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# Factors Affecting the Activation of Rabbit Muscle Phosphofructokinase by Actin†

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*Received July 1, 1985*

ABSTRACT: 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 subpopulations of rabbit muscle phosphofructokinase which differ in their catalytic properties and endogenous covalent phosphate content. A band of "high"-phosphate enzyme (fraction II) flanked by regions of "low"-phosphate enzyme (fractions I and III) is an unusual feature of the final purification profile. Fractions I (containing in this case 0.42 mol of P/82 000 g of enzyme) and II (containing 1.26 mol of P/82 000 g of enzyme) exhibit the most pronounced functional differences of the fractions. Following our original report [Liou, R.-S., & Anderson, S. R. (1980) *Biochemistry 19*, 2684], both are activated by the addition of rabbit skeletal muscle F-actin. Under the assay conditions, half-maximal stimulation of phosphofructokinase activity occurs at 15.4 nM actin (in terms of monomer) for fraction I and 9.7 nM for fraction II. The low-phosphate enzyme is synergistically activated in the presence of 0.12 $\mu$M actin plus 3.0 $\mu$M fructose 2,6-bisphosphate, 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 fructose 2,6-bisphosphate. Phosphorylation of fraction I in an in vitro reaction catalyzed by the cAMP-dependent protein kinase causes its properties to become more like those of fraction II. The total amount of covalent phosphate present after in vitro phosphorylation approaches 2 mol of P/82 000 g of enzyme for both fractions.

The enzyme 6-phosphofructo-1-kinase (phosphofructokinase) (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is considered to be the controlling enzyme in the glycolytic pathway of yeast and mammals, catalyzing the phosphorylation of fructose 6-phosphate by Mg-ATP to give fructose 1,6-bisphosphate and Mg-ADP [cf. review by Hofmann (1978)]. As such, the enzyme is subject to close regulation by a number of allosteric effectors, including inhibitors such as nucleotide triphosphates and citrate and positive effectors such as glucose 1,6-bisphosphate and fructose 1,6-bisphosphate [cf. reviews by Kemp & Foe (1983), Uyeda (1979), and Goldhammer & Paradies (1979)]. A novel sugar phosphate, fructose 2,6-bisphosphate, is a potent activator of phosphofructokinase and is synthesized from and degraded to fructose 6-phosphate by a specific kinase and phosphatase, respectively. The intracellular levels of fructose 2,6-bisphosphate are influenced by a variety of hormonal and metabolic factors which also affect the activity of phosphofructokinase. The synthesis and degradation of fructose 2,6-bisphosphate by a specific kinase and phosphatase are directly controlled by the level of cyclic AMP via the cAMP-dependent protein kinase [cf. reviews by Hers & Van Schaftingen (1982), Pilkis et al. (1982), Furuya et al. (1982), and Hers & Hue (1983)].

Accumulating evidence shows that macromolecular interactions also affect the catalytic activity of the enzyme. In the case of rabbit muscle phosphofructokinase, experiments have shown that the catalytic activity (Hofer, 1970; Lad et al., 1973) and substrate binding (Hill & Hammes, 1975) are both affected by the self-association of the enzyme. Although the tetramer appears to be the smallest active species of the enzyme, the self-associated forms may play some role in the regulation (Luther et al., 1983).

Like several other glycolytic enzymes, phosphofructokinase can be covalently modified by phosphorylation, suggesting the existence of a specific protein kinase and phosphatase. Initial reports of phosphorylation applied to liver extracts (Brand & Soling, 1975). Hofer and Furst (1976) later isolated $^{32}$P-labeled phosphofructokinase from the skeletal muscle of mice that had been injected with [$^{32}$P]$P_i$. However, phosphorylation of rabbit muscle phosphofructokinase has little effect upon enzyme activity [cf. reviews by Söling & Brand (1981), Clark

†Supported by research grants from the National Institutes of Health (AM13912), the Muscular Dystrophy Association, and the Oregon Affiliate of the American Heart Association.

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& Patten (1984), Foe & Kemp (1982), and Sakakibara & Uyeda (1983)]. The exact role of phosphorylation remains unclear; the kinetic effects seen with phosphofructokinase phosphorylated by the cAMP-dependent protein kinase are small, and its rate of phosphorylation is low in comparison to the rates obtained with other protein kinase substrates.

Enzymes often exhibit a reversible intracellular partitioning between soluble and particulated forms [cf. review by Wilson (1978)]. Several of the glycolytic enzymes exhibit this trait with respect to muscle proteins [cf. reviews by Clarke & Masters (1976), Walsh et al. (1977), and Masters (1978)]. Histochemical experiments showed that the glycolytic and glycogenolytic enzymes are located within the I band of the muscle fiber, corresponding to the site of the thin filaments of the relaxed myofibril (Sigel & Pette, 1969; Arnold et al., 1969). Experiments with pressed juice extracts as well as purified enzymes have demonstrated in vitro adsorption of several glycolytic enzymes, with phosphofructokinase being the most strongly adsorbed (Clarke & Masters, 1976; Westrin & Blackman, 1983; Clarke et al., 1984). The major criticism of the adsorption studies pertains to the observed dependence on ionic strength. While adsorption does decline as the ionic strength increases, the associations also depend on the protein concentrations (Clarke & Masters, 1976). When physiological protein concentrations are used, efficient binding persists at $\mu = 0.15$ M (Masters, 1978). Bronstein and Knull (1981) noted that enzyme–enzyme interactions also play a role in stabilization of complexes with F-actin or the thin filament. Starr and Offer (1982) reported copurification of rabbit skeletal phosphofructokinase with myosin while Choate et al. (1985) described a form of phosphofructokinase that is tightly associated with cardiac myofibrils.

The binding of enzymes to various structural elements keeps the intermediate products at hand for the next enzymatic step, in what is termed substrate tunneling (Gaertner, 1978), and may even alter their kinetic and regulatory properties (Arnold & Pette, 1970; Walsh et al., 1977; Meyer et al., 1970). In the case of skeletal muscle phosphofructokinase, F-actin can directly influence the activity of the enzyme in vitro as well as modulate the effects of other allosteric agents (Liou & Anderson, 1980).

Since actin is a nearly universal protein comprising 10–20% of the cell protein in eucaryotes (Korn, 1978), its association with glycolytic enzymes may be a widespread phenomenon occurring in both muscle and nonmuscle cels. In view of the key regulatory role of phosphofructokinase, we have extended our previous observations on factors involved in the interaction of the enzyme with actin. The present report considers the effects of the state of actin polymerization, of chemical modification of actin, of the phosphorylation state of phosphofructokinase, both in vivo and in vitro, and of the activator fructose 2,6-bisphosphate.

## Materials and Methods

*Materials.* Fructose 6-phosphate, fructose 2,6-bisphosphate, ATP, dithiothreitol, and NADH were purchased from Sigma Chemical Co., as were the enzymes aldolase, glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, and alkaline phosphatase (bovine intestinal mucosa). Sephacryl S-200 and S-300 superfine were obtained from Pharmacia; DE-51, DE-52, and DE-53 were from Whatman. All other reagents were of the highest grade commercially available.

*Protein Purification and Assays.* The catalytic subunit of type II cAMP-dependent protein kinase was prepared from bovine heart (Peters et al., 1977). It was stored frozen (−70 °C) after dialysis against 1 volume of glycerol.

Rabbit skeletal muscle actin was purified from an acetone powder (Pardee & Spudich, 1982). After purification, G-actin was passed through a 0.45-$\mu$m Millipore filter (Grazi & Magri, 1981) and stored under a thymol atmosphere at 4 °C. F-Actin was prepared by polymerization of G-actin in the presence of 50 mM KCl and 1 mM $Mg^{2+}$. After high-speed centrifugation (100000$g$ for 3 h), the F-actin pellet was dissolved in 50 mM 3-(*N*-morpholino)propanesulfonic acid/potassium hydroxide (Mops/KOH);[1] 3 mM $MgCl_2$; 2.5 mM dithiothreitol, and 0.5 mM $(NH_4)_2SO_4$ (pH 7.0). Actin concentrations were determined by the Bradford method (Bradford, 1976) or from absorption using a value of $A^{0.1\%}_{290nm} = 0.63$.

The actin core was prepared according to the method of Jacobson and Rosenbusch (1976) with a few modifications. The chymotryptic digestion was stopped by the addition of phenylmethanesulfonyl fluoride (3 mM). The mixture was then filtered and passed over an affinity column of 4-phenylbutyl Sepharose in order to remove the last traces of chymotrypsin (Le Peuch et al., 1978). The core was then passed over an S-200 column (1.5 × 90) that had been equilibrated with 20 mM Tris-HCl, pH 8.0, 0.2 mM calcium acetate, 0.5 mM 2-mercaptoethanol, and 0.3 M NaCl. The actin core peak was identified by sodium dodecyl sulfate gel electrophoresis, concentrated by Amicon ultrafiltration, and used immediately.

The actin trimer preparation followed the procedure of Gilbert and Frieden (1983). The cross-linking agent phenylenebis(maleimide) was added to the actin as described. After 10 min, the reaction was stopped by the addition of 2-mercaptoethanol to a final concentration of 40 mM. The cross-linked pellet was spun for 3 h at 100000$g$ and dissolved in a depolymerizing buffer containing 5 mM Tris-HCl, 0.2 mM calcium acetate, 0.2 mM ATP, and 0.01% azide (Pardee & Spudich, 1982). After another polymerization–depolymerization cycle, the cross-linked actin was applied to an S-300 column equilibrated in the preceding buffer. Appropriate pools of actin trimer were made on the basis of sodium dodecyl sulfate gel electrophoresis.

Rabbit muscle phosphofructokinase was purified from fresh rabbit skeletal muscle according to the method of Kemp (1975) with slight modifications. The muscle was ground in a meat grinder and extracted by stirring for 20 min in 3 volumes of 30 mM KF, 4 mM EDTA, and 15 mM 2-mercaptoethanol. After centrifugation at 2000$g$ for 10 min, the clear supernatant fraction was brought to 5 mM sodium pyrophosphate and the pH adjusted to 6.8 by the addition of 1 M Tris. After being cooled to 4 °C, the supernatant solution was immersed in a −10 °C constant-temperature bath, and the dropwise addition of $^1/_5$th volume of 2-propanol was begun. At the end, the temperature of the solution was −4 °C. Stirring was continued another 10 min and the material centrifuged at 3000$g$ for 20 min. The pellet was dissolved in 100 mM Tris–phosphate, 0.2 mM EDTA, 0.2 mM fructose 1,6-bisphosphate, 1 mM 2-mercaptoethanol, and 5 mM sodium pyrophosphate (pH 8.0). After several hours dialysis against this buffer, the material was brought to 57 °C and held at this temperature for 3 min; it is then rapidly cooled and centrifuged. The supernatant was collected and a 38–55% ammonium sulfate fractionation performed in order to concentrate the enzyme. After centrifugation of the final precipitate, the pellet was taken up in

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; F2,6-$P_2$, fructose 2,6-bisphosphate; $NaDodSO_4$, sodium dodecyl sulfate.

a solution containing 50 mM Tris–phosphate, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol plus 5 mM sodium pyrophosphate (pH 8.0) and dialyzed in preparation for ion-exchange column chromatography. DE-51, DE-52, and DE-53—each equilibrated in the preceding buffer—were packed into three columns (2.5 cm × 15 cm) and connected in series. These anion-exchanging celluloses differ in their degree of substitution with the diethylaminoethyl functional group. After application of the protein sample and washing with the equilibration buffer, a linear gradient of 0.1–0.3 M Tris–phosphate (total volume 1000 mL) was applied. Each sample was assayed for catalytic activity and analyzed for phosphate content (see below). Appropriate samples were pooled, dialyzed against a buffer containing 50% glycerol, 0.1 M potassium phosphate, 0.2 mM EDTA, 5 mM pyrophosphate, and 1 mM $\beta$-mercaptoethanol (pH 8.0), and stored at −70 °C. The specific activity of the pooled samples was greater than 200 units/mg and judged homogeneous from $NaDodSO_4$ gel electrophoresis. The protein concentration was determined by using the Bradford method with bovine serum albumin as a standard (Bradford, 1976) or by using the absorbance value of $A^{0.1\%}_{280nm} = 1.07$ (Hesterberg & Lee, 1980).

The enzyme was assayed spectrophotometrically by coupling fructose 1,6-bisphosphate formation to the oxidation of NADH through the use of the enzymes aldolase, triosephosphate isomerase, and $\alpha$-glycerol-phosphate dehydrogenase. The optimal activity is generated in a buffer containing 50 mM glycylglycine (pH 8.2), 1 mM EDTA, 2.5 mM dithiothreitol, 5 mM ammonium sulfate, 0.16 mM NADH, 5 mM $MgCl_2$, 1 mM ATP, 1 mM fructose 6-phosphate, aldolase (0.4 unit), triosephosphate isomerase (2 units), and $\alpha$-glycerol-phosphate dehydrogenase 0.4 unit) in a total volume of 1 mL (Racker, 1947). The activity was also measured at pH 7.0 where the allosteric effects are enhanced by use of a 50 mM Mops/KOH (pH 7.0) buffer containing 2.5 mM dithiothreitol, 0.5 mM ammonium sulfate, and 3.0 mM $MgCl_2$ plus the previously specified concentrations of coupling enzymes. Typically, the assay mixture contained ca 0.5 $\mu$g of phosphofructokinase. After addition of the indicated fructose 6-phosphate concentrations, ±fructose 2,6-bisphosphate, ±F-actin, and ±G-actin (70-fold molar excess over phosphofructokinase when either of the latter was used), the reaction mixture was incubated for 1 min and the reaction initiated with ATP (1.0 mM final concentration).

*Miscellaneous Procedures.* Protein-bound phosphate was determined according to the method of Hasegawa et al. (1982). Purified phosphorylase *a* and/or phosphoserine were used as standards. F-Actin was used as a control to show that noncovalently bound nucleotides etc. were removed in the washing procedures. After trichloroacetic acid precipitation, the pellet was washed with water. Resuspension and washing were repeated 3 times before the samples were ashed and phosphate determined.

$NaDodSO_4$–acrylamide gels were prepared and run according to Pollard (1982). Gels (7.5%) were run with myosin heavy chain, phosphorylase, bovine serum albumin, actin, carbonic anhydrase, troponin C, and parvalbumin as molecular weight standards.

DNase I, purified according to the procedure of Wang et al. (1978), was assayed spectrophotometrically by using solutions containing 30 $\mu$g/mL DNA, 6.5 nM DNase I, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.5 (Hitchcock et al., 1977).

Phosphorylation reactions employing the catalytic subunit of the cAMP-dependent protein kinase were performed in a buffer containing 50 mM Mops/KOH (pH 7.0), 0.5 mM EGTA, 5 mM $MgCl_2$, 0.5 mM ATP, 2.5 mM dithiothreitol, and 1 mg/mL phosphofructokinase. The reaction was initiated by the addition of 5 $\mu$g/mL catalytic subunit. Aliquots were taken out at various times for catalytic assay and phosphate determination. Two-hour incubation periods were used for the preparation of maximally phosphorylated samples.

## Results

*Problems in the Purification of Phosphofructokinase.* Our initial preparations of rabbit muscle phosphofructokinase followed the procedure of Ling et al. (1966). Assay of the fractions obtained from column chromatography on (diethylaminoethyl)cellulose (DE-52) revealed a distribution of activity in which about 30% of the enzyme units typically appear in the break-through fraction (100 mM Tris–phosphate, pH 8.0) and the remaining 70% in the eluate obtained on application of 300 mM Tris–phosphate. $NaDodSO_4$ gel electrophoresis experiments show that the enzyme which was bound is electrophoretically homogeneous while the break-through fraction contains additional components, including a prominent protein of apparent molecular weight 42 000 which is present in approximate equimolar ratio with the enzyme. No additional adsorption occurs when the pool of unbound protein is applied to a reequilibrated column of DE-52. Since the molecular weight of the contaminant is close to that of the actin monomer, we investigated the effect of this fraction on the catalytic activity of DNase I. Mannherz et al. (1975) found that DNase I forms a high-affinity complex with G-actin, promoting depolymerization of F-actin. This association is accompanied by inhibition of DNase I activity (Lindbergh, 1967; Lazarides & Lindbergh, 1974). The addition of 25 $\mu$g/mL break-through protein fraction to the standard DNase I assay results in 95% inhibition, suggesting that the 42 000-dalton contaminant is indeed actin. (Pure phosphofructokinase alone has no effect on DNase I.) The apparent copurification of actin and phosphofructokinase points to an obstacle to overcome in the efficient fractionation of the enzyme as well as to the stability of its complex with actin. Actin itself would also bind to the DE-52 medium under the above conditions.

Adoption and modification of the purification procedure of Kemp (1975) allowed us to obtain actin-free phosphofructokinase which is partially resolved into fractions exhibiting varying degrees of phosphorylation. The addition of a final chromatographic separation, utilizing columns of DE-51, DE-52, and DE-53 connected in series, gives the resolution depicted in Figure 1. Determinations of total phosphate showed that the most heavily phosphorylated enzyme occurs in fractions 89–93; the average composition for this pool is 1.29 mol of phosphate/82 000 g of enzyme. Charcoal treatments and/or gel filtration of the enzyme have no effect on its phosphate content. This fraction is flanked by enzyme exhibiting lower levels of phosphorylation. $NaDodSO_4$ gel electrophoresis (inset to Figure 1) demonstrated the homogeneity of all active fractions. Most of our experiments were performed with fractions I and II, designated respectively as "low"- and "high"-phosphate phosphofructokinase. Fraction III was examined in less detail. The addition of 5 mM pyrophosphate, a phosphatase inhibitor, to the supernatant extracts of rabbit muscle and to the solutions used in all subsequent fractionations is necessary for consistency in the preparation of the different enzyme fractions.

*Association of Phosphofructokinase with Actin.* The association of phosphofructokinase with actin is observable on several levels. The addition of phosphofructokinase to solutions containing 0.2 mg/mL F-actin (previously polymerized) in 5

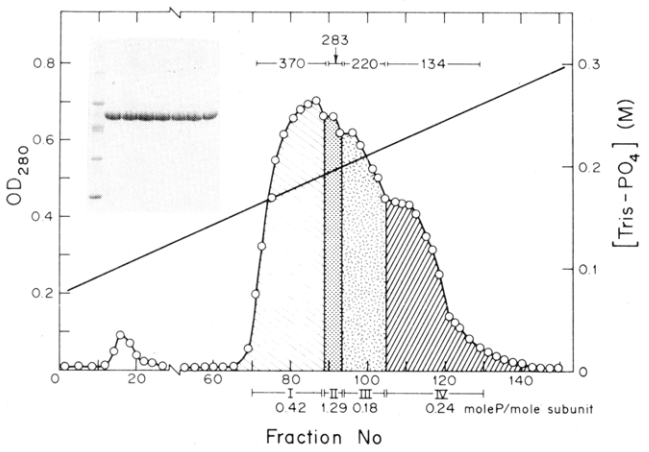


FIGURE 1: Fractionation of rabbit muscle phosphofructokinase on a connected series of DE-51, DE-52, and DE-53 columns. The enzyme was eluted with a gradient ranging from 0.1 to 0.3 M Tris–phosphate in 5 mM *sodium* pyrophosphate, 0.2 mM EDTA, and 1 mM β-mercaptoethanol, pH 8.0. The fractions contained *6.5* mL each. The Roman numerals designate pools containing 0.42 mol of P/82 000 g of enzyme (I), 1.3 mol of P/82 000 g (II), 0.18 mol of P/82 000 g (III), and 0.24 mol of P/82 000 g (IV). The figures above the peaks show the corresponding specific activities determined under standard assay conditions (see Materials and Methods). The inset shows photos of polyacrylamide–$NaDodSO_4$ electrophoresis gels representing the four major fractions. From left to right, the channels correspond to the standards and to fractions IV, III, III, II, II, I, and I.

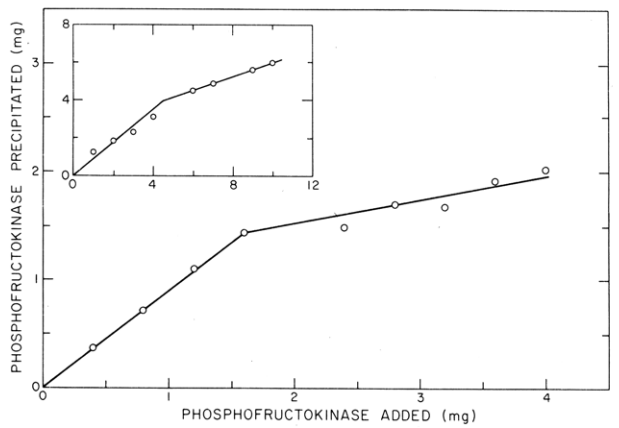


FIGURE 2: Precipitation of phosphofructokinase by F-actin. Various amounts of enzyme were added to a 1.0-mL solution containing 0.20 mg/mL previously polymerized rabbit skeletal muscle F-actin plus 5 mM glycylglycine, 5 mM glycerophosphate, 5 mM KCl, and 1.0 mM $MgCl_2$, pH 7.0 (25 °C). The plot shows the milligrams of enzyme present in the washed precipitate as a function of the total milligrams added. See text for details. The inset shows the ratio of enzyme to actin (mol/mol) in the precipitate vs. the ratio initially present. The enzyme was obtained by the procedure of Ling et al. (1966).

mM glycylglycine, 5 mM glycerophosphate, 5 mM KCl, and 1 mM $MgCl_2$, pH 7.0 (25 °C), results in precipitation. Sodium dodecyl sulfate gel electrophoresis on the resulting protein pellets, which are collected by centrifugation at 12000*g* and washed with a volume of buffer equal to twice the supernatant volume, shows that nearly total precipitation occurs up to the addition of 0.25 mol of phosphofructokinase tetramers/mol of actin monomer (Figure 2). Still further association takes place, although incomplete, as additional enzyme is added. Possibly the actin-bound enzyme associates with free phosphofructokinase or with other phosphofructokinase molecules bound to actin, producing a cross-linked network which precipitates. The solubility of this complex is enhanced by low concentrations of ADP and ATP and also by fructose 6-phosphate, fructose 1,6-bisphosphate, and citrate (Figure 3). The distinctive effects of F-actin on activity measurements in

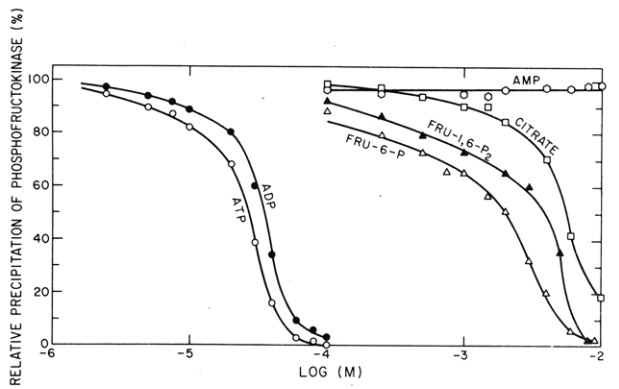


FIGURE 3: Solubilization of the phosphofructokinase–F-actin adsorbate by enzyme effectors and other metabolites. The solution initially contained 0.20 mg/mL previously polymerized F-actin plus 0.40 mg/mL phosphofructokinase prepared by the procedure of Ling et al. (1966). The molar concentrations of effector (*M*) are plotted on a logarithmic scale. AMP, creatine, and phosphocreatine have minimal effects through concentrations of 0–0.01 M. See Figure 2 for details.

the presence of ATP or citrate (Liou & Anderson, 1980) indicate that the interaction between the two proteins may still occur after solubilization. The solubility of the complex is *not* enhanced by AMP or phosphocreatine, which were tested at concentrations up to 0.01 M. 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: 33% with NaCl and 5–10% with KCl. The relative insolubilities at the protein concentrations used in analytical ultracentrifugation and other hydrodynamic or spectroscopic procedures limit the range of direct binding measurements obtainable with the system. Note that the concentration of F-actin in muscle is much higher—25–30 mg of actin/g (Hasselback & Schneider, 1951)—than that used here. Considering the physiological concentration of the enzyme, 0.33 mg/g or less (DeDuve, 1972), association in vivo would occur at the maximum rate of about one phosphofructokinase tetramer per 640 actin monomers. This ratio was approximated in our original activity measurements (Liou & Anderson, 1980).

One of the major effects of F-actin on phosphofructokinase activity is reflected in a decrease in the $K_m$ for the substrate fructose 6-phosphate (Liou & Anderson, 1980). In view of the tedious nature of the pH-stat assay used in our first report, we have applied the coupled assay method of Racker (1947) in the present study. This method also was used by Uyeda et al. (1977) to determine effects of the inner erythrocyte membrane on phosphofructokinase activity. Figure 4 shows the increases in phosphofructokinase activity measured at a nonsaturating level of fructose 6-phosphate (0.1 mM) when varying proportions of F-actin are added to solutions containing 1.5 nM enzyme tetramer. Even though the binding equilibrium is likely to be complex, the concentration dependence can be described simply. In the case of fraction I, the apparent $K_d$ = 15.4 ± 1.7 nM actin (given in terms of monomer), the Hill coefficient ($n$) = 1.3, and maximum activation = 3.3-fold. With fraction II, $K_d$ = 9.7 ± 1.9 nM actin, $n$ = 1.3, and maximum activation = 2.3-fold. Since these actin concentrations are ~60-fold smaller than the critical level necessary for polymerization (ca. 0.04 mg/mL) [cf. review by Cooper & Pollard (1982)], a certain amount of dissociation of F-actin to monomeric actin must occur in this experiment. Rapid dissociation of actin filaments is known to take place on dilution (Walsh et al., 1982). However, the distribution of small oligomers and monomers in the disassembled state remains undetermined (Maruyama & Tsukagashi, 1984). Differences

Table I: Summary of Catalytic Properties of Fraction I and II Phosphofructokinase When Fructose 6-Phosphate Is the Variable Substrate[a]

| additions | $K_{m\,app}$ (mM) | | $V_{max}$ (units/mg) | | $n_H$ | |
|---|---|---|---|---|---|---|
| | I | II | I | II | I | II |
| none | 0.26 | 0.27 | 23.8 | 21.5 | 3.0 | 3.0 |
| F2,6-$P_2$ | 0.081 | 0.105 | 29.6 | 24.9 | 1.7 | 1.6 |
| F-actin | 0.15 | 0.22 | 27.7 | 24.6 | 2.0 | 2.5 |
| F-actin + F2,6-$P_2$ | 0.086 | 0.093 | 53.8 | 25.1 | 1.4 | 1.6 |
| G-actin | 0.25 | 0.26 | 24.9 | 23.1 | 2.1 | 1.8 |
| G-actin + F2,6-$P_2$ | 0.13 | 0.19 | 27.2 | 25.8 | 2.2 | 1.5 |
| actin trimer | 0.15 | 0.13 | 24.0 | 21.5 | 3.0 | 3.1 |
| trimer + F2,6-$P_2$ | 0.060 | 0.096 | 26.3 | 25.0 | 1.8 | 1.5 |
| actin core | 0.30 | 0.32 | 22.5 | 21.2 | 3.2 | 3.3 |
| actin core + F2,6-$P_2$ | 0.12 | 0.078 | 25.4 | 28.8 | 1.8 | 1.7 |

[a] 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 $(NH_4)_2SO_4$, 2.5 mM dithiothreitol, and 50 mM Mops/KOH, pH 7.0 (2 °C). 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.

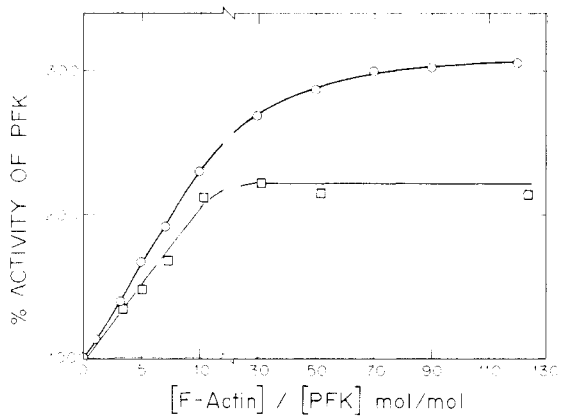


FIGURE 4: Activation of rabbit muscle phosphofructokinase as a function of the concentration of added F-actin (expressed as protomer concentration). Results are shown for phosphofructokinase fractions I (○) and II (□) isolated according to our modification of the procedure of Kemp (1975). Conditions: 0.5 μg/mL enzyme, 50 mM Mops/KOH, 0.10 mM fructose 6-phosphate, 1.0 mM ATP, 3.0 mM $MgCl_2$, 0.5 mM $(NH_4)_2SO_4$, and 2.5 mM dithiothreitol (pH 7.0, 25 °C). See Materials and Methods for other details.

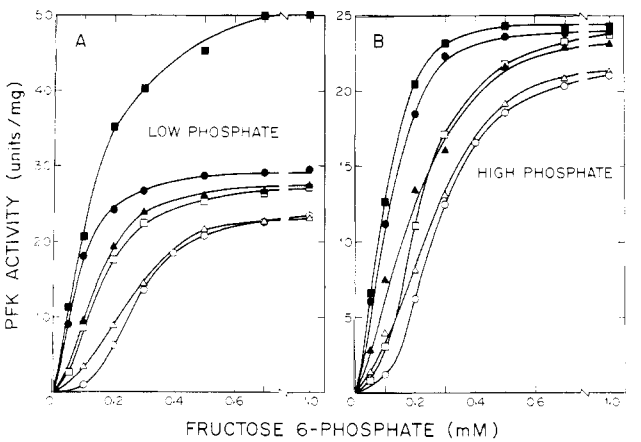


FIGURE 5: Influence of F-actin, G-actin, and fructose 2,6-bisphosphate on the catalytic activity of two major fractions of rabbit muscle phosphofructokinase prepared according to our modification of the procedure of Kemp (1975). Reaction rates were determined as a function of varied concentrations of fructose 6-phosphate and fixed concentrations of ATP (1 mM) and enzyme (0.5 μg/mL). 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 fraction I (0.42 mol of phosphate/82 000 g) and panel B on fraction II (1.3 mol of phosphate/82 000 g). Conditions: 50 mM Mops ($K^+$), 3 mM $MgCl_2$, 2.5 mM dithiothreitol, and 0.5 mM $(NH_4)_2SO_4$, pH 7.0 (25 °C). See Materials and Methods for other details.

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 are performed at an excess actin concentration, 0.12 μM or 70 mol of actin/mol of enzyme, giving nearly maximal enhancement of activity.

There was no sign of the precipitation which takes place when higher concentrations of the enzyme and F-actin are mixed. The apparent avidity of the 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.* Fluorescence binding measurements had previously demonstrated the binding of 3 mol of 1,$N^6$-ethenoadenosine triphosphate (εATP)/82 000 g of enzyme and the enhancement of εATP binding in the presence of citrate (Liou & Anderson, 1978). The inhibition of rabbit muscle phosphofructokinase by either citrate or high concentrations of ATP is partially reversed in the presence of F-actin. The allosteric activator AMP and F-actin have similar effects on the activity of the enzyme. The *maximum* activation observed in mixtures of the two effectors is about the same as that obtained with either AMP or F-actin alone (Liou & Anderson, 1980). Exploration of the role of all known effectors of either actin or phosphofructokinase on the interaction of the two proteins is a multidimensional problem. In the present work, we have concentrated on the effects of the state of actin polymerization, on the role of variously phosphorylated states of the enzyme, and on the action of fructose 2,6-bisphosphate. Activity measurements were obtained as a function of the fructose 6-phosphate concentration using a noninhibitory concentration of ATP (1 mM) which gives nearly optimal reaction rates. Figure 5 shows the results obtained with the low- and high-phosphate phosphofructokinase samples (fractions I and II). These two fractions behave very similarly in the absence of added effectors. The addition of F-actin (0.12 μM) stimulates the activity of both forms of the enzyme: the apparent $K_m$'s and Hill coefficient for fructose 6-phosphate decrease while $V_{max}$ increases by ca. 15% (Table I). G-Actin (0.12 μM) has minimal effect on either of the enzymes.

The largest difference between the two phosphofructokinase fractions appears when added F-actin and fructose 2,6-bisphosphate are studied in combination. Figure 5 demonstrates the marked increase in $V_{max}$ obtained with the low-phosphate enzyme and actin plus fructose 2,6-bisphosphate but not with the high-phosphate enzyme. Observations on three different enzyme preparations have confirmed this substantial difference

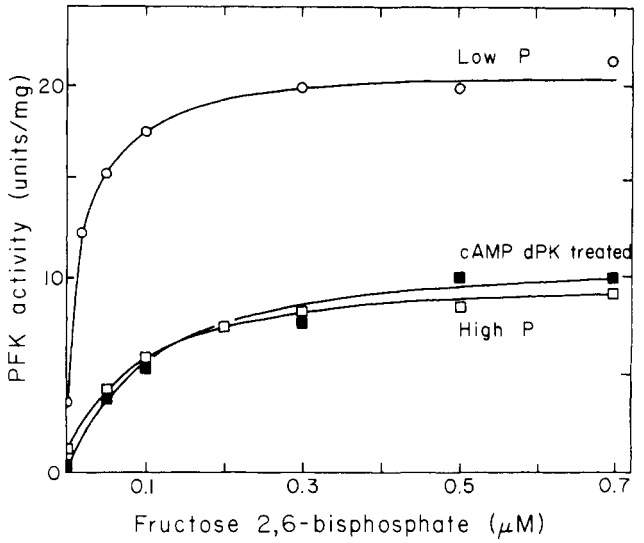


FIGURE 6: Effect of in vitro phosphorylation, catalyzed by the cAMP-dependent protein kinase, on the sensitivity of fraction I to fructose 2,6-bisphosphate. Results are shown for fraction II (□) and for fraction I before (○) and after (■) phosphorylation. Conditions: 0.5 μg/mL enzyme, 50 mM Mops/KOH, 0.10 mM fructose 6-phosphate, 1.0 mM ATP, 3.0 mM $MgCl_2$, 0.5 mM $(NH_4)_2SO_4$, and 2.5 mM dithiothreitol (pH 7.0, 25 °C). See Materials and Methods for other details.

in the properties of low- and high-phosphate phosphofructokinase. Parallel experiments with G-actin indicate that it also associates with phosphofructokinase, but with completely different consequences. 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 that determined with fructose 2,6-bisphosphate alone.

Fraction III, containing 0.18 mol of phosphate/82 000 g, was characterized in less detail. It is generally similar to fractions I and II. However, its sensitivity to the combined effects of F-actin and fructose 2,6-bisphosphate is not as pronounced as that of fraction I. The value of $V_{max}$ 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.

*Relationship between the Low- and High-Phosphate Enzyme Fractions.* The cAMP-dependent protein kinase is the enzyme most likely to be responsible for the in vivo phosphorylation of phosphofructokinase. Foe and Kemp (1982) reported phosphorylation experiments on pooled fractions of phosphofructokinase in which 0.4 mol of P/82 000 g could be incorporated in vitro. We find that the total phosphate present after incubation with the purified catalytic subunit of the cAMP-dependent protein kinase, as described under Materials and Methods, approaches 2 mol of phosphate/mol of enzyme protomer (2.0 ± 0.1) with both fraction I and fraction II. Activity measurements on the fraction which had originally contained 0.42 mol of endogenous P/mol of protomer (I) show that its catalytic properties approach but do not equal those of the endogenous high-phosphate enzyme. The stimulatory effect of fructose 2,6-bisphosphate in the standard assay is diminished after phosphorylation, with the enzyme becoming more like the native high-phosphate enzyme than it is like low-phosphate enzyme (Figure 6). The joint effects of F-actin and fructose 2,6-bisphosphate in the activation of the treated enzyme are intermediate between those found with the original low- and high-phosphate fractions. In a set of parallel experiments, the $V_{max}$ of the fraction I enzyme decreased from 180% of its basal value (determined in the absence of effectors)

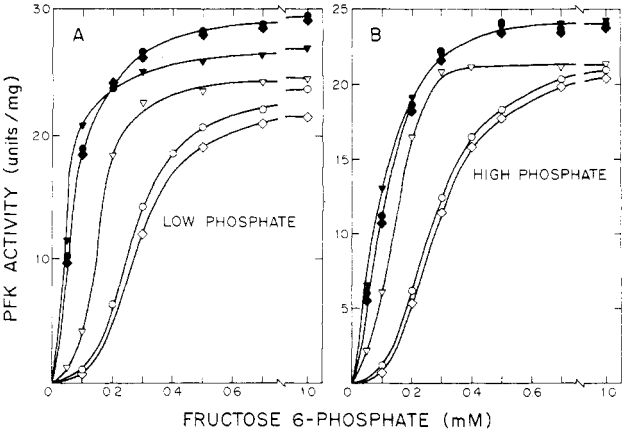


FIGURE 7: Effects of the cross-linked trimer and the chymotrypsin-resistant actin core on the catalytic activity of phosphofructokinase. Reaction rates were determined as a function of varied concentrations of fructose 6-phosphate and fixed concentrations of ATP (1 mM) and enzyme (0.5 μg/mL). Results 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, 5 μg/mL + 3 μM (◆); and no additions (○). See Figure 5 for other details.

to 140% following phosphorylation catalyzed by the cAMP-dependent protein kinase.

*Effects of Actin Modification.* Removal of the first 67 or so amino acid residues from actin leaves a chymotrypsin-resistant core which fails either to polymerize or to activate myosin but retains high-affinity ATP binding (Jacobson & Rosenbusch, 1976). This core, tested at 1.5 μM, has minimal effects on the activities of both the low- and high-phosphate fractions of phosphofructokinase (Figure 7). It also fails to inhibit DNase I at concentrations where G-actin gives ~50% inhibition (5 nM). This suggests that the N-terminal region of actin is involved in the binding of myosin, DNase I, and phosphofructokinase and that the tightly bound ADP or ATP molecule of actin does not contribute solely to the activation of our enzyme. The interaction of phosphofructokinase with the core is apparently much less than that found with G-actin, which is moderately inhibitory under certain conditions (Figure 5).

Covalently cross-linked actin trimers and dimers, prepared by using the reagent phenylenebis(maleimide), 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 purified trimer, tested at 5 μg/mL, activates both fractions of phosphofructokinase very efficiently (Figure 7). Apparently, it is a nearly minimal unit capable of activating both myosin ATPase activity (Knight & Offer, 1978) and phosphofructokinase phosphotransferase activity. However, the reduction of the Hill coefficeint obtained with F-actin and the synergistic activation of fraction I are absent in the case of the trimer (Table I).

## DISCUSSION

A connected series of DE-51, DE-52, and DE-53 chromatography columns used in the final purification of rabbit muscle phosphofructokinase facilitates the resolution of the enzyme into electrophoretically homogeneous[2] subpopulations which differ both in their catalytic properties and in their endogenous covalent phosphate content. The incorporation of phosphatase inhibitors, potassium fluoride in the initial extraction (Ling et al., 1966; Kemp, 1975; Hofer et al., 1982)

[2] According to $NaDodSO_4$ gel electrophoresis.

and sodium pyrophosphate through all remaining purification steps, is essential for the isolation of phosphofructokinase of reasonably uniform phosphate content from preparation to preparation. Pyrophosphate is avoided in the original extraction since its use there leads to marked contamination of the enzyme with actin and myosin. We note that the standard isolation procedure of Ling et al. (1966) results in a highly variable phosphate content as well as a substantial fraction (~30%) of phosphofructokinase that is apparently bound to actin. A band of high-phosphate enzyme (fraction II) flanked by regions of low-phosphate enzyme (fractions I and III) is an unusual feature of the purification profile. We have concentrated on the characterization of fractions I and II because they exhibit the most pronounced functional differences.

The quantity of phosphate in fraction II, 1.0–1.6 mol of P/82000 g in various preparations of the enzyme, exceeds that of the phosphorylated rabbit muscle phosphofructokinase described by Uyeda et al. (1978). The latter, which corresponds to the DE-52-bound fraction of enzyme obtained in the procedure of Ling et al. (1966), contained 0.2 mol of P/82000 g. Foe and Kemp (1982) were able to incorporate only 0.4 mol of P/82000 g through in vitro phosphorylation of a pooled phosphofructokinase preparation. However, Hofer and Sorensen-Ziganke (1979) found 0.5 mol of P/82000 g when phosphofructokinase is prepared from resting muscle which was stored on ice immediately prior to extraction, 1.2 mol of P/82000 g when resting muscle was quickly frozen in liquid nitrogen, and 1.98 mol of P/82000 g when the muscle was quickly frozen after nervous stimulation which had caused the muscle to contract rhythmically for 10 min. Trypsin digestion of their enzyme preparation followed by fingerprinting revealed two different phosphopeptides. Muscle phosphofructokinase from *Ascaris* contains up to 2 mol of P per subunit (Hofer et al., 1982). Labeling experiments with [$\gamma$-$^{32}$P]ATP suggested that all of the phosphate incorporated into rabbit muscle phosphofructokinase may be introduced in vivo by the cAMP-dependent protein kinase (Kemp et al., 1981). However, experiments with propranolol- and epinephrine-treated rabbits showed that glycogen synthase exhibits large differences in phosphorylation under conditions where phosphofructokinase does not (Parker et al., 1982). Phosphorylase kinase, myosin light chain kinase (Kemp & Foe, 1983), and epidermal growth factor dependent tyrosine kinase (T. Hunter, personal communication) are unable to phosphorylate phosphofructokinase in vitro.

Our fractions display variations in specific activity and other kinetic properties. The largest differences are seen between pools I and II, which contained 0.42 and 1.3 mol of P/82000 g in the present study. The low-phosphate phosphofructokinase is synergistically activated in the presence 0.12 $\mu$M added F-actin plus 3.0 $\mu$M fructose 2,6-bisphosphate, with a marked increase in $V_{max}$, while the high-phosphate enzyme is not.[3] The total amount of covalently bound phosphate approaches 2 mol of P/82000 g when either fraction I or fraction II is phosphorylated in vitro in reactions catalyzed by the isolated subunit of the cAMP-dependent protein kinase. Although in vitro phosphorylation of fraction I causes its properties to become more like those of the original fraction II, they are not exactly the same. Fraction III, containing 0.18 mol of P/82000 g, has a lower specific activity than fraction I and is less sensitive to synergistic activation. We are apparently dealing with a heterogeneous population of enzyme molecules, where the subpopulation within each group behaves somewhat differently. Differences in phosphorylation state may account for some or most of the heterogeneity. The difficulty of assessing the role of various phosphorylation sites was illustrated in the case of glycogen synthase (Parker 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 phosphorylated if it is accessible.

Our observations are consistent with the known role of cAMP in the regulation of fructose 2,6-bisphosphate levels. Through the actions of protein kinase on fructose-6-phosphate 2-kinase and fructose 2,6-bisphosphatase, cAMP both prevents the formation and favors the destruction of fructose 2,6-bisphosphate (Richards et al., 1982; Van Shaftingen et al., 1982). When fructose 2,6-bisphosphate levels are high, the phosphorylation level of the enzyme is expected to be low and its activation by F-actin maximal. The apparently slow rates of phosphorylation and dephosphorylation of phosphofructokinase (Foe & Kemp, 1983) would not allow rapid response to changing metabolic conditions in the cell. However, they could be compatible with a structural role. For example, the level of phosphofructokinase activity in fertilized *Urechis* eggs increases markedly over the 30-min time interval where substantial cytoskeletal organization occurs (Tazawa & Yasumasu, 1977). That low concentrations of added F-actin activate rabbit muscle phosphofructokinase while G-actin exhibits minimal, even slightly inhibitory, effects suggests that cytoskeletal organization could play a role in the regulation of the enzyme. Although reorganization does not occur in striated muscle, preliminary work shows that smooth muscle F-actin—the isoactin characteristic of nonmuscle cells (Rubenstein & Spudich, 1977)—also binds and activates phosphofructokinase (unpublished data).

The contrasting effects obtained on the addition of F- and G-actin to phosphofructokinase 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 are presently under investigation by us: (1) Small oligomers of actin activate 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; Fouayrenc & 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 nonmuscle cells, proteins such as profilin stabilize the pool of unpolymerized actin (Carlson et al., 1977).

Classically, the glycolytic enzymes are known as soluble or cytoplasmic proteins. However, increasing evidence points to a more complicated situation in which the enzymes are de-

[3] Luther and Lee (1985) have reported preliminary studies on the binding of phosphorylated phosphofructokinase by actin.

scribed as *ambiguitous* [cf. review by Wilson (1978)]. Certain enzymes exhibit intracellular partitioning between soluble and particulate forms. Skeletal muscle has provided the most detail regarding these interactions, primarily because the system is well-defined in terms of both protein composition and structure. Among the glycolytic enzymes, phosphofructokinase and aldolase have been the most extensively studied [cf. reviews by Clarke & Masters (1976), Arnold & Pette (1968, 1970), and Clarke et al. (1984)]. Liou and Anderson (1980) originally showed that the activity of phosphofructokinase is affected both by F-actin and by the reconsituted thin filament, that these effects occurred at low and high ionic strengths, and that the effects of F-actin are modulated by allosteric effectors. Adsorption to the inner surface of erythrocyte membranes alters the catalytic properties of rabbit and human phosphofructokinase (Karadsheh & Uyeda, 1977). Although actin is a major component of the membrane (Marchesi et al., 1976), Richards et al. (1979) ascribed this activation to the band 3 protein.

In skeletal muscle that has been frozen (Kemp, 1975) or in heart tissue for which there was a delay of 20 min before processing (Choate et al., 1985), phosphofructokinase activity occurs exclusively in the particulate fraction. These enzymes can be subsequently solubilized by millimolar concentrations of ATP. This observation is consistent with our results in the F-actin–phosphofructokinase "precipitation" experiments, where precipitation is prevented by the addition of ATP. Phosphofructokinase and aldolase are also bound to the thin filaments within a short time after onset of artificially induced anoxia and ischemia in hearts (Clarke et al., 1984).

**Registry No.** F2,6-$P_2$, 79082-92-1; phosphofructokinase, 9001-80-3; fructose 6-phosphate, 643-13-0.

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# $\alpha$-Aminoboronic Acid Derivatives: Effective Inhibitors of Aminopeptidases†

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*Received May 23, 1985*

ABSTRACT: $\alpha$-Aminoboronic acids and their derivatives have been synthesized as stable white solids. These compounds are effective inhibitors of human enkephalin degrading aminopeptidase, microsomal leucine aminopeptidase (EC 3.4.11.2), and cytosolic leucine aminopeptidase (EC 3.4.11.1) at micro- to nanomolar concentrations. The inhibition of cytosolic leucine aminopeptidase has been studied in some detail. Kinetic data correspond to the mechanism for biphasic slow-binding inhibition: $E + I \rightleftarrows E{\cdot}I \rightleftarrows E{\cdot}I^*$, in which a rapid initial binding is followed by a slow transformation to a stable enzyme inhibitor complex. The initial and final binding constants are dependent on the nature of the side chain at the $\alpha$-carbon atom but are independent of the protecting group on the boronic acid moiety and follow the trend for the hydrolysis of the corresponding amino acid amides. The first-order rate constant for the transformation of E·I to E·I* is similar for all four compounds studied. These data suggest that the slow-binding step represents the formation of tetrahedral boronate species from trigonal boronic acid.

Aminopeptidases are a group of metalloenzymes that catalyze the hydrolysis of the $NH_2$-terminal peptide bonds in polypeptides. Aminopeptidases with similar or identical properties have been found in many tissues, including the lens, kidney, pancreas, muscle, and liver [for reviews, see Smith & Hill (1960) and Delang & Smith (1971)]. Although their mechanism of action is unclear, the primary amino acid sequence of leucine aminopeptidase from bovine eye has been reported (Cuypers et al., 1982). Studies on the mechanism of action of aminopeptidases have been reported (Lin & Van Wart, 1982; Makinen et al., 1982; Taylor et al., 1982; Carson & Carpenter, 1983; Baker & Prescott, 1980; Allen et al., 1983; Baker et al., 1983). Effective inhibitors reported for aminopeptidases include amino ketones and derivatives (Birch et al., 1972; Kettner et al., 1974), small peptide analogues from microbial sources (Umezawa et al., 1976; Aoyagi et al., 1978; Rich et al., 1984), aliphatic and aromatic boronic acids (Baker et al., 1983), amino acid hydroxamates (Chan et al., 1982; Baker et al., 1983; Wilkes & Prescott, 1983), $\alpha$-aminoaldehydes (Andersson et al., 1982), and amino acid thiols (Chan, 1983). Of these, aliphatic boronic acids and $\alpha$-aminoaldehydes are thought to act as "transition-state analogues".

In the course of synthesis of enzyme inhibitors, we prepared a number of $\alpha$-aminoboronic acids and their derivatives, **1** (Figure 1), using a synthesis scheme similar to the one reported by Matteson et al. (1981). These compounds, in which the boron atom has trigonal geometry, can form a tetrahedral boronate ion and are expected to act as transition-state analogues for proteases (Koehler & Leinhard, 1971; Philipp & Bender, 1971). Indeed, [2-(*N*-acetylamidophenyl)ethyl]boronic acid (Matteson et al., 1981), as well as peptide boronic acids (Kettner & Shenvi, 1984), have been shown to be effective inhibitors of chymotrypsin (a serine protease). Intrigued by the possibility that $\alpha$-aminoboronic acids may act as transition-state analogues for aminopeptidases, we synthesized several of these compounds and tested them as inhibitors of human enkephalin degrading aminopeptidase (Coletti-Previero et al., 1982), microsomal leucine amino-

† Contribution No. 3784.

0006-2960/86/0425-1286$01.50/0 