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Some characteristics of Rabbit muscle phosphofructokinase-1 inhibition by ascorbate

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Abstract
These studies relate to a working hypothesis that glycogen storage is facilitated in resting muscle by inhibiting glycolysis via inhibition of LDH, AK, and PFK-1 by ascorbate; when muscle is active, these isozymes combine with muscle proteins and are released and protected from inhibition by ascorbate and glycolysis proceeds. Focus in these studies is on the ability of G-actin and aldolase to prevent PFK-1 inhibition by ascorbate. We found that inhibition by ascorbate was PFK-1 concentration dependent; ascorbate does not inhibit above 200 nM PFK-1. We conclude that ascorbate inhibits PFK-1 dimers (and perhaps monomers) but not PFK-1 tetramers. Separation of PFK-1 dimers from tetramers was achieved with centrifugal filter devices and differences in their sensitivity to ascorbate inhibition were demonstrated. Some comparisons are made with attributes of AK inhibitions by ascorbate that, like PFK-1, are also enzyme concentration dependent. Discussions relate findings to cellular infrastructure and the role of ascorbate in glycogen synthesis.

Keywords: *PFK-1, inhibition by ascorbate, aldolase, G-actin, glycogen synthesis*

Introduction

Based upon observations that vitamin C specifically inhibits muscle isozymes of AK (adenylate kinase), LDH, and PFK-1 [1–4], the following hypothesis was developed: when muscle is resting glycolysis is inhibited: ascorbate inhibits LDH, AK, and PFK-1, thus facilitating synthesis and storage of glycogen; when muscle is active, these enzymes combine with muscle protein and are relieved and protected from inhibition by ascorbate and glycolysis proceeds. We showed previously that muscle isozymes AK, LDH, and PFK-1 are inhibited by ascorbate [2] and that AK [4] and LDH [3] are protected from inhibition by the presence of G-actin or aldolase. Characteristics of inhibitions by ascorbate differ among the AK, LDH, and PFK-1 muscle isozymes. At 200 nmolar or greater, when PFK-1 exists as a tetramer and AK exists as a dimer, these oligomermic forms are only slightly inhibited or not at all by ascorbate but become more sensitive to inhibition with decreasing enzyme concentrations. These behaviors are attributed to sensitivity of PFK-1 dimers and AK monomers [4] to inhibitions by ascorbate. Muscle LDH does not show this concentration effect [3]. While PFK-1 and AK [4] show similar activity losses and sensitivity to inhibitions by ascorbate at low concentrations, they show differences in the effects of G-actin and aldolase in preventing activity losses and in protecting and reversing inhibitions by ascorbate. Both aldolase and G-actin prevent activity losses at low PFK-1 and low AK concentrations, aldolase being much more effective. Aldolase protects PFK-1 from inhibition by ascorbate but G-actin does not. By contrast, G-actin both protects AK from inhibitions by ascorbate and reverses inhibitions by ascorbate [4].

This paper examines further effects of low PFK-1 concentrations on sensitivity to inhibitions by ascorbate, compares PFK-1 concentration effects with AK,

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and proposes that increased sensitivities to ascorbate inhibition are due to the dissociation of PFK-1 tetramers to ascorbate-sensitive dimers and the dissociation of AK dimers to ascorbate-sensitive monomers. Centrifugal filter devices separated PFK-1 tetramers from PFK-1 dimers that are sensitive to ascorbate inhibition. The instability of AK monomers precluded separation of monomers from dimers with centrifugal filter devices.

Materials and methods

Materials

Biologicals. These studies used enzymes from rabbit unless stated otherwise. Sigma-Aldrich Co. was also the source of enzymes used in assay systems below. Rabbit muscle G-actin (A 2522) had no aldolase, LDH or AK activities. Rabbit muscle aldolase (A 8811) was free of AK and LDH activities under our conditions.

Phosphofructokinase-1. Rabbit muscle PFK-1 used in these experiments was prepared by our laboratory from frozen tissue according to the method of Kemp [5]. All rabbit muscle PFK-1 samples used in these experiences were shown to have a single band in SDS PAGE, as given below, and were devoid of AK, LDH and aldolase activities.

Centrifugal filter devices. Centrifugal filter devices by Amicon, with molecular weight cut off (MWCO) values of 300 kD, and with 2 mL capacities were used. Prior to use, each filter was washed with 0.1 N NaOH by centrifuging 1 mL at 1000 × g for 2 min followed by washing with 0.1 mM potassium phosphate buffer, pH 8.0. PFK-1 samples that varied from 1 to 2 mL were centrifuged at 1000 × g for 30 min, resulting in about 90 percent of the sample volume in the filtrate. Recovery of the holdup volume was at 1000 × g for 2 min. Recoveries of total volumes ranged between 94 and 98 percent.

Criteria of purity. Purity of proteins was determined using SDS polyacrylamide gel electrophoresis (PAGE, not shown). SDS PAGE was run with 12 percent cross-linked gels and the Mini-Protean II Cell assembly and Bio-Rad. Gels were silver stained for proteins using the procedure of Morrissey [6].

Methods

All operations were at 25°C unless otherwise stated. All buffers used in these studies were 0.1 m*M* potassium phosphate buffer, pH 8.0 except when G-actin was present. When G-actin was present, all final PFK-1 solutions and reagents were in 0.02 m*M* potassium phosphate buffer, pH 8.0 to avoid polymerization to F-actin.

Phosphofructkinase-1 (PFK-1) assay. We measured PFK-1 activity, F 6-P + ATP = F 1,6-BP, with a modification of the method by Anderson et al. [7]. A 1 mL assay mixture contained 2 mM fructose 6-phosphate; 1 mM ATP (A 7699); 3.0 mM MgCl2; 0.13 mM NADH (N 1161); 1.7 eu/mL glyceraldehyde 3-phosphate dehydrogenase (G 0763); 18 eu/mL triose phosphate isomerase (T 2391); 1.3 eu/mL aldolase (A 8811); and 100 mM Tris-phosphate buffer, pH 8.0. In order to determine the activities of low concentrations of PFK-1, the assay components above were concentrated 10 times the assay final concentrations into 0.1 mL thus allowing PFK-1 samples up to 0.9 mL. This allowed reasonable activity measurements of low concentration-PFK-1 solutions that we defined as rate changes below 0.05 absorbancy units/min in 100 μL samples. A molar absorptivity value of 6,220 was used to convert NADH absorbance changes to μmoles of product formed. One enzyme unit (eu) of aldolase activity is defined as 1 μmole of NAD^+ formed per min.

Aldolase assay. Reagents for measurement of aldolase activity were the same as for PFK-1 assay except that 2 mM fructose 6-phosphate and 1 mM ATP were omitted and replaced by 2 mM fructose 1,6-bisphosphate.

Pre-incubations of low PFK-1 concentrations. It can be shown that the magnitude of activity loss was related to low PFK-1 concentration and stabilized in about 40 min (see Figure 5). Unless stated otherwise, a routine procedure was to allow test solutions of PFK-1 diluted from a stock solution to stand for 1 h at 25°C before other additions were made. Other additions were made in 5 to 10 percent of total volume.

Inhibition assays. Inhibitions of various PFK-1 concentrations by ascorbate were conducted as follows unless specified otherwise. Dilutions were made with 0.1 M Tris-phosphate, pH 8 or 0.02 μM Tris-phosphate when G-actin was present. Activities were measured both immediately after the dilution and again after 1 h so that the loss of activity due to dilution was stabilized [4]. Ascorbate was added to yield the final concentrations indicated on the graphs, solutions were incubated for an additional 1 h, and activities were then determined.

Time course of ascorbate inhibition. Ascorbate and PFK-1 were mixed at the time of dilution from the stock PFK-1 and aliquots removed to assay activity

remaining at indicated times. Under our conditions, it was determined that no significant changes in ascorbate inhibition occurred after 0.5 h of incubation and then held steady up to 2 h.

Ascorbate titrations against a constant PFK-1 concentration. Fixed PFK-1 concentrations with varying ascorbate concentrations were incubated at least 1 h. The PFK-1 activity remaining was then determined.

PFK-1 titrations against a constant ascorbate concentration. Fixed concentrations of ascorbate against varying PFK-1 concentrations were incubated 1 h. The PFK-1 activity remaining was then determined.

Measurements of protein concentrations. Two methods of determining protein were used.

1. During PFK-I purifications [5] protein concentrations were measured using the following formula to estimate protein concentrations

$$\text{mg protein/mL} = 1.55A_{280} - 0.76\,A_{260},$$

where A_{280} and A_{260} are absorbencies at 280 nm and 260 nm, respectively [8]. The spectrophotometric protein determinations were comparable to the Bradford method [9].
2. As a matter of convenience, protein concentrations of purified PFK-1 were measured as the absorbancy at 280 nm. Total protein in a sample, for example, is the $A_{280\,nm}$ × the volume.

Results

Effect of some proteins on activity at various PFK-1 concentrations

Figure 1 shows controls below 150 nM PFK-1 (■) result in concave deviations from linearity, typical for allosteric enzymes at low concentrations. These deviations are considered due to dissociation of PFK-1 tetramers to dimers that are either not active or less active than tetramers [10–13]. Several proteins were tested for their ability to prevent activity loss due to dilution. As shown in Figure 1, the presence of actin during dilution (Δ) resulted in a linear relationship while the presence of myosin (•) and BSA (◦) have little or no effect. Aldolase (▲) showed a small but consistent convex stimulatory effect at concentrations below 150 nM PFK-1; at concentrations above 200 nM PFK-1, the presence of aldolase was indistinguishable from controls.

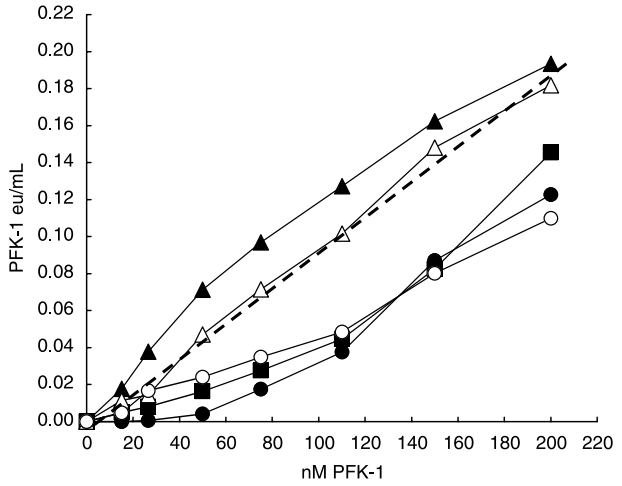

Figure 1. *Effects on PFK-1 dilutions in the presence of some proteins*. A stock solution of 2 μM PFK-1 (2.60 eu/mL) was diluted with 0.1 M Tris-phosphate, pH 8 to concentrations indicated above containing 5 μmolar of each of the following: myosin (•) BSA (◦), or aldolase (▲). Dilutions containing G-actin (Δ) were obtained by with 0.02 M Tris-phosphate, pH 8 (see Methods). The control (■) contained PFK-1 only. Diluted solutions were then incubated for 1 h to insure that losses of activity due to dilution were stable [13]. Each point is an average of at least 4 separate experiments with an average deviation of ±7% from the mean.

Effect of G-actin on PFK-1 activity losses at low concentrations. Below 200 nM PFK-1 activity loses become detectable. Figure 2 shows small restorations of activity by G-actin at several PFK-1 concentrations. Stabilized activities obtained after 1 h incubation are given in parentheses and are much lower than expected values based on dilution. The lower the PFK-1 concentration the greater is deviation from

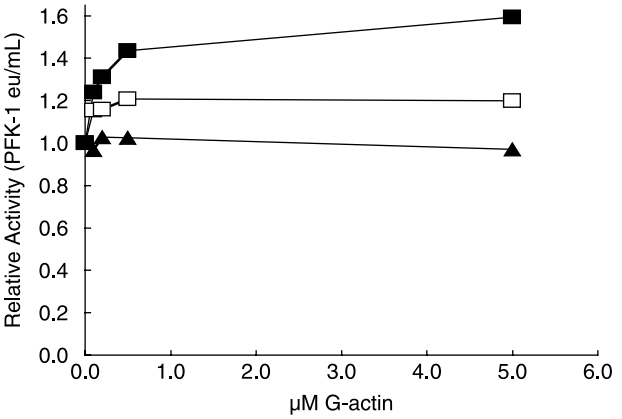

Figure 2. *Effect of G-actin concentrations at various PFK-1 concentrations*. In the presence of G-actin at concentrations indicated, 2 μM PFK-1 (2.1 eu/mL) was diluted with 0.02 M Tris-phosphate buffer and then incubated for 1 h to stabilize activity losses due to dilution (**14**). The PFK-1activities shown in parentheses were then determined as follows: 10 nmolar, *v* (0.006 eu/mL); 25 nmolar, □ (0.015 eu/mL); and 50 nmolar □ (0.041 eu/mL) and (0.092 eu/mL) 100 nmolar were indistinguishable, ▲.

estimated activity based on the dilution. Figure 2 shows that G-actin prevents activity losses due to low concentrations. For example, dilution to 25 nM PFK-1 (□) from a 2 μM PFK-1 stock solution should yield an estimated activity of 0.033 eu/mL (an 80-fold dilution) but after 1 h incubation yielded instead a stable value of 0.015 eu/mL. In a separate experiment, when diluted in the presence of 500 nM G-actin, the 25 nM PFK-1 retains the estimated activity of 0.033 eu/mL. It is noteworthy that G-actin appears to have an effect only at the lower PFK-1 concentrations; as shown in Figure 2, G-actin effects at 50 nM PFK-1 and 100 nM PFK-1 (▲) were indistinguishable.

Figure 3 shows that the effects of aldolase appears similar to G-actin in Figure 2 but differs in that at 10 nM (■) and 25 nM PFK-1 (□) aldolase enhances the activity much above estimated values based on dilution and at much lower concentrations than G-actin. This enhanced activity was also evident in Figure 1 where the presence of aldolase gave rise to a slight convexity at low PFK-1 concentrations and G-actin merely restored the linear relationship of PFK-1 concentration to activity.

It was shown previously [4] that 200 nM AK or higher showed little or no inhibition by ascorbate and that sensitivity to inhibition by ascorbate increased as concentrations of AK are lowered. Figure 4 shows that the relative inhibition of PFK-1 by ascorbate also increases as concentrations of PFK-1 are lowered. At concentrations above 200 nM PFK-1 (■), little or no inhibition by ascorbate occurs.

Figure 5 shows that 1 μM G-actin (□) did not protect 20 nM PFK-1 from activity losses but not from inhibition by ascorbate (Δ), 5 μM G-actin was as equally ineffective. Neither substrates nor products of PFK-1 (F 6-P, F 1,6-BP, ATP, Mg-ATP, ADP) at

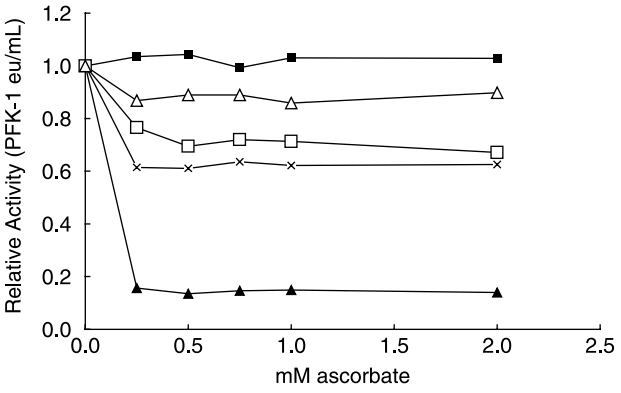

Figure 4. *Inhibition ascorbate at various PFK-1 concentrations*. Dilutions with 0.1 M Tris-phosphate, pH 8 and conditions were similar to those in Figure 3. The PFK-1 activities were determined after 1 h incubation: 25 nM, ▲ (0.016 μeu/mL); 50 nM, **X** (0.042 eu/mL); 100 nM, □ (0.100 μeu/mL); 200 nM, Δ (0.21 μeu/mL); and 400 nM, ■ (0.51 eu/mL). Ascorbate was added at the concentrations indicated and the solutions were incubated for an additional 1 h and then activities were determined.

5 μmolar levels prevented activity losses due to low PFK-1 concentration or protected from inhibition by ascorbate; similarly 1 mM AMP did not prevent ascorbate inhibition. This absence of effect by PFK-1 substrates contrasts with 25 nM AK where substrates caused markedly increased sensitivity to inhibitions by ascorbate [4].

Figure 6 shows that 5 μM aldolase (□) protected 20 nM PFK-1 from both activity loss due low concentration and from inhibition by ascorbate (Δ).*Separation of PFK-1 oligomers by centrifugal filtration*

It has been suggested by several investigators [10–13] that PFK-1 exists primarily as a tetramer at high concentrations but can dissociate to a dimer at low concentrations. Our studies suggest that noticeable

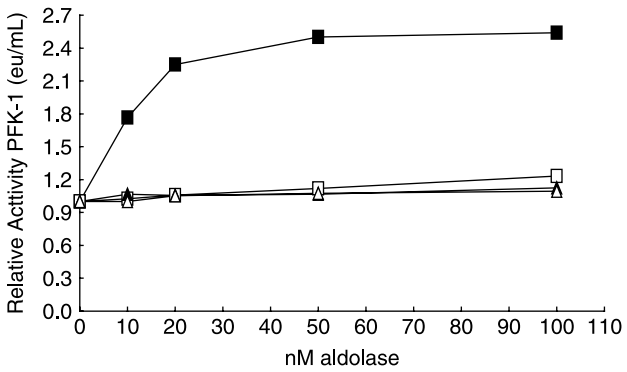

Figure 3. *Effect of aldolase concentrations at various PFK-1 concentrations*. Dilution of PFK-1 and PFK-1 concentrations and conditions were the same as in Figure 2, except that 0.1 M Tris-phosphate, pH 8 was used. Zero-time was after 1 h incubation activities were as follows: 10 nmolar, ■ (0.005 eu/mL); 25 nmolar, □ (0.018 eu/mL); 50 nmolar, ▲ (0.046 eu/mL) and 100 nmolar, Δ (0.098 eu/mL) are virtually identical.

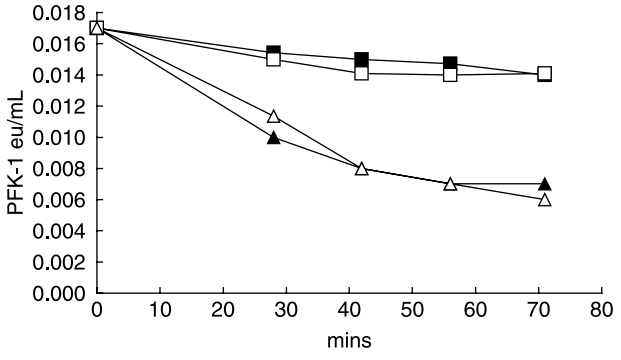

Figure 5. *Effect of 1.0 μM actin on inhibition of 20 nM PFK-1 by 2 mM ascorbate*. As in Figure 2, Dilutions to 20 nM PFK-1 was with 0.02 M Tris-phosphate and incubated for 15 min instead of the usual 1 h incubation to stabilize activity losses at which time when the following additions were made to their final concentrations: no additions (■); 1 μM G-actin (□); 2 mM ascorbate (▲); 1 μM G-actin plus 2 mM ascorbate (Δ). Activities were then followed with time.

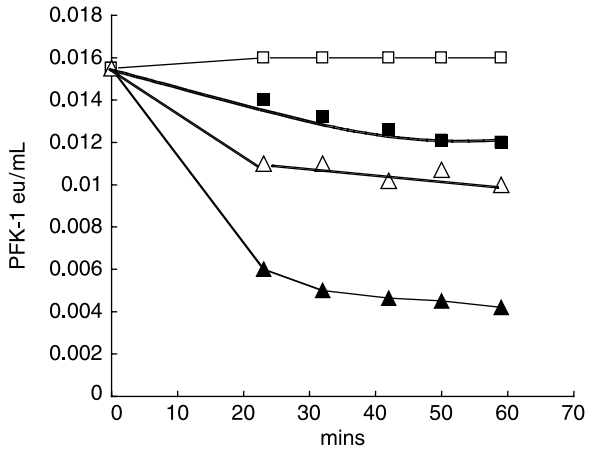

Figure 6. *Protection of 20 nM PFK-1 from ascorbate inhibition by aldolase.* The conditions were similar to Figure 5 except that 0.1 M Tris-phosphate, pH 8 buffer was used. The following additions were at 15 min and activities were followed with time: no additions (□); 5 μM aldolase (□); 2 mM ascorbate (▲); 5 μM aldolase and plus 2 mM ascorbate (Δ).

dissociations can begin below 200 nM PFK-1 (see Figure 4); below that concentration, PFK-1 begins a relatively rapid loss of activity followed by a relatively stable, lower level of activity It should be noted that the studies here were conducted at pH 8, where we found the activities of PFK-1 at low concentrations were more stable than at pH 7.4 [13]. Taking advantage of the differences in the size of a tetramer (320 kD) and dimer (160 kD), we passed solutions of varying concentrations of PFK-1 through centrifugal filter devices with molecular weight cut-off values (MWCO) of 300 kD.

Table I shows that with increasing concentrations less PFK-1 activity and protein filters through; at 400 nM PFK-1 (not shown) no protein or activity entered the filtrate. These outcomes are consistent with dissociation of PFK-1 tetramers (320 kD) to dimers (160 kD) or monomer (80 kD). Also shown is that the PFK-1 activity remaining in the holdup is not inhibited by ascorbate while the PFK-1 activity in the filtrate is very sensitive to ascorbate inhibition. Attempts to restore the PFK-1 tetramer and its associated resistance to ascorbate inhibition by concentrating filtrates through a centrifugal filter with a MWCO of 50 kD were not successful because losses of both activity and protein were so great that no definitive conclusions could be made. Conditions for restoration of PFK-1 tetramers using centrifugal filter devices is under current study. The nature of the filter material, centrifugal force, and the length of centrifugation time all appear to be significant contributing factors in recoveries of activity and protein at low PFK-1 concentrations.

Discussion

These studies were based upon a proposed general working hypothesis [3] that introduces a new function for ascorbate. It proposes that in resting muscle, ascorbate inhibits specific glycolytic enzymes and so facilitates glycogen synthesis; in active muscle, glycolytic enzymes are protected from ascorbate inhibition by forming complexes with muscle proteins. The specificity of ascorbate inhibitions for the muscle isozymes of AK, LDH, and PFK-1 [1–4] and the ascorbate concentrations found in muscle tissues [14–15] are consistent with the working hypothesis. The simplicity of this hypothesis relative to the very complex biochemistry is acknowledged.

Both G-actin and aldolase, respectively, are capable of preventing activity losses due to low PFK-1 concentrations; aldolase is about 10-fold more effective (Figures 2 and 3). Losses of PFK-1 activity at low concentrations and prevention of activity losses by aldolase were previously observed by others [10–13]. These results are also interpreted as due to dissociation of PFK-1 tetramers to dimers, resulting in loss of activity and prevention activity losses by aldolase due to PFK-1 dimer-aldolase complex formation [13]. Aldolase also protects PFK-1 from activity losses due to ascorbate inhibition (Figure 6) and enhances activity at low PFK-1 concentrations (Figures 1 and 3). G-actin also protects low PFK-1

Table I. Separation of PFK-1 oligomers at various concentrations with centrifugal filter devices[a].

	20 nM PFK-1	40 nM PFK-1	50 nM PFK-1	200 nM PFK-1
% recovered eu in filtrate	98	75	52	32
eu/mL in filtrate	0.009	0.026	0.043	0.13
% eu of recovered in holdup	2	25	48	68
eu/mL in holdup	0.063	0.033	0.081	0.18
Control eu/mL	0.016	0.028	0.060	0.21
% Control recovered	51	59	54	66
% of recovered protein in filtrate[b]	87	71	66	50
% of recovered protein in holdup	13	29	34	50
Control protein (PFK-1)/mL	0.023	0.040	0.061	0.290
% of control protein recovered	46	59	69	73
% inhibition by ascorbate: filtrate	96	–	99	–
% inhibition by ascorbate: holdup	4	–	1	–

[a] Conditions for the centrifugation are given in Methods; [b] Protein concentrations were determined by $A_{280\,nm}$ as given in Methods.

concentrations (Figure 2) from activity losses, but it has no effect against ascorbate inhibition (Figure 5, Δ). It can also be shown that 5 μM BSA resulted in effects similar to G-actin, i.e., BSA protected low PFK-1 concentrations from activity losses but not against ascorbate inhibition. Unlike G-actin, however, BSA had no effect on deviations from linear relationships of PFK-1 activities vs. low PFK-1 concentrations (Figure 1, ▲). The protective effect of BSA against activity losses due to low PFK-1 concentrations is presumed due to general, non-specific protective effects of BSA for low protein concentrations [26].

Interpretations of these PFK-1 behaviors are as follows. At low concentrations, PFK-1 tetramers dissociate and are in equilibrium with dimers [13]. Dimers then undergo conformational changes that result in less activity, less stability and are sensitivity to ascorbate inhibition. G-actin and BSA can form complexes with the dimers that are then protected from activity losses due to low PFK-1 concentrations but not from ascorbate inhibitions. On the other hand, aldolase-dimer complexes result in PFK-1 activities that are greater than values expected from dilution alone and are also protected from ascorbate inhibitions (Figure 1, □; Figure 3, ▲; and Figure 6, □).

Some success in separating PFK-1 tetramers (320 kD) from dimers (160 kD) was achieved, using centrifugal filter devices with varying MWCO (molecular weight cutoff) values. Table I shows that with decreasing PFK-1 concentrations an increasing amount of protein and activity enters the filtrate and that PFK-1 activity entering the filtrate shows a greater inhibition by ascorbate than in the holdup volume that is a mixture of both PFK-1 tetramers and dimers. Separated dimers are very labile and attempts to reconstitute PFK-1 tetramers by passage of filtrates containing PFK-1 dimers (160 kD) through filters with MWCO 100 values that would retain PFK-1 dimers were unsuccessful; an almost complete loss of activity and a considerable loss of protein resulted in both the filtrate and holdup volumes. The PFK-1 dimers in filtrates may dissociate further into even more unstable PFK-1 monomers (80 KD) that denature completely. Further studies with centrifugal filter devices are current and include LDH and AK in particular [4] because its behavior upon dilution is similar to PFK-1.

The infrastructure scaffold of the cytoskeleton is primarily composed of F-actin, tubulin, and intermediate filaments forming an enormous surface area with a high negative charge [16]. The glycolytic enzymes are among the best-characterized cytoskeleton binding enzymes [10,17–19]. Evidence exists on several levels for the structural organization of the glycolytic enzymes [11,20–23]. For example, electrical stimulation of bovine psoas produced increased binding of several glycolytic enzymes to particulate matter, in addition to a decrease in muscle glycogen and an increase in tissue lactate [24]. Most of the glycolytic enzymes are positively charged at physiological pH [11]; PFK-1 is not with a pI value between 5.0 and 5.3 [17]. The interaction of tubulin with PFK-1 partially inactivates the enzyme by forming a complex with dimers in equilibrium with PFK-1 tetramers; binding of PFK-1 to tubulin decreases PFK-1 intracellular concentrations that in turn leads to more dimer formation [10]. Aldolase also forms a complex with PFK-1 dimers and is capable of restoring the activity lost due to dimer formation at low concentrations; PFK-1-dimer complexes do not appear to bind to tubulin [11].

We show as others [13] have shown that the PFK-1 tetramer dissociates at low concentrations into dimers with diminished activity and susceptibility to further loses of activity by forming complexes with tubulin [13] or ascorbate (Figure 4). Aldolase at nmolar levels is capable of restoring activity losses due to low PFK-1 concentrations (Figure 3). In addition, aldolase prevents inhibition by ascorbate. Aldolase always increases the activity of PFK-1 concentrations below 200 nmolar, presumably by restoring the activity of PFK-1 dimers. At high PFK-1 concentrations (above 200 μmolar) the enhancement of activity is small probably because dimer formation is small.

In summary, tubulin [10–13] and ascorbate diminish PFK-1 activity; G-actin and aldolase ([13], Figure 6), on the other hand, protect PFK-1 from these activity losses; aldolase also has the ability to restore activity losses due to low PFK-1 concentrations. Aldolase also protects low concentrations of muscle AK [4] and muscle LDH [3] isozymes from ascorbate inhibitions.

We propose that muscle tubulin [10,11,13] and ascorbate assist in the diminution of glycolysis when the muscle is at rest while actin and aldolase promote the activation of glycolysis when the muscle is active. Both tubulin [10,11,13] and ascorbate interact with the dimer form of PFK-1. Ascorbate inhibits only very low concentrations of LDH [3] and AK [4], the dimer and monomer forms, respectively. The concentrations of PFK-1, LDH, and AK become an important factor in relation to the proposed working hypothesis. It is doubtful that any of these enzymes exist in high concentration *in situ*. It is more probable that these enzymes are docked on one or another cellular infrastructure networks and such sequestrations would favor dissociations. It seems plausible that enzymes shuttle from one network structure to another depending cellular needs. Our current interest is movement between tubulin and F-actin. These current studies are with G-actin and not F-actin and we are developing methods for studying the binding of PFK-1, LDH, and AK or higher molecular weight forms of F-actin using centrifugal filter devices.

We consider that these multifaceted series of interactions provide some insight on conditions that

favor glycogen synthesis or glycolysis. This is stated with awareness that up to this point important phosphorylation reactions have not been taken into account. Phosphorylated PFK-1 has been studied [25] but the effect of phosphorylated PFK-1 on its reactions with ascorbate, tubulin, actin and aldolase have yet to be determined.

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