

# Comparisons of Phosphofructokinases-1 from Rabbit, Chicken, and Fish

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## ABSTRACT

**Background:** Previous studies suggested that glycogen storage was facilitated by ascorbate inhibition of phosphofructokinase-1 (PFK-1) in resting mammalian muscle; these studies showed that purified PFK-1 from fish or chicken muscle has properties similar to PFK-1 from mammalian muscles.

**Materials and Methods:** The enzymes utilized in the assay systems came from Sigma-Aldrich Co. Rabbit (*Oryctolagus cuniculus*) muscle G-actin (A 2522) was free of aldolase, LDH (EC 1.1.1.28) or AK (EC 2.7.4.3) activity and rabbit muscle aldolase (EC 4.1.2.13) was free of AK (EC 2.7.4.3) and LDH activity. Rabbit muscle PFK-1 (RPFK-1), chicken (*Gallus gallus*) muscle PFK-1 (CPFK-1) and Pacific red snapper (*Lutjanus peru*) muscle PFK-1 (FPFK-1) used in these tests were prepared from frozen tissues with modifications of a method. AK, LDH, and aldolase activity were absent in purified FPFK-1 and CPFK-1 preparations. **Results:** It can be shown that the following enzymes associated with glycolysis are not inhibited by 0.1 M ascorbate under our conditions: rabbit muscle aldolase (EC 4.1.2.13); rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); rabbit muscle phosphoglucose isomerase (EC 5.3.1.9); rabbit muscle pyruvate kinase

(EC 2.7.1.40); yeast hexokinase (EC 2.7.1.1); and yeast 3-phosphoglyceric phosphokinase (EC 2.7.2.3). Rabbit muscle enolase (EC 4.2.1.11) is inhibited under our conditions. **Conclusion:** In summary, FPFK-1 and CPFK-1, possess characteristics and behaviors similar to RPFK-1, e.g., losses of activities due to dilutions and protections of some of these activity losses by rabbit muscle aldolase. These interactions of a mammalian aldolase with fish and a bird PFK-1's suggests a conservative evolutionary relationship among aldolases and PFK-1's.

**Keywords:** Aldolase, Ascorbate, Chicken, Fish, Phosphofructokinase-1.

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## INTRODUCTION

Various Studies showed that ascorbate inhibited rabbit muscle phosphofructokinase-1 (RPFK-1), the putative controlling enzyme of glycolysis. The aim of current research was to determine whether the proposed ascorbate function of facilitating glycogen storage in resting muscle by inhibiting PFK-1, as shown in mammals,<sup>1,2</sup> occurs also in bird and fish. Because of instabilities during both isolation and storage of chicken CPFK-1 and Red Snapper FPFK-1, development of a rapid PFK-1 purification became a part of this study. Rapid purification was enabled by eluting columns with a vacuum instead of by gravity or pump and by avoiding ammonium sulfate precipitations until the step before storage. Among properties of PFK-1 from chicken and fish investigated were activity losses due to dilution; inhibitions by ascorbate; inhibitions by lithium, potassium, and sodium salts; and protection from these activity losses by rabbit muscle aldolase. Results were compared with the properties previously shown for RPFK-1.<sup>3-4</sup>

## MATERIALS AND METHODS

### Materials

The enzymes utilized in the assay systems came from Sigma-Aldrich Co. Rabbit (*Oryctolagus cuniculus*) muscle G-actin (A 2522) was free of aldolase, LDH (EC 1.1.1.28) or AK (EC 2.7.4.3) activity and rabbit muscle aldolase (EC 4.1.2.13) was free of AK (EC 2.7.4.3) and LDH activity.

### Phosphofructokinase-1 (PFK-1, EC 2.7.1.11)

Rabbit muscle PFK-1 (RPFK-1), chicken (*Gallus gallus*) muscle PFK-1 (CPFK-1) and Pacific red snapper (*Lutjanus peru*) muscle PFK-1 (FPFK-1) used in these tests were prepared from frozen tissues with modifications of a method by Kemp.<sup>5</sup> AK, LDH, and aldolase activity were absent in purified FPFK-1 and CPFK-1 preparations.

### Methods

#### Dilutions of RPFK- to 30 Nmolar

For PFK-1, following standard protocols were used to prepare its low concentrations.

Stock solutions of 3  $\mu$ M RPFK-1 (3.0 eu/mL) in 10 mM Tris-phosphate, pH 8 (10TP8) were diluted to final concentrations of 30 nM PFK-1 in the same buffer at 25°C for 0.5 hr (hour) to permit activity losses. For more than 2 h, activities remained constant. The dilution of PFK-1 samples by adding 1/20<sup>th</sup> of the volume of test sample with final concentration with 20-fold. PFK-1 activity was determined at 25°C for 1 h by incubation of test sample.<sup>3</sup>

### PFK-1 Assays

#### Standard PFK-1 Assay

We measured RPFK-1 activity, F6-P + ATP = F1,6-BP, with a modification of the method by Anderson.<sup>6</sup> A 1.0 mL assay mixture contained the following as final concentrations: 2 mM fructose 6-phosphate; 1

mM ATP; 3 mM MgCl<sub>2</sub>; 0.13 mM NADH; 1.7 eu/mL glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); 18 eu/mL triose phosphate isomerase (EC 5.3.1.1); 1.3 eu/mL aldolase (EC 4.1.2.13); and 10 mM Tris-phosphate buffer, pH 8.0.

### 30 nM PFK-1 Assays

In a 100 µL sample, the PFK-1 activity was below than the 0.05 absorbance unit/min while if it were concentrated more than ten times as in Standard PFK-1 assay in 0.1 mL. Therefore, we are using 0.9 mL of test samples for more accuracy.

### Aldolase Assay

To determine the aldolase activity in muscles rabbit, the same reagents were used which used in *Standard RPFK-1 Assay* above except 2 mM fructose 1,6-bisphosphate was used in place of 2 mM fructose 6-phosphate and 1 mM ATP.

### LDH Assay

LDH assay was determined according to the method which is mentioned in Vassault.<sup>7</sup> One enzyme unit (eu) of LDH activity is defined as 1 µmole of NAD<sup>+</sup>/min.

## RESULTS

### Modification of PFK-1 Purifications

Purification of PFK-1 from chicken (CPRK-1) and fish (FPRK-1) were not so stable to procedures as rabbit muscle (RPFK-1).<sup>5</sup> Elution by vacuum, described below, reduced preparation time and permitted elimination of desalting with Sephadex G 25, which caused considerable activity losses.

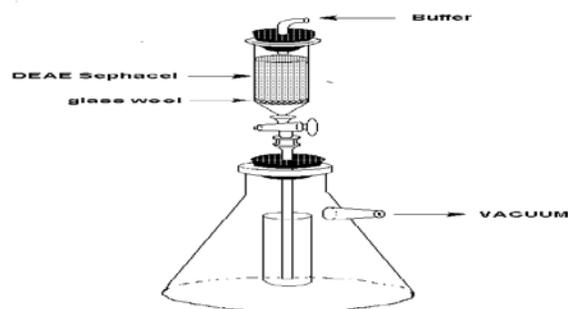
### Rapid purifications of CPFK-1, and FPFK-1.

Mince 50 g of muscle tissue. Homogenize minced muscle in 3 volumes (1 mL/g) of 1 mM DTT, 0.1 mM EDTA, 1 mM NaF, pH 7.5, for 3 min at the highest homogenizer setting; then homogenize again with a Polytron for 3 min with intermittent cooling. Centrifuge the homogenate at 10,000 x g for 20 min at 4°C. Discard supernatants contain LDH, aldolase and little or no PFK-1 activity. The pellet containing PFK-1 may be stored at 4°C. Suspend the pellet in 2 volumes of 1 mM DTT, 1 mM EDTA, 50 mM MgSO<sub>4</sub>, 0.5 mM ATP, and Tris-HCl, pH 8.0. In a water bath at 70°C, bring the suspended pellet to 57°C for 3 min with stirring, then plunge into an ice bath and swirl until the temperature approaches 10°C. Centrifuge at 10,000 x g for 20 min at 4°C. Save the supernatant; wash the pellet in 1 volume of the same buffer and centrifuge. Add the wash to the supernatant and save. Generally, FPFK-1 preparations contained little or no aldolase and LDH activities.

### Vacuum Elution of DEAE Sephacel

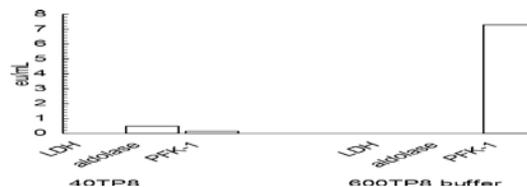
To a 30 mL DEAE Sephacel column equilibrated with 40 mM TP8 (40TP8), up to 120 mL of heat-step preparation is percolated through by gravity, followed by 100 mL of 40TP8 under slight vacuum, collected at about 10 mL/min (Figure 1). Then add 100 mL 600mM TP8 to the column and collect under slight vacuum at the same rate. PFK-1 activity is in this fraction. An example of a vacuum apparatus outcome is shown in Figure 2.

Table 1 shows average yield results from RPFK-1, CPFK-1, and FPFK-1 purifications using the vacuum method given above. Yields were sacrificed for speed because of instability of FPFK-1 and CPFK-1 with time, storage and desalting. The procedure as described above reduced purification time from about 25 h total to about 6 h. RPFK-1 is stable for several months at 4°C as a 60% saturated ammonium sulfate precipitate.



**Figure 1:** Vacuum elution apparatus.

The 30 mL DEAE Sephacel column is a 30 mL or 50 mL plastic syringe barrel and the filter is glass wool. The stopcock below the column or another stopcock between the vacuum flask outlet and the vacuum may better control the elution rate.



**Figure 1:** Final Purification of FPFK-1 with DEAE Sephacel column.

A 100 mL heat-step preparation containing 24 eu of FPFK-1 was placed on a 30 mL DEAE-Sephacel column and eluted by gravity; followed by 100 mL of 40TP8 eluted under a slight vacuum; and finally followed by 100 mL of 600 TP8 under vacuum. The 600 TP8 contained 7.3 eu of FPFK-1 free of aldolase activity.

**Table 1:** Comparison of purification from 50 g rabbit, chicken, and fish muscle.

PFK-1	Teu Crude extract	Teu After Heat-step	Teu After DEAE- Sephacel	% Recovery
Rabbit	1600	110	34	30
Chicken	552	584	15	8
Fish	262	194	22	11

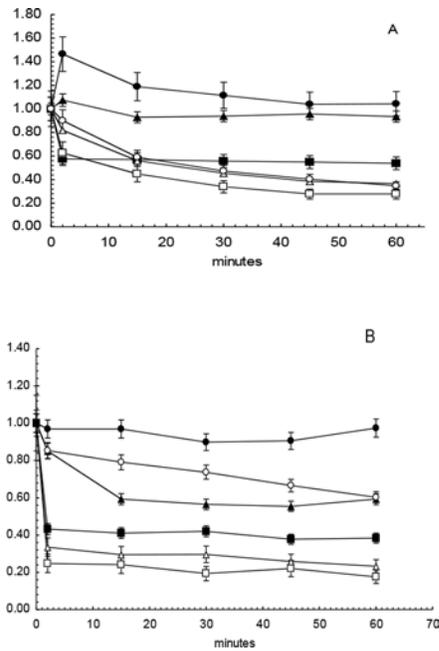
Under the same conditions, CPFK-1 is stable for about a week and FPFK-1 loses more than 50 percent of its activity in a couple of days.

### Criteria for PFK-1 purity

The primary criterion for PFK-1 purity was absence aldolase and LDH activities. This criterion was used to qualify a PFK-1 preparation for experiments in this report. RPFK-1<sup>1</sup> was the control preparation that yielded an absence of LDH, aldolase and a single protein band by PAGE.<sup>8</sup>

### Effect of dilution on CPFK-1 and FPFK-1 activities

It was reported previously<sup>1,9-12</sup> that dilutions of RPFK-1 result in substantial losses of activity. Figure 3 compares losses activity due to dilution of FPFK-1 (A) and CPFK-1 (B) Figure 3 (Unfilled symbols) and the protective effect of 5 µM rabbit muscle aldolase (filled symbols). In order



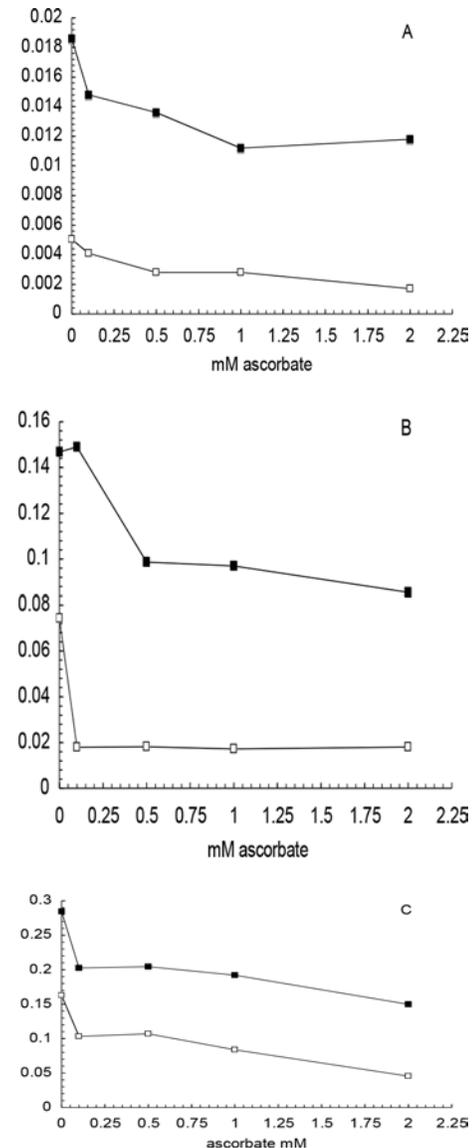
**Figure 3:** A and B. Relative activities of the effect of 5  $\mu\text{M}$  rabbit muscle aldolase on dilution to 30 nM, 100 nM, and 300 nM FPFK-1 and CPFK-1.

Relative activities were determined by using one hundredth of activities of 3  $\mu\text{M}$  FPFK-1 and CPFK-1 stock solutions as the base (see Methods). The absence and presence of 5  $\mu\text{M}$  rabbit muscle aldolase are denoted by unfilled and filled symbols, respectively. The concentrations of FPFK-1 (A) and CPFK-1 (B) are as follows: 30 nmolar (c,  $\blacksquare$ ); 100 nmolar (D,  $\blacktriangle$ ); and 300 nmolar (O,  $\bullet$ ).

to compare PFK-1 at different dilutions, relative activities are plotted. As shown in Figure 3 A at 45 minutes, FPFK-1 at all concentrations show about 70 percent activity losses due to dilution (c, D, O) in the absence of 5  $\mu\text{M}$  rabbit muscle aldolase. In Figure 3 B by comparison, CPFK-1 activity losses due to dilution (c, D, O) in the absence of 5  $\mu\text{M}$  rabbit muscle aldolase differed depending up the CPFK-1 concentration. At 30 nM (c) and at 100 nM (D) CPFK-1, activity losses due to dilution were about an 80 percent, but at 300 nM CPFK-1 (O), activity loss was about 40 percent, indicating that FPFK-1 is more sensitive to activity losses due to dilution than the CPFK-1. It was shown previously<sup>1</sup> that there were no losses due to dilution in rabbit muscle PFK-1 above 200 nmolar. The filled symbols in Figure 3 A shows that 5  $\mu\text{M}$  rabbit muscle aldolase offers some protection of FPFK-1 from activity losses due to dilutions (open symbols), but to different extents. For 30 nM FPFK-1 (c,  $\blacksquare$ ), the presence of rabbit muscle aldolase increased activity by 25 percent; for 100 nM nM FPFK-1 (D,  $\blacktriangle$ ), by 50 percent; and for 300 nM FPFK-1 (O,  $\bullet$ ), by 70 percent higher activity. The consistent stimulation in the early phases of incubation times in Figure 3A is not understood. In Figure 3 B, CPFK-1 differences in activities due to the absence (open symbols) and increased activity in the presence of rabbit muscle aldolase (filled symbols) were as follows: for 30 nM CPFK-1 (D,  $\blacktriangle$ ); 35 percent; for 100 nM CPFK-1 (D,  $\blacktriangle$ ), 35 percent; and for 300 nM CPFK-1 (O,  $\bullet$ ), 40 percent higher – distinctly different from FPFK-1 in Figure 3A.

### Inhibitions of FPFK-1 and CPFK-1 by ascorbate and the protective effect of rabbit muscle aldolase

Figure 4 and Figure 5 show that rabbit muscle aldolase protects several concentrations of FPFK-1 and CPFK-1 from inhibitions by ascorbate. In A, B, and C of Figure 4 and Figure 5, dilutions to 30 nM, 100 nM, and 300 nM FPFK-1 or CPFK-1 with ascorbate were made in the absence ( $\gamma$ ) and presence of 5  $\mu\text{M}$  rabbit muscle aldolase ( $\blacksquare$ ) showing its protective



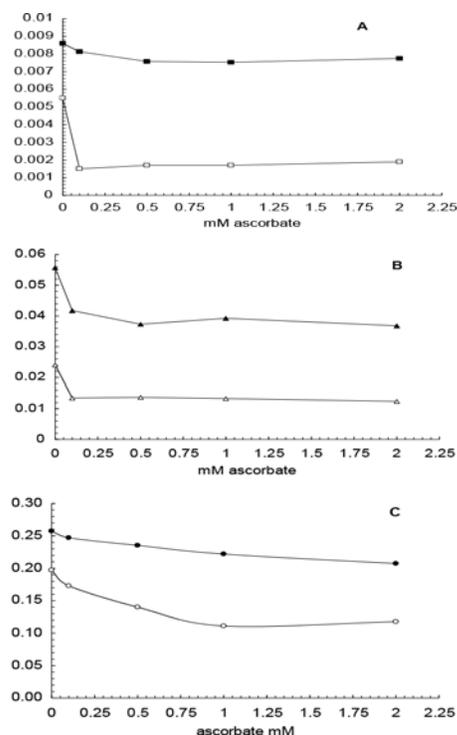
**Figure 4:** Effect of 5  $\mu\text{M}$  rabbit muscle aldolase on (A) 30 nM FPFK-1, (B) 100 nM FPFK-1 and (C) 300 nM FPFK-1 inhibitions by ascorbate.

Solutions of FPFK-1 in the presence ( $\blacksquare$ ) and absence (c) of 5  $\mu\text{M}$  rabbit muscle aldolase were prepared as given in Materials and Methods. The preparations were incubated for 1 hr at the concentrations of ascorbate indicated and activities then determined.

effect. Above 200 nM RPFK-1, inhibitions by ascorbate do not occur<sup>1</sup>. The results here show that FPFK-1 and CPFK-1 are similarly sensitive to inhibitions by ascorbate and protection by rabbit muscle aldolase.

### Inhibition of FPFK-1 and CPFK-1 by Lithium Salts

Earlier studies<sup>3</sup> showed that some salts could inhibit RPFK-1. Because of use in treating manic-depressive disorder<sup>13-15</sup> and because glucose is the major energy source for the brain, we were particularly interested in effects of lithium salts on PFK-1. The tacit conclusions appear to be that “lithium” is the therapeutic factor. Others showed that lithium carbonate inhibited RPFK-1 and concluded that lithium was the inhibitor.<sup>16</sup> We showed that RPFK-1 was inhibited by lithium carbonate and lithium sulfate but not by lithium acetate nor by lithium chloride and showed further with other monovalent cations that associated anions should be investigated before assigning inhibition to the cation or to the anion.<sup>17</sup>



**Figures 5 A-C:** Inhibitions of CPFK-1 by ascorbate and the protective effect of rabbit muscle aldolase.

Shows 30 nM CPFK-1, 100 nM CPFK-1, and 300 nM CPFK-1 inhibitions by ascorbate and protection from inhibitions by rabbit muscle aldolase in a manner similar to PPFK-1 above.

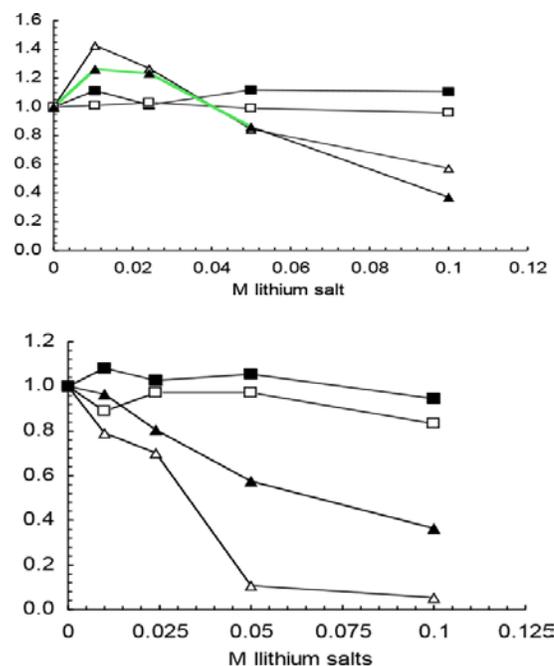
Figures 6 A-B: Show lithium salts effects on PPFK-1 and CPFK- 1 activities, Figure 6 respectively. Figure 6 A shows that lithium acetate and lithium chloride are not inhibitors of PPFK-1 and that lithium carbonate (D) and lithium sulfate (▲) are both stimulators below 0.04 molar but are inhibitors above that concentration. A 40 percent inhibition obtains at 0.1 M lithium carbonate and 60 percent inhibition obtains at 0.1 M lithium sulfate. Figure 6 B shows that lithium salts affect CPFK-1 differently from PPFK-1 in Figure 6 A. Lithium acetate (c) and lithium chloride (■) are slight inhibitors, 15 percent and 5 percent, respectively; lithium carbonate (D) and lithium sulfate inhibit CPFK-1 (▲) 98 percent and 65 percent, respectively, with very little or no stimulation at low lithium salt concentrations.

### Rabbit muscle aldolase prevention of 30 nM PPFK-1 and 30 nM CPFK inhibitions by lithium salts

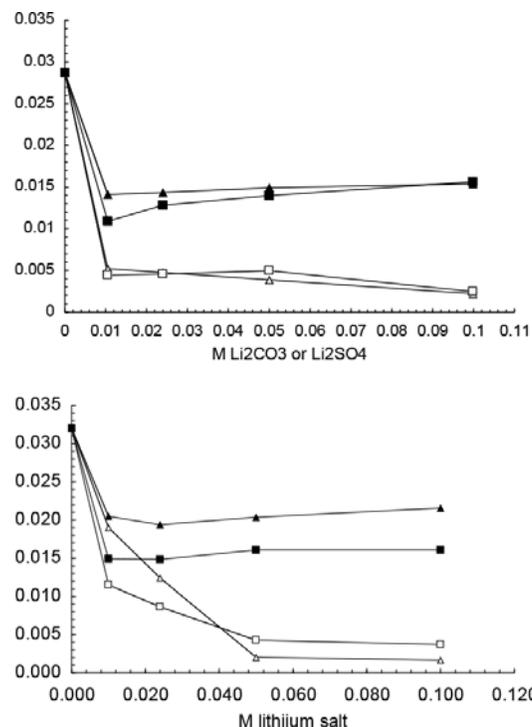
Figure 7 A-B: Show the inhibition patterns of 30 nM PPFK-1 and 30 nM CPFK- Figure 7

1, respectively, in the absence ( $\Delta$ ,  $\chi$ ) and presence ( $\blacktriangle$ ,  $\blacksquare$ ) of  $\text{Li}_2\text{CO}_3$  and  $\text{Li}_2\text{SO}_4$  were similar. At 0.1 M lithium salt concentrations, both enzymes lost about 90 percent their activity estimates due to dilution but in the presence rabbit muscle aldolase lost about 50 percent of their activity estimates.

Previous studies showed that lithium, potassium, and sodium salts of acetate and chloride had little or no inhibitory effect on RPFK-1 while the salts of carbonate and sulfate were substantial inhibitors.<sup>17</sup> The effects of these salts on PPFK-1 and CPFK-1 were investigated. Depending upon the associated anion, monovalent cations show wide extents of inhibitions in Table 2 A Table B for PPFK-1 and CPFK-1, respectively. For example, Table 2 in Table 2A, acetates and chlorides of monovalent



**Figure 6 A-B:** Inhibitions of 30 nM PPFK-1 and 30 nM CPFK-1 by lithium salts. Dilutions were made from a stock solution as given in Methods and Materials. Solutions of 30 nM PPFK-1 and 30 nM CPFK-1 were incubated for 1 h at the concentrations of the lithium salts given and then activities were determined. Symbols for the lithium salts are as follows: lithium acetate, c; lithium carbonate, D; lithium chloride, ■; lithium sulfate, ▲.



**Figure 7 A-B:** Effect of 5  $\mu\text{M}$  rabbit muscle aldolase on lithium carbonate and lithium sulfate inhibitions of 30 nM PPFK-1 and 30 nM CPFK-1. Activity values at 0-salt concentrations shown were the expected estimated values from 0.01 activity of 3  $\mu\text{M}$  stock solutions activities (see Materials and Methods). Symbols are as follows: lithium carbonate,  $\Delta$ ; lithium carbonate plus 5  $\mu\text{M}$  rabbit muscle aldolase,  $\blacktriangle$ ; lithium sulfate, c; and lithium sulfate plus 5  $\mu\text{M}$  rabbit muscle aldolase,  $\blacksquare$ .

**Table 2 A: Percent inhibition of 30 nM FPFK-1 by 0.1 M monovalent salt.**

Cations	Anions			
	Acetate	Carbonate	Chloride	Sulfate
Lithium	4	43	6	63
Potassium	7	47	13	67
Sodium	18	46	4	58

**Table 2B: Percent inhibition of 30 nM CPFK-1 by 0.1 M monovalent salt.**

Cations	Anions			
	Acetate	Carbonate	Chloride	Sulfate
Lithium	5	95	4	72
Potassium	-5	60	-13	48
Sodium	9	-29	12	31

**Table 3: Effect of 5  $\mu$ M rabbit muscle aldolase on inhibition of 30 nM FPFK-1 and 30 nM CPFK-1 by 0.1 M carbonates and sulfates of potassium and sodium.**

	Control	0.1 M $K_2CO_3$	0.1 M $K_2CO_3$ + 5 $\mu$ M rabbit muscle aldolase	0.1 M $Na_2CO_3$	0.1 M $Na_2CO_3$ + 5 $\mu$ M rabbit muscle aldolase	0.1 M $K_2SO_4$	0.1 M $K_2SO_4$ + 5 $\mu$ M rabbit muscle aldolase	0.1 M $Na_2SO_4$	0.1 M $Na_2SO_4$ + 5 $\mu$ M rabbit muscle aldolase
30 nM FPFK-1 eu/mL	0.0084	0.0039	0.015	0.0042	0.044	0.0031	0.015	0.0039	0.027
30 nM CPFK-1 eu/mL	0.0087	0.0040	0.027	0.020	0.034	0.005	0.032	0.0061	0.029

cations show comparatively little or no inhibition of FPFK-1 while carbonates and sulfates show considerable inhibition. In terms of inhibition effectiveness, anions rank as sulfate > carbonate > acetate = chloride. Table 2 B shows inhibitions of CPFK-1 by monovalent cationic salts vary greatly. For example, potassium acetate, potassium chloride, and sodium carbonate show stimulations of activity, not inhibitions. Inhibitions of CPFK-1 by carbonates and sulfates show a wide range compared to FPFK-1. In terms of inhibition effectiveness for CPFK-1 order is  $Li^+ > K^+ > Na^+$  for both carbonate and sulfate salts.

### Effect of rabbit muscle aldolase on FPFK-1 and CPFK-1 inhibitions by salts

Previous studies<sup>3</sup> showed that inhibition of RPFK-1 by potassium and sodium carbonates and sulfates were prevented by rabbit muscle aldolase. Table 3 shows that rabbit muscle aldolase protected 30 nM FPFK-1 from inhibitions by carbonate and sulfate salts. In the case of CPFK-1, the presence of rabbit muscle aldolase protected against inhibition by 0.1 M  $K_2SO_4$  and increased the activity above that stimulated by  $Na_2CO_3$ .

## DISCUSSION

Our working hypothesis is that ascorbate facilitates storage of glucose as glycogen in resting muscle by inhibiting glycolysis; glycolysis is inhibited through specific inhibitions of muscle PFK-1, LDH, and AK by ascorbate.<sup>1-4,17</sup> It can be shown that the following enzymes associated with glycolysis are not inhibited by 0.1 M ascorbate under our conditions: rabbit muscle aldolase (EC 4.1.2.13); rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); rabbit muscle phosphoglucose isomerase (EC 5.3.1.9); rabbit muscle pyruvate kinase (EC 2.7.1.40); yeast hexokinase (EC 2.7.1.1); and yeast 3-phosphoglyceric phosphokinase (EC 2.7.2.3). Rabbit muscle enolase (EC 4.2.1.11) is inhibited under our conditions. In active muscle, PFK-1, LDH<sup>18</sup> and aldolase<sup>19-21</sup> form complexes with contractile proteins that would protect them from inhibition by ascorbate; we view aldolase protection of these enzymes from inhibitions by ascorbate as a microcosm of *in situ* events.<sup>18</sup> Since PFK-1 is the putative controlling enzyme of glycolysis, it was chosen for scrutiny in this study. This study explored whether muscle PFK-1 from species other than mammals showed similar characteristics and behaviors with respect to ascorbate inhibitions and prevention of or protection from inhibitions by aldolase.

With respect to concentrations, Figure 3 A shows that 300 nM FPFK-1 (O) loss about a 70 percent activity due to dilution, making FPFK-1

more sensitive to activity losses due to dilution than RPFK-1 that showed no activity losses of above 200 nmolar; activity losses due to dilutions have been attributed to tetramer to dimer formations.<sup>1,9-12</sup> The initial but consistent stimulatory effect by the presence of rabbit muscle aldolase 100 nM and 300 nM RPFK-1 is not understood. The protective effect of 5  $\mu$ M rabbit muscle aldolase from activity losses due to dilution of FPFK-1 was not so great as that shown for RPFK-1 previously.<sup>1</sup> Figure 3 B shows that CPFK-1 is more like RPFK-1 than FPFK-1; response to dilution was graded and devoid of an initial stimulatory effect with rabbit muscle aldolase. Loss of CPFK-1 activity was more rapid upon dilution than FPFK-1, probably reflecting a more rapid transition from tetramer to dimer.<sup>1</sup>

Figure 4 and Figure 5 show that FPFK-1 and CPFK-1 were inhibited by ascorbate and again that rabbit muscle aldolase also protected against ascorbate inhibition with resemblances to one another and to RPFK-1; the most pronounced protections were at the lower PFK-1 concentrations. The variation of the protective effect of rabbit muscle aldolase with PFK-1 concentration is attributed to interactions of aldolase with PFK-1 dimers and little or no interaction with or effect on the tetramer as shown in RPFK-1.<sup>12</sup>

In summary, the PFK-1's from rabbit, fish and chicken are all inhibited by ascorbate, and all are protected from inhibition with rabbit muscle aldolase. It can be shown that rabbit muscle aldolase must be present at the time of dilution or ascorbate addition and does not have the ability to reverse PFK-1 losses of activity due to dilutions or ascorbate.<sup>12</sup>

Inhibitions of RPFK-1 by lithium, potassium and sodium salts are other characteristics with which to compare with FPFK-1 and CPFK-1. Lithium salts are of particular interest because of their therapeutic use in manic-depressive disorder.<sup>13-15</sup> Figure 6 shows that, neither lithium acetate (c) nor lithium chloride (■) inhibits at concentrations that lithium carbonate (D) and lithium sulfate (▲) inhibit, similar to RPFK-1.<sup>17</sup> Figure 6 also shows CPFK-1 more sensitive to inhibition by lithium carbonate than FPFK-1.

In Figure 7, the zero-salt concentration value of the activity, 0.032 eu/mL, was a calculated one from dilutions of CPFK-1 and FPFK-1 stock solutions values, in order to permit separate views of activity losses due to dilution, activity losses by salts, and effects of 5  $\mu$ M rabbit muscle aldolase. Figure 7A shows the effect of 5  $\mu$ M rabbit muscle aldolase on inhibitions of 30 nM FPFK-1 by lithium carbonate (D) or lithium sulfate (▲). Rabbit muscle aldolase (▲, ■) partially prevented inhibitions by the salts, resulting in about 50 percent of the calculated activity compared to 10 percent of the calculated activity in the absence of rabbit

muscle aldolase. Figure 7 B shows similar results for 30 nM CPRK-1. The presence of rabbit muscle aldolase with lithium carbonate (▲) resulted in 60 percent of the calculated activity compared to 6 percent of the activity in its absence (D). The presence of rabbit muscle aldolase with lithium sulfate with 30 nM CPRK-1 (■) resulted 45 percent of the calculated activity compared to 16 percent of the activity in its absence (c). These results were similar those obtained with RPFK-1.

In summary, rabbit muscle aldolase gives partial protections from FPFK 1 and CPFK-1 activity losses due the presence of lithium carbonate or lithium sulfate similar to those shown for RPFK-1. In RPFK-1, inhibitory effects of salts were prevented by the presence of rabbit muscle aldolase.<sup>3,17</sup>

Table 2 A shows that in general, effects of individual anion salts were similar. For example, the acetate and chloride salts inhibitions of FPFK 1 were slight or almost zero while carbonate and sulfate salts showed substantial similar inhibitions. As shown in Table 2 B, CPFK-1 shows dissimilarities among inhibitions by carbonate and sulfate salts. For example, under conditions given, lithium carbonate showed a nearly complete inhibition while sodium carbonate showed a stimulation of activity. Though effects were modest, there were other dissimilarities; potassium acetate and potassium chloride were stimulatory while sodium carbonate was stimulatory and sodium sulfate was as inhibitory. With respect to the inhibitory effects of carbonate and sulfate salts of lithium, potassium and sodium, FPFK-1 showed characteristics more like RPFK-1 than did CPFK-1.

## CONCLUSION

FPFK-1 and CPFK-1, possess characteristics and behaviors similar to RPFK-1, e.g., losses of activities due to dilutions and protections of some of these activity losses by rabbit muscle aldolase. These interactions of a mammalian aldolase with fish and a bird PFK-1's suggests a conservative evolutionary relationship among aldolases and PFK-1's.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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