UNIVERSITY OF CALIFORNIA, SAN DIEGO

Inhibitions of ascorbate fatty acid derivatives on three rabbit muscle glycolytic enzymes

A thesis submitted in partial satisfaction of the
requirements for the degree of Master of Science

in

Biology

by

Duyen-Anh Pham

Committee in charge:

Professor Percy Russell, Chair
Professor Paul Price, Co-Chair
Professor Edward Alexander
Professor James Golden

2011

The Thesis of Duyen-Anh Pham is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2011

DEDICATION

This thesis is dedicated to Professor Percy Russell, his staffs Anita Williams and Ami Abbott and students. Because of their relentless care, guidance and love for more than two years, I have growed academically and personally.

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LIST OF ABBREVIATIONS

PFK-1	Phosphofructokinase -1 of rabbit muscle
LDH	Lactate dehydrogenase of rabbit muscle
AK	Adenylate kinase or myosine of rabbit muscle
AMP	Adenosine monophosphate or 5'-adenylic acid
AA	L-ascorbate or ascorbic acid or (5*R*)-[(1*S*)-1, 2-dihydroxyethyl]-3, 4-dihydroxyfuran-2 (5*H*)-one
AAS	6 stearyl ascorbate
AAP	6 palmityl ascorbate
AADP	2, 6 dipalmityl ascorbate
AADB	2, 6 dibutyryl ascorbate
MB	Methyl butyrate
EB	Ethyl butyrate
MP	Methyl palmitate
EP	Ethyl palmitate
MS	Methyl stearate
ES	Ethyl stearate
LGGL	L-gulono-gamma-lactone

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ACKNOWLEDGEMENTS

First and foremost, I am deeply indebted to my advisor, Professor Percy Russell, for his constant support, exceptional care and plentiful encouragement. Without his guidance, this work would not be possible. Second, I would also like to extend my special thanks to Professor Edward Alexander who introduced me to this project and has been my research mentor in the past two years. Third, many thanks are offered to Anita Williams and Ami Abbott for teaching me scientific methods and providing technical support. Their assistance is invaluable to my study. Fourth, I want to express my appreciation to the thesis committee members Professor Paul Price and Professor James Golden for spending their valuable time to learn about my project and giving me such constructive feedbacks for both my oral presentation and thesis paper.

Last but not least, I am not who I am today without the love, caring, understanding and patience of my family. I cannot express enough the gratitude for my mother and brother who are always with me to share my happiness and lend me a hand over those challenging times.

ABSTRACT OF THE THESIS

Inhibitions of ascorbate fatty acid derivatives

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by

Duyen-Anh Pham

Master of Science in Biology

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Professor Percy Russell, Chair
Professor Paul Price, Co-Chair

This thesis relates to a working hypothesis that inhibition of three isozymes rabbit muscle PFK-1, LDH and AK by ascorbate shuts down glycolysis pathway and facilitates glycogen storage in resting muscle; when muscle is active these isozymes combine with skeletal muscle proteins such as aldolase and actin and are protected from inhibition by ascorbate and glycolysis proceeds. Dibutyryl- (DB), dipalmityl- (DP), palmityl- (P), and stearyl- (S) fatty acid derivatives of ascorbic acid (AA) were shown to plot as irreversible

inhibitors of rabbit muscle PFK-1 and LDH. Inhibition potencies of all these inhibitors on PKF-1, LDH and AK were measured as I_{50} values, concentrations of inhibitor required for 50% inhibition of enzyme activity, and compared to I_{50} values for AA. All AA-fatty acid derivatives were more inhibitory than AA except for AADB, which was less inhibitory for PFK-1 but the most inhibitory for LDH. Most AA fatty acid derivatives showed inhibitory effect on AK but were not better inhibitors than AA. Studies on substituents of AA fatty acid inhibitors suggested double bond in the lactone ring of AA and its derivatives contribute to the inhibitory characteristics. Similar to AA inhibitions of PFK-1 and LDH, rabbit muscle aldolase protected against activity losses due to dilution and AA-fatty acid derivatives. AMP, a PFK-1 activator, also demonstrated protecting PFK-1 from AA and its derivative inhibitors. Inhibition properties of AA-fatty acid derivatives shown here are discussed in terms of other possible fatty acid derivatives and possible therapeutic values in cancer and diabetes.

Chapter 1- Introduction

Glycolysis is a universal process among most living organisms and its multiple metabolic steps function in breaking down glucose molecules to generate ATP as cellular energy sources during catabolism by digestive system enzymes. Glucose can also undergo an anabolism process resulting in the synthesis of polymer chains of glycogen.

As a result of earlier investigations [1-3], a hypothesis was developed that ascorbate (ascorbic acid or vitamin C) facilitates glycogen storage in resting muscle by inhibiting degradation of glucose through inhibition of muscle glycolytic isozymes – phosphofructokinase-1 (PFK-1), lactate dehydrogenase (LDH), and adenylate kinase (AK). In active muscle, these isozymes form a multi-enzyme complex with contractile muscle protein actomyosin [4-7] that we posit protects them from inhibition by ascorbate. Muscle aldolase, which has a firm association with actin [4, 6], protects muscle isozymes PFK-1, LDH, and AK from inhibition by ascorbate [3, 8]; this protection is considered a microcosmic reflection of the larger complex formed in active muscle.

Ascorbate (5*R*)-[(1*S*)-1, 2-dihydroxyethyl]-3, 4-dihydroxyfuran-2 (5*H*)-one is an essential nutrient for the biosynthesis of collagen, L-cartinine, and the conversion of dopamine to norepinephrine [9] in addition to its role as a water-soluble antioxidant. Although most animals are able to synthesize large amount of ascorbate endogenously from glucose, some mammals including humans and other primates lost this function due to inactivating mutations on a gene encoding L-gulonolactone oxidase (GULO), an important enzyme in vitamin C biosynthetic pathway [10, 11]. For those animals that cannot synthesize, ascorbate is a vitamin. Acute ascorbic acid deficiency leads the nutritional condition called scurvy, characterized by blood vessel fragility, connective tissue damage, fatigue, and ultimately to death [11].

Ascorbate is composed of a double-bond ester ring and four hydroxyl groups. Like glucose, ascorbic acid is a significantly large and polar compound which cannot diffuse freely across the amphipathic phospholipid bilayer of the cell membrane into the intracellular compartment. Therefore, in order to cross the cell membrane, like glucose, vitamin C needs, a glucose transporter (GLUT) coupled or the sodium vitamin C cotransporter (SVCT). The presence of GLUT and SVCT types varies in different tissues. Ascorbate can also enter cells initially in its oxidized form, dehydroascorbic acid (DHA). GLUT facilitates DHA across membranes without SVCT; DHA is then converted to ascorbate inside the cell [12-13]. Ascorbic acid can also cross the membrane barrier as an ascorbyl ester.

Previous studies showed that amphipathic compounds including ascorbyl stearate (Asc-S) [14] and ascorbic acid-2-O-phosphate-6-O-laureate (AA2P6L) [15] were nontoxic, easily cross the blood-brain barrier due to its lipophilic property, and were able to inhibit cell proliferation, transformation and upregulate apoptosis in human pancreatic cancer and glioma cells *in vitro* and *in vivo*.

Recent studies showed high-dose intravenous vitamin C administration was applicable in cancer therapy, despite a controversial history in cancer treatment which suggested high ascorbate doses of 10 g daily prolonged the survival of cancer patients in one study and the opposite in the other [16-17]. Pharmacological concentrations of ascorbate (0.3-20 mmol/L) that are comparable to those attained by intravenous therapy selectively target and kill human lymphoma cancer cells but not normal human lymphoma cells in vitro [18]. Ascorbate at physiologic concentrations (0.1mmol/L) showed no effect on either cancer or normal cells [18]. In both *in vitro* and *in vivo* experiments, pharmacologic ascorbate is a prodrug for preferential steady-state formation of ascorbyl radical and hydrogen peroxide (H_2O_2) in the extracellular space,

but not in blood [19]. Production of H_2O_2 from oxidized ascorbate may potentially cause DNA damage, glycolysis downregulation, and mitochondrial damage leading to reduction of ATP synthesis [19], the significant role of glycolysis in cancer cells is not well enough defined to elucidate possible mechanisms on how tumor cells are specifically eliminated.

In this research project, inhibitory characteristics of fatty acid derivatives of L-ascorbate 2, 6 dibutyryl- (AADB), 2, 6 dipalmityl- (AADP), 6 palmityl- (AAP) and 6 stearyl- (AAS) were studied and compared with inhibitory characteristics of ascorbate under similar conditions. Characteristics investigated for comparisons of ascorbate fatty acid derivatives with ascorbate are effects of muscle PFK-1, LDH, and AK concentrations on inhibitions, activity losses due to dilutions, inhibitor concentrations yielding 50 percent inhibition (I_{50}) and the ability of rabbit muscle aldolase or adenosine monophosphate (AMP) to prevent activity losses were studied. The results of these studies are discussed in terms of possible relationships these fatty acid derivatives of ascorbate to diabetes and certain types of cancer.

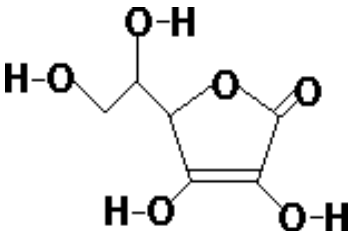
Chapter 2 - Materials and Methods

Materials

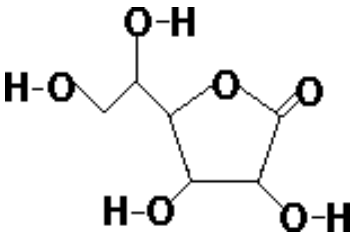
Biologicals and chemicals

Ascorbate-fatty acid derivatives

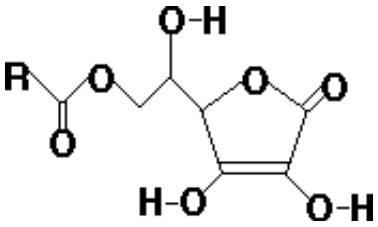
The L-ascorbate (AA-) fatty acid derivatives were obtained from TCI and Alfa Aesar. The following were obtained from Aldrich and Sigma: adenosine monophosphate (AMP), L(+)-gulonic acid gamma-lactone (LGGL); ethyl butyrate (EB); methyl butyrate (MB); ethyl palmitate (EP); methyl palmitate (MP); ethyl stearate (ES); and methyl stearate (MS). Structures of the AA-fatty acid derivatives are shown in Figure 1.



L-ascorbate

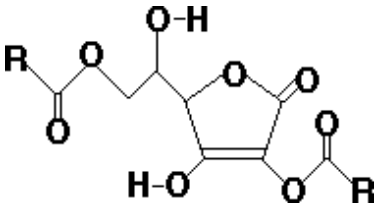


L(+)-gulonic acid gamma-lactone (LGGL)



6 palmityl ascorbate (AAP)
R = $CH_3(CH_2)_{14}$–

6 stearyl ascorbate (AAS)
R = $CH_3(CH_2)_{16}$–



2, 6 dibutyryl ascorbate (AADB)
R = $CH_3(CH_2)_2$–

2, 6 dipalmityl ascorbate (AADP)
R = $CH_3(CH_2)_{14}$–

Figure 1: Structure of ascorbate fatty acid derivatives

Sigma-Aldrich Co was the source of chemicals and enzymes used unless stated otherwise. Rabbit muscle lactic dehydrogenase (LDH, EC 1.1.1.28), adenylate kinase (AK, EC 2.7.4.3) and rabbit muscle aldolase (EC 4.1.2.13) were free of PFK-1, LDH, AK activities under our conditions.

Purified phosphofructokinase-1 enzyme (EC 2.7.1.56) was prepared from frozen rabbit muscle tissue (PFK-1) by a minor modification of a method of Kemp [20] protocol except for small changes due to different physical properties. Purified PFK-1 samples used in these experiments were determined as free of aldolase and LDH by PAGE and enzyme assay as described below.

Phosphofructokinase-1 Purification

Extraction

50 grams of rabbit muscle were minced and then passed through a blender. Muscle tissue was homogenized in an Osterizer for 3 minutes at highest speed in 3 volumes (3 mL/g of tissue) of 1 mM disodium ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol (DTT), and 1 mM NaF pH 7.5. The PFK-1 activity of the homogenate was determined. The mixture was centrifuged at 4^{o} C and 10,000 X g for 30 minutes. The supernatant contained no or little PFK-1 activity was discarded. The sediment was resuspended in 2 volumes (2 mL/g of tissue) of the same solution with a Polytron and the mixture was centrifuged again at 4^{o} C and 10,000 X g for 30 minutes. The supernatant was discarded after testing negative for PFK-1 activity. The sediment was then suspended with a Polytron in 1.5 volumes (1.5 mL/g of tissue) of 50 mM Tris-Base pH 8.0 (TP8), 50 mM $MgSO_4$, 0.1 mM EDTA, 0.5 mM adenosine 5’ triphosphate (ATP),

and 1 mM DTT and heated in a 70^{o} C water bath. The suspension was rapidly stirred until the temperature of the thick slurry reaches 57^{o} C. The temperature was maintained between 57^{o} C and 59^{o}C for 3 minutes. The flask was removed and placed in ice slurry with stirring until the temperature dropped to 10^{o}C. The mixture was centrifuged at 4^{o} C and 10,000 X g for 20 minutes. The first supernatant was retained and the PFK-1 activity determined, and the sediment was resuspended by Polytron in 1.5 volumes of the Tris-$MgSO_4$-EDTA-ATP-DTT-NaF buffer and the mixture was centrifuge for 20 minutes at 10,000 X g. The second supernatant was combined with first supernatant and measured for activities of PFK-1, LDH and AK in the extract.

Diethylene Amino Ethanol (DEAE) Sephacel column (I-6505)

The collected PFK-1 extract solution was run through a 100 mL anion exchange DEAE-Sepharose column (2.5 X 9 cm) that was equilibrated with 40 mM TP8. The PFK-1 extract was passed through the column and washed with 50 mL of 40 mM TP8/ 1 mM DTT/ 30 mM NaF, followed by 100 mL of 40mM TP8/ 1mM DTT/ 30nM NaF pH 8.0 which removed large amounts of aldolase and LDH from the column. Then, 100 mLs of 600mM TP8/ 1mM DTT/ 30mM NaF, pH 8.0 washed the column to remove PFK-1 (see Figure 2.1). Samples with highest PFK-1 activity and lowest LDH and aldolase activity were combined and the rest was discarded.

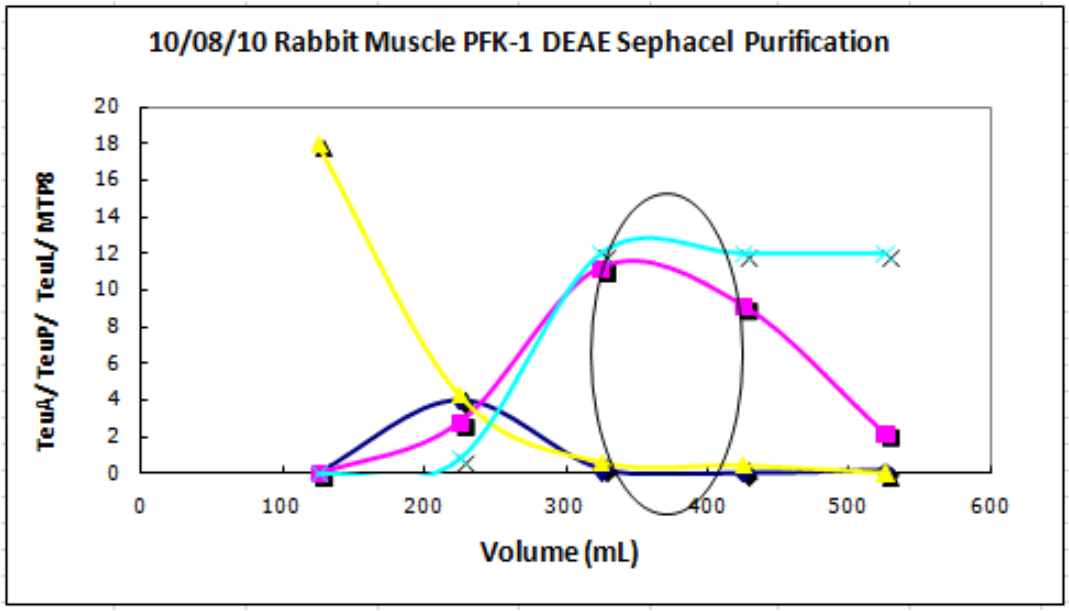


Figure 2.1: Enzyme activity levels of PFK-1 (■); LDH (▲) and aldolase (◆) were present after different washes of TP8/ DTT/ NaF. PFK-1 was washed with 150 mL of 40 mM TP8/ 1mM DTT/ 30 mM NaF and several 100 mL of 600 mM TP8/ 1 mM DTT/ 30 mM NaF, at pH 8.0 in order to remove aldolase and LDH. As the result, most aldolase and LDH were removed by 325-425 mL of 600 µM Tris phosphate.

For each PFK-1 enzyme, the combined fractions were put in the ice bath and brought to 70 percent saturated ammonium sulfate by addition of solid until all ammonium sulfate ($AmSO_4$) dissolved, stirred for 20 minutes and then centrifuged at 10,000 X g and 4^oC for 30 minutes. The PFK-1 samples of $AmSO_4$ precipitates were stored at 4^oC until used.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Criteria for PFK-1 purity were determined by the absence of aldolase activity and of PFK-1 and SDS PAGE with a 5.2 percent stacking gel and a 12 percent cross-linked separating gel in the Mini-Protean II Cell assembly. The developed Bio-Rad gels

were silver stained for proteins using the procedure of Morrissey [21]. All PFK-1 preparations used in these studies were devoid of LDH and aldolase activities and showed a single band in SDS PAGE (Figure 2.2).

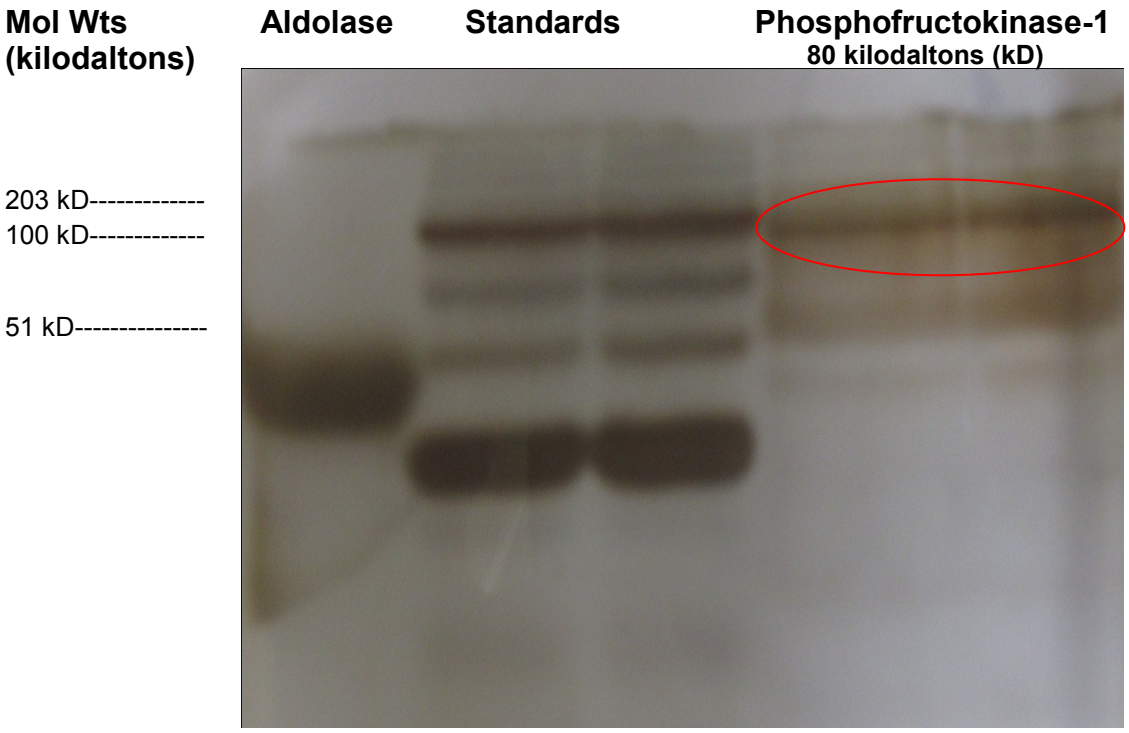


Figure 2.2: SDS PAGE image of PFK-1 purification. The sample demonstrates thick lines of PFK-1 (80kD/ subunit of a tetramer) and the presence of very low concentration of other proteins. The sample contained no aldolase or LDH activities (see Figure 2.1).

Methods

All operations were at 25 °C and pH 8 unless otherwise stated. All measurements were done in duplicate with n=6 as a minimum. All experimental values reported in this study were within ± 10% of the mean indicated by error bars in the

figures. All buffers used in these studies were 0.1 M TP8 for PFK-1 studies and 0.1 M potassium phosphate, pH 8.0 (KP8) for LDH and AK studies.

Standard PFK-1 assay

We determined PFK-1 activity, F 6-P + ATP = F 1, 6-BP, with a modification of the method by Anderson et al. [21]. A 1.0 mL assay mixture contained 2 mM fructose 6-phosphate (F 6-P); 1 mM adenosine 5' triphosphate (ATP, A 7699); 3 mM magnesium chloride ($MgCl_2$); 0.13 mM Nicotinamide adenine di-nucleotide reduced form (NADH, N 1161); 1.7 eu/mL glyceraldehyde 3-phosphate dehydrogenase (G 0763); 18 eu/mL triose phosphate isomerase (G1881); 1.3 eu/mL aldolase (A 8811); and 100 mM TP8 as final concentrations.

30 nM PFK-1 Assay

When diluted PFK-1 solutions had rates below (0.05-absorbancy unit)/min in 100 µL of samples, standard PFK-1 assay components were concentrated 10 times the final assay concentrations into 0.1 mL [8]. This allowed up to 0.9 mL PFK-1 samples for more accurate rate measurements. A molar absorptivity value of 6,220 converted NADH absorbance changes to µmoles of product formed. One PFK-1 enzyme unit (eu) of activity is defined as 1 µmole of NAD^+ formed per minute at 25^{o}C.

Standard LDH assay

The LDH assay medium contained 0.4 mM NADH, 0.7 mM pyruvate, 20 mM potassium phosphate, pH 8.0. Measurement of LDH activity was according to Vassault [23]. A molar absorptivity value of 6,220 was used to convert NADH absorbance changes to µmole of product 1 enzyme unit (eu) of aldolase activity is defined as 1 µmole of NAD+ formed per minute at 25^{o}C.

30nM LDH Assay

When diluted LDH solutions had rates below 0.05 absorbancy unit/min in 100 µL of samples, standard LDH assay components were treated the same as given for 30 nM PFK-1 assay above.

Standard AK assay

We measured AK activity, AMP + Mg.ATP = ADP + Mg.ADP, according to Adam [24]. Other kinases and reactions producing ADP were appropriately coupled and adapted to the same pyruvate kinase-lactate dehydrogenase assay system as AK. A 1.0 mL assay mixture contained 0.3mM phosphoenolpyruvate (PEP), 0.4 mM NADH, 8.0 mM ATP, 0.9 mM AMP, 8.1 mM $MgCl_2$ and 20 mM potassium phosphate buffer, pH 8. There were sufficient amounts of LDH-h4 and pyruvate kinase (PK) so the coupling system was not rate limiting. Reactions were initiated by additions of test enzymes. Initial reaction rates were determined by measuring decreased absorbance of NADH at 340 nm with time. One AK enzyme unit (eu) of activity is defined as formation 1 µmole of NAD+ formed per min at 25^{o}C.

30nM AK assay

When diluted AK solutions had rates below 0.05-absorbancy unit/min in 100 µL of samples, standard AK assay components were treated the same as given for 30 nM PFK-1 and LDH assays above.

Aldolase Assay

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

enzyme unit (eu) of aldolase activity is defined as 1 μmole of NAD^+ formed per minute at 25^{o}C.

Measurement of protein concentration

Protein concentrations are 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 [25]. The spectrophotometric protein determinations are comparable to the Bradford method [26].

Dilutions of and additions to PFK-1, LDH and AK

Standard procedures for preparing low concentrations of all PFK-1, LDH, and AK preparations were as follows unless stated otherwise. Purified 3 μM solutions of PFK-1 (3 eu/mL) in 0.1 M TP8, 3 μM LDH (60 eu/mL) in 0.1 M KP8, and 3 μM AK (12.5 eu/mL) in 0.1 M KP8 were diluted in their respective buffers to final 30 nM concentrations desired and allowed to stand at 25 °C for at least 0.5 hour (h) to allow activity losses due to dilution to stabilize; PFK-1, LDH and AK activities remained constant for more than 2 h under these conditions [8]**.** Additions of AA-fatty acid derivatives in 95% ethanol to diluted PFK-1, LDH and AK test samples were achieved by adding a maximum 1/20 test sample volume of the 20-fold final AA-fatty acid derivative concentration desired; it was determined that final concentrations of ethanol in test samples did not inhibit PFK-1, LDH and AK (will check to confirm AK). Test samples were then incubated at 25 °C for 1 h and PFK-1, LDH and AK activities then determined.

Variable concentration of fatty acid derivatives of ascorbate against 30 nM RMPFK-1, commercial 30 nM RMLDH and commercial 30 nM RMAK (Chapter 4)

A range of increasing concentrations of ascorbate derivatives were incubated for 1 hour with constant concentration of 30nM RMPFK-1, commercial 30nM RMLDH, and commercial 30nM RMAK. The RMPFK-1, RMLDH, RMAK activity remaining were then determined by utilizing PFK-1, LDH, AK assays and spectrophotometry and later relative enzyme activities were calculated by taking ratio of each derivative and ascorbate. Finally, calculations of 50% inhibition of each substrate on PFK-1, LDH, and AK (I_{50}) were performed to determine PFK-1, LDH and AK specificities and inhibitory functions.

Aldolase and RMPFK-1 or RMLDH incubated with AA derivatives

Ascorbate derivatives at predetermined I_{50} were incubated in 5 μM aldolase for 1 hour. The RMPFK-1 and RMLDH activity recoveries were then determined by utilizing PFK-1, LDH assays and spectrophotometry.

Adenosine Monophosphate (AMP) and RMPFK-1 incubated with AA derivatives

AA derivatives at predetermined I_{50} were incubated with PFK-1 in various AMP concentrations to determine the maximum protective effect of AMP; ascorbate derivatives at predetermined I_{50} were then incubated at this AMP concentration. After 1 hour the PFK-1 activity remaining was determined by utilizing PFK-1 assays and spectrophotometry.

Chapter 3 – Ascorbic acid derivatives acting as reversible and irreversible inhibitors on different concentration ranges of glycolytic PFK-1 and LDH

Previous studies [8] show that at concentrations found naturally in cells ascorbate was a specific inhibitor of muscle PFK-1 and LDH [1]. Preliminary studies determined whether or not ascorbic acid derivatives used in these studies were inhibitors. Also, types of compounds, reversible or irreversible inhibitors were shown in figure 3. Parallel patterns in Figure 3A suggest that inhibition of PFK-1 by AADP is irreversible [26]. However, at lower concentrations of PFK-1, a deviation from the parallel pattern obtains. In Figure 3B, lower concentration ranges show diverging patterns suggestive of reversible inhibitions [26] and are consistent with evidence that at low concentrations PFK-1 transitions from tetramers to dimers; dimers show less activity and inhibition characteristics different from tetramers [8]. Though not shown, other AA-fatty acid inhibitors, AADB, AAP and AAS show similar inhibition patterns at low and higher PFK-1 concentrations. Complexities of PFK-1 polymer formations precluded definitive Michaelis-Menten kinetics inhibition studies.

Figure 3A

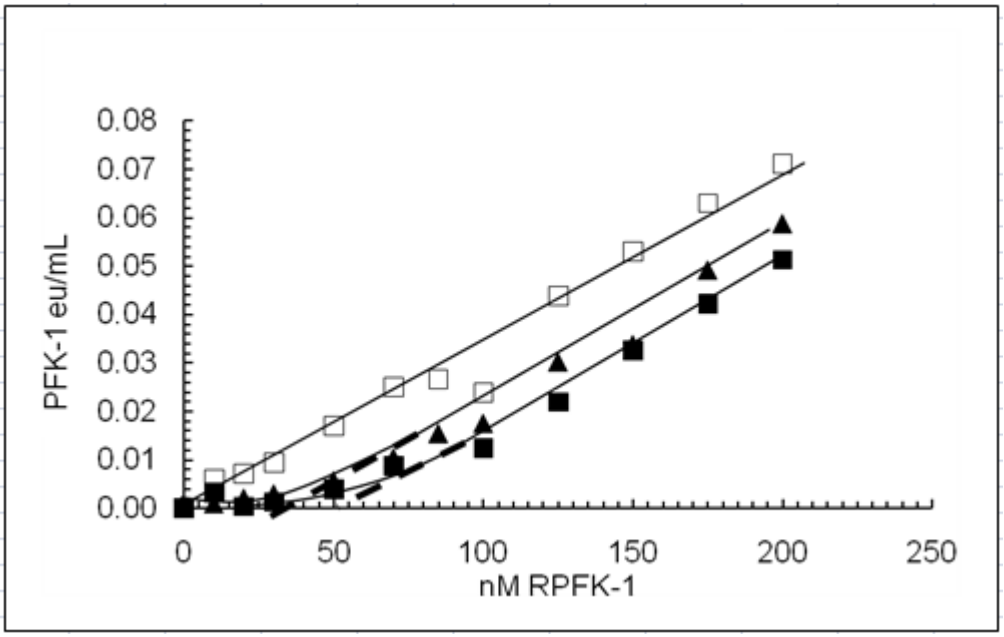


Figure 3B

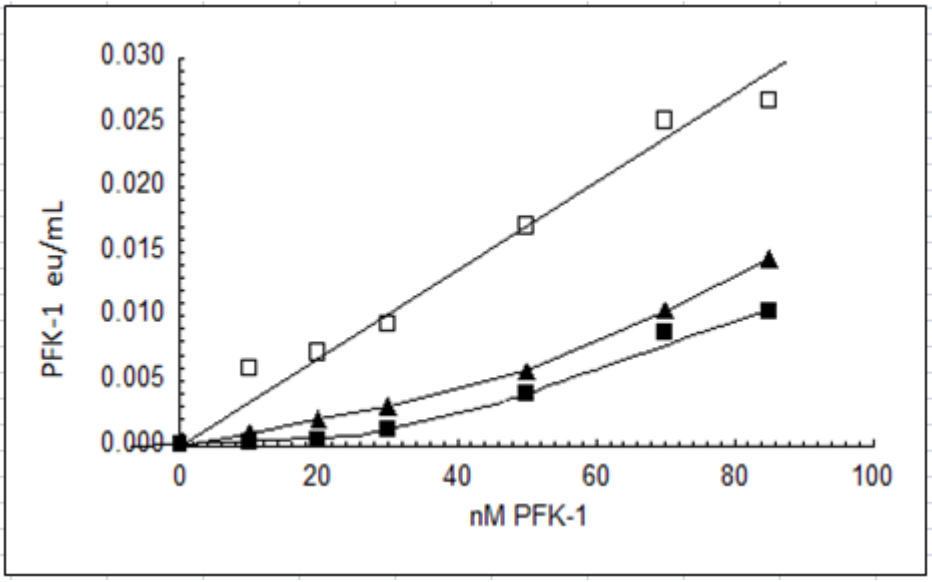


Figure 3: Determination of the reversibility of PFK-1 inhibition by AADP

A. Titration of PFK-1 against 0.5 μM AADP (▲) and 1.0 μM AADP (■) compared to control (□). B. Titration of PFK-1 against 0.5 μM AADP (▲) and 1.0 μM AADP (■) compared to control (□) over a narrower nM PFK-1 concentration range. Conditions are given in Materials and Methods.

The parallel pattern in Figure 4 shows no deviation at lower LDH concentrations, which suggests that AADP is an irreversible inhibitor [26] for monomeric, dimeric forms or tetrameric forms [27-28].

Figure 4

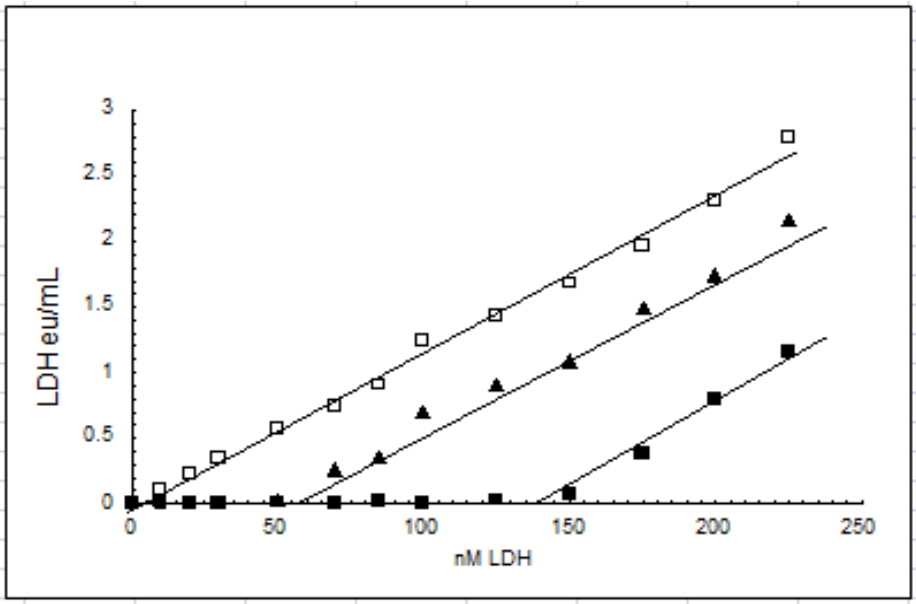


Figure 4: Determination of the reversibility of LDH inhibition by AADP conditions. AADP concentrations and symbols were the same as in Figure 3A.

Chapter 4 – Inhibitory characteristics of various fatty acid derivatives of ascorbate on glycolytic PFK-1, LDH and AK (myokinase) in muscle tissues

In order to compare the abilities of AA-fatty acids to inhibit PFK-1, LDH and AK with the ability of AA to inhibit, inhibitor concentrations that produce 50% inhibition (I_{50} values) at 30nmolar enzyme concentrations were determined.

Figure 5, 6, and 7 show how I_{50} values were determined for these studies.

Figure 5A

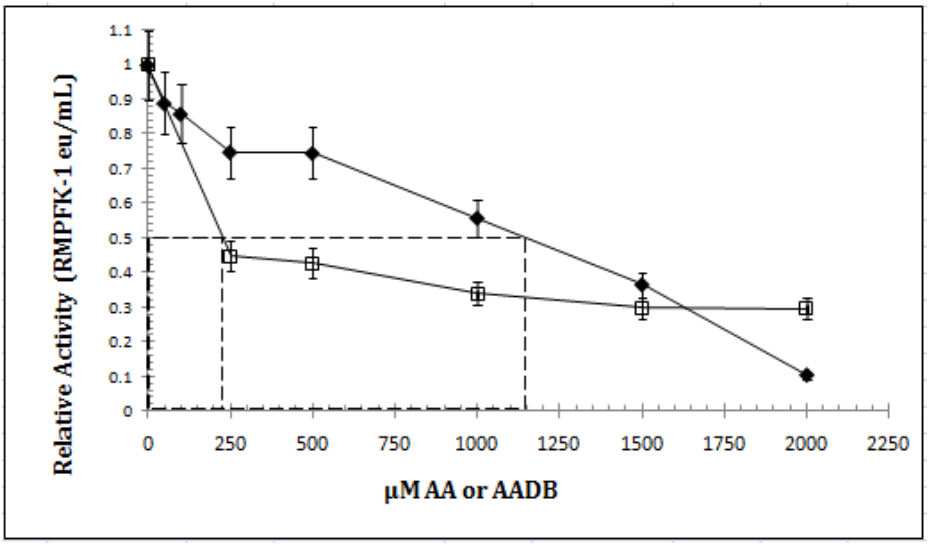


Figure 5B

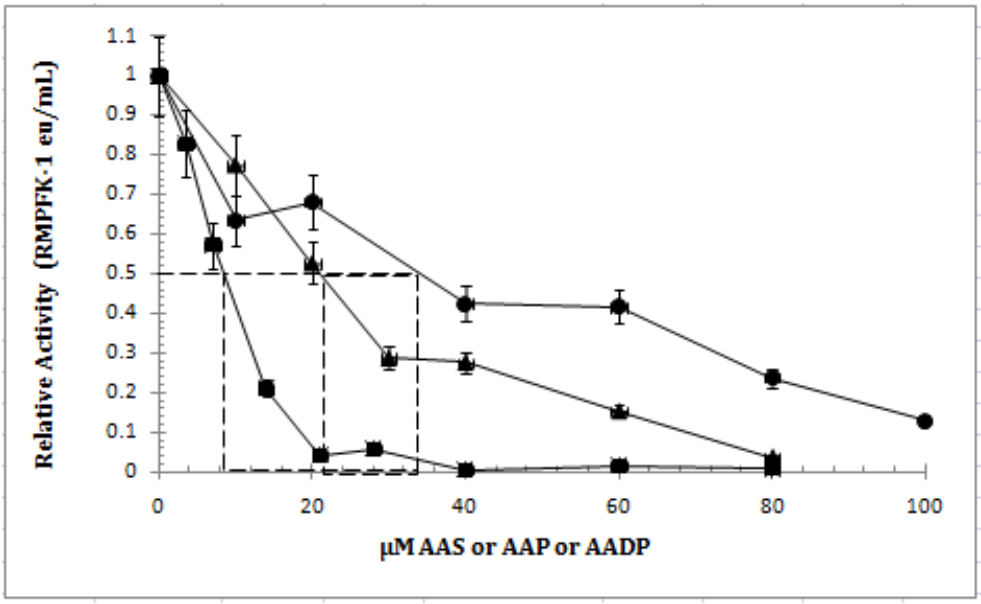


Figure 5: Estimation of I_{50} values of PFK-1 and inhibition behaviors of AA derivatives on PFK-1. The titrations of 30 nM PFK-1 against ascorbate and AADB whose concentrations at I_{50} are greater than 100µmolar (Figure 5A); other AA-fatty acid derivatives with less- than-100µmolar I_{50} concentrations (Figure 5B) are described in the Materials and Methods. Symbols are as follows: AA (□); AADB (◆); AADP (■), AAP (▲); and AAS (●). Dotted lines show extrapolations to determine I_{50} values. Experimental values are within ± 10% of the mean with n=6 as a minimum.

Figure 6A

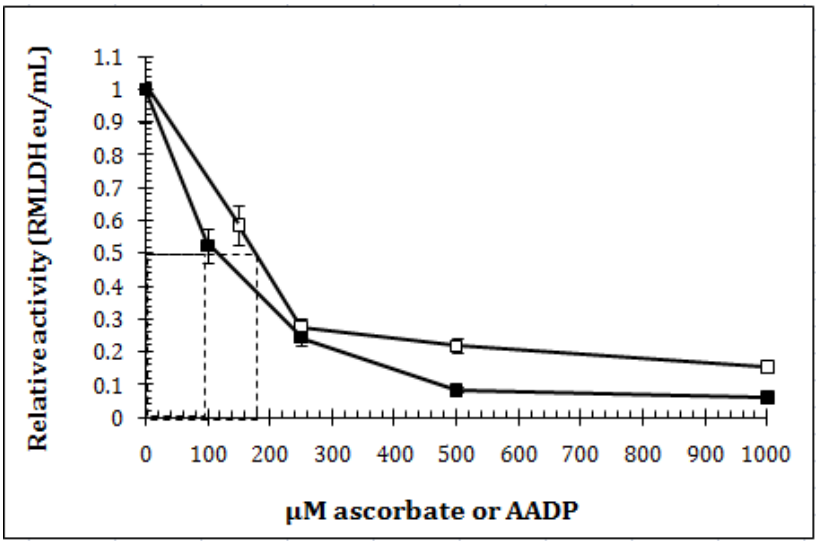


Figure 6B

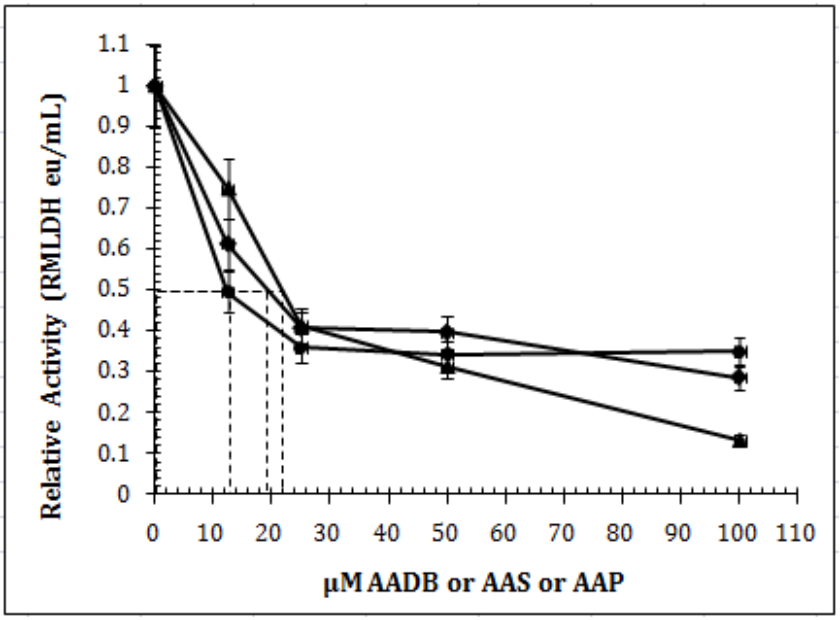


Figure 6: Estimation of I_{50} values of LDH and AA derivatives' behaviors on LDH

The titrations of 30 nM LDH against ascorbate and AADP whose concentrations at I_{50} are greater than 100µmolar (figure 6A); other AA-fatty acid derivatives with less-than-100µmolar I_{50} concentrations (figure 6B) are described in the Materials and Methods. Symbols are as follows: AA (□); AADP (■); AADB (●); AAS (◆) and AAP (▲). Other conditions were the same as in Figure 5.

Figure 7A

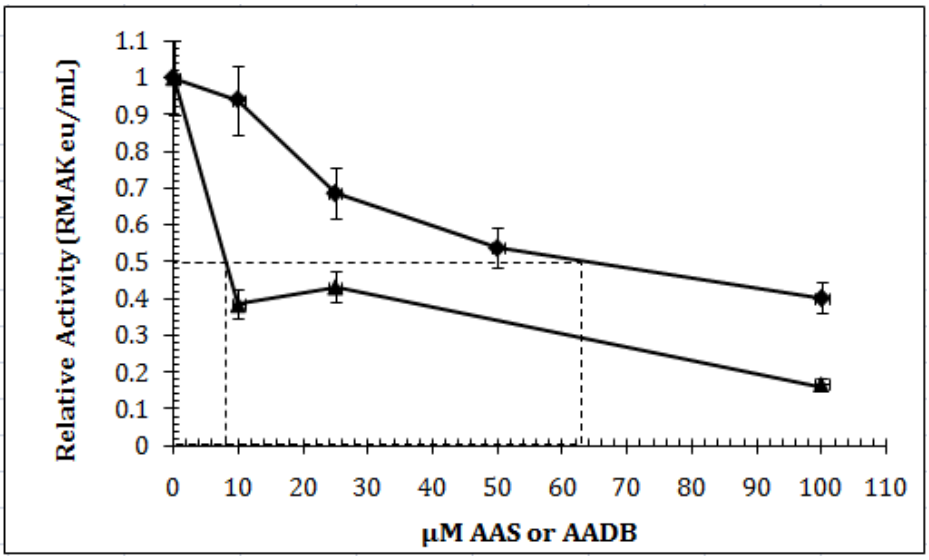


Figure 7B

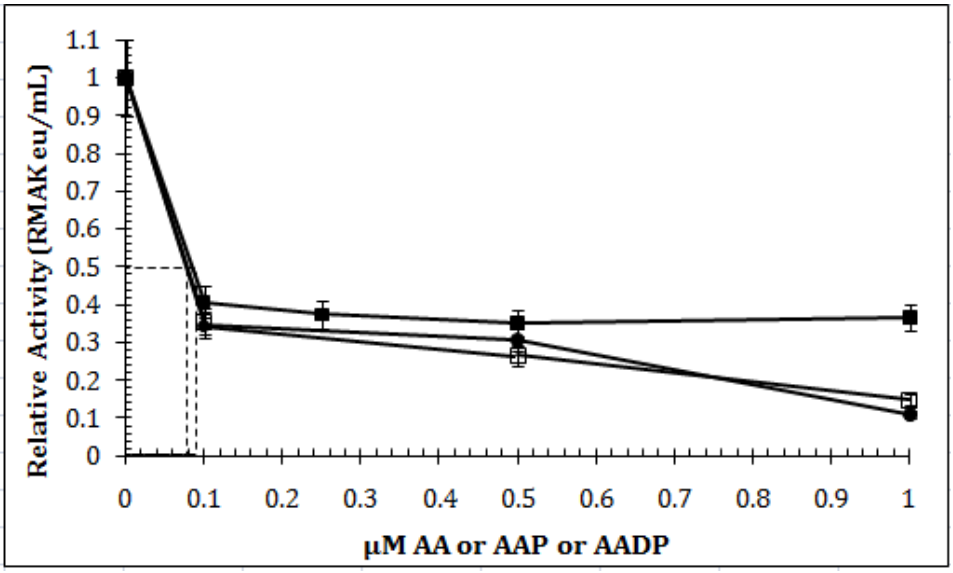


Figure 7: Estimation of I_{50} values of AK and inhibitory behaviors of AA derivatives on AK. The titrations of 30 nM AK against AAS and AADB whose concentrations at I_{50} are greater than 1µmolar (Figure 7A); other AA-fatty acid derivatives with less-than-1µmolar I_{50} concentrations (Figure 7B) are described in the Materials and Methods. Symbols are as follows: AA (■); AADP (●); AAP (□); AAS (◆) and AADB (▲). Other conditions were the same as in Figure 5.

It should be noted that the I_{50} value of AADP estimation for PFK-1 in Figure 5B is not accurate due to the steepness of the curve but were similarly estimated for PFK-1 and LDH over a narrower AADP concentration range (not shown). In contrast to both PFK-1 and LDH, AK demonstrated narrower concentration ranges of inhibitory ascorbate and AA derivatives. In particularly, Figure 7 showed these compounds inhibited 50% of original AK activity at concentrations that are less than 100μmolar. AAP and AADP seemed to behave on AK similarly within 1μmolar of substrates and closely resembled to results in AA. Measures of inhibition effectiveness by AA-fatty acid derivatives were made relative to the I_{50} value for AA inhibition. The relative I_{50} values are shown in Table 1.

The I_{50} values for AA estimated were 250 μmolar for PFK-1, 175 μM for LDH and 85 nmolar for AK. Other conditions are given in Materials and Methods. All I_{50} concentrations of other AA derivatives were standardized to AA I_{50} values in all PFK-1, LDH, and AK titrations. In PFK-1 cases, beside AADB most other AA derivatives were more effective than AA to display inhibitory characteristics, meaning their relative I_{50} ratio is larger than that of AA and their concentrations at I_{50} were lower than that of AA when interacting with PFK-1 to reduce 50% PFK-1 activity. The best molecule was AADP and the least effectiveness was of AADB. In contrast, these two di-substitute groups traded places when reacting with LDH to show AADB as the best inhibitor and AADP as the least effective inhibitor while both AAS and AAP remained similarly in inhibitory effects on both PFK-1 and LDH. Interestingly, unlike both PFK-1 and LDH, AK didn't render any better candidate compared to that of AA. More detailed, AAP and AADP yielded similar results to that seen in AA while AAS and AADB were not as great inhibitors as AA on AK.

Table 1: depicts comparison of inhibitory effects (relative I_{50} values) of ascorbate and AA derivatives on glycolytic PFK-1, LDH, and AK. All I_{50} concentrations of other AA derivatives were calibrated to AA standard I_{50} value when titrated with PFK-1, LDH, and AK.

Table 1

Relative I_{50} values of AA-fatty acid derivatives

Inhibitor	30 nM PFK-1 Relative I_{50}	30 nM LDH Relative I_{50}	30 nM AK Relative I_{50}
AA	1.00	1.00	1.00
AAS	7.14	8.75	0.00135
AAP	10.87	8.75	1.13
AADB	0.24	13.46	0.017
AADP	31.25	1.04	1.13

Chapter 5 – The roles of various fatty acid side chains and LGGL on glycolytic enzyme PFK-1, LDH and AK activities

The high specificities of the AA derivatives shown in Table 1 suggested that the contribution of individual substituents be investigated. To examine what substituents of ascorbate and AA derivatives contribute to the inhibitions of these glycolytic enzymes, the fatty acid side chains and L(+)-gulonic acid gamma-lactone (LGGL), the oxidized ring of ascorbate, were incubated with these 3 glycolytic enzymes. Enzyme activities were measured and converted to activities relative to the absence of the substituents. Concentration ranges of each substituent are similar to the concentration ranges of corresponding AA derivatives to determine I_{50} values. Figures 8, 9, and 10 indicate the interactive effects of the derivative substrates on PFK-1, LDH, AK, respectively.

Figure 8A

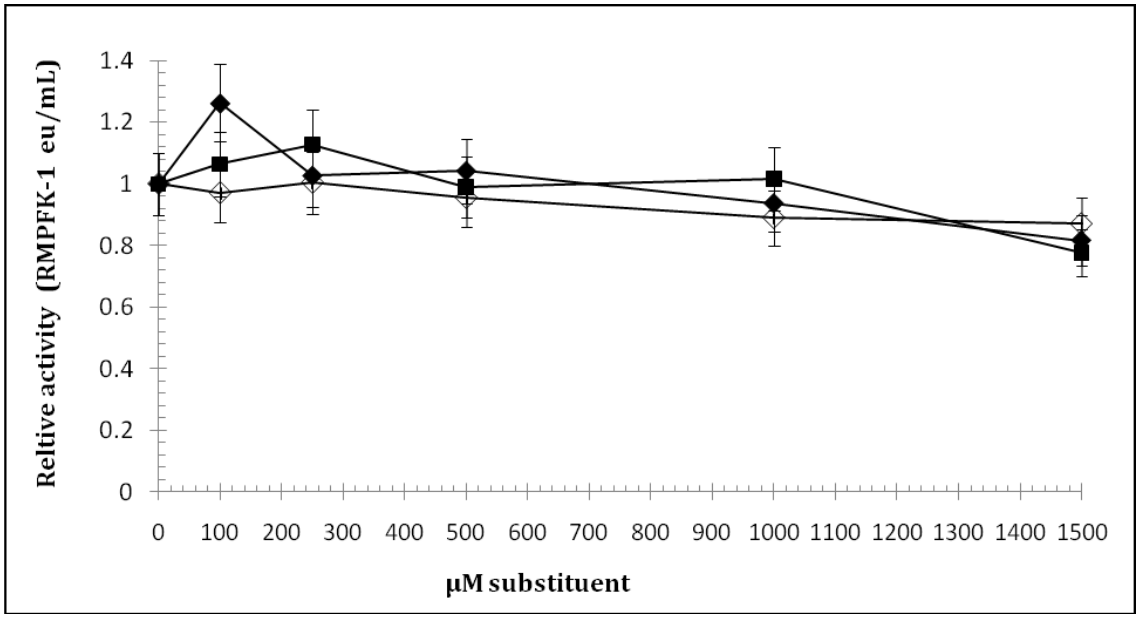


Figure 8B

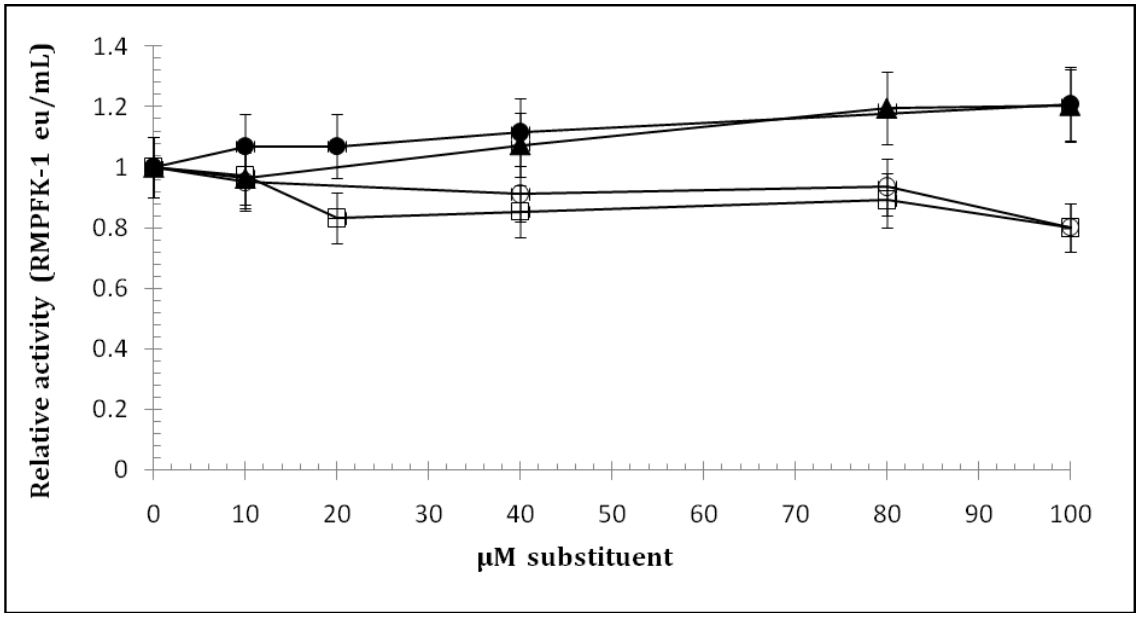


Figure 8: Effect of AA derivative substituents and LGGL on 30 nM PFK-1 activity relative to Controls. The average value of the controls was 0.0090eu/mL. Symbols are as follows: in (A) EB (◆); MB (■); LGGL (◇) and in (B) MS (○); ES (□); MP (▲); and EP (●). Other conditions are in Figure 5 and Materials and Methods.

Figure 9A

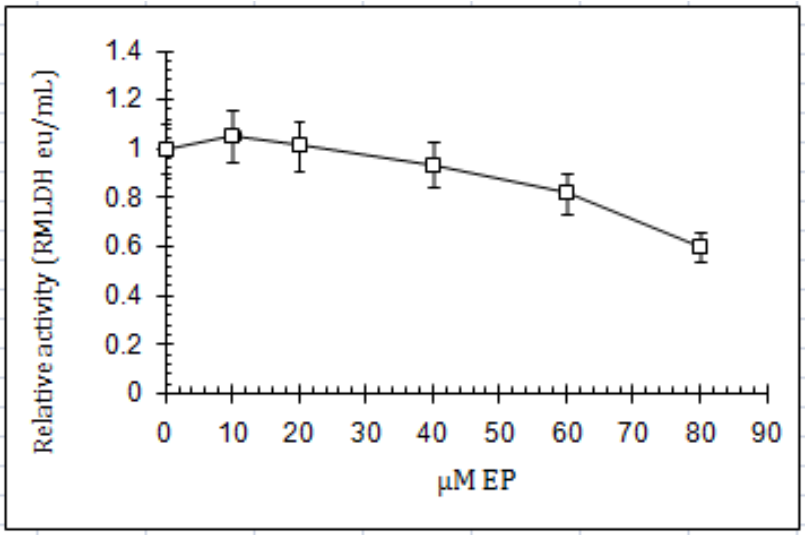


Figure 9B

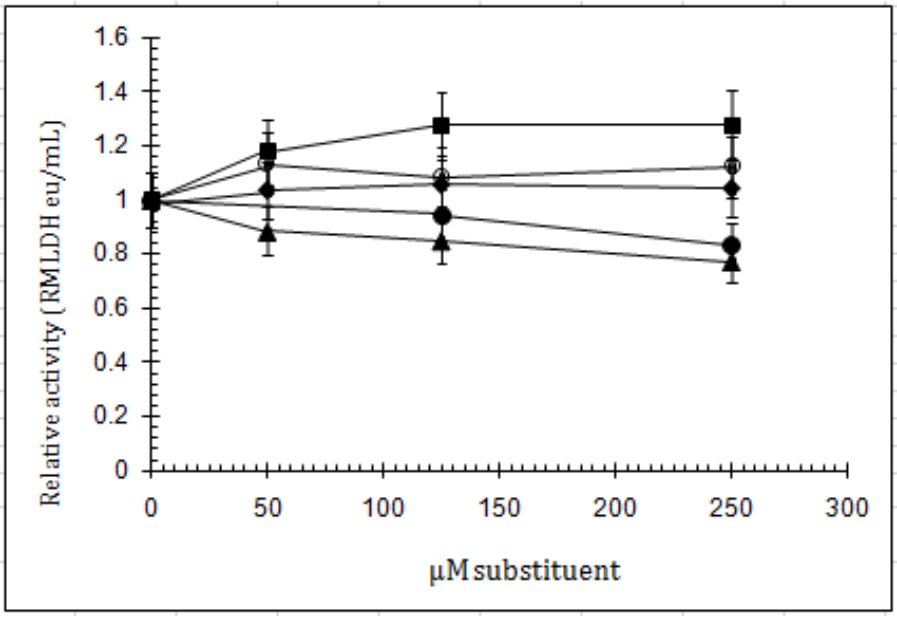


Figure 9: Effect of AA derivative substituents and LGGL on 30 nM LDH activity relative to Controls. The average value of the controls was 0.084eu/mL. Symbols are as follows: in (A) EP (□) and in (B) MB (◆); EB (▲); MS (■); ES (○); and LGGL (●). Other conditions are in Figure 5 and Materials and Methods.

Figure 10A

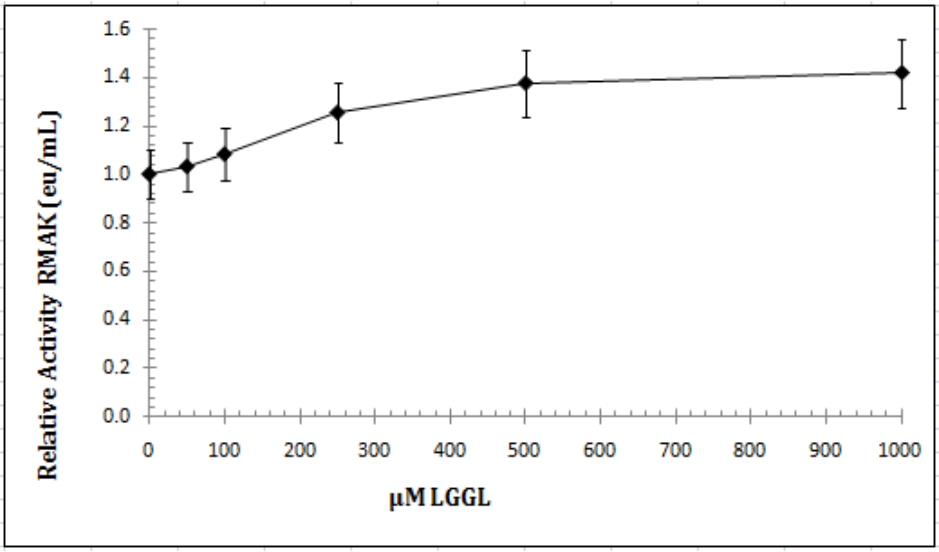


Figure 10B

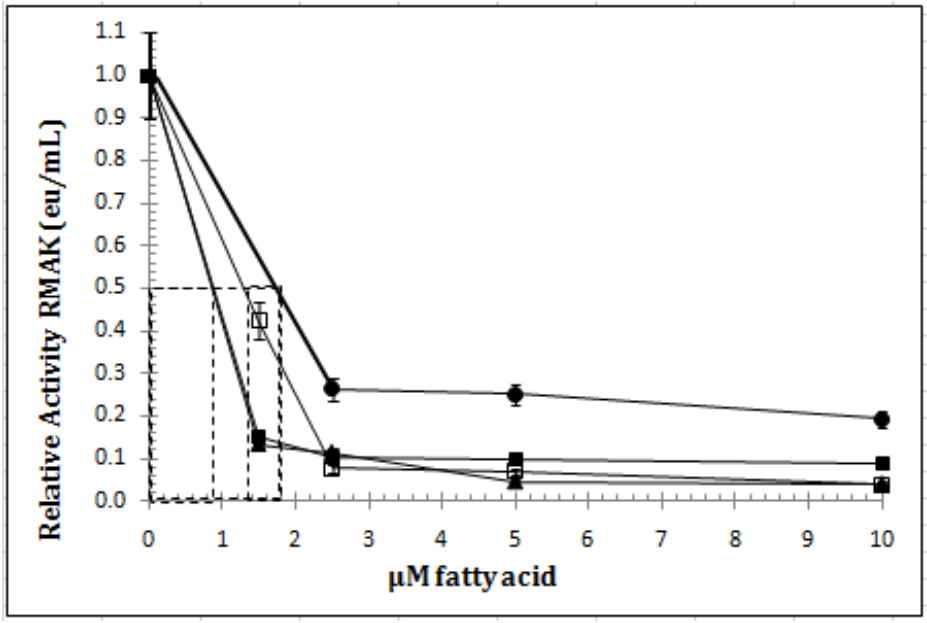


Figure 10: Effect of AA derivative substituents on 30 nM AK activity relative to Controls. The average value of the controls was 0.0098eu/mL. Symbols are as follows: in (A) LGGL (◆) and (B) MB (●); EB (■); MP (▲); and EP (□). Other conditions are in Figure 5 and Materials and Method.

The possibility that inhibitions of AA derivatives were due to substituents was tested. Table 2 shows effects of substituents on activities of 30 nM PFK-1, 30 nM LDH and 30 nM AK. Substituents of AA derivatives have little or no effect on PFK-1 and LDH activities, except for stimulatory effect of EP on PFK-1 and inhibitory effect on LDH activities; still, AAP is 8-times more inhibitory than AA for PFK-1 and LDH and AADP 31-times more inhibitory than AA for PFK-1 and about the same effect as AA for LDH. These results suggest that inhibitory effects of AA derivatives on PFK-1 and LDH do not come from substituents. However, AK interaction with these substituents proved otherwise. Most of the substituents, except LGGL, presented significant inhibition effect on 30 nM AK at very low concentrations of substrates (less than 1.5 μmolar) and suggest more refined studies on the sensitivity of AK to fatty acids.

Table 2 shows that a double bond in the 5-membered ring (Figure 1) of AA is important for inhibitions. While 250 μmolar LGGL shows a small inhibitory effect on PFK-1 and LDH, it shows no inhibitory effect for AK (Figure 8A◇; 9B●; and 10A◆). The oxidized form of AA, dehydroascorbate (DHA), was previously shown not to inhibit either PFK-1 or LDH [1].

Table 2: shows comparison of inhibitory and stimulatory effects (% inhibition) of all substituents on various glycolytic isozymes PFK-1, LDH, and AK. Positive numbers indicate % inhibition and negative numbers indicate % stimulation. The concentrations of substituents were tested with 30nM PFK-1, 30nM LDH, 30nM AK under conditions given in Materials and Methods.

Table 2

Effect of substituents of AA derivatives on

30 nM PFK-1, 30 nM LDH and 30nM AK activities after 1 hour incubation

Substituent	% Inhibition 30 nM PFK-1, concentration	% Inhibition 30 nM LDH, concentration	% Inhibition 30 nM AK, concentration
methyl butyrate (MB)	28 at 1500 μM	-4 at 250 μM	80 at 10 μM
ethyl butyrate (EB)	18 at 1500 μM	23 at 250 μM	90 at 10 μM
methyl stearate (MS)	20 at 100 μM	-27 at 250 μM	Not done
ethyl stearate (ES)	20 at 100 μM	-12 at 250 μM	Not done
ethyl palmitate (EP)	-21 at 100 μM	40 at 80 μM	90 at 10 μM
L-gulono-γ-lactone (LGGL)	13 at 100 μM	17 at 250 μM	-42 at 1000 μM

Chapter 6 – Protective effect of aldolase on PFK-1 and LDH from ascorbate and AA derivatives inhibition and long-period dilutions

Figure 11 shows the effect of rabbit muscle aldolase on fatty acid inhibitors of PFK-1 and LDH. Attempts to inhibit the enzyme near 50% using predetermined I_{50} values met with reasonable success. While Figure 11A and Figure 11B appear similar, there are distinct differences. Control for PFK-1 is stimulated by aldolase more than control for LDH. This difference represents a prevention of some PFK-1 activity losses due to dilution [8]. Additionally, AADP is a much more powerful inhibitor of PFK-1 than of LDH, 8 μM compared to 168 μM AADP, respectively. The inhibitors AAP and AAS are nearly equivalent with AAP slightly more inhibitory to PFK-1 than to AAS. Lastly, inhibitor AADB is about 50-times more inhibitory to LDH than to PFK-1. Ascorbate-fatty acid inhibitors clearly show a specificity of inhibition between these PFK-1 and LDH. These data show substantial AA derivative inhibition specificity.

Figure 11A

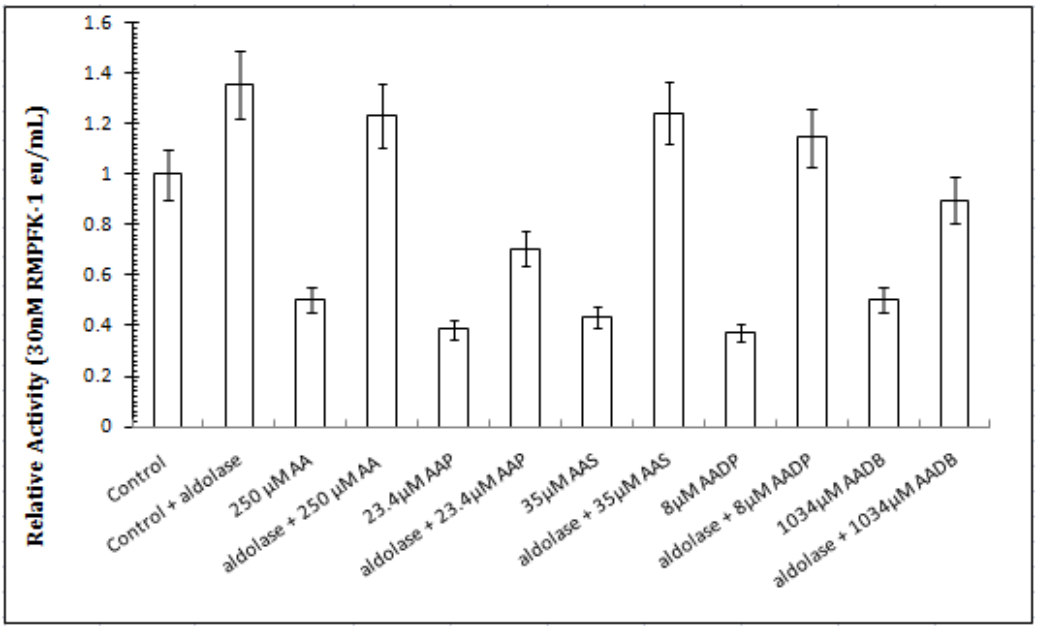


Figure 11B

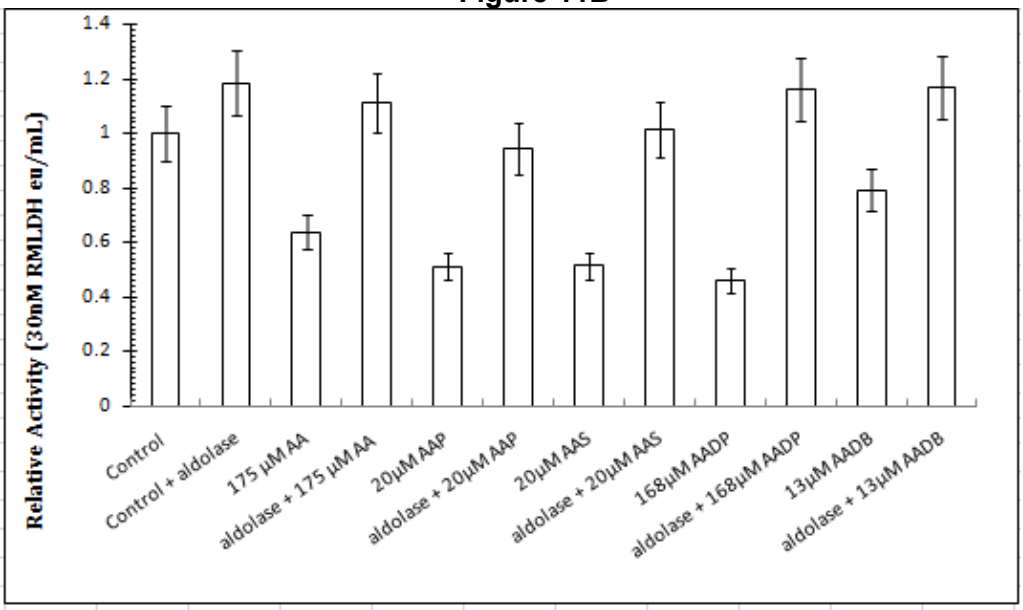


Figure 11: Aldolase effect on ascorbate-fatty acid inhibitors of PFK-1 (A) and LDH (B). PFK-1 and LDH stock solutions were diluted to 30 nmolar (Control) with the additions to final concentrations of 5 µmolar aldolase and 250 µmolar of AA, other AA derivatives whose concentrations are at I_{50} and allowed to stand for at least 1 h after which activities stabilized and determined. Control values for 30 nM PFK-1 were 0.012eu/mL and for 30 nM LDH was 0.87eu/mL. Other details are in Materials and Methods.

Chapter 7 – The effect of adenosine monophosphate or 5'-adenylic acid (AMP) on PFK-1 activity incubated with AA and its derivatives

Adenosine monophosphate (AMP) along with fructose 2, 6- bisphosphate (F2, 6BP) acts as allosteric in glycolytic pathways. While F 2,6BP can activates PFK-1, AMP also activates and competes with ATP at the allosteric effector site to promote a stimulation of PFK-1 activity. The question was posed whether or not AMP could protect PFK-1 from AA derivative inhibitors. To find the optimal concentration of AMP at which is used to stabilize or recover activity loss of PFK-1 due to dilution and inhibitors, titrations of 30 nM PFK-1 and AA I_{50}, AAP I_{50} values with various concentrations of AMP. Preliminary studies suggested the highest percentage of PFK-1 activity recovery from AA and other AA derivative inhibitors were at 80μmolar AMP (not shown). This AMP concentration was then incubated without or with various ascorbate-fatty acids to learn specificities between derivatives and AMP. Figure 12 compared the protective AMP effects on Control, AA and other AA-fatty acids. While control and AADB with no or significantly low recovery of PFK-1 relative activity, AA and other ascorbate derivatives expressed little relative activity. In particular, Table 3 quantified % of PFK-1 activity for Control, AA and AA derivatives shown in Figure 12. Ascorbate demonstrated the highest % PFK-1 recovery in AMP incubation and was more 24 times effective than those of AADB and Control. Interestingly, AMP provided 4 fold less % PFK-1 recovery than recovery from aldolase. Similar to AMP stimulators, aldolase did not benefit Control samples. However, aldolase was 2 times more effective to protect PFK-1 activities from AA, AAS and AADP inhibitors than from other AA derivatives.

Figure 12

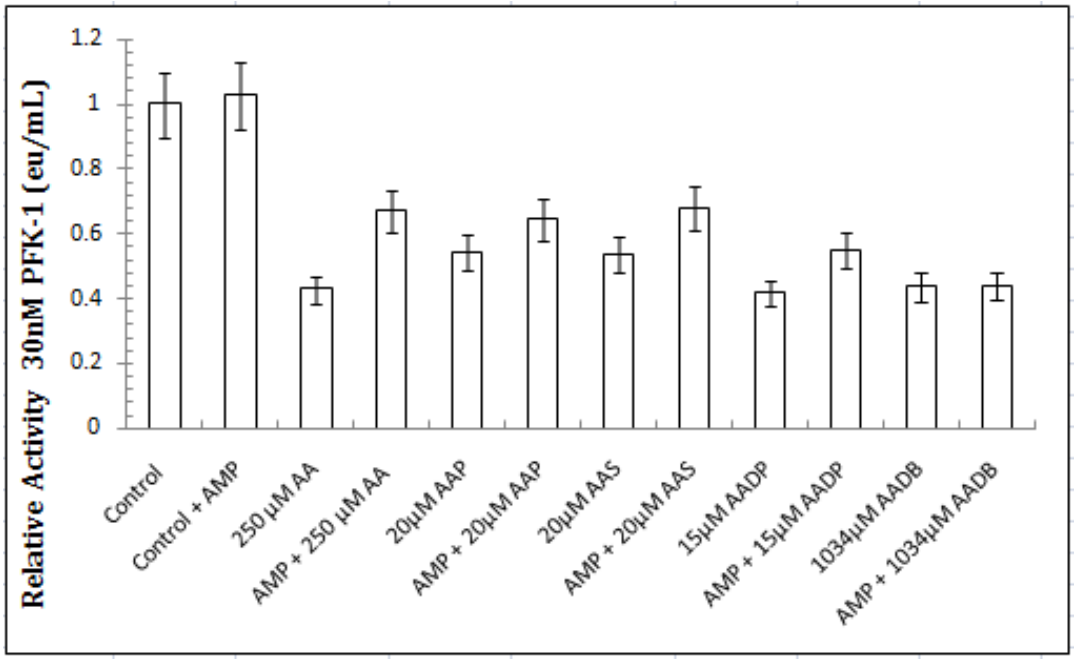


Figure 12: AMP effect on ascorbate-fatty acid inhibitors of PFK-1.
PFK-1 stock solutions were diluted to 30 nmolar (Control) with the additions to final concentrations of 80 µmolar AMP and 250 µmolar of AA, other AA derivatives whose concentrations are at I_{50} and allowed to stand for at least 1 h after which activities stabilized and determined. Control values for 30 nM PFK-1 were 0.014eu/mL. Other details are in Materials and Methods.

Table 3: Comparison of PFK-1 activity recovery due to inhibitors ascorbate and AA derivatives when incubating with aldolase versus AMP.

Table 3

Protective effects of 5 μM aldolase vs. 80 μM AMP

on 30 nM PFK-1 from various I_{50} inhibitors

Inhibitors	5 μM aldolase % Recovery	80 μM AMP % Recovery
Control	35.8	2.57
AA	73.3	24.2
AAP	32.0	18.9
AAS	80.8	14.3
AADP	77.5	13.3
AADB	39.2	0.38

Chapter 8 – Discussion

Previous studies [1-3] showed that ascorbate at cellular concentrations specifically inhibited three enzymes – PFK-1, LDH, and AK, which would have the effect of inhibiting glycolysis; ATP produced through glycolysis is the primary source of energy for muscle contraction. Out of these studies a hypothesis was developed [1] that ascorbate facilitates storage of glycogen in muscle by inhibiting glycolysis when the muscle is at rest. Others [8] have shown that in active muscle glycolytic enzymes form a complex with contractile proteins. We believe that the protective effect of aldolase on PFK-1, LDH, and AK from ascorbate inhibitions [3, 8] demonstrated is a reflection of the in situ formation complex formation.

With the report that ascorbic acid-O-phosphate-6-O-laurate (AA2P6L) was reported to prevent the growth of tumors in live mice and cell cultures with low cytotoxicity [15] but not affect normal cells, this study focused on the effects of ascorbate fatty acid derivatives on glycolytic enzymes.

The results showed that the ascorbate derivatives in these studies were essentially irreversible (Figures 3 and 4) and that the fatty acid substituents showed marked inhibition specificities relative to ascorbate inhibitions (Table 1). Since the inhibitions among the ascorbate derivatives were distinctive, the possibility that the distinctions came from individual substituents alone was tested. The results indicated the following: that LGGL and individual fatty acid esters had no inhibitory effects on PFK-1 (Figure 8), except for EP (Figure 9A); fatty acid substituents and LGGL (Figure 9B) had little or no inhibitory effect on LDH; and AK was substantially inhibited by all of the fatty acid esters (Figure 10B) but not by LGGL. In summary, except for AK, individual substituents of ascorbate derivatives use in these experiments do not appear

to be major inhibition factors. It is clear that the double bond in ascorbic acid is essential for inhibitions of PFK-1, LDH, and AK (Figures 8A, 9B, and 10A).

We believe that these results have some relationship to cancer and diabetes. Glycolytic phenotype is one of key differences between normal and cancer cells in biochemical metabolism. Glycolysis' vital function in human body plays a significant role in cancer diagnosis. In clinical tumor imaging using fluorodeoxyglucose and positron emission tomography (FdG PET), primary and secondary metastatic cancer cells exhibit upregulated aerobic glycolysis and result in increased glucose consumption [30]. Most cancer cells shut down oxidative phosphorylation process which is capable of generating 36 ATP per glucose and have to compensate their ATP loss by an increase of glycolysis utilization. This phenomenon is known as Warburg effect [31]. Compared to normal cells, malignant cells use glycolysis in a higher rate and quantity of glucose due to mitochondrial defects, adaptation to hypoxic environment in cancer tissues, oncogenic signals or altered metabolic enzymes [31]. These biological alterations present a major challenge in cancer treatment such that cancer cells in a hypoxic environment become resistant to chemotherapeutic agents and radiation. A hypothesis that ATP generation should be severely depleted via inhibition of glycolysis in cancer cells only, without killing normal cells [32] was consistent with the study of Liu et. al. and our results. Glycolytic inhibitors for cancer treatment face challenges because they require high concentrations to cause toxicity to the cancerous tissues, are not very potent and are unstable in solution. These inhibitors do not selectively target only cancer cells but also normal cells [32]. In addition, normal tissues including brain, retina, and testis frequently that need glucose replenishment will be affected by these glycolytic inhibitors [31].

The ascorbate fatty acid derivative AA2P6L was reported to prevent the growth of tumors in live mice and cell cultures with low cytotoxicity [15] but not affect normal cells. No mechanism for this effect was solidified. Previously proposed mechanisms [14-15] are reactive oxygen species or superoxide anion released from ascorbate in the vicinity of cancer cells causing membrane degradation and significant reduction in insulin-like growth factor 1 receptor expression leading to inhibit cancer development signaling cascade. Based upon our results, we posit that the ascorbate derivative AA2P6L [15] profoundly inhibited glycolysis, selectively targeting the cancer cells with mitochondrial damage [31]. Table 1 shows that lipophilic ascorbic acid derivatives with a variety of fatty acid side chains inhibited three glycolytic PFK-1, LDH and AK enzymes with a demonstrable specificity. It is not unreasonable to propose that the anticancer growth effects of AA2P6L was due to an inhibition of glycolysis or the idea chemotherapeutic agents directed at glycolysis may have broad implications for cancer treatment [31].

With PFK-1, results indicate that strong and specific inhibitory effects by the ascorbate derivatives are due to the intact molecule rather than the individual fatty acid substituents. For example, the palmitate substituent at 100 μM EP is stimulatory (Figure 8B and Table 2), while AADP (I_{50} = 8 μM) is 30-times more inhibitory than AA (Table 1), representing 23 μM AAP for a 50% inhibition. Similarly, results show that the ethyl ester of palmitate inhibits 50% LDH activity at about 90 μM (Figure 9A) while inhibitions of AAP and AADP derivatives inhibit in the micromolar ranges are 8.8-times and 1.0 times as inhibitory as AA (I_{50} = 250 μM). Unlike PFK-1 and LDH, AK appears to be sensitive to inhibitions by the fatty acid substituents and the AA derivatives are either equal to or less inhibitory than AA inhibition. The removal of the double bond

from the ascorbate ring was only slightly inhibitory to PFK-1 and LDH but quite stimulatory to AK.

It was shown previously [3, 8] that 5 μM rabbit muscle aldolase protected PFK-1, LDH, and AK from losses of activity due to inhibitions by ascorbate, activity losses due to dilutions, and activity losses due the presences of carbonates and sulfates. It was of interest to determine the effect of rabbit muscle aldolase on loses of activity due to the presence of AA derivatives. Figure 11A and 11B show that rabbit muscle aldolase protects PFK-1 with the same protective effect against AA-fatty acid derivatives inhibitions as it does against AA.

At cellular concentrations, energy carriers including ATP and ADP are regularly recycled during glycolysis process; thus AMPs are produced from one of the three mechanisms: (1) during ATP synthesis by the aid of adenylate kinase (AK) and combining 2ADP molecules, (2) hydrolysis of one high energy phosphate bond of ADP, and (3) hydrolysis of ATP into AMP and pyrophosphate. Also, like F 2,6BP, AMP competes with ATP to stimulate PFK-1 at an allosteric site. To examine whether AMP in the microenvironment protects PFK-1 from losses of activity due to inhibitions by ascorbate and AA fatty acid derivatives and activity losses due to dilutions, preliminary experiments were to incubate 80 μM AMP with the same conditions of PFK-1 and AA and AA derivatives I_{50}. Figure 12 show some specificities of AMP in protecting PFK-1 from various AA derivatives. However, AMP slightly protects PFK-1 from most AA derivatives and shows 4-fold less protective function than aldolase on those compounds (Table 3). Only PFK-1 activity loss due to AADB inhibition was not improved after the sample was added with AMP, as shown with 0.3% PFK-1 recovery. Future studies to test the protective effect of AMP on LDH and AK are needed to

compare with the results of PFK-1 and how AMP affects glycolysis and glycogen pathways in skeletal muscles during the times at rest and active.

Inhibitory properties of AA-fatty acid derivatives may have some benefit in uncompensated diabetes studies also. Type-2 diabetes is associated with a faulty glycogen synthesis [34-36]. The uptake of AA by muscle uses the same insulin-dependent GLUT 4 transport system as glucose giving rise to a condition characterized by Cunningham as “tissue scurvy” [33]. Based on the proposed role for AA of inhibiting glycolysis in resting muscle to facilitate glycogen synthesis [8], faulty glycogen synthesis may in part be due to the absence of AA. Absence of AA in resting muscle should allow glycolysis to continue, competing with and compromising glycogen synthesis. The lipophilic character of AA-fatty acid derivatives may also allow circumvention of GLUT 4 transport by crossing through membranes. Inhibition of glycolysis by an AA-fatty acid derivative could then facilitate glycogen synthesis; administration of AA2P6L was by intraperitoneal injection [15]. With the large number of possible AA-fatty acid derivatives and the report of AA2P6L anticancer effects [15], AA-lipophilic derivatives should be a productive direction for research [31].

Chapter 9 – Conclusion

In summary, AAP, AAS, AADB, and AADP were tested for their abilities to inhibit rabbit muscle PFK-1, LDH and AK enzymes essential for glycolysis. These AA derivatives are suggested as irreversible inhibitors. Inhibition potency was compared with the previously established AA inhibitor [1, 8] and inhibition potency varied greatly with the fatty acid chain. For PFK-1, AADP was the most potent inhibitor and AADB the least potent; for LDH, AADB was the most potent inhibitor and AADP the least potent; for AK, AAP and AADP were the most potent inhibitors and comparable to AA, and AAS the least potent (see Table 1). The specificity of these inhibitors affecting each enzyme PFK-1, LDH, or AK and all three key enzymes suggested future studies to look at more different substituents of ascorbate. Inhibitory characteristics come from the double bond of lactone ring of ascorbate and fatty acid substituents are not essential to the inhibition of AA. Both aldolase and AMP demonstrated protective effect on PFK-1, LDH, and AK from activity losses due to dilutions and AA derivatives inhibition. While aldolase significantly improved PFK-1 activity losses with 80% PFK-1 activity recovery, AMP improved activity losses as low as 24%. The possible therapeutic value of AA-fatty acid derivatives in cancer and diabetes are presented.

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