Reduction of Dehydroascorbate to Ascorbate by the Selenoenzyme Thioredoxin Reductase*

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James M. Mayé, Shalu Mendiratta, Kristina E. Hill, and Raymond F. Burk

From the Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6303

Recycling of ascorbate from its oxidized forms is essential to maintain stores of the vitamin in human cells. Whereas reduction of dehydroascorbate to ascorbate is thought to be largely GSH-dependent, we reconsidered the possibility that the selenium-dependent thioredoxin system might contribute to ascorbate regeneration. We found that purified rat liver thioredoxin reductase functions as an NADPH-dependent dehydroascorbate reductase, with an apparent Km of 2.5 mM for dehydroascorbate, and a kcat of 90 min⁻¹. Addition of 2.8 mM purified rat liver thioredoxin lowered the apparent Km to 0.7 mM, without affecting the turnover (kcat of 71 min⁻¹). Since thioredoxin reductase requires selenium, we tested the physiologic importance of this enzyme for dehydroascorbate reduction in livers from control and selenium-deficient rats. Selenium deficiency lowered liver thioredoxin reductase activity by 88%, glutathione peroxidase activity by 99%, and ascorbate content by 33%, but did not affect GSH content. NADPH-dependent dehydroascorbate reductase activity due to thioredoxin reductase, on the basis of inhibition by aurothioglucose, was decreased 88% in dialyzed liver cytosolic fractions from selenium-deficient rats. GSH-dependent dehydroascorbate reductase activity in liver cytosol was variable, but typically 2–3-fold that of NADPH-dependent activity. These results show that the thioredoxin system can reduce dehydroascorbate, and that this function is required for maintenance of liver ascorbate content.

Ascorbic acid, or vitamin C, is important as a cofactor in several enzyme reactions, and in the defense against oxidant stress (1). Mammalian cells efficiently regenerate ascorbic acid from its two-electron oxidized form, dehydroascorbate (DHA). This recycling of DHA to ascorbate is known to be mediated by GSH (2), either by direct chemical reduction (3), or with the assistance of one or more GSH-dependent enzymes (4–6). However, a recent report showing that rapid DHA reduction in HL-60 cells was unaffected by GSH depletion (7) brings up the possibility that other mechanisms may contribute to DHA recycling. For example, NADPH-dependent DHA reduction has been reported to occur in rat liver through the action of 3α-hydroxysteroid dehydrogenase (8). Another potential NADPH-dependent DHA reductase activity, the thioredoxin system, was reported not to reduce DHA (9).

Mammalian thioredoxin reductase (TR) (EC 1.6.4.5) is a selenoprotein (10) that, in conjunction with thioredoxin (Trx), forms an effective system for reduction of protein disulfides (11). TR can also reduce many other substrates without the assistance of Trx, including lipico acid (12), vitamin K₁ (13), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (14), and alloxan (15). DHA resembles alloxan in size and also has a cyclic triketone structure. Given the broad substrate specificity of TR, and the chemical similarities of alloxan and DHA, it was surprising that DHA was reported not to be a substrate of TR (9). We reinvestigated the possibility that rat liver TR alone or in combination with TRx can function as an NADPH-dependent DHA reductase. Our results show that the thioredoxin system can reduce DHA in the presence of NADPH, and that a decrease in this reduction may contribute to lowered ascorbate concentrations in livers of selenium-deficient rats.

EXPERIMENTAL PROCEDURES

Materials—DHA, d-isoascorbate, and ascorbate-6-palmitate were from Aldrich. Where noted, DHA or its derivative was prepared by bromine oxidation of the reduced form (16). Recombinant human placental glutaratedoxin (lot 3A) was purchased from IMCO Corporation (Stockholm, Sweden). Glutathione reductase (lot 1211H80701), glutathione S-transferase (lot 75H8030), and protein disulfide isomerase (lot 66H4072) were purchased from Sigma. Trx from Escherichia coli (lot B11094) was purchased from Calbiochem. 6-Sulphophenyl-ascorbate was a generous gift from Dr. K. Wimalasena at Wichita State University, Wichita, KS.

Purification of TR and Trx—TR and Trx were purified from rat liver by the method of Luthman and Holmgren (17) and stored at −80 °C. The purity of both preparations was judged to be >95% by SDS-polyacrylamide gel electrophoresis. The specific activity of the TR preparation was 90 ± 6 A₄₅₀ units/mg (mg of protein)⁻¹ (S.D., n = 4) in the insulin assay for TR (14). In the presence of 0.2 mM NADPH, purified rat liver TR reduced 0.5 mM DTNB at a rate of 4630 nmol/min per mg of enzyme, which is similar to that observed by Holmgren (14). As noted by Holmgren (13), it was necessary to reduce purified rat liver Trx by a 1 h incubation with 2 mM dithiothreitol just before use. The dithiothreitol was separated from Trx on Bio-Rad Bio-Spin 6 columns.

Assays of DHA Reductase Activity—Two assays were used to measure NADPH-dependent DHA reductase activity. In the first assay, conversion of DHA to ascorbate was measured by incubating purified TR with 0.4 mM NADPH and freshly prepared DHA in 50 mM Tris-HCl (pH 7.4). Following incubation at room temperature for 10 min, the reaction was stopped by addition of 10 volumes of ice-cold 80% methanol (v/v) that contained a final concentration of 1 mM EDTA. After incubation for 5 min on ice, the mixture was microcentrifuged and aliquots of the supernatant were assayed for ascorbate by HPLC with electrochemical detection (18). Under these conditions, ascorbate generation was linear for at least 20 min, and increased linearly with an increase in the TR concentration from 10–80 ng (results not shown). NADPH alone did not reduce DHA. Commercial DHA contained a small amount of ascorbate (~0.5%), which was measured and corrected for as a blank in each assay. When prolonged stability of DHA was required, or when a DHA derivative was the substrate, bromine oxidation was used to oxidize the ascorbate-containing form (16).

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In the second assay of NADPH-dependent DHA reductase activity, the disappearance of NADPH was followed spectrophotometrically. The reaction mixture consisted of the indicated concentration of TR, 0.2 mM NADPH, and the substrate to be reduced in the Tris-EDTA buffer. The reaction was started by adding the substrate to a cuvette with mixing, and followed at room temperature by recording the absorbance at 340 nm in a Beckman model 640 spectrophotometer. The rate of NADPH disappearance was linear for at least 3 min, and the nanomoles of NADPH consumed were calculated using an extinction coefficient of 6.22 × 10^3 M^-1 cm^-1. For both assays, the rate of reaction was normalized to the amount of TR present.

NADPH-dependent DHA reductase activity of purified commercial glutaredoxin and of protein disulfide isomerase was determined by measurement of the rate of appearance of ascorbate at 265 nm (4). The amount of ascorbate generated was calculated using a molar extinction coefficient of 14.7 × 10^3 M^-1 cm^-1 (4).

Animals and Diets—From the weanling stage, male Sprague-Dawley rats were fed either a Torula yeast diet that was deficient in selenium, or the same diet that was supplemented with 0.25 ppm selenium as Na2SeO3 to provide an adequate amount of dietary selenium (19). Rats were fed the diet for at least 14 weeks, during which time they were allowed free access to food and water. In a typical experiment with three animals in each group, the weights of selenium-deficient animals averaged 244 ± 17 g at the time of the experiment, compared with 254 ± 16 g for control animals (mean ± S.D.). Following an overnight fast, rats were each injected with pentobarbital and exsanguinated by removal of blood from the aorta. Tissues were immediately removed, frozen in liquid nitrogen, and stored at −70 °C until assay of ascorbate content and DHA reductase activity.

Preparation of Liver Homogenates and Dialyzed Cytosol—For measurement of ascorbate, frozen tissue samples (100 mg) were homogenized in 1 ml of ice-cold 50 mM perchloric acid in a hand-held Teflon homogenizer. After a 5-min incubation on ice, the samples were microcentrifuged for 5 min at 4 °C, and stored on ice until assay. Tissue content of ascorbate is expressed as a concentration, assuming that cellular water comprises 80% by weight of the tissue.

For preparation of dialyzed cytosolic fractions, frozen liver (400 mg) was homogenized in 1 ml of ice-cold 50 mM Tris-HCl that contained 1 mM EDTA, pH 7.4. The homogenate was microcentrifuged for 15 min at 4 °C, and the supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was placed in dialysis tubing (molecular weight cut-off 3400) and dialyzed overnight against three changes of 250 ml of the Tris-EDTA buffer at 4 °C. The HPLC assay that was used to measure ascorbate generation from DHA also detects GSH. In dialyzed cytosol before addition of DHA, neither ascorbate nor GSH were detectable by HPLC. The lower limits for HPLC detection in the incubated cytosol were 0.1 μM for ascorbate and for 2 μM for GSH.

Other Assays—Protein was measured by the BCA method (Pierce). The GSH concentrations shown in Table III were measured by the DTNB-glutathione reductase recycling assay of Tietz (20). Glutathione peroxidase activity was measured using H2O2 as substrate in 13,000 × g supernatants of livers that were homogenized in 100 mM sodium phosphate buffer, pH 7.5 (21). TR activity in 13,000 × g supernatants was measured as recently described (22).

Data and Statistical Analysis—Data are shown as mean ± S.E., except where otherwise indicated. Michaelis-Menten kinetic parameters were determined by nonlinear least-squares regression of initial rates of reaction using Global Analysis as described previously (23). The enzyme activity from multiple experiments was determined from the minimum value of each kinetic parameter in the χ^2 distribution. Differences between kinetic parameters were established from the χ^2 distribution on the basis of an F test (24) and were considered significant when there was no overlap of their χ^2 values below a confidence level of 0.67. Statistical significance of biochemical data was assessed using the statistical software package Sigmastat 2.0 (Jandel Scientific, St. Louis, MO).

RESULTS

Purified rat liver TR used NADPH to reduce DHA to ascorbate with a 1:1 stoichiometry, when either the appearance of ascorbate was measured by HPLC or the disappearance of NADPH was measured by spectrophotometry. As shown in Fig. 1, increasing amounts of DHA caused a saturable increase in the rate of DHA reduction by purified rat liver TR alone and by the combination of TR and Trx. The kinetic parameters for these reactions are shown in Table I. Addition of rat liver Trx more than tripled the apparent affinity of TR for DHA, without affecting the turnover number. Trx from E. coli, on the other hand, both tripled the affinity and doubled the catalytic capacity of the enzyme. The measured kinetic parameters of two GSH-dependent enzymes, protein disulfide isomerase and recombinant human placental glutaredoxin, are also shown in Table I. These were similar to the values observed for rat liver TR + Trx. Commercial glutathione S-transferase and glutathione reductase failed to reduce DHA under these conditions (results not shown).

TR from rat liver also reduced several derivatives of ascorbate and alloxan, as shown in Table II. Whereas DHA and its alloxan iso-DHA were reduced at comparable rates, the more hydrophobic ascorbate derivatives showed much greater rates of reaction. When ascorbate 6-palmitate was oxidized with bromine to the uncharged DHA derivative, a fine precipitate formed that was added directly to the enzyme assay. Differences in solubility from experiment to experiment probably account for the large variability in reaction rates observed. Alloxan and ninhydrin were also reduced manyfold more rapidly by TR than was DHA. Barbitaluric acid, which lacks the

![Fig. 1](image-url.com)
TABLE II
Reduction of triketone derivatives by TR

| Agent         | Rate Control | Selenium-deficient |
|---------------|--------------|--------------------|
|               | mw | min | %     | %     |
| Aurothioglucose| 1.0| 62±13| 5.4±0.5| 2.0±0.7*|
| Alloxan        | 1.0| 61±7 | 5.4±0.5| 2.0±0.7*|
| 6-S-Phenyl-DHA | 1.0| 624±58*| 3.4±0.3| 1.4±0.2*|
| 6-Palmitoyl-DHA| 1.0| 1263±772*| 3.4±0.3| 1.4±0.2*|
| Ninhydrin      | 0.2| 3999±1139*| 5.4±0.5| 2.0±0.7*|

Central carbonyl group of alloxan, was not reduced at a concentration of 0.2 mM (data not shown). These results confirm the broad substrate specificity of TR for several different redox-active DHA derivatives and related triketones.

To test the physiologic importance of TR as a DHA reductase, the effect of selenium deficiency on tissue ascorbate content and on NADPH-dependent DHA reduction in dialyzed liver cytosolic fractions was studied. As shown in Table III, selenium deficiency markedly decreased the activities of the selenoenzymes TR and glutathione peroxidase, which documents the severity of the selenium deficiency. Selenium deficiency also decreased the liver ascorbate content by one-third compared with control rats. The concentration of GSH in liver was unaffected by selenium deficiency.

NADPH-dependent DHA reductase activity in cytosolic fractions prepared from livers of selenium-deficient rats was less than half that measured in controls (Table IV). Aurothioglucose, an inhibitor of TR (22), was used to enhance the specificity of the assay for TR. At a concentration of 10 μM, aurothioglucose inhibited the NADPH-dependent DHA reductase activity of purified rat liver TR by 85% (n = 7). Similarly, addition of 10 μM aurothioglucose decreased the NADPH-dependent DHA reductase activity of control cytosol by 75%. Treatment of cytosol from livers of selenium-deficient animals with aurothioglucose caused a smaller decrease in activity, leaving a non-inhibited activity that was similar to that in control cytosol after incubation with aurothioglucose. The difference between total NADPH-dependent DHA reductase activity and the residual activity after inhibition by aurothioglucose is shown in the third row of Table IV. This aurothioglucose-sensitive DHA reductase activity was decreased 88% in liver cytosols from selenium-deficient animals compared with controls.

Liver cytosolic fractions from control and selenium-deficient rats were prepared as described under "Experimental Procedures" and incubated in Tris-EDTA buffer with 1 mM DHA in the presence of either 0.4 mM NADPH or 2 mM GSH. The amount of ascorbate generated was measured by HPLC. Aurothioglucose-inhibitable activity represents the difference between the control NADPH-dependent DHA reductase activity and that remaining after incubation of the sample with 10 μM aurothioglucose. GSH-dependent DHA reductase activity is corrected for nonenzymatic DHA reduction that was measured in the absence of cytosol. Data are shown from three livers as mean ± S.D., with an asterisk (*) indicating p < 0.05 compared to control analysis of variance of the NADPH-dependent data. Similar results were observed in another selenium deficiency experiment.
gest that hydrophobic interactions of the substrate with TR can enhance activity, and that a vicinal triketone structure is required for reduction. We have no explanation for the earlier negative results with regard to the DHA reductase activity of TR (9).

The turnover number for DHA reduction by TR, with or without rat liver Trx, was no different than that found for GSH-dependent DHA reduction by recombinant human placental glutaredoxin and protein disulfide isomerase. Maximal rates of DHA reduction by GSH-dependent thioredoxinases (glutaredoxins) from different tissues and species vary widely (4, 9). For example, recombinant porcine liver thioredoxin has been reported to have a turnover number of 374 min⁻¹ (4), whereas a value of 68 min⁻¹ can be calculated for glutaredoxin purified from human neutrophils (25). The apparent affinity of recombinant human glutaredoxin for DHA of 0.27 mM was similar to that of 0.2 mM reported for purified porcine liver glutaredoxin (9).

Addition of rat liver Trx to TR increased the apparent affinity of the enzyme system for DHA (Fig. 1 and Table I), as previously noted for allooxan (15). This suggests that reduction of DHA by Trx occurs, and that the high affinity of TR for Trx (Kₘ = 2.5 μM) (17) facilitates the overall reaction. We also tested Trx from E. coli with rat liver Trx and found both an increase in apparent affinity for DHA and a doubling of the maximal catalytic capacity (Table I). The increased apparent affinity of TR for DHA in the presence of E. coli Trx can again be attributed to a high affinity of TR for the bacterial Trx (Kₘ = 35 μM) (17). The increase in turnover number may reflect an ability of the E. coli Trx to enhance the relatively low catalytic efficiency of TR for DHA.

Selenium deficiency decreased the ascorbate content of rat liver by one-third, even though this tissue can synthesize ascorbate. The concentration of GSH in liver was not affected, as expected in this model of moderate selenium deficiency (9). The fall in ascorbate content, therefore, supports a role for selenoenzymes in ascorbate regeneration from DHA. In diazoyl liver cytosol from selenium-deficient rats, NADPH-dependent ascorbate regeneration was decreased to about 40% of that in controls (Table IV). The decrease was even greater when considered as aurothioglucose-sensitive activity. Gold-containing compounds are known to inhibit selenoenzymes (26). As expected from its inhibition of DTNB and insulin disulfide reduction by rat liver TR (22), aurothioglucose was also a potent inhibitor of DHA reduction by purified rat liver TR. Since glutathione peroxidase activity is unaffected by 10 μM aurothioglucose (22), we consider the gold-sensitive, NADPH-dependent, DHA reductase activity to reflect that of TR. Based on this reasoning, selenium deficiency decreased TR-dependent DHA reductase activity in liver cytosol by almost 90% (Table IV), or to a similar extent as it did TR-dependent disulfide reduction (Table III). Furthermore, the apparent Kₘ of the DHA reductase activity in diazoyl liver cytosol was 1.0 mM (Fig. 2), which is similar to that of purified rat liver TR + Trx (Table I).

The NADPH-dependent DHA reductase activity measured in liver cytosol of control rats of about 5 nmol/mg of protein⁻¹ min⁻¹ was in between values of 3.6 and about 8 nmol/mg of protein⁻¹ min⁻¹ that were measured by Del Bello et al. (8) and by Rikans et al. (27), respectively. The former authors went on to purify a NADPH-dependent DHA reductase activity from liver that was identified as 3α-hydroxysteroid dehydrogenase (8). However, since 3α-hydroxysteroid dehydrogenase does not code for selenocysteine (28), the enzyme should not be affected by selenium deficiency or by aurothioglucose. Thus, it is unlikely to have contributed more than 25% of the NADPH-dependent DHA reductase activity measured in the current experiments. Further, the apparent Kₘ measured for 3α-hydroxysteroid dehydrogenase from rat liver was about 4 mM, with a calculated kₘ of 59 min⁻¹ (8). These kinetic parameters also suggest a lesser role for this enzyme in reducing DHA than either glutaredoxin or TR in liver.

Direct comparison of GSH- and NADPH-dependent DHA reductase activities in diazoyl liver cytosol was complicated by the variability of GSH-dependent reduction. This variability was related in part to the high rate of nonenzymatic DHA reduction by GSH. GSH-dependent DHA reductase activity was reported in rat liver many years ago (29), although a recent study failed to detect this activity in crude rat liver cytosol (27). Whereas our results support a GSH-dependent DHA reductase activity in liver, more detailed studies will be needed to determine the relative contributions of direct and enzymatic GSH-dependent and NADPH-dependent DHA reduction in liver.

These studies show that mammalian TR has DHA reductase activity and that this activity is diminished in liver cytosols from selenium-deficient rats. Decreased TR activity may therefore contribute to the observed decrease in liver ascorbate content of selenium-deficient rats.

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