The Catalytic Mechanism of the Glutathione-Dependent Dehydroascorbate Reductase Activity of Thioltransferase (Glutaredoxin)†,‡

Michael P. Washburn and William W. Wells*

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Received March 2, 1998; Revised Manuscript Received October 7, 1998

ABSTRACT: The catalytic mechanism of the glutathione (GSH)-dependent dehydroascorbic acid (DHA) reductase activity of recombinant pig liver thioltransferase (RPLTT) was investigated. RPLTT and the C25S mutant protein had equivalent specificity constants (k_{cat}/K_m) for both DHA and GSH. Iodoacetamide (IAM) inactivated the DHA reductase activities of RPLTT and C25S, confirming the essential role of cysteine in the reaction mechanism. When preincubated with DHA, RPLTT but not C25S was protected against IAM inactivation, suggesting that RPLTT has the ability to chemically reduce DHA forming ascorbic acid (AA) and the intramolecular disulfide form of the enzyme. Electrochemical detection of AA demonstrated the ability of both reduced RPLTT and C25S to chemically reduce DHA to AA in the absence of GSH. However, RPLTT had an initial rate of DHA reduction which was 4-fold greater than that of C25S, and after 10 min, RPLTT resulted in an AA concentration 11-fold greater than that of C25S. Isoelectric focusing analysis revealed that the product of reaction of reduced RPLTT but not C25S with DHA was consistent with the oxidized form of the enzyme. This result suggested that even though both RPLTT and the C25S mutant had equivalent specificity constants for DHA and GSH, they may have different catalytic mechanisms. On the basis of the experimental results, a catalytic mechanism for the DHA reductase activity of RPLTT is proposed. This is the first description of a catalytic mechanism of a glutathione:dehydroascorbate oxidoreductase (EC 1.8.5.1).

Ascorbic acid (AA)[1] is an important antioxidant (*1*−*6*) and electron donor for enzymes such as dopamine-*β*-hydroxylase, prolyl-4-hydroxylase, and lysyl hydroxylase (*7*). The function of AA as an electron donor is highlighted by its importance in collagen biosynthesis (*8*) and norepinephrine biosynthesis (*9*). In addition, AA has recently been demonstrated to be essential for the release of insulin from the pancreatic islets of scorbutic guinea pigs (*10*) and as a cofactor for mitochondrial glycerol-3-phosphate dehydrogenase (*11*).

When AA carries out its function, it undergoes two successive one-electron oxidations to semidehydroascorbic acid (semiDHA) and dehydroascorbic acid (DHA). A relatively stable radical, semiDHA may disproportionate to AA and DHA (*12*) or be directly reduced to AA by an NADH-semiDHA reductase (*13*−*15*). DHA can be reduced to AA in an NADPH-dependent manner by both 3α-hydroxysteroid dehydrogenase (*16*) or thioredoxin reductase (*17*). Glutathione (GSH) chemically reduces DHA to AA, and three mammalian GSH-dependent DHA reductases have been identified: thioltransferase (glutaredoxin) (*18*), protein disulfide isomerase (PDI) (*18*), and a 32 kDa enzyme (32kDHAR) identified in rat liver (*19*) and human erythrocytes (*20*). In vivo experiments demonstrated the role of GSH in DHA reduction (*21*), but whether in vivo DHA reduction is primarily chemical, enzymatic, or both remains unclear with evidence existing for both mechanisms (*22*, *23*). Recently, Park and Levine (*24*) provided evidence for enzymatic recycling of DHA in human neutrophils, catalyzed in large part by thioltransferase. Despite the potential importance of GSH-dependent DHA reductases in the maintenance of cellular AA, the only catalytic mechanism information available is derived from the DHA reductase activity of recombinant pig liver thioltransferase (RPLTT) (*25*).

Thioltransferase is an 11.7 kDa protein belonging to a class of enzymes, containing the CXXC active site motif, known as thiol:disulfide oxidoreductases (*26*). Thioltransferase catalyzes thiol−disulfide exchange reactions (for reviews, see refs *26* and *27*), the reduction of alloxan to dialuric acid (*28*), and the reduction of DHA to AA (*18*), all in a GSH-dependent manner. The active site of RPLTT has been previously identified as Cys-22 which has a pK_a of 3.8 (*25*, *29*) and is the essential amino acid mediating the enzyme's thioltransferase and DHA reductase activities (*25*).

In this study, we investigated the catalytic mechanism of the DHA reductase activity of thioltransferase using RPLTT

† This work was supported in part by NIH Grant DK-44456, NIEHS Grant ES-7255, and a grant from the Center for Improvement of Human Functioning International, Inc. (Wichita, KS).

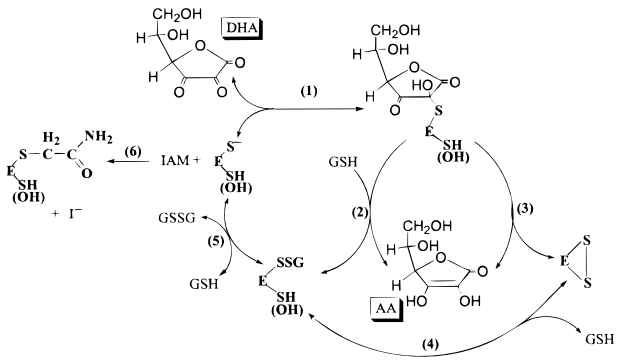
‡ The results of this study were presented in part at the ASBMB/ASIP/AAI meeting in New Orleans, LA (*FASEB J. 10*, A2220, 1996), and at the ICBMB/ASBMB meeting in San Francisco, CA (*FASEB J. 11*, A2679, 1997).

* To whom correspondence should be addressed. E-mail: wellsw@pilot.msu.edu. Fax: (517) 353-9334.

[1] Abbreviations: A_1, amplitude or the maximal amount of inhibition; AA, ascorbic acid; C25S, C25S mutant of RPLTT; DHA, dehydroascorbic acid; DTT, dithiothreitol; GSH, glutathione; GSSG, glutathione disulfide; HED, hydroxyethyl disulfide; IAA, iodoacetic acid; IAM, iodoacetamide; IEF, isoelectric focusing; IPTG, isopropyl thiogalactoside; *k*, rate constant of inactivation; PDI, protein disulfide isomerase; RPLTT, recombinant pig liver thioltransferase; semiDHA, semidehydroascorbic acid; 32kDHAR, 32 kDa GSH-dependent DHA reductase.

10.1021/bi980480v CCC: $18.00
Published on Web 12/12/1998

Scheme 1: Proposed Mechanism of GSH-Dependent DHA Reduction Catalyzed by RPLTT



and the C25S mutant protein. The results provided experimental evidence for a proposed mechanism (Scheme 1) for the DHA reductase activity of RPLTT, which can be used as a model for studying other GSH-dependent DHA reductases.

MATERIALS AND METHODS

Materials. Coomassie Brilliant Blue R-250, iodoacetic acid (IAA), iodoacetamide (IAM), EDTA, and glutathione disulfide (GSSG) were purchased from Sigma (St. Louis, MO). GSH, dithiothreitol (DTT), glutathione disulfide reductase, and NADPH were products of Boehringer Mannheim (Indianapolis, IN). Metaphosphoric acid and hydroxyethyl disulfide (HED) were purchased from Aldrich (Milwaukee, WI). Thiourea was obtained from Matheson Coleman and Bell (Cincinnati, OH), and bromine was purchased from Acros Organics (Pittsburgh, PA). AA, ammonium sulfate, and NaCl were purchased from J. T. Baker (Phillipsburg, NJ), and isopropyl thiogalactoside (IPTG) was purchased from Calbiochem (San Diego, CA). CM Sepharose, Sephacryl S-200, and Sephadex G25 were products of Amersham Pharmacia Biotech (Piscataway, NJ). Centriprep-3 concentrators were purchased from Amicon, Inc. (Beverly, MA). Serva Servalyt Precotes for pH 3−10 and p*I* test mix were acquired from Crescent Chemical Co., Inc. (Hauppage, NY). DHA was prepared as described previously (*30*). *S*-Sulfocysteine was prepared by the method of Segel and Johnson (*31*).

Expression of the Thioltransferase Gene and Purification of Pig Liver Thioltransferases. Expression of the gene and purification of RPLTT and C25S were carried out as described previously (*32*), with the following modifications. After ammonium sulfate precipitation, the pellets were resuspended in 2 mL of 20 mM sodium phosphate (pH 6.5) (buffer A) containing 2 mM DTT and dialyzed overnight (2 × 2 L) against the same buffer. After dialysis, the sample was loaded onto a Sephacryl S-200 column (2.5 cm × 89 cm) equilibrated and eluted with buffer A containing 2 mM DTT. The pooled active fractions were incubated with 10 mM HED for 30 min at room temperature and loaded onto a CM-Sepharose column (5 cm × 38 cm) equilibrated with buffer A. The column was washed with 1 L of buffer A followed by 1 L of 20 mM sodium phosphate (pH 7.5) (buffer B). The bound disulfide enzyme was eluted with an 800 mL 0.0 to 1.0 M NaCl linear gradient in buffer B. The active fractions were pooled and concentrated using Centriprep 3 concentrators and stored at −70 °C until they were used. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co.) according to the manufacturer's directions. During the purification, the thioltransferase activity was assayed as described previously (*35*).

Dehydroascorbic Acid Reductase Assay and Enzyme Kinetics. DHA reductase activity was assayed as described previously (*30*). Assays performed to determine the kinetic constants were carried out in 200 mM sodium phosphate and 1 mM EDTA (pH 6.9) in a final volume of 500 μL at 30 °C and recorded for 3 min at 265.5 nm. A blank without enzyme was run simultaneously with each assay, and the difference gave the activity in nanomoles per minute. Kinetic constants for DHA were determined by varying the DHA concentration and holding the GSH concentration at 6.0 mM, and kinetic constants for GSH were determined by varying the GSH concentration and holding the DHA concentration at 4.5 mM. The following enzyme amounts were used in their respective kinetic studies: 0.72 μg of RPLTT and 0.44 μg of C25S. K_m(app) and V_{max}(app) values were calculated with a nonlinear least-squares fit to the velocity versus substrate concentration data using the PSI-Plot 3.5 software. The values for k_{cat} were calculated by dividing V_{max}(app) by the molar concentration of the enzymes.

Preparation of Reduced Thioltransferases. RPLTT and C25S samples, purified to homogeneity, were separately adjusted to 20 mM DTT and reduced for 30 min at room temperature, subjected to Sephadex G25 (2.5 cm × 78 cm) filtration, and eluted with triply distilled H_2O to remove the excess DTT. Fractions with thioltransferase activity were pooled and lyophilized. The lyophilized enzyme was resuspended in triply distilled H_2O, and protein concentrations were determined.

Iodoacetamide Inactivation of Thioltransferases' DHA Reductase Activity. Reduced RPLTT (7 μM) and C25S (7 μM) were incubated with 0.1 mM IAM, separately, in 100 mM sodium phosphate (pH 7.5). Aliquots were removed at various times up to 45 min and assayed for DHA reductase activity in 200 mM sodium phosphate (pH 6.9) containing 1 mM EDTA and 3.0 mM GSH. Upon the addition of 1.5 mM DHA, reductase activity was recorded at 265.5 nm for 2 min at 30 °C. The percent activity was determined for each enzyme by running a concurrent control with IAM absent. The IAM inactivation experiments were also performed after separate preincubation of each enzyme with 0.05 mM DHA and 0.1 mM GSH, 0.05 mM AA, 0.05 mM GSSG, 0.5 mM HED, 0.05 mM GSH, or 0.5 mM DHA in 100 mM sodium phosphate (pH 7.5) for 10 min at room temperature. IAM was then added to a concentration of 0.1 mM, and the experiments were conducted as described above.

Reduction of DHA to AA by Thioltransferases in the Absence of GSH. Equal amounts (6 μM) of reduced RPLTT and C25S were separately incubated in a 100 μL reaction volume with 600 μM DHA for 0.5, 1, 2, 5, and 10 min in 200 mM sodium phosphate and 1 mM EDTA (pH 6.9). In addition, control reactions containing DHA and no enzyme were run. At each time point, 100 μL of 10% metaphosphoric acid, 1 mM thiourea, and 1 mM EDTA was added to the reaction tube. The samples were then centrifuged at 14 000 rpm for 2 min in an Eppendorf microcentrifuge. AA concentrations were determined with the supernatant as described previously (*33*). Separation and quantitative analysis were accomplished using a 3.9 mm × 150 mm Waters

Table 1: Wild Type and Mutant Pig Liver Thioltransferase Kinetic Parameters

parameter	RPLTT[a]	C25S[a]
k_{cat} (min^{-1})[b]	454 ± 17	686 ± 24[d]
K_m(app) (mM)[c]		
DHA	0.32 ± 0.02	0.51 ± 0.02[d]
GSH	3.7 ± 0.3	5.2 ± 0.3[d]
k_{cat}/K_m (M^{-1} s^{-1})		
DHA	(2.34 ± 0.15) × 10^4	(2.24 ± 0.13) × 10^4
GSH	(2.04 ± 0.18) × 10^3	(2.19 ± 0.17) × 10^3

[a] Kinetic constants were the mean ± standard deviation of three or more separate experiments as described in Materials and Methods. [b] k_{cat} values were calculated by dividing V_{max}(app) by the molar concentration of the enzymes. [c] K_m(app) and V_{max}(app) values were calculated by nonlinear least-squares fit to the velocity vs substrate concentration data using the PSI-Plot 3.5 software. [d] Significantly different from corresponding RPLTT value with a p of <0.01 (GraphPAD InSTAT statistical program).

Delta Pak-5μ C18 reversed phase column with a mobile phase of 50 mM sodium phosphate (pH 3.0) and a flow rate of 0.7 mL/min. AA was detected using an ESA model 5200A Coulochem II electrochemical detector 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 AA were compared with those from standard solutions of 50 nM AA to 3 μM AA dissolved in 5% metaphosphoric acid, 0.5 mM thiourea, 1.0 mM EDTA, and 100 mM sodium phosphate (pH 2).

Isoelectric Focusing. Reduced RPLTT and C25S, 12 μg each, were incubated alone and with 1 mM DTT, 1 mM HED, 1 mM DHA, 1 mM GSH, 0.5 mM DHA and 1 mM GSH, 0.5 mM GSSG, or 0.5 mM AA separately in 40 mM sodium phosphate (pH 7.5) for 30 min in a 30 °C water bath. IAA was added to a final concentration of 2 mM, and the samples were incubated for an additional 30 min at 30 °C. Controls included enzyme alone, enzyme and 1 mM DTT, enzyme and 1 mM HED, or enzyme and 1 mM DHA. Each of the incubation mixtures (in a total volume of 10 μL) was then analyzed directly on a Servalyt Precotes isoelectric focusing gel at pH 3−10 (150 μm thick, 125 mm × 125 mm) following the manufacturer's instructions for 2666 volt hours on an LKB 2217 Ultraphor Electrofocusing Unit with a Lauda K-2/R cooling system. After focusing, the gel was fixed with a solution of 30% ethanol and 10% glacial acetic acid, stained with a solution of 0.001% Coomassie Brilliant Blue R-250, 10% glacial acetic acid, and 50% ethanol, and washed with the fixing solution.

RESULTS AND DISCUSSION

Kinetic Characterization of Wild Type and Mutant RPLTT Dehydroascorbic Acid Reductase Activity. Previously, Yang and Wells (*25*) compared the relative DHA reductase activities of an equal amount of C25S to that of RPLTT. With RPLTT activity defined as 100%, C25S had a relative activity of 194%, and the amino acid Cys-25 was not an essential amino acid for either the DHA reductase or thiol:disulfide oxidoreductase activities of thioltransferase (*25*). In this study, the kinetic parameters for C25S were determined and compared to those of RPLTT (Table 1). The kinetic plots for each enzyme were best fit by the Michaelis−Menten equation (data not shown, EZ-Fit Kinetic Program; *34*). C25S had a 50% higher maximum rate at saturating substrate levels, the k_{cat}, than RPLTT (686 ± 24 vs 454 ± 17 min^{-1}, respectively). However, RPLTT and C25S had equivalent specificity constants for both DHA [(2.34 ± 0.15) × 10^4 M^{-1} s^{-1} for RPLTT vs (2.24 ± 0.13) × 10^4 M^{-1} s^{-1} for C25S] and GSH [(2.04 ± 0.18) × 10^3 M^{-1} s^{-1} for RPLTT vs (2.19 ± 0.17) × 10^3 M^{-1} s^{-1} for C25S]. Essentially, the mutation of Cys-25 to serine had a minor kinetic effect on the ability of the enzyme to function as a GSH-dependent DHA reductase.

Proposed Mechanism of RPLTT's DHA Reductase Activity. On the basis of our knowledge of RPLTT's DHA reductase activity (*25*) and on the reported mechanism of RPLTT's thiol−disulfide exchange activity (*26*, *27*), a mechanism for RPLTT's GSH-dependent DHA reductase activity is proposed (Scheme 1). Reaction 1 is the formation of a thiohemiketal intermediate between RPLTT and DHA. With the wild type enzyme, the reaction can then proceed in two possible ways: (i) in reaction 2, GSH displaces AA from RPLTT and a mixed disulfide between RPLTT and GSH is formed; and (ii) in reaction 3, Cys-25 attacks the thiohemiketal intermediate at Cys-22 and displaces AA, forming an intramolecular disulfide bond. Reaction 3 is followed by reaction 4 during which GSH attacks the intramolecular disulfide bond to form a mixed disulfide between RPLTT and GSH at Cys-22. In reaction 5, a second molecule of GSH attacks the mixed disulfide, ES-SG, forming GSSG and the original reduced form of RPLTT. The alteration in the C25S mutant protein is indicated by **(OH)**. Since C25S cannot proceed via reactions 3 and 4, only reactions 1, 2, and 5 are feasible. In the thiol−disulfide exchange mechanism, a disulfide compound like HED or GSSG, RSSR, would replace DHA, and RSH would be released upon formation of a mixed disulfide between Cys-22 and RSSR, i.e., RPLTT-S-SR (*35*). This species could have either Cys-25 attacking Cys-22 and forming an intramolecular disulfide bond as in reaction 3 or GSH attacking Cys-22 and displacing RSH as in reaction 2 (Scheme 1). Initial reaction of RPLTT and RSSG would release RSH and form RPLTT-S-SG. This species would then undergo reaction 5 to restore reduced enzyme. In vivo, thioltransferase would most likely reduce protein glutathionated substrates, RSSG, to RSH and GSH (*36*, *37*).

Iodoacetamide Inactivation of the Dehydroascorbic Acid Reductase Activity of Wild Type and Mutant RPLTT. To investigate substrate binding to RPLTT, IAM inactivation studies of the DHA reductase activity of reduced RPLTT and C25S were carried out. Only Cys-22 of pig liver thioltransferase reacted with iodo[1-^{14}C]acetic acid at pH 6.5 (*29*), and treatment of reduced RPLTT and C25S inactivated each enzyme's thiol:disulfide oxidoreductase activity (*25*). IAM inactivated the DHA reductase activity of reduced RPLTT (Figure 1A) and C25S (Figure 1B). For each enzyme, the inactivation profile best fit the exponential decay function $y = y_0 + A_1e^{-tk}$, where A_1 is the maximal amount of inhibition, with a k (the rate constant of inactivation) of 0.16 ± 0.02 min^{-1} and a y_0 (the remaining activity at infinite time) of 15 ± 2% for RPLTT, versus a k of 0.2 ± 0.02 min^{-1} and a y_0 of 8 ± 2% for C25S. IAM inactivation of the DHA reductase activity of reduced RPLTT and C25S confirmed the role of cysteine as the key catalytic residue in the mechanism (*25*). Preincubation of reduced RPLTT (Figure

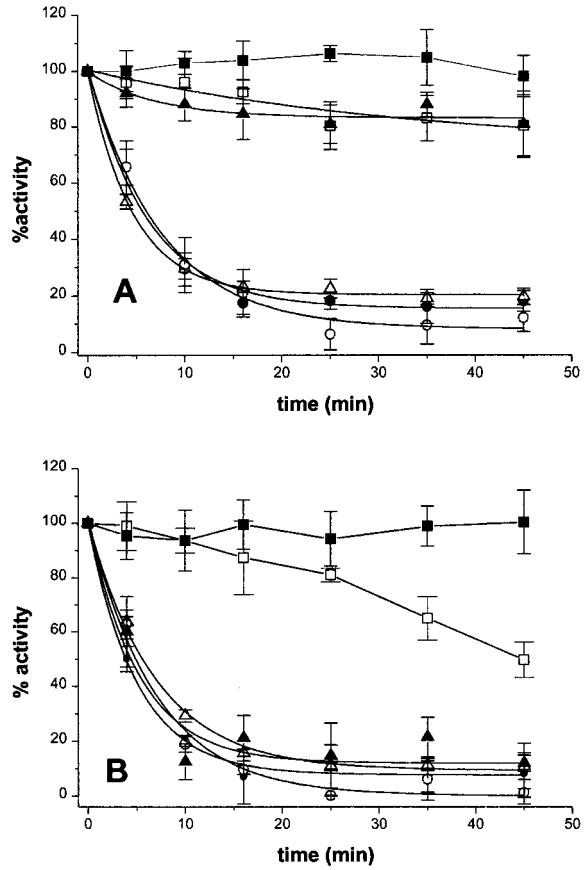


FIGURE 1: IAM inactivation of reduced RPLTT and C25S with and without preincubation with substrates. Equal amounts (7 μM) of reduced RPLTT (A) and C25S (B) were separately incubated with 0.1 mM IAM (●) in 100 mM sodium phosphate (pH 7.5) or preincubated with 0.05 mM GSH (○), 0.05 mM GSSG (■), 0.05 mM DHA and 0.1 mM GSH (□), 0.5 mM DHA (▲), or 0.05 mM AA (△) for 10 min in buffer prior to IAM addition. Aliquots were removed at the time points shown and assayed for DHA reductase activity as described in Materials and Methods. Each data point is the average of three separate experiments. Error bars represent one standard deviation of the data and are not seen if the values are smaller than the size of the symbols.

1A) and C25S (Figure 1B) for 10 min with 0.05 mM GSH had little effect on the IAM inactivation of either RPLTT or C25S. The inactivation profiles best fit the exponential decay function with a k of 0.13 ± 0.02 min^{-1} and a y_0 of 8 ± 1% for RPLTT and a k of 0.15 ± 0.01 min^{-1} and no residual activity for C25S.

Preincubation of reduced RPLTT (Figure 1A) and C25S (Figure 1B) for 10 min with 0.05 mM DHA and 0.1 mM GSH significantly protected the enzyme's DHA reductase activity from IAM inactivation. RPLTT and C25S retained 80 ± 11 and 50 ± 7% of their respective DHA reductase activities after 45 min. When each enzyme was separately preincubated with the reaction products, 0.05 mM AA (Figure 1A,B) or 0.05 mM GSSG (Figure 1A,B), only preincubation with GSSG protected the enzyme's DHA reductase activity from IAM inactivation. When each enzyme was preincubated with AA, the inactivation profiles best fit the exponential decay equation with a k of 0.20 ± 0.01 min^{-1} and a y_0 of 20 ± 1% for RPLTT and a k of 0.15 ± 0.01 min^{-1} and a y_0 of 9 ± 2% for C25S. Preincubation of RPLTT and C25S with another disulfide reagent, 0.5 mM HED, completely protected each enzyme's DHA reductase activity from IAM inactivation (data not shown). Reduced RPLTT

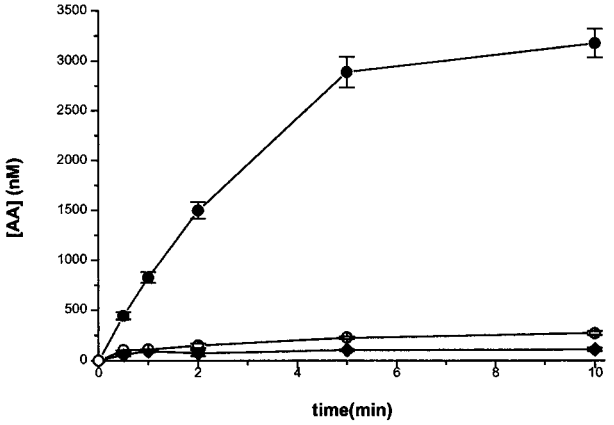


FIGURE 2: HPLC analysis of AA production by DHA incubation with reduced RPLTT and C25S. Equal amounts (6 μM) of reduced RPLTT (●) and C25S (○) were separately incubated in a 100 μL reaction volume with DHA (600 μM) for various lengths of time in 200 mM sodium phosphate and 1 mM EDTA (pH 6.85). In addition, DHA alone control reactions were run (◆). At the time points shown, 100 μL of 10% metaphosphoric acid, 1 mM thiourea, and 1 mM EDTA was added to the reaction tube, and AA concentrations were determined by an HPLC procedure as described in Materials and Methods. Each data point is the average of at least three measurements of one to three separate experiments. Error bars represent one standard deviation of the data and are not seen if the values are smaller than the size of the symbols.

and C25S were protected from IAM inactivation by forming a mixed disulfide bond with GSSG or HED, to generate ES-SG or ES-S-$(CH_2)_2$-OH, respectively. When the thiol:disulfide oxidoreductase activity of thioltransferase was studied, RPLTT was protected from IAM inactivation by preincubation with the substrate *S*-sulfocysteine or HED (*35*). In addition, RPLTT and C25S were shown to be oxidized or form enzyme−substrate intermediates with the disulfide species cystine or HED (*35*). Because Cys-25 is present, RPLTT could also be protected from IAM inactivation by forming an intramolecular disulfide bond between Cys-22 and Cys-25, i.e., oxidized enzyme, a form of RPLTT shown to exist by X-ray crystallography (*38*).

In contrast to reduced RPLTT (Figure 1A), the DHA reductase activity of C25S (Figure 1B) was not protected from IAM inactivation by a 10 min preincubation with 0.5 mM DHA. The inactivation profile of C25S best fit the exponential decay equation with a k of 0.20 ± 0.04 min^{-1} and a y_0 of 12 ± 1%. The fact that C25S was not protected suggested that the postulated thiohemiketal intermediate (reaction 1, Scheme 1) was not stable enough as an enzyme−substrate intermediate to prevent IAM inactivation. If this intermediate existed, it probably dissociated into the reduced enzyme and DHA, and IAM then reacted irreversibly with the reduced enzyme (reaction 6, Scheme 1). Because the postulated thiohemiketal intermediate did not protect C25S, the protection of RPLTT from IAM inactivation by DHA suggested that DHA and reduced RPLTT may form AA and oxidized enzyme.

Ability of Reduced Wild Type and Mutant RPLTTs To Chemically Reduce Dehydroascorbic Acid to Ascorbic Acid. Because RPLTT contains Cys-25, we hypothesized that reduced RPLTT could chemically reduce DHA to AA in the absence of GSH, forming oxidized enzyme (reactions 1 and 3, Scheme 1). Both reduced RPLTT and C25S could chemically reduce DHA to AA in the absence of GSH (Figure 2). DHA alone was also spontaneously reduced to

AA and L-erythroascorbic acid as seen by Jung and Wells (*39*) with an initial rate of 122 ± 34 nmol of AA/min, reaching a maximum AA concentration of 109 ± 17 nM after 10 min. C25S had a slightly higher initial rate of 201 ± 4 nmol of AA/min and reached a maximum AA concentration of 277 ± 18 nM after 10 min, double that of the control reaction. RPLTT was a more robust DHA reductant with an initial rate of 883 ± 76 nmol of AA/min, 4.4-fold greater than that of C25S, and a maximum AA concentration of 3.18 ± 0.14 μM after 10 min, 11-fold greater than that of C25S.

The results (Figure 2) indicated that reduced RPLTT can undergo reactions 1 and 3 (Scheme 1) where 1 mol of reduced enzyme reduces 1 mol of DHA to 1 mol of AA and 1 mol of oxidized enzyme. While C25S cannot undergo reaction 3 because it lacks Cys-25 to form an intramolecular disulfide bond, it still reduced DHA to AA at a rate faster than that of DHA alone, but at a rate much slower than that of RPLTT, suggesting a weak ability of C25S to form an intermolecular disulfide bond upon the reduction of DHA.

Isoelectric Focusing Analysis of RPLTT and C25S. The goal of the isoelectric focusing (IEF) studies was to determine the state of the enzyme that afforded protection from iodoacetamide. In the IEF studies, we used iodoacetic acid (IAA) to modify the enzymes instead of IAM. IAM would form carbamidomethylated enzyme which would be indistinguishable from oxidized enzyme in IEF analysis because the net result would be the loss of the negative charge on Cys-22. For example, Yang et al. (*35*) determined the p*I* values of oxidized RPLTT and RPLTT treated with IAM to be identical (pI = 8.0). IAA and IAM behaved identically with respect to their inactivation of the DHA reductase activities of RPLTT and C25S (data not shown). Using DTT to determine the p*I* of the reduced state of each enzyme and HED to determine the oxidized state of each enzyme gave results similar to those of Yang and Wells (*25*). Reduced RPLTT and C25S both yielded a p*I* of 7.0 (data not shown). Oxidized RPLTT yielded a p*I* of 8.0 (data not shown). Oxidized RPLTT yielded a more basic p*I* because of the formation of an intramolecular disulfide bond between Cys-22 and Cys-25. HED-treated C25S focused to p*I* values of primarily 7.0 and 8.0 which correspond to reduced enzyme and enzyme with a mixed disulfide with HED, respectively (data not shown).

IAA treatment of each enzyme allowed us to trap the reduced form of the enzyme because the process of isoelectric focusing was itself slightly oxidizing. Untreated RPLTT (Figure 3A, lane 1) focused to a p*I* corresponding to both reduced and oxidized species, whereas C25S, lacking the ability to form an intramolecular disulfide bond, focused only to a reduced p*I* (Figure 3B, lane 1). When treated with IAA after a 30 min preincubation in buffer, RPLTT focused to bands of 5.9 and 4.9 (Figure 3A, lane 2) and C25S focused to a p*I* of 6.0 (Figure 3B, lane 2). The addition of IAA to the reduced enzyme resulted in species which are unique from the reduced or oxidized form of the enzyme and trapped the reduced form of the enzyme, preventing oxidation of enzyme during IEF itself.

Preincubation with HED, DHA, DHA and GSH, or GSSG protected against the IAM inactivation of the DHA reductase activity of RPLTT, whereas preincubation with GSH or AA did not. The predominant form of RPLTT (Figure 3A) when preincubated with HED (data not shown), DHA and GSH (lane 6), or GSSG (lane 7) was oxidized enzyme (pI = 8.0), protecting against the formation of IAA-modified species. The primary form of RPLTT, when preincubated with DHA prior to IAA treatment (lane 4), was at pI = 7.3, possibly indicating the trapping of an enzyme−substrate intermediate. These results confirm that the formation of oxidized RPLTT results in the protection of the enzyme from IAA modification and IAM inactivation. In agreement with the IAM inactivation studies, preincubation of RPLTT (Figure 3A) with GSH (lane 5) or AA (lane 8) prior to IAA treatment did not protect against IAA modification and resulted in the appearance of reduced enzyme (pI = 7.0) and IAA-modified enzyme (pI = 4.9 and 5.9).

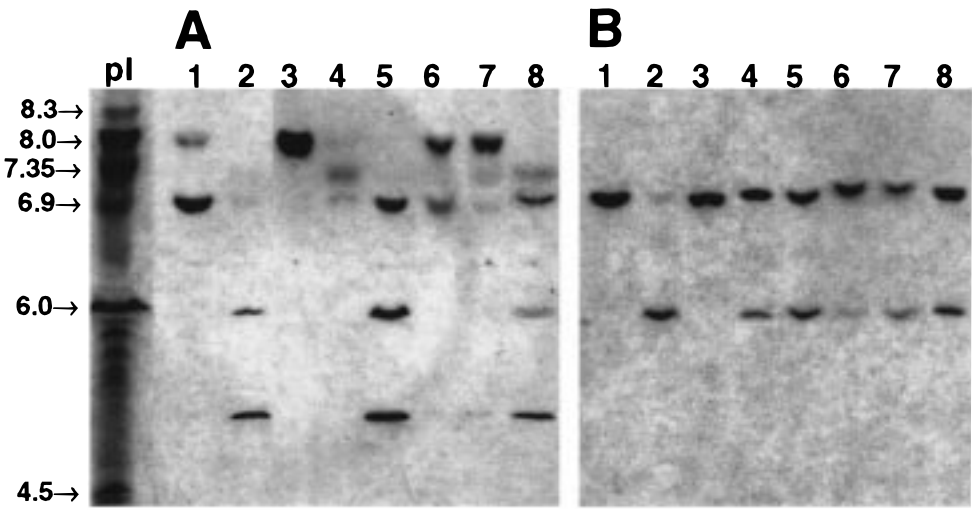


FIGURE 3: Isoelectric focusing analysis of incubation of reduced RPLTT and C25S with various substrates. Equal amounts (12 μg) of reduced RPLTT (A) and C25S (B) were separately incubated alone (lane 2) or with 1 mM DHA (lane 4), 1 mM GSH (lane 5), 0.5 mM DHA and 1 mM GSH (lane 6), 0.5 mM GSSG (lane 7), or 0.5 mM AA (lane 8) in 40 mM sodium phosphate (pH 7.5) for 30 min in a 30 °C water bath. IAA was then added to a final concentration of 2 mM, and the samples were incubated for an additional 30 min at 30 °C. No IAA controls included untreated enzyme (lane 1) or enzyme treated with 1 mM DHA (lane 3). Isoelectric point standards are 5 μL of Serva p*I* test mix. The p*I* value for each of the reaction mixtures was measured on a Servalyt Precote pH 3−10 isoelectric focusing gel according to the manufacturer's instructions. The gels shown are typical of two or more analyses.

The DHA reductase activity of C25S was protected from IAM inactivation by HED, DHA and GSH, or GSSG but not by GSH, DHA, or AA. In agreement with these results, when C25S (Figure 3B) was preincubated with GSH (lane 5), AA (lane 8), and DHA (lane 4), both the reduced enzyme (pI = 7.0) and the band indicative of carboxymethylation (pI = 6.0) appeared in significant quantities. When C25S was preincubated with HED (data not shown) or DHA and GSH (lane 6) prior to IAA treatment, no species with a pI of 6.0 was seen. A slight amount of a species with a pI of 6.0 was seen when C25S was preincubated with GSSG (lane 7) even though GSSG afforded complete protection against IAM inactivation of the DHA reductase activity of C25S (Figure 1B).

As shown in Figure 2, reduced RPLTT was capable of chemically reducing DHA to AA in the absence of GSH. When reduced RPLTT (Figure 3A, lane 3) was incubated with DHA alone, oxidized enzyme (pI = 8.0) was formed. This is consistent with the ability of reduced RPLTT to chemically reduce DHA, forming AA and oxidized enzyme (reactions 1 and 3, Scheme 1). In support of this, DHA has previously been shown to be a thiol oxidant of reduced bovine pancreatic ribonuclease (*40*−*43*).

CONCLUSIONS

The work described in this paper supports the catalytic mechanism proposed in Scheme 1. When the reduced form of the enzyme was the starting point, IAM inactivation studies demonstrated the ability of DHA to bind to enzyme (reaction 1, Scheme 1), prior to GSH binding (reaction 3 or 4, Scheme 1) because DHA protected against IAM inactivation whereas GSH did not. The ability of RPLTT to chemically reduce DHA to AA and form oxidized RPLTT in the absence of GSH (reactions 1 and 3, Scheme 1) was demonstrated by electrochemical analysis and IEF. In addition, IEF analysis demonstrated the ability of reduced RPLTT to react with GSSG and form oxidized enzyme (reverse of reactions 5 and 4 in Scheme 1). The relative inability of C25S to chemically reduce DHA to AA and its robust DHA reductase activity strongly suggested that reaction 2 (Scheme 1) is a part of this mechanism. Further studies in progress involve the detection of the thiohemiketal intermediate and the study of its catalytic capacity. The results of this paper suggested that RPLTT and C25S may have different catalytic mechanisms. However, on the basis of the kinetic constants k_{cat} and k_{cat}/K_m, RPLTT and C25S were equally competent as GSH-dependent DHA reductases. In the presence of GSH, whether reaction 2 or 3 predominates in the mechanism of RPLTT has yet to be determined. Previously proposed models of the DHA reductase activity of thioltransferase (*22*, *23*, *26*, *35*) and for the DHA reductase activity of *Trypanosoma cruzi* p52 (*44*) have not included reactions 1 and 3 in Scheme 1, but the work described in this paper clearly demonstrates the potential of thioltransferase to undergo these reactions.

ACKNOWLEDGMENT

We thank Drs. Tom Deits, Che-Hun Jung, Laurence Florens, Claire Vielle, and Honggao Yan for helpful discussions about the manuscript.

REFERENCES

1. Frei, B., England, L., and Ames, B. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6377−6381.
2. Halliwell, B., and Gutteridge, J. M. C. (1990) *Arch. Biochem. Biophys. 280*, 1−8.
3. Buettner, G. R. (1993) *Arch. Biochem. Biophys. 300*, 535−543.
4. Sciamanna, M. A., and Lee, C. P. (1993) *Arch. Biochem. Biophys. 305*, 215−224.
5. May, J. M., Qu, Z., and Whitesell, R. R. (1995) *Biochemistry 34*, 12721−12728.
6. Ozaki, M., Fuchinoue, S., Teraoka, S., and Ota, K. (1995) *Arch. Biochem. Biophys. 318*, 439−445.
7. Englard, S., and Seifter, S. (1986) *Annu. Rev. Nutr. 6*, 365−406.
8. Lyons, B. L., and Schwarz, R. I. (1984) *Nucleic Acids Res. 12*, 2569−2579.
9. Levine, M., Morita, K., and Pollard, H. (1985) *J. Biol. Chem. 260*, 12942−12947.
10. Wells, W. W., Dou, C. Z., Dybas, L. N., Jung, C. H., Kalbach, H. L., and Xu, D. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11869−11873.
11. Jung, C. H., and Wells, W. W. (1997) *Biochem. Biophys. Res. Commun. 239*, 457−462.
12. Bielski, B. H. J. (1982) in *Ascorbic Acid: Chemistry, Metabolism, and Uses* (Seib, P. A., and Tolbert, B. M., Eds.) Advances in Chemistry Series 200 (Comstock, M. J., Ed.) pp 81−100, American Chemical Society, Washington, DC.
13. Coassin, M., Tomasi, A., Vannini, V., and Ursini, F. (1991) *Arch. Biochem. Biophys. 290*, 458−462.
14. Villalba, J. M., Canalejo, A., Burón, M. I., Córdoba, F., and Navas, P. (1993) *Biochem. Biophys. Res. Commun. 192*, 707−713.
15. Bando, M., and Obazawa, H. (1994) *Jpn. J. Ophthalmol. 38*, 1−9.
16. Del Bello, B., Maellaro, E., Sugherini, L., Santucci, A., Comporti, M., and Casini, A. F. (1994) *Biochem. J. 304*, 385−390.
17. May, J. M., Mendiratta, S., Hill, K. E., and Burk, R. F. (1997) *J. Biol. Chem. 272*, 22607−22610.
18. Wells, W. W., Xu, D. P., Yang, Y., and Rocque, P. A. (1990) *J. Biol. Chem. 265*, 15361−15364.
19. Maellaro, E., Del Bello, B., Sugherini, L., Santucci, A., Comporti, M., and Casini, A. F. (1994) *Biochem. J. 301*, 471−476.
20. Xu, D. P., Washburn, M. P., Sun, G. P., and Wells, W. W. (1996) *Biochem. Biophys. Res. Commun. 221*, 117−121.
21. Meister, A. (1994) *J. Biol. Chem. 269*, 9397−9400.
22. Wells, W. W., and Xu, D. P. (1994) *J. Bioenerg. Biomembr. 26*, 369−377.
23. Wells, W. W., and Jung, C. H. (1997) in *Vitamin C in Health and Disease* (Packer, L., and Fuchs, J., Eds.) pp 109−121, Marcel Dekker, Inc., New York.
24. Park, J. B., and Levine, M. (1996) *Biochem. J. 315*, 931−938.
25. Yang, Y., and Wells, W. W. (1991) *J. Biol. Chem. 266*, 12759−12765.
26. Wells, W. W., Yang, Y., Deits, T. L., and Gan, Z. (1993) *Adv. Enzymol. Relat. Areas Mol. Biol. 66*, 149−201.
27. Mieyal, J. J., Gravina, S. A., Mieyal, P. A., Srinivasan, U., and Starke, D. W. (1995) in *Biothiols in Health and Disease* (Packer, L., and Cadenas, E., Eds.) pp 305−372, Marcel Decker, Inc., New York.
28. Washburn, M. P., and Wells, W. W. (1997) *Free Radical Biol. Med. 23*, 563−570.
29. Gan, Z.-R., and Wells, W. W. (1987) *J. Biol. Chem. 262*, 6704−6707.
30. Wells, W. W., Xu, D. P., and Washburn, M. P. (1995) *Methods Enzymol. 252*, 30−38.
31. Segel, I. H., and Johnson, M. J. (1963) *Anal. Biochem. 5*, 330−337.
32. Yang, Y., and Wells, W. W. (1990) *J. Biol. Chem. 265*, 589−593.

33. Xu, D. P., and Wells, W. W. (1996) *J. Bioenerg. Biomembr. 28*, 77−85.
34. Perrella, F. W. (1988) *Anal. Biochem. 174*, 437−447.
35. Yang, Y., and Wells, W. W. (1991) *J. Biol. Chem. 266*, 12766−12771.
36. Gravina, S. A., and Mieyal, J. J. (1993) *Biochemistry 32*, 3368−3376.
37. Jung, C. H., and Thomas, J. A. (1996) *Arch. Biochem. Biophys. 335*, 61−72.
38. Katti, S. K., Robbins, A. H., Yang, Y., and Wells, W. W. (1995) *Protein Sci. 4*, 1998−2005.
39. Jung, C. H., and Wells, W. W. (1998) *Arch. Biochem. Biophys. 355*, 9−14.
40. Venetianer, P., and Straub, F. B. (1963) *Acta Physiol. Acad. Sci. Hung. 24*, 41−53.
41. Givol, D., Goldberger, R. F., and Anfinsen, C. B. (1964) *J. Biol. Chem. 239*, PC3114−PC3116.
42. Krause, E. G., Venetianer, P., and Straub, F. B. (1965) *Acta Physiol. 27*, 295−302.
43. Venetianer, P., and Straub, F. B. (1965) *Acta Physiol. 27*, 303−315.
44. Moutiez, M., Quéméneur, E., Sergheraert, C., Valérie, L., Tartar, A., and Davioud-Charvet, E. (1997) *Biochem. J. 322*, 34−48.

BI980480V