Binding of Phosphofructokinase to Filamentous Actin†

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ABSTRACT: Phosphofructokinase (PFK) and filamentous actin from rabbit skeletal muscle form a specific association as demonstrated by electron microscopy of the negatively stained proteins. Actin paracrystals have distinct cross-striations when PFK is present. The periodicity of these striations, 37 ± 1.0 nm, corresponds to the crossover spacing of the actin helix, 36 ± 1.0 nm. Assays based on the sedimentation of actin indicate that PFK binds to actin in a concentration-dependent manner with no indication of saturation at a PFK:actin ratio 33 times higher than the ratio in mammalian skeletal muscle. This binding is maintained at physiological ionic strength. Increasing the pH from 6.7 to 7.5 causes a gradual elution of PFK from purified actin filaments. The binding of PFK to actin decreases the rate and extent of activity loss caused by the dissociation of PFK tetramers at low pH and low temperature. The reversible association of PFK with actin may play a role in regulating PFK activity and, therefore, glycolysis during periods of metabolic acidosis.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) plays an important role in the regulation of glycolysis; therefore, the allosteric properties of PFK[1] have been the focus of intensive research (Kemp & Foe, 1983). Recently, a new dimension has been added to the control of PFK activity: the discovery of the enzyme's ability to associate reversibly with the structural elements of cells. Liou and Anderson (1980) originally described the kinetic properties of PFK when associated with actin, and Luther and Lee (1986) and Kuo et al. (1986) have since shown that the phosphorylated form of the enzyme has a higher affinity for actin than the dephosphorylated enzyme. The presence of actin in the PFK assay medium decreases the enzyme's K_M for fructose 6-phosphate and reduces the inhibition of the enzyme at high concentrations of ATP. Higashi et al. (1970) conducted similar studies on the association of PFK with erythrocyte membranes and described kinetic effects analogous to the effects seen when PFK binds actin. Subsequent work using erythrocytes has shown that PFK binds to the acidic amino terminus of the membrane protein band 3, which also binds the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and aldolase (Jenkins et al., 1985).

The binding of PFK to structural proteins also may influence the tetramer–dimer equilibrium, which plays an important role in regulating PFK activity. The work of Jenkins et al. (1985) shows that band 3 protein in erythrocyte membranes initiates the slow, reversible loss of PFK activity. This inactivation is apparently a consequence of the dimerization of the catalytically active PFK tetramer. Membranes preferentially bind the inactive dimers and therefore enhance the dissociation of tetramers in solution.

The tetramer–dimer equilibrium of PFK is exquisitely sensitive to pH and temperature (Bock & Frieden, 1974). Since this inactivation is readily reversible and responsive to physiological shifts in pH, it has been postulated to function as a mechanism for decreasing glycolytic metabolism during hibernation in mammals (Hand & Somero, 1983) and during ischemia in the rat heart (Carpenter & Hand, 1986).

This study investigates the association of PFK with filamentous actin, the major protein of the I band. The I band has been identified as the site of glycolytic enzyme localization in skeletal muscle (Sigel & Pette, 1969; Dolken et al., 1975). By use of a new binding assay based on the low-speed sedimentation of actin paracrystals, the reversible binding of PFK to actin is demonstrated. Electron micrographs of these PFK–actin paracrystals indicate that the binding occurs only at specific sites along the actin filament. In addition, the effects of pH and ionic strength on the association of PFK with actin are described, and evidence for the stabilization of PFK tetramers by actin is presented.

Materials and Methods

Materials. Phosphofructokinase was obtained as a crystalline suspension in ammonium sulfate (type III) from Sigma Chemical Co. or purified according to the procedure of Hesterberg and Lee (1980). Comparable results were obtained with both of these enzyme preparations. After PFK was desalted into the appropriate buffer by passage through Sephadex G-25, the absorbance of PFK at 290 nm was used to calculate the concentration of PFK, assuming an extinction coefficient of 0.87 L cm^{-1} g^{-1} (Paetkau & Lardy, 1967). Glycerol-3-phosphate dehydrogenase, aldolase, and triose phosphate isomerase used in the coupled assay of PFK were purchased from Calbiochem (La Jolla, CA). All substrates were purchased from Sigma Chemical Co. (St. Louis, MO). Actin was extracted from an acetone powder of rabbit skeletal muscle as described by Pardee and Spudich (1982). The concentration of actin was determined spectrophotometrically, assuming an extinction coefficient at 280 nm of 1.11 L cm^{-1} g^{-1} (Houk & Ue, 1974). Tropomyosin was extracted by the method of Smillie (1982), and troponin was extracted by the method of Potter (1982). The protein concentrations of the tropomyosin and the troponin solutions were determined by the microbiuret method (Itzhaki & Gill, 1964).

Electron Microscopy. Solutions of F-actin or F-actin plus PFK were prepared in binding assay buffer [50 mM HEPES[1]–KOH, pH 7.3 at 20 °C, 0.2 mM $CaCl_2$, 0.5 mM

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; PFK, phosphofructokinase.

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fructose 6-phosphate, 2.0 mM dithiothreitol (DTT), and 0.005% sodium azide]. Shortly before the proteins were to be used for microscopy, $MnCl_2$ was added to a final concentration of 7.5 mM. Divalent cations such as Mn^{2+} induce filamentous actin to form a side-by-side array of filaments known as a paracrystal, with the crossover (or turn) of the actin helix aligned in a regular pattern. We found that day-old paracrystals lacked the uniformity of the freshly prepared ones. In some preparations, catalase crystals were included for internal size standardization. Drops of a 10-fold dilution of the protein preparations were placed onto ionized, carbon-coated, Formvar films and negatively stained with 2% uranyl acetate, pH 4.5. Micrographs were taken at several magnifications in a Philips 300 electron microscope operated at 80 kV.

Measurements, by use of a Bausch & Lomb measuring magnifier, were made on micrographs enlarged 2.5 times from the negative. Magnification was calibrated with catalase crystals (Wrigley, 1968). At least 14 micrographs, taken at three magnifications from several different grids and protein preparations, were used in determining the periodicity of both the actin helix and the PFK banding pattern.

Paracrystal Binding Assay. PFK and actin were prepared in binding assay buffer. The presence of fructose 6-phosphate in the buffer kept the specific activity of PFK constant during the experiments. Actin solutions of 4.0 mg/mL were polymerized with 2.5 mM $MgCl_2$ and diluted to 1.0 mg/mL for the binding assays. A quantity of a concentrated PFK solution was added to the other proteins, initiating a 30-min incubation at 20 °C. At the end of this incubation, $MnCl_2$ was added to a final concentration of 7.5 mM; this induces paracrystal formation. After 1 h at 20 °C, aliquots of the paracrystal solutions were centrifuged for 10 min at 13000*g* in a Fisher microcentrifuge. The supernatants were removed, and the pellets were resuspended in an equal volume of binding assay buffer. The initial paracrystal solution, supernatant, and resuspended pellets were assayed at 20 °C for maximal PFK activity according to the previously described fructose 1,6-bisphosphate coupled method (Bock & Frieden, 1974). Under these conditions the specific activity of the enzyme was 140-160 units/mg. Greater than 90% of the total enzyme activity could be accounted for in the supernatant and pellet fractions. For some experiments the enzyme assay results were confirmed by densitometric analysis of samples electrophoresed with sodium dodecyl sulfate on 10% polyacrylamide gels stained with Coomassie Blue.

To quantify this binding assay further, actin was isotopically labeled with *N*-[2-^{3}H]ethylmaleimide (Detmers et al., 1981; Murray et al., 1981). Control experiments in which the specific activity of the actin was varied showed that *N*-ethylmaleimide labeling did not affect the ability of actin to bind PFK. In all PFK–actin binding assays, 90% of the paracrystalline actin sedimented during the 10-min centrifugation at 13000*g*.

Effect of pH. A binding assay based on the ultracentrifugal sedimentation of actin was used for these experiments. Aliquots of the PFK–actin mixture were centrifuged for 30 min at 30 psi (175000*g*) in a Beckman Airfuge. Supernatants were assayed for PFK activity, and these values were used to calculate the percentage of PFK bound to actin. At the low concentrations of PFK used in these experiments, PFK did not sediment in the absence of actin. PFK (0.05 mg/mL) and actin (1.0 mg/mL) were incubated for 30 min at 20 °C in 50 mM BES–KOH, 0.5 mM fructose 6-phosphate, 0.2 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 1.0 mM DTT adjusted to the following pH values: 6.7, 6.9, 7.1, 7.3, and 7.5. PFK did not lose activity during the 30-min incubation in the samples at low pH due to the presence of fructose 6-phosphate and the moderate temperature (20 °C).

Effect of Ionic Strength. The ultracentrifugal binding assay described above was also used to study the effects of increasing ionic strength on the binding of PFK to actin. KCl or potassium acetate at 25 or 50 mM were added to aliquots of PFK (0.08 mg/mL) and F-actin (1.0 mg/mL) prepared in binding assay buffer. After a 30-min incubation at 20 °C, the samples were centrifuged and the supernatants assayed to determine the fraction of PFK bound relative to a control without added KCl or potassium acetate.

Specific Activity Measurements. PFK and various concentrations of actin were incubated for 25 min at 20 °C in the assay cuvette. The reaction was initiated by the addition of the PFK substrates. Fructose 6-phosphate and ATP, at the concentrations used in the substrate mixture, did not affect the binding of PFK to actin (data not shown). The assay buffer was 42 mM HEPES–KOH, pH 7.7, 51 mM KCl, and 5.1 mM NH_4Cl. PFK had been dialyzed against 0.1 mM potassium phosphate, pH 8.0, with 2.0 mM DTT. Actin was polymerized in the standard binding assay buffer without fructose 6-phosphate.

Phosphofructokinase Inactivation Studies. Concentrated stocks of PFK and F-actin were prepared in the binding assay buffer without fructose 6-phosphate as previously described. F-Actin was diluted into inactivation buffer (50 mM BES–KOH, pH 6.45, pH 6.3, or pH 6.15, 2.5 mM $MgCl_2$, 7.5 mM $MnCl_2$, 0.2 mM $CaCl_2$, 0.005% sodium azide), and then PFK was added, starting the inactivation. Samples were incubated at 6 °C, but PFK activity was assayed at 20 °C throughout the time course of the experiment. The enzyme solution was diluted 1:200 when assayed; this dilution prevents the reassociation of PFK dimers at the higher pH and temperature of the assay medium. Percent initial PFK activity was calculated from the activity at each time point during the inactivation relative to the activity measured at the time of mixing. Less than a 10% loss of PFK activity occurred in the 20 °C, pH 7.8, control after a 45-min incubation.

RESULTS

Electron Microscopy of PFK–Actin Aggregates. The addition of phosphofructokinase to actin gives the paracrystalline structure a striated appearance (Figure 1). Electron micrographs of negatively stained preparations show that striated paracrystals predominate at both the lower (0.006:1, Figure 1B) and the higher 0.08:1, Figure 1C) molar ratios of PFK to actin used in these studies. The repeat distance of the PFK striations is 37 nm (SD 1.0 nm, $n = 62$). This is close to the crossover spacing of the actin helix, 36 nm (SD 1.0 nm, $n = 16$), as measured on micrographs calibrated with catalase crystals (Wrigley, 1968). Other studies, using optical diffraction techniques, give a value of 35–36 nm for the spacing of the actin helix (Spudich et al., 1973; O'Brien et al., 1971). The transverse banding of the paracrystals appears to be caused by the linear alignment of PFK tetramers. The width of the bands, 11 nm (SD 2 nm, $n = 20$), corresponds to the 10 × 10 nm dimensions of a PFK tetramer (Foe & Trujillo, 1980), and tetrameric structures can be distinguished in several micrographs (Figure 1C, inset). Although some paracrystals have striations perpendicular to the filament axis, others in the same preparation have striations angled 28–32° relative to the filament axis. It is possible that PFK tetramers or linear aggregates of tetramers bridge adjacent actin filaments and that the angle (pitch) of the striations reflects the alignment of the filaments within the paracrystal (see Discussion).

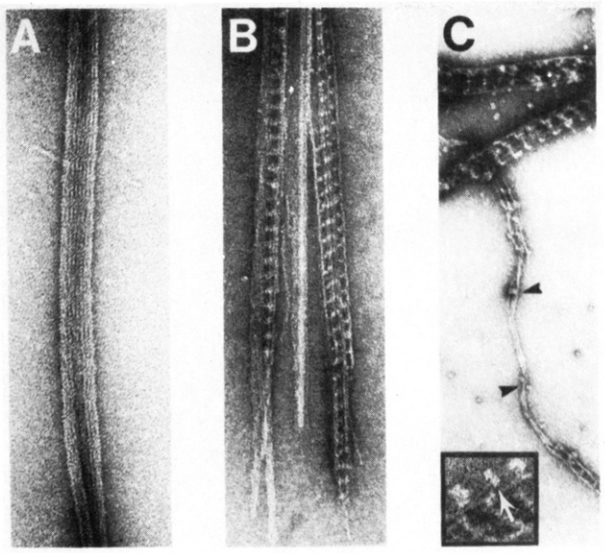

FIGURE 1: Electron micrographs of paracrystalline actin and phosphofructokinase (PFK). All concentrations of proteins given below refer to values determined before the samples were diluted 10-fold for microscopy in binding assay buffer with 7.5 mM $MnCl_2$. Samples were stained with 2% uranyl acetate (pH 4.5) for 30 s just prior to viewing. (A) Actin paracrystal. Faint striations perpendicular to the filament axis are indicative of the crossover of the actin helix. Actin concentration 1.0 mg/mL. Magnification 90750×. (B) Actin–PFK paracrystal. A distinct banding pattern appears on the paracrystalline actin filaments in the presence of PFK. Bands are roughly the width of a PFK tetramer (9.0–10.0 nm). Actin concentration 1.0 mg/mL; PFK concentration 0.05 mg/mL. Magnification 60500×. (C) Actin–PFK paracrystal. At a higher concentration of PFK (0.6 mg/mL), "bridges" composed of PFK tetramers connecting actin filaments (black arrows) and tetrameric structures (white arrow) can be distinguished on some micrographs (inset, magnification 90750×). Actin concentration 1.0 mg/mL; PFK concentration 0.6 mg/mL. Magnification 60500×.

Concentration Dependence of PFK Binding. To establish a binding curve for the association of PFK with actin, we developed an assay based on the low-speed sedimentation of Mn^{2+}-induced paracrystals. Using the paracrystalline form of actin allows us to compare results of the biochemical assay directly with the electron micrographs. Also, compared to the assay based on the sedimentation of F-actin during ultracentrifugation, the paracrystal assay avoids an artifact caused by the ultracentrifugal sedimentation of PFK aggregates formed at high PFK concentrations (Arnold et al., 1971; Clarke & Masters, 1975). No PFK, other than that bound to actin, sedimented at the low *g* forces used to sediment paracrystals. This low-speed sedimentation assay differs from that used by Kuo et al. (1986). In their assay, actin is precipitated by the addition of an excess of PFK over actin (by weight). In our assay, the sedimentation of actin is not dependent on the concentration of PFK; hence binding could be examined at lower, more physiological ratios of PFK and actin. Direct comparisons of the amount of PFK bound to paracrystals and filaments were made at low PFK concentrations, where the amount of PFK sedimented during ultracentrifugation in the absence of actin was a minor fraction of the total PFK. With constant concentrations of PFK and actin, about 10% more PFK bound to paracrystalline actin than to filamentous actin.

Binding as a function of PFK concentration in binding assay buffer with and without 5 mM $(NH_4)_2SO_4$ is linear [slope = 0.66, *y* intercept = –0.003, and $R = 0.99$ without $(NH_4)_2SO_4$, and slope = 0.60, *y* intercept = –0.019, and $R = 0.99$ with $(NH_4)_2SO_4$] and shows no indication of saturation. Because PFK forms large aggregates at concentrations greater than 1.0 mg/mL in this assay system, the concentration of PFK required to saturate the actin binding sites could not be determined. Even at 0.6 mg/mL PFK, aggregates of PFK larger than the tetramer were visible in electron micrographs. The increase in the proportion of these aggregates as PFK concentration increases precludes analysis of binding as a simple function of PFK concentration.

Table I: Comparison of Actin Binding Properties of PFK Fractionated with the PFK–Actin Binding Assay[a]

		PFK concn before fractionation (mg/mL)	
		0.55	0.045
$PFK_{initial}$	[PFK] (mg/mL)	0.55	0.045
	% PFK bound	63.2	59.1
PFK_{pellet}	[PFK] (mg/mL)	0.38	0.03
	% PFK bound	69.6	40.6
$PFK_{supernatant}$	[PFK] (mg/mL)	0.044	0.010
	% PFK bound	44.9	46.5

[a] Three fractions of PFK were assayed for their ability to bind to paracrystalline actin. The first fraction, $PFK_{initial}$, is the standard preparation of PFK added to 1.0 mg/mL actin. The second fraction, PFK_{pellet}, is the resuspended actin–PFK pellet remaining from the assay of $PFK_{initial}$. The third fraction, $PFK_{supernatant}$, is the supernatant from the assay of $PFK_{initial}$ desalted over Sephadex G-25. After desalting, 1.0 mg/mL actin was added to the supernatant.

To show that the binding equilibrium is not complicated by the presence of subpopulations of PFK with different affinities for actin, experiments using the bound and unbound fractions of PFK remaining after the binding assays were conducted to see if there was a significant difference in their abilities to bind actin (Table I). Although there is some change in the percent of PFK bound, especially at the higher concentrations of PFK, both fractions have a high affinity for actin. Luther and Lee (1986) have shown that the phosphorylation of PFK increases its affinity for actin. Since the commercially obtained PFK used in these experiments was less than 19% phosphorylated (0.75 mol of PO_4/mol of PFK tetramer) as measured by the inorganic phosphate assay (Buss & Stull, 1983), this enzyme preparation may be considered relatively homogeneous. Also, the affinity of PFK for actin described here reflects the low-phosphate, hence low-affinity form of the enzyme.

The ionic strength of the buffer used in these experiments was approximately 0.1 M. Since physiological ionic strength is about 0.15 M and previous studies of glycolytic enzyme binding to myofibrils have been questioned due to inhibition of the interaction by physiological ionic strengths, the effect of ionic strength on the binding of PFK to actin was tested with two potassium salts: KCl because it is the salt most frequently used to adjust the ionic strength of buffers and potassium acetate because it more closely resembles a physiological salt. With the addition of 25 mM of either salt (ionic strength = 0.125 M), there is no significant change in the amount of PFK bound to F-actin. At 50 mM added salt (ionic strength = 0.15 M), there is a 23.5% decrease in the amount of PFK bound relative to the control without added salt. KCl and potassium acetate have identical effects on binding (Table II).

Previous studies of the kinetics of PFK in the presence of actin (Liou & Anderson, 1980; Luther & Lee, 1986) indicated that the V_{max} of the PFK reaction did not change in the presence of low concentrations of F-actin. The effect of increasing actin concentrations on the specific activity of the enzyme was studied to see if maximal activity was a function of how much PFK is associated with actin. To test this, a single concentration of PFK was incubated in the assay cuvette

Table II: Effect of Ionic Strength on Binding

	% of control[a]	
	25 mM (μ = 125 mM)	50 mM (μ = 150 mM)
potassium chloride	94	76
potassium acetate	110	77

[a] Percent of control equals the percent sedimented at the indicated salt concentration divided by the percent sedimented in binding assay buffer without additional salt. Ionic strength is indicated in parentheses.

with actin concentrations ranging from 0 to 1.0 mg/mL. Substrate concentrations were chosen to yield V_{max}, as in the standard assay used throughout the study. If binding to actin has no effect on the specific activity of PFK, the maximal rate of the reaction should be the same regardless of the actin concentration. The results of this experiment (data not shown) confirmed that the specific activity of PFK is not affected by its association with actin.

Inactivation of Phosphofructokinase at Low pH and Temperature. The amount of PFK bound to actin increases by a small percentage as pH decreases within the physiological range at 20 °C (data not shown). Seventeen percent more PFK bound to actin at pH 6.7 than at pH 7.5. When both pH and temperature are lowered, PFK rapidly loses enzymatic activity; this instability has been attributed to the dissociation of the active tetramer to inactive dimers (Bock & Frieden, 1976). Actin decreases the rate and extent of PFK inactivation at all the pHs tested and at the various concentrations of PFK (Figure 2). The troponins or tropomyosin at the same concentrations as actin were added to incubations of PFK without actin for comparison. These two myofibrillar proteins were chosen to test whether the effect is unique to actin or whether it is simply due to a charge effect caused by the presence of another protein. Troponins are soluble proteins with isoelectric points ranging from 4.1 to 7.2. Tropomyosin, under these conditions, is a filamentous protein with an isoelectric point similar to that of actin, 5.4 and 5.6 for the different subunits (Hartshorne & Dreizen, 1972). As shown in Figure 2A, PFK in the presence of actin loses activity at a slower rate and retains a greater percentage of the total activity. PFK (0.048 mg/mL), after a 19-h incubation at low pH and temperature, retained 35% of the original activity when actin was present while an equivalent sample without actin retained only 7% of the initial activity (data not shown). The dependence of the rate of inactivation on PFK concentration can be seen in Figure 2A. Inactivation occurs at a significantly faster rate at the lower PFK concentrations with or without actin, consistent with the hypothesis that the loss of activity is due to a shift in the tetramer–dimer equilibrium. Troponin also offsets the inactivation of PFK although not as effectively as actin (data not shown). Tropomyosin accelerates the rate of activity loss relative to PFK without added protein (Figure 2B). Aliquots of PFK, PFK–troponin, or PFK–tropomyosin were centrifuged at 13000*g* for 5 min midway through the inactivation (at 50% initial activity). When PFK or PFK–troponin aliquots were centrifuged, no protein sedimented and the residual activity remained in the supernatant. When PFK–tropomyosin aliquots were centrifuged, all of the tropomyosin and 50% of the PFK sedimented (determined electrophoretically). Less than 5% of the PFK activity was recovered in either the supernatant or the tropomyosin pellet that had been resuspended in pH 7.8 buffer. In the PFK–actin incubations, >90% of the PFK sedimented with the actin (determined electrophoretically), there was no PFK activity in the supernatant, and 30% of the initial activity was recovered in the resuspended actin pellet.

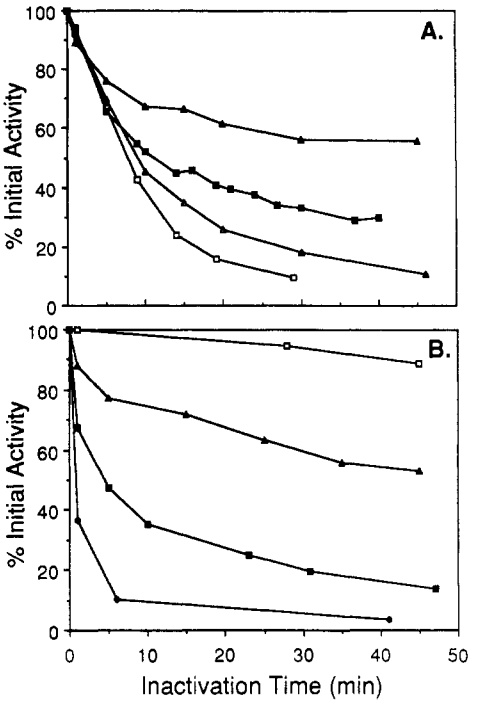

FIGURE 2: Effects of actin, tropomyosin, and troponin on the low-pH, low-temperature inactivation of phosphofructokinase (PFK). (A) Effect of PFK concentration on the rate of inactivation in the presence or absence of actin. Incubations were at 6 °C in inactivation buffer, pH 6.3. Open squares, 0.044 mg/mL PFK; solid squares, 0.066 mg/mL PFK; open triangles, 0.044 mg/mL PFK with 1.0 mg/mL actin; solid triangles, 0.066 mg/mL PFK with 1.0 mg/mL actin. (B) Comparison of the rates of inactivation in the presence of actin or tropomyosin. PFK, 0.06 mg/mL, was incubated in inactivation buffer at pH 7.8 at 20 °C (open squares); pH 6.15 at 6 °C (solid squares); pH 6.15 at 6 °C with 1.0 mg/mL actin (solid triangles); pH 6.15 at 6 °C with 1.0 mg/mL tropomyosin (solid diamonds).

Thus the actual binding of PFK to actin seems to be responsible for the stabilizing effect.

Discussion

The contribution of structural proteins to cellular function has long been recognized in muscle tissue, where the precise orientation of contractile proteins is essential for translating microscopic movements to macroscopic forces. However, the implications of such organization for the cellular distribution of enzymes has not often been addressed. There are two extreme possibilities: one, that enzymes are randomly distributed within the available space of the sarcoplasm, and, two, that enzymes are precisely arranged in relation to the units of contraction. The latter describes the case for skeletal muscle creatine phosphokinase, which regenerates ATP from ADP and phosphocreatine. Not only is this enzyme located at the M line, but also it is the major structural component of the M line (Turner et al., 1973; Walliman et al., 1978). The localization of creatine phosphokinase on the contractile apparatus allows for the continuous supply of ATP in the area of highest ATP consumption. The juxtaposition of several of the glycolytic enzymes including PFK, located on the I band of striated muscle in immunofluorescent micrographs (Dolken et al., 1975), implies that these enzymes are also organized into a spatial as well as a functional unit. In this study, the association of one glycolytic enzyme, phosphofructokinase, with actin is examined; this allows a more detailed characterization of the structural and functional attributes of enzyme organization in muscle.

Much of what is known about the structure of the actin filament and its association with tropomyosin and troponin

has been determined from electron micrographs of actin paracrystals. In the paracrystal, filaments are arranged in a side-by-side array with the crossover of the actin helices in transverse register (Hanson, 1973). This enhances any regular repeating features of the filaments, making them particularly suited for analysis by optical diffraction and three-dimensional image reconstruction. Paracrystals of muscle proteins have proven useful in determining structural and functional relationships. For example, the striations seen on micrographs of thin filament paracrystals reconstituted from purified proteins are analogous to the striations seen in the I band region of skeletal muscle. These striations have been attributed to the tropomyosin–troponin complex that lies in the groove of the actin helix (O'Brien et al., 1971; Spudich et al., 1972; Hanson, 1973). When the glycolytic enzyme fructose-bisphosphate aldolase is added to paracrystals of actin or thin filaments, transverse bands of aldolase appear to cross-bridge the filaments into a regular lattice (Clarke & Morton, 1976; Morton et al., 1977; Stewart et al., 1980). Aldolase has been shown to bind to both actin and thin filaments, setting a precedent for the interaction of glycolytic enzymes with myofibrillar proteins.

Electron micrographs provide direct evidence for the physical association of PFK and actin (Figure 1). Distinct bands of PFK visible on actin paracrystals suggest a stoichiometry of binding and imply that PFK–PFK interactions, as well as PFK–actin interactions, are involved in this assembly. The end-on-end aggregation of tetramers as seen in previously published micrographs of PFK (Foe & Trujillo, 1980) could also be responsible for the striated pattern associated with the binding of PFK to actin paracrystals. If PFK associates with both actin and other PFK molecules, then PFK could connect adjacent actin filaments through PFK–PFK interactions. This requires the precise alignment of PFK binding sites on the neighboring filament and may explain the correspondence between the actin helical repeat and the periodicity of the PFK banding pattern. Arguments that detail similar constraints on the bridging of filaments into bundles by cross-linking proteins have been given by DeRosier and Tilney (1982). The binding stoichiometry derived from micrographs of PFK–actin paracrystals is one PFK binding site per turn of the actin helix (approximately 14 actin monomers).

The nonsaturating binding curve indicates that the binding of PFK to actin does not fit a simple binding isotherm. Besides its interaction with actin, PFK may aggregate with free PFK or with PFK already bound to actin. Since the self-aggregation of PFK changes the concentration of enzyme required to saturate actin, saturation may not be reached at realistic concentrations of PFK and actin. There is no indication of saturation at PFK:actin ratios 33 times higher than those found in mammalian skeletal muscle (Liou & Anderson, 1980). Considering the low concentration of PFK in muscle and the number of other proteins that bind to actin, the amount of PFK bound to actin in muscle may depend primarily on competition for available binding sites. Competition for binding sites has been demonstrated to be a factor in the binding of enzymes to band 3 protein in erythrocyte membranes (Higashi et al., 1979).

A criticism of earlier studies on the association of glycolytic enzymes with the cytoskeleton was that binding only occurred at low ionic strength; at physiological ionic strength the interaction was completely inhibited. However, this inhibition can be offset by increasing the protein concentration to a more physiological level (Masters, 1981). It is difficult to compare results between studies because other factors (i.e., pH, temperature, actin concentration, the presence or absence of substrates) also affect the binding equilibrium. In addition, ionic strength affects the association of PFK with actin. The ionic strength of the buffer used in our standard assays was higher than in previous studies of PFK and actin, but still below physiological ionic strength. Increasing the ionic strength of the medium with either potassium chloride or potassium acetate had only a small effect on the amount of PFK that bound to actin (Table II). Thus, physiological ionic strength does not prevent the interaction of PFK with actin.

There are two ways in which the reversible association of enzymes with structural elements of a cell could affect flux through a pathway: formation of a multi-enzyme cluster that has more efficient catalytic properties (Gaertner, 1975) or modification of the activity of individual enzymes. We have only studied the binding of one enzyme to actin and so have limited our interests to the possible change in enzyme activity caused by actin. As previously discussed, actin and erythrocyte membranes effect the kinetic activation of PFK. Jenkins et al. (1985) have shown that binding of PFK to band 3 in the erythrocyte membrane favors the dissociation of tetramers to dimers. Apparently, band 3 has a higher affinity for dimeric PFK and gradually pulls the equilibrium toward this inactive form. Also, earlier studies in our laboratory on elasmobranch PFK suggested that actin may protect the tetrameric enzyme from dissociation by urea (Hand & Somero, 1984). F-Actin does not affect the maximal catalytic rate of PFK when PFK is assayed under optimal conditions (data not shown). However, under the destabilizing conditions of low pH and low temperature, actin affords PFK significant protection from the loss of catalytic activity associated with the protonation and subsequent dimerization of the tetrameric enzyme (Bock & Frieden, 1976). These experiments also show that troponin decreases the rate and degree of inactivation although not to the same extent as does actin. Tropomyosin, however, enhances the dissociation and inactivation of PFK. The stabilization of PFK tetramers by actin filaments is apparently mediated by the binding of PFK to actin because all of the residual PFK activity is associated with the sedimented actin pellet. Also, low pH increases the amount of PFK bound to actin, suggesting that more PFK is bound when conditions favor the dissociation of the enzyme.

The dissociation of PFK and subsequent loss of catalytic activity has been postulated to determine the kinetic properties of PFK (Luther et al., 1985) and also to explain the decrease in PFK activity associated with tissue acidification (Bock & Frieden, 1976). Therefore, the question of what fraction of the enzyme dissociates under true cellular conditions needs to be answered. The results of the current study indicate that actin stabilizes the active, tetrameric form of PFK, and previous reports document the kinetic activation of PFK by actin (Liou & Anderson, 1980; Luther & Lee, 1985, Kuo et al., 1986). It is possible that the kinetic activation is partially due to the stabilization of the active, tetrameric enzyme. PFK activity would then reflect the equilibrium determined both by the self-association of PFK subunits and the association of PFK with actin. Studies on the effects of poly(ethylene glycols) on PFK subunit assembly have shown that stabilizing the PFK tetramer causes a decrease in the K_M for fructose 6-phosphate (Reinhart, 1980).

The properties of PFK seen in dilute buffer solutions may partially misrepresent the importance of the tetramer–dimer equilibrium when compared to those for the solution conditions that exist in cells. Studies by Minton (1981), Pittz and Timasheff (1978), Clegg (1984), and others have shown that

cellular conditions differ significantly from ideality because of the amount of water bound by the hydration of macromolecules. Entropy-driven protein–protein interactions reduce the hydration volume and will be favored in solutions such as the cytoplasm. However, the nonideal solution conditions of cells have rarely been considered in studies of PFK aggregation. Since cellular conditions should favor both the self-association of PFK and the binding of PFK to actin, the dynamics of PFK subunit assembly and hence the catalytic properties of the enzyme in vivo may be quite different from what have been determined for isolated PFK. Future studies on the correlation between the level of glycolytic activity and the amount of PFK associated with actin will help reveal what role cytoskeletal association has in the regulation of PFK and glycolysis.

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