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Aldolase and Actin Protect Rabbit Muscle Lactate Dehydrogenase from Ascorbate Inhibition

PERCY J. RUSSELL*, ANITA WILLIAMS, XAVIER AMADOR† and REYNALDO VARGAS†

Department of Biology, University of California 0690, San Diego, La Jolla, CA 92093-0690, USA

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Muscle-type LDH (LDH-m4) activity is critical for efficient anaerobic glycolysis. The results here show that rabbit LDH-M4 is inhibited by concentrations of ascorbate normally found in tissues. Aldolase and muscle G-actin were found to protect and to reverse inhibitions of LDH-m4 by ascorbate. G-actins showed some species specificity. Myosin, tropomyosin and troponin from rabbit muscle and muscle proteins from other animal sources had no affect on the inhibitions by ascorbate. The substrate inhibition of LDH-m4 by pyruvate is partially relieved by the presence of aldolase and lowers the Km without affecting the Vm. G-actin under similar conditions has no affect. It is believed that these studies reflect some of the resting properties of glycolytic enzymes that bind and unbind to contractile elements. It is proposed that ascorbate facilitates the storage of glycogen in muscle at rest by inhibiting glycolysis.

Keywords: LDH-m4; ascorbate inhibition; aldolase; G-actin; glycogen synthesis

INTRODUCTION

Normal ascorbate concentrations in striated muscles of most mammals are approximately 0.3 mM[1–3] and that may more than double with the equivalent of grams per day human intakes.[4] It was shown previously that much less than 1 mM ascorbate concentrations[5] greatly inhibited commercial, highly purified rabbit muscle L-lactate dehydrogenase (LDH-m4, EC 1.1.1.27), muscle 6-phosphofructokinase (PFK-1, EC 2.7.1.11) and muscle adenylate kinase (AK, EC 2.7.4.3) and that sulfhydryl reagents or oxidation of ascorbate completely reversed these inhibitions.[1] It was also determined that the LDH, PFK-1 and AK isozymes associated with liver or heart tissues were not inhibited by ascorbate.[5]

When commercial, purified PFK-1 was no longer available, we found that LDH-m4, PFK-1, and AK activities in crude rabbit muscle extracts were not inhibited by 10 mM ascorbate and higher. Sensitivity to inhibition by ascorbate returned with purification. The course of the purifications suggested that proteins in the crude muscle extracts were protecting LDH-m4, PFK-1, and AK from inhibition by ascorbate. In this report we show that aldolase or G-actin from rabbit muscle completely protect purified rabbit muscle LDH-m4 from inhibition by ascorbate.

We suggest that a function of ascorbate is to facilitate synthesis of muscle glycogen during periods of rest by inhibiting key enzymes promoting glycolysis. Other muscle proteins tested did not protect LDH-m4 from inhibition by ascorbate. Protections from ascorbate inhibitions by rabbit muscle aldolase and rabbit muscle G-actin show some tissue and species specific. The role of ascorbate as a facilitator of glycogen storage in muscle is discussed.

MATERIALS AND METHODS

Materials

Rabbit tissue enzymes were used in these studies unless stated otherwise. The following enzymes were obtained commercially from Sigma-Aldrich Co: muscle adenylate kinase (AK, EC 2.7.4.3); muscle

*Corresponding author. Tel.: +1-858-534-4184. Fax: +1-858-534-1513. E-mail: prussell@ucsd.edu
†Participants in the CURE Program of the UCSD Cancer Center.

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aldolase (EC 2.4.1.13); muscle and liver 6-phosphofructokinases (PFK-1 EC 2.7.1.11); muscle and heart lactate dehydrogenases (LDH-m4 and LDH-h4, EC 1.1.1.27). The following rabbit muscle proteins (Sigma-Aldrich product numbers are in parentheses) were used in these experiments: G-actin (A 2522), myosin (M 1636), myosin heavy chain (M 7659), and troponin (T 3515). The following proteins from other animals sources were used: bovine muscle G-actin (A 3653); bovine muscle tropomyosin (T 4770); chicken muscle G-actin (A 0398); chicken gizzard myosin (M 1270); chicken muscle myosin (M 7266); chicken muscle troponin (T 1771); chicken gizzard tropomyosin (T 2036); porcine muscle G-actin (A 0541), porcine heart myosin (M 0531); porcine muscle myosin (M 0273); porcine muscle tropomyosin (T 2400); porcine muscle troponin (T 2275); human heart troponin I (T 9924); human heart troponin (T 0175).

All enzymes and proteins in these experiments were tested for cross contaminations with LDH-m4 and aldolase. Some myosin lots contained LDH-m4 activity and in these cases additional controls were run to compensate for the added LDH-m4 activities.

Ascorbic acid was also obtained from Sigma Chemical-Aldrich Co.

Methods

LDH Assay

LDH activity, (pyruvate + NADH + H^+ = lactate + NAD^+), was measured according to previously published procedures.[6] The 1 mL assay mixture contained 0.6 mM pyruvate; 0.2 mM NADH; and 50 mM potassium phosphate buffer, pH 8.0. Addition of enzyme initiated reactions and reaction rates were determined by measuring decreased absorbance of NADH at 340 nm with time. A molar absorptivity value of 6,220 was used to convert the NADH absorbance change to micromoles of product formed. One enzyme unit (eu) of activity is the formation of 1 μmole of NAD^+ per min at 25°C.

AK Assay

AK activity, (AMP + Mg.ATP = ADP + Mg.ADP), was measured according to Adam.[7] The 1 mL assay mixture contained 0.3 mM phosphoenolpyruvate; 0.4 mM NADH; 8.0 mM ATP and AMP each; 8.5 mM $MgCl_2$; and 20 mM potassium phosphate buffer, pH 8.0. Addition of enzyme initiated reactions. Reaction rate measurements and the definition of enzyme unit (eu) are the same as for the LDH assay.

Phosphofructokinase Assay

PFK was assayed by a modification of the coupled assay used by Roberts *et al.*[8] All solutions were made up in 100 mM Tris–potassium phosphate buffer, pH 8. The assay medium contained the following: 2 mM fructose 6-phosphate; 1 mM ATP; 0.13 mM NADH; 30 mM $MgCl_2$; 1.7 and 18 eu/mL of glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase, respectively; and 1.3 eu/mL of aldolase. One eu of PFK-1 activity is the formation of 1 μmole of NAD^+ per min at 25°C.

Aldolase Assay

Aldolase activity was measured using modifications of a method previously published.[9] All solutions were made up in 100 mM Tris–potassium phosphate buffer, pH 8. The assay medium contained the following: 2 mM fructose 1,6-bisphosphate; 0.13 mM NADH; and 1.7 and 18 eu/mL of glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase, respectively. One enzyme unit (eu) of aldolase activity is the formation of 1 μmole of NAD^+ per min at 25°C.

Inhibition Assays

Conditions for determining inhibitions by ascorbate were as follows unless stated otherwise. All reaction mixtures were in potassium phosphate, buffer, pH 8.0 unless stated otherwise. All reactions involving actin were at a final concentration of 0.02 M potassium phosphate buffer, pH 8.0. Indicated concentrations of ascorbate were added to approximately 0.5 μM LDH-m4 initially, followed by test proteins at the concentrations given. Samples were incubated for 0.5 h or more, after which it was determined that no additional significant inhibitions occurred. The activity remaining was then determined by addition of microliter aliquots of the reaction mixtures to assay mixtures.

Initial Velocity Studies

When determining kinetic constants, Km, Vm and Ki, ascorbate or test proteins were in assay mixtures at the given concentrations. Reactions were initiated by addition of the test enzyme.

Measurements of Protein Concentrations

Protein concentrations were measured using the following formula: mg protein/mL = 1.55 A_{280} − 0.76 A_{260}, where A_{280} and A_{260} are absorbencies at 280 nm and 260 nm, respectively.[10] The spectrophotometric protein determinations were comparable to the Bradford method.[11]

Criteria of Purity

The purity of proteins was determined using SDS polyacrylamide gel electrophoresis (SDS PAGE, not shown), and LDH assays. Rabbit muscle myosin (Sigma, M1636) was the only protein addition that sometimes contained detectable LDH activity under our conditions. When LDH activity was detected in myosin, additional controls were run to account for any added activity. SDS PAGE used 12% cross-linked gels and the Mini-Protean II Cell assembly and directions of Bio-Rad. Gels were silver stained for proteins using the procedure of Morrissey.[12]

RESULTS

Characteristics of LDH-m4 Inhibition by Ascorbate

It was previously shown[5] that ascorbate specifically inhibited rabbit muscle-type lactate dehydrogenase (LDH-m4) and had no effect on rabbit heart-type lactate dehydrogenase (LDH-h4). Ascorbate also inhibited highly purified muscle adenylate kinase (AK) and muscle phosphofructokinase-1 isozymes. Several other rabbit muscle enzymes associated with glycolysis were shown not to be inhibited by 10 mM ascorbate or higher.[5]

Several conditions for LDH-m4 inhibition by ascorbate were investigated. Figure 1 shows that between pH 7 and pH 8 the rates of inhibition of 0.54 μM LDH-m4 and out-comes were similar with more than 90 percent of the final inhibition occurring well within 10 min. There was no increased inhibition after 30 min. Similarly, the concentrations of potassium phosphate buffer, pH 8 between 0.1 and 0.02 M had no effect on the rate or the extent of inhibition at the concentrations of ascorbate and LDH-m4 used.

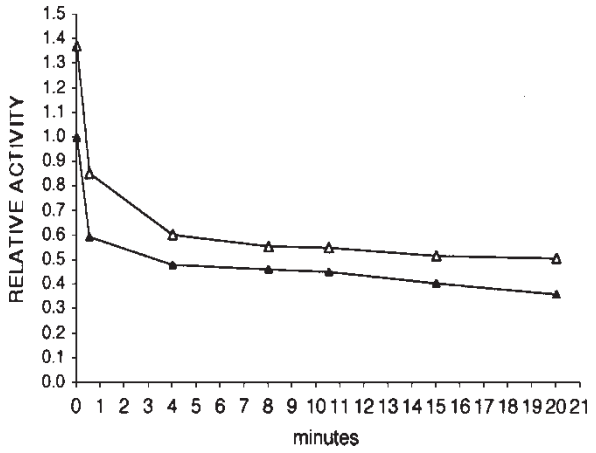

FIGURE 1 *Rate of ascorbate inhibition of LDH-m4 at pH 7 and pH 8.* A final concentration of 0.54 μM LDH-m4 was added to 20 mM KPO4 buffer at pH 7 (Δ) and pH 8 (▲) and 2 mM ascorbate. The activity remaining was measured at the times indicated. The relative activity of 1.00 had an average activity of 10.2 ± 0.6 eu/mL.

Aldolase and Actin Protect LDH-m4 from Inhibition by Ascorbate

We found previously that highly purified muscle isozymes PFK-1, AK, and LDH-m4 were greatly inhibited by 1 mM ascorbate or less.[5] Alternatively, these same isozymes in crude rabbit muscle extracts were not inhibited at ascorbate concentrations in excess of 20 mM. It became apparent during purification processes that other proteins were protecting these enzymes from inhibition by ascorbate. As these enzymes from crude extracts become purified they were again susceptible to inhibition by ascorbate.

A report[13] that rabbit muscle aldolase protected muscle PFK-1 from loss of activity as a result of extensive dilution encouraged us to test muscle aldolase for protection of LDH-m4 from inhibition by ascorbate. As shown in Figure 2A, aldolase protects LDH-m4 inhibition by ascorbate and rabbit muscle G-actin also has an ability to protect that is comparable to aldolase. Experiments, not shown here, with mixtures of aldolase and G-actin suggest that the protective effects of the two proteins are additive. Figure 2B shows the effects of aldolase and actin over a narrower range. An estimated ratio of about one LDH mole per mole of aldolase and one LDH mole per three or four moles of G-actin is obtained at the intersections of the biphasic curves.

The order of addition is not important. As shown in Figure 3, when LDH-m4 is inhibited first by ascorbate, inhibition can be reversed by subsequent additions of aldolase or G-actin under our conditions. Heat-treated (100°C for 10 min) muscle aldolase or G-actin loses activity and the ability to protect LDH-m4 from ascorbate inhibition. Under the same heat-treated conditions, G-actin showed no loss of its ability to protect LDH-m4 from ascorbate inhibition (not shown). Rabbit muscle aldolase inhibited by glyceraldehyde 3-phosphate[14] was fully protective.

Other Muscle Proteins

Other muscle proteins were tested for their ability to protect LDH-m4 from inhibition by ascorbate. Figure 4 shows that rabbit muscle myosin did not protect LDH-m4 from inhibition by ascorbate under conditions similar to those in which G-actin or aldolase gave protection. Other muscle proteins that did not protect LDH-m4 against inhibition by ascorbate were rabbit heavy chain myosin, rabbit troponin, and rabbit tropomyosin. Proteins from sources other than rabbit that also had no ability to protect LDH-m4 against inhibition by ascorbate were

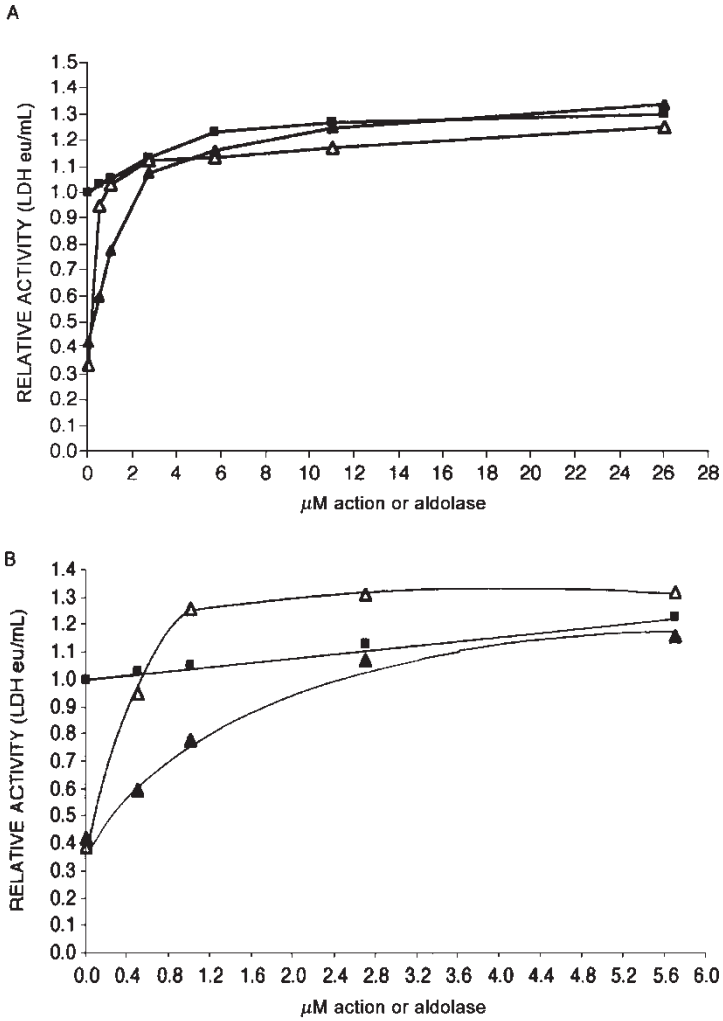

FIGURE 2 *Rabbit muscle actin and aldolase protection of LDH-m4 against ascorbate inhibition.* (A) The figure is a composite average of a minimum of three experiments. The 0.02 M K-PO_4, pH 8 mixtures were incubated for 0.5 h containing 0.5 μM LDH-m4 and actin (▲) or aldolase (Δ) at the concentrations given. The mixtures were then made 2 mM ascorbate and incubated for an additional 0.5 h. The LDH activity remaining was measured. The average 8.3 eu/mL control was similarly treated but contained no ascorbate but did contain either actin or aldolase at concentrations given (■). (B) The same conditions as in (A), but over a narrower range.

as follows: chicken gizzard myosin, porcine muscle myosin, porcine heart myosin, chicken gizzard tropomyosin, bovine muscle tropomyosin, chicken muscle troponin, porcine muscle troponin, human heart troponin I and human heart troponin T.

G-actins from Other Sources

Figure 5 shows that G-actins from other animal muscle sources also have varying abilities to protect LDH-m4 from inhibition by ascorbate; this suggests a species specificity for G-actins.

Effect of Aldolase on the Km of LDH-m4

The addition of aldolase to LDH-m4 usually results in a 25–35 percent activity increase and prompted an investigation of the effect of aldolase on LDH-m4 kinetic constants, Km and Vm. Pyruvate is a substrate inhibitor of LDH, its effect being much more pronounced on LDH-h4 than LDH-m4. Figure 6A shows that LDH-m4 never approaches the maximum velocity. An analysis of a Lineweaver–Burk plot shows that aldolase decreases the Km without effect on the Vm of LDH-m4 as shown in Figure 6B. The Km values of pyruvate, estimated from the intercepts along the abscissas of the double reciprocal plots of these data, are 0.67 mM in the absence of aldolase and a Km′ of 0.26 mM in the presence of aldolase. Figure 6C shows that under conditions similar to these for Figure 6B actin had little or no effect on the Km value, estimated as 0.72 mM, or the Vm.

DISCUSSION

The pathway leading to glycogen synthesis in muscle is relatively less understood than the pathway of anaerobic glycolysis. In anaerobic glycolysis, LDH, PFK-1 and AK activities have roles that

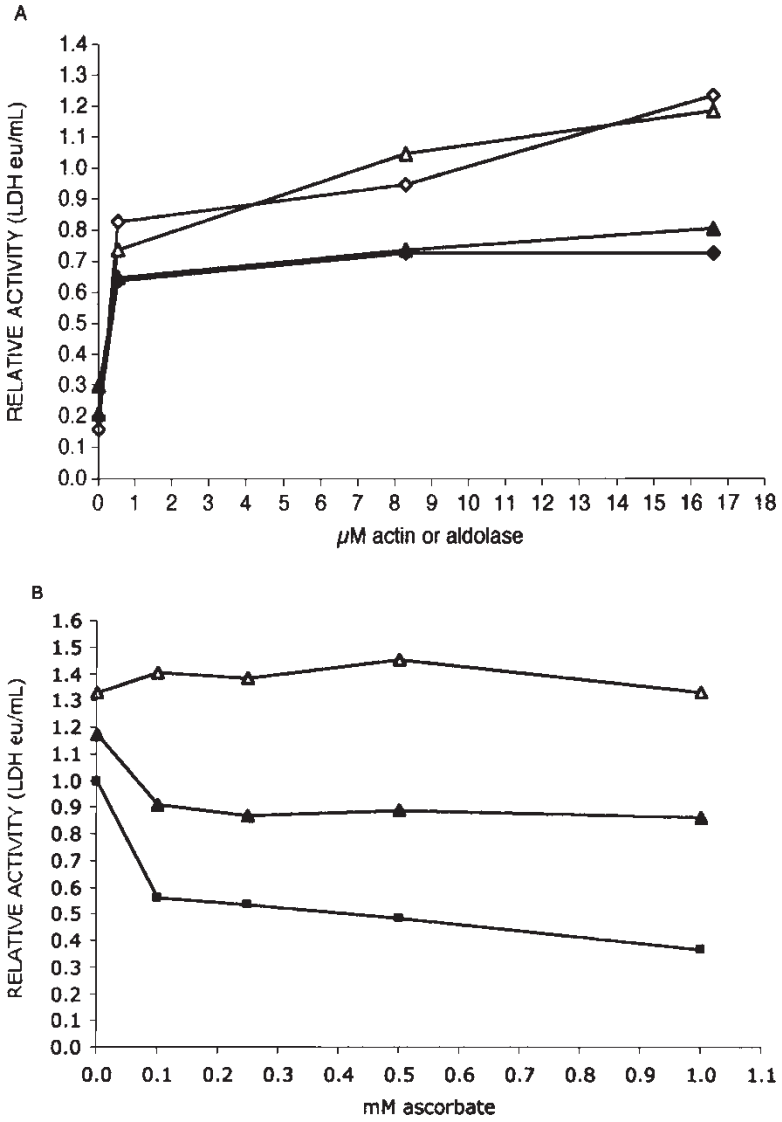

FIGURE 3 *Ability of muscle actin and aldolase to reverse ascorbate inhibitions.* (A) The ability to reverse ascorbate inhibition was tested in two ways. Activities were measured after 0.5 μM LDH was inhibited by 2 mM ascorbate after 0.5 h incubation and then aldolase (Δ) and actin (▲) were added at final concentrations indicated, incubated an additional 0.5 h and activities then determined. Aldolase (◇) and actin (◆) first added to 0.5 μM LDH at the concentrations indicated, incubated for 0.5 h. The mixtures were then made 2 mM ascorbate, incubated an additional 0.5 h and the activities determined. Both the inhibited LDH controls with and without 2 mM ascorbate were unchanged over the time frame. (B) Mixtures of 0.5 μM LDH containing 2.5 μM aldolase (Δ) or actin (▲) were incubated for 0.5 h and then ascorbate was added to the final concentrations indicated. After 0.5 h the activities were determined. The control (■) was similarly treated but contained no aldolase or actin.

facilitate the process. By reducing pyruvate to lactate, LDH facilitates anaerobic glycolysis by continuous provision of NAD^+ for anaerobic oxidations of 3-phosphoglyceraldehyde to 1,3-bisphosphoglycerate. PFK-1 forms fructose 1,6-bisphosphate and is the step that putatively commits to pyruvate formation. AK provides AMP, one of several PFK-1 activators. In rabbit muscle, these three isozymes are inhibited by ascorbate at concentrations found normally in mammalian muscle tissues.[2–4] So far, inhibitions by ascorbate appear confined to these three muscle-type isozymes.[5,20]

The results here show that muscle aldolase and muscle G-actin protect LDH-m4 from inhibition by ascorbate. Aldolase immediately follows PFK-1 in the glycolytic pathway. Others[13] showed that muscle aldolase prevented muscle PFK-1 inactivation resulting from dilutions and proposed the formation of a complex with aldolase. Though not shown here, it can be shown that aldolase also protects highly purified PFK-1 and AK from ascorbate inhibition.[5]

G-actin, which accounts for more than 20 percent of total muscle protein, is effective in protecting LDH-m4 against ascorbate inhibition but not so effective as aldolase (Figure 2B). Myosin (Figure 4),

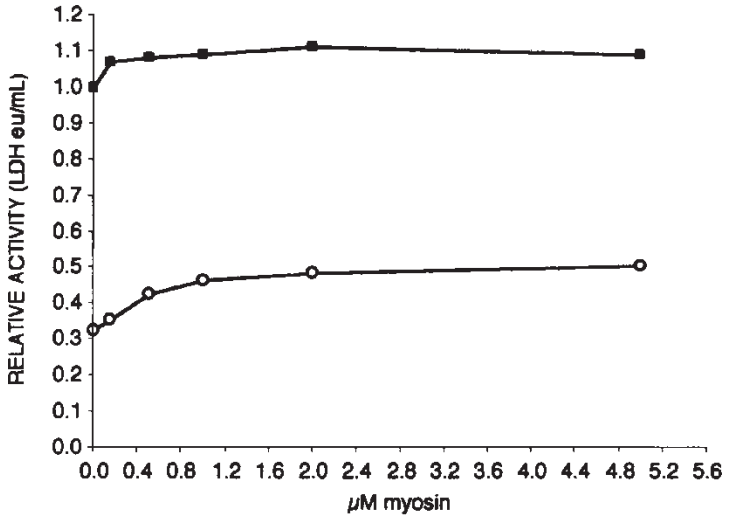

FIGURE 4 *Lack of myosin protection of LDH-m4 against ascorbate inhibition.* The symbols and conditions are the same as in Figure 2 except for the substitution of myosin (○) for actin or aldolase.

which accounts for more than 65 percent of total muscle protein, tropomyosin, and troponin do not protect LDH-m4 against ascorbate inhibition. Preliminary experiments indicate that G-actin is nearly as effective as aldolase in protecting highly purified PFK-1 from inhibition by ascorbate. Highly purified muscle AK, on the other hand, is protected from ascorbate inhibition by aldolase but not by G-actin. Experiments with mixtures of aldolase and G-actin, not shown here, suggest that the protective effect of the two proteins is additive as opposed to synergistic. High purity of muscle-type isozymes of LDH, AK and PFK-1 is critical for inhibition by ascorbate because they are protected by low concentrations of aldolase or G-actin. The possibility also exists that other proteins have similar protective effects.

There is evidence[15–19] that muscle glycolytic enzymes bind and unbind with striated muscle elements during contraction and rest, respectively. Such an arrangement allows rapid and direct deployment of ATP to the contractile proteins during contraction. We believe that the studies here reflect some of the resting properties of the contractile elements and the glycolytic enzymes. LDH-m4, aldolase and muscle PFK-1 are known to combine with both F-actin and G-actin.[15–19] Muscle AK, on the other hand, appears not to combine with either form[19] and though not shown here, G-actin does not protect AK against ascorbate inhibition.

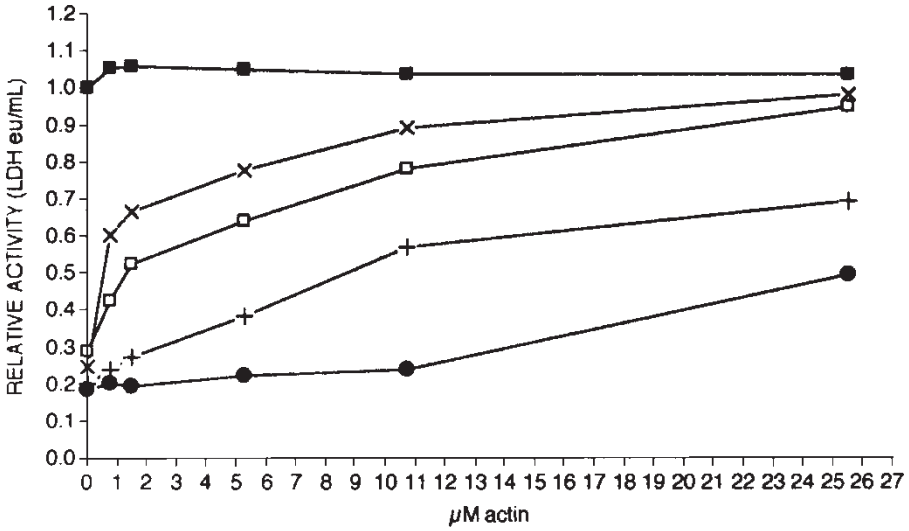

FIGURE 5 *Protection of LDH-m4 against ascorbate inhibition by G-actins from various muscle sources.* The concentration of ascorbate was 2 mM and the concentrations of LDH-m4 were 0.50 ± 0.05 μM. The conditions were the same as those in Figure 2. The actin sources are as follows: porcine muscle, (+); bovine muscle,(×); chicken muscle, (●); and rabbit muscle, (□). The control (■) is an average from separate experiments with 0.54 μM LDH-m4 in the absence of ascorbate but in the presence of the individual actins.

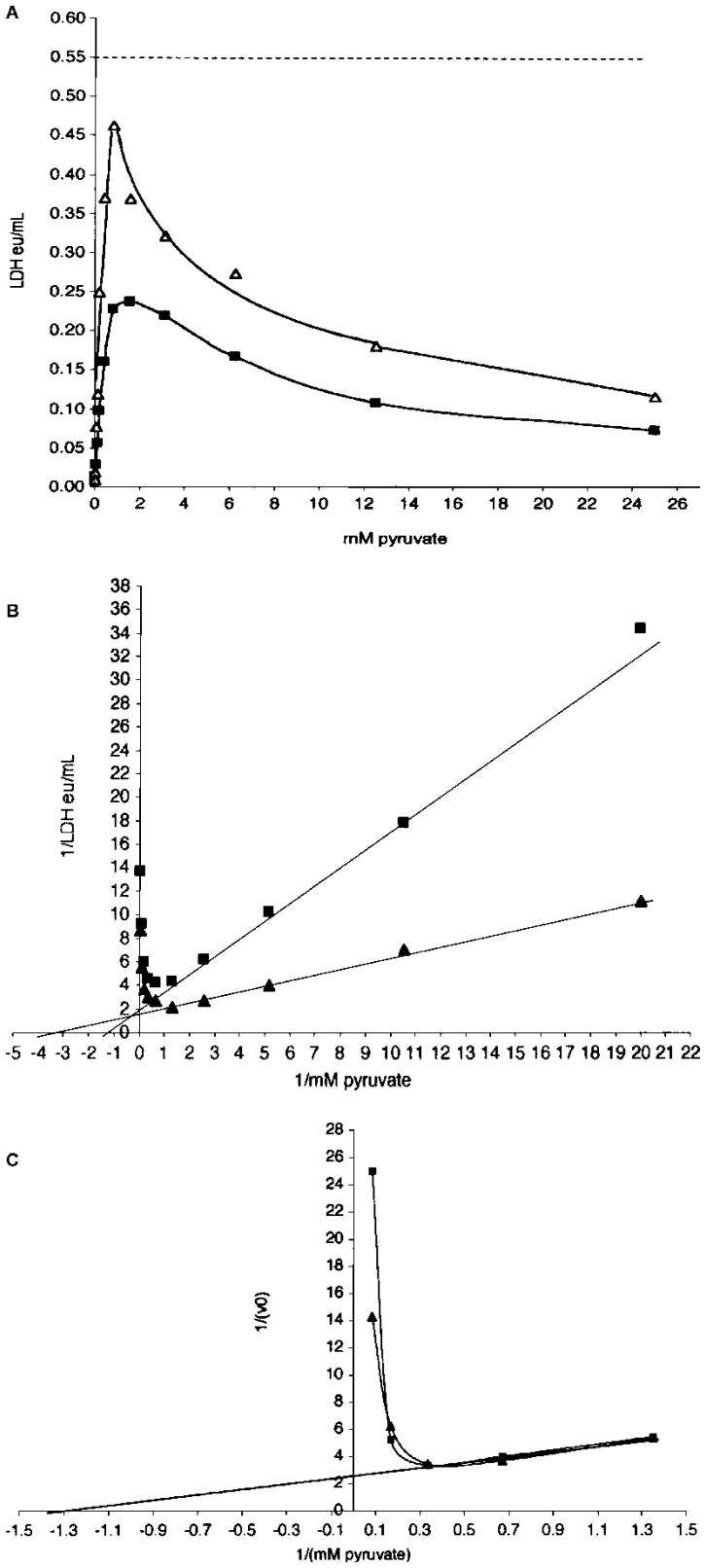

FIGURE 6 *Effect of aldolase on pyruvate (substrate) inhibition of LDH-m4 and the kinetic constants.* (A) Initial velocities of four determinations were estimated for pyruvate concentrations indicated at 25°C in potassium phosphate buffer, pH 8: (Δ) contained 0.085 μM LDH-m4 and 0.34 μM aldolase. The control (■) was without aldolase. The dotted line is the Vm (maximum velocity) determined from double reciprocal plots of the data. (B) Double reciprocal plots of the data shown in (A) from which a Km = 0.76 mM and a Km′ = 0.32 for pyruvate were estimated from the extrapolated lines in the absence (■) and presence of aldolase (▲), respectively. (C) The conditions and the symbols are the same as in (B) except that 0.26 μM actin (▲) was substituted for the aldolase. The increase in LDH activity due to the presence of actin was insufficient to significantly affect the Km or the Vm. An estimated Km = 0.74 mM for pyruvate was determined from these data. Regression analyses were $P < 0.05$.

Based on the inhibition by ascorbate of LDH, AK and PFK-1 muscle isozymes,[5] we propose that ascorbate facilitates the storage of glycogen in muscle at rest by inhibiting glycolysis. In the studies here, the combination of LDH-m4 with G-actin or aldolase protects LDH-m4 from ascorbate inhibition. We propose that during contraction, in addition to allowing rapid access to ATP generated by glycolysis, binding also prevents ascorbate from inhibiting glycolytic isozymes. During rest, these isozymes unbind and are subject to inhibition by ascorbate. The inhibition of LDH-m4, PFK-1, and LDH-m4 would greatly diminish glycolytic activity and thus facilitate glycogen synthesis and storage.

The demonstration that ascorbate inhibitions can also be reversed by aldolase or actin (Figure 3) is consistent with our proposal. We recognize the proposal as a simplification of more complex processes. For example, there is evidence that glycolytic enzymes bind and unbind from the contractile muscle elements,[15–18] but little is known about biochemical events that precipitate these actions.

It is of interest that ascorbate inhibits LDH-m4, the predominant LDH isozyme in muscle and liver tissues. Muscle and liver account for more than 90 percent of the total glycogen storage with about two-thirds occuring in muscle and about one-third in liver. Muscle stores glycogen as an immediate, rapid source of energy; liver stores glycogen as a reservoir for blood glucose delivery to other tissues. Most tissues utilize fat oxidation as a major energy source while muscle, brain and erythrocytes use glucose as a major energy source. The blood–brain barrier limits most energy sources except glucose. In the brain, about half of the LDH isozyme activity is LDH-m4; the other half is the LDH-h4 isozyme that is not inhibited by ascorbate.

Erythrocytes depend on anaerobic glycolysis as a major energy source and the predominate isozyme in erythrocytes is LDH-m4. This argues against the hypothesis that ascorbate inhibits glycolysis but crude cell extracts of erythrocytes LDH, AK and PFK-1 are not inhibited by 20 mM ascorbate. In muscle, aldolase appears to bind firmly to F-actin[16,18,19] and may not be available to protect LDH except during contraction when other glycolytic enzymes also bind to F-actin. If ascorbate does indeed facilitate storage of glycogen by inhibiting glycolysis in muscle at rest, then facilitation of glycogen synthesis in liver must proceed by a somewhat different mechanism because isozymes of liver PFK-1 and liver AK are not inhibited by ascorbate.[5] We are currently purifying PFK-1 and AK isozymes from heart muscle, erythrocytes and brain with the view of determining their sensitivity to ascorbate inhibitions in relation to glycolytic activity.

Acknowledgements

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