substrates suggest that the Arg-Arg-Leu-Ser sequence at positions 206-209 (Poorman et al., 1984) would also be a site for phosphorylation if it is accessible. The existence of two different phosphorylation sites could contribute to the heterogenous behavior of phosphofructokinase. The distribution of phosphate between two different phosphorylation sites in a tetramer of identical subunits would result in a heterogenous population in the case of
partial phosphorylation \(0 < P < 2\) mol/protomer).

Attempted phosphorylation with rabbit muscle phosphorylase kinase, myosin light chain kinase and smooth muscle myosin light chain kinase resulted in negligible phosphate incorporation. Phosphorylation with the cGMP-dependent protein kinase resulted in the incorporation of one mol P/mol protomer. Any effects on catalytic activity and possible overlap of this phosphorylation site with the two recognized by the cAMP-dependent protein kinase remain to be determined.

The time course experiments indicate that phosphofructokinase is a better substrate for the cGMP-dependent protein kinase than for cAMP-dependent protein kinase, since 50% of maximal incorporation can be achieved within 2 minutes for cGMP-dependent protein kinase but only within 6 minutes for the cAMP-dependent protein kinase. The initial phosphorylation rate of phosphofructokinase by either cAMP- or cGMP-dependent protein kinases are only about 2.5% and 25%, respectively, of the rates obtained when cardiac troponin I is the substrate. This rate is apparently not enough to phosphorylate all the phosphofructokinase in vivo within a short time period. However, the phosphorylation of the enzyme could have a structural role requiring a relatively slow response. For example, the level of phosphofructokinase activity in fertilized \textit{Urechis} eggs increases markedly over the 30 min time interval where substantial cytoskeletal organization occurs (Tazawa & Yasumasu, 1977). Variations in the phosphorylation state of the enzyme with the accompanying changes in sensitivity to F-actin and fructose 2,6-bisphosphate could be involved in the assembly or disassembly of the cytoskeleton and the concomitant changes in glycolytic rate occurring.
4. **Pilot Studies Demonstrating Functionally Unique Fractions of Phosphofructokinase in the Muscle of Fasting Rabbits**

Short term (4-days) fasts using two different rabbits, each of which was processed separately, show that both the "low" and "high" phosphate enzyme fractions differ from corresponding fractions prepared from well fed rabbits. The "high" phosphate fraction, containing 4.45 mol P/mol protomer, is completely insensitive to fructose 2,6-bisphosphate and/or F-actin. The phosphate levels suggest additional phosphorylation sites--either in the form of serine(P) or threonine(P). Possibly another type of modification such as ADP-ribosylation is involved. Phosphotyrosine seems less likely as T. Hunter (personal communication to Malencik and Anderson) finds that phosphofructokinase is not a substrate for the epidermal growth factor receptor. The fact that the "low" phosphate enzyme differs from that of the well fed-rabbit indicates that other types of covalent modification, or even an unidentified non-covalently bound effector, participate in the regulation of the enzyme. Further characterization of these unique fractions will be one of the more interesting extension of this project.

5. **Comparison of the Effects of F-actin and G-actin**

My experiments show that F-actin is an efficient activator of phosphofructokinase while G-actin has minimal--even slightly inhibitory effects on the enzyme. These contrasting results suggest at first that the state of actin polymerization is a major determinant
in the activation of the enzyme. Yet, maximum activation takes place within a concentration range where actin alone is known to be extensively depolymerized. Little further change in enzyme activity occurs at actin concentrations above the critical level for polymerization. Three different interpretations of these results should be considered in future investigations. 1. Small oligomers of actin activates phosphofructokinase. 2. An F-actin-like conformational state of the monomer is capable of enzyme binding and activation. 3. Association with phosphofructokinase stabilizes filamentous actin. The activation of the enzyme by the covalently cross-linked actin trimer indicates either that the degree of actin polymerization does not need to be large or that cross-linking has stabilized an essential conformation. The possible existence of an F-actin monomer distinct from G-actin was suggested by the sequential conformational states detected before and during incorporation of "activated" monomers into filaments (Rich & Estes, 1976; Frieden et al., 1980; Rouayrenc & Travers, 1981; Pardee et al., 1982). However, appreciable quantities of unbound monomers or small oligomers probably do not occur in vivo. In the case of non-muscle cells, proteins such as profilin stabilize the pool of unpolymerized actin (Carlsson et al., 1977).

The actin "core", lacking the first 67 amino acid residues, has negligible effects on phosphofructokinase. This part of the actin sequence—known to be involved in the inhibition of DNAseI (Mannherz et al., 1980) and the association with myosin (Jacobson & Rosenbusch, 1976)—may contain a protein interaction site recognized by the phosphofructokinase.
Two actin binding proteins—α-actinin and filamin—had unexpected effects on the activity of phosphofructokinase in the absence of actin. Filamin activates the "low" phosphate enzyme from well fed rabbits to about the same extent as F-actin does alone. However, filamin and fructose 2,6-bisphosphate failed to act synergistically in the stimulation of enzyme activity. α-Actinin proved an effective inhibitor of the "low" phosphate enzyme. The opposing effects are consistent with a model in which either filamin or F-actin stabilizes the tetramer of phosphofructokinase, which is active, and α-actinin stabilizes the enzyme dimer, which is inactive (Bock & Frieden, 1974). Significantly, phosphofructokinase has been found to associate with the band-3 protein of the erythrocyte membrane which is a 95,000-dalton glycoprotein that spans the bilayer of the erythrocyte plasma membrane (Macara & Cantley, 1983)—with an accompanying enhancement of catalytic activity (Richards et al., 1979; Jenkins et al., 1985). The native "high" phosphate enzyme from well fed rabbits is comparatively insensitive to either filamin or α-actinin. The experiments suggest that association of phosphofructokinase with either filamin or α-actinin is unlikely to occur in vivo unless the concentration of F-actin is limiting. The addition of excess concentrations of F-actin to the assays largely reverses the effects of filamin and α-actinin.


Beitner, R. & Cohen, T.J. (1980) Opposite effects of dibutyryl cyclic GMP and dibutyryl cyclic AMP on glucose 1,6-diphosphate levels and the activities of glucose 1,6-diphosphate phosphatase and phosphofructokinase in diaphragm muscle. FEBS Lett. 115, 197-120.


