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CHARACTERIZATION OF THE CATALYTIC MECHANISM OF THIOLTRANSFERASE TOWARD THE NON-DISULFIDE SUBSTRATES OF DEHYDROASCORBIC ACID AND ALLOXAN: THE POTENTIAL TO PROTECT AND THE POTENTIAL TO HARM

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# CHARACTERIZATION OF THE CATALYTIC MECHANISM OF THIOLTRANSFERASE TOWARD THE NON-DISULFIDE SUBSTRATES OF DEHYDROASCORBIC ACID AND ALLOXAN: THE POTENTIAL TO PROTECT AND THE POTENTIAL TO HARM

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## ABSTRACT

CHARACTERIZATION OF THE CATALYTIC MECHANISM OF THIOLTRANSFERASE TOWARD THE NON-DISULFIDE SUBSTRATES OF DEHYDROASCORBIC ACID AND ALLOXAN: THE POTENTIAL TO PROTECT AND THE POTENTIAL TO HARM

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Michael P. Washburn

Unable to synthesize the antioxidant ascorbic acid (AA), humans, other primates, and guinea pigs rely on dietary intake and AA regeneration from either its one or two electron oxidation products semidehydroascorbate and dehydroascorbate (DHA) to maintain cellular levels of this essential vitamin. A rapidly growing class of enzymes catalyze NADPH-dependent or glutathione (GSH)-dependent DHA reduction. Based on the comparison of their catalytic efficiencies, the GSH-dependent DHA reductases, recombinant pig liver thioltransferase (RPLTT) and a 32kDa enzyme isolated from human erythrocytes, are the most robust DHA reductases. Several DHA reductases including protein disulfide isomerase, the thioredoxin/thioredoxin reductase system, and thioltransferase belong to a class of enzymes known as thiol:disulfide oxidoreductases, which contain -CXXC- at the active site. The thiol:disulfide exchange activities of these enzymes are well understood, but little is known about their DHA reductase activities.

The catalytic mechanism of the GSH-dependent DHA reductase activity by thioltransferase was characterized. Kinetic comparisons demonstrated RPLTT and the C25S mutant protein to have equivalent catalytic efficiencies, in excess of those of the R26V and K27Q variants with DHA as a substrate. Iodoacetamide (IAM) inactivation studies confirmed the role of cysteine in the reaction mechanism and demonstrated the ability of

DHA to bind to reduced enzyme and protect RPLTT and the R26V and K27Q variants but not the C25S enzyme from IAM inactivation. As demonstrated by isoelectric focusing (IEF) and electrochemical analysis, reduced RPLTT and the R26V and K27Q variants but not the C25S enzyme could reduce DHA to AA in the absence of GSH and form oxidized enzyme. The inability of the C25S enzyme to chemically reduce DHA to AA in the absence of GSH suggested the possibility of an alternate route of thioltransferase catalysis where a postulated thiohemiketal intermediate between DHA and the enzyme would be cleaved by GSH, releasing AA and forming and enzyme/GSH mixed disulfide.

The diabetogenic agent alloxan has a similar structure to DHA, and RPLTT was shown to be a GSH-dependent alloxan reductase. Based on catalytic efficiencies, thioltransferase was a more robust alloxan reductase than DHA reductase. Essential to alloxan's toxicity, after the reduction of alloxan to dialuric acid, dialuric acid oxidizes back to alloxan generating reactive oxygen species. RPLTT catalysis of the formation of dialuric acid resulted in increases in oxygen consumption and production of superoxide and hydrogen peroxide in the alloxan/GSH system, suggesting a role for thioltransferase in alloxan's toxicity. Like DHA, reduced RPLTT and the K27Q variant but not the C25S enzyme could chemically reduce alloxan forming oxidized enzyme, suggesting similar catalytic mechanisms for the alloxan and DHA reductase activities of RPLTT. Interestingly, IEF of C25S incubated with alloxan resulted in reduced enzyme and a more acidic species which may be the formation of a thiohemiketal intermediate between alloxan and C25S.

***To the most important people of them all: my family***

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## LIST OF ABBREVIATIONS

$A_1$ - amplitude
Abs-absorbance
Alx- alloxan
$AlxH_2$-dialuric acid
•AlxH-alloxan radical
AA - ascorbic acid
BSA-bovine serum albumin
BSO - buthionine sulfoximine
C25S - C25S mutant of RPLTT
DHA - dehydroascorbic acid or dehydroascorbate
DTT- dithiothreitol
$E^{o\prime}$ - standard reduction potential
Grx - glutaredoxin
GSH - glutathione
3-Glu-Cys-Gly - glutathione
GSSG - glutathione disulfide
HED - hydroxyethyldisulfide
$H_2O_2$-hydrogen peroxide
HOCl - hypochlorous acid
IAA - iodoacetic acid
IAM - iodoacetamide
IEF - isoelectric focusing
IPTG - isopropylthiogalactoside
K27Q - K27Q mutant of RPLTT
PDI - protein disulfide isomerase
RPLTT - wild type recombinant pig liver thioltransferase
R26V- R26V mutant of RPLTT
semiDHA - semidehydroascorbic acid or semidehydroascorbate
•OH- hydroxyl radical
$O_2^{\bullet -}$-superoxide
PMA - phorbol myristate acetate
PMSF - phenylmethylsulfonyl flouride
RBC - red blood cell
SOD - superoxide dismutase
TOH- K-tocopherol, vitamin E
TO • - K-tocopheroxyl radical
TR - thioredoxin reductase
Vp-16-O • - etoposide
32kDHAR - a 32 kDa GSH-dependent DHA reductase

# INTRODUCTION

The nemesis of long distance sea explorations from the 1400s to the whaling industry of the 1800s, scurvy may have been first described in the Thebes Ebers papyrus of approximately 1500 B.C. (*1*). Scurvy is a condition initially marked by lethargy, malaise, and weakness, followed by skin lesions on the lower leg, easy bruisability, bleeding gums, and poor wound healing (*2*). The first scientific attempt to devise a cure for scurvy at sea was carried out by James Lind in 1746 on the H.M.S. Salisbury. Dr. Lind described the alleviation of the scorbutic condition by lemons and oranges on this particular voyage in " A Treatise of the Scurvy" published in 1753. In spite of Dr. Lind's discovery, scurvy continued to plague long distance sea exploration until the 1800s and was a cause of thousands of deaths during the American Revolutionary War and the American Civil War when approximately 3000 Union soldiers imprisoned in Andersonville, Georgia, died from scurvy. (*1*)

In 1895, Theobald Smith became the first person to induce scurvy in guinea pigs by feeding them a diet of oats and bran, and he prevented scurvy in guinea pigs by adding clover, grass, or cabbage to their diets (*1*). The development of the guinea pig as an animal model led to the discovery and synthesis of ascorbic acid (vitamin C, AA) by Charles Glen King and Albert Imre Szent-Györgyi in 1932 (*1*). Since the discovery of AA and the development of animal models to investigate AA deficiency, multiple roles of AA in biology have been characterized.

## I. Roles of Ascorbic Acid in Biology

*I. A. Role of Ascorbic Acid as a Ferric Iron Reductant.*

In the AA deficient state of scurvy, the medical problems of bleeding gums and poor wound healing are most likely the result of a defect in collagen biosynthesis (*3*). Prolyl-4-hydroxylase and lysyl hydroxylase are involved in the post-translational modification of collagen, respectively forming hydroxyproline and hydroxylysine (*3*). Hydroxyproline is essential for the stabilization of the collagen triple helix (*4*), while hydroxylysine is necessary for the formation of collagen intermolecular cross-links (*5*). Both enzymes are K-ketoglutarate dependent dioxygenases which undergo the following generalized reaction (*6*):

$$1) \quad O_2 + \text{K-ketoglutarate} + \text{specific substrate} \xrightarrow[\text{(Reductant)}]{(Fe^{2+})} \text{succinate} + CO_2 + \text{hydroxylated substrate}$$

Upon the oxidation of $Fe^{2+}$ to $Fe^{3+}$, the enzyme is no longer functional and ferric ion must be reduced to ferrous ion (*6*). AA is the best known iron reductant for both prolyl-4-hydroxylase and lysyl hydroxylase, and AA's importance *in vivo* in the maintenance of these enzyme activities is demonstrated by the condition scurvy (*3, 6*).

AA's role in collagen biosynthesis surpasses its function as a cofactor for prolyl-4-hydroxylase and lysyl hydroxylase. Incubation of AA with normal human skin fibroblasts stimulated collagen synthesis independent of hydroxylation (*7*), and AA incubation with PAT cells from chicken embryos resulted in a three fold rate of procollagen gene transcription (*8*). This evidence suggests that AA may act as a signal to induce collagen production.

AA also functions as a ferric ion electron donor for the enzymes prostaglandin synthases I and II (*9*), the K-ketoglutarate dependent dioxygenases 3-butyrobetaine

dehydrogenase and 6-N-trimethyl-1-lysine hydroxylase in the carnitine biosynthetic pathway (*6*), and the monooxygenases peptidylglycine K-amidating monooxygenase and dopamine 2-hydroxylase (*6*). Dopamine 2-hydroxylase catalyzes the formation of norepinephrine from dopamine (*10*):

2) Dopamine + $O_2$ + 2 AA ——➤ Norepinephrine + 2 Semidehydroascorbic acid

As an example of AA's importance *in vivo* in the maintenance of these enzyme's activities, norepinephrine biosynthesis is enhanced by incubating cultured bovine chromaffin cells with AA (*10*). Finally, AA stimulated the chlorinating activity of myeloperoxidase (*11*) by reducing the inactive form, compound II, of myeloperoxidase back to active form of the enzyme (*12*).

AA has been demonstrated to be essential for insulin release from the islets of scorbutic guinea pigs (*13*). Treatment of normal and scorbutic guinea pig islets with membrane depolarizing additions of KCl and the secretagogue D-glyceraldehyde demonstrated the site of AA action to fall between the triose-phosphate level of glycolysis and the increase in ATP levels leading to insulin release (*14*). AA may stimulate insulin release by functioning as a ferric ion reductant for mitochondrial glycerol-3-phosphate dehydrogenase. Addition of AA stimulated mitochondrial glycerol-3-phosphate dehydrogenase from scorbutic guinea pig brain, liver, and skeletal muscle several fold, an effect inhibited by iron chelators (*15*). Thus, the role AA plays in insulin release may also be mediated by AA's ability to reduced ferric ion to ferrous ion.

*I. B. Antioxidant Functions of Ascorbic Acid.*

Another major function of AA in addition to that of a ferric ion reductant, is as an antioxidant. An antioxidant can be defined as " any substance that, when compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate, " (*16*). Antioxidants provide an important defense against oxidative stress. Reactive oxygen species such as superoxide ($O_2^{\bullet -}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($^{\bullet}OH$) can irreversibly modify and damage proteins, lipids, and nucleic acids (reviewed in *17*). Free radical damage has been proposed to play a role in ageing, atherosclerosis, carcinogenesis, and diabetes. AA is considered a primary defense because it is able to directly interact with $O_2^{\bullet -}$, which is the first one-electron reduction product of oxygen (reviewed in *17*). Other primary antioxidant defense systems include the vitamins E and A, glutathione (GSH), uric acid, and antioxidant scavenging enzymes including catalase, glutathione peroxidase, and superoxide dismutase (reviewed in *17*).

AA readily oxidizes by consecutive one electron oxidations to semidehydroascorbate (semiDHA) and dehydroascorbate (DHA) as shown in Figure 1 (*18*). The reaction of AA to semiDHA has an $E^{o\prime}$ of -282 mV at pH 7.0 and semiDHA to DHA an $E^{o\prime}$ of 174 mV at pH 7.0 (*18*). AA is an excellent small molecule chain-breaking antioxidant because semiDHA is neither a strongly oxidizing nor a strongly reducing radical, semiDHA reacts poorly with superoxide, and only relatively small amounts of ascorbic acid need to be present to serve as an effective antioxidant (*18, 19*). In solution, AA will primarily be in the form of the ascorbate monoanion with a $pK_1 = 4.1$ (*19*). The ascorbate monoanion, (AA-) scavenged $^{\bullet}OH$ with a rate constant ($k$) = $1.28 \times 10^{10}$ $M^{-1}$ $s^{-1}$ at pH 7.0, the quinone radical hydroxyacetophenone- $O^{\bullet}$ with a $k = 1.7 \times 10^9$ $M^{-1}$ $s^{-1}$ at pH 9.5, and the phenoxy radical of the chemotherapeutic agent etoposide (Vp-16-$O^{\bullet}$) with a $k = 3.5 \times 10^7$ $M^{-1}$ $s^{-1}$ at pH 8.5 (*19*).

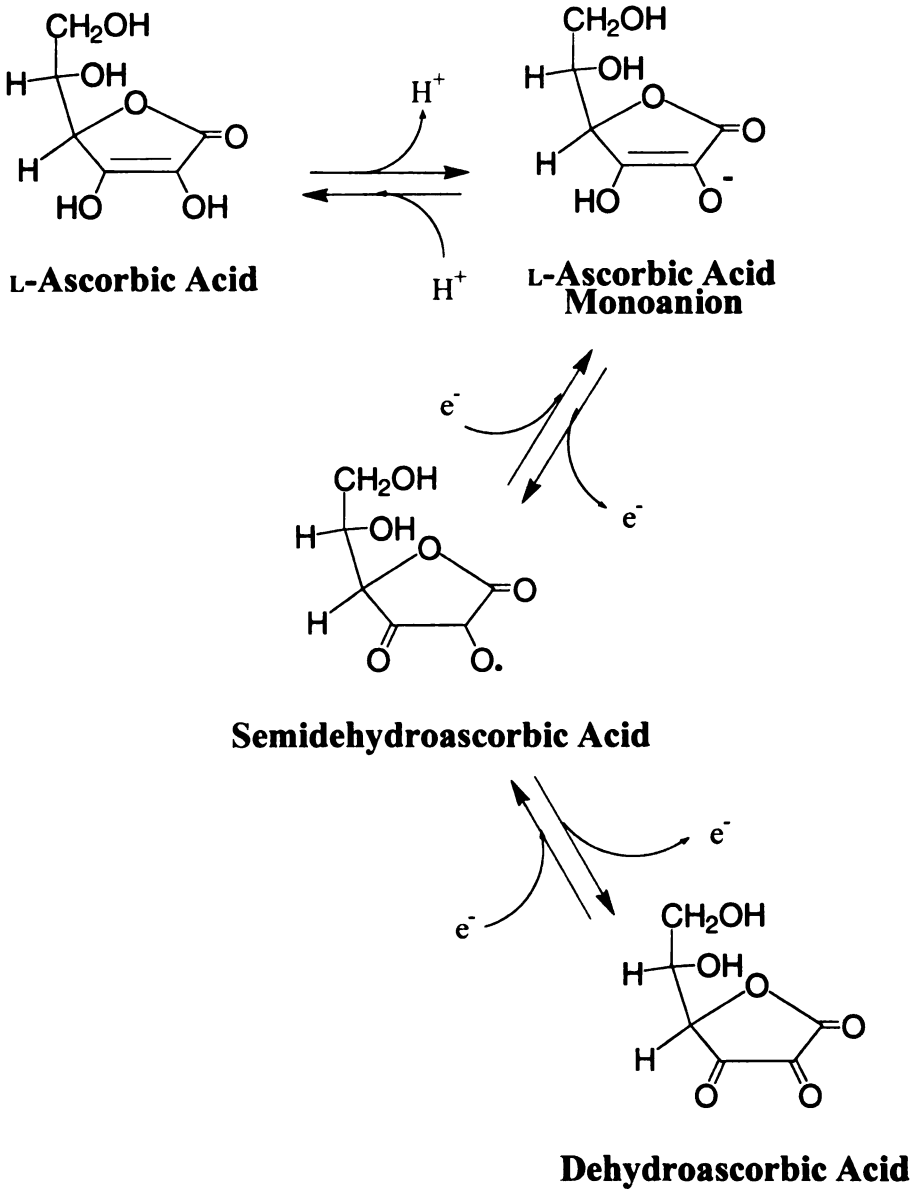



**Figure 1**: Structures of L-ascorbic acid, semidehydroascorbic acid, and dehydroascorbic acid.

Under more physiologically relevant conditions, AA reduced Vp-16-O• in rat hepatocyte and retinal cell homogenates (*20*). *In vitro* AA effectively scavenged $O_2^{\bullet -}$ (*21*) because of the combination of the following reactions:

3) $O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2$ ( $E^{o\prime} = 940$ mV)

4) AA → semiDHA• + $H^+$ ($E^{o\prime} = -282$ mV)

combining into

5) $O_2^{\bullet -} + 2H^+ + AA \rightarrow H_2O_2$ + semiDHA• + $H^+$

The sum of the two reactions has an $E^{o\prime} = 658$ mV making it a spontaneous reaction (*18*). The importance of AA as an antioxidant in physiology is demonstrated by protection by AA against lipid peroxidation (*22, 23*), providing antioxidant capacity for the blood (*24, 25*), and protecting against ischemia reperfusion injury (*26, 27*).

*I. B.1. Ascorbic Acid Protection Against Lipid Peroxidation.*

AA protected against lipid peroxidation in a variety of systems. Chakrabatty *et al.* (*22*) found that marginal AA deficiency in guinea pigs resulted in lipid peroxidation in the cardiac tissue, and this damage was independent of decreases of other reactive oxygen species scavengers such as superoxide dismutase, catalase, and glutathione peroxidase. In a different system, AA protected against lipid peroxidation in human plasma induced by gas phase oxidants of cigarette smoke (*23*). In contrast, in the presence of free metal ions like iron, AA was prooxidative and induced lipid oxidation (*28*). It is generally believed that AA does not directly protect against lipid peroxidation but carries out this function because of its ability to regenerate vitamin E (K-tocopherol, TOH) from the tocopheroxyl radical (TO•) (*18*). Vitamin E is an important chain-breaking antioxidant in the lipid bilayer, and when it

carries out its function it is oxidized by one electron to TO• (*18*). AA has been demonstrated to regenerate TOH from TO• *in vitro* (*29, 30*). The regeneration of TOH by AA from TO• is a thermodynamically favorable reaction (*18*):

6) $TO^{\bullet} + H^{+} \longrightarrow TOH \qquad (E^{o\prime} = 500\ mV)$

7) $AA \longrightarrow semiDHA^{\bullet} + H^{+} \quad (E^{o\prime} = -282\ mV)$

Combining into the following reaction

8) $TO^{\bullet} + AA \longrightarrow TOH + semiDHA^{\bullet} \quad (E^{o\prime} = 218\ mV)$

As a result, for example, the protection against lipid peroxidation of rat liver microsomes by AA was dependent on vitamin E (*31*).

*I. B. 2. Antioxidant Function of Ascorbic Acid in the Plasma.*

Extracellular fluids of the human body like blood plasma contain very low levels of glutathione (GSH), superoxide dismutase, glutathione peroxidase, and catalase activities which are all important intracellular antioxidant defenses (*32*). Substantial evidence exists that AA is a pivotal plasma antioxidant. AA was the primary defense against lipid peroxidation when human plasma was treated either with a water-soluble radical initiator or by activated polymorphonuclear leukocytes (*24, 25*). AA is an effective scavenger of $O_2^{\bullet -}$ (*21*) and hypochlorous acid (HOCl, *33, 34*), which are both released by activated polymorphonuclear leukocytes (*2, 35*). Human plasma devoid of AA, but no other endogenous antioxidants like vitamin E or uric acid, was unable to prevent initiation of lipid peroxidation (*25*). These results suggest that AA is an important antioxidant in the plasma.

In addition to being an important antioxidant in the plasma, AA protects against ischemia/reperfusion injury. Sciamanna and Lee (*26*) demonstrated a dose dependent

protection of AA on ischemia/reperfusion injury in the rat forebrain. In this study, AA provided partial protection even if given after the onset of reperfusion, but maximal protection occurred when AA was administered prior to the onset of ischemia (*26*). In addition, AA protected against lipid peroxidation in rat liver when AA was administered prior to ischemia/reperfusion (*27*). Recall that when AA functions as an antioxidant it is first oxidized to the relatively stable radical semiDHA. SemiDHA has been identified as a biological marker of ischemia/reperfusion injury in isolated rat hearts (*36*), and as a biological marker of oxidative stress in human open heart surgery patients (*37*).

*I. B. 3. Role of Ascorbic Acid in Immune System Function.*

As a result of its antioxidant capacity, AA also plays an important role in the immune system. Cells of the immune system such as neutrophils and macrophages accumulate at a site of inflammation to engulf and destroy microbes in a process known as phagocytosis (*2*). The process of microbial killing is predominantly carried out by oxygen dependent mechanisms. The phagocytic vacuole contains NADPH oxidase which produces $O_2^{\bullet -}$ also resulting in the production of $H_2O_2$ by the spontaneous dismutation of $O_2^{\bullet -}$. In addition, myeloperoxidase generates HOCl from $O_2^{\bullet -}$ and $Cl^-$. Both $O_2^{\bullet -}$ and HOCl are important in microbial killing. This process is known as the respiratory burst. The contents of the phagocytic vacuole can also be released into the extracellular space producing a highly oxidative environment around the immune cells. (*2, 35*).

Existing in a potential highly oxidative environment, lymphocytes and erythrocytes must be able to protect themselves against both endogenous and exogenous oxidative stresses and therefore require antioxidant defenses. In erythrocytes, using nitroxides as a model of

persistent free radicals, Melhorn concluded that AA was the only reducing agent for nitroxides in erythrocytes (*38*), a result confirmed by Zhang and Fung (*39*). Using the membrane-impermeable oxidant ferricyanide, May *et al.* (*40*) found that AA in erythrocytes was capable of protecting against ferricyanide damage with the subsequent formation of DHA, which is reduced back to AA to provide ongoing antioxidant protection.

Erythrocytes have long been known to have impressive DHA uptake capacity and GSH-dependent DHA reduction activity (*41-43*). Melhorn (*38*) and May *et al.* (*40*) both found the DHA reductive capacity of erythrocytes to be essential for the erythrocyte's ability to defend themselves against oxidative stress. When erythrocytes were depleted of AA and incubated with DHA, their ability to reduce ferricyanide and nitroxides was restored (*38, 40*). Furthermore, AA depleted, DHA treated cells have greater nitroxide reductive capacity than buffered solutions of DHA plus GSH, suggesting the important role of a DHA reductase in erythrocyte AA regeneration (*38, 39*).

Neutrophils also require robust antioxidant capacity to defend themselves against endogenously produced oxidants. As discussed earlier, activated neutrophils undergo the "respiratory burst" whereby they produce reactive oxygen species like $O_2^{\bullet -}$, $H_2O_2$, and HOCl. Human neutrophils stimulated with opsonized zymosan or phorbol myristate acetate (PMA) oxidized 30-40% of their intracellular AA to DHA (*44*). Activated neutrophils, but not quiescent neutrophils, consumed extracellular AA in a superoxide and HOCl dependent process (*45*). Human neutrophils, incubated with a physiological plasma concentration of AA (200 $\mu$M) and stimulated with PMA, N-formyl-methionyl-leucyl-phenylalanine, or NaF, accumulated AA intracellularly above the levels of unstimulated neutrophils from 2 mM to as high as 10 mM AA (*46*). Unstimulated neutrophils incubated with 200 $\mu$M AA in the

presence of the $O_2^{\bullet -}$ generating system xanthine/xanthine oxidase increased their intracellular AA concentration from 2 mM to 7 mM (*46*). Activated chronic granulomatous disease neutrophils, which cannot produce $O_2^{\bullet -}$, did not accumulate AA (*46*). These results suggested that neutrophils preferentially take up DHA and reduce it to AA, and Welch *et al.* (*47*) demonstrated that DHA transport in human neutrophils was at least 10-fold faster than AA transport, and DHA and AA were taken up by separate mechanisms. Furthermore, once DHA was transported into the neutrophil, it was immediately reduced to AA (*47*).

Recently, AA recycling in human neutrophils was induced by incubation of neutrophils with Gram-positive and Gram-negative bacteria and the fungal pathogen *Candida albicans* (*48*). Recycling was dependent on extracellular AA oxidation to DHA because incubation of neutrophils, bacteria, and AA with superoxide dismutase and catalase completely inhibited AA recycling (*48*). In addition, when neutrophils unable to make oxidants from chronic granulomatous disease patients were incubated with bacteria, no AA increase in neutrophils was seen (*48*). However, neutrophils from chronic granulomatous disease patients incubated with DHA accumulated AA intracellularly. Wang *et al.* (*48*) suggested that the pronounced ability of activated human neutrophils to transport DHA, reduce it to AA and accumulate intracellular AA may be an integral component of the antimicrobial function of neutrophils. In conclusion, both erythrocytes and neutrophils accumulate AA intracellularly by transporting DHA across the plasma membrane and rapidly reducing DHA to AA. This process provides antioxidant protection to these cells against endogenously and exogenously produced oxidative stress and may be important in the body's ability to fight bacterial infection.

*I. B. 4. Antioxidant Function of Ascorbic Acid in Glutathione Deficient Animals.*

Perhaps the best *in vivo* evidence for AA as an essential antioxidant comes from studies in glutathione deficient animals (GSH). GSH deficiency is induced in experimental animals by administering buthionine sulfoximine (BSO) which is a transition state inhibitor of 3-glutamylcysteine synthetase, an enzyme in the GSH synthesis pathway (*49-50*). GSH is also an important antioxidant because it is a substrate of glutathione peroxidases which scavenge $H_2O_2$ and lipid peroxides (*51*). In adult mice, severe GSH deficiency induced by BSO led to damage in skeletal muscle, lung, and mucosal degeneration in the jejunum and colon (*51*). In newborn rats, severe GSH deficiency induced by BSO led to 83% mortality after seven days. In these animals, there were fewer mitochondria in brain, liver, lung , and kidney, evidence of mitochondrial swelling and degeneration in all tissues except the heart, and cellular damage in the lung, liver and brain (*52*). Mårtensson *et al.* (*52*) concluded that BSO treated newborn rats, which do not synthesize AA, are excellent models for endogenously produced oxidative stress. When AA was given to these animals along with BSO, mortality after seven days decreased to 11% and none of the tissue damage was seen (*52*). In another study using adult mice, severe GSH deficiency induced by BSO led to swelling and destruction of lung type 2 cell leamellar bodies and severe mitochondrial degeneration, decreased phosphatidylcholine in the lung, decreased bronchioalveolar lining fluid, and in the skeletal muscle, severe mitochondrial swelling and vacuolization, and mitochondrial membrane disintegration ( *53*). Concomitant addition of AA with BSO abrogated all of these effects, and caused an increase in GSH levels in the lung, lung mitochondria, muscle, and muscle mitochondria, but not to their original control levels (*53*). Thus, AA had a sparing effect on GSH levels as part of its antioxidant function. These

studies by Meister and colleagues clearly demonstrated the interrelationship of GSH and AA and the function of AA *in vivo* as an antioxidant.

## II Ascorbic Acid Synthesis and Regeneration.

When AA carries out its function as an antioxidant or as a ferric ion reductant, it undergoes two successive one electron oxidations to semiDHA and DHA (Figure 1). In order to maintain sufficient tissue AA levels, AA must be synthesized , absorbed from the diet, or its oxidation products recycled. Most species of animals are able to synthesize AA from glucose (*54*). Humans, other primates, and guinea pigs cannot synthesize AA endogenously because they lack the enzyme L-gulono-3-lactone oxidase which is required for the conversion of L-gulono-3-lactone to 2-oxo-L-gulono-3-lactone, a tautomer of L-ascorbic acid that spontaneously transforms into AA (*54*). Therefore, in species like humans and guinea pigs, dietary vitamin C and recycling semiDHA and DHA are vital for the maintenance of AA levels.

### *II. A. Semidehydroascorbate Regeneration.*

SemiDHA is a relatively stable radical, neither strongly oxidizing nor strongly reducing (*18*). SemiDHA may either be reduced back to AA by an NADH-semiDHA reductase (*55-57*) or it rapidly disproportionates to AA and DHA with a rate constant ($k$) = $1.4 \times 10^6$ $M^{-1}$ $s^{-1}$ at pH 7.4 (*19*). NADH-semiDHA reductase has been identified in the microsomal fraction of pig heart (*55*) and rat liver (*56*). In addition, a soluble NADH-semiDHA reductase has been identified in human lens (*57*). However, NADH-semiDHA

reductase activity from any of these sources has not yet been purified and kinetically characterized.

*II. B. Dehydroascorbic Acid Regeneration.*

DHA is potentially toxic to cells and tissues as highlighted by its *in vitro* toxicity to pancreatic islets (*58-59*) and erythrocytes (*60*). As discussed previously, DHA is taken up preferentially by cells like human erythrocytes (*61*), neutrophils (*47*), and fibroblasts (*47*) and rapidly reduced to AA. May *et al.* (*61*) determined that DHA reduction by human erythrocytes was GSH-dependent and did not involve semiDHA. In addition, Melhorn (*38*) and Zhang and Fung (*39*) concluded that the DHA reductive capacity of erythrocytes could not be accounted for by GSH alone, suggesting the importance of a DHA reductase activity in erythrocyte AA recycling. Therefore, recycling of DHA is important especially in species like humans whose only source of AA is from the diet.

*II. B. 1. NADPH-dependent Dehydroascorbic Acid Regeneration.*

Evidence exists that both NADPH-dependent and GSH-dependent DHA reduction are important *in vivo*. DHA can be reduced to AA in an NADPH dependent manner by both 3K-hydroxysteroid dehydrogenase (*62*) or the selenoprotein thioredoxin reductase (*63*). Rats fed a selenium deficient diet had an 88% reduction in thioredoxin reductase activity and a 33% reduction in liver ascorbate levels providing evidence for selenium dependent enzymes, like thioredoxin reductase, playing a role in DHA recycling (*63*). However, 67% of control levels of liver ascorbate remained in selenium deficient rats, suggesting a role for GSH-dependent DHA reductases, whose activities were not affected by selenium deficiency (*63*).

*II. B. 2. GSH-dependent Dehydroascorbic Acid Regeneration.*

Unable to initiate synthesis of AA, when newborn rats were made GSH deficient by administering the 3-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO), drastic decreases in tissue ascorbate levels and increases in DHA levels occurred (*64*). Similar results were seen when GSH deficiency was induced in guinea pigs after BSO administration (*50*), and when GSH monethyl ester, which is transported and converted intracellularly into GSH, was administered to GSH deficient guinea pigs, the onset of scurvy was delayed suggesting the sparing effect of GSH on ascorbate (*65*). In adult mice, which are able to synthesize AA, GSH deficiency resulted in increased AA synthesis in the liver (*66*). This result has also been seen in isolated murine hepatocytes (*67*). Meister and coworkers have concluded that the drastic decrease in tissue ascorbic acid levels in GSH deficiency demonstrated the importance of GSH dependent AA recycling (*51*). These studies demonstrated a role of GSH in the regeneration of AA *in vivo*, but they did not resolve the question of whether the regeneration of AA from DHA by GSH was enzymatic or chemical.

*II. B. 3. Chemical vs. Enzymatic GSH-dependent Dehydroascorbic Acid Reduction.*

GSH reduction of DHA to AA has been estimated to have a $t_{1/2}$ of 15 min at pH 7.5 and at 25°C (*68*). However, under the same conditions DHA degrades to non recyclable products, like 2,3-diketogulonic acid with a $t_{1/2}$ of 2 min (*69*). This strongly supports the importance of catalytic regeneration of AA from DHA. Evidence for GSH-dependent DHA reductases in animal tissues have existed for many years (*70-72*). Bigley *et al.* (*73*) demonstrated that GSH-dependent DHA reductase activity was greatest in human neutrophils, followed by monocytes, lymphocytes, and fibroblasts. Several GSH-dependent

DHA reductases have been identified including the thioltransferases from vaccinia virus (*74*), pig liver (*75-76*), human placenta (*63*), and human neutrophil (*77*), the 32kDa-DHA reductase isolated from rat liver (*78*) and human erythrocytes (*79*), bovine liver protein disulfide isomerase (PDI) (*63, 75, 76*), the p52 enzyme from *Trypanosoma cruzi* (*80*), a DHA reductase from spinach chloroplasts homologous to plant trypsin inhibitor (*81*), and a 24 kDa DHA reductase from spinach leaves (*82*).

In a study by Park and Levine (*77*), the reducing activity of neutrophils after cell lysis was non-dialysable and could not be accounted for by chemical reduction of DHA by GSH. They found that the purified DHA reducing activity was thioltransferase (*77*). In rat liver cytosol, up to 70% of the DHA reductase activity was immunotitrateable using an antibody directed towards the rat liver 32 kDa DHA reductase (*83*). These experiments demonstrate the importance of GSH-dependent DHA reductases in DHA reduction.

The emerging picture in the field of DHA reductases is one in which there are several potential systems to regenerate AA from DHA. NADPH-dependent DHA reductases like 3K-hydroxysteroid dehydrogenase (*62*) or thioredoxin/thioredoxin reductase (*63*) and GSH-dependent DHA reductases like thioltransferase (*75*) or the 32 kDa DHA reductase (*78-79*) may all play roles *in vivo*. It is likely that the importance of each enzyme in DHA reduction will vary from species to species and cell to cell. Meister and coworkers have provided strong evidence for GSH-dependent DHA reduction *vs.* NAD(P)H dependent DHA reduction (*50-51*). However, the NADPH-dependent DHA reduction from the thioredoxin/thioredoxin reductase system may also be important. Thioredoxin reductase (*63*) and the 32 kDa DHA reductase (*83*) have been separately shown to be responsible for 33% and 70% of the DHA reductase activity in rat liver, completely accounting for the DHA reductase activity in this

tissue. On the other hand, thioltransferase has been proposed to be the only DHA reductase in human neutrophils (*77*).

The wide variety of DHA reductases, especially the GSH-dependent DHA reductases raises several enzyme structure/function questions. Many of these enzymes have several different activities including thioltransferase, thioredoxin/thioredoxin reductase, and PDI which belong to the class of enzymes known as thiol:disulfide oxidoreductases (*84*). Whether each enzyme has an identical, similar or different catalytic mechanism for DHA reduction is unknown.

## III. Thiol:disulfide Oxidoreductases.

Thiol:disulfide oxidoreductases catalyze the oxidation of protein thiols and the reduction and isomerization of protein disulfide bonds. These reactions are important for both the structural integrity of proteins and the enzymatic activity of other enzymes (*85*). Thiol:disulfide oxidoreductases like PDI (*86*), DsbA and DsbB (*87*), thioredoxin (*88*), and thioltransferase (glutaredoxin) (*84*), all contain the -CXXC- active site motif, which is essential for their activity. GSH is the principal reductant of PDI and thioltransferase, whereas NADPH, via thioredoxin reductase, is the principal reductant of thioredoxin (*120*). The environment in which the -CXXC- active site exists in each of these enzymes dictates its redox potential and the subsequent specialization of each enzymatic activity (*89-90*).

### *III. A. DsbA, DsbB, and Protein Disulfide Isomerase.*

DsbA, DsbB, and DsbC in *E. coli* and PDI in eukaryotes are believed to be essential

enzymes for the proper folding of newly synthesized proteins which contain disulfide bonds (*86-87*). DsbA is a relatively oxidizing enzyme compared to other thiol:disulfide oxidoreductases with a redox potential of -0.089 V (Figure 2; *91* ). Of the essential cysteines for DsbA's activity, the $pK_a$ of Cys-30 in DsbA is approximately 3.5 with Cys-33 being relatively unreactive (92).

PDI is a highly abundant homodimer (57 kDa each, 114 kDa total) located in the lumen of the endoplasmic reticulum (*93-94*). Like DsbA, PDI is also a relatively oxidizing thiol:disulfide oxidoreductase with a redox potential of -0.11 V (Figure 2: *95*). The sequence of PDI reveals two domains that share approximately 30% amino acid identity to thioredoxin (*96*). The N-terminal cysteines in each of the thioredoxin-like domains of PDI possess a $pK_a$ of 6.7 as determined by iodoacetic acid inactivation (*97*) and are essential for disulfide isomerase activity ( *98*). The essential function of PDI has been proposed to be its ability to unscramble non-native disulfide bonds (*99*), but bovine liver PDI has also been demonstrated to be a GSH-dependent DHA reductase which may also be an important function of PDI (*75*).

*III. B. Thioredoxin.*

The thiol:disulfide oxidoreductase, thioredoxin, is a 12 kDa enzyme found in a wide variety of organisms ranging from archaea to humans (*100*). Thioredoxins have the conserved active site sequence of -Trp-Cys-Gly-Pro-Cys-, and a disulfide bond is formed in the active site upon thioredoxin carrying out its function (*100*). The oxidized form of thioredoxin is reduced to a dithiol by NADPH and the flavoprotein thioredoxin reductase (*100*). The thioredoxin/thioredoxin reductase system has a wide variety of activities

primarily as a catalyst for protein disulfide bond reduction or oxidation of a dithiol to a disulfide (*88*), but the thioredoxin/thioredoxin reductase system also functions as an alloxan

| Enzyme or chemical | Redox potential (V) |
|---|---|
| *E. coli* DsbA (*91*) | -0.089 |
| Bovine liver PDI (*95*) | -0.11 |
| Pig liver thioltransferase (*149*) | -0.159 |
| *E. coli* thioltransferase 3 (*150*) | -0.198 |
| cysteine (*203*) | -0.22 |
| *E. coli* thioltransferase 1 (*150*) | -0.233 |
| GSH (*203*) | -0.24 |
| *E. coli* thioredoxin (*88*) | -0.26 |
| NADPH (*88*) | -0.31 |
| dihydrolipoate (*203*) | -0.32 |

**Figure 2**. Redox potentials of chemicals and thiol:disulfide oxidoreductases.

reductase (*101*), a DHA reductase (*63*), and catalyzes the cleavage of S-nitrosoglutathione to GSH and nitric oxide (*102*). Thioredoxin has been shown to increase proliferation of human B-cell lines presumably due to its thiol:disulfide oxidoreductase activity (*103*). In addition, thioredoxin/thioredoxin reductase have been demonstrated to function as an endogenous regeneration system for heat shock factor during oxidative stress (*104*) and

promote AP-1 activity and inhibit NF-κβ activity in intact cells (*105*).

*E. coli* thioredoxin has a redox potential of -0.26V at pH 7.0, making it a relatively strongly reducing thiol:disulfide oxidoreductase (Figure 2, *88*). The essential amino acid for *E. coli* thioredoxin's activity is Cys-32 whose titration curve displays $pK_a$s at 7.5 and 9.2 (*106*). The solvent inaccessible Cys-35 has a $pK_a$ of 11.1 in *E. coli* thioredoxin, but Cys-35 is believed to act as a nucleophile in the thioredoxin catalytic mechanism (*107*). Asp-26 is a highly conserved residue in thioredoxins (*100*), and has been shown to act as a general acid/base in the catalysis of *E. coli* thioredoxin (*108*). The proposed catalytic mechanism of thioredoxin is shown in Figure 3 (*108*). To complete the mechanism, oxidized thioredoxin is regenerated to reduced thioredoxin by thioredoxin reductase and NADPH (*88*).

*III. C. Thioltransferase (Glutaredoxin).*

The final well characterized thiol:disulfide oxidoreductase is thioltransferase, otherwise known as glutaredoxin. The term thioltransferase was first used in 1974 by Askelöf *et al.* (*109*) to describe a partially purified rat liver enzyme which catalyzes the following reaction:

9) RSSR + GSH ⟶ RSSG + RSH

10) RSSG + GSH ⟶ GSSG + RSH

Previously, the term transhydrogenase had been used to describe enzymes which catalyze the combination of reactions (9) and (10)

11) RSSR + 2GSH ⟶ GSSG + 2RSH

(*110-111*), but Askelöf, *et al.* (*112*), provided the first evidence of a two step mechanism. Axelsson, *et al.*, first purified thioltransferase from rat liver as an 11,000 dalton enzyme in

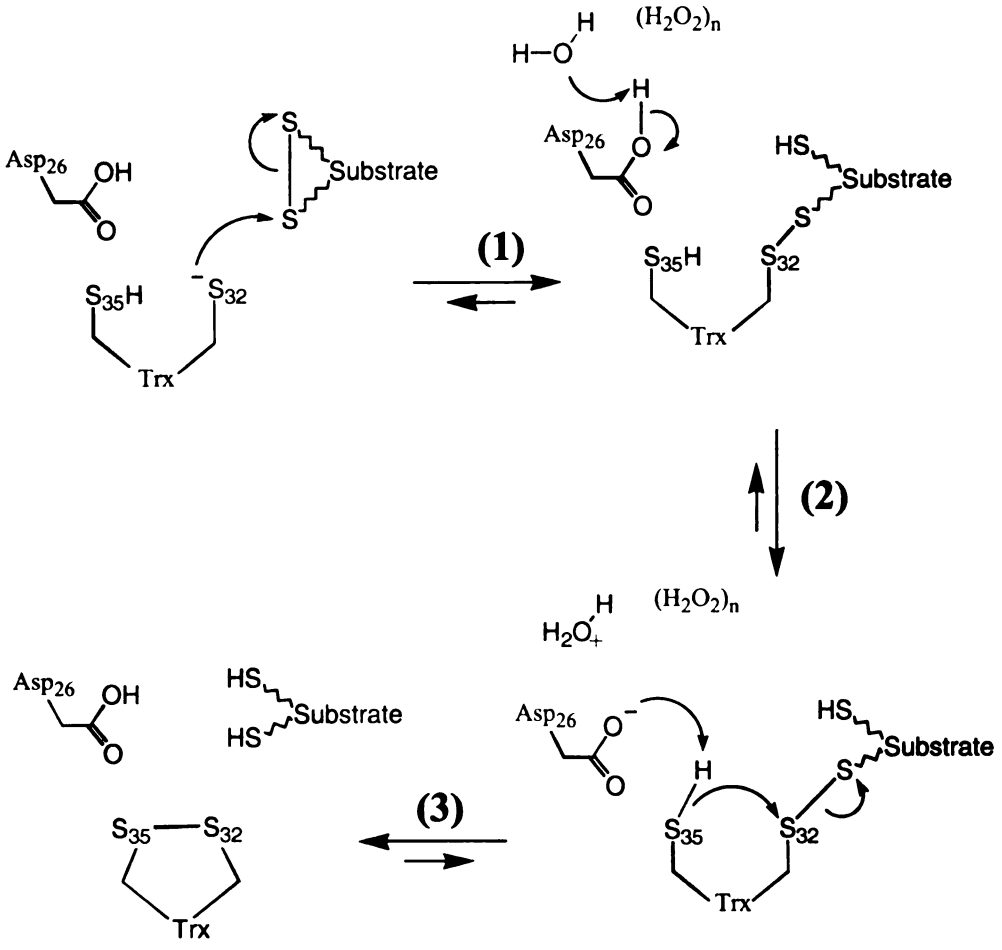



**Figure 3**: Proposed catalytic mechanism of thioredoxin from Chivers and Raines (*108*). In this mechanism, Cys-32 of *E. coli* thioredoxin attacks a sulfur group of the substrate froming a mixed disulfide between thioredoxin and the substrate (reaction 1). After deprotonation by water (reaction 2), Asp-25 then deprotonates Cys-35 of thioredoxin which attacks Cys-32 releasing reduced substrate and forming oxidized thioredoxin (reaction 3). Oxidized thioredoxin is reduced by thioredoxin reductase and NADPH.

1978 and characterized thioltransferase's ability to catalyze the GSH-dependent reduction of S-sulfoglutathione, S-sulfocysteine, L-cystine, L-homocystine, cystamine, oxidized trypsin, ribonuclease, oxytocin, or insulin, and mixed disulfides of GSH and coenzyme A, L-cysteine, cysteamine, and hen egg white lysozyme.

In 1976 Holmgren isolated an *E. coli* mutant strain lacking thioredoxin activity, but retaining the ability to provide reducing equivalents to ribonucleotide reductase (*113*). Holmgren named this heat stable factor glutaredoxin, and found it to catalyze the hydrogen transfer from GSH to ribonucleotide reductase (*113*). *E. coli* glutaredoxin was determined to be a protein of 11,600 daltons (*114*), and was shown to catalyze reaction (12) in an *in vitro* reconstituted system with the B1 and B2 subunits of ribonucleotide reductase:

(12) $2GSH + CDP \longrightarrow GSSG + dCDP + H_20$ (*115*)

Glutaredoxin has also been suggested to play a role in ribonucleotide reductase function in calf thymus (*116*) and vaccinia virus (*117*).

Until 1987, thioltransferase and glutaredoxin were considered distinct enzymes. In 1987, Gan and Wells determined the primary structure of pig liver thioltransferase, a 12 kDa enzyme, and found it to be 82% identical to calf thymus glutaredoxin (*118*). Then in 1989, when the amino acid sequence of calf thymus glutaredoxin was reexamined by mass spectroscopy and compared to pig liver thioltransferase and rabbit bone marrow glutaredoxin, each possible comparison yielded at least an 83% sequence identity (*119*). It is now generally accepted that glutaredoxin and thioltransferase are two names for the same enzyme (*120, 84*). For the purpose of the discussion, from this point on this class of enzymes will be solely referred to as thioltransferase.

*III. C. 1. Purification, Cloning, and Sequences of Thioltransferases.*

Thioltransferase has been purified and its amino acid sequence determined from human red blood cells (*121*), human placenta (*122*), pig liver (*118*), calf thymus (*119*), rabbit bone marrow (*123*), rice (*124*), yeast (*125*), *E. coli* (*126*), vaccinia virus (*127*), and bacteriophage T4 (*128*). Each enzyme possesses the common active site -CPF/YC- (amino acid alignment in Figure 4). Thioltransferase has been cloned from human brain (*129*), human placenta (*130*), pig liver (*131*), rice (*132*), *E. coli* (*133*), and vaccinia virus (*74*).

## IV. 3D Structures of Thiol:disulfide Oxidoreductases.

Thioredoxins, PDI, DsbA, DsbB, and DsbC are related enzymes based on the similarity of their active site sequences (Figure 5). The conserved Asp at position 26 in *E. coli* thioredoxin is a Glu in PDI and DsbA suggesting that a Glu at this position may function as a general acid/base in PDI and DsbA catalysis. Thioredoxins, DsbA-C and PDI, also show conserved 3D structural characteristics beyond the similarity of their active sites.

| | |
|---|---|
| human | MAQEFVN CKIQPGKWV FI KPT**CPYC**RR AQEILSQLPI KQGLLEFVDI TATNH |
| pig | AQAFVN SKIQPGKVVV FI KPT**CPFC**RK TQELLSQPF KEGLLEFVDI TATSD |
| bovine | AQAFVN SKIQPGKVVV FI KPT**CPYC**RK TQELLSQLPF KQGLLEFVDI TAAGN |
| rabbit | AQEFVN SKIQPGKVVV FI KPT**CPYC**RK TQEILSELPF KQGLLEFVDI TATSD |
| yeast | VSQETVAHVKDLIGQKEVFVAAKTY**CPYC**KA TLSTLFQELN VPKSKALVLE LDEMS |
| vaccinia | MAEEFVQ QRLANNKVTI FV KYT**CPFC**RN ALDILNKFSF KRGAYETVDI KEFKP |
| rice | MALAKAK ETVASAPVVV YS KSY**CPFC**VR VKKLFGQLGA TFKAIELDGE SDG |
| *E. coli* | MQTVIFG RSG**CPYC**VR AKDLAEKLSN ERDDFQYQYV DIRAEGI |
| T4 | MFKVYGYDSN IHK**CVYC**DN AKRLLTVKKQ PFEFINIMPE KGVFDDEK |
| | |
| human | TNEIQDYLQQ LTGARTVPRV FIGKDCIGGC SDLVSLQQSGE LLTRLKQIGA LQ ..... |
| pig | TNEIQDYLQQ LTGARTVPRV FIGKECIGGC TDLESMHKRGE LLTRLQQIGA LK ..... |
| bovine | ISEIQDYLQQ LTGARTVPRV FIGQECTGGC TDLVNMHERGE LLTRLKQMGA LO ..... |
| rabbit | MSEIQDYLQQ LTGARTVPRV FLGKDCIGGC SDLIAMQEKGE TLARLKEMGA LRQ ..... |
| yeast | NGSEIQDALE EISGQKTVPNV YINGKHIGGN SDLETLKKNGK LAEILKPVFQ ..... |
| vaccinia | ENELRDYFEQ ITGGRTVPRI FFGKTSIGGY SDLLEIDNMDA LGDILSSIGV LRTC |
| rice | SELQSALAE WTGQRTVPNV FINGKHIGGC DDTLALNNEGK LVPLLTEAGA |
| *E. coli* | TKEDLQQKA GKPVET**V**PQI FVDQQHIGGY **TD**FAAWVKENLDA ..... |
| T4 | IAELLTKLGRDTQIGLTMPQV FAPDGSHIGG FDQLREYFK |
| | |
| human | |
| pig | |
| bovine | |
| rabbit | |
| yeast | |
| vaccinia | |
| rice | ITA |
| *E. coli* | |

**Figure 4.** Amino acid sequence alignment of thioltransferases. The active site -CPF/YC- is in bold and underlined. Val-59, Thr-73, and Asp-74 in *E. coli* thioltransferase are also in bold and underlined because these residues have been shown to be involved in binding GSH (*143*).

| Enzyme | Active Site Sequence |
|---|---|
| E coli DsbA (*137*) | EFFSFF**CPHC**YQFE |
| E coli DsbB (*204*) | VMLLK**CVLC**IYER |
| E coli DsbC (*205*) | VFTDIT**CGYC**HKLH |
| Rat PDI domain I (*100*) | EFYAPW**CGHC**KALA |
| Rat PDI domain II (*100*) | EFYAPW**CGHC**KQLA |
| E coli thioredoxin (*100*) | DFWAES**CGPC**KMIA |
| Mouse thioredoxin (*100*) | DFSATW**CGPC**KMIK |
| Human thioredoxin (*100*) | DFSATW**CGPC**KMIK |

**Figure 5.** The active site sequences of the thiol:disulfide oxidoreductases DsbA, DsbB, DsbC, PDI and thioredoxin. The active sites of each enzyme are in bold and underlined.

*IV. A. 3D Structure of Thioredoxin.*

The crystal structure of the oxidized form of *E. coli* thioredoxin at 4.5 Å was first reported in 1974 by Söderberg *et al.* (*134*), and further refined to 1.68Å by Kati *et al.* (*135*) in 1990 ( Figure 6). The secondary structure of *E. coli* thioredoxin is $\beta_1\alpha_1\beta_2\alpha_2\beta_3\alpha_3\beta_4\beta_5\alpha_4$ and is composed of a core of a twisted β-sheet flanked on either side by helices (Figure 6, *135*). The active site forms a protrusion on the surface of the molecule and is at the amino terminal end of helix $\alpha_2$. The -SG including Cys-32 is solvent accessible, whereas Cys-35 is not. The proximity of Cys-32 to the positive electrostatic field of the N-terminal end of an α helix may contribute to the low $pK_a$ of Cys-32 and its subsequent reactivity (*135*). In support of this concept, Kortemme and Creighton (*136*) demonstrated with model α-helical peptides that a decrease in the thiol $pK_a$ of cysteine occurs when cysteine is near the N-terminus of the α-helix, whereas at the C-terminus the thiol $pK_a$ value increases slightly.

*IV. B. 3D Structures of DsbA and Protein Disulfide Isomerase.*

Oxidized DsbA has been crystallized and its structure determined (*137*). A portion of the structure of DsbA closely resembles that of thioredoxin (Figure 6). In Figure 6, the structure of DsbA is given with the thioredoxin-like structure facing the reader and on the right hand side of the molecule. The α-helices behind the active site are not part of the thioredoxin fold. In addition, the structure of the N-terminal domain, residues 1-120, of human PDI in the oxidized form has been determined by NMR and it's structure also closely resembles that of thioredoxin (Figure 6, *138*). In each of the described structures, the -CXXC- active site is located at the N-terminus of an α helix most likely contributing to the nucleophilicity of the N-terminal cysteine. The common structural motif seen in these

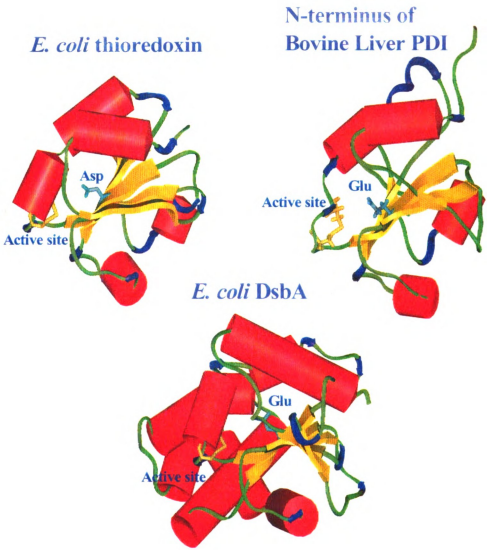



**Figure 6.** Kabsch-Sander representation of the structures of *E. coli* thioredoxin, the N-terminus of protein disulfide isomerase, and *E. coli* DsbA. The active site disulfide of each enzyme is labeled and is found at or near the N-terminus of an alpha helix (designated by the red cylinders).

enzymes of a central core composed of a β-sheet surrounded by α-helices has become known as the thioredoxin fold. The structure of reduced *E. coli* thioredoxin has been determined via NMR, and the overall structure is very similar to the crystal structure of oxidized thioredoxin (*139*).

*IV. C. 3D Structures of Thioltransferases.*

The 3D structures of oxidized pig liver thioltransferase (*140*), oxidized *E. coli* thioltransferase (*141*), reduced *E. coli* thioltransferase (*142*), *E. coli* thioltransferase mutant C14S in complex with GSH (*143*), and oxidized T4 bacteriophage glutaredoxin (*144*) have been determined.

*IV. C. 1. 3D Structure of Bacteriophage T4 Thioltransferase.*

The structure of oxidized T4 thioltransferase is very similar to that of thioredoxin with a central β-sheet surrounded by α-helices except for the first 20 amino acids in thioredoxin which form a β-strand and α-helix not found in T4 thioltransferase, and the active site of *E .coli* thioredoxin protrudes more from the molecule whereas the active site of T4 thioltransferase is located more in an occluded cleft (Figure 7, *144, 140*). The active site disulfide of Cys-14 and Cys-17 is found at the N-terminus of helix α1, and Cys-17 is solvent inaccessible (Figure 7, *144*).

*IV. C. 2. 3D Structures of E. coli Thioltransferase.*

Both reduced and oxidized *E. coli* thioltransferase were found to have very similar structures, except the solvent accessible surface area in the active site of the reduced enzyme

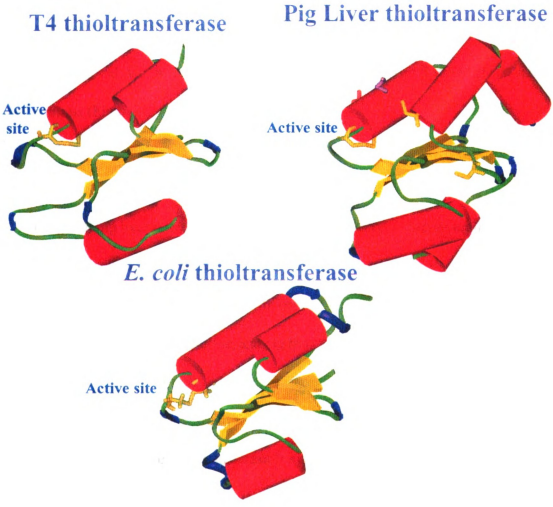



**Figure 7**: Kabsch-Sander representations of the structures of T4 thioltransferase, pig liver thioltransferase, and *E. coli* thioltransferase. The active site disulfide of each enzyme is shown and is near the N-terminus of an alpha helix (represented by red cylinders).

was larger than that of the oxidized enzyme (*141*). Again, the active site cysteines were located at the N-terminus of α-helix 1 and Cys 11 is solvent accessible whereas Cys 14 is not (Figure 7, *141*). Xia *et al.* (*141*) found that the structures of oxidized *E. coli* thioltransferase and oxidized T4 thioltransferase were very similar to each other with a βαβαββα structure and to *E. coli* thioredoxin, which is βαβαβαββα possessing an extra β strand and α-helix at the N-terminus (Figure 7).

*IV. C. 3. 3D Structure of Pig Liver Thioltransferase.*

Oxidized pig liver thioltransferase also possesses a very similar overall fold to other thioltransferases and the thioredoxins, with a central β-sheet surrounded by α-helices (figure 7, *140*). Major differences include *E. coli* thioredoxin having an additional β-strand prior to α-helix 1 of pig liver thioltransferase, and pig liver thioltransferase having an α-helix prior to β-strand 1 not found in T4 or *E. coli* thioltransferase. As a result, thioltransferases are also referred to as having the "thioredoxin fold", as seen in Figure 8 where the structures of *E. coli* thioredoxin and pig liver thioltransferase are compared. Note how the active sites are at the N-terminus of an α-helix and the overall structure is that of a central β-sheet surrounded by α-helices.

The active site in pig liver thioltransferase of -Cys22-Pro23-Phe24-Cys25- is found at the amino terminus of helix α2 and Cys 22 is solvent accessible and forms a hydrogen bond with the main-chain nitrogen atom N 25 (3.3 Å) (Figure 9, *140*). Katti *et al.* (*140*) compared the disulfide bridge of pig liver thioltransferase to those of *E. coli* thioredoxin and T4 thioltransferase finding the 14-membered rings to be geometrically very similar. It is believed that the 14-membered disulfide ring of -CXXC- is evolutionarily favored because

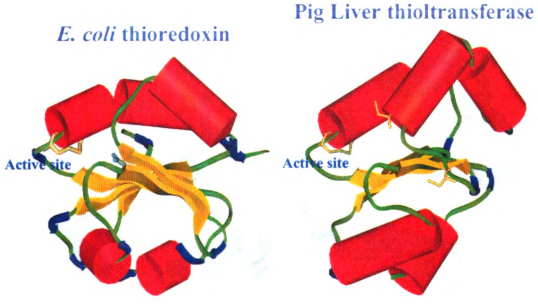



**Figure 8.** Kabsch-Sander representations of the structures of *E. coli* thioredoxin and pig liver thioltransferase. The active site disulfide of each enzyme is shown and is near the N-terminus of an alpha helix (represented by red cylinders).

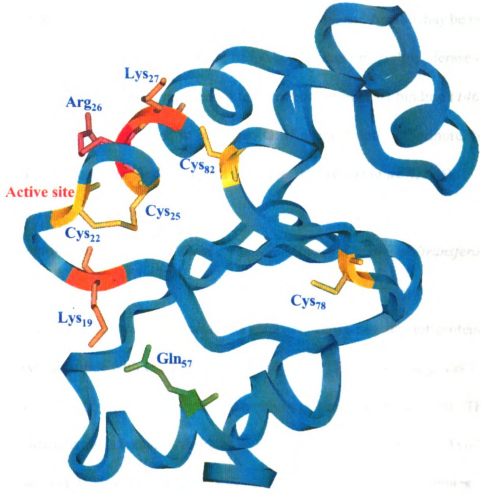



**Figure 9.** Crystal structure of pig liver thioltransferase. The active site disulfide -$C_{22}$-X-X-$C_{25}$- is shown as are $Lys_{19}$, $Arg_{26}$, $Lys_{27}$, $Gln_{57}$, $Cys_{78}$, and $Cys_{82}$.

*IV. C. 4. Glutathione Binding to Thioltransferase.*

The GSH binding site of thioltransferase has been explored in T4 thioltransferase and *E. coli* thioltransferase. Modeling of GSH into the crystal structure of T4 thioltransferase suggested that His-12, Cys-14, Val-15, Cys-17, Tyr-16, Thr-64, and Asp-80 may be involved in GSH binding (*146*). Cys-14 was shown to be the key residue for thioltransferase activity and residues His-12 and Tyr-16 were shown to be important in GSH binding (*146*). The mutation of Asp-80 to Ser resulted in enhanced GSH affinity, and furthermore, in other thioltransferases the comparable residue is a threonine or serine (Figure 4, *146*).

*IV. C. 5. Solution Structure of the Mixed Disulfide Between E. coli Thioltransferase and Glutathione.*

The solution structure of *E. coli* thioltransferase active site mutant protein C14S forming a mixed disulfide with GSH has been solved (*143*). The -SG moiety was found to be localized in a cleft (*143*), which is not seen in pig liver thioltransferase (*140*). The cleft in *E. coli* thioltransferase is bound by residues Tyr-13, Thr-58, Val-59, Tyr-72, Tyr-73, and Asp-74. The overall structure of the C14S-mixed disulfide closely resembles that of oxidized *E. coli* thioltransferase, more so than reduced *E. coli* thioltransferase (*143*). The strongest interactions of the enzyme with -SG were hydrogen bonds seen between the carbonyl oxygen atom of the Cys residue in -SG and the amide proton of Val-59 and the carboxylate group of γ-Glu in -SG and the amide proton of Thr-73 (*143*). In addition, the carboxylate group of γ-Glu of -SG was located at the N-terminus of α helix 3, and therefore the partial positive charge located at the N-terminus of an α-helix may contribute to the binding of GSH to the enzyme (*143*). Weaker interactions between the enzyme and -SG

included hydrogen bonds between the carboxylate group of -SG and the amide protons of Tyr-72 and the OH of Thr-73 and salt bridges between the $NH_3^+$ group of Glu in -SG and the side chain carboxylate of Asp-74 and between the carboxylate group of Gly in -SG and the side chain NH3+ of Lys-45 (*143*). In other thioltransferases, Val and Asp are completely conserved in positions comparable to Val-59 and Asp-74 in the *E. coli* enzyme (Figure 4 ). Tyr-73 in *E. coli* thioltransferase corresponds to either a Tyr or a Ser in nearly all the other thioltransferases (Figure 4). Lys-45 in *E. coli* thioltransferase does not appear to be conserved, but in the crystal structure of pig liver thioltransferase Gln-57 seems to correspond to the position of Lys-45 in *E. coli* thioltransferase (Figure 9).

Most of the interactions between GSH and the C14S mutant *E. coli* thioltransferase outside of the disulfide bond itself were between the γ-Glu moiety of GSH and the enzyme with only one contact between the Gly residue and the enzyme (*143*). Rabenstein and Mills (*147*) studied the thioltransferase catalyzed GSH/GSSG interchange reaction by NMR using pig liver thioltransferase finding the γ-Glu-Cys moiety of GSSG and GSH-containing mixed disulfides to be essential for recognition by thioltransferase. However, the rate constant for catalysis of the GSH/GSSG interchange was 10 fold faster than the rate constant for the catalysis of γ-Glu-Cys/γ-Glu-Cys disulfide interchange (*147*). These results were supported by Srinivasan *et al.* (*148*) who found the γ-Glu-Cys moiety of GSH to be essential for reactivity by human erythrocyte thioltransferase, but thioltransferase catalysis was enhanced further by GSH. These results suggest that the structure of a mixed disulfide between GSH and a -CXXS- mutant of a mammalian thioltransferase would show similar enzyme/GSH interactions to those seen in *E. coli* thioltransferase and GSH (*143*).

## V. Function and Mechanism of Thioltransferase.

### *V. A. Redox Potentials and $pK_a$ of Active Site Cysteines in Thioltransferases.*

Thioltransferases have redox potentials which are more reducing than DsbA or PDI, but not as reducing as thioredoxin, ranging from -0.159 V for pig liver thioltransferase (*149*) to -0.233V for *E. coli* thioltransferase 1 (*150*) (Figure 2). A hallmark of thioltransferase as compared to other thiol:disulfide oxidoreductases is the extremely low $pK_a$ of the N-terminal active site cysteine. Cys-22 in pig liver thioltransferase has a $pK_a$ of 3.8 (*151*), Cys-22 in human thioltransferase a $pK_a$ of 3.8 (*152*), and Cys-26 in yeast thioltransferase a $pK_a$ of 3.5 (*153*). The N-terminal cysteine of pig liver thioltransferase, Cys-22, has been demonstrated to be essential for thioltransferase activity (*151*).

Several factors contribute to the extremely low $pK_a$ of Cys-22. In the crystal structure of pig liver thioltransferase, Cys-22 is situated near the amino terminal end of helix α2, which provides a partial positive charge to stabilize the negative charge on the sulfur atom (*140*). Also, the sulfur atom of Cys-22 forms a hydrogen bond with the main-chain nitrogen atom of Cys-25 (*140*). Yang and Wells carried out a site-directed mutagenesis study of pig liver thioltransferase and determined that Arg-26 was a major contributor to the low $pK_a$ of Cys-22 because the R26V and R26V:K27Q mutant proteins were weakly sensitive to carboxymethylation with an undetermined $pK_a$ for Cys-22 that was pH independent from 2.5 to 8.5 (*151*). Katti *et al.* (*140*) concluded from the crystal structure that Arg-26 had the potential to swing its side chain into the active site without much steric hindrance. The combination of Arg-26, the position of Cys-22 at the amino-terminal end of helix α2, and the sulfur atom hydrogen bond to main-chain nitrogen atom of Cys-25 most likely act

synergistically resulting in the low $pK_a$ of Cys-22 thiol.

*V. B. Protein Deglutathionylation Activity of Thioltransferase.*

Although thioltransferase is capable of catalyzing the GSH-dependent reduction of small molecular weight molecules like S-sulfocysteine and the disulfides cystine and hydroxyethyldisulfide (*112, 152*) this is probably not a significant or important role of thioltransferase *in vivo*. When Axelsson *et al.* (*112*) first described thioltransferase, they also demonstrated its ability to catalyze the reduction of a mixed disulfide of a protein such as lysozyme and GSH. This process now named protein deglutathionylation is where thioltransferase catalyzes the following reaction:

(13) protein-SSG + GSH ———➤protein-SH + GSSG

Protein glutathionylation is reversible, occurs as an early cellular response to oxidative stress (*154-159*), and has been proposed to be an important mechanism to protect proteins from irreversible modification by oxidative stress (*160-161*). The activities of many enzymes including glycogen phosphorylase, glycogen synthase, phosphofructokinase, pyruvate kinase, and protein kinase are modified by thiol:disulfide exchange (*162-163*).

Thioltransferase has been demonstrated to catalyze the GSH-dependent reactivation of oxidatively inactivated pyruvate kinase (*164-165*), ornithine decarboxylase (*166*), human and rat pi class GSH-S-transferases (*167-168*), phosphofructokinase (*169*), and fructose 1,6-bisphosphatase (*170*). Recently, Zheng *et al.* (*171*) demonstrated the autoregulation of the *E. coli* transcription factor OxyR by *E. coli* thioltransferase. When *E. coli* cells were subjected to peroxide stress, OxyR was activated by oxidation and induced the production of thioltransferase (*172*). After induction of thioltransferase and restoration of the

GSH/GSSG ratio, OxyR was reduced and inactivated by thioltransferase (*171*). In none of these cases was reactivation shown to be determined by deglutathionylation of these enzymes.

Gravina and Mieyal (*173*) have argued that thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. They proposed that thioltransferase catalyzes the following reaction:

(14) GSSR + GSH ———➤RSH + GSSG

where the first step in GSH-dependent RSSR reduction is nonenzymatic and forms the GSSR substrate, which thioltransferase then catalytically reduces (*173*). Gravina and Mieyal demonstrated that thioltransferase catalyzed the deglutathionylation of papain-S-SG, BSA-S-SG, and oxyhemoglobin-S-SG but not papain-S-S-cysteine, BSA-S-S-cysteine, hemoglobin-S-S-cysteine, or hemoglogin-S-S-cysteamine (*173*). Gravina and Mieyal's work suggests that the GSH-dependent reactivation of oxidatively inactivated enzymes catalyzed by thioltransferase discussed previously is indeed deglutathionylation. In support of this concept, thioltransferase reactivated inactivated glutathionylated HIV-I protease in a GSH-dependent manner (*174*), and the DNA-binding activity of oxidatively inactivated nuclear factor I (*175*). A shortcoming of Gravina and Mieyal's work is they did not analyze thioltransferase catalyzed GSH-dependent dethiolation of small molecular weight disulfides like cystine or HED. It is possible that a mixed disulfide containing GSH, RSSG, is a favored substrate due to the proposed GSH binding site in thioltransferase. With very large substrates like protein mixed disulfides, steric hindrance may only allow thioltransferase to catalyze the reduction of protein-S-SG bonds, but small disulfides like HED may not face an active site accessibility problem. Most likely, thioltransferase is a specific glutathionyl

mixed protein disulfide oxidoreductase and also a catalyst of GSH-dependent reduction of small molecular weight disulfides.

An additional role for thioltransferase and its catalysis of deglutathionylation has been demonstrated *in vitro* in accelerating PDI catalysis of GSH-dependent folding of ribonuclease A (*176, 177*). The role of thioltransferase in the refolding of ribonuclease resides in thioltransferase's ability to catalyze both the formation and reduction of mixed disulfides containing GSH which are then converted rapidly to intramolecular disulfides in the presence of PDI (*177*).

Under conditions of oxidative stress, thiol:disulfide oxidoreductases like thioltransferase, thioredoxin, and protein disulfide isomerase may all play roles in protein dethiolation. For example, human erythrocyte membranes damaged by diamide treatment were reduced to free sulfhydryls in the presence of thioltransferase (*169*). Thioltransferase and thioredoxin appear to have different substrate specificities with respect to regenerating oxidatively damaged proteins (*178*). Yoshitake *et al.* (*178*) found that thioltransferase regenerated phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase inactivated with mixed disulfide bonds, whereas thioredoxin regenerated phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase inactivated by monothioloxidation to sulfenic or sulfinic acid. In support of these results, Jung and Thomas (*149*) also found thioltransferase to more efficiently catalyze the deglutathionylation of protein mixed disulfides than thioredoxin or protein disulfide isomerase.

*V. C. The Catalytic Mechanism of Thioltransferase.*

The catalytic mechanism of thioltransferase has been studied in detail. Yang and

Wells (*151*) demonstrated by site directed mutagenesis of the gene encoding pig liver thioltransferase that Cys at position 22 is the essential amino acid for activity. Cys-25 in pig liver thioltransferase was not essential for activity because its mutation to a serine led to a 10% increase in relative activity, but the C25A mutant protein had a 91% loss in relative activity (*151*). This result suggested that either a sulfur or an oxygen atom is required at position 25 as part of the catalytic mechanism, but the placement of the hydrophobic amino acid alanine at position 25 may have disrupted the stability of the enzyme complicating the interpretation of these results (*151*). Arg at position 26 and Lys at position 27 were also shown to be important in the mechanism because the R26V, K27Q, and R26V:K27Q mutant proteins had a loss in relative activity of 68%, 33%, and 95%, respectively (*151*). As discussed earlier, Arg-26 was determined to play a major role in the nucleophilicity of Cys-22, but the role of Lys-27 in the mechanism was not determined.

Using proteins isolated from these site directed mutants, Yang and Wells (*179*) then determined the catalytic mechanism of pig liver thioltransferase using S-sulfocysteine (Cys-$SO_3^-$) and the small molecular weight disulfides cystine and hydroxyethyldisulfide (HED). Preincubation of wild type recombinant pig liver thioltransferase (RPLTT) and the C25S, R26V, and K27Q mutant proteins with iodoacetamide (IAM) yielded residual activities of approximately 10%, 0%, 70%, and 15%, respectively (*179*). Thus, R26V was only partially inactivated which was expected based on the importance of Arg-26 in the nucleophilicity of Cys-22. Preincubation of RPLTT, C25S, R26V, and K27Q with GSH prior to the addition of IAM did not provide protection against IAM inactivation and actually potentiated the inactivation of RPLTT, R26V, and K27Q (*179*). RPLTT, R26V, and K27Q were partially or fully protected from IAM inactivation by preincubation with Cys-$SO_3^-$, GSH + Cys-$SO_3^-$,

and HED (*179*). In contrast, C25S was only protected from IAM inactivation by preincubation with GSH + Cys-$SO_3^-$ (*179*).

RPLTT, R26V, and K27Q have different isoelectric points in the oxidized and reduced form because of the formation of an intramolecular disulfide bond upon oxidation with the subsequent loss of the negative charge of Cys-22 (*151*). To analyze the oxidation state of the enzymes and possibly demonstrate the presence of enzyme-substrate intermediates from the preincubation mixtures, Yang and Wells (*179*) carried out isoelectric focusing studies on RPLTT and the C25S mutant. RPLTT has a pI of 7.0 and a pI of 8.0 in the reduced and oxidized forms, respectively (*151*). Oxidized RPLTT was found by IEF when reduced RPLTT was treated with HED, cystine, Cys-$SO_3^-$, cystine + GSH, and GSH + Cys-$SO_3^-$ (*179*). Reduced RPLTT was also detected when RPLTT was incubated with Cys-$SO_3^-$, cystine + GSH, and GSH + Cys-$SO_3^-$ (*179*). Finally, a possible enzyme-substrate intermediate was seen when RPLTT was incubated with Cys-$SO_3^-$ (*179*). The protection against IAM inactivation by preincubating RPLTT with HED, Cys-$SO_3^-$ , and Cys-$SO_3^-$ + GSH was most likely due to the formation of oxidized enzyme, but may also be due to the formation of an enzyme-substrate intermediate when RPLTT was preincubated with Cys-$SO_3^-$ (*179*). These results demonstrated the ability of the disulfide substrate (RSSR) to bind to reduced enzyme forming oxidized enzyme and releasing 2 RSH (reactions 1 and 2 in Figure 10).

The C25S mutant protein does not possess the ability to form an intramolecular disulfide at the active site. IEF of C25S preincubated with HED, cystine, and Cys-$SO_3^-$ demonstrated the formation of enzyme-substrate intermediates, but HED and Cys-$SO_3^-$ preincubation with C25S did not protect against IAM inactivation (*179*). These results

suggested that the enzyme-substrate intermediates were in equilibrium with reduced enzyme + substrate, and reduced enzyme was susceptible to IAM inactivation (reactions 1 and 6 in Figure 10). Enzyme-substrate intermediates of RPLTT and C25S were demonstrated by radioactive labeling of each enzyme with L-[$^{14}$C]-cystine and Cys-$SO_3^-$ + [$^3$H]-GSH but not [$^3$H]-GSH alone demonstrating the ability of the disulfide substrate to bind to thioltransferase before GSH, a result supported by IAM inactivation studies (*179*).

Yang and Wells (*179*) propose a mechanism for thioltransferase that is shown in Figure 10. In this mechanism, the disulfide substrate RSSR binds first to the enzyme releasing RSH and forming the mixed disulfide ES-SR (reaction 1, Figure 10). Enzymes possessing both cysteines at the active site could then undergo two possible pathways where (1) Cys-25 attacks Cys-22 releasing RSH and forming an intramolecular disulfide (reaction 2, Figure 10) which is then attacked by GSH forming the mixed disulfide ES-SG (reaction 3, Figure 10), or (2) the first molecule of GSH attacks ESSR forming ES-SG and releasing RSH (reaction 4, Figure 10). Lacking the second active site cysteine, the C25S mutant protein can only undergo reaction 4. ES-SG is then reduced by the second molecule of GSH to form $ES^-$ releasing GSSG to complete the catalytic cycle (reaction 5, Figure 10). Reaction 6 demonstrates the irreversible modification of reduced enzyme by IAM.

Gravina and Mieyal (*173*) proposed a different mechanism for thioltransferase as shown in Figure 11. Recall that Gravina and Mieyal provided evidence for thioltransferase being a specific glutathionyl mixed disulfide oxidoreductase (*173*), but they only studied protein mixed disulfides. They proposed that of reactions (15) and (16) thioltransferase only catalyzes reaction (16)

(15) RSSR′ + GSH ⇌ RSH + GSSR′

(16) GSSR′ + GSH ⇌ GSSG + R′SH

with reaction (15) occurring spontaneously (*173*). This may be true for protein mixed disulfides due to steric constraints because of the size of the substrate, but Yang and Wells provide strong evidence that thioltransferase catalyzes both reactions (15) and (16) with small molecular weight molecules like Cys-$SO_3^-$ or the disulfides HED and cystine (*173*). The Gravina and Mieyal mechanism for thioltransferase (Figure 11) is a ping pong mechanism consistent with the observed parallel lines in two-substrate kinetic studies (*173*). As stated previously most likely a major *in vivo* function of thioltransferase is to catalyze protein deglutathionylation, and therefore the mechanism proposed by Gravina and Mieyal may be important.

*V. D. The Dehydroascorbate Reductase Activity of Thioltransferase.*

It was long believed that thioltransferase was only a GSH-dependent thiol:disulfide oxidoreductase, but in 1990 Wells *et al.* (*75*) demonstrated that human placenta, bovine thymus, and pig liver thioltransferases possessed GSH-dependent DHA reductase activity. Subsequently, thioltransferase from vaccinia virus (*74*) and human neutrophils (*77*) were demonstrated to possess GSH-dependent DHA reductase activity. There are conflicting reports concerning the DHA reductase activity of plant thioltransferase. Sha *et al.* (*124*) reported that purified rice thioltransferase has DHA reductase activity, although the same group reported that cloned rice thioltransferase does not have DHA reductase activity (*132*). In addition, Morell *et al.* (*180*) reported that purified spinach thioltransferase lacks DHA reductase activity.

The identification of thioltransferase as a DHA reductase raised the fundamental

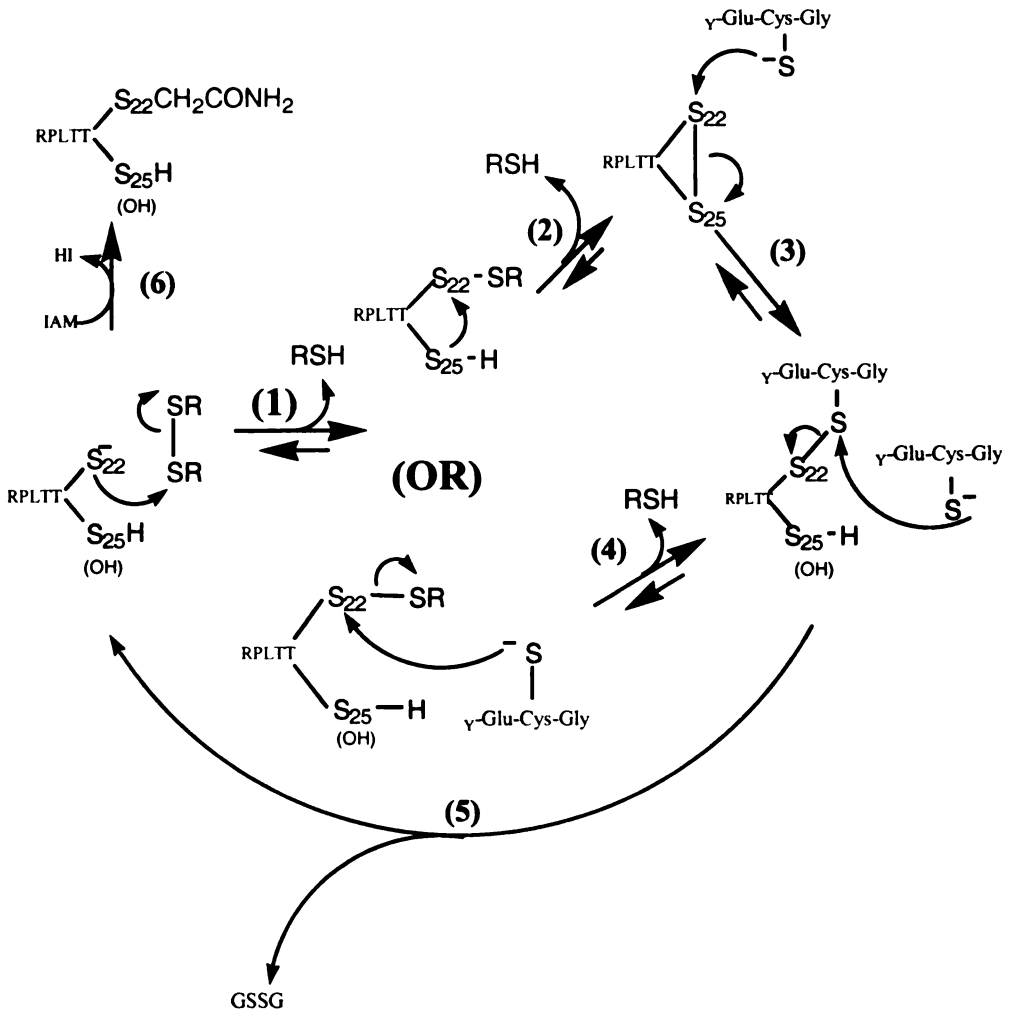



**Figure 10**. Proposed catalytic mechanism of thioltransferase by Yang and Wells (*179*). The C25S mutant is represented by (OH) placed under Cys-25.

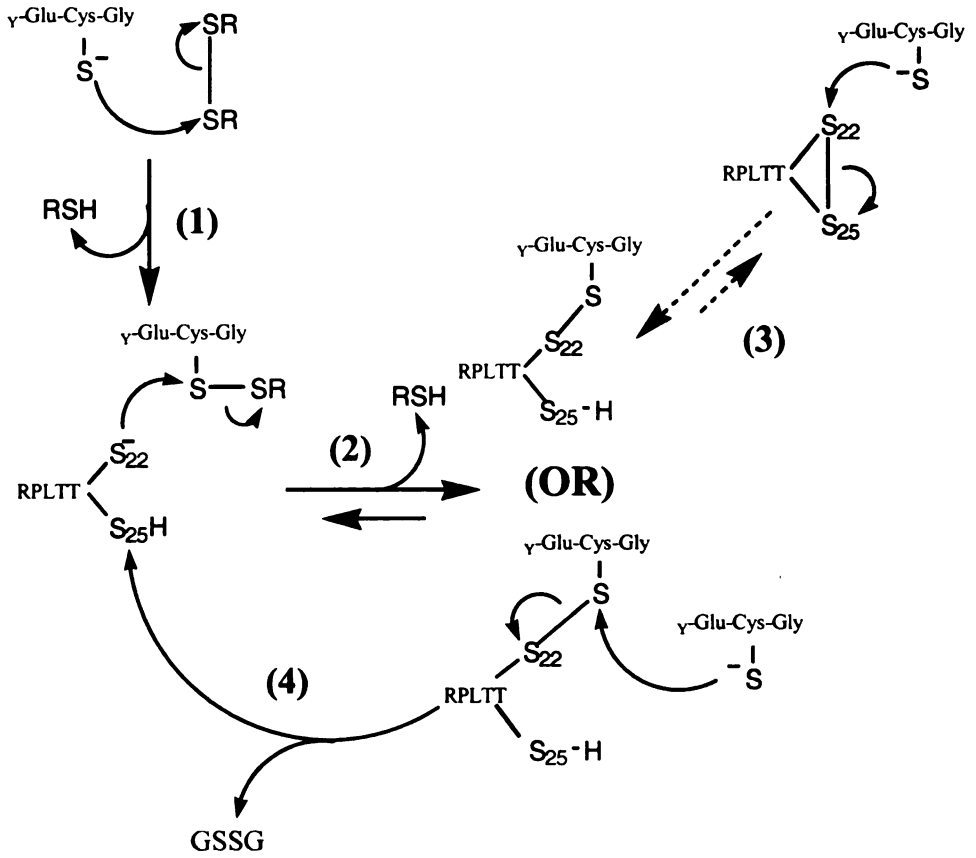



**Figure 11**. Mechanism of thioltransferase proposed by Gravina and Mieyal (*173*). In this reaction mechanism, Gravina and Mieyal propose that GSH chemically reacts first with the disulfide substrate RSSR forming RSSG and releasing RSH (reaction 1). Cys-22 of thioltransferase then attacks the sulfur group of GSH in the RSSG substrate releasing RSH forming a mixed disulfide between enzyme and -SG (reaction 2). The mixed disulfide is then reduced by a second molecule of GSH forming GSSG and reduced enzyme completing the reaction cycle ( reaction 4).

enzyme structure/function question, does thioltransferase have analogous/different catalytic mechanisms for its DHA reductase and thiol:disulfide oxidoreductase activities? Yang and Wells (*151*) provided initial evidence supporting analogous mechanisms when they compared the relative DHA reductase activities of RPLTT to the variant proteins and found: C22S (no activity), C25S, (191% activity), R26V (30% activity), K27Q (73% activity), R26V:K27Q and C25A (both with no activity). These results demonstrated that Cys-22 is the essential amino acid for both the thiol:disulfide oxidoreductase and DHA reductase activities of thioltransferase (*151*). In addition, Arg-26 and Lys-27 are important for both activities and Ser can replace Cys at position 25 in both activities, but Ala cannot (*151*). Surprisingly, the C25S mutant protein had nearly double the relative DHA reductase activity of the wild type but only 10% more thioltransferase activity (*151*). Yang and Wells proposed a DHA reductase mechanism analogous to that of thioltransferase as shown in Figure 12 (*179*). In this mechanism, they proposed the formation of a thiohemiketal intermediate which is releasable by GSH, but not by attack of Cys-25 on Cys-22, forming AA and the mixed disulfide ES-SG (*179*). Similarly, Mieyal *et al.* (*84*) proposed the formation of the intramolecular disulfide and release of AA in the DHA reductase activity of thioltransferase as one possible mechanism. However, based on their studies which suggested that thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase, Mieyal *et al.* (*84*) also proposed that the true substrate for thioltransferase is DHA-SG (Figure 13).

*V. E. Other Potential Non-disulfide Substrates for Thioltransferase.*

It is possible that other non-disulfide substrates for thioltransferase exist. For example, the thioredoxin/thioredoxin reductase system has been shown to catalyze the

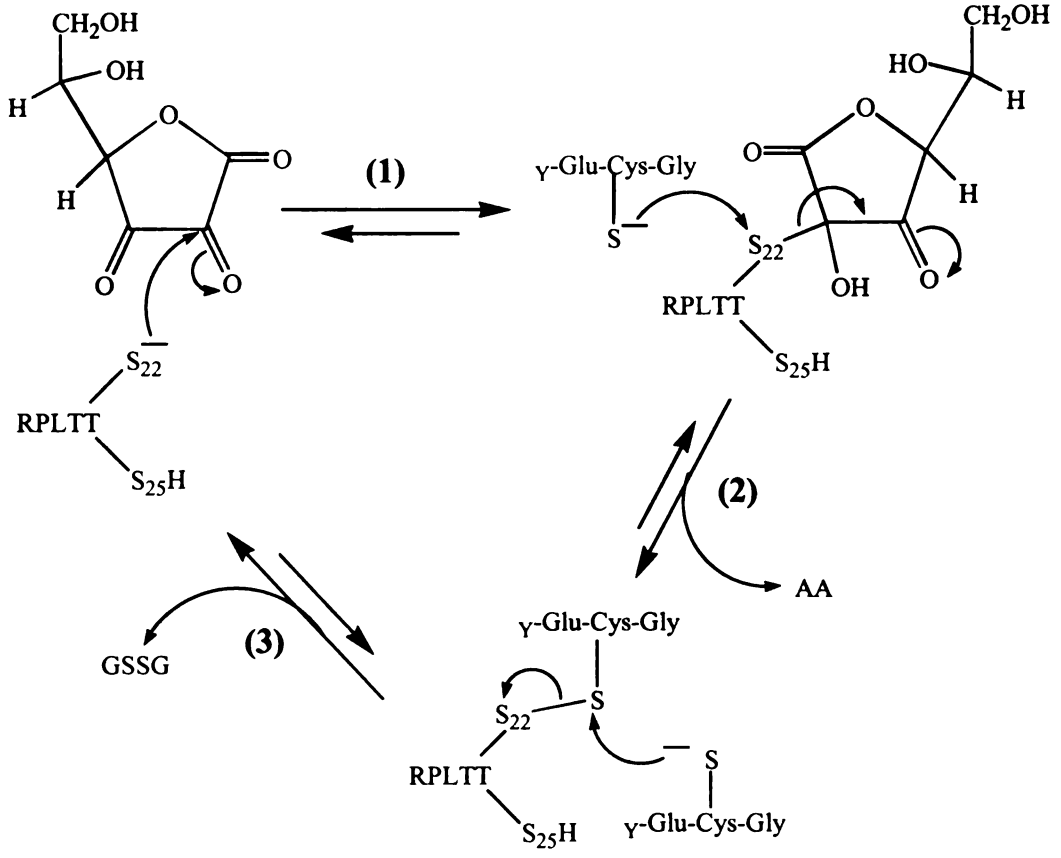



**Figure 12.** Mechanism of thioltransferase's DHA reductase activity proposed by Yang and Wells (*179*). In this mechanism, reduced enzyme attacks C1 of DHA forming a thiohemiketal intermediate (reaction 1). GSH then attacks Cys-22 releasing AA and forming a mixed disulfide between enzyme and -SG (reaction 2). A second molecule of GSH then attacks the sulfur of the GSH in the mixed disulfide releasing GSSG and restoring the reduced form of the enzyme (reaction 3).

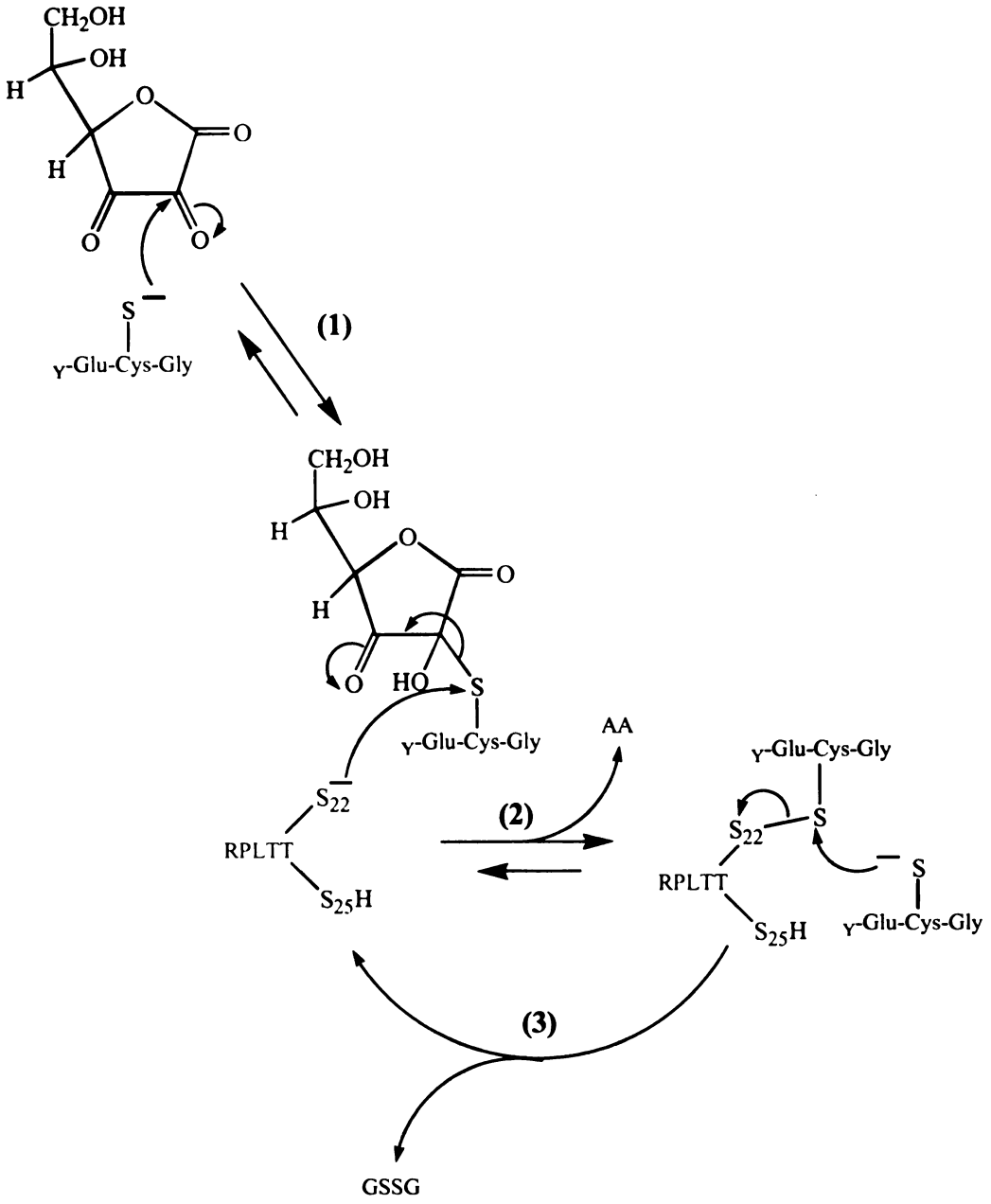


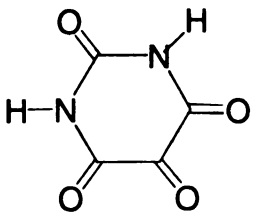

**Figure 13.** Alternative mechanism for the DHA reductase activity of thioltransferase proposed by Mieyal *et al.*(*84*). In this mechanism, DHA reacts with GSH chemically forming a thiohemiketal intermediate in reaction (1). Cys-22 of thioltransferase attacks the sulfur atom of GSH forming a mixed disulfide between enzyme and -SG releasing AA in reaction (2). A second molecule of GSH attacks the sulfur atom of the GSH in the mixed disulfide releasing GSSG and forming reduced enzyme to complete the catalytic cycle.

NADPH dependent reduction of the diabetogenic agent alloxan to dialuric acid (*101*). Based on the structural similarities between alloxan and DHA (Figure 14), thioltransferase may be a GSH-dependent alloxan reductase.

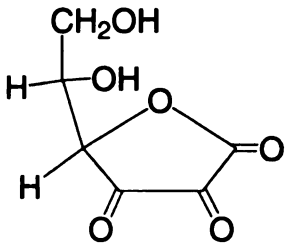
*V. E. 1. Alloxan Toxicity to the 2-cells of the Pancreas.*

Alloxan (2,4,5,6 [1H,3H] pyrimidinetetrone) is selectively toxic to the 2-cells of the pancreas of certain animals, e.g., mice and rats (*181-183*). This toxicity has been attributed in part to accumulation of alloxan in the islets (*184. 185*) and 2-cells (*186*). Furthermore, the pancreas possesses relatively lower levels of enzymes such as catalase, Cu-Zn superoxide dismutase (SOD), Mn-SOD, and glutathione peroxidase, which protect cellular components against reactive oxygen species (*185, 187*). The toxic mechanism of alloxan involves redox cycling between alloxan and dialuric acid. Reduction of alloxan to dialuric acid by physiological reagents has been demonstrated with NAD(P)H (*188, 189*) and GSH (*190-193*) and enzymatically by the thioredoxin/NADPH-thioredoxin reductase system (E.C. 1.6.4.5) (*101*). Dialuric acid oxidation generates superoxide, hydrogen peroxide, and hydroxyl radical, in the presence of an appropriate metal catalyst like iron (*194-197*). Specifically, reaction of alloxan with GSH generated superoxide and hydrogen peroxide (*190-193*), hydroxyl radical in the presence of iron (*197*), and released iron from ferritin and subsequently formed hydroxyl radical (*198*). Evidence supporting this mechanism includes protection against alloxan toxicity in isolated islets by SOD, catalase, hydroxyl radical scavengers, metal chelators (*199-201*), and in mice, *in vivo*, by SOD (*202*). If thioltransferase does catalyze the GSH-dependent reduction of alloxan to dialuric acid, then thioltransferase may play a role in the toxicity of alloxan.

**Oxidized Forms:**

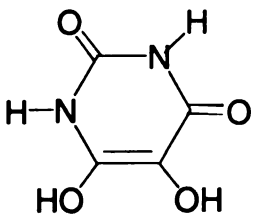


**Alloxan**

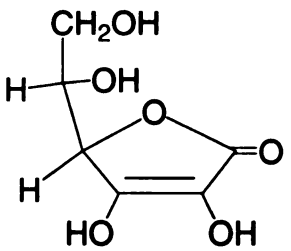


**Dehydroascorbic Acid**

**Reduced Forms:**



**Dialuric Acid**



**L-Ascorbic Acid**

**Figure 14**. Structural comparison of alloxan to dehydroascorbic acid and dialuric acid to L-ascobic acid

## VI. Objectives of this Thesis.

The objectives of my work in this thesis are threefold. First, I wish to develop a reproducible GSH-dependent DHA reductase activity assay and to kinetically characterize and compare the rapidly growing class of mammalian DHA reductases. Second I seek to characterize the catalytic mechanism of the GSH-dependent DHA reductase activity associated with thioltransferase. This will provide a foundation for studies of other GSH-dependent DHA reductases and will answer the enzyme structure/function question, does thioltransferase have analogous/different catalytic mechanisms for its GSH-dependent DHA reductase and thiol:disulfide oxidoreductase activities. Third I will determine whether other non-disulfide substrates for thioltransferase exist, specifically alloxan, and if thioltransferase is a GSH-dependent alloxan reductase, might thioltransferase contribute to the toxicity of alloxan.

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# Chapter II

## Kinetic Characterization and Comparison of Mammalian Dehydroascorbate Reductases

This chapter is the combination of the contributions made to the following papers: (1) Wells, W.W., Xu, D.P., and Washburn, M.P. (1995) *Methods. Enzymol.* **252**, 30-38 and (2) Xu, D.P., Washburn, M.P., Sun, G.P., and Wells, W.W. (1996) *Biochem. Biophys. Res. Commun.* **221**, 117-121.

ABSTRACT

Several glutathione (GSH)-dependent dehydroascorbate (DHA) reductases (EC 1.8.5.1) were purified to homogeneity and their DHA reductase activities kinetically characterized. Recombinant pig liver thioltransferase (RPLTT) was the most robust DHA reductase at pH 6.9 with a $k_{cat}/K_m$ for DHA of 2.43 ± 0.85 x $10^4$ $M^{-1}$ $sec^{-1}$ followed by human erythrocyte 32 kDa DHA reductase with a $k_{cat}/K_m$ for DHA of 1.49 ± 0.21 x $10^4$ $M^{-1}$ $sec^{-1}$, with bovine liver protein disulfide isomerase the weakest DHA reductase with a $k_{cat}/K_m$ for DHA of 93 ± 14 $M^{-1}$ $sec^{-1}$. Human erythrocyte 32kDa DHA reductase and RPLTT were equally robust DHA reductases at pH 7.2 with a $k_{cat}/K_m$ for DHA of 2.47 ± 0.64 x $10^4$ $M^{-1}$ $sec^{-1}$ and a $k_{cat}/K_m$ for DHA of 2.01 ± 0.13 x $10^4$ $M^{-1}$ $sec^{-1}$. The DHA reductase activities of these enzymes and several others from mammals are compared and contrasted. Based on kinetic comparisons, the most efficient and most likely to play an important *in vivo* role in DHA reduction are thioltransferase and the 32kDa GSH-dependent DHA reductase.

Ascorbic acid (AA) is an important antioxidant (*1-6*) and electron donor for enzymes like 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 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 rapidly disproportionate to AA and DHA (*12*) or be directly reduced to AA by an NADH-semiDHA reductase (*13-15*). Currently, NADH-semiDHA reductase has not been purified to homogeneity nor kinetically characterized.

DHA is toxic to cells and tissues as highlighted by its in vitro toxicity to pancreatic islets (*16-17*) and erythrocytes (*18*). DHA is taken up preferentially by cells like human erythrocytes (*19*), neutrophils (*20*), and fibroblasts (*20*) and rapidly reduced to AA. May *et al* (*19*) determined that DHA reduction by human erythrocytes does not involve semidehydroascorbate. Therefore, recycling of DHA is important especially in species like humans whose only source of AA is from the diet.

DHA can be reduced to AA in an NADPH dependent manner by both 3α-hydroxysteroid dehydrogenase (*21*) and thioredoxin reductase (*22*). However, Glutathione (GSH) deficiency in newborn rats, which compared with adults synthesize AA at a reduced rate, caused drastic ascorbate decreases in tissues highlighting the importance of GSH in the recycling of AA (*23*). GSH chemically reduces DHA to AA, especially at alkaline pH, and three mammalian GSH-dependent DHA reductases have been identified: thioltransferase

(glutaredoxin, *24*), protein disulfide isomerase (PDI, *24*), and a 32kDa enzyme (32kDHAR) identified in rat liver (*25*) and human erythrocytes (*26*). Whether DHA reduction *in vivo* is primarily chemical, enzymatic, or both remains unclear with evidence existing for both mechanisms (*27-28*). However, GSH reduction of DHA to AA has been estimated to have a $t_{1/2}$ of 15 min at pH 7.5 and at 25°C (*29*), but under the same conditions the degradation of DHA to non recyclable products like 2,3-diketogulonic acid has a $t_{1/2}$ of 2 minutes (*30*) strongly supporting the importance of catalytic regeneration of AA from DHA. In addition, recent work by Park & Levine (*31*) provided evidence for enzymatic recycling of DHA in human neutrophils, catalyzed in large part by thioltransferase. In this chapter, the GSH-dependent DHA reductase activities of several mammalian enzymes are kinetically characterized and compared.

## MATERIALS AND METHODS

*Materials.* EDTA, glutathione disulfide (GSSG), bovine pancreatic insulin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO. GSH, dithiothreitol (DTT), glutathione disulfide reductase, and NADPH were products of Boehringer Mannheim, Indianapolis, IN. Bromine was purchased from Acros Organics, Pittsburgh, PA. Hydroxyethyldisulfide (HED) was a product of Aldrich, Milwaukee, WI. Isopropylthiogalactoside (IPTG) was acquired from Calbiochem, San Diego, CA. CM-Sepharose, CM-Sephadex C50, DEAE-Sephacel, Sephacryl S-200, and Sephadex G75 were products of Amersham Pharmacia Biotech, Piscataway, NJ. Sodium phosphate was purchased from EM Science, Gibbstown, NJ. Triton X-100 was a product of Research Products International, Elk Grove Village, IL. S-sulfocysteine was prepared by the method of Segel and Johnson (*32*). DHA was prepared as described previously (*33*). Human

erythrocyte 32 kDA GSH-dependent DHA reductase was purified by Dian Peng Xu, as described (*26*).

*Purification of Pig Liver Thioltransferase.* Expression of the thioltransferase gene and purification of RPLTT was carried out as described previously (*34*) 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 x 2L) against the same buffer. After dialysis, the sample was loaded onto a Sephacryl S-200 column ( 2.5 cm x 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 x 38 cm) equilibrated with buffer A. The column was then 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 0.0-1.0 M NaCl linear gradient in buffer B. The active fractions were pooled and concentrated using Centriprep 3 concentrators and stored at -70°C until use. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Company) according to the manufacturer's directions.

*Assay of thioltransferase activity.* During the purification, the enzyme activity was assayed as described previously (*34*). Samples were assayed in 0.318 mM sodium phosphate, 1.0 mM EDTA, 0.5 mM GSH, 0.34 mM NADPH, 5 $\mu$g GSSG reductase, and 2.5 mM S-sulfocysteine in a final volume of 500 $\mu$l. Upon the addition of S-sulfocysteine , the mixture was assayed at 340 nm for 3 min at 30°C. A control without sample was run simultaneously for each assay and the difference (($\Delta Abs_{340nm}$/min) x 0.5 x 1000/6.22)) gave activity in nmoles/min.

*Purification of Bovine Liver Protein Disulfide Isomerase.* Bovine liver PDI was purified through a modification of the procedure described by Hilson *et al* (*35*). Frozen bovine liver was thawed and washed in 0.9% w/v NaCl. It was homogenized in a Waring blender in 1 L of 100 mM sodium phosphate, 5 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, pH 7.50. After straining of the homogenate through two layers of muslin, the sample was centrifuged at 18,000 x *g* for 30 min. The supernatant was decanted through glass wool and heated to 58°C for 15 min in a 70°C water bath. The supernatant was transferred to an ice bath to cool and centrifuged at 18,000 x *g* for 40 min. Ammonium sulfate was added to the supernatant from the heat denaturation to reach a final concentration of 55% saturation. After stirring for 30 min the sample was centrifuged at 18,000 x g for 30 min and ammonium sulfate was further added to the supernatant to reach 85% saturation. After stirring for 30 min the sample was centrifuged at 18,000 x *g*. The pellet was resuspended in a minimal volume of 25 mM citrate, pH 5.3, and dialyzed overnight against the same buffer (2 x 5L).

Dialyzed extract was applied to a Sephadex G75 column equilibrated with 25 mM citrate, pH 5.3, and eluted with the same buffer. Active fractions were pooled and applied to a CM-Sephadex C50 column equilibrated with 25 mM citrate, pH 5.3 and eluted with the same buffer. Active fractions were pooled and ammonium sulfate added to reach a final concentration of 90%. After stirring for 30 min the sample was centrifuged at 18,000 x *g* for 30 min. The pellet was resuspended in a minimal volume of 25 mM sodium phosphate, 1 mM EDTA, 0.1 M NaCl, pH 8.0 and dialyzed 2 x 2L overnight against the same buffer.

Dialyzed sample was loaded onto a DEAE-Sephacel column equilibrated with 25 mM sodium phosphate, 1 mM EDTA, 0.1 M NaCl, pH 8.0. After loading, the column was washed with two volumes of 25 mM sodium phosphate, 1 mM EDTA, 0.1 M NaCl, pH 8.0.

PDI was eluted with 0.1-0.5 M NaCl gradient in 25 mM sodium phosphate, 1 mM EDTA, pH 8.0. Active fractions were concentrated in Centriprep 10 concentrators, diluted with 25 mM sodium phosphate, 2.5 mM EDTA, pH 7.50, and concentrated again.

*Assay of Protein Disulfide Activity.* Bovine liver PDI activity was assayed by the insulin reduction assay of Holmgren (*36*). Sample was assayed in 100 mM potassium phosphate, 2 mM EDTA, pH 7.0, 1mM DTT, and 0.06 mM insulin in a final volume of 500 $\mu$l. Upon the addition of insulin the sample was assayed at 650 nm at 30°C for 30 min. A control without sample was run simultaneously for each assay. An increase in absorbance at 650 nm was indicative of activity.

*Dehydroascorbic Acid Reductase Assay and Enzyme Kinetics.* DHA reductase activity was assayed as described previously (*33*). Initial reaction velocities were compared at various pH values using 200 mM sodium phosphate, 1 mM EDTA buffers between pH 5.5 and 7.5. Assays performed to determine the kinetic constants for RPLTT ( 0.72 $\mu$g), bovine liver PDI (69$\mu$g), and human erythrocyte 32kDa-DHA reductase (1.85 $\mu$g for DHA profile and 1.575 $\mu$g for GSH profile) were run in 200 mM sodium phosphate, 1 mM EDTA, pH 6.9, in a final volume of 500 $\mu$l at 30°C for 3 min and recorded at 265.5 nm. Assays performed to determine the kinetic constants for RPLTT (1 $\mu$g) and human erythrocyte 32kDa DHA reductase (2.3 $\mu$g) were also run in 100 mM sodium phosphate, pH 7.2, in a final volume of 500 $\mu$l at 30°C for 3 min and recorded at 265.5 nm. A blank without enzyme was run simultaneously with each assay and the difference gave the activity in nmoles/min. Kinetic constants for DHA were determined by varying [DHA] and holding [GSH] = 3.0 mM, and kinetic constants for GSH were determined by varying [GSH] and holding [DHA] = 1.5 mM. $K_m$(app) and $V_{max}$(app) values were calculated by nonlinear least-squares fitting

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.

## RESULTS

*DHA reductase activity of bovine liver PDI, RPLTT, and human erythrocyte 32kDa DHA reductase.* The kinetic plots for the DHA reductase activity of bovine liver PDI at pH 6.9 (Figure 1A and 1B), RPLTT at pH 6.9 (Figure 2A and 2B) and 7.2 (Figure 3A and 3B), and human erythrocyte 32 kDa DHA reductase at pH 6.9 (Figure 4A and 4B) and at pH 7.2 (Figure 5A and 5B) best fit the Michaelis-Menten equation (EZ-Fit Kinetic Program, *37*). The results of these kinetic analyses are found in Table I. Although L-cysteine and cysteamine chemically reduce DHA to AA, neither bovine liver PDI, RPLTT, or human erythrocyte 32 kDa DHA reductase was able to catalyze this reaction demonstrating a specificity of GSH.

## DISCUSSION

The results in this chapter contribute to the growing body of literature concerning DHA reductases. A large number of DHA reductases in many species have been identified and kinetically characterized. These include the GSH-dependent DHA reductases like thioltransferases from vaccinia virus (*39*) pig liver (*24, 33*), human placenta (*22*), and human neutrophil (*31*), the 32kDa-DHA reductase isolated from rat liver (*25*) and human erythrocytes (*26*), bovine liver PDI (*22, 24, 33*), the p52 enzyme from *Trypanosoma cruzi* (*38*), a DHA reductase from spinach chloroplasts homologous to plant trypsin inhibitor (*40*), and a 24 kDa DHA reductase from spinach leaves (*41*). In addition, NADPH-dependent DHA reductases have been identified which include rat liver thioredoxin/thioredoxin

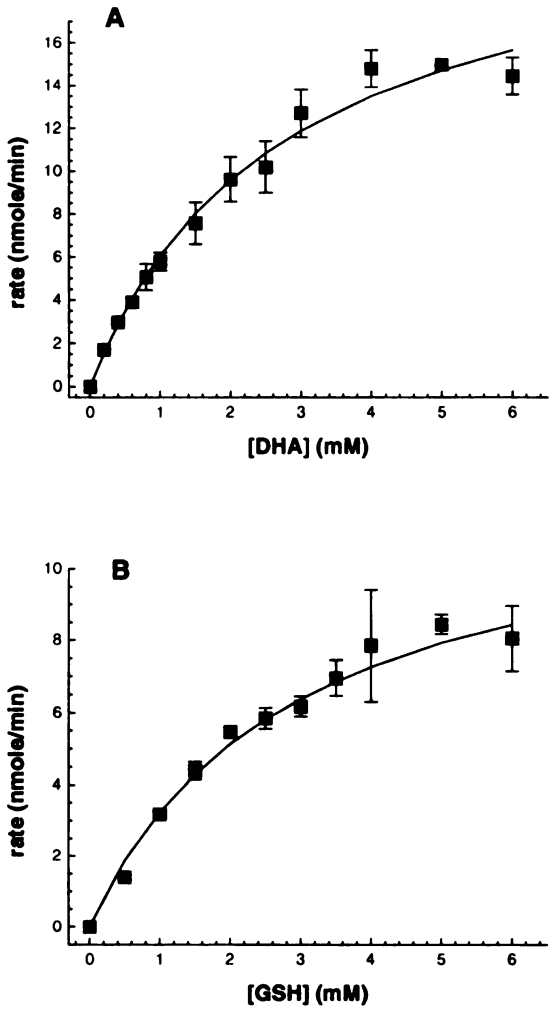


**Figure 1.** Kinetic analysis of the DHA reductase activity of bovine liver PDI at pH 6.9. Bovine liver PDI was assayed for DHA reductase activity as described in the Methods section. Kinetic constants for DHA were determined by varying [DHA] as shown and holding [GSH] = 3.0 mM (A), and kinetic constants for GSH were determined by varying [GSH] as shown and holding [DHA] = 1.5 mM (B). Each data point (-■-) is the average of 3 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.

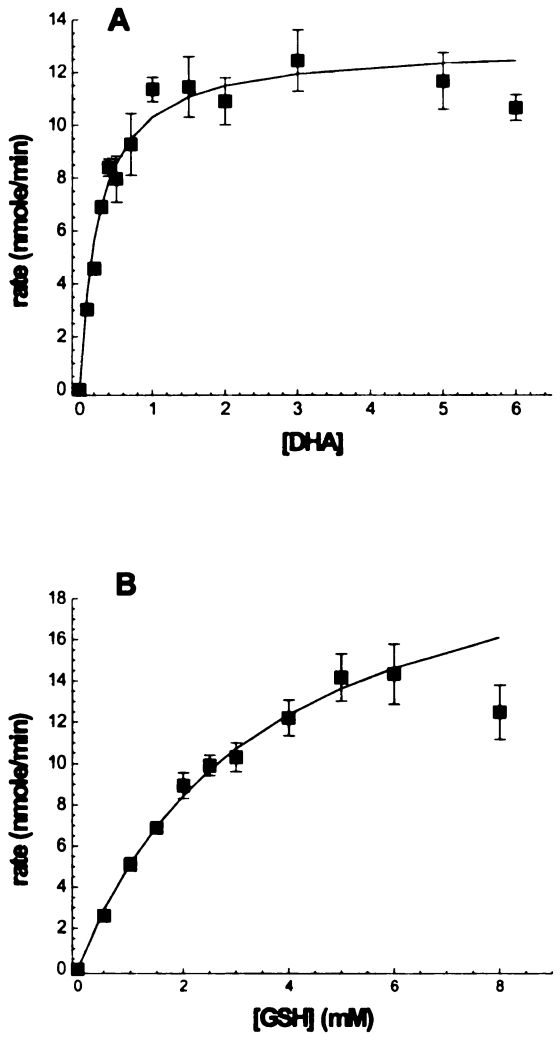


**Figure 2.** Kinetic analysis of the DHA reductase activity of RPLTT at pH 6.9. RPLTT was assayed for DHA reductase activity as described in the Methods section and Figure 1. Each data point (-■-) is the average of 3 separate experiments. Error bars represent one standard deviation of the data.

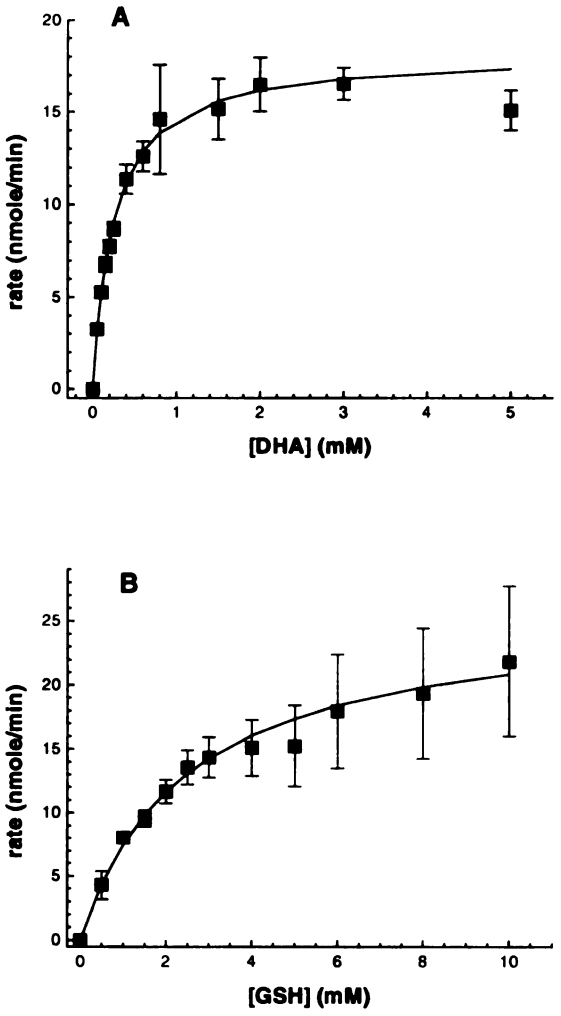


**Figure 3.** Kinetic analysis of the DHA reductase activity of RPLTT at pH 7.2. RPLTT was assayed for DHA reductase activity as described in the Methods section and Figure 1. Each data (-■-) point is the average of 3 separate experiments. Error bars represent one standard deviation of the data.

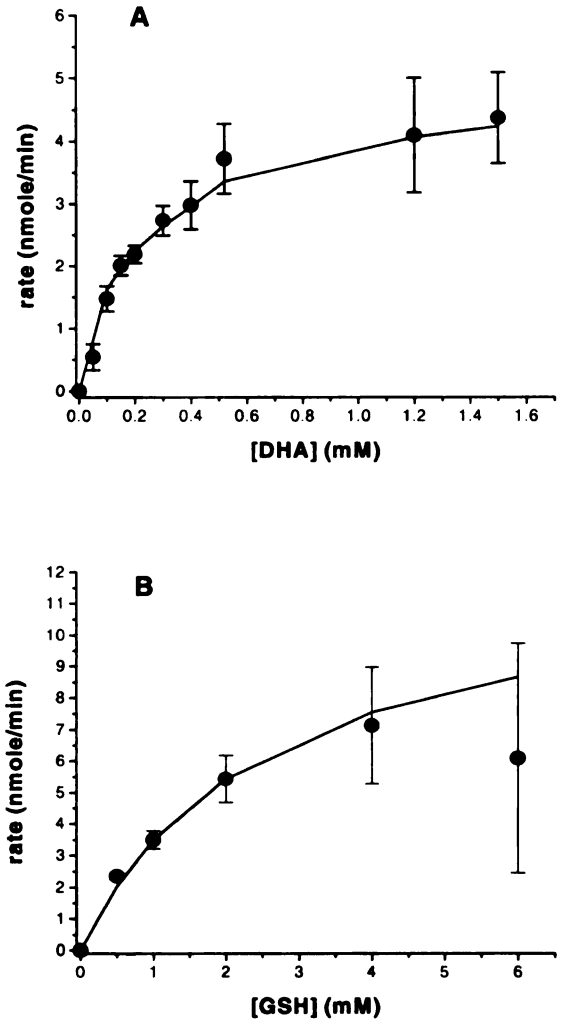


**Figure 4.** Kinetic analysis of the DHA reductase activity of human erythrocyte 32kDa DHA reductase at pH 6.9. Human erythrocyte 32kDa DHA reductase (1.85 $\mu$g for DHA profile and 1.575 $\mu$g for GSH) profile was assayed for DHA reductase activity as described in the Methods section and Figure 1. Each data point (-●-) is the average of 3 separate experiments. Error bars represent one standard deviation of the data.

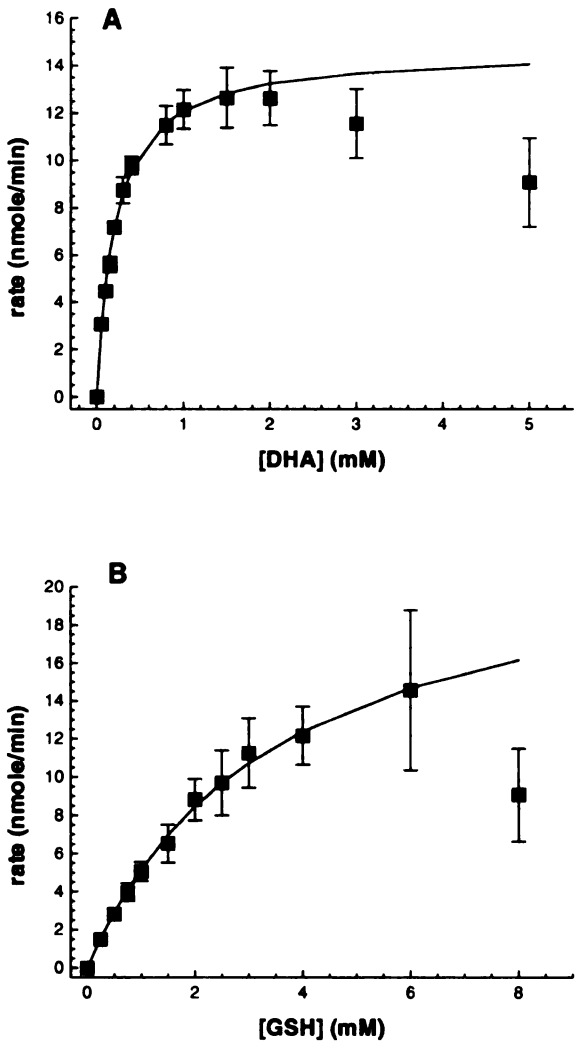


**Figure 5.** Kinetic analysis of the DHA reductase activity of human erythrocyte 32kDa DHA reductase at pH 7.2. Human erythrocyte 32kDa DHA reductase (2.3 $\mu$g) was assayed for DHA reductase activity as described in the Methods section and Figure 1. Each data point (-■-) is the average of 3 separate experiments. Error bars represent one standard deviation of the data.

Table I
Kinetic Parameters for Mammalian Dehydroascorbate Reductases

| Parameter | Human RBC DHA Reductase[a] | Human RBC DHA Reductase[b] | Pig Liver Thioltransferase[a] | Pig Liver Thioltransferase[b] | Bovine Liver Protein Disulfide Isomerase[a] |
|---|---|---|---|---|---|
| $k_{cat}$ ($min^{-1}$)[c] | 251 ± 2 | 316 ± 1 | 374 ± 20 | 305 ± 10 | 16 ± 1 |
| $K_m$(app) (mM)[d] | | | | | |
| DHA | 0.28 ± 0.04 | 0.21 ± 0.06 | 0.26 ± 0.09 | 0.25 ± 0.01 | 2.8 ± 0.4 |
| GSH | 2.53 ± 0.13 | 3.5 ± 0.3 | 3.5 ± 0.3 | 2.5 ± 0.2 | 2.9 ± 0.4 |
| $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) | | | | | |
| DHA | $1.49 \pm 0.21 \times 10^4$ | $2.47 \pm 0.64 \times 10^4$ | $2.43 \pm 0.85 \times 10^4$ | $2.01 \pm 0.13 \times 10^4$ | 93 ± 14 |
| GSH | $1.65 \pm 0.09 \times 10^3$ | $1.51 \pm 0.11 \times 10^3$ | $1.81 \pm 0.2 \times 10^3$ | $2.05 \pm 0.19 \times 10^3$ | 91 ± 14 |

[a] Kinetic constants determined as described in Methods at pH 6.9.
[b] Kinetic constants determined as described in Methods at pH 7.2.
[c] $k_{cat}$ values were calculated by dividing $V_{max}$(app) by the molar concentration of enzymes.
[d] $K_m$(app) values were calculated by nonlinear least-squares fit to the velocity versus substrate concentration data using the PSI-Plot 3.5 software.

reductase (*22*) and 3α-hydroxysteroid dehydrogenase (*21*). Many of these enzymes have several different activities including thioltransferase, thioredoxin/thioredoxin reductase, and PDI which belong to the class of enzymes known as thiol:disulfide oxidoreductases, which contain -CXXC- at the active site (*42*).

The newly measured and reported kinetic constants of the known DHA reductases are found in Tables I and II. Of these enzymes, bovine liver PDI is the weakest DHA reductase with a $k_{cat}/K_m$ for DHA of 93 ± 14 $M^{-1} sec^{-1}$ (*33*) although reported values vary (*22*). The most robust DHA reductases identified to date are RPLTT (*33*) and human erythrocyte 32kDa-DHA reductase (*26*) each with a $k_{cat}/K_m$ for DHA of approximately 2 x $10^4$ $M^{-1} sec^{-1}$ at both pH 6.9 and 7.2. Even though there are a wide variety of enzymes represented, analysis of the kinetic constants yield some common themes. The $K_m$ values for DHA for all the enzymes range from 0.2 to 4.6 mM with the majority of the enzymes having $K_m$ values for DHA in the range of 0.2 to 0.7 mM. The lower limit of $K_m$ values for DHA is apparently 0.2 mM, even for an enzyme like the 32kDa GSH dependent DHA reductase which is presumably a specific DHA reductase having no known activities other than as a DHA reductase (*25, 26*). In addition, no enzyme has a $k_{cat}/K_m$ for DHA in excess of 2.5 x $10^4$ $M^{-1}$ $sec^{-1}$, a value that is substantially lower than the theoretical limit for a $k_{cat}/K_m$ of 1 x $10^8$ $M^{-1}$ $sec^{-1}$. Based on kinetic comparisons, the 32kDa-DHA reductase from human erythrocytes (*26*) would be the primary human DHA reductase because it had a $k_{cat}$ and a $k_{cat}/K_m$ for DHA which were 6 fold higher than those of human placental thioltransferase (*22*) (both measured at pH 6.9) and a $k_{cat}$ and a $k_{cat}/K_m$ for DHA which were 5 fold higher than those of human neutrophil thioltransferase (*31*) (measured at pH 7.2 and 7.4, respectively). However, the *in*

Table II
Kinetic Parameters for DHA Reductases

| | | Values for DHA | | | Values for GSH | |
|---|---|---|---|---|---|---|
| enzyme | pH | $k_{cat}$ ($min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) |
| bovine liver PDI | 6.9 | 16 | 2.8 | 93 | 2.9 | 91 |
| bovine liver PDI (*22*) | 6.9 | 94 | 1.8 | 870 | | |
| RPLTT | 6.9 | 374 | 0.26 | 24,300 | 3.5 | 1810 |
| RPLTT | 7.2 | 305 | 0.25 | 20,100 | 2.5 | 2050 |
| Human placental Grx (recombinant) (*22*) | 6.9 | 41 | 0.27 | 2531 | | |
| Human neutrophil Grx | | | | | | |
| Native (*31*) | 7.4 | 66 | 0.25 | 4400 | 2.0 | 550 |
| Recombinant (31) | 7.4 | 66 | 0.20 | 5500 | 2.0 | 550 |
| Human erythrocyte 32kDa-DHA reductase | 6.9 | 251 | 0.28 | 14,900 | 2.53 | 1650 |
| Human erythrocyte 32kDa-DHA reductase | 7.2 | 316 | 0.21 | 24,700 | 3.5 | 1510 |
| Rat liver 32kDa-DHA Rase (*25*) | 7.2 | 140 | 0.245 | 9520 | 2.8 | 833 |
| *Tryp Cruzi* p52 (*38*) | 7.0 | 358 | 0.67 | 8910 | 2.6 | 2290 |
| | | | | | NADPH | NADPH |
| Human Thioredoxin Reductase (TR) (*22*) | 7.4 | 90 | 2.5 | 600 | | |
| TR + rat liver thioredoxin (*22*) | 7.4 | 71 | 0.7 | 1690 | | |
| Rat 3α-hydroxysteroid dehydrogenase (*21*) | 6.9 | 58.1 | 4.6 | 211 | 0.0043 | 22,500 |

*vivo* concentrations of these enzymes are not known, which would better indicate the importance of each enzyme in DHA reduction.

Perhaps the best characterized DHA reductase is thioltransferase. Thioltransferase has been purified and characterized as a DHA reductase from pig liver (*24, 33*), human placenta (*22*), human neutrophils (*31*), rice (43), spinach leaves (*44*), *E. coli.* (*24*), and vaccinia virus (*39*). Of these enzymes, even though pigs can synthesize AA and would presumably not depend on this DHA reductase system, pig liver thioltransferase was the most robust DHA reductase with a $k_{cat}/K_m$ for DHA of 2.4 x $10^4$ $M^{-1}$ $sec^{-1}$ compared to a $k_{cat}/K_m$ for DHA for the human enzymes of 2.5-5.5 x $10^3$ $M^{-1}$ $sec^{-1}$ (Table II) making the pig liver enzyme 4-10 fold more efficient. In contrast, there are conflicting reports concerning the activity of plant thioltransferase. Sha *et al.* (*43*) reported that purified rice thioltransferase has DHA reductase activity although the same group reported that cloned rice thioltransferase does not (*45*). In addition, Morell *et al.* (44) reported that purified spinach thioltransferase lacks DHA reductase activity.

Strong evidence exists that both NADPH-dependent and GSH-dependent DHA reduction is important *in vivo*. The selenoprotein thioredoxin reductase possesses NADPH-dependent DHA reductase activity (*22*). Rats fed a selenium deficient diet had an 88% reduction in thioredoxin reductase activity and a 33% reduction in liver ascorbate levels providing evidence for selenium dependent enzymes, like thioredoxin reductase, playing a role in DHA recycling (*22*). However, 67% of control levels of liver ascorbate remained in selenium deficient rats, suggesting a role for GSH-dependent DHA reductases, whose activities were not affected by selenium deficiency (*22*).

Unable to synthesize AA, when newborn rats were made GSH deficient by

administering the γ-glutamylcysteine synthetase inhibitor, buthionine sulfoximine (BSO), drastic decreases in tissue ascorbate levels occurred (*23*). Similar results are seen when GSH deficiency is induced in guinea pigs after BSO administration (*46*), and when GSH monethyl ester, which is transported and converted intracellularly into GSH, was administered to GSH deficient guinea pigs, the onset of scurvy was delayed suggesting a sparing effect of GSH on ascorbate (*48*). In adult mice, which are able to synthesize AA, GSH deficiency resulted in increased AA synthesis in the liver (*47*). These studies demonstrated a role of GSH in the regeneration of AA, *in vivo*, but they did not resolve the question of whether the regeneration of AA from DHA by GSH was enzymatic or chemical, suggesting a need for knockout transgenic mice studies in the future.

GSH reduction of DHA to AA has been estimated to have a $t_{1/2}$ of 15 min at a pH of 7.5 and at 25°C (*29*). However, under the same conditions DHA degrades to non recyclable products, like 2,3-diketogulonic acid, with a $t_{1/2}$ of 2 min (*30*), strongly supporting the importance of catalytic regeneration of AA from DHA. Evidence for GSH-dependent DHA reductases in animal tissues have existed for many years (*49-51*). Bigley *et al.* (*52*) demonstrated that GSH-dependent DHA reductase activity was greatest in human neutrophils, followed by monocytes, lymphocytes, and fibroblasts. In a study by Park and Levine (*31*), the reducing activity of neutrophils after cell lysis was non-dialysable and could not be accounted for by chemical reduction of DHA by GSH. In addition they found that the purified DHA reducing activity was thioltransferase (*31*). In rat liver cytosol, up to 70% of the DHA reductase activity was immunotitratable using an antibody directed towards the rat liver 32 kDa DHA reductase (*53*). These experiments demonstrate the importance of GSH-dependent DHA reductases in DHA reduction *in vivo*.

The emerging picture in the field of DHA reductases is one in which there are several potential enzymatic systems to regenerate AA from DHA. NADPH-dependent DHA reductases like 3α-hydroxysteroid dehydrogenase (*21*) and thioredoxin/thioredoxin reductase (*22*), and the more catalytically efficient GSH-dependent DHA reductases thioltransferase (*24*) and the 32 kDa DHA (*25, 26*) reductase may all play roles *in vivo*. It is likely that the importance of each enzyme in DHA reduction will vary from species to species and cell to cell. For example, thioredoxin reductase (*22*) and the 32 kDa DHA reductase (*53*) have been separately shown to account for 33% and 70% of the DHA reductase activity in rat liver, completely accounting for the DHA reductase activity in this particular tissue. On the other hand, thioltransferase has been proposed to be the major DHA reductase in human neutrophils (*31*).

The wide variety of DHA reductases, especially the GSH-dependent DHA reductases, raises several enzyme structure/function questions. In addition, thioltransferase, protein disulfide isomerase and thioredoxin all belong to the class of enzymes known as thiol:disulfide oxidoreductases which contain the -CXXC- motif in their respective active sites (*42*). Whether each of these enzymes has an identical, similar or different catalytic mechanism for DHA reduction is unknown. The only catalytic mechanism information available is derived from the DHA reductase activity of RPLTT. Yang and Wells (*54*) compared the relative DHA reductase activities of equal amounts of C25S, R26V, and K27Q variants to that of the native RPLTT. With RPLTT activity defined as 100%, C25S had a relative activity of 194%, R26V a relative activity of 30%, and K27Q a relative activity of 73% (*54*). Furthermore, the active site of RPLTT has been previously identified as Cys-22 which has a $pK_a$ of 3.8 (*55*) and is the essential amino acid mediating RPLTT thioltransferase

and DHA reductase activities (*54*). In the following chapter the first detailed description of a GSH-dependent DHA reductase mechanism is presented; i.e., that of thioltransferase.

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# Chapter III

## The Catalytic Mechanism of the Glutathione-Dependent Dehydroascorbate Reductase Activity of Thioltransferase (Glutaredoxin)

This chapter has been submitted to *Biochemistry* and is currently under review.

## ABSTRACT

The catalytic mechanism of the glutathione (GSH) dependent dehydroascorbic acid (DHA) reductase activity of recombinant pig liver thioltransferase (RPLTT) was investigated. Kinetic analysis of the GSH-dependent DHA reductase activities of native RPLTT and the R26V, K27Q, and C25S variants indicated that Arg-26 and Lys-27 are involved in catalysis, but Cys-25 appears to lower the catalytic capacity of the enzyme since its substitution with a serine enhances the $k_{cat}$(app). Iodoacetamide (IAM) inactivated the DHA reductase activities of RPLTT, and its C25S, R26V, and K27Q variants confirming the essential role of cysteine in the reaction mechanism. When preincubated with DHA, RPLTT or the R26V and K27Q but not C25S variants, proteins were protected against IAM inactivation, suggesting that RPLTT and R26V and K27Q enzymes have the ability to chemically reduce DHA forming AA and the intramolecular disulfide form of the enzyme. Electrochemical detection of AA demonstrated the ability of reduced RPLTT and the R26V K27Q, and C25S variants to chemically reduce DHA to AA in the absence of GSH. Also in the absence of thiol substrate, RPLTT and the R26V and K27Q proteins had nearly identical initial rates of DHA reduction which were 4 to 5 fold greater than that of the C25S variant. Isoelectric focusing analysis revealed that the product of reaction of reduced RPLTT and the R26V and K27Q enzymes but not the C25S variant with DHA was indeed oxidized enzyme. Based on 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) 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 32kDa 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 & 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 *26, 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 (*29*) and is the essential amino acid mediating RPLTT thioltransferase and DHA reductase activities (*25*).

In the present study, we investigated the catalytic mechanism of the DHA reductase activity of thioltransferase using RPLTT and several site directed mutant proteins. The results provided experimental evidence for a proposed mechanism 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 hydroxyethyldisulfide (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 isopropylthiogalactoside (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 pI 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 the C25S variant 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 x 2L) against the same buffer. After dialysis, the sample was loaded onto a Sephacryl S-200 column ( 2.5 cm x 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 x 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 0.0-1.0 M NaCl linear gradient in buffer B. The active fractions were pooled and concentrated using Centriprep 3 concentrators and stored at -70°C until use. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Company) according to the manufacturer's directions. During the purification, the enzyme activity was assayed as described previously (*35*).

The R26V and K27Q encoding genes were subcloned from the pKK233-2 vector (*25*) into pET23d(+) (Novagen, Inc.) using pET23d(+) *Hind*III and *Nco*I sites and standard molecular biology techniques (*33*). In these constructs, R26V and K27Q proteins were

synthesized without the His•Tag®. The two proteins were produced in *E. coli* strain BLD21(DE3) (Novagen, Inc.) transformants that were grown overnight at 37°C in Luria-Bertani medium containing 50 $\mu$g/ml ampicillin. These cultures were diluted 1/200 in 1 L of fresh LB medium with 50 $\mu$g/ml ampicillin and shaken at 37°C until an $A_{550}$ of 0.4-0.7 was reached. Protein expression was induced by adding 0.45 mM IPTG to the culture, and after 3.5 h the cells were harvested by centrifugation at 10,000 x *g* for 10 min. From this point, R26V and K27Q proteins were purified as described for RPLTT and C25S enzyme except that for the R26V and K27Q variants, 20 mM sodium phosphate, pH 6.0, and 20 mM sodium phosphate, pH 7.0, were used instead of buffers A and B, respectively.

*Dehydroascorbic Acid Reductase Assay and Enzyme Kinetics.* DHA reductase activity was assayed as described previously (*30*). Assays performed to determine the kinetic constants were run in 200 mM sodium phosphate, 1 mM EDTA, pH 6.9, in a final volume of 500 $\mu$l 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 nmoles/min. Kinetic constants for DHA were determined by varying [DHA] and holding [GSH] = 3.0 mM, and kinetic constants for GSH were determined by varying [GSH] and holding [DHA] = 1.5 mM. The following enzyme amounts were used in their respective kinetic studies: 0.72 $\mu$g RPLTT, 2.12 $\mu$g R26V, 1.45 $\mu$g K27Q, and 0.44 $\mu$g C25S. $K_m$(app) and $V_{max}$(app) values were calculated by nonlinear least-square 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 the C25S, R26V, and K27Q variant 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 x 78 cm) filtration, and eluted with triply distilled $H_2O$ to remove the excess DTT. Fractions with thioltransferase activity were pooled and lyophilized. Lyophilized enzyme was resuspended in triple distilled $H_2O$, and protein concentration were determined.

*Iodoacetamide Inactivation of Thioltransferases DHA Reductase Activity.* Reduced RPLTT (7$\mu$M) and its C25S ( 7$\mu$M), R26V (10 $\mu$M), and K27Q (10 $\mu$M) variants 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 6.85, 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. Percent activity was determined for each enzyme by running concurrent control with IAM absent. The IAM inactivation experiments were also performed after preincubation of each enzyme separately with 0.05 mM DHA plus 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 0.1 mM and the experiments conducted as described above.

*Reduction of DHA to AA by Thioltransferases in the Absence of GSH.* Equal amounts (6 $\mu$M) of reduced RPLTT or the C25S, R26V, and K27Q variants were separately incubated in a 100 $\mu$l reaction volume with 600 $\mu$M DHA for 0.5, 1, 2, 5, and 10 min in 200 mM sodium phosphate, 1 mM EDTA, pH 6.85. In addition, control reactions containing DHA and no enzyme were run. At each time point, 100 $\mu$l of 10% metaphosphoric acid, 1 mM thiourea, 1 mM EDTA was added to the reaction tube. The samples were centrifuged at 14,000 rpm for 2 min in an Eppendorf Microcentrifuge. AA concentrations were determined

on the supernatant as described previously (*34*). Separation and quantitative analysis were accomplished using a 3.9 x 150 mm Waters Delta Pak-5$\mu$ C18 reversed phase column with a mobile phase of 50 mM sodium phosphate, pH 3.0, and a flow rate of 0.7 ml per 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 by 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 $\mu$M AA dissolved in 5% metaphosphoric acid, 0.5 mM thiourea, 1.0 mM EDTA, 100 mM sodium phosphate (pH 2).

*Isoelectric Focusing.* Reduced RPLTT or its C25S, R26V, and K27Q variants, 12 $\mu$g each, were incubated alone and with 1 mM DTT, 1 mM HED, 1 mM DHA, 1 mM GSH, 0.5 mM DHA plus 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 30 additional min at 30°C. Controls included enzyme alone, enzyme + 1mM DTT, enzyme + 1 mM HED, or enzyme + 1 mM DHA. Each of the incubation mixtures (in a total volume of 10 $\mu$l) was then analyzed directly on a Servalyt Precotes isoelectric focusing gel, pH 3-10 (150 $\mu$m thick, 125 mm x 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 equal amounts of C25S, R26V, and K27Q mutant proteins to that of RPLTT. With RPLTT activity defined as 100%, C25S had a relative activity of 194%, R26V a relative activity of 30%, and K27Q a relative activity of 73% (*25*). In the current study, the kinetic parameters for C25S, R26V, and K27Q variants were determined and compared to those of RPLTT (Table I). The kinetic plots for each enzyme were best fit by the Michaelis-Menten equation (RPLTT (Figure 2 in Chapter II), C25S (Figure 1), R26V (Figure 2), and K27Q, (Figure 3), EZ-Fit Kinetic Program, *35*). The catalytic constant, $k_{cat}$(app), for R26V and K27Q proteins were 2.1 and 1.8 fold lower, respectively, than that of RPLTT. The affinity constant, $K_m$(app), for DHA of R26V enzyme was 1.6 fold higher than that of RPLTT. As a result, the specificity constants ($k_{cat}/K_m$) of R26V protein were 3.3 fold lower for DHA and 1.9 fold lower for GSH than those of RPLTT. Because of the decrease in $k_{cat}$(app) for K27Q enzyme , the specificity constants were 1.9 fold lower for DHA and 1.5 fold lower for GSH than those of RPLTT. The C25S variant had a 2.7 fold higher $k_{cat}$ (app), a 3.2 fold higher $K_m$(app) for DHA, and a 1.5 fold higher $K_m$(app) for GSH than those of RPLTT. As a result of the increased $K_m$(app) for DHA, the specificity constant of C25S protein for DHA was not significantly different than that of RPLTT. However, the specificity constant for GSH was 1.9 fold higher than that of RPLTT. Arg-26 and Lys-27 participated in catalysis because both R26V and K27Q variants had lower values for $k_{cat}$(app) than RPLTT. In contrast, a cysteine at position 25 lowered the catalytic capacity of the enzyme since the placement of a serine at position 25 enhanced the $k_{cat}$(app).

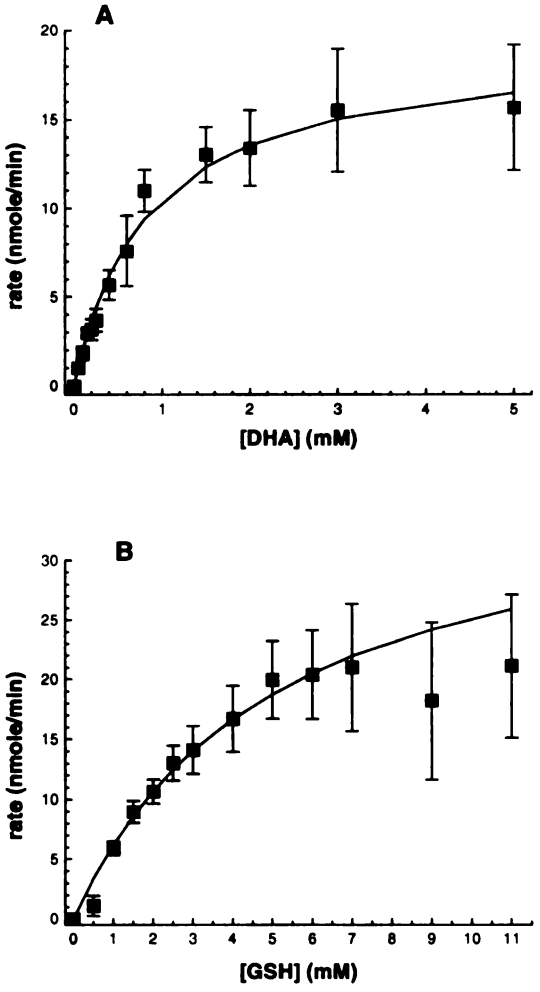


**Figure 1.** Kinetic analysis of the DHA reductase activity of the C25S enzyme. C25S variant ( 0.44 $\mu$g) was assayed for DHA reductase activity in 200 mM sodium phosphate, 1 mM EDTA, pH 6.9. A blank without enzyme was run simultaneously with each assay and the difference gave the activity in nmoles/min (-■-). Kinetic constants for DHA were determined by varying [DHA] as shown and holding [GSH] = 3.0 mM, (A) and kinetic constants for GSH were determined by varying [GSH] as shown and holding [DHA] = 1.5 mM (B). Each data point is the average of 3 separate experiments. Error bars represent one standard deviation of the data.

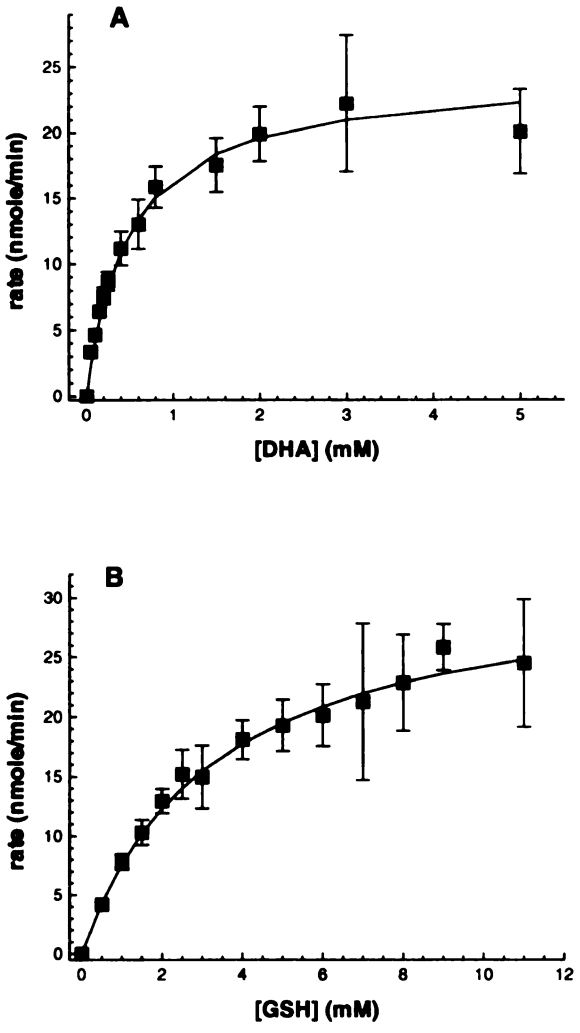


**Figure 2.** Kinetic analysis of the DHA reductase activity of the R26V variant. R26V enzyme ( -■-) was assayed for DHA reductase activity and kinetic constants determined as described in Figure 1.

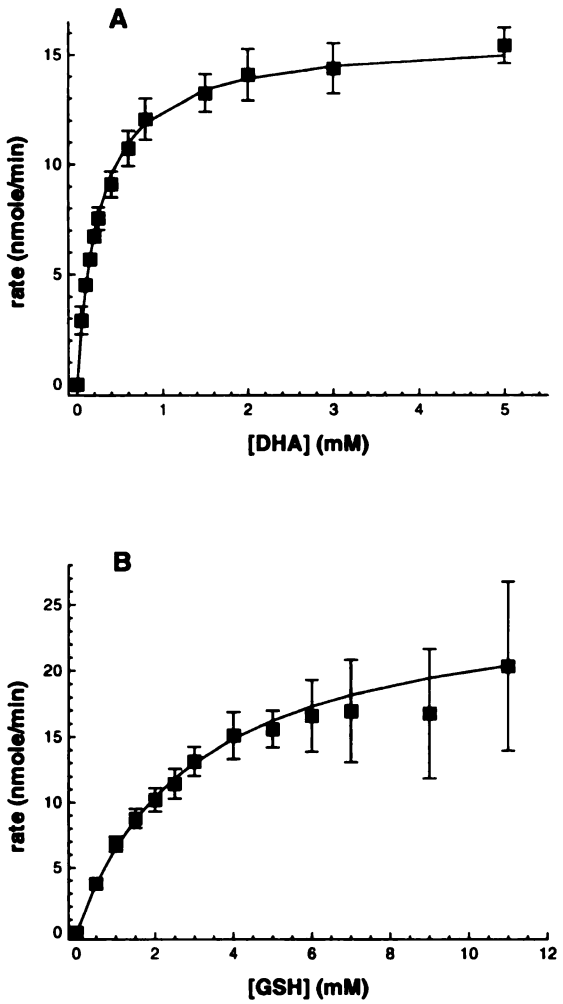


**Figure 3.** Kinetic analysis of the DHA reductase activity of the K27Q variant. K27Q enzyme (-■-) was assayed for DHA reductase activity and kinetic constants determined as described in Figure 1.

Table I
Wild Type and Mutant Pig Liver Thioltransferase Kinetic Parameters

| Parameter | RPLTT[a] | C25S[a] | R26V[a] | K27Q[a] |
|---|---|---|---|---|
| $k_{cat}$(min$^{-1}$)[b] | 374 ± 20 | 1009 ± 101* | 179 ± 15* | 209 ± 4* |
| $K_m$(app) (mM)[c] | | | | |
| DHA | 0.26 ± 0.09 | 0.83 ± 0.10* | 0.41 ± 0.09* | 0.27 ± 0.04 |
| GSH | 3.4 ± 0.3 | 5.1 ± 0.9** | 3.1 ± 0.7 | 3.0 ± 0.1** |
| $k_{cat}/K_m$ (M$^{-1}$sec$^{-1}$) | | | | |
| DHA | 2.43 ± 0.85 x $10^4$ | 2.01 ± 0.31 x $10^4$ | 7.3 ± 1.7 x $10^3$* | 1.29 ± 0.19 x $10^4$ ** |
| GSH | 1.8 ± 0.2 x $10^3$ | 3.3 ± 0.69 x $10^3$* | 0.96 ± 0.23 x $10^3$* | 1.18 ± 0.05 x $10^3$* |

[a]Kinetic constants were the mean ± standard deviation of three or more separate experiments as described in the methods section. Kinetic constants for RPLTT have been previously published (*30*).

[b] $k_{cat}$ values were calculated by dividing $V_{max}$(app) by the molar concentration of the enzymes.

[c] $K_m$(app) values were calculated by nonlinear least-square fit to the velocity versus substrate concentration data using the PSI-Plot 3.5 software.

* Significantly different from corresponding RPLTT value with a $p<0.01$ (GraphPAD InSTAT statistical program).

**Significantly different from corresponding RPLTT value with a $p<0.05$ (GraphPAD InSTAT statistical program).

*Proposed Mechanism of RPLTT's DHA Reductase Activity.* Based on 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 (Figure 4). 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; (ii) in reaction 3, Cys-25 attacks the mixed disulfide 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 enzyme 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-SSR (*36*). 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 (Figure 4) (*36*). 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 (*37, 38*).

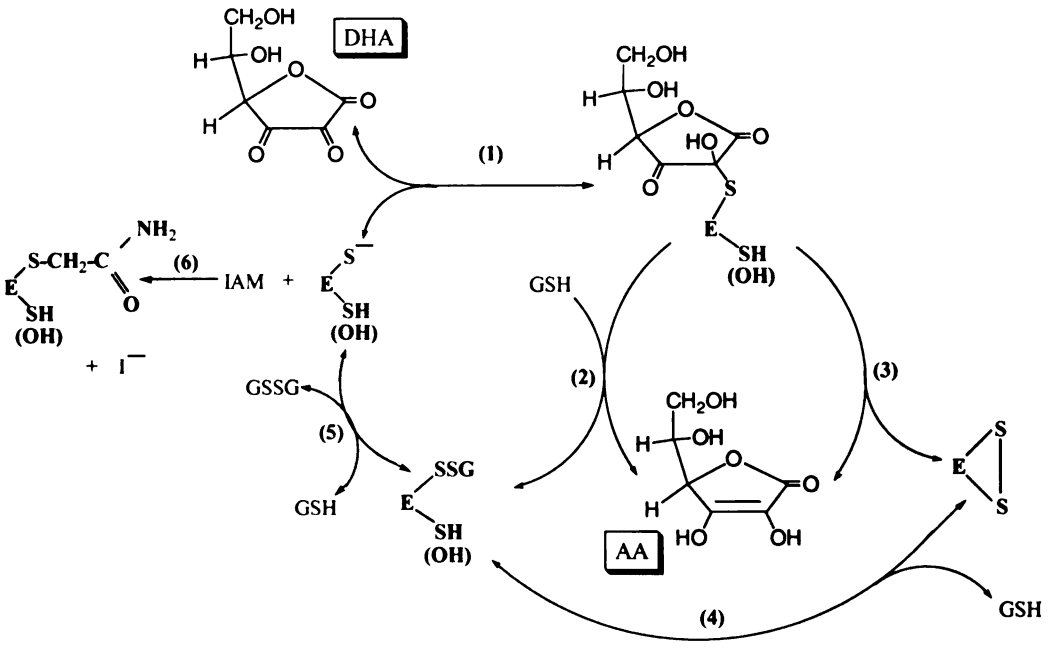


**Figure 4**: Proposed mechanism of GSH-dependent DHA reduction catalyzed by RPLTT

*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 its C25S, R26V, and K27Q variants were carried out. Only Cys-22 of pig liver thioltransferase reacted with iodo-[1-$^{14}$C]acetic acid (*39*), and treatment of reduced RPLTT or its C25S, R26V, and K27Q variants inactivated each enzyme's thiol:disulfide oxidoreductase activity (*25*). IAM inactivated the DHA reductase activity of reduced RPLTT (Figure 5) as well as the reduced C25S (Figure 6), R26V (Figure 7), and K27Q (Figure 8) enzymes. For each enzyme, the inactivation profile best fit the exponential decay function, $y = y_0 + A_1e^{(-xk)}$, with a *k* (the rate constant of inactivation) = 0.163 ± 0.02 min$^{-1}$ for RPLTT, versus 0.202 ± 0.023 min$^{-1}$ for C25S, 0.196 ± 0.092 min$^{-1}$ for R26V, and 0.146 ± 0.028 min$^{-1}$ for K27Q enzymes. Both R26V (Figure 7) and K27Q (Figure 8) variants were incompletely inhibited with 40% residual activity after 45 minutes of incubation with IAM. In contrast, RPLTT (Figure 5) and C25S enzyme (Figure 6) had residual activities of 15% and 8%, respectively. Several attempts were made to prepare reduced R26V and K27Q proteins to increase the inactivation of their respective DHA reductase activities, but the results were the same as in Figures 7 and 8 with incomplete inactivation of both mutants (data not shown). IAM inactivation of the DHA reductase activity of reduced RPLTT and the C25S, R26V, and K27Q variants confirmed the role of cysteine as the key catalytic residue in the mechanism (*25*).

Preincubation of reduced RPLTT (Figure 5) or the C25S (Figure 6), R26V (Figure 7), or K27Q (Figure 8) variants for 10 minutes with 0.05 mM GSH enhanced the IAM inactivation of RPLTT and K27Q enzymes and had no effect on the IAM inactivation of C25S and R26V variants. The inactivation profiles best fit the exponential decay function

with a $k = 0.133 \pm 0.015$ $min^{-1}$ and an $A_1$ (the maximal amount of inhibition) = 93 ± 4 % for RPLTT, a $k = 0.146 \pm 0.014$ $min^{-1}$ and an $A_1 = 100 \pm 1$ % for C25S enzyme, a $k = 0.105 \pm 0.012$ $min^{-1}$ and an $A_1 = 80 \pm 4$ % for R26V protein, and a $k = 0.108 \pm 0.01$ $min^{-1}$ and an $A_1 = 89 \pm 4$ % for K27Q variant. The *k* for C25S, R26V, and K27Q enzymes were significantly lower than the k when enzyme was inactivated with IAM with no preincubation. However, preincubation with GSH enhanced the % inactivation of K27Q protein by 30% (Figure 8), and RPLTT by 8% (Figure 5). These results indicated that the resistance of K27Q enzyme to IAM inactivation is due to this protein being in the oxidized form. GSH can reduce the oxidized enzyme to expose Cys-22 to reaction with IAM. As shown in Figure 4, GSH could react with the oxidized enzyme (reaction 4) forming ES-SG. A second molecule of GSH could react with ES-SG (reaction 5) forming GSSG and the reduced enzyme, which is susceptible to IAM inactivation (reaction 6). When studying the thiol:disulfide oxidoreductase activity of RPLTT, Yang & Wells (*36*) found that GSH enhanced RPLTT and K27Q variant inactivation by IAM, as seen in the current studies. In addition, Yang & Wells (*36*) found the R26V protein to be partially resistant to IAM inactivation, with little effect of GSH preincubation on IAM inactivation. Arg-26 has been determined to be the key amino acid for the low pK*a* of Cys-22, however, its pK*a* is as yet undetermined (*25*). At pH 7.5, Cys-22 in R26V protein will primarily be in the protonated form rendering Cys-22 resistant to IAM inactivation because IAM will only react with the unprotonated or thiolate form of cysteine.

Preincubation of reduced RPLTT (Figure 5) or its C25S (Figure 6), R26V (Figure 7), or K27Q (Figure 8) variants for 10 minutes with 0.05 mM DHA + 0.1 mM GSH significantly protected the enzyme's DHA reductase activity from IAM inactivation. R26V and K27Q

variants were completely protected, whereas RPLTT and C25S enzyme 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 (Figures 5-8) or 0.05 mM GSSG (Figures 5-8), only preincubation with GSSG protected the enzyme's DHA reductase activity from IAM inactivation. Again, the inactivation profiles when each enzyme was preincubated with AA best fit the exponential decay equation with a $k = 0.203 \pm 0.008$ $min^{-1}$ and an $A_1 = 80 \pm 1$ % for RPLTT, a $k = 0.146 \pm 0.011$ $min^{-1}$ and an $A_1 = 92 \pm 3$ % for C25S enzyme, a $k = 0.205 \pm 0.035$ $min^{-1}$ and an $A_1 = 63 \pm 5$ % for R26V protein, and a $k = 0.238 \pm 0.029$ $min^{-1}$ and an $A_1 = 59 \pm 3$ % for the K27Q variant. Preincubation of the enzymes with another disulfide reagent, 0.5 mM HED, completely protected the enzyme's DHA reductase activity from IAM inactivation (data not shown). Reduced RPLTT or the C25S, R26V, and K27Q variants 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 studying the thiol:disulfide oxidoreductase activity of thioltransferase, RPLTT or R26V and K27Q proteins were protected from IAM inactivation by preincubation with the substrate S-sulfocysteine or HED (*36*). In addition, RPLTT and C25S enzyme were shown to be oxidized or form enzyme-substrate intermediates with the disulfide species cystine or HED (*36*). Because Cys-25 is present, RPLTT or R26V and K27Q proteins 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 (*40*).

In contrast to reduced RPLTT (Figure 5) or the R26V (Figure 6) or K27Q (Figure 7) variants, the DHA reductase activity of C25S protein (Figure 8) was not protected from

IAM inactivation by a 10 min preincubation with 0.5 mM DHA. The inactivation profile of C25S enzyme best fit the exponential decay equation with a $k = 0.195 \pm 0.04$ $min^{-1}$ and an $A_1 = 90 \pm 8$ %. The fact that C25S enzyme was not protected suggests that the postulated thiohemiketal intermediate (reaction 1, Figure 4) is not stable enough as an enzyme-substrate intermediate to prevent IAM inactivation. If this intermediate exists, it probably dissociates into the reduced enzyme and DHA, and IAM then reacted irreversibly with the reduced enzyme (reaction 6, Figure 4).

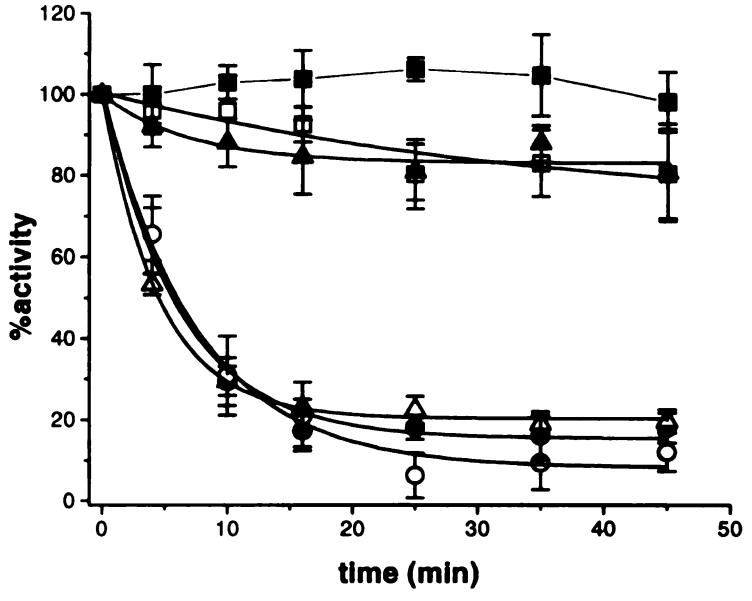


**Figure 5.** IAM inactivation of reduced RPLTT with and without preincubation with substrates. Reduced RPLTT (7$\mu$M) was separately incubated either with 0.1 mM IAM (-●-) in 100 mM sodium phosphate, pH 7.5 or preincubated with 0.05 mM GSH (-○-), 0.05 mM GSSG (-■-), 0.05 mM DHA + 0.1 mM GSH (-□-), 0.5 mM DHA (-▲-), or 0.05 mM AA (-Δ-) 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 the Methods section. Each data point is the average of 3 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.

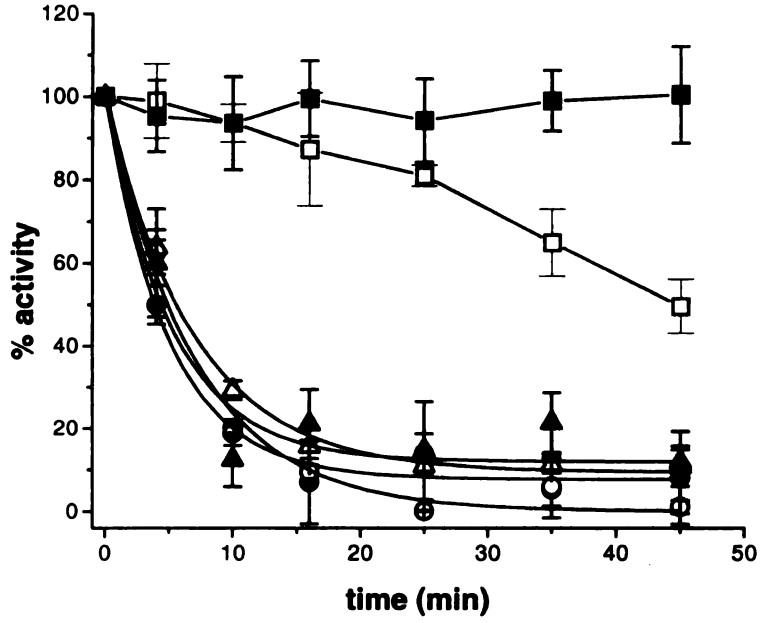


**Figure 6**. IAM inactivation of reduced C25S protein with and without preincubation with substrates. Reduced C25S (7$\mu$M) was separately incubated either with 0.1 mM IAM (-●-) in 100 mM sodium phosphate, pH 7.5 or preincubated with 0.05 mM GSH (-○-), 0.05 mM GSSG (-■-), 0.05 mM DHA + 0.1 mM GSH (-□-), 0.5 mM DHA (-▲-), or 0.05 mM AA (-Δ- ) 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 the Methods section. Each data point is the average of 3 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.

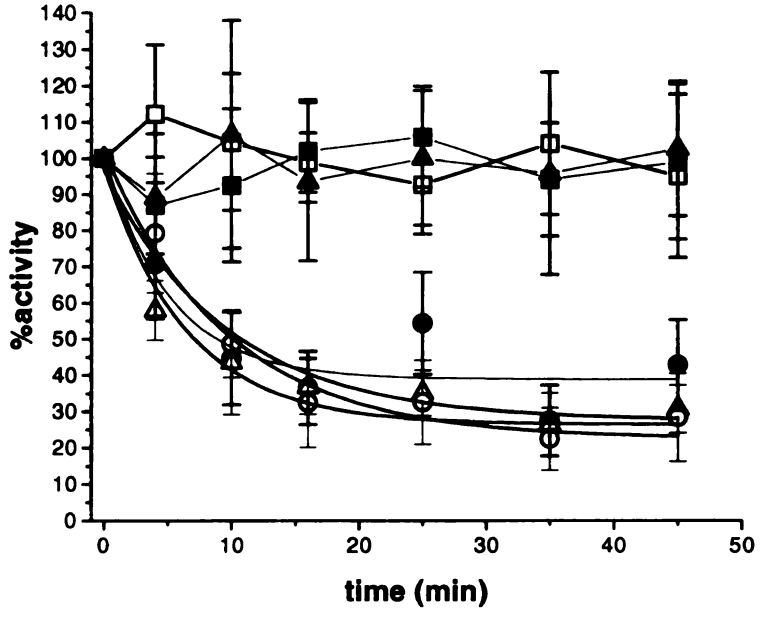


**Figure 7.** IAM inactivation of reduced R26V protein with and without preincubation with substrates. Reduced R26V (7$\mu$M) was separately incubated either with 0.1 mM IAM (-●-) in 100 mM sodium phosphate, pH 7.5 or preincubated with 0.05 mM GSH (-○-), 0.05 mM GSSG (-■-), 0.05 mM DHA + 0.1 mM GSH (-□-), 0.5 mM DHA (-▲-), or 0.05 mM AA (-Δ-) 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 the Methods section. Each data point is the average of 3 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.

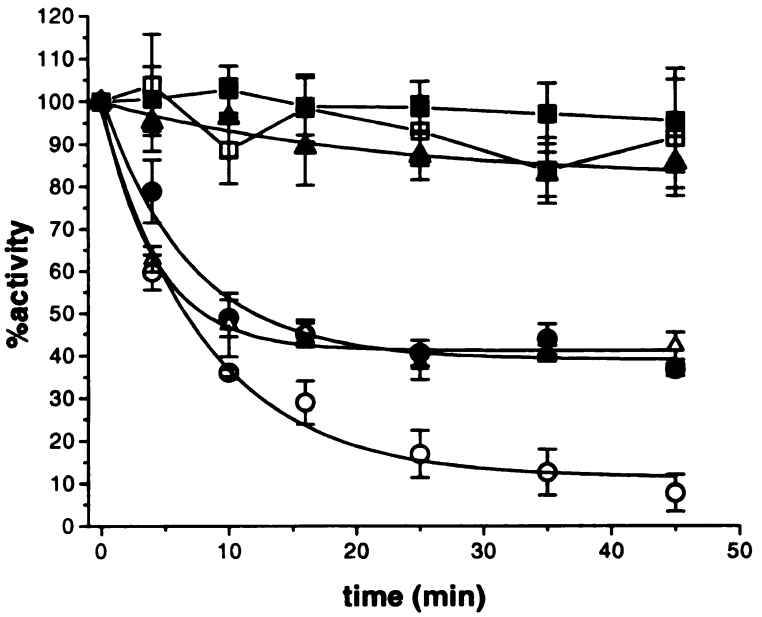


**Figure 8.** IAM inactivation of reduced K27Q protein with and without preincubation with substrates. Reduced K27Q (7$\mu$M) was separately incubated either with 0.1 mM IAM (-●-) in 100 mM sodium phosphate, pH 7.5 or preincubated with 0.05 mM GSH (-○-), 0.05 mM GSSG (-■-), 0.05 mM DHA + 0.1 mM GSH (-□-), 0.5 mM DHA (-▲-), or 0.05 mM AA (-Δ- ) 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 the Methods section. Each data point is the average of 3 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.

*Ability of Reduced Wild Type and Mutant RPLTTs to Chemically Reduce Dehydroascorbic Acid to Ascorbic Acid.* Because they contain Cys-25, we hypothesized that reduced RPLTT or its R26V and K27Q variants could chemically reduce DHA to AA in the absence of GSH forming oxidized enzyme (reactions 1 and 3, Figure 4). Reduced RPLTT and the C25S, R26V, and K27Q proteins all could chemically reduce DHA to AA in the absence of GSH (Figure 9). DHA alone was also spontaneously reduced to AA and L-erythroascorbic acid as seen by Jung & Wells (*41*) with an initial rate of 122 ± 34 nM AA/min reaching a maximum AA concentration of 109 ± 17 nM after 10 min. C25S enzyme had an initial rate of 201 ± 4 nM AA/ min and reached a maximum AA concentration of 277 ± 18 nM after 10 min. RPLTT and its R26V and K27Q variants were the most robust DHA reductants with initial rates of 883 ± 76 nM AA/min, 896 ± 144 nM AA/min, and 1053 ± 60 nM AA/min, respectively, and maximum AA concentrations of 3.18 ± 0.14 $\mu$M, 2.09 ± 0.79 $\mu$M, and 2.62 ± 0.25 $\mu$M, respectively.

The results (Figure 9) indicated that reduced RPLTT and its R26V and K27Q variants can undergo reactions 1 and 3 (Figure 4) where one mole of reduced enzyme reduces one mole of DHA to one mole of AA and one mole of oxidized enzyme. While C25S protein cannot undergo reaction 3 because it lacks Cys-25 to form an intramolecular disulfide bond, it still reduced DHA to AA at a rate greater than that of DHA alone, but at a much lower rate than that of RPLTT and its R26V and K27Q variants, suggesting a weak ability of C25S protein to form an intermolecular disulfide bond upon the reduction of DHA.

The initial rates of reactions 1 and 3 (Figure 4) for RPLTT and its R26V and K27Q variants were nearly identical. However, when RPLTT and its R26V and K27Q variants are compared kinetically in the presence of GSH (Table I), R26V and K27Q mutant proteins

have decreased DHA reductase activity. These results suggest that Arg-26 and Lys-27 do not play a role in DHA binding to the enzyme (reaction 1, Figure 4), or the conversion of the postulated thiohemiketal intermediate to AA and oxidized enzyme (reaction 3, Figure 4). Recently, Srinivasan et al. (*42*) provided evidence that the rate-limiting step in the thiol:disulfide oxidoreductase activity of thioltransferase is the nucleophilic attack of GSH on ES-SG forming GSSG and reduced enzyme (reaction 5, Figure 4). If this step is also the rate limiting step of RPLTT's DHA reductase activity, one would expect to see an effect of the R26V and K27Q variants only at this point and not elsewhere in the reaction mechanism. The evidence presented in this paper supports the hypothesis that the rate limiting step in RPLTT's DHA reductase activity is reaction 5 (Figure 4), because neither mutation of Arg-26 to Val or Lys-27 to Gln affected the ability of the enzyme to undergo reactions 1 and 3 (Figure 4), while both mutant proteins had decreased DHA reductase activity (Table I).

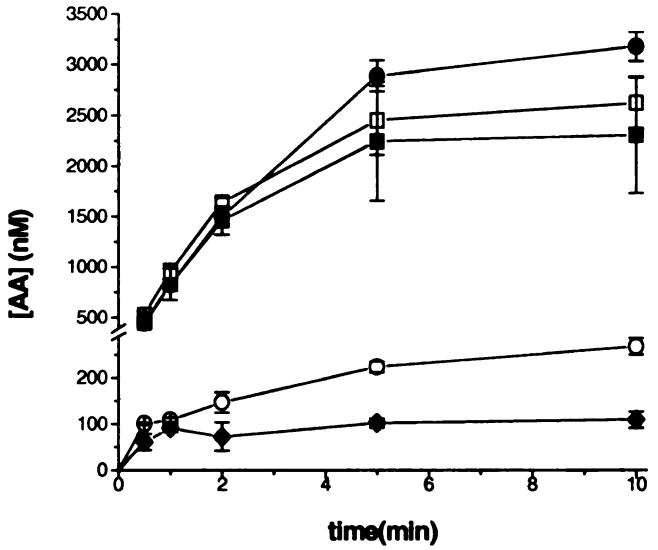


**Figure 9**. HPLC analysis of AA production by DHA incubation with reduced RPLTT and the C25S, R26V, and K27Q variants. Equal amounts (6 $\mu$M) of reduced RPLTT (-●-) and the C25S (-○-), R26V (-■-), and K27Q (-□-, 10 $\mu$M) proteins were separately incubated in a 100 $\mu$l reaction volume with DHA (600 $\mu$M) for various lengths of time in 200 mM sodium phosphate 1 mM EDTA, pH 6.85. In addition, DHA alone control reactions were run (-◆-). At the time points shown, 100 $\mu$l of 10% metaphosphoric acid, 1 mM thiourea, 1 mM EDTA was added to the reaction tube and AA concentrations were determined by an HPLC chromatography procedure as described in the Methods section. 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.

*Isoelectric Focusing Analysis of RPLTT and the C25S, R26V, and K27Q variants.* 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 carboxamidomethylated 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* (*36*) determined the pI 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 the C25S, R26V, and K27Q mutant proteins (data not shown). Using DTT to determine the pI of the reduced state of each enzyme and HED to determine the oxidized state of each enzyme gave similar results to those seen by Yang and Wells (*25*). Reduced RPLTT (Figure 10, lane 3) and the C25S (Figure 11, lane 3), R26V (Figure 12, lane 3), K27Q (Figure 13, lane 3) variants, yielded pIs of 7.0, 7.0, 5.7, and 5.9 respectively. Oxidized RPLTT (Figure 10, lane 5) and the R26V (Figure 12, lane 5) and K27Q (Figure 13, lane 5) mutant proteins, yielded pIs of 8.0, 7.0, and 7.0 respectively. Oxidized RPLTT and the R26V and K27Q variants yield more basic pIs because of the formation of an intramolecular disulfide bond between Cys-22 and Cys-25. HED treated C25S protein focused to pIs of 7.0, 7.3 and 8.0 which corresponds to reduced enzyme (pI 7.0) and enzyme with a mixed disulfide with HED (Figure 11, lane 5).

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 10, lane 1) and the R26V (Figure 12, lane 1) and K27Q (Figure 13, lane 1) proteins focused to pIs corresponding to both reduced and oxidized species, whereas C25S enzyme,

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lacking the ability to form an intramolecular disulfide bond, focused only to a reduced pI (Figure 11, lane 1). When treated with IAA after a 30 minute preincubation in buffer, RPLTT focused to bands of 5.9 and 4.9 (Figure 10, lane 2), the C25S variant focused to a pI of 6.0 (Figure 11, lane 2), R26V protein focused to bands of 4.4, 4.9, and 5.7 (Figure 12, lane 2), and K27Q enzyme focused to bands of 4.6, 4.9, 5.0, 5.9, and 7.0 (Figure 13, lane 2). For each enzyme pretreatment with DTT prior to IAA treatment yielded similar results except that reduced enzyme (pI = 7.0) was also seen with RPLTT (Figure 10, lane 4) and C25S protein (Figure 11, lane 4). In each case, 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 + GSH, or GSSG protected against the IAM inactivation of the DHA reductase activity of RPLTT and the R26V and K27Q mutant proteins whereas preincubation with GSH or AA did not. In agreement with the IAM inactivation studies, preincubation of R26V (Figure 12) or K27Q (Figure 13) proteins with HED (lane 6), DHA (lane 8), DHA + GSH (lane 10), or GSSG (lane 11) prior to IAA addition resulted in the formation of oxidized enzyme (pI = 7.0). The predominant form of RPLTT (Figure 10) when preincubated with HED (lane 6), DHA + GSH (lane 10), or GSSG (lane 11) was oxidized enzyme (pI 8.0), protecting against the formation of IAA modified species, although a small amount of pI = 4.9 species was seen when RPLTT was preincubated with HED. The primary form of RPLTT (Figure 10), when preincubated with DHA prior to IAA treatment (lane 7), was at pI 7.3, possibly indicating the trapping of an enzyme-substrate intermediate. These results confirm that the formation of oxidized RPLTT and its R26V and K27Q variants results in the protection of the enzyme from IAA

modification and IAM inactivation.

Preincubation of RPLTT (Figure 10) or the R26V enzyme (Figure 12) with GSH (lane 9) or AA (lane 12) prior to IAA treatment resulted in the appearance of reduced enzyme ( pI = 7.0 for RPLTT and pI = 5.7 for R26V protein) and IAA modified enzyme (pIs = 4.9 and 5.9 for RPLTT and pIs = 4.4 and 4.9 for R26V protein). In contrast to RPLTT and R26V protein, K27Q enzyme treated with IAA (Figure 13, lane 2)and K27Q enzyme preincubated with AA prior to IAA treatment (Figure 13, lane 12) yielded some oxidized enzyme, indicating that the prepared reduced K27Q enzyme contained some oxidized enzyme. Preincubation of the K27Q variant with GSH yielded species with pIs = 6.0 and 5.0 (Figure 13, lane 9) indicating the reaction of enzyme with IAA and supporting the finding that GSH enhances the IAM inactivation of the DHA reductase activity of K27Q enzyme.

The DHA reductase activity of the C25S protein was protected from IAM inactivation by HED, DHA + GSH, or GSSG but not by GSH, DHA, or AA. In agreement with these results, when the C25S enzyme (Figure 11) was preincubated with GSH (lane 9), AA (lane 12), and DHA (lane 8) both reduced enzyme (pI = 7.0) and the band indicative of carboxymethylation (pI = 6.0) appeared in significant quantities. When the C25S mutant protein was preincubated with HED (lane 6) or DHA + GSH (lane 10) prior to IAA treatment, no pI 6.0 species was seen. Some 6.0 species was seen when the C25S enzyme was preincubated with GSSG (lane 11) even though GSSG afforded complete protection against IAM inactivation of the DHA reductase activity of C25S enzyme (Figure 6).

As shown in Figure 9, reduced RPLTT and the R26V and K27Q variants were capable of chemically reducing DHA to AA in the absence of GSH. When reduced RPLTT (Figure 10, lane 7) and the R26V (Figure 12, lane 7) or K27Q (Figure 13, lane 7) mutant

proteins were incubated with DHA alone, oxidized enzyme (pI = 8.0 for RPLTT and pI = 7.0 for the R26V and K27Q enzymes) was formed. This is consistent with the ability of reduced RPLTT or the R26V and K27Q variants to chemically reduce DHA forming AA and oxidized enzyme (Figure 4, reactions 1 and 3). Previously, DHA had been shown to be a thiol oxidant of reduced bovine pancreatic ribonuclease (*43-46*).

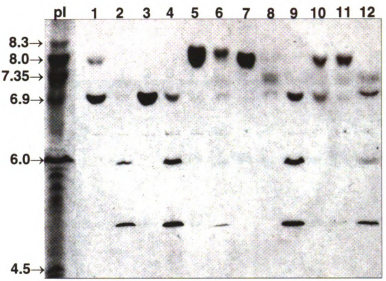


**Figure 10**. Isoelectric focusing analysis of incubation of reduced RPLTT with various substrates. Reduced RPLTT (12$\mu$g) was separately incubated alone (lane 2), with 1 mM DTT (lane 4), 1 mM HED (lane 6), 1 mM DHA (lane 8), 1 mM GSH (lane 9), 0.5 mM DHA plus 1 mM GSH (lane 10), 0.5 mM GSSG (lane 11), or 0.5 mM AA (lane 12) 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 incubated for 30 additional minutes at 30°C. No IAA controls included untreated enzyme (lane 1), enzyme treated with 1 mM DTT (lane 3), 1 mM HED (lane 5), 1 mM DHA (lane 7). Isoelectric point standards are 5$\mu$l of Serva pI test mix. The pI 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 gel shown is typical of two or more analyses.

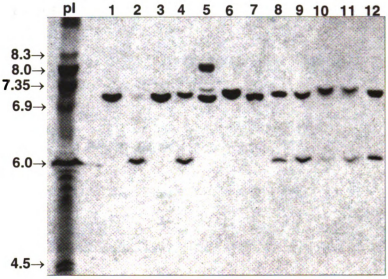


**Figure 11**. Isoelectric focusing analysis of incubation of reduced C25S with various substrates. Reduced C25S (12$\mu$g) was separately incubated alone (lane 2), with 1 mM DTT (lane 4), 1 mM HED (lane 6), 1 mM DHA (lane 8), 1 mM GSH (lane 9), 0.5 mM DHA plus 1 mM GSH (lane 10), 0.5 mM GSSG (lane 11), or 0.5 mM AA (lane 12) 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 incubated for 30 additional minutes at 30°C. No IAA controls included untreated enzyme (lane 1), enzyme treated with 1 mM DTT (lane 3), 1 mM HED (lane 5), 1 mM DHA (lane 7). Isoelectric point standards are 5$\mu$l of Serva pI test mix. The pI 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 gel shown is typical of two or more analyses.

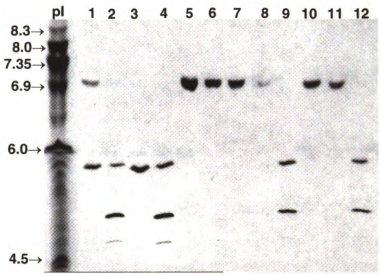


**Figure 12**. Isoelectric focusing analysis of incubation of reduced R26V with various substrates. Reduced R26V (12$\mu$g) was separately incubated alone (lane 2), with 1 mM DTT (lane 4), 1 mM HED (lane 6), 1 mM DHA (lane 8), 1 mM GSH (lane 9), 0.5 mM DHA plus 1 mM GSH (lane 10), 0.5 mM GSSG (lane 11), or 0.5 mM AA (lane 12) 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 incubated for 30 additional minutes at 30°C. No IAA controls included untreated enzyme (lane 1), enzyme treated with 1 mM DTT (lane 3), 1 mM HED (lane 5), 1 mM DHA (lane 7). Isoelectric point standards are 5$\mu$l of Serva pI test mix. The pI 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 gel shown is typical of two or more analyses.

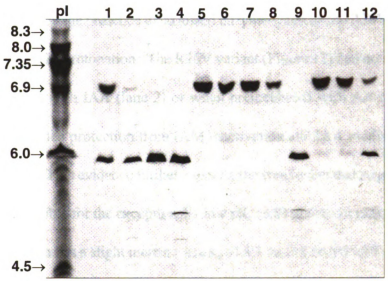


**Figure 13**. Isoelectric focusing analysis of incubation of reduced K27Q with various substrates. Reduced K27Q (12$\mu$g) was separately incubated alone (lane 2), with 1 mM DTT (lane 4), 1 mM HED (lane 6), 1 mM DHA (lane 8), 1 mM GSH (lane 9), 0.5 mM DHA plus 1 mM GSH (lane 10), 0.5 mM GSSG (lane 11), or 0.5 mM AA (lane 12) 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 incubated for 30 additional minutes at 30°C. No IAA controls included untreated enzyme (lane 1), enzyme treated with 1 mM DTT (lane 3), 1 mM HED (lane 5), 1 mM DHA (lane 7). Isoelectric point standards are 5$\mu$l of Serva pI test mix. The pI 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 gel shown is typical of two or more analyses.

*Proposed functions of R26 and K27 in thioltransferase.* The DHA reductase activity of both the R26V and K27Q variants were incompletely inhibited by IAM with 40% residual activity after 45 minutes. GSH only enhanced the IAM inactivation of the DHA reductase activity of the K27Q protein but not that of the R26V protein. This suggested that the K27Q variant was protected by the presence of oxidized enzyme whereas the R26V mutant protein was protected by Cys-22 protonation. The R26V variant (Figure 12) had no oxidized protein present when reacted with IAA (lane 2) or when preincubated with AA (lane 12). Thus R26V protein is afforded protection from IAM inactivation and IAA modification because Cys-22 is protonated. This evidence further supports the conclusion that Arg-26 is the amino acid primarily responsible for the exceptionally low $pK_a$ (3.8) of Cys-22 (*25*). Although Cys-22 in the K27Q protein has a slight increase in $pK_a$ of 4.3 vs. 3.8 for RPLTT, the role of Lys-27 was previously unknown (*25*). The current studies suggest that the role of lysine at position 27 is to stabilize the reduced form of RPLTT. In contrast to the other enzymes, when the K27Q variant was treated with IAA (Figure 13, lane 2) or preincubated with AA prior to IAA addition (Figure 13, lane 12), a significant amount of oxidized enzyme was seen. Finally, K27Q protein had the greatest initial rate of chemical reduction of DHA to AA, a reaction which includes the oxidation of the active site sulfhydryls (reaction 3, Figure 4). These data suggest that the K27Q enzyme is much more readily oxidized than RPLTT or the R26V mutant protein indicating a role of lysine at position 27 in the stabilization of the reduced form of the enzyme.

## CONCLUSIONS

The work described in this paper supports the catalytic mechanism proposed in Figure 4. When starting with the reduced form of the enzyme, IAM inactivation studies

demonstrated the ability of DHA to bind to enzyme (reaction 1, Figure 4), prior to GSH binding (reaction 3 or 4, Figure 4) because DHA protected against IAM inactivation whereas GSH did not. The ability of RPLTT and the R26V and K27Q variants to chemically reduce DHA to AA in the absence of GSH (reactions 1 and 3, Figure 4) was demonstrated by electrochemical analysis and IEF. In addition, IEF analysis demonstrated the ability of reduced RPLTT and the R26V and K27Q variants to react with GSSG and form oxidized enzyme (reverse of reactions 5 and 4 in Figure 4). The relative inability of the C25S protein to chemically reduce DHA to AA, but its robust DHA reductase activity strongly suggest that reaction 2 (Figure 4) is a part of this mechanism. Further studies in progress involve the detection of the thiohemiketal intermediate and the study of its catalytic capacity. In addition, in the presence of GSH, whether reaction 2 or 3 predominates in the mechanism of RPLTT and the R26V and K27Q variants has yet to be determined. Previously proposed models of the DHA reductase activity of thioltransferase (*22, 23, 26, 27, 36*) and for the DHA reductase activity of *Trypanosoma cruzi* p52 (*47*) have not included reactions 1 and 3 in Figure 4, but the work described in this paper clearly demonstrates the potential of thioltransferase to undergo these reactions.

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# Chapter IV

## Glutathione Dependent Reduction of Alloxan to Dialuric Acid Catalyzed by Thioltransferase (Glutaredoxin): a Possible Role for Thioltransferase in Alloxan Toxicity

The majority of this chapter has been published: Washburn, M.P. and Wells, W.W. (1997) *Free Rad. Biol. Med*. **23**, 563-570.

## ABSTRACT

Recombinant pig liver thioltransferase (RPLTT) catalyzes the reduction of alloxan to dialuric acid by glutathione (GSH). This is the second non-disulfide substrate, after dehydroascorbic acid, described for thioltransferase. The reaction kinetics, measured by a coupled assay including glutathione disulfide reductase and NADPH, yielded a $K_m$ = 82 $\mu$M for alloxan, a $k_{cat}$ = 37 $sec^{-1}$, and a $k_{cat}/K_m$ = 4.5 x $10^5$ $M^{-1}sec^{-1}$. The presence of RPLTT suppressed the competitive formation of compound 305, an alloxan-GSH conjugate of unknown structure, and at GSH concentrations between 0.05 mM and 1.5 mM, oxygen consumption was greater than that recorded in the uncatalyzed reaction. Both superoxide dismutase and catalase inhibited oxygen consumption in 1.0 mM GSH and 0.2 mM alloxan in the presence of RPLTT. This study suggests that thioltransferase (glutaredoxin) plays a significant role in the cytotoxicity of alloxan in vulnerable tissues.

Alloxan (2,4,5,6 [1H,3H] pyrimidinetetrone) is selectively toxic to the 2-cells of the pancreas of certain animals, e.g., mice and rats (*1-3*). This toxicity has been attributed in part to accumulation of alloxan in the islets (*4, 5*) and 2-cells (*6*). Furthermore, the pancreas possesses relatively lower levels of enzymes such as catalase, Cu-Zn superoxide dismutase (SOD), Mn-SOD, and glutathione (GSH) peroxidase, which protect cellular components against reactive oxygen species (*5, 7*). The toxic mechanism of alloxan involves redox cycling between alloxan and dialuric acid. Reduction of alloxan to dialuric acid by physiological reagents has been demonstrated with NAD(P)H (*8, 9*), GSH (*10-13*), and enzymatically by the thioredoxin/NADPH-thioredoxin reductase system (E.C. 1.6.4.5) (*14*). Dialuric acid oxidation generated superoxide, hydrogen peroxide, and hydroxyl radical, in the presence of an appropriate metal catalyst like iron (*15-18*) Specifically, reaction of alloxan with GSH generated superoxide and hydrogen peroxide (10-13), hydroxyl radical in the presence of iron (*18*), and released iron from ferritin and subsequently formed hydroxyl radical (*19*). Evidence supporting this mechanism includes protection against alloxan toxicity in isolated islets by SOD, catalase, hydroxyl radical scavengers, metal chelators (*20-22*), and in mice by SOD (*23*).

The chemical reaction of alloxan and GSH yields two distinct spectrophotometric products, dialuric acid and compound 305 (*10-13*). Dialuric acid, the reduced form of alloxan, has a characteristic absorbance at 275 nm (13) and is rapidly formed upon the addition of GSH to alloxan (*10*). Compound 305 is a more gradually appearing alloxan-GSH conjugate of undetermined structure which absorbs maximally at 305 nm (*10, 12*). The formation of compound 305 is in competition with the reduction of alloxan to dialuric acid (*10, 12*), and the production of dialuric acid is favored when GSH is in excess of alloxan (*10,*

*12*).

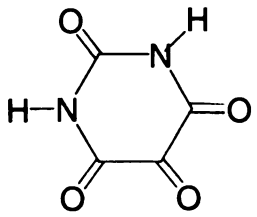
Thioltransferase (EC1.8.4.1) (glutaredoxin), a 12 kDa enzyme, catalyzes GSH-dependent thiol-disulfide exchange reactions (*24*) Furthermore, this enzyme isolated from human placenta, bovine thymus, and recombinant pig liver thioltransferase (RPLTT) catalyze the GSH-dependent reduction of dehydroascorbic acid (DHA) to ascorbic acid (*25*). The active site of RPLTT has been determined to be -Cys22-Pro23-Phe24-Cys25-, with Cys22 the essential amino acid for both thioltransferase and DHA reductase activity (*26*).

Thioredoxin, shown to oxidize NADPH in the presence of thioredoxin reductase and alloxan (*14*) shares a similar active site to that of thioltransferase, -CXXC- (*24, 27*). Furthermore, alloxan and DHA and their reduction products, dialuric acid and ascorbic acid, have similar structures (Figure 1). Based on thioredoxin/NADPH-thioredoxin reductase system reactivity with alloxan and thioltransferase catalysis of DHA reduction, we examined thioltransferase as a GSH-dependent alloxan reductase and tested the potential role of thioltransferase in alloxan's cytotoxicity.

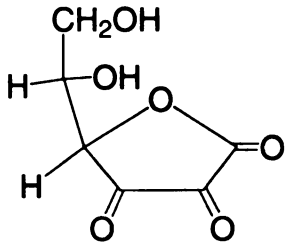
## MATERIALS AND METHODS

*Materials.* GSH, alloxan monohydrate, bovine erythrocyte SOD (4400 units/mg protein), and bovine liver catalase (1600 units/mg protein) were purchased from Sigma Chemical Company (St. Louis, MO USA). NADPH, dithiothreitol (DTT), bovine serum albumin (BSA), and yeast glutathione disulfide (GSSG) reductase were from Boehringer Mannheim Corporation (Indianapolis, IN USA). RPLTT was purified as described previously (*28*). In all of the following studies RPLTT was stored in 20% glycerol, 20 mM sodium phosphate, pH 7.5. Reduced RPLTT, C25S, and K27Q for IEF were prepared in the absence of glycerol as described in Chapter III. GSSG reductase stock solution was 1.25

## Oxidized Forms:

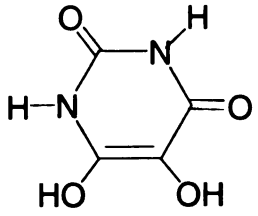



**Alloxan**

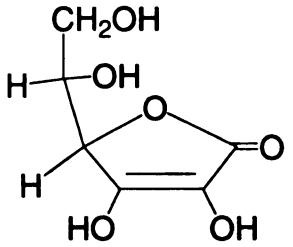



**Dehydroascorbic Acid**

## Reduced Forms:




**Dialuric Acid**




**L-Ascorbic Acid**

**Figure 1**. Structural comparison of alloxan to dehydroascorbic acid and dialuric acid to L-ascobic acid

mg/ml in 1.3 M $(NH_4)_2SO_4$. Stock solutions of alloxan were in $N_2$ sparged 1 mM HCl and kept on ice. This method of preparation and storage maintained alloxan for up to 1.5 h.

*Kinetic characterization of RPLTT as an alloxan reductase.* Kinetic analysis of RPLTT catalysis of the GSH-dependent reduction of alloxan to dialuric acid was based on the following assay system.

Thioltransferase

$$\text{Alloxan} + 2\text{GSH} \rightleftharpoons \text{Dialuric Acid} + \text{GSSG}$$

GSSG Reductase

$$\text{GSSG} + \text{NADPH} + H^+ \rightleftharpoons 2\text{GSH} + \text{NADP}^+$$

Initial reaction velocities were compared at various pH values using 200 mM sodium phosphate, 1 mM EDTA buffers, between pH 5.5 and 7.5, and 200 mM Tris-HCl, 1 mM EDTA buffers, between pH 8.0 and 9.5. The optimal assay conditions consisted of 1.0 mM GSH, 0.5 mM NADPH, 8.75 $\mu$g GSSG reductase, 200 mM sodium phosphate, 1 mM EDTA, pH 7.5 in a 500 $\mu$l reaction volume. This assay is similar to those used previously for thioltransferase studies (*29, 30*). Kinetic studies were carried out in the presence of 0.375 $\mu$g RPLTT. The reaction was initiated upon the addition of varying amounts of alloxan and followed as a decrease in absorbance at 340 nm at 25°C for 1.5 min. A blank without enzyme was run simultaneously for each assay and the difference (($\acute{C}Abs_{340\ nm}$/min) x 0.5 x 1000/ 6.22) gave activity in nmoles/min. Using this assay, the initial velocity of RPLTT was linear over a range up to 2 mg protein/ml.

*Spectrophotometric study of uncatalyzed and RPLTT catalyzed reactions.* The effect of RPLTT on the alloxan and GSH reaction from 250 nm to 320 nm was monitored (scan run

at 2 nm/sec). GSH (1.5 mM) and alloxan (0.2 mM) in 200 mM sodium phosphate, 1 mM EDTA, pH 7.5, were mixed in a final volume of 500 $\mu$l in the presence or absence of 1.25 $\mu$g RPLTT at 23 °C. The difference in uncatalyzed and RPLTT catalyzed production of dialuric acid increased linearly up to 2.5 $\mu$g/ml RPLTT. Spectra were taken at multiple time points up to 50 min after initiating the reaction by addition of alloxan.

The extinction coefficient for dialuric acid was determined to be 14,500 $M^{-1}cm^{-1}$ at 275 nm by taking the absorbance multiple times of 50 $\mu$M alloxan reacted with 1.0 mM DTT in 200 mM sodium phosphate, 1 mM EDTA, pH 7.5. The reaction was initiated by the addition of alloxan and analyzed from 250 nm to 320 nm until reaching a peak absorbance value at 275 nm.

*Oxygen consumption during the GSH-dependent reduction of alloxan.* Oxygen consumption was determined at 25 °C using a YSI Model 5331 oxygen probe and a YSI Model 53 biological oxygen monitor. Baseline oxygen uptake was measured in a 3 ml reaction volume of 200 mM sodium phosphate, 1 mM EDTA, pH 7.5, in the presence or absence of 7.5 $\mu$g RPLTT and various concentrations of GSH. Alloxan was then added to a final concentration of 0.2 mM and maximal rates of oxygen consumption were measured. In experiments investigating the effects of SOD and catalase, the enzymes were dissolved in 100 mM sodium phosphate, 0.5 mM EDTA, pH 7.5, and added prior to baseline oxygen uptake measurement. Oxygen consumption in the presence of bovine serum albumin (BSA, 2.5 $\mu$g/ml) was measured as a control for non-specific protein interactions. Oxygen consumption was measured on a Linseis Flatbed Recorder model L6522 B. Statistical analysis was carried out with the GraphPAD InStat program using a one way analysis of variance with selected comparisons subjected to the Bonferroni post hoc test.

*Isoelectric Focusing.* Reduced RPLTT and its C25S and K27Q variants, 12 $\mu$g each, were separately incubated alone or with 1 mM alloxan or 0.5 mM alloxan + 1.0 mM GSH in 40 mM sodium phosphate, pH 7.5, for 30 min in a 30°C water bath. Each of the incubation mixtures (in a total volume of 10 $\mu$l) was then analyzed directly on a Servalyt Precotes isoelectric focusing gel, pH 3-10 (150 $\mu$m thick, 125 mm x 125 mm), following the manufacturer's instructions for 2666 volt hours on a 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

*Spectrophotometric study of the effect of RPLTT on the GSH reduction of alloxan.* At 22 sec after reaction initiation, the uncatalyzed reaction yielded 0.11 ± 0.01 mM dialuric acid measured spectrophotometrically at 275 nm indicating a 55% reduction of alloxan to dialuric acid (Figure 2A). The RPLTT catalyzed reaction yielded 0.16 ± 0.01 mM dialuric acid, equivalent to 82% reduction of alloxan to dialuric acid, 45% more dialuric acid than in the uncatalyzed reaction (Figure 2A). By 4 min, the reduction of alloxan to dialuric acid in both the RPLTT catalyzed and the uncatalyzed reaction was 90%-95% complete (Figure 2B). From 4-50 min, the levels of dialuric acid in the RPLTT catalyzed and the uncatalyzed reaction were not significantly different and remained 90%-95% complete, never reaching 100% completion (Figure 2C-2D). The absorbance at 305 nm for the uncatalyzed and RPLTT catalyzed reactions at various time points are plotted in Figure 3. The rate of formation of compound 305 from 0-4 min was faster in the uncatalyzed reaction mixture than

in the RPLTT catalyzed reaction mixture. By 2 min, a significant difference in absorbance at 305 nm occurred; the uncatalyzed reaction had an absorbance of 0.40 ± 0.12 greater than the RPLTT catalyzed reaction, and this trend continued for 50 min. However, from 4-50 min, the rate of production of compound 305 was similar in both reaction mixtures.

*Kinetics.* The coupled assay system was chosen because under certain alloxan:GSH ratios necessary for kinetic characterization, compound 305 generation interfered with accurate measurement of dialuric acid formation at 275 nm, whereas no interference occurred at 340 nm. Although NADPH does reduce alloxan to dialuric acid at a low rate (*8, 9*) this was subtracted as part of the control without RPLTT, and RPLTT did not utilize NADPH as a substrate. Up to 1 mM alloxan did not affect GSSG reductase activity (data not shown). Optimal pH for the coupled assay was in the range of 7.5-8.0 (Figure 4). The kinetic profile yielded a sigmoidal curve with inhibition at high substrate concentration (Figure 5). The best fit was to the Hill equation (n=2.7) (EZ-Fit kinetic program (*31*)). The kinetic parameters of the GSH dependent reduction of alloxan are reported in Table I. Based on these kinetic values, alloxan compares favorably with DHA, hydroxyethyldisulfide, and S-sulfocysteine as a thioltransferase substrate.

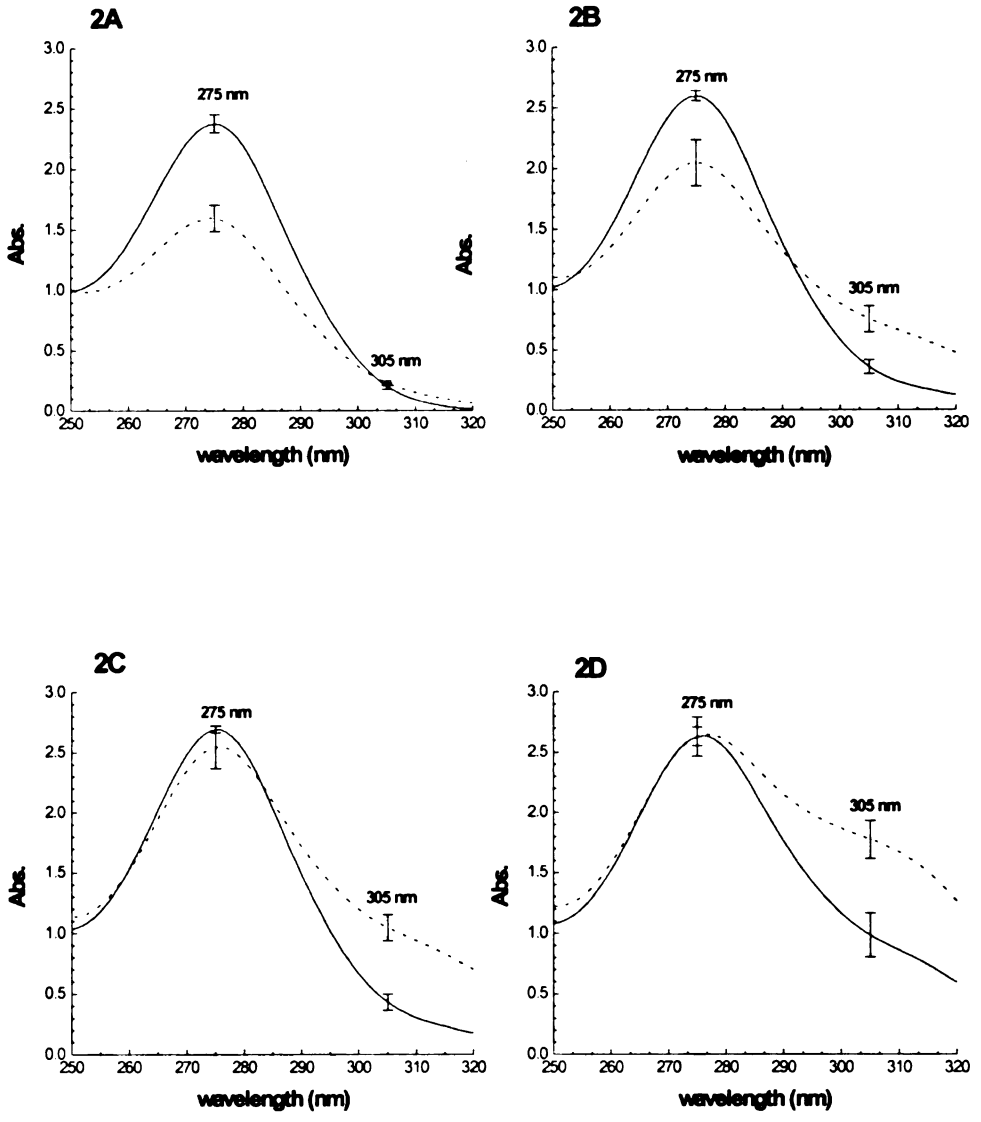



**Figure 2.** Spectrophotometric (250 nm - 305 nm) time course study of the effect of RPLTT on the alloxan and GSH reaction. GSH (1.5 mM) and 0.2 mM alloxan in 200 mM sodium phosphate, 1 mM EDTA, pH 7.5, were mixed in a final volume of 500 $\mu$l in the presence (solid lines) or absence (dashed lines) of 1.25 $\mu$g RPLTT1 at 23°C and analyzed from 250 nm to 320 nm. Spectra were taken at multiple times over 50 min. The spectra were taken at A) 22 sec (first opportunity for measurement), B) 2 min, C) 4 min, and D) 30 min. Each chemical and enzymatic spectra shown at each time point are the average of three or four separate experiments. Error bars represent one standard deviation of the data at 275 nm and 305 nm.

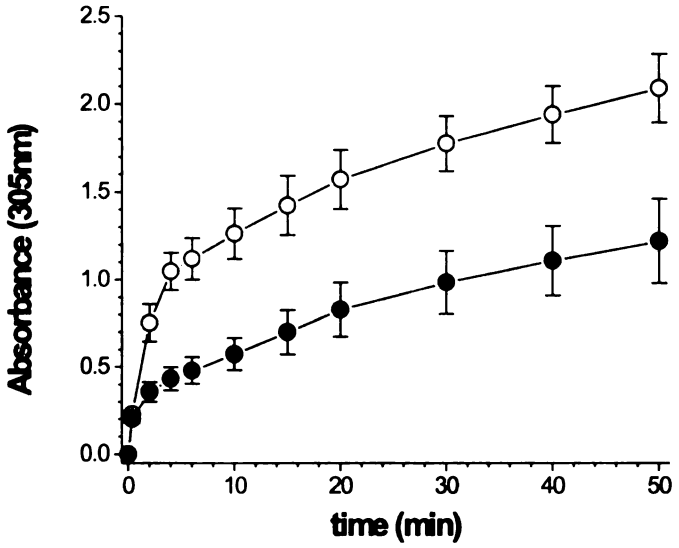



**Figure 3.** Time course study of the effect of RPLTT on the alloxan and GSH reaction at 305 nm. GSH (1.5 mM) and 0.2 mM alloxan in 200 mM sodium phosphate, 1 mM EDTA, pH 7.5, were mixed in a final volume of 500 $\mu$l in the presence (-●-) or absence (-○-) of 1.25 $\mu$g RPLTT at 23°C and analyzed from 250 nm to 320 nm. Spectra were taken at multiple times over 50 min. Data points are the averages of three or four separate experiments. Error bars represent one standard deviation of the data at 305 nm and are not seen if the values are smaller than the size of the symbols.

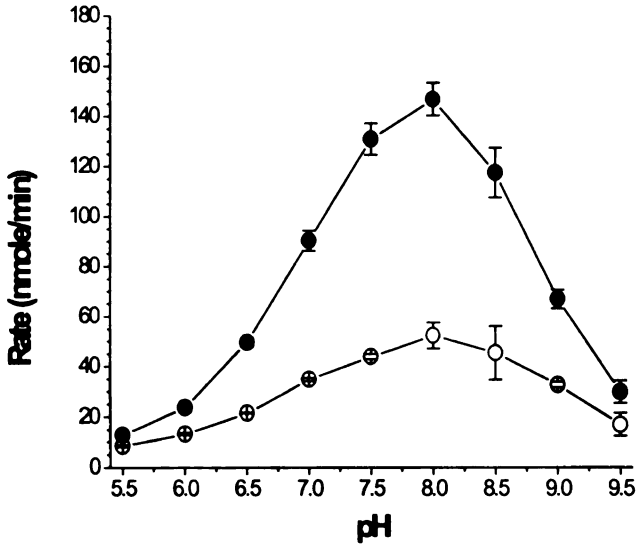



**Figure 4.** pH profile of the GSH-dependent alloxan reductaes activity of RPLTT. RPLTT (0.7491 $\mu$g) was assayed for alloxan reductase activity in 1.0 mM GSH, 0.34 mM NADPH, 5 $\mu$g GSSG reductase, and various buffer conditions in a 500 $\mu$l reaction volume. The reaction was initiated by the addition of alloxan and was followed at 340 nm at 25°C for 15 min. The pH was varied as shown using 200 mM sodium phosphate containing 1mM EDTA between pH 5.5 and 7.5., and 200 mM Tris-HCl with 1mM EDTA between pH 8.0 and 9.5. Reaction rates in the presence (-●-) or absence (-○-) of RPLTT are shown. Data points are the averages of at least three separate experiments. Error bars represent one standard deviation of the data and are not seen if values are smaller than the size of the symbols.

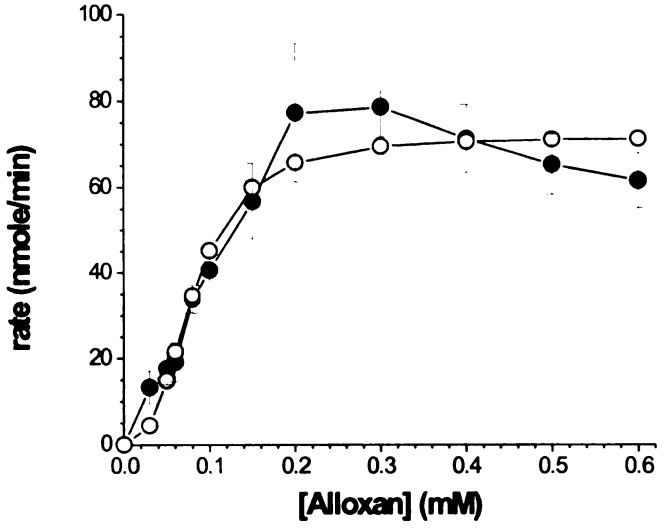



**Figure 5**. Kinetic analysis of GSH dependent alloxan reductase activity of RPLTT. RPLTT (0.375 $\mu$g) was assayed for alloxan reductase activity in 1.0 mM GSH, 0.5 mM NADPH, 8.75 $\mu$g GSSG reductase, 200 mM sodium phosphate, 1 mM EDTA, pH 7.5, in a 500 $\mu$l reaction volume. The reaction was initiated by the addition of alloxan and followed at 340 nm at 25°C for 1.5 min. Alloxan concentration was varied as shown and initial rates calculated. Data points (-●-) are the averages of four 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. Kinetic parameters were obtained via the Hill equation. The Hill equation (-○-) (n = 2.7) yielded the best fit of a wide range of kinetic models tested in the EZ-FIT kinetic program.

Table I

Recombinant pig liver thioltransferase kinetic parameters

| Substrate | $K_m$ (mM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) |
|---|---|---|---|
| alloxan* | 0.082 ± 0.009 | 37 ± 2 | (4.5 ± 0.6) x $10^5$ |
| dehydroascorbic acid† | 0.26 ± 0.09 | 6.2 ± 0.3 | (2.4 ± 0.9) x $10^4$ ref. 32 |
| hydroxyethyldisulfide‡ | 2.95 ± 0.51 | 68 ± 5 | (2.3 ± 0.4) x $10^4$ |
| S-sulfocysteine‡ | 1.61 ± 0.35 | 54 ± 6 | (3.6 ± 0.9) x $10^4$ |

* RPLTT (0.375 $\mu$g) was assayed for alloxan reductase activity in 1.0 mM GSH, 0.5 mM NADPH, 8.75 $\mu$g GSSG reductase, 200 mM sodium phosphate, 1 mM EDTA, pH 7.5, in a 500 $\mu$l reaction volume. The reaction was initiated by the addition of alloxan and followed at 340 nm at 25°C for 1.5 min. Best fit was with the Hill equation with a coefficient of n = 2.7, and kinetic constants were determined using the EZ-Fit kinetic program.[31]

† assay system used to obtain these kinetic parameters was a direct spectrophotometric analysis of ascorbic acid at 265.5 nm (*32*).

‡ Kinetic constants for hydroxyethyldisulfide and S-sulfocysteine were determined using a coupled assay system similar to the system described for alloxan. The assay system included 1.6 mM GSH, 0.25 $\mu$g RPLTT, 5 $\mu$g GSSG reductase, and 0.61 mM NADPH. In these assay systems, the reactions were initiated upon the addition of S-sulfocysteine or hydroxyethyldisulfide and followed for 3 min at 340 nm at 30°C.

*Oxygen consumption in the RPLTT catalyzed and uncatalyzed GSH reduction of alloxan.* Neither alloxan, RPLTT, GSH + RPLTT nor RPLTT + alloxan consumed oxygen. By contrast, the addition of GSH to alloxan or to alloxan + RPLTT caused significant oxygen consumption (Figure 6). The presence of RPLTT caused an increase of oxygen consumption above the uncatalyzed reaction with 0.05 mM GSH to 1.5 mM GSH. At 2.0 mM GSH and above, oxygen consumption declined, and the uncatalyzed and RPLTT catalyzed oxygen consumptions converged. Maximal oxygen consumption occurred at 1.5 mM GSH where the presence of 2.50 $\mu$g/ml RPLTT was associated with an oxygen consumption of $148 \pm 8$ $\mu$M $O_2$/min. Maximal difference between the RPLTT catalyzed and the uncatalyzed reactions ($52.6 \pm 9.2$ $\mu$M $O_2$/min) occurred at 0.5 mM GSH. Enhancement of oxygen consumption was specific for RPLTT. Oxygen consumption experiments with BSA (2.50 $\mu$g/ml BSA, data not shown) behaved identically to those without BSA.

*Catalase and SOD inhibition of oxygen consumption in the RPLTT catalyzed and uncatalyzed GSH reduction of alloxan.* In the uncatalyzed and RPLTT catalyzed reactions, catalase (5 $\mu$g/ml to 80 $\mu$g/ml) significantly inhibited oxygen consumption when compared with the control (Figure 7A, $p<0.01$ in each case). In contrast, SOD did not inhibit oxygen consumption in the uncatalyzed reaction, but did inhibit oxygen consumption in the RPLTT catalyzed reaction (Figure 7B, 2.5 $\mu$g/ml to 80 $\mu$g/ml SOD, $p<0.01$ in each case). From 10 µg/ml to 80 µg/ml SOD there was no significant difference in oxygen consumption between the uncatalyzed and RPLTT catalyzed reaction. Oxygen consumption in the RPLTT catalyzed reaction with SOD (40 $\mu$g/ml) and catalase (40 $\mu$g/ml) was significantly lower than with both SOD(40 $\mu$g/ml) alone and catalase (40 $\mu$g/ml) alone (Figures 7A and 7B, $p<0.001$). The addition of SOD (40 $\mu$g/ml) and catalase (40 $\mu$g/ml) to the RPLTT catalyzed

reaction was the only instance where oxygen consumption was decreased by 50%. Furthermore, oxygen consumption in the RPLTT catalyzed reaction was not significantly different from the uncatalyzed reaction with catalase (40 $\mu$g/ml), but it was significantly different from the uncatalyzed reaction with SOD (40 $\mu$g/ml) + catalase (40 $\mu$g/ml) ($p<0.05$). Finally, oxygen consumption in the uncatalyzed reaction with SOD (40 $\mu$g/ml) + catalase (40 $\mu$g/ml) was not significantly different from catalase (40 $\mu$g/ml) alone, but it was significantly lower than with SOD (40 $\mu$g/ml) alone (Figures 7A and 7B, $p<0.01$).

At 1.0 mM GSH and 0.2 mM alloxan, the lag time (time to maximal rates) was 25 ± 2 sec. In the presence of 2.50 $\mu$g/ml RPLTT, the time of initial rates was 17 ± 2 sec. SOD increased the lag time incrementally in the uncatalyzed reaction to 36 ± 4 sec at 80 $\mu$g/ml. SOD had no effect on the lag time in the presence of RPLTT, and catalase had no effect on the lag time in either case (data not shown). The maximal rate of oxygen consumption was always completed 2 min after reaction initiation (data not shown).

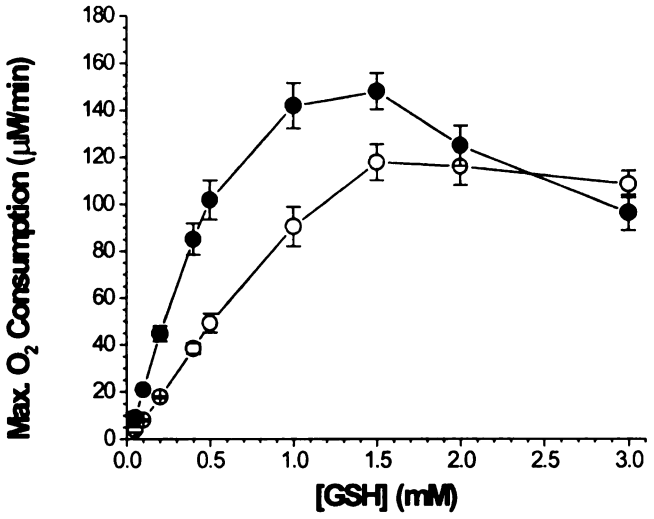



**Figure 6**. Oxygen uptake in the uncatalyzed and RPLTT catalyzed alloxan + GSH reaction. Oxygen consumption was determined at 25°C using a YSI Model 5331 oxygen probe and a YSI Model 53 biological oxygen monitor. Baseline oxygen uptake was measured in a 3 ml reaction volume in 200 mM sodium phosphate, 1 mM EDTA, pH 7.5 in the presence (-●-) or absence (-○-) of 7.5 $\mu$g RPLTT and varied [GSH]. Alloxan was then added to a final concentration of 0.2 mM and maximal rates of oxygen consumption calculated (values plotted). Data points are the averages of three or four separate experiments. Error bars represent one standard deviation of the data and are not seen if values are smaller than the size of the symbols.

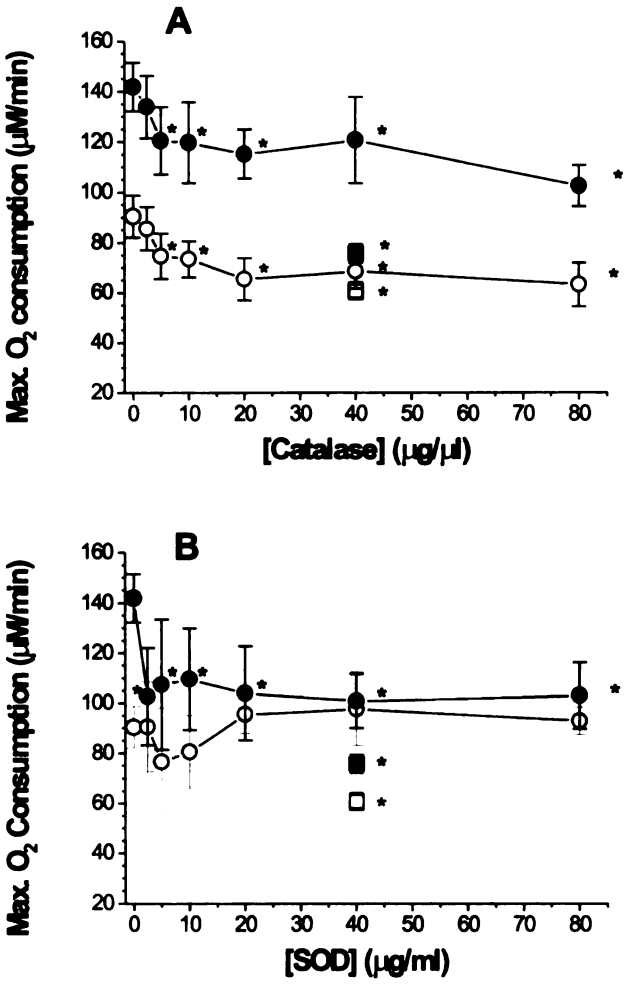



**Figure 7.** Catalase and SOD inhibition of oxygen uptake in the uncatalyzed and RPLTT catalyzed alloxan + GSH reaction. Oxygen consumption was determined as described in Figure 6. Baseline oxygen uptake was measured in a 3 ml reaction volume in 200 mM sodium phosphate, 1 mM EDTA, 1 mM GSH, pH 7.5 in the presence (-●-) or absence (-○-) of 7.5 $\mu$g RPLTT and in the presence of varied [catalase] (A) or varied [SOD] (B). The effect of both 40 $\mu$g/ml catalase and 40 $\mu$g/ml SOD on oxygen consumption is plotted on A and B as -■- in the presence of RPLTT and -□- in the absence of RPLTT. Maximal rates began 36 seconds or less after reaction initiation (80 $\mu$g/ml SOD in the chemical reaction). The 0 $\mu$g/ml SOD and/or catalase data points are taken directly from the 1.0 mM GSH data points in Figure 6. Data points are the averages of three or more 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. (*Statistically different from oxygen consumption in the absence of catalase or SOD; $p<0.01$ Bonferroni *post hoc* test.)

*Isoelectric focusing of RPLTT and the C25S and K27Q variants.* In the previous chapter (III), reduced RPLTT and the K27Q protein but not the C25S protein were demonstrated to have pronounced abilities to chemically reduce DHA to AA. The product of this reaction was oxidized enzyme, which was identified via IEF. Because of the structural similarities between alloxan and DHA, we investigated the ability of alloxan to oxidize reduced protein. Reduced RPLTT and the C25S and K27Q variants were seperately incubated alone or with 1 mM alloxan or 0.5 mM alloxan + 1.0 mM GSH (Figure 8). As seen previously in Chapter III, IEF itself was slightly oxidizing with reduced RPLTT (lane 1) and reduced K27Q enzyme (lane 7) each focusing to reduced (7.0 for RPLTT and 5.9 for K27Q enzyme) and oxidized pIs (8.0 for RPLTT and 7.0 for K27Q enzyme). Upon the addition of alloxan to either RPLTT (lane 2) or the K27Q variant (lane 8) only oxidized enzyme was seen (pI = 8.0 for RPLTT and pI = 7.0 for K27Q enzyme). When RPLTT (lane 3) or the K27Q variant (lane 9) was incubated with alloxan + GSH, reduced enzyme was primarily seen with a slight amount of oxidized enzyme seen with the K27Q variant. C25S enzyme incubated with alloxan resulted in focused pIs of 7.0, corresponding to reduced enzyme, and 6.0 (lane 5). When the C25S variant was preincubated with alloxan + GSH (lane 6) it focused to pIs of 7.0 and 5.9.

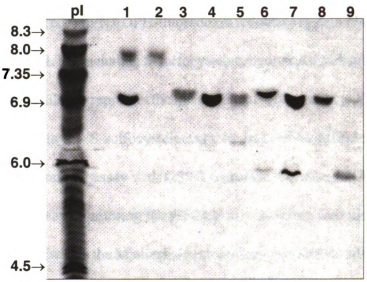



**Figure 8**. Isoelectric focusing analysis of incubation of reduced RPLTT and the C25S and K27Q variants. Equal amounts (12$\mu$g) of reduced RPLTT and the C25S and K27Q variants were separately incubated alone (lanes 1, 4, and 7, respectively), with 1 mM alloxan (lanes 2, 5, and 8, respectively), or with 0.5 mM alloxan plus 1.0 mM GSH (lanes 3, 6, and 9, respectively). Isoelectric point standards are 5$\mu$l of Serva pI test mix. The pI 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.

## DISCUSSION

Direct spectral analysis demonstrated that RPLTT catalyzed the GSH-dependent reduction of alloxan to dialuric acid. This is the second nondisulfide substrate, after DHA, described for thioltransferase, and the second demonstration of enzyme catalyzed alloxan reduction after the thioredoxin/NADPH-thioredoxin reductase system (*14*). Compared with other substrates for RPLTT, including S-sulfocysteine, hydroxyethyldisulfide and DHA, the kinetic parameters for alloxan represent the lowest $K_m$ and the largest $k_{cat}/K_m$ measured (Table I). As in the case of alloxan, S-sulfocysteine, and hydroxyethyldisulfide, kinetic constants were measured via a coupled assay with GSSG reductase, whereas DHA kinetic constants were obtained in an assay monitoring the production of ascorbic acid directly (*32*). In the direct assay, the best fit was to the Michaelis-Menten equation with substrate inhibition. The kinetics for alloxan and S-sulfocysteine (*29*) in the coupled assay yielded a best fit to a Hill plot with coefficients of n = 2.7 and n = 2.5, respectively. The sigmoidal nature of the curve is most likely a side effect of the coupled assay and not a result of cooperativity.

The formation of compound 305, an alloxan-GSH conjugate of unidentified structure, was in competition with the reduction of alloxan to dialuric acid (*10-12*). Since a greater rate of reduction to dialuric acid was catalyzed by RPLTT between 0-4 min, there was more alloxan available to form compound 305 in the uncatalyzed reaction. From 4-50 minutes, however, there were equal and constant amounts of dialuric acid and alloxan in both reactions, suggesting the reaching of reaction equilibrium with respect to GSH-dependent dialuric acid formation. The remaining alloxan and alloxan formed from dialuric acid oxidation in both reactions will either achieve equilibrium or form compound 305. Because enzymes catalyze reactions but do not alter equilibrium constants, the rate of formation of

compound 305 was equal in both the RPLTT catalyzed and uncatalyzed reaction. The evidence presented here supports the view that alloxan is a preferred substrate for RPLTT and that compound 305 formation is the product of a competing side reaction. Although RPLTT led to the suppression of the formation of compound 305, compound 305 increased with time in both the uncatalyzed and RPLTT catalyzed reaction. Furthermore, 82% of the alloxan was reduced independently to dialuric acid 22 sec after initiation of the reaction in the presence of RPLTT with negligible absorbance at 305 nm. If compound 305 was a substrate or intermediate in the reaction forming dialuric acid, an increased absorbance at 305 nm would be expected prior to development of the 275 nm peak.

To assess a potential role for thioltransferase in alloxan toxicity, oxygen uptake in the uncatalyzed and RPLTT catalyzed reaction was analyzed. The presence of RPLTT caused a significant increase in oxygen consumption compared to the chemical reaction from 0.05 mM GSH to 1.5 mM GSH. At higher concentrations of GSH (2.0 and 3.0 mM), there was a decrease in oxygen consumption compared with the 1.0 and 1.5 mM GSH levels. This was most likely due to the inhibitory effect of high GSH:alloxan ratios on dialuric acid oxidation and oxygen consumption, as observed previously (*12*).

The production of reactive oxygen species is essential for the toxicity of alloxan in the 2-cells of the pancreas (*2, 3, 20-23*). If thioltransferase is to play a role in the toxicity of alloxan, not only should there be an increase in oxygen consumption, but superoxide and hydrogen peroxide should be produced by the RPLTT catalyzed reaction mixture. In the RPLTT catalyzed reaction, SOD and catalase significantly inhibited oxygen uptake. The presence of both SOD (40 $\mu$g/ml) and catalase (40 $\mu$g/ml) further enhanced inhibition of oxygen uptake, and was the only instance where maximal oxygen consumption was

decreased by 50%. These results indicate that both superoxide and hydrogen peroxide were generated by the RPLTT catalyzed reaction and subsequent oxidation of dialuric acid. In the uncatalyzed reaction, only catalase inhibited oxygen consumption. These results appear to conflict with previous work where both SOD and catalase inhibited oxygen consumption during the chemical reaction (*10, 11, 13*) For example, Munday, *et al.* (*13*) determined that in 50 mM potassium phosphate, pH 7.4, 1 mM GSH, 0.05 mM alloxan, and at 25°C, the presence of 10 $\mu$g/ml SOD inhibited oxygen uptake by 91%.

According to Winterbourn, *et al.* (*33*), dialuric acid oxidation can occur via two mechanisms, a superoxide dependent chain mechanism and an autocatalytic mechanism. As a result, under the appropriate conditions, dialuric acid oxidation can occur as rapidly in the presence of SOD as in the absence of SOD because of the ability of dialuric acid to oxidize via the autocatalytic mechanism (*33*). In the proposed reaction scheme for the alloxan-dialuric acid cycle (Figure 9), SOD can suppress reactions 2 ($O_2^{\bullet-} + H^+ + AlxH_2 \rightarrow {}^{\bullet}AlxH + H_2O_2$) and 4, but oxidation can persist if reaction 3 keeps pace with the alloxan radical produced in reactions 1 ($AlxH_2 + O_2 \rightarrow {}^{\bullet}AlxH + O_2^{\bullet-} + H^+$) and 5 (*33*). In the presence of SOD, maximal rates of oxidation occurred with increasing concentrations of alloxan and dialuric acid and when [alloxan] = [dialuric acid] (*33*). In the experiments described in the current paper a 4-fold higher level of alloxan was used than was used in references *10*, *11*, and *13*, resulting in favorable conditions for rapid dialuric acid oxidation by the autocatalytic reaction in the presence of SOD.

In contrast to the uncatalyzed reaction , SOD inhibited oxygen consumption in the presence of RPLTT because dialuric acid oxidation by the autocatalytic mechanism is minimized in the presence of RPLTT. The autocatalytic mechanism depends on the

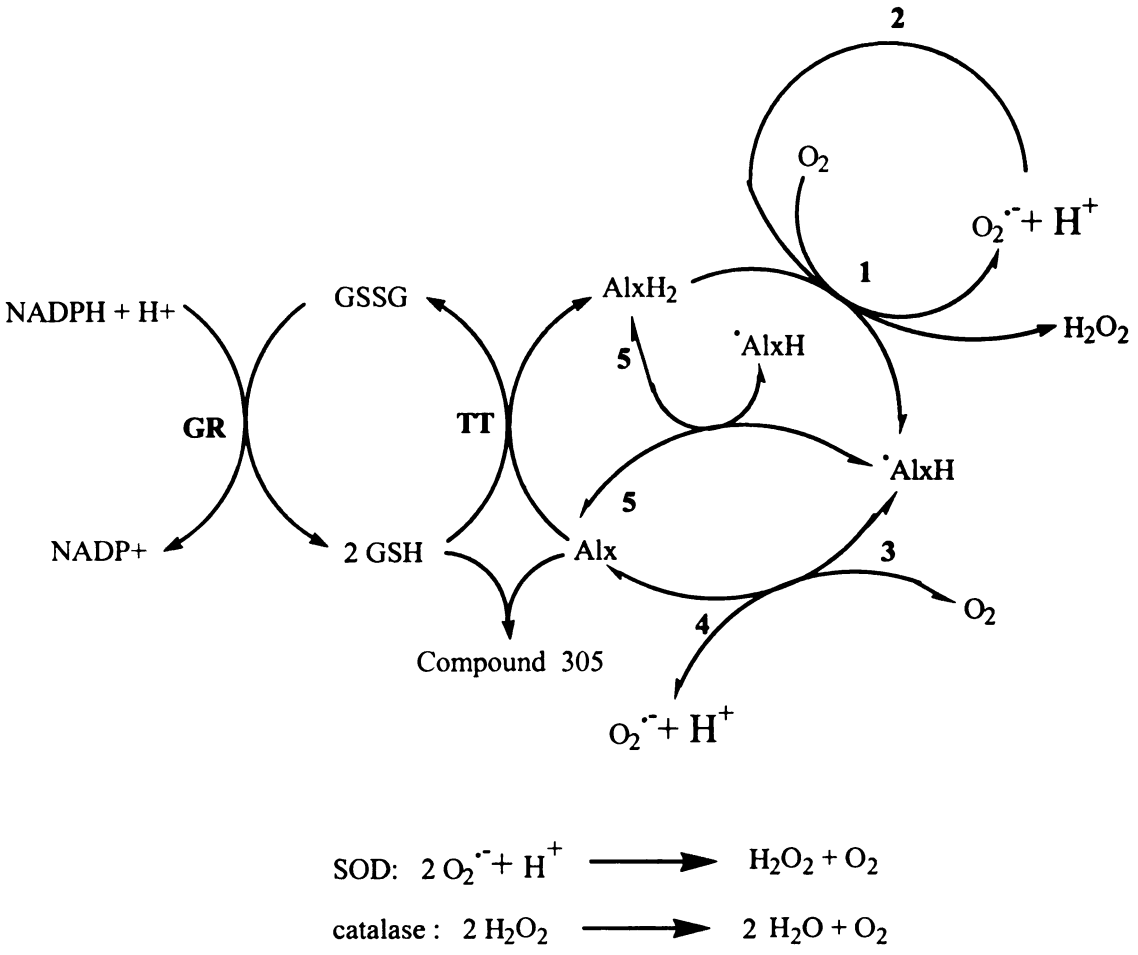



**Figure 9**: Interactions between oxygen and dialuric acid. Reversible reactions are designated by numbers placed near their reactants. Reaction 1 is $AlxH_2 + O_2 \longrightarrow {}^{\cdot}AlxH + O_2^{\cdot-} + H^+$ and reaction 2 is $O_2^{\cdot-} + H^+ + AlxH_2 \longrightarrow {}^{\cdot}AlxH + H_2O_2$. GR, GSSG reductase: TT, thioltransferase;

production of alloxan radical by reactions 1 and 5, and the consumption of alloxan radical by reaction 3 (Figure 9). However, in the presence of RPLTT the GSH-dependent reduction of alloxan to dialuric acid was favored over the formation of alloxan radical by reaction 5. Winterbourn, *et al.* (*33*) report that the rate constant for reaction 5 ($Alx + AlxH_2 \rightarrow 2\ ^{\bullet}AlxH$, Figure 8) is 45 $M^{-1}$ $sec^{-1}$ whereas in this paper we report the $k_{cat}/K_m$ for RPLTT catalysis of $Alx + 2GSH \rightarrow AlxH_2 + GSSG$ as $4.5 \times 10^5$ $M^{-1}$ $sec^{-1}$. Alloxan concentrations in the reaction mixture were low since RPLTT rapidly catalyzed the GSH-dependent reduction of alloxan to dialuric acid. Therefore, oxidation of dialuric acid depended predominantly on the superoxide dependent chain mechanism because the comproportionation of alloxan and dialuric acid forming alloxan radical was not favored. In the presence of both RPLTT and SOD, oxygen consumption was inhibited because dialuric acid oxidation by the autocatalytic mechanism was minimized.

In the previous chapter, reduced RPLTT and the R26V and K27Q variants but not the C25S enzyme were demonstrated to be able to reduce DHA to AA in the absence of GSH. The product of this reaction is oxidized enzyme, which was identified via isoelectric focusing. Due to the structural similarities between DHA and alloxan, the ability of RPLTT and the K27Q and C25S variants to reduce alloxan in the absence of GSH was investigated. When reduced RPLTT and K27Q enzyme were incubated with alloxan, oxidized enzyme was formed, suggesting the ability of RPLTT and K27Q enzyme to reduce alloxan chemically. This result suggests that thioltransferase has similar catalytic mechanisms for both its DHA and alloxan reductase activities.

In contrast, the C25S enzyme was unable to form oxidized enzyme, and when incubated with DHA no species besides reduced enzyme was seen. However, when the

C25S variant was incubated with alloxan, reduced enzyme and a species at pI = 6.2 were seen. The pI = 6.2 species may be the formation of a thiohemiketal intermediate. When the C25S enzyme was preincubated with alloxan plus GSH, reduced enzyme and a species focusing at a pI = 5.9 was seen. The reaction of alloxan plus GSH has been demonstrated to produce oxygen radicals (Figure 7 and refs *10-13*). The species focusing to a pI = 5.9 is perhaps the formation of oxidized sulfur like sulfenic or sulfonic acid.

Reduction of alloxan to dialuric acid was previously demonstrated with GSH *(10-13)*, NAD(P)H (*8, 9*) and enzymatically by the thioredoxin/NADPH-thioredoxin reductase system (*14*). In this paper, we demonstrated thioltransferase catalysis of the GSH dependent reduction of alloxan to dialuric acid. We provided evidence suggesting that thioltransferase may play a role in the toxicity of alloxan by enhancing redox cycling between alloxan and dialuric acid. In studies of calf (*34*) and pig (*35*) thioltransferases, although the pancreas had lower levels than other tissues like the stomach and lung, moderate activity was demonstrated. Thioltransferase levels in the 2-cells of the pancreas is unknown. Eizirik, *et al.* (*36*) reported that human islets were not destroyed by alloxan, *in vitro*, nor when grafted into nude mice, whereas they confirmed the toxicity of alloxan in rat and mouse islets. This raises the question of species differences in susceptibility to alloxan and the reasons for these species differences.

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# Chapter V

## Summary and Perspectives

In the completion of this thesis I have developed a reproducible GSH-dependent DHA reductase activity assay and kinetically characterized and compared mammalian DHA reductases, a rapidly growing class of enzymes. Prior to this thesis, very little was known about the catalytic mechanism of any DHA reductase. The only information known was that Cys-22 was essential for the DHA reductase activity of thioltransferase (*1*). Furthermore, Cys at position 25 was known not to be essential, and the placement of a serine at this position enhanced the relative DHA reductase activity of thioltransferase (*1*). I characterized the catalytic mechanism of a GSH-dependent DHA reductase for the first time, that of thioltransferase. Finally, I characterized the alloxan reductase activity of thioltransferase and provided evidence that thioltransferase may contribute to the toxicity of alloxan. Alloxan is the second non-disulfide substrate described for thioltransferase.

Chapter II describes the kinetic characterization of the DHA reductase activity of RPLTT, bovine liver PDI , and the human erythrocyte 32kDA DHA reductase. The emerging picture in the field of DHA reductases is one in which there are several potential systems to regenerate AA from DHA. NADPH-dependent DHA reductases like 3K-hydroxysteroid dehydrogenase (*2*) and thioredoxin/thioredoxin reductase (*3*) and GSH-dependent DHA reductases like thioltransferase (*4*) and the 32 kDa DHA (*5, 6*) reductase may all play roles *in vivo*. It is likely that the importance of each enzyme in DHA reduction will vary from species to species and cell to cell. Based on their respective catalytic activities, RPLTT and the human erythrocyte 32kDa DHA reductase were the most robust DHA reductases when compared to all the known mammalian DHA reductases.

It is interesting that human and pig liver thioltransferases had very similar $K_m$ values for DHA of 0.2 to 0.27 mM but their $k_{cat}$ values varied from 41 to 374 $min^{-1}$ resulting in pig

liver thioltransferase having a $k_{cat}/K_m$ for DHA of 2.4 x $10^4$ $M^{-1}$ $sec^{-1}$ compared to a $k_{cat}/K_m$ for DHA for the human enzymes of 2.5 to 5.5 x $10^3$ $M^{-1}$ $sec^{-1}$ (Table II ,Chapter II) making the pig liver enzyme 4 to 10 fold more efficient. Both pig liver and human thioltransferases contain 105 amino acids. With a sequence identity of 83% there are 18 amino acids different between the two enzymes (Figure 4, Chapter I). A thorough site directed mutagenesis study of the human enzyme should allow one to identify which amino acid(s) account for this difference in activities.

When the 32kDa DHA reductase from human erythrocytes was purified by Xu *et al.* (*6*), the following peptide fragments were sequenced (unpublished results): (1) HEVININLK, (2) NKPEWFFKK, (3) MILELFSK, (4) PSLVGSFIR, (5) WQFLELYL, and the C-terminal fragment (6) LKQIGALQ. A recent search in the data bank by Dr. Wells identified fragments (1) to (5) to be identical to fragments in a partial clone of a human homolog to a mouse protein that is differentially expressed in lymphoma cells that have different susceptibilities to radiation induced apoptosis. The cloning was carried out under the direction of Michael Story at the University of Texas MD Anderson Cancer Center. The full length protein is 32 kDa (personal communication from Dr. Story), making it possible that the 32kDa DHA reductase from human erythrocytes is differentially expressed in lymphoma cells, and that its expression may provide defense's against damage induced by radiation.

Thioltransferase has been demonstrated to have a 4 fold enhanced expression in MCF-7 adriamycin resistant cells compared to MCF-7 adriamycin sensitive cells (*7*). It was postulated that the DHA reductase activity of thioltransferase contributed to the resistant phenotype. In the Ph.D. thesis of Dr. Elizabeta Borer-Meyer, Dr. Borer-Meyer transfected

MCF-7 adriamycin sensitive cells with human thioltransferase and found that thioltransferase transfection indeed increased the resistance of these cells to adriamycin, but not to the level of resistance of the MCF-7 adriamycin resistant cells. Furthermore, the increased resistance of the transfected cell lines was most likely not due to the DHA reductase activity of thioltransferase. The latest results concerning the 32kDa DHA reductase suggest that cancer cells may possess enhanced DHA reductase activities due to elevated levels of this enzyme, and enhanced DHA reductase activity may provide cancer cells with enhanced resistance to damage induced by radiation.

The wide variety of DHA reductases, especially the GSH-dependent DHA reductases, raises several enzyme structure/function questions. Whether each enzyme has an identical, similar or different catalytic mechanism for DHA reduction is unknown. Several catalytic mechanisms for DHA reductases have been proposed prior to this thesis, but no one had specifically studied the catalytic mechanism of a DHA reductase. In Chapter III, the first description of a catalytic mechanism of a DHA reductase is presented.

When starting with the reduced form of the enzyme, IAM inactivation studies demonstrated the ability of DHA to bind to enzyme, prior to GSH binding because DHA protected against IAM inactivation whereas GSH did not. The ability of RPLTT and the R26V and K27Q variants to chemically reduce DHA to AA in the absence of GSH was demonstrated by electrochemical analysis and IEF. In addition, IEF analysis demonstrated the ability of reduced RPLTT and the R26V and K27Q variants to react with GSSG and form oxidized enzyme. The relative inability of the C25S enzyme to chemically reduce DHA to AA, but its robust DHA reductase activity suggest that the postulated formation of a thiohemiketal intermediate is reduced by GSH releasing AA and forming an enzyme mixed

disulfide with GSH.

Although Chapter III provides strong evidence for the proposed catalytic mechanism of the DHA reductase activity of thioltransferase, many more studies will be needed to completely determine the catalytic mechanism. The major aspect of the proposed mechanism that needs to be experimentally demonstrated is the presence of a thiohemiketal intermediate between enzyme and a non-disulfide substrate like DHA. It is clear that in order to carry out this work, the C25S enzyme of the enzyme must be used since wild type enzyme is capable of binding and chemically reducing DHA to AA forming oxidized enzyme. It is also important to determine what the mechanism of thioltransferase is in the presence of GSH. Does wild type enzyme form an intramolecular disulfide as an intermediate, or does GSH displace DHA from the enzyme prior to Cys-25 attack on Cys-22 releasing AA. Finally, in *E. coli* thioredoxin, Asp-26 has been demonstrated to act as a general acid/base and deprotonate Cys-35 which can then attack Cys-32 to release reduced substrate forming oxidized enzyme (*8*). RPLTT was capable of chemically reducing DHA to AA forming oxidized enzyme. It is likely that Cys-25 in RPLTT must be deprotonated by a general base to attack Cys-22 to form an intramolecular disulfide in this reaction, but a residue acting as a general base has not been found in thioltransferase.

During the study of the catalytic mechanism of the DHA reductase activity of thioltransferase, a possible function of Lys-27 in thioltransferase was elucidated. The DHA reductase activity of the K27Q variant was incompletely inhibited by IAM with 40% residual activity after 45 minutes. GSH enhanced the IAM inactivation of the DHA reductase activity of the K27Q enzyme. Although Cys-22 in the K27Q enzyme has a slight increase in pKa of 4.3 vs. 3.8 for RPLTT, the role of Lys-27 was previously unknown (*1*). The

current studies suggested that the role of lysine at position 27 is to stabilize the reduced form of RPLTT. In contrast to the other enzymes, when the K27Q variant was treated with IAA or preincubated with AA prior to IAA addition, a significant amount of oxidized enzyme was seen. Finally, the K27Q enzyme had the greatest initial rate of chemical reduction of DHA to AA, a reaction which includes the oxidation of the active site sulfhydryls. These data suggest that the K27Q variant is much more readily oxidized than RPLTT indicating a role of lysine at position 27 in the stabilization of the reduced form of the enzyme.

Many DHA reductases have several different activities including thioltransferase, thioredoxin/thioredoxin reductase, and PDI which belong to the class of enzymes known as thiol:disulfide oxidoreductases, which contain the -CXXC- active site (*9*). Nothing is known about the catalytic mechanism of the DHA reductase activity of any other enzyme besides thioltransferase. Because they are thiol:disulfide oxidoreductases, it will be interesting to determine whether PDI or the thioredoxin/thioredoxin reductase system have identical, similar or different catalytic mechanisms for DHA reduction, and how their respective catalytic mechanisms compare to that of thioltransferase. Since nothing is known about the active site of the recently identified 32kDa DHA reductase, this is perhaps the most exciting enzyme to study, especially because of its potential role in cancer.

It was long believed that thioltransferase was only a GSH-dependent thiol:disulfide oxidoreductase, but in 1990 Wells *et al.* (*10*) demonstrated that human placenta, bovine thymus, and pig liver thioltransferases possessed GSH-dependent DHA reductase activity. With the advent of this discovery, I asked the question, what other non-disulfide substrates for thioltransferase might exist?

Reduction of alloxan to dialuric acid was previously shown to occur with GSH (*11*-

*14*), NAD(P)H (*15, 16*) and enzymatically by the thioredoxin/NADPH-thioredoxin reductase system (*17*). Because thioltransferase is GSH dependent, thioltransferase and thioredoxin share the active site -CXXC-, and alloxan and DHA share a similar triketone structure, I investigated the GSH-dependent alloxan reductase activity of thioltransferase. In Chapter IV, I demonstrated thioltransferase catalysis of the GSH dependent reduction of alloxan to dialuric acid. I provided evidence suggesting that thioltransferase may play a role in the toxicity of alloxan by enhancing redox cycling between alloxan and dialuric acid. Presumably because of the similarities between alloxan and DHA, thioltransferase would have similar catalytic mechanisms for their GSH-dependent DHA reduction. In support of this, when alloxan was incubated with RPLTT or the K27Q variant , oxidized enzyme was formed. When alloxan was incubated with the C25S enzyme and analyzed via IEF, both reduced the C25S variant (pI = 7.0) and a species focusing at a pI of 6.2 were seen (Figure 8, Chapter IV). This species at pI = 6.2 may be the postulated enzyme-substrate intermediate thiohemiketal which would be very useful for future investigations.

Finally, the alloxan reductase activities of thioltransferase and the thioredoxin/thioredoxin reductase system suggest that these enzymes may play a role in alloxan induced diabetes. Alloxan must be reduced to dialuric acid in order to be toxic because the oxidation of dialuric back to alloxan generates reactive oxygen species like superoxide, hydrogen peroxide, and hydroxyl radical in the presence of an appropriate metal catalyst (*18-21*). There may be other as yet unstudied redox cycling compounds similar in structure to alloxan that may be substrates for thioltransferase and/or thioredoxin making these enzymes important in their toxic action on cells and tissues.

The substrate profile of thioltransferase demonstrates that depending on the

conditions, the presence of thioltransferase could be either beneficial or harmful. As a DHA reductase and a thiol:disulfide oxidoreductase, thioltransferase is believed to contribute to the homeostasis of a cell. However, in the presence of a redox cycling substrate like alloxan, thioltransferase could potentiate the compound's toxicity. Therefore, depending on the conditions, thioltransferase may either contribute to the protection or add to the damage of a cell.

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