Journal of Bioenergetics and Biomembranes, Vol. 28, No. 1, 1996

Original Article

α-Lipoic Acid Dependent Regeneration of Ascorbic Acid from Dehydroascorbic Acid in Rat Liver Mitochondria

Dian Peng Xu[1] and William W. Wells[1]

Received August 3, 1995; accepted September 11, 1995

Rat liver mitochondria were examined for their ability to reduce dehydroascorbic acid to ascorbic acid in an α-lipoic acid dependent or independent manner. The α-lipoic acid dependent reduction was stimulated by factors that increased the NADH dependent reduction of α-lipoic acid to dihydrolipoic acid in coupled reactions. Optimal conditions for dehydroascorbic acid reduction to ascorbic acid were achieved in the presence of pyruvate, α-lipoic acid, and ATP. Electron transport inhibitors, rotenone and antimycin A, further enhanced the dehydroascorbic acid reduction. The reactions were strongly inhibited by 1 mM iodoacetamide or sodium arsenite. Mitoplasts were qualitatively similar to intact mitochondria in dehydroascorbate reduction activity. Pyruvate dehydrogenase and α-ketoglutarate dehydrogenase reduced dehydroascorbic acid to ascorbic acid in an α-lipoic acid, coenzyme A, and pyruvate or α-ketoglutarate dependent fashion. Dehydroascorbic acid was also catalytically reduced to ascorbic acid by purified lipoamide dehydrogenase in an α-lipoic acid ($K_{0.5}$ = 1.4 ± 0.8 mM) and lipoamide ($K_{0.5}$ = 0.9 ± 0.3 mM) dependent manner.

KEY WORDS: Mitochondria; mitoplasts; dehydroascorbic acid; ascorbic acid; α-lipoic acid; lipoamide; dihydrolipoic acid; lipoamide dehydrogenase; pyruvate dehydrogenase; α-ketoglutarate dehydrogenase.

INTRODUCTION

Ascorbic acid (AA)[2] is closely associated with biological reactions that produce dehydroascorbic acid (DHA) directly (Groden and Beck, 1979) or indirectly in sequential one-electron exchanges via semidehydroascorbic acid radical disproportionation (Kersten *et al.*, 1958; Bielski, 1982). For a number of years, it was known that glutathione (GSH) was able to reduce dehydroascorbic acid in a two-step process resulting in ascorbic acid and glutathione disulfide (GSSG) (Szent Györgyi, 1928; Crook and Hopkins, 1938). Enzymes (glutathione: dehydroascorbate oxidoreductases (EC 1.8.5.1), also called DHA reductases) that catalyze this reaction have been identified in plants (Hossain and Asada, 1984) and animal tissues (Hughes, 1964). Thioltransferase (glutaredoxin), a cytosolic enzyme, and protein disulfide isomerase (PDI), a lumenally associated endoplasmic reticulum protein, were shown to have intrinsic dehydroascorbic acid reductase activity (Wells *et al.*, 1990). A third protein of 31 kDa from rat liver cytoplasm was recently reported to have GSH-dependent DHA reductase activity (Maellaro *et al.*, 1994). In addition, a NADPH-dependent DHA reductase has also been found in rat liver cytoplasm and identified as 3α-hydroxy steroid dehydrogenase (Del Bello *et al.*, 1994). That cellular AA regeneration from DHA was in large part dependent on GSH, *in vivo*, was shown in a series of informative studies from Meister's laboratory (Meister, 1992). However, it remains to be established to what extent the reaction between GSH and DHA in the cytoplasm at a pH of ca. 6.9 (Roos and Boron, 1981) is enzymatically or nonenzymatically mediated.

Mitochondria contain a pool of GSH and GSSG that represents between 10–20% of the total cellular

[1] Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824.

[2] Abbreviations used: AA, ascorbic acid; DHA, dehydroascorbic acid; PDI, protein disulfide isomerase; LDH, lipoamide dehydrogenase; BSA, bovine serum albumin.

0145-479X/96/0200-0077$09.50/0

GSH + GSSG (Jocelyn and Kamminga, 1974; Meredith and Reed, 1982; Wahlländer *et al.*, 1979; Griffith and Meister, 1985). Since the pH of the mitochondrial matrix is estimated to be ca. 7.5 under state 4 (succinate based) respiration (Addanki, 1968), it is conceivable that only chemical regeneration of ascorbic acid from dehydroascorbic acid by GSH is sufficient at that pH. Indeed, there is no evidence for a GSH-dependent dehydroascorbate reductase in mammalian mitochondria (unpublished observations).

Dihydrolipoic acid ($E_0' = -0.32$ V) (Searls and Sanadi, 1960), a more powerful reductant than GSH ($E_0' = -0.24$ V) (Scott *et al.*, 1963), is present, largely in conjugate form, in very low concentrations in rat liver mitochondria (Stockstad *et al.*, 1956). Thus, only minor mitochondrial ascorbate regeneration is likely to occur under normal conditions via dihydrolipoic acid. However, recent studies of the efficacy of lipoic acid in alleviation of oxygen-linked cytotoxicity (Burkart *et al.*, 1993) or in disease states such as diabetic polyneuropathy (Packer *et al.*, 1995) have raised the possibility that under these conditions, sufficient dihydrolipoic acid may be present in tissues to significantly influence the level and redox status of ascorbic acid, especially in cells under severe oxygen stress. In mitochondria, the reduction of pharmacological doses of lipoic acid would likely be mediated by the lipoamide dehydrogenase activity of the α-keto acid dehydrogenases and electrons derived from substrate oxidation (Bunik and Follmann, 1993; Kagan *et al.*, 1992). In microsomes or other organelles (Matuda and Saheki, 1982), lipoamide dehydrogenase coupled with NADH, available in the organelle involved, may also lead to significant interconversion of lipoic acid to dihydrolipoic acid (Icén, 1967).

Mitochondria generate abundant superoxide free radicals as a product of oxidative reactions with molecular oxygen (Chance *et al.*, 1979). Ascorbic acid may scavenge these reactive radicals or participate in the reduction of α-tocopheryl radicals, hence it is important to evaluate to what extent mitochondria may potentially regenerate ascorbic acid from dehydroascorbate via a dihydrolipoic acid coupled pathway. In the present study, we asked whether DHA could be converted to AA by either GSH dependent or independent reactions in rat liver mitochondria, *in vitro*. These studies show the reduction of DHA in both a GSH dependent and independent manner. The latter process is stimulated by dihydrolipoic acid generated by the reactions catalyzed by pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, or lipoamide dehydrogenase.

MATERIALS AND METHODS

Materials

Sprague Dawley rats were obtained from Charles River Laboratories, Wilmington, Massachusetts. The rats were fed Teklad 8640 Rodent Chow, *ad libitum*. L-Ascorbic acid, BSA (fraction V), mannitol, digitonin, pyruvate, α-ketoglutarate, ATP, thiamine pyrophosphate, α-lipoic acid, DL-lipoamide, glutathione, hydroxyurea, sodium azide, rotenone, antimycin A, pyruvate dehydrogenase (porcine heart), α-ketoglutarate dehydrogenase (porcine heart), and lipoamide dehydrogenase (bovine intestinal mucosa) were purchased from Sigma Chemical Co. Metaphosphoric acid, bromine, and iodoacetamide were obtained from Aldrich Chemical Co. HEPES, sucrose, Coenzyme A, NAD^+, and NADH were products of Boehringer Mannheim Corp., Indianapolis, Indiana. Sodium arsenite was from Mallinckrodt Chemical Co., EDTA from J. T. Baker, Inc., and thiourea from Matheson Company, Inc. DHA was freshly prepared from AA by bromine oxidation (Wells *et al.*, 1995).

Preparation of Rat Liver Mitochondria and Mitoplasts

Mitochondria were isolated from adult male Sprague Dawley rats (250–300 g) by the procedure of Greenawalt (1974). The rats were starved overnight prior to mitochondria preparation. Briefly, the mitochondria were pelleted at 7,000 × g for 15 min after the previous removal of a pellet containing nuclei and unbroken cell debris at 600 × g for 10 min. The mitochondrial fraction was washed twice in a buffer of 2 mM HEPES, pH 7.4, 70 mM sucrose, and 220 mM mannitol. The mitochondria were resuspended in the same medium, and protein was analyzed by the bicinchoninic acid method according to the manufacturer's direction (Pierce Chemical Co.) with bovine serum albumin as standard. Estimation of the mitochondrial enrichment was obtain by comparing the cytochrome *c* oxidase activity of the mitochondria preparation relative to that of the crude homogenate. Cytochrome *c* oxidase was assayed by the method of Storrie and Madden (1990). Mitoplasts were prepared

by the method of Greenawalt (1974) using 0.12 mg of digitonin per mg of mitochondrial protein.

Reduction of DHA to AA by Rat Liver Mitochondria and Mitoplasts

Freshly prepared mitochondria were incubated at 37°C for 5 min. The reactions were terminated by the addition of 500 μl of 20% metaphosphoric acid, 2 mM thiourea, and 2 mM EDTA to 500 μl of reaction mixture. Denatured protein was pelleted in 1.5 ml polyethylene centrifuge tubes at high speed. The ascorbic acid was determined by a HPLC chromatographic procedure that measured ascorbic acid electrochemically as described previously (Wells *et al.*, 1995). Separation and quantitative analysis were accomplished using a 3.9 × 150 mm Waters Delta Pak-5μ C18 reversed-phase column with a mobile phase of 100 mM sodium phosphate, pH 3.0 and a flow rate of 0.7 ml per min. Ascorbic acid was detected using an ESA Model 5200A Coulochem II electrochemical detector (ESA, Inc., Bedford, Massachusetts), and an ESA Model 5011 analytical cell. Detector one, −150 mV, detector two, +125 mV, and a guard cell, +200 mV were optimal for quantification. The areas of the ascorbic acid peaks were determined using a Hewlett-Packard Model 3395 integrating recorder. Areas of sample ascorbic acid were compared with those from standard solutions of 1–64 μM ascorbic acid dissolved in 10% metaphosphoric acid containing 1 mM thiourea and 1 mM EDTA. In other experiments, mitochondria or mitoplasts were centrifuged at appropriate times and AA determined in the pellet and in the 7,000 × *g* supernatant fraction by the addition of equal volumes of 20% metaphosphoric acid, 2 mM thiourea, and 2 mM EDTA.

Reduction of DHA to AA by Pyruvate and α-Ketoglutarate Dehydrogenases

The reduction of DHA to AA was analyzed using commercially (Sigma) available pyruvate and α-ketoglutarate dehydrogenases from porcine heart. In each case, the appropriate system was provided with either 4.0 mM pyruvate or 2.0 mM α-ketoglutarate and for a complete mixture, 100 mM potassium phosphate, pH 7.0, 1.2 mM $MgCl_2$, 0.4 mM thiamine pyrophosphate, 0.8 mM coenzyme A, 1 mM DHA, and 4.0 mM DL-α-lipoic acid. The activity of the complete mixture was corrected for a high blank due to chemical reduction of DHA to AA by coenzyme A and the low level of β-mercaptoethanol (50 μM) derived from the commercial enzyme preparations.

Reduction of DHA to AA by Dihydrolipoic Acid and Dihydrolipoamide Catalyzed by Lipoamide Dehydrogenase

Bovine intestine lipoamide dehydrogenase (Sigma) (1.65 μg) was incubated in a total volume of 1 ml containing 100 mM potassium phosphate, pH 5.9, 0.3 mM EDTA, 0.7 mg BSA, 0.1 mM NAD^+, 0.15 mM NADH, 1 mM DHA, and variable levels of α-lipoic acid (0.025–4 mM) for 5 min at 37°C (Massey, 1960). The reaction was stopped by adding an aliquot of the reaction mixture to an equal volume of ice-cold 20% metaphosphoric acid, 2 mM EDTA, and 2 mM thiourea in a 1.5 ml conical plastic centrifuge tube. The tubes were centrifuged at 10,000 × *g* for 10 min, and the supernatant was analyzed for AA formation as described above. For experiments using DL-lipoamide as substrate, reactions of 1 ml were incubated exactly as described for α-lipoic acid except that the 100 mM potassium phosphate was pH 6.5, the lipoamide dehydrogenase was 41.25 ng, and the DL-lipoamide varied from 0.025–2.0 mM. In each case, the amount of enzyme selected was within the linear range for reduction of DHA to AA in the coupled system. The commercial lipoamide dehydrogenase (Sigma) was assayed spectrophotometrically using the method of Komuniecki and Saz (1979) modified by the addition of 0.7 mg/ml of bovine serum albumin. The incubations were carried out at 37°C under substrate saturation conditions and with the enzyme in the linear range of oxidation of NADH over the first minute of reaction.

Analysis of Total Mitochondrial GSH + GSSG

As a potential source of a chemical reductant, mitochondrial GSH + GSSG was determined by the method of Roberts and Francetic (1993).

RESULTS

Mitochondrial Dehydroascorbate Reduction

Mitochondrial fractions were typically enriched 14.7-fold over the whole homogenate based on cyto-

chrome *c* oxidase specific activities (data not shown). Over extended periods, linearity of DHA reduction with time decreased due to the decay of the DHA at pH 7.0, and two electrochemically reactive unknown degradation products of DHA were produced (data not shown). Therefore, a 5-min period was selected for the current studies of cofactors and inhibitors, in which the DHA degradation was minimal and the DHA reduction rate was linear with time. Rat liver mitochondria contained 5.1 nmol total GSH + 2 GSSG/mg protein which might largely contribute to the basal rate of DHA reduction, 1.10 ± 0.04 nmol/min/mg protein (Table I). Including 3.2 mM pyruvate or 1.2 mM α-lipoic acid individually did not stimulate the DHA reduction. A small increase in DHA reduction (122%) occurred with the addition of 0.64 mM ATP possibly due to an inhibition of electron transport, and increased electron availability for lipoamide dehydrogenase or semidehydroascorbate reductase activity. A synergistic increase in DHA reduction (440%) was seen when pyruvate, lipoate, and ATP were combined with the basal conditions. Only minor reduction of DHA due to NADH occurred in the absence of α-lipoic acid. The addition of NADH (427%) but not NAD^+ (94%) when supplemented with 1.2 mM extramitochondrial lipoate resulted in increased reduction of DHA compared with the basal rate. The ability of extramitochondrial NADH to stimulate reduction may demonstrate the action of either a transhydrogenase or semidehydroascorbate reductase. A basal control omitting DHA revealed only a small amount of mitochondrial ascorbic acid. Other controls included NADH, rotenone, and antimycin A in the absence of α-lipoic acid. All values in Table I were corrected for blanks when appropriate.

We speculated that mitochondria, provided with exogenous lipoate, ATP, and pyruvate, would increase DHA reduction if inhibitors of electron transport were added to block the oxidation of metabolically generated NADH. Indeed, rotenone, which inhibits complex I, caused a 312% increase above the uninhibited rate, and this increase was totally dependent on α-lipoic acid. Antimycin A, an inhibitor of complex III, likewise afforded increased reduction (332%) and was also dependent on α-lipoic acid. However, 10 mM sodium azide, an inhibitor of Complex IV, elicited only a minor response (120%). In order to implicate the site of dihydrolipoate formation, sodium arsenite, a strong inhibitor of lipoamide dehydrogenase was tested at the 1 mM level, and resulted in significant inhibition of DHA reduction (15% activity remaining). Similarly, 1 mM iodoacetamide, capable of reacting with reactive thiols at pH 7.0, strongly inhibited the reduction reaction (17% remaining). The free radical scavenger,

Table I. Formation of Ascorbic Acid from DHA in Rat Liver Mitochondria

Sample[a]	Assay composition	AA formation (nmol/min/mg protein)	Relative activity (%)
1	Basal: 0.5 mg mitochondria protein,[b] 1 mM DHA, 50 mM sodium phosphate, pH 7.0, 50 mM KCl, 6.4 mM $MgCl_2$	1.10 ± 0.04	100[c]
2	Basal + 3.2 mM pyruvate	1.05 ± 0.12	96[c]
3	Basal + 1.2 mM α-lipoic acid	1.13 ± 0.28	105[c]
4	Basal + 0.64 mM ATP	1.32 ± 0.18	122[c]
5	Basal + pyruvate, α-lipoic acid, and ATP	4.83 ± 0.27	440[c]
6	Basal + 0.6 mM NADH + 1.2 mM α-lipoic acid	4.65 ± 0.70	427[c]
7	Basal + 0.6 mM NADH	1.85 ± 0.04	168[c]
8	Basal + 0.6 mM NAD^+ + 1.2 mM α-lipoic acid	1.02 ± 0.20	94[c]
9	Sample No. 5 + 10 μM rotenone	15.14 ± 2.14	312[d]
10	Sample No. 5 + 10 μg antimycin A	16.05 ± 0.98	332[d]
11	Sample No. 5 + 10 mM sodium azide	5.85 ± 2.65	120[d]
12	Sample No. 5 + 1 mM iodoacetamide	0.80 ± 0.50	17[d]
13	Sample No. 5 + 1 mM sodium arsenite	0.73 ± 0.30	15[d]
14	Sample No. 5 + 10 mM hydroxyurea	5.18 ± 0.41	107[d]

[a] Incubations were for 5 min at 37°C. The reaction was stopped by addition of an equal volume of 20% metaphosphoric acid, 2 mM thiourea, and 2 mM EDTA. Ascorbic acid was measured by HPLC/electrochemical detection.

[b] Mitochondria contained 5.1 nmol total glutathione/mg protein.

[c] Activity relative to basal activity.

[d] Activity relative to Sample No. 5. Values are the mean ± standard deviation of three experiments.

hydroxyurea (10 mM), had no inhibitory effect on the basal DHA reduction reaction supplemented with pyruvate, lipoate, and NADH (107%, Table I). That reduction of DHA to AA occurred within the mitochondrial matrix or at the inner membrane was supported by the use of mitoplasts. Incubation of mitoplasts with DHA under basal conditions resulted in the reduction of DHA to AA (0.42 ± 0.1 nmol/min/mg protein, Table II). As in the case of intact mitochondria, the supplementation of mitoplasts with pyruvate, ATP, or lipoate individually produced little change in the rates of DHA reduction. The addition of NADH increased the DHA reduction by 250%. However, combining 1.2 mM α-lipoic acid and 0.6 mM NADH led to a significant reduction rate, i.e., 4076% compared with the basal rate. The addition of all supplements resulted in a maximum stimulation of the DHA reduction rate (81.19-fold). A blank was run for the basal conditions plus all supplements, but containing no mitoplasts. The value was 0.15 ± 0.02 nmol/min as compared with 34.25 nmol/min/mg protein when mitoplasts were included.

α-Keto Acid Dehydrogenase Catalyzed Reduction of Dehydroascorbic Acid

Two of the major α-keto acid dehydrogenases of mitochondria, pyruvate and α-keto-glutarate dehydrogenase, showed substantial substrate-dependent reduction of DHA to AA (Tables III and IV). The amount of enzyme selected was within the linear range of velocity. Each dehydrogenase behaved similarly with respect to the effect of omitting the respective α-keto acid substrates (2.6% activity relative to the complete assay). Moreover, each dehydrogenase showed an absolute requirement for coenzyme A and α-lipoic acid for reduction of DHA to AA. Blanks were always run for both series for the complete system without

Table III. Reduction of DHA by Pyruvate Dehydrogenase

Sample[a]	Assay composition	AA formation (nmol/min/unit)[b]	Relative activity (%)
1	Complete	18.8 ± 8.1	100[c]
2	Complete minus pyruvate	0.5 ± 0.7	2.6[c]
3	Complete minus coenzyme A	0	0
4	Complete minus α-lipoic acid	0	0
5	Complete minus NAD^+	19.0 ± 7.3	101[c]

[a] Incubations were for 5 min at 37°C. The complete reaction mixture consists of 46.5 mU PDH ml, 100 mM potassium phosphate, pH 7.0, 1.2 mM $MgCl_2$, 0.4 mM thiamine pyrophosphate, 0.8 mM coenzyme A, 0.6 mM NAD^+, 4 mM pyruvate, 4.0 mM α-lipoic acid, and 1 mM DHA. The reactions were stopped by the addition of an equal volume of 20% metaphosphoric acid, 2 mM thiourea, and 2 mM EDTA.

[b] Pyruvate dehydrogenase from Sigma Chem. Co. One unit is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of pyruvate/min/mg protein at 30°C. Values are the mean ± standard deviation of five experiments.

[c] Relative to the complete system.

Table II. Formation of Ascorbic Acid from DHA by Rat Liver Mitoplasts

Sample[a]	Assay composition	AA formation (nmol/min/mg protein)	Relative activity (%)
1	Basal: 0.5 mg mitoplasts protein,[b] 1 mM DHA, 50 mM sodium phosphate, pH 7.0, 50 mM KCl, 6.4 mM $MgCl^2$	0.42 ± 0.10	100[c]
2	Basal + 3.2 mM Pyruvate	0.40 ± 0.09	95[c]
3	Basal + 0.64 mM ATP	0.32 ± 0.07	76[c]
4	Basal + 0.6 mM NAD^+	0.45 ± 0.09	107[c]
5	Basal + 0.6 mM NADH	1.18 ± 0.07	280[c]
6	Basal + 1.2 mM α-lipoic acid	0.41 ± 0.06	98[c]
7	Basal + 1.2 mM α-lipoic acid + 0.6 mM NADH	17.12 ± 0.82	4076[c]
8	Basal + all supplements	34.10 ± 2.66	8119[c]

[a] Incubations were for 5 min at 37°C. Reactions were stopped by the addition of an equal volume (500 μl) of 20% metaphosphoric acid, 2 mM thiourea, and 2 mM EDTA.

[b] Mitoplasts were prepared as described for mitochondria with 0.12 mg digitonin/mg protein.

[c] Activity relative to basal activity. Values are the mean ± standard deviation of three experiments.

Table IV. Reduction of DHA by α-Keto-Glutarate Dehydrogenase

Sample[a]	Assay composition	AA formation (nmol/min/unit[b])	Relative activity (%)
1	Complete	35.0 ± 3.1	100[c]
2	Complete minus α-keto-glutarate	0.9 ± 1.0	2.6[c]
3	Complete minus coenzyme A	0	0
4	Complete minus α-lipoic acid	0	0
5	Complete minus NAD^+	12.4 ± 4.3	35.4[c]

[a] Incubations were for 5 min to 37°C. The complete reaction mixture consists of 72.8 mU α-KDH. 100 mM potassium phosphate, pH 7.0, 1.2 mM $MgCl_2$, 0.4 mM thiamine PP, 0.8 mM coenzyme A, 0.6 mM NAD^+, 2 mM α-keto-glutarate, 4.0 mM α-lipoic acid, and 1 mM DHA. The reactions were stopped by the addition of an equal volume (500 μl) of 20% metaphosphoric acid, 2mM EDTA, and 2 mM thiourea.

[b] α-Ketoglutarate dehydrogenase (Sigma Chemical Co.). One unit is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of α-ketoglutarate/min/mg protein at 30°C. Values are the mean ± standard deviation of three experiments.

[c] Activity relative to the complete system.

enzymes, since CoASH reduced DHA to AA at a significant rate and for a minor reduction afforded by the low level of β-mercaptoethanol in the commercial enzyme preparations. No modifying effect on pyruvate dehydrogenase was observed when NAD^+ was omitted from the incubation mixture (Table III). However, a decrease to 35.4% of the complete activity of α-keto-glutarate dehydrogenase was observed with the omission of 0.6 mM NAD^+ from the complete mixture (Table IV).

Lipoamide Dehydrogenase Catalyzed Reduction of Dehydroascorbic Acid

Since the previous results implicated lipoamide dehydrogenase in the catalytic reduction of DHA to AA, a purified preparation of the enzyme from bovine intestinal mucosa (Sigma Chemical Co.) was examined. The ability to engage in the catalytic reduction of lipoic acid or lipoamide to dihydrolipoic acid and dihydrolipoamide, respectively, leading to the chemical reduction of DHA to AA, was observed in a concentration dependent manner (Table V). These results showed that the half maximal rate of DHA reduction ($K_{0.5}$) occurred at 1.4 ± 0.8 mM for lipoic acid and at 0.9 ± 0.3 mM for lipoamide (Table V). The apparent

Table V. α-Lipoic Acid and Lipoamide Dependent Reduction of Dehydroascorbate to Ascorbate Catalyzed by Lipoamide Dehydrogenase[a]

Parameters	α-Lipoic acid[b]	Lipoamide[c]
$K_{0.5}$ (mM)	1.4 ± 0.8	0.9 ± 0.3
k_{cat} (sec^{-1})	16	661
$k_{cat}/K_{0.5}$ (M^{-1} sec^{-1})	1.2×10^4	7.3×10^5

[a] The reaction was carried out at 37°C for 5 min in a volume of 500 μl, and stopped by the addition of an equal volume of 20% metaphosphoric acid, 2 mM thiourea, and 2 mM EDTA. Ascorbic acid was determined as described in Materials and Methods. Values are mean ± standard deviation; $n = 3$.

[b] The reaction composition was 100 mM potassium phosphate, pH 5.9, 0.3 mM EDTA, 0.7 mg/ml BSA, 0.15 mM NADH, 0.1 mM NAD^+, 1 mM DHA, 1.65 μg/ml lipoamide dehydrogenase (bovine intestine, Sigma), and variable α-lipoic acid (0.025–4.0 mM).

[c] The reaction composition was 100 mM potassium phosphate, pH 6.5, 0.3 mM EDTA, 0.7 mg/ml BSA, 0.15 mM NADH, 0.1 mM NAD^+, 1 mM DHA, 41.25 ng/ml lipoamide dehydrogenase (bovine intestine, Sigma), and variable lipoamide (0.025–2 mM).

k_{cat} (μmol AA/min/mole enzyme) for α-lipoic acid and lipoamide was 16 and 661 sec^{-1}, respectively and the $k_{cat}/K_{0.5}$ was 1.2×10^4 and 7.3×10^5 $M^{-1}sec^{-1}$, respectively (Table V). The lipoamide dehydrogenase (Sigma), assayed at 37°C using lipoamide as substrate, had an activity of 1,400 μmol/min/mg protein, i.e., about 2-fold greater than the combined reactions as measured by the reduction of DHA to AA. The maximum velocity of the DHA reduction was substantially greater (>40-fold) with lipoamide as the substrate (Table V).

DISCUSSION

Earlier studies have shown the dihydrolipoate dependent reduction of DHA to AA, *in vitro* (Bast and Haenen, 1990). Kagan, *et al.* (1992) suggested that mitochondrial reduction of lipoic acid to dihydrolipoic acid by α-keto acid dehydrogenases may largely account for the cellular α-lipoic acid reduction process. This concept was also discussed in a recent review by Packer *et al.* (1995). The results of the present study provide evidence that the reduction of lipoic acid in mitochondria is mediated by lipoamide dehydrogenase associated with α-keto acid oxidation by α-keto acid dehydrogenase complexes. The dihydrolipoic acid produced, in turn, reduced DHA to AA rapidly. A similar reaction was demonstrated in a recent study by Bunik

and Follmann (1993) in which lipoic acid, reduced to dihydrolipoate, promoted the reduction of oxidized thioredoxin from *E. coli*. The present studies demonstrate that α-keto acid dehydrogenase complexes of mitochondria and mitoplasts and isolated lipoamide dehydrogenase have α-lipoic acid dependent dehydroascorbic acid reducing activity mediated by the catalytic formation of dihydrolipoic acid (Scheme A). The rate of dihydrolipoic acid or dihydrolipoamide reduction of DHA to AA (Scheme A, reaction 2) is believed to be rapid since the $K_{0.5}$ for lipoic acid and lipoamide in the lipoamide dehydrogenase catalyzed reduction of DHA is similar to the K_m for these two substrates (Massey, 1963).

1) α-Lipoic Acid (Lipoamide) + NADH + H^+ $\overset{\text{LDH}}{\rightleftharpoons}$ Dihydrolipoic Acid (Dihydrolipoamide) + NAD^+

2) Dihydrolipoic Acid (Dihydrolipoamide) + DHA → AA + α-Lipoic Acid (Lipoamide)

Scheme A.

In the intact mitochondria, the effect of various metabolites observed in this study may involve enhanced levels of NADH by various mechanisms. For example, the addition of NADH but not NAD^+ plus lipoic acid to mitochondria stimulated AA production. This action may be explained by the NADH:NAD^+ transhydrogenase activity of lipoamide dehydrogenase (Massey, 1963; Green and O'Brien, 1973). Alternatively, the mitochondrial preparations may have had some membrane damage leading to penetration of the NADH into the mitochondrial matrix. Similarly, a source of electrons, pyruvate, with a blockade of the coupled oxidative phosphorylation complexes by, e.g., rotenone or antimycin A, quite likely resulted in increased NADH levels and increased AA formation. The complex IV inhibitor, sodium azide, only slightly stimulated AA formation, and the smaller stimulation by azide may be due to difficulty in transport of azide anion across the inner mitochondrial membrane. Severe inhibition of mitochondrial AA formation by 1 mM iodoacetamide and 1 mM sodium arsenite are consistent with the known inhibition of lipoamide dehydrogenase by these agents (Massey, 1963). Experiments with mitoplasts confirmed the submitochondrial location of the regeneration system. Further, purified α-keto acid dehydrogenases and lipoamide dehydrogenase appear to be largely responsible for significant lipoic acid dependent mitochondrial AA regeneration from DHA.

Purified mitochondria contain GSH and GSSG, and in the absence of exogenous lipoic acid and a supplementary source of NADH, we suggest that the basal DHA reducing activity seen in Tables I and II is largely due to the chemical reduction of DHA by GSH. The increase in AA formation from basal levels due to NADH (Tables I and II) suggest that NADH may be utilized by lipoamide dehydrogenase via possible transhydrogenase or by semidehydroascorbate reductase activities. The latter may be considered since DHA together with AA generate low levels of semidehydroascorbate, i.e., the reverse of the disproportionation reaction.

In the α-keto acid dehydrogenase systems, a model for exogenous α-lipoic acid may be drawn to illustrate the satellite reactions observed in the present study (Fig. 1A). Consistent with this model, the reduction of DHA to AA by the pyruvate dehydrogenase system was not influenced by the presence of NAD^+. In contrast, NAD^+ had a substantial stimulatory effect on the same process in the case of α-ketoglutarate dehydrogenase. In this case, the effect of NAD^+ may be related to the absolute requirement for NAD^+ in the lipoyl dehydrogenase reaction. The mechanism is believed to involve preventing the enzyme from converting to the fully reduced form which is catalytically inactive (Massey, 1963). Figure 1B illustrates a proposed reaction between α-lipoic acid, lipoamide, NADH, and DHA catalyzed by isolated lipoamide dehydrogenase. In the absence of α-lipoic acid or lipoamide, DHA may react directly to accept electrons from the SH groups of the reduced lipoamide dehydrogenase, but at a much slower rate, i.e., <1% of the activity observed when 50–100 μM lipoic acid was provided (data not shown). If the process described in this report is replicated *in vivo*, when lipoic acid is administered therapeutically, a significant contribution to cellular AA regeneration from DHA would be expected. The present study offers an explanation for the ability of α-lipoic acid to ameliorate the symptoms of scurvy in guinea pigs fed an ascorbic acid deficient

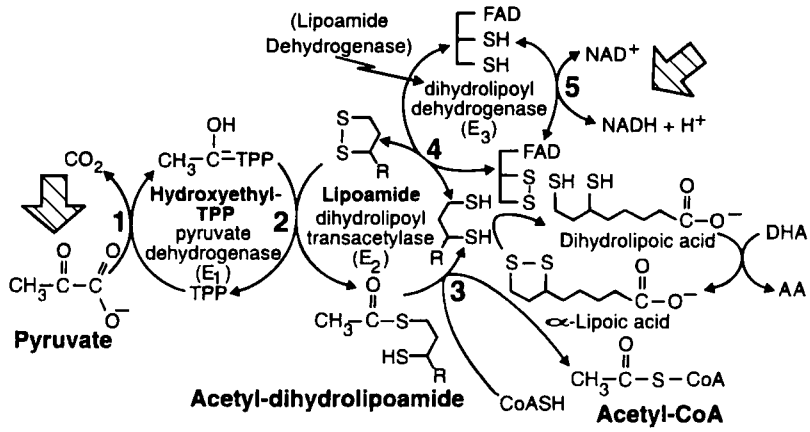

Fig. 1A. A model illustrating the proposed interaction between α-lipoic acid, DHA, and the pyruvate dehydrogenase complex. Reducing equivalents may enter the system from substrates, pyruvate, or NADH (large arrows) to stimulate dihydrolipoate mediated reduction of DHA to AA.

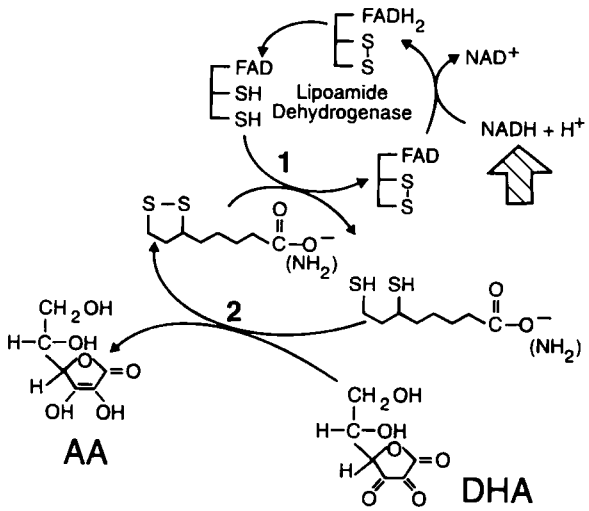

Fig. 1B. A model illustrating the α-lipoate or lipoamide mediated reduction of DHA to AA catalyzed by lipoamide dehydrogenase. Reducing equivalents are derived from NADH (large arrow).

diet (Rosenberg and Culik, 1959). As dihydrolipoic acid receives increasing attention as an antioxidant in biology, it is essential to include evaluation of its effects on ascorbic acid recycling as well as its effects on GSH and vitamin E in cellular redox economy (Zimmer *et al.*, 1991; Scholich *et al.*, 1989; Bast and Haenen, 1988; Maitra *et al.*, 1995).

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grants DK 44456 and CA 51972. The authors thank Carol McCutcheon for typing the manuscript.

REFERENCES

Addanki, S., Cahill, F. D., and Sotos, J. F. (1968). *J. Biol. Chem.* **243**, 2337–2348.

Bast, A., and Haenen, G. R. M. M. (1988). *Biochim. Biophys. Acta* **963**, 558–561.

Bast, A., and Haenen, G. R. M. M. (1990). In *Antioxidants in Therapy and Preventative Medicine* (Emerit *et al.*, eds.), Plenum Press, New York pp 111–116.

Bielski, B. H. J. (1982). In *Ascorbic Acid: Chemistry, Metabolism and Uses* (Sieb, P. A., and Tolbert, B. M., eds.), Advances in Chemistry Series 200 (Comstock, M. J., ed.), American Chemical Society, pp 81–100.

Bunik, V., and Follmann, H. (1993). *FEBS Lett.* **336**, 197–200.

Burkart, B., Koike, T., Brenner, H.-H., Imai, Y., and Kolb, H. (1993). *Agents Actions* **38**, 60–65.

Chance, B., Sies, H., and Boveris, A. (1979). *Physiol. Rev.* **59**, 527–604.

Crook, E. M., and Hopkins, F. G. (1938). *Biochem. J.* **32**, 1356–1363.

Del Bello, B., Maellaro, E., Sugherini, L., Santucci, A., Comporti, M. and Casini, A. F. (1994). *Biochem. J.* **304**, 385–390.

Green, R. C., and O'Brien, P. J. (1973). *Biochim. Biophys. Acta* **293**, 334–342.

Greenawalt, J. W. (1974). *Methods Enzymol.* **31**, 310–323.

Griffith, O. W., and Meister, A., (1985). *Proc. Natl. Acad. Sci. USA* **82**, 4668–4672.

Groden, D., and Beck, E. (1979). *Biochim. Biophys. Acta* **546**, 426–435.

Hossain, M. A., and Asada, K., (1984). *Plant Cell Physiol.* **25**, 85–92.

Hughes, R. E. (1964). *Nature* **203**, 1068–1069.

Icén, A. (1967). *Scand. J. Clin. Lab. Invest. (Suppl. 2)* **96**, 1–67.

Jocelyn, P. C., and Kamminga, A. (1974). *Biochim. Biophys. Acta* **343**, 356–362.

Kagan, V. E., Shvedova, A., Serbinova, E., Khan, S., Swanson, C., Powell, R., and Packer, L. (1992). *Biochem. Pharmacol.* **44**, 1637–1649.

Kersten, H., Kersten, W., and Staudinger, Hj. (1958). *Biochim. Biophys. Acta* **27**, 598–608.

Komuniecki, R., and Saz, H. J. (1979). *Arch. Biochem. Biophys.* **196**, 239–247.

Maellaro, E., DelBello, B., Sugherini, L., Santucci, A., Comporti, M., Casini, A. F., (1994). *Biochem. J.* **301**, 471–476.

Maitra, I., Serbinova, E., Trischler, H., and Packer, L. (1995). *Free Rad. Biol. Med.* **18**, 283–829.

Massey, V. (1960). *J. Biol. Chem.* **235**, PC47–48.

Massey, V. (1963). In *The Enzymes*, 2nd edn. (Boyer, P. D., Lardy, H., and Myrbäck, K., eds.), Academic Press, New York, pp 275–306.

Matuda, S., and Saheki, T. (1982). *J. Biochem.* **91**, 553–561.

Meister, A. (1992). *Biochem. Pharmacol.* **44**, 1905–1915.

Meredith, M. J., and Reed, D. J. (1982). *J. Biol. Chem.* **257**, 3747–3753.

Packer, L., Witt, E. H., and Tritschler, H. J. (1995). *Free Rad. Biol. Med.* **19**, 227–250

Roberts, J. C., and Francetic, D. J. (1993). *Anal. Biochem.* **211**, 183–187.

Roos, A., and Boron, W. F. (1981). *Physiol. Rev.* **61**, 296–434

Rosenberg, H. R., and Culik, R. (1959). *Arch. Biochem. Biophys.* **80**, 86–93.

Scholich, H., Murphy, M. E., and Sies, H. (1989). *Biochim. Biophys. Acta* **1001**, 256–261.

Scott, E. M., Duncan, I. W., and Ekstand, V. (1963). *J. Biol. Chem.* **238**, 3928–3933.

Searls, R. L., and Sanadi, D. R., (1960). *J. Biol. Chem.* **235**, 2485–2491.

Storrie, B., and Madden, E. A. (1990). *Methods Enzymol.* **182**, 214–215.

Stockstad, E. L. R., Seaman, G. R., Davis, R. J., and Hunter, S. H. (1956). In *Methods of Biochemical Analysis*, Vol. 3 (Glick, D., ed.), Wiley Interscience, New York, pp, 23–47.

Szent György, A. (1928). *Biochem. J.* **22**, 11387–1409.

Wahlländer, A., Soboll, S., and Sies, H. (1979). *FEBS Lett.* **97**, 138–140.

Wells, W. W., Xu, D-P., Yang, Y., and Rocque, P. A. (1990). *J. Biol. Chem.* **265**, 15361–15364.

Wells, W. W., Rocque, P. A., Xu, D-P., Meyer, E. B., Charamella, L. J., and Dimitrov, N. V. (1995). *Free Rad. Biol. Med.* **18**, 699–708.

Zimmer, G., Mainka, L., and Krüger, E. (1991). *Arch. Biochem. Biophys.* **288**, 609–613.