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INHIBITION OF RABBIT MUSCLE ISOZYMES BY VITAMIN C

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The ability of vitamins C, E and K to inhibit enzymes directly has been investigated. It was found that vitamin E and some analogs and menadione (vitamin K_3) inhibited several enzymes irreversibility at concentrations below one millimolar. Ascorbate inhibits rabbit muscle 6-phosphofructokinase (MPFK-1; EC 2.7.1.11), muscle type LDH (EC 1.1.1.27), and muscle AK (EC 2.7.4.3) at low concentrations that do not inhibit equivalent liver isozymes. Ascorbate K_i values for muscle-type LDH and heart-type LDH isozymes are 0.007 and 3 mM, respectively. The ascorbate K_i value for rabbit skeletal muscle PFK-1 is 0.16 mM; liver PFK-1 is not inhibited by ascorbate. Dehydroascorbate does not inhibit any enzyme at ascorbate concentrations normally found in cells. All ascorbate inhibitions are completely reactivated or nearly so by L-ascorbate oxidase, CYS, GSH, or DTT. We propose a hypothesis that ascorbate facilitates glycogen storage in muscle by inhibiting glycolysis. The relationship between ascorbate metabolism and diabetes is discussed.

Keywords: Vitamins; Specific ascorbate inhibitions; Sulfhydryl reactivations; Glycolysis controls

INTRODUCTION

Vitamins generally serve as cofactors in enzyme reactions or as factors in metabolic processes. The water soluble vitamins manifest as cofactors for a variety of specific biochemical reactions. Other than our report[1] on ascorbate inhibition of rabbit muscle adenylate kinase (RMAK, EC 2.7.4.3), no direct enzyme inhibitions by an unmodified water soluble vitamin have been

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found. Enzyme inhibition by tocopherol analogs[2 8] have been reported. Adenyl cyclase appears to be inhibited by ascorbate in adrenal cells derived from tocopherol-deficient rats.[9] There are other reports suggesting enzyme inhibitions by α-tocopherol or tocopherol analogs *in vitro*,[9,10] in tissue,[6,10] in heart muscle cell culture,[7,8] and in whole animal systems.[5]

This paper reports the specificity of ascorbate inhibitions associated with rabbit skeletal muscle and liver isozymes. The main theme of this report is ascorbate inhibition at concentrations near or below normal, human cellular ascorbate concentrations.[11,12] We offer the views that ascorbate has specific enzyme inhibitor functions in muscle and that other vitamins may have direct, specific inhibitor functions.

MATERIALS AND METHODS

Materials

These studies used enzymes from rabbit unless stated otherwise. The following enzymes were obtained commercially from Sigma-Aldrich Co.: muscle and *B. stearothermophilus* adenylate kinase (AK, EC 2.7.4.3); muscle creatine kinase (CK, EC 2.7.3.2), muscle phosphorylase b kinase (EC 2.4.1.1), muscle 3′,5′-cyclic AMP phosphodiesterase (EC 3.1.4.17), muscle adenyl cyclase (EC 2.7.4.3), muscle and liver pyruvate kinase (PK, EC 2.7.1.40), muscle and liver fructose 6-phosphate kinase (PFK-1 EC 2.7.1.11), muscle fructose 1,6-bisphosphatase (EC 3.1.3.11), *Escherichia coli* glycerol kinase (GK, EC 2.7.1.30), *S. cerevisiae* hexokinase (HK, EC 2.7.1.1), muscle and heart lactate dehydrogenase (LDH-m4 and LDH-h4, EC 1.1.1.27), liver glucose 6-phosphatase (EC 3.1.3.9), bovine brain 3′,5′-cyclic AMP phosphodiesterase (EC 3.1.4.17) and ascorbate oxidase (EC 1.10.3.3) from *Cucurbita* (gourd). Rabbit liver adenylate kinase (RLAK) was purified as previously described.[13]

The following chemicals were also obtained from Sigma Chemical-Aldrich Co.: ascorbic acid, dehydroascorbic acid (DHA), GSH, dithiothreitol (DTT), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), ethanolamine and cysteine.

Methods

Assay Conditions

It was determined that the vitamins tested in these studies had no effect on the assay systems under the conditions used. AK activity was measured,

AMP + MgATP = ADP + MgADP, according to Adam,[14] and modified elsewhere.[15] Other kinases and reactions producing ADP were appropriately coupled and adapted to the same pyruvate kinase–lactate dehydrogenase assay system as AK.[14,15] A 1 mL assay mixture contained 0.3 mM phosphoenolpyruvate, 0.4 mM NADH, 8.0 mM ATP, 8.1 mM $MgCl_2$ and 20 mM potassium phosphate or Tris buffer, pH 8. There were sufficient amounts of heart LDH and/or PK so that the coupling system was not rate limiting. Reactions were initiated by the addition of enzyme. Initial reaction rates were determined by measuring the decrease in absorbance of NADH at 340 nm with time. A molar absorptivity value of 6220 was used to convert the NADH absorbance change to micromoles of product formed. One enzyme unit (eu) of activity is the formation of 1 μmol of ADP per min at 25°C.

Inhibition Assays

Three inhibition assays were used in these studies: (1) *Vitamin titrations* against a constant enzyme concentration were used to provided a crude estimate of inhibition. We define a vitamin as an inhibitor when a concentration of 1 mM or less yields 50% or more inhibition of the control. (2) *Enzyme titrations* against a constant vitamin concentration were used to determine whether or not the inhibition was kinetically reversible (3). *Inhibition kinetics* were used only when the vitamin was shown to inhibit reversibly. The initial velocity measurements were used to estimate K_i and K_m values. The conditions for each of the inhibition assays were as follows.

Vitamin titrations Approximately 10 eu/mL of enzymes, in 100 mM potassium phosphate or Tris buffer, pH 8.0, were tested with each vitamin ranging from 0.2 to 4 mM. Samples were incubated for 0.5 h, after which no additional significant inhibitions occurred. The activity remaining was determined by addition of microliter aliquots to assay mixtures described above. The concentration of vitamin that yielded 50% inhibition was used as an estimate of its relative potencies under these conditions (see Figure 1 below).

Enzyme titrations A vitamin concentration, in 100 mM potassium phosphate or Tris buffer, pH 8.0, at an estimated 50% inhibition, as described above, was titrated with enzyme. Samples were incubated for 0.5 h, after which no additional significant inhibition occurred. The activity remaining was determined by addition of microliter aliquots to assay mixtures described above. The reversibility was determined by the distinguishing pattern of convergence of the uninhibited and inhibited plots at the origin, as described elsewhere.[1]

Inhibition kinetics When determining inhibition by initial velocity studies, vitamins at given concentrations were in the assay mixtures. Reactions were initiated by addition of the enzyme concerned. Other conditions are given in the text (see Table II).

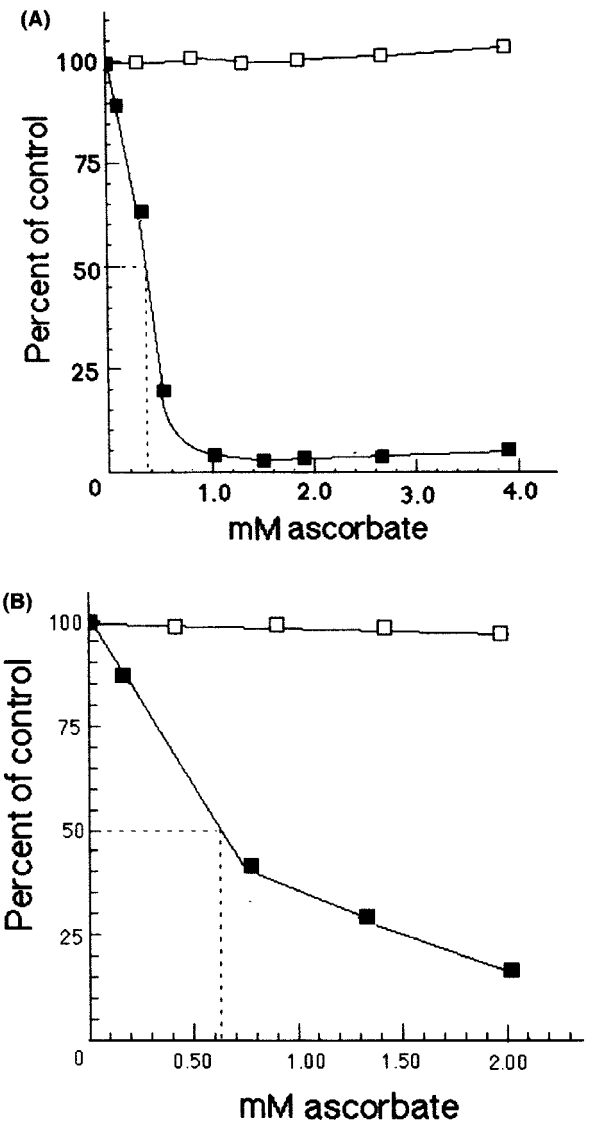

FIGURE 1(A) and (B)

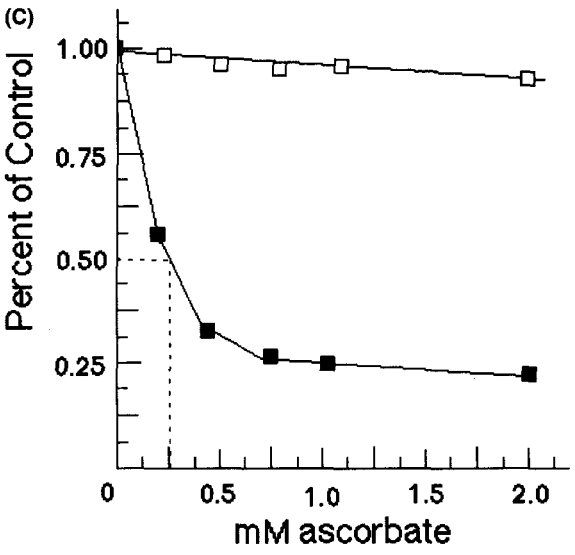

FIGURE 1(C)

FIGURE 1 Specificity of ascorbate inhibitions of AK, LDH and PFK-1 from rabbit muscle and liver. (A) Inhibitions of AK isozymes by ascorbate. About 10 eu/mL of RMAK (■) and RLAK (□) were incubated for about 0.5 h in 0.05 M K-PO4, pH 8.0 at ascorbate concentrations given. The activities remaining were then determined by a spectrophotometric assay.[14,15] The rectangular figure indicates how the 50% inhibition (about 0.4 mM) criteria used in Table I were determined. (B) Inhibitions of LDH isozymes. Conditions the same as those in (A) After incubation, activities remaining LDH-m4 (■) and LDH-h4 (□) were then determined. (C) Inhibitions of PFK-1 isozymes. About 10 eu/mL of MPFK-1 (■) and LPFK-1 (□) were treated the same as in (A), except that 0.05 M Tris-buffer, pH 8.0 was used. Other conditions are given in Table I and Materials and Methods.

Reactivation of Vitamin Inhibitions

After determining that an enzyme was inhibited by a vitamin, an aliquot was made 10 mM with respect to DTT, GSH, CYS or mercapoethanol. At this concentration of sulfhydryl compound, reactivation of the enzyme is nearly instantaneous. If an inhibition was not reactivated immediately, incubation for 1 h or more had no effect. The activity remaining was determined by addition of microliter aliquots to assay mixtures as described above.

RESULTS

Inhibition of Enzymes by Vitamins C, E, and K

Table I shows the enzymes tested for inhibition by vitamins as given in the Materials and Methods. The results show that α-tocopherol derivatives and

TABLE I Inhibition of enzymes by vitamins. The conditions for determining whether a vitamin is inhibitory or not is given in Materials an Methods and shown in Figure 1

Enzyme	*Vitamin*				
	C	E	Es	Ep	K-3
RMAK	+	−	+	+	+
RLAK	−	−	−	−	−
LDH-m4	+	−	−	+	−
LDH-h4	−	−	−	+	−
MPFK-1	+	ND	ND	ND	ND
LPFK-1	−	ND	ND	ND	ND
MCK	−	−	−	−	+
LPK	−	−	−	+	+
MPK	−	−	−	+	+
eGK	−	−	−	+	−
bAK	−	−	−	+	−
yHK	−	−	−	−	−

Abbreviations: C, ascorbate; E, α-tocopherol; Es, α-tocopherol succinate; Ep, 2 α-tocopherol phosphate; K-3, menadione; RMAK, RLAK, rabbit muscle and liver adenylate kinase; LDH-m4, LDH-h4, muscle and heart lactate dehydrogenase; MPFK-1, LPFK-1, rabbit muscle and liver fructose 6-phosphate kinase; MPK, LPK, rabbit muscle and liver pyruvate kinase; CK, creatine phosphokinase; eGK, *E. coli* glycerokinase; bAK, *B. stearothermophilus* AK; yHK, *S. cerevisiae* hexokinase.
Symbols: (+), inhibited more than 50% by 1 mM vitamin or less; (−), less than 50% inhibited by 4 mM vitamin or greater; (ND), not determined.

vitamin K_3 were inhibitors of RMAK. Vitamins K_1 (2-methyl-3-phytyl-1,4-napthoquinone) and K_2 (menaquinone 4) did not inhibit any of the enzymes tested. All of the inhibitions by the tocopherols and various forms of vitamin K were irreversible.

We have reported[1] that RMAK was inhibited by ascorbate. We report now that purified rabbit liver AK isozyme is not inhibited by ascorbate. All ascorbate inhibitions were kinetically reversible. Ascorbate inhibits muscle AK-1 and LDH-m4, while liver AK-2, LDH-h4, muscle CK (MCK), and muscle pyruvate kinase (MPK) are not inhibited. This suggests that ascorbate inhibitions are specific. In addition to the enzymes in Table I, the following also showed no inhibition at 5 mM ascorbate or greater: rabbit muscle phosphorylase a, rabbit muscle phosphorylase kinase, rabbit liver glucose-6 phosphatase, fructose 1,6-bisphosphatase, bovine heart hexokinase, and bovine brain 3′,5′-cyclic AMP phosphodiesterase.

Ascorbate Inhibition of AK, LDH and PFK-1 from Rabbit Muscle and Liver

In Figure 1A, titration of ascorbate against RMAK and RLAK shows that the former is inhibited and the latter is not. In Figure 1B, a similar titration

against LDH-m4 and LDH-h4 shows an inhibition specificity for the muscle isozyme; LDH-h4 is the major isozyme in liver with near 2 mM ascorbate, LDH-m4 is more than 85% inhibited while LDH-h4 retains more than 90% activity. Figure 1C shows a similar ascorbate inhibition specificity for MPFK-1 when compared to LPFK-1.

Dehydroascorbate

Dehydroascorbate (DHA) was not a good inhibitor of any enzyme in muscle or liver tested. Enzymes inhibited by ascorbate, RMAK, LDH-m4, and MPFK-1, showed a small but significant inhibition that did not exceed 20% at 10 mM DHA. In our consideration of how ascorbate inhibitions might be controlled *in vivo*, we were interested in determining whether or not DHA competes with ascorbate. As shown in Figure 2, DHA does not interfere with ascorbate inhibition of RMAK. It can be shown that DHA, in 20-fold excess of ascorbate concentrations that yield 80% inhibition neither reverses, reduces, nor interferes with ascorbate inhibitions of LDH-m4, muscle AK, or MPFK-1.

Reactivation of Ascorbate Inhibitions

Figure 3 shows that L-ascorbate oxidase, CYS, GSH, or DTT completely reactivate ascorbate inhibition of LDH-m4. L-ascorbate oxidase and these

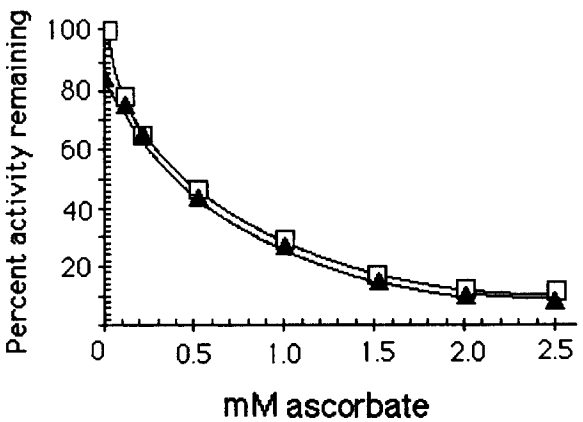

FIGURE 2 Ascorbate inhibition of RMAK in the presence of DHA. About 10 eu/mL of RMAK (□) was incubated for about 0.5 h in 0.01 M K-PO4, pH 8.0 at 25°C in 2.0 mM DHA (▲) and ascorbate was then added to final concentrations given above and incubated an additional 0.5 h. The activity remaining was then determined.

sulfhydryl compounds did not reactivate any of the other inhibitions by vitamins shown in Table I.

Kinetics of Ascorbate Inhibitions

Figure 4A–C shows the double reciprocal plots from initial velocity data for LDH-m4, MPFK-1 with fructose 6-phosphate as the variable substrate

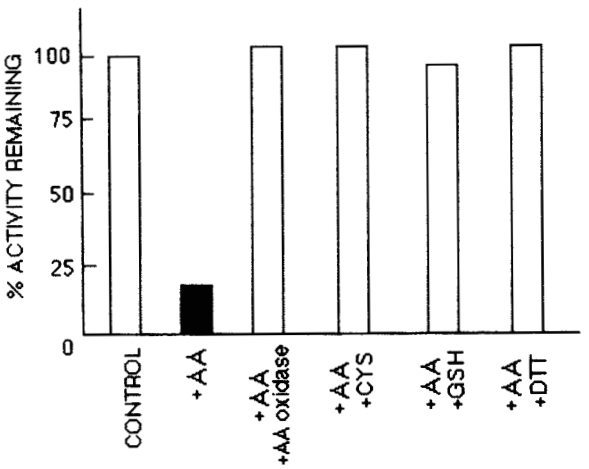

FIGURE 3 Reactivation of ascorbate inhibitions of LDH-m4 by sulfhydryl compounds and ascorbate oxidase. Samples were taken from experiments similar to that given in Figure 1 where ascorbate inhibition was approximately 80%. The samples were then made 10 mM with respect to CYS, GSH and DTT, as indicated, and 10 eu/mL with respect to ascorbate oxidase (AA Ox). After 15 min, the activity remaining was determined. Addition of the reactivating agents to controls resulted in average activities of 112% for LDH-m4. Percent recoveries were determined with the appropriate controls. The data were derived from three experiments with duplicates.

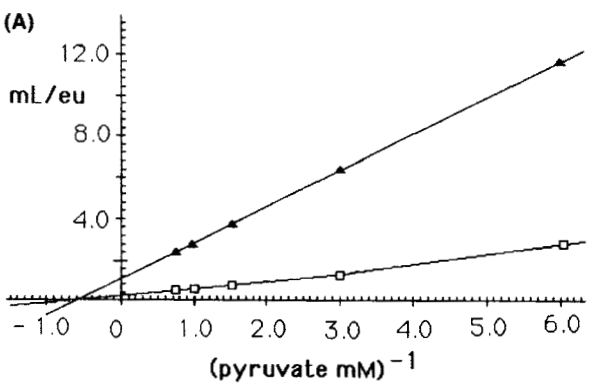

FIGURE 4(A)

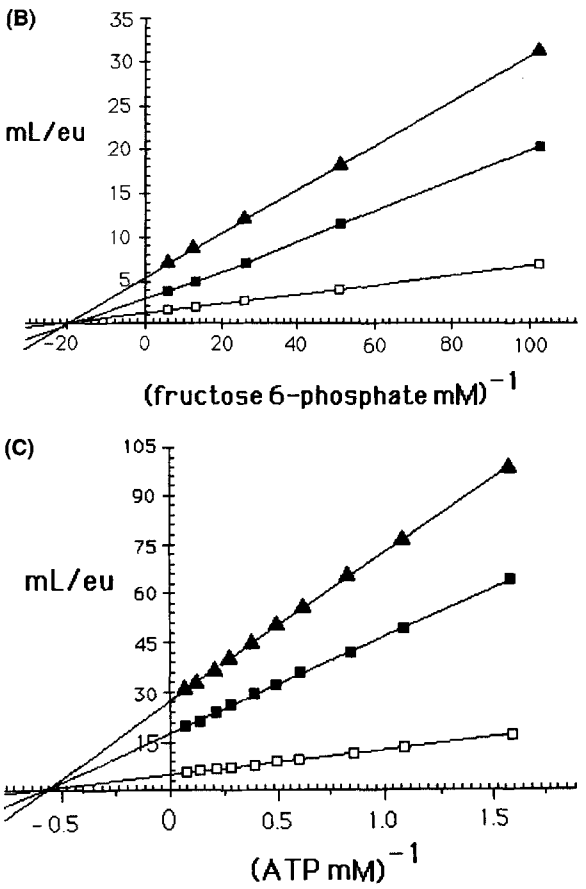

FIGURE 4(B) and (C)

FIGURE 4 Ascorbate inhibition kinetics of LDH-m4 and MPFK-1. (A) LDH-m4. The symbols are 12.7 μM ascobate (▲) and a control (□) without ascorbate. The regression plot derives from a minimum of six data sets with a $p < 0.05$ for the linear fit. Assay conditions are given in Methods and Materials. (B) MPFK-1 with fructose 6-phosphate as the variable substrate. The symbols are 12.7 μM ascobate (▲), 5.0 μM ascorbate (■) and a control (□) without ascorbate. The conditions and data analyses were the same as in (C) MPFK-1 with ATP as the variable substrate. The symbols are 12.7 μM ascorbate (▲), 5.0 μM ascorbate (■) and a control (□) without ascorbate. The conditions and data analyses were the same as in (A).

and MPFK-1 with ATP as the variable substrate, respectively. The inhibition patterns are similar and suggest a classical non-competitive inhibition.

Kinetic Constants and K_i Values

Table II shows K_m values and ascorbate K_i values for LDH muscle and liver isozymes as estimated from initial velocity data. Under the conditions for

TABLE II The determination of K_m and ascorbate K_i values for LDH isozymes and MPFK-1. Each value derives from more than six Lineweaver–Burke plots and regression analyses using the least squares method. Only analyses with $p < 0.05$ were used in estimations of K_m and K_i values. The K_i values were determined using the relationship $V_{m'}/K_{m'} = (V_m/K_m)/(1 + [i]/K_i)$, where $V_{m'}$ and $K_{m'}$ are the apparent maximum velocities and apparent Michaelis–Menton constants in the presence of [i], millimolar ascorbate, respectively. Other conditions are given in Figure 3

Enzyme	K_m (mM)	K_i (mM)
LDH-m4	1.8 ± 0.1	0.007 ± 0.002
LDH-h4	0.26 ± 0.06	2.7 ± 0.3
MPFK-1[a]	0.16 ± 0.02	0.157 ± 0.045
MPFK-1[b]	0.036 ± 0.04	0.090 ± 0.007

[a]Values for fructose 6-phosphate as variable substrate. [b]Values for ATP as variable substrate.

determining K_i values, the PFK-1 isozyme from rabbit liver showed no inhibition at 8 mM ascorbate. Estimated normal concentrations of ascorbate in human muscle and liver are about 0.3 and 1 mM, respectively. Ascorbate concentrations do not appear to vary widely with species but cell concentrations[12,16,17] can vary 2- to 3-fold with grams per day intakes of ascorbate.

DISCUSSION

The concentrations of ascorbate in mammal tissues,[12,16,17] including man, are similar, approximately 0.3 and 1 mM in muscle and liver, respectively.[11] In blood, ascorbate is carried by albumin. The total ascorbate in blood is about 1 mM, concentrated mostly in fibroblasts, neutrophils, and lymphocytes. These concentrations can double with grams per day ascorbate intakes. We found two rabbit isozymes, putatively critical for muscle glycolysis, that ascorbate inhibited. MPFK-1 has ascorbate K_i values of 0.16 and 0.09 mM against fructose 6-phosphate and ATP, respectively. LDH-m4 has an ascorbate K_i value of 0.007 mM. The K_i values are below the estimated tissue ascorbate concentrations.

Ascorbate has functions in specific hydroxylation reactions and a less specific function as a water soluble antioxidant.[17] Based on our studies, we propose that ascorbate also has a control function in muscle glycolysis. PFK-1 activity, which forms fructose 1,6-bisphosphate, is considered the major controlling step in glycolysis. LDH activity, which forms NAD^+, is required for glyceraldehyde 3-phosphate oxidation in order to continue glycolysis under the relatively anaerobic conditions of muscle activity. We propose that ascorbate inhibitions of MPFK-1 and LDH-m4 in rabbit muscle

facilitate storage of glycogen by limiting glycolysis during periods of muscle inactivity.

Based on ascorbate K_i values for LDH-m4 (0.007 mM) and MPFK-1 (0.09 mM) and the estimated 0.3 mM ascorbate in muscle,[12,16,17] these enzymes should be inhibited in muscle at all times. On the other hand, the GSH, in all its forms, varies from 0.1 to 10 mM depending upon the tissue, with the [GSSG]/[GSH] ratio varying from 1/30 to 1/300. There is also a small but significant concentration of mixed disulfide proteins.[18] According to our studies, these concentrations of GSH are more than enough for reactivation of any ascorbate inhibition.

We show that DHA is a very poor inhibitor, if at all, and does not interfere with ascorbate inhibitions. We considered that control mechanisms for [DHA]/[ascorbate] ratios relate to cellular redox status. Other reports[19,20] suggest a close, complex relationship among ascorbate, DHA, GSSG/GSH system, and glucose metabolism.[20–22] Glutathione-dependent DHA reductions[19,21,22,24,25] occur both non-enzymatically[21] and enzymatically in association with a thiotransferase;[19,22] the reaction rates are similar. The relationships among DHA reductions, GSH, and NADPH are shown in Figure 5. The $E^{0'}$ value of [DHA]/[ascorbate] is +0.06 V. Based on $E^{0'}$ values, [cystine]/[CYS], [NADP]/[NADPH], and [GSSG]/[GSH] enzyme systems reduce DHA.[25] The intracellular distribution of DHA reductase activity is not known. One source of DHA reductase activity is the mitochondrial compartment where pyruvate dehydrogenase, in concert with lipoamide dehydrogenase, reduces DHA to ascorbate and oxidizes lipoamide[25] in the process. DHA reductase activities also occur in liver and erythrocytes[22] and lens.[23] There is no evidence of a specific dehydroascorbate reductase or a specific L-ascorbate oxidase in mammals. We suspect that a control mechanism exists for [DHA]/[ascorbate] ratios based upon the redox status of the cell and DHA reductase activity. Since DHA has no effect on ascorbate inhibitions, we suggest that ascorbate concentration is more important than [DHA]/[ascorbate] ratios.

An inhibition mechanism involving free radical products of ascorbate oxidations by heavy metal contaminants is seemingly ruled out. We show that ascorbate inhibitions are rapidly reversed by ascorbate oxidase, supporting our conclusion that DHA is not an inhibitor. Inhibitions by free radical oxidation products may be reversed by sulfhydryl compounds[21] but would not be by ascorbate oxidase. Kinetic studies showed that ascorbate inhibitions present similar classical non-competitive inhibition patterns. This pattern also applies to LDH-h4 which is inhibited with a relatively high ascorbate K_i value of 3 mM.

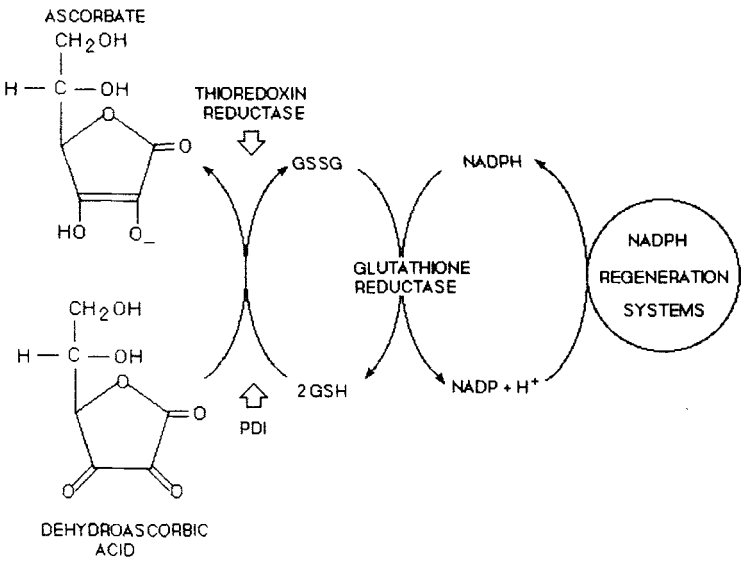

FIGURE 5 Relationships among ascorbate, glutathione and NADPH. The reduction of DHA occurs both non-enzymatically and enzymatically.[19,21] Enzymatic reduction of DHA may involve both thioredoxins (thioltransferases) in the cytoplasm and protein disulfide isomerase (PDI, EC 1.6.4.4) in the endoplasmic reticulum; both have dehydroascorbate reductase activity[19] in concert with reduced glutathione (GSH). Oxidized glutathione (GSSG) is reduced by a glutathione reductase (EC 1.6.4.2) that relies on NADPH regenerating systems, such as the hexose monophosphate shunt.

Ascorbate inhibition of rabbit liver isozymes LDH-h4 and LPFK-1 is very poor and not at all, respectively. The differences among isozymes from muscle and liver shown here suggest that other tissues may have different ascorbate inhibition profiles. We are currently exploring ascorbate as an inhibitor of PFK-1, PFK-2, AK and LDH isozymes in brain and erythrocytes. We predict that brain and erythrocyte isozymes will not be inhibited by ascorbate. Brain is aerobic, using glucose as the major oxidative energy source. Erythrocytes are anaerobic, using glycolysis as the energy source. Active muscle is relatively anaerobic when active, using glycogen reserves as an emergency energy source. The resistance of liver isozymes to ascorbate inhibition is consistent with the function of supplying blood glucose for brain and other tissues.

We believe that this report is relevant to the association of ascorbate and diabetes. Scorbutic guinea pigs present with abnormal glucose tolerance tests,[26] an observation consistent with our hypothesis that ascorbate inhibits muscle isozymes in the glycogenolysis pathway. Also, Islet Cells from scorbutic guinea pigs require ascorbate to release insulin.[27] Grams per day

intakes of ascorbate by normal humans show significantly delayed glucose tolerance responses,[28] though still within the normal range. All diabetics have high DHA concentrations in their erythrocytes[12,24,29,30,32] as do their offspring.[32] High concentrations of DHA in plasma are also found in animals with chemically induced diabetes.[24,30] Normals do not have DHA in the plasma. DHA can form a 1 : 1 complex with GSH[33] and it has been suggested[24,29,31,34,35] that some pathology associated with diabetes is due to interaction of DHA with membrane sulfhydryl groups. We found no evidence or suggestion that DHA is a good inhibitor of enzymes associated with glycolysis in the liver or muscle.

These studies show that ascorbate specifically inhibits enzymes associated with glycogen metabolism in muscle but not in liver. Dehydroascorbate, on the other hand, is not considered an inhibitor. The general theme of these studies is that other vitamins may also have specific inhibitory functions. Prefatory experiments show that less than 1 mM vitamin E, and some derivatives, inhibit muscle AK, LDH m-4, and liver PK while vitamin K_3 inhibits muscle AK, CK, PK, and liver PK.

Acknowledgments

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