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Original Article

Inhibition by Ascorbate among Phosphofructokinase-1, Aldolase, Enolase, and Lactate Dehydrogenase in Rabbit Muscle

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ABSTRACT

Background: These studies examined mutual protective relationships among rabbit muscle aldolase, enolase, phosphofructokinase-1 (PFK-1) and LDH from inhibitions by ascorbate (AA). It was proposed earlier that specific inhibitions of PFK-1 and LDH by AA faciltated glycogen storage in resting muscle by inhibiting glycolysis. **Materials and Methods:** The L-ascorbate (AA), L-ascorbyl dibutyrate (AADB), L-ascorbyl dipalmitate (AADP), L-ascorbyl palmitate (AAP), and L- ascorbyl stearate (AS) are shown in Figure 1 and were obtained from TCI and Alfa Aesar. Unless otherwise stated, all enzymes come from rabbit and all experimental temperatures were 25°C, pH 8.0. **Results:** Rabbit muscle enolase was examined for its protective effect on other rabbit muscle glycolytic enzymes against inhibitions by ascorbate (AA) and some AA-faty acid derivatives. The IC_{50} values of enolase by ascorbate (AA) and IC_{50} values of AA-fatty acid derivatives were compared to estimate inhibition potency. For example, ascorbyl dipalmitate (AADP) was 156 times more inhibitory to enolase than AA. It was previously shown that rabbit muscle aldolase prevented LDH activity loses due to AA inhibition and prevented PFK-1 activity losses due both to dilution and AA inhibition; enolase was found to have the same effects as aldolase. Additionally, PFK-1 prevented enolase and LDH inhibitions by AA. LDH did not prevent enolase or PFK-1 from inhibition by AA. LDH did stimulate enolase activity but not PFK-1 activity. **Conclusion:** The results suggest that interactions among glycolytic enzyme serve to mutually protect one another from activity losses. The inhibition properties of the AA-fatty acid derivatives are discussed in relation to their possible roles in cancer and diabetes.

Keywords: Phosphofructokinase-1, Aldolase, Enolase, Ascorbate, Rabbit.

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INTRODUCTION

These studies examined mutual protective relationships among rabbit muscle aldolase, enolase, phosphofructokinase-1 (PFK-1) and LDH from inhibitions by ascorbate (AA). It was proposed earlier[1-2] that specific inhibitions of PFK-1 and LDH by AA faciltated glycogen storage in resting muscle by inhibiting glycolysis. A ten step metabolic pathway i.e. Glycolysis is catablozed by the pyruvate that leads to the production of energy i.e. adenosine tri phosphate (ATP) and nicotine adenine dinucleotide (NADP). The breakdown of aaldol cleavage is the fourth step of glycolysis in which 6 free carbon of fructise 1,6 bisphosphate in to 2. The two are glyceraldehyde 3- phosphatte and dihydoxyacetone phosphate.[3] Aldolase were found in various types of bacteria and eukaryotes. It has important role not only in glycolysis but also in gluconeogenesis and metabolism of fructise.[4-5]

Earlier studies[4] also showed that activity losses due to inhibition by AA and due to dilution,[5-6] were prevented by the presence of rabbit muscle aldolase. Rexaminations of enolase and other glycolytic enzymes inhibitons by AA were undertaken to determine whether or not inhibitions of enolase by AA were also prevented by aldolase.

A report by others[7-8] that a AA-fatty acid derivative inhibited cancer growth effects of other AA-fatty acid derivatives on enolase activity. Mutual protection of PFK-1, aldolase, enolase and LDH from inhibitions were investigated. Comparitive studies of enolase and aldolase effects on PFK-1 activity loses by dilutions, by AA inhibitions, and by AA fatty acid inhibitions were made.

MATERIALS AND METHODS

Materials

The L-ascorbate (AA), L-ascorbyl dibutyrate (AADB), L-ascorbyl dipalmitate (AADP), L-ascorbyl palmitate (AAP), and L- ascorbyl stearate (AS) are shown in Figure 1 and were obtained from TCI and Alfa Aesar. Enzymes and materials given below were from Sigma (catalog numbers) unless stated otherwise.

Methods

Unless otherwise stated, all enzymes come from rabbit and all experimental temperatures were 25°C, pH 8.0. Compared to the main enzyme activity, it was determined that enzyme enolase (E0379) contained activities ≥ 0.05% by LDH or by PFK-1; LDH contained ≥ 0.01% enolase activity; and aldolase (A8811) contained ≥ 0.05% PFK-1.

AA-fatty acid derivatives were dissolved in ethanol or dimethyl sulfoxide (DMSO, D8418). It can be shown that neither 15% ethanol nor 15% DMSO inhibited any enzymes used in these studies, more than the upper limit of the solvents used. Minimum six experiemnts were performed. Less than ± 10% standard deviation from the mean (SEM) than Data were acceptable. ± 10% error bars represent the SEM.

Rabbit Muscle PFK-1 Preparation

Purified rabbit muscle PFK-1 in these experiments was presented previously[5] according to the method of Kemp[9] and stored as a 60% saturated ammonium precipitate until ready for use. A single band in SDS PAGE was used for PFK-1 samples in all muscle of rabbit as shown

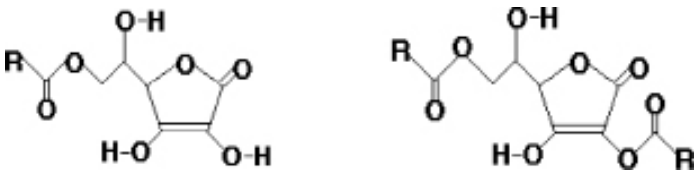


Figure 1: Structure of L- ascorbate fatty acid derivatives.
6 palmityl ascorbate (AAP) 2,6 dibutyryl ascorbate (AADB)
$R = CH_3(CH_2)_{14}–R = CH_3(CH2)_2–$
6 stearyl ascorbate (AAS)2,6 dipalmityl ascorbate (AADP)
$R = CH_3(CH2)_{16}–R = CH_3(CH2)_{14}–$

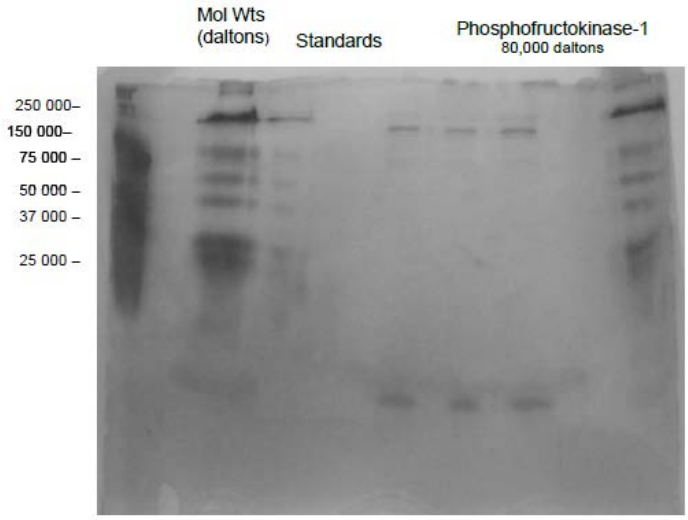


Figure 2: Polyacrylamide gel electrophoresis of a typical PFK-1 preparation. On the right are the protein standards and on the left are their molecular weights. In the center is a sample of a typical PFK-1preparation.

in Figure 2, and were devoid of adenylate kinase, aldolase (A8811), enolase, and LDH activities.

Standard PFK-1 Assay

We measured PFK-1 activity, F 6-P + ATP = F 1,6-BP (fructose 1,6-bisphosphate) + ADP, with a modification of the method by Anderson *et al.*[10] A 1 mL assay mixture contained 2 mM fructose 6-phosphate (F 6-P, F1502); 1 mM ATP (A7699); 3.0 mM $MgCl_2$; 0.13 mM NADH (N1161); 1.7 eu/mL glyceraldehyde 3-phosphate dehydrogenase (G0763); 18 eu/mL triose phosphate isomerase (T2391); 1.3 eu/mL aldolase; and 100 mM Tris-phosphate buffer, pH 8.0 (TP8).

30 nM PFK-1 Assay

When PFK-1 activity rates were below 0.05 absorbancy eu/min in a 100 µL sample, Standard PFK-1 assay system components above were concentrated to 10 times final assay concentrations into 0.1 mL; this allowed PFK-1 test samples up to 0.9 mL in the assay for more accurate rate activity measurements.

Enolase Assay

We measured enolase activity, 2-phosphoglycerate + H_2O = phospho-enolpyruvate, as follows.[11] A 1.0 mL assay mixture contained 0.01 mM TP8; 2 mM 2-phosphoglycerate (79470); 1 mM ADP (A1386); 0.13 mM NADH; 5 eu LDH (L13780); and 3 eu of pyruvate kinase (P1506) as final concentrations. One enolase enzyme unit (eu) of activity is defined as 1 µM NAD^+/min formed.

Dilutions to 30 nM PFK-1 and to 30 nM Enolase

Procedures for diluting PFK-1 and enolase to desired concentrations were as follows unless stated otherwise. Prepared stock solutions of purified 3 µM PFK-1 (3.1 eu/mL ± 0. 1) and commercial 3 µM enolase (1.6 ± 0.1 eu/mL) in 0.01 M Tris-phosphate, pH 8 were diluted with the same buffer to final concentrations and allowed to stand at least 0.5 h (hour) to allow activity losses due to dilution to stabilize. For 2 h, all activities are same under these conditions.[12]

Aldolase Assay

The reagents used for aldolase activity in rabbit muscle was same as Standard PFK-1. The only difference was using 2 mM fructose 1,6 bisphosphate (F6803) in replace of 2 mM fructose 6-phosphate and 1 mM ATP. One enzyme unit (eu) of aldolase activity is defined as 1 µM NAD^+/ min formed.

RESULTS

Reversible Inhibition of Enolase by AA

Figure 3 shows titrations of enolase versus constant AA concentrations resulted in inhibition plots that diverge from the uninhibited Control. Such divergence is associated with reversible inhibitions.[13] The Control shows small concave deviations from linearity (dotted line) at lower enolase concentrations similar to PFK-1 where it was shown that tetramers dissociated to lower, less active oligomers below 100 nM PFK-1;[14] in this case dimers would dissociate to less active monomer.

Anti-competitive character of AA inhibition of enolase

Having established the reversible character of AA inhibition (Figure 3) of enolase permitted determination of a K_i value using Michaelis-Menten kinetics.[15] As shown in Figure 4, AA inhibition of enolase is an anti-competitive or uncompetitive inhibition. Double reciprocal plots of 30 nM enolase in the presence of 2.5 mM AA (n) and 5 mM AA (s) formed parallel lines with the uninhibited Control (n), a characteristic of a reversible, anti- or un-competitive inhibition,[9] interpreted as addition of an inhibitor (I) to an enzyme-substrate complex (ES) only to form (ESI). The average AA K_i value for 30 nM enolase inhibitions was 3.8 ± 0.8 mM AA (n =6). The AA K_i values for PFK-1 was 0.16 ± 0.05 AA mM with F 6-P as the variable substrate and 0.09 ± 0.01 mM AA with ATPMg as the variable substrate,[16] two and three orders of magnitude lower than the AA K_i for enolase.

Because cancer cells rely heavily on glycolysis,[17-21] the report that a fatty acid derivative of AA, ascorbyl 2-O-phospho-6-O laurate (AA2PL6), inhibited cancer growths in mice and cell cultures[22] suggested a study of enolase inhibitions by other AA-fatty acid derivatives. Comparisons of inhibitions by AA with inhibitions by AA-fatty acid derivatives was used as a measure of inhibition potency. Plots of 100 mM AAS and 500 mM AAS inhibitions were parallel with the Control in Figure 5 indicated that AAS is an irreversible inhibitor, unlike AA (Figure 4). Though not shown, the irreversible character of all AA fatty acid inhibitors tested here gave similar irreversible plots that precluded K_i value determinations; AADB was not inhibitory.

In order to compare inhibition potencies of AA-fatty acid derivatives with AA, the AA IC_{50} and the AA fatty acid derivatives IC_{50} value were determined under the same conditions. Figure 6A and Figure 6B show how IC_{50} values[23-24] were determined and how IC_{50} values vary with enzyme concentrations. Because lC_{50} values vary with enzyme concentration, conditions for lC_{50} values have to be specified. Determinations of IC_{50} values at 30 nM enolase (Table 1) were made over

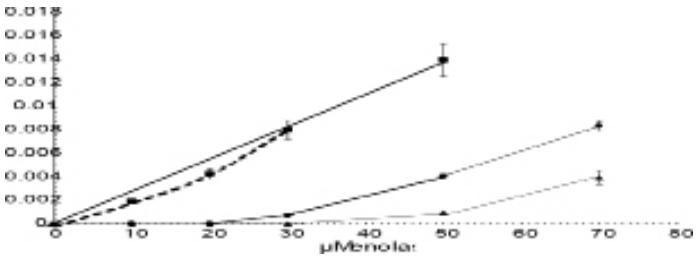


Figure 3: Titration of enolase against 2 mM AA and 5 mM AA (n = 5). Concentrations of enolase were incubated for 0.5 h to allow activities to stabilize; 2 mM AA (ν) and 5 mM AA (σ) were added and activities were then determined. Other conditions are in Methods.

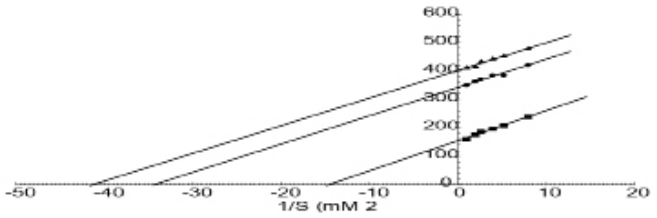


Figure 4: Michaelis-Menton plots [8] of 30 nM enolase inhibition by AA (*n* = 6).
The symbols are as follows: Control, n; 2.5 mM AA, n; and 5.0 mM AA, s.

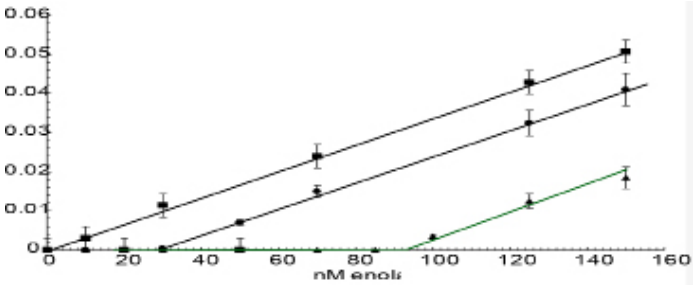
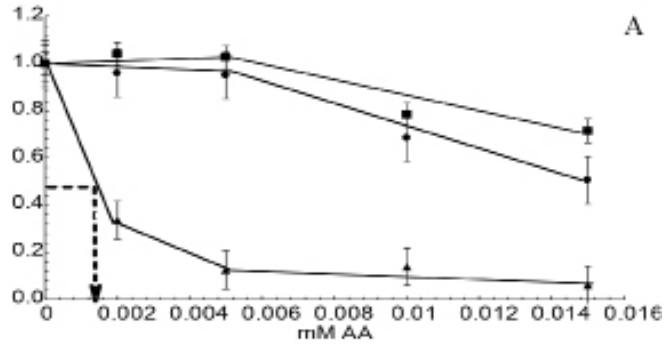


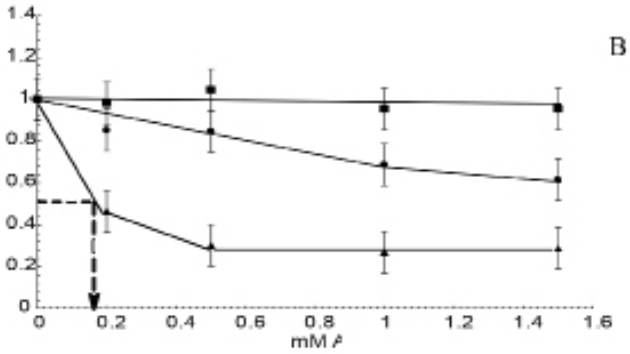
Figure 5: Titration 100 mM AADP and 500 mM AADP vs enolase (*n* =5).
The conditions were the same as that given in Figure 3 except for the inhibitors and their concentrations. The symbols are as follows: Control, n; 100 mM AADP, l; and 500 mM AADP, s.

narrower inhibitor concentration ranges than shown in Figure 6A and Figure 6B. Figure 6B also shows that at 200 nM enolase is not inhibited up to 1.5 mM AA, suggesting that higher polymeric forms, in this case dimers, are not inhibited by AA, similar to what was found for tetramers of PFK-1.[25-28] The results of these studies are shown in Table 1. The AA IC_{50} value of 0.28 mM AA for 30 nM enolase in Table 1 contrasts with the AA IC_{50} value of 0.009 mM AA for 30 nM PFK-1 (not shown). Table 1 also shows AA-fatty acid derivatives IC_{50} values relative to the AA IC_{50} value.

PFK-1 and enolase mutually protect from AA inhibition.

It was determined that aldolase, enolase, and PFK-1 were mutually free of contaminations of one by the others. Aldolase protected 30 nM PFK-1 from activity losses due to dilution and to inhibition by AA[29-31] and preliminary experiments suggested a test for effects of enolase on PFK-1 activity losses.





Figures 6A and 6B: Determination of I_{50} values of enolase for AADP and AA (*n* = 6). The symbols indicate the concentrations of enolase as follows: s, 30 nM enolase; l, 100 nM enolase; and n, 200 nM enolase. Other conditions are in Methods.

Table 1: The IC_{50} values (n ≥ 6) were determined as illustrated in Figures 2 A and B. Under the same conditions the 30 nM PFK-1 IC_{50} value was 0.009 mM AA.

AA and AA-fatty acid derivatives IC_{50} values for 30 nM enolase		
Inhibitor	Ratio	IC_{50} value mM
AA	1.00	0.28 ± 0.1
AAS	0.36	0.101 ± 0.05
AAP	10.0	0.028 ± 0.0 1
AADP	156	0.002 ± 0.001

Figure 7A shows that enolase also protects 30 nM PFK-1 from inhibition by 10 mM AA. It can be shown that, like aldolase, enolase cannot reverse AA inhibitions.[32-34] Figure 7B shows that enolase protects 30 nM PFK-1 from inhibition by AA and compares that ability to protect with aldolase. Both enolase and aldolase have about the same potential for protecting PFK-1 from AA inhibition. Figure 7C shows that PFK-1 also protects enolase from inhibition by AA. In other words, enolase and PFK-1 protect each other against inhibition by AA.

Figure 8A shows that enolase prevents LDH inhibition by AA. The loss of activity due to dilution is not a property of LDH as it is for PFK-1 (Figure 7B) or enolase (Figure 7C). However, the presence of enolase does stimulate LDH activity. It appears AA inhibits that portion of LDH activity that is stimulated by enolase. Aldolase also prevents LDH

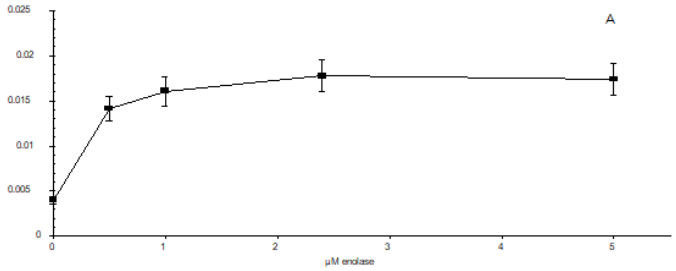


Figure 7A: Enolase protects PFK-1 against inhibition by AA ($n = 4$).
Conditions were the same as in Figure 3. Loses of activity due to dilution to 30 nM PFK-1 were 45.7 ± 0.3% on average; addition of 2 mM AA and an additional 30 min incubation brought the total activity lost to 89.4%. There was no detectable PFK-1 activity in 5 µM enolase. Other details are in Methods.

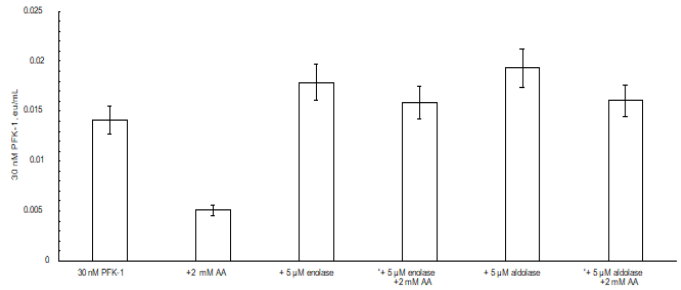


Figure 7B: Comparison of enolase and aldolase prevention of PFK-1 inhibition by AA ($n = 5$).
Enolase and aldolase were added to 30 nM PFK-1 initially, incubated for 0.5 h, AA was then added and incubated for an additional 0.5 h to allow activities to stabilize.[2] The theoretical activity is based on estimated dilutions of 3 µM PFK-1 (0.031 ± 0.001 eu/mL, $n = 6$) to 30 nM PFK-1. Activity loss due to dilution was 64%. It was determined there was no PFK-1 activity in enolase preparations. Other conditions are given in Methods.

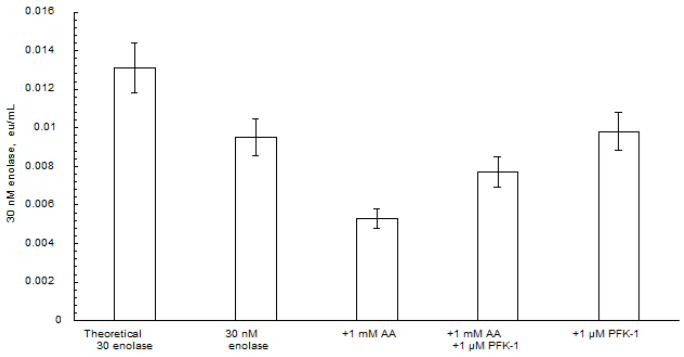


Figure 7C: PFK-1 protects enolase from AA inhibition ($n = 6$).
PFK-1 was added to 30 nM enolase and incubated for 0.5 h; AA was added and incubated for an 0.5 hr.[2] Theoretical activities were based on estimated dilutions of 30 µM enolase (2.06 ± 0.38 eu/mL) to 30 nM enolase. Average activity loss due to dilution was 34%. It was determined there was no enolase activity in PFK-1 preparations. Other conditions are given in Methods.

inhibition by AA but does not stimulate LDH activity. Figure 8B shows that LDH does not prevent enolase inhibition by AA but is equally effective as aldolase in preventing activity loss due to dilution.

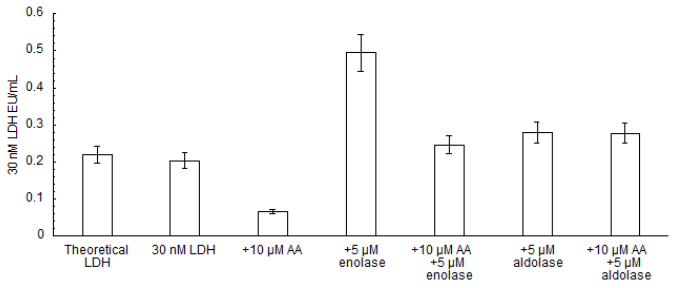


Figure 8A: Enolase and aldolase protect LDH from inhibition by AA ($n = 5$).
Enolase and aldolase were added to 30 nM LDH, incubated for 0.5 h, AA was then added and incubated for an additional 0.5 h.[2] The theoretical activity was estimated from dilutions of 3 µM LDH (23 ± 0. 01 eu/mL)) to 30 nM. It was determined that 5 µM enolase contains 0.001 eu/mL of LDH activity and 5 µM aldolase contains 0.003 eu/mL of LDH activity -- these activities were subtracted to arrive at values above containing enolase and aldolase. Other conditions are given in Methods.

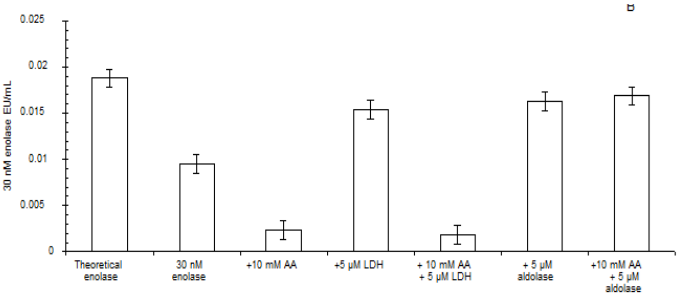


Figure 8B: LDH does not protect enolase from inhibition by AA.
LDH and aldolase were added to 30 nM enolase, incubated for 0.5 h, AA was then added and incubated for an additional 0.5 h;[2] 5 µM LDH and 5 µM aldolase contained 0.002 eu/mL and 0.003 eu/mL of enolase activity, respectively. Other conditions are the same as in Methods.

DISCUSSION

Based on inhibition of the putative controlling enzyme of glyclolysis, PFK-1, a hypothesis was proposed that AA inhibiton faciliated glycogen formation in resting muscle by inhibiting PFK-1 and therefore glycolysis.[1-2] It was shown that normal muscle AA concentrations specifically inhibited PFK-1, LDH, and adenylate kinase muscle isozymes,[1-3,13] all of which could be prevented by the presence of muscle aldolase. Others showed[14-21] that active contractile muscle proteins form complexes with glycolytic enzymes. That aldolase prevented PFK inhibitions by AA was viewed as a microcosm of *in situ* events when glycolytic enzymes combine with contractile proteins.[14-20] Glycolytic enzymes were re-examined for inhibition by AA along with other properties associated with PFK-1.[2-4] It was found that AA also inhibited 30 nM enolase (Figure 4) but with a K_i or an AA IC_{50} value (Table 1) more than an order of magnitude higher AA than the K_is or IC_{50} values for PFK-1 or LDH.[1] Figure 4 shows that AA inhibitions of 30 nM enolase yielded double reciprocal plots associated with un- or anti-competitive inhibition.[9] The higher K_i value for enolase AA inhibition relative to K_i values for PFK-1 or LDH suggest that enolase inhibition by AA is not a factor in facilitation of glycogen formation.

The hypothesis initially considered that glycolytic enzymes formed complexes with contratile muscle proteins that prevented AA inhibitions. However, mutual protective effects from AA inhibitions among PFK-1, enolase, and aldolase (Figures 7B-8A) suggest that interactions among

glycolytic enzymes themselves provide prevention of inhibitions that would allow glycolysis to produce ATP for contractions, in addition to any protection that might arise from a complex with contractile muscle proteins.[16-20,31]

That ascorbyl 2-O-phospho-6-O laurate (AA2PL6) inhibited cancer growths in mice and cell cultures[5] suggested our study of enolase inhibitions by other AA-lfatty acid derivatives. The AA-fatty acid derivatives studied were irreversible inhibitiors against enolase (Figure 5). In a comparison of IC_{50} values, AAS was less inhibitory than AA, AAP was 10 times more inhibitory than AA, and AADP was 156 times more inhibitory than AA (Table 1). The results suggest a wide range of fatty acid compounds as AA fatty acid derivatve inhibitors or glycolytic enzymes. Oxidative phosphorylation appears to be faulty in most cancer cells,[21-25] which rely on glycolysis as a major energy source.[26] Since most cancer cells depend on glycolysis as a energy source,[22-23,27-28] the highly inhibitory nature of AADP to enolase suggests that AA2P6L may have inhibited cancer growth and metastasis[5] by inhibiting glycolysis in lieu of or in addition to a decrease in the reactive oxygen species (ROS) as the authors propose.

It was shown that aldolase prevented PFK-1 activity losses due to dilution and due to inhibitions by AA;[2-4] enolase was examined for the same protective properties. Figures 7B shows that enolase prevents PFK-1 inhibiton by AA similar to aldolase; enolase also prevents activity losses due to dilution. Figure 7C shows that PFK-1 mutually prevents enolase from inhibition by AA but has less effect on enolase activity losses due to dilution.

Enolase prevented 30 nM LDH inhibition by AA (Figure 8A) equal to aldolase but does not stimulate enolase activity. The presence of aldolase enhances LDH activity. No properties of enolase, PFK-1, LDH and aldolase examined conflicted with the hypothesis that AA facilitates the storage of glycogen by inhibiting glycolysis when muscle is at rest[1-2] Aldolase prevents enolase, PFK-1, and LDH inhibitions by AA and was not inhibited by AA at concentrations used in these studies. Enolase was similar to aldolase in protection of PFK-1 and LDH from inhibition by AA. LDH did not mutually prevent enolase inhibition by AA. Aldolase is associated with actin[30-31] and it was assumed that glycolytic enzymes protections from AA inhibition was the function of aldolase. Mutual protection among glycolytic enzymes themselves makes it possible that when contracting muscle forms a complex with glycolytic enzymes that they protect each other from inhibiitons and glycolysis proceeds.

At an IC_{50} value of 2 nM AADP, the AA-fatty acid derivative was a potent inhibitor of enolase (Table 1). These studies agree with suggestions[27-28] that development of novel glycolytic inhibitors as a new class of anticancer agents is likely to have broad therapeutic applications.

These studies may be related to diabetes. Diabetics apparently have a faulty glycogen synthesis.[32-36] The hypothesis on which this study was based implicated AA as a positive factor in glycogen synthesis.[1-4,13] In diabetes, AA does not get into muscle tissue[34] because AA as dehydroascorbate (DHA); DHA uses the same insulin-dependent GUT 1 system as glucose[35] resulting in what has described[36-39] as «tissue scurvy» in an uncompensated diabetic.

CONCLUSION

In summary, glycolytic enzymes in association protect one another from inhibtions due to dilution, AA inhibition, and inhibitions by AA fatty acid derivatives. Because of irreversible nature of the latter, IC_{50} values were determined to compare inhibition potencies. Although not considered a member of the glycolytic enzymes, LDH was also protected from inhibitions by PFK-1, aldolase and enolase but it did not protect in turn protect these enzymes from inhibitions. The highly inhibitory nature of the AA fatty acid derivatives, the report of an AA fatty acid derivative inhibiting cancer growth,[5] and the large number of fatty acids available suggests that AA fatty acid derivatives have a potential as chemotherapeutic agents.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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