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STUDIES ON THE ROLE OF PROTEIN DISULFIDE ISOMERASE IN THE PROLYL 4-HYDROXYLASE REACTION

By

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ABSTRACT

STUDIES ON THE ROLE OF PROTIEN DISULFIDE ISOMERASE IN THE PROLYL 4-HYDROXYLASE REACTION

By

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The involvement of protein disulfide isomerase (PDI), the β subunit of prolyl 4-hydroxylase (P4H), in recycling ascorbic acid, a cofactor of P4H, was investigated. Purified chicken embryo P4H was assayed based on the decarboxylation of [1-^{14}C]α-ketoglutarate. An ascorbate regeneration system consisting of glutathione (GSH), NADPH and glutathione disulfide reductase, stimulated basal P4H activity by 150%. To differentiate the chemical regeneration of ascorbate from dehydroascorbic acid (DHA) by GSH from the proposed enzymatic regeneration by PDI, the effect of potential inhibitors of PDI's DHA reductase activity were tested. 17 β-estradiol and antibodies to bovine liver PDI did not inhibit chicken liver PDI's DHA reductase activity. Glutathione sulfonic acid (GSO_3^-), inhibited chicken liver PDI's DHA reductase activity competitively, the apparent K_i being 2.2 mM. GSO_3^- (5 mM), inhibited the stimulation in P4H activity seen at 0.5 mM ascorbate in the presence of GSH and the regeneration system by 55% and 53% respectively. Basal P4H activity, in the absence of GSH was also inhibited by GSO_3^-, at 0.5 mM ascorbate but not at 1 mM and 2 mM ascorbate. These observations suggest that PDI, the β subunit of P4H, may be involved in recycling the cofactor ascorbic acid..

To my parents

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LIST OF ABBREVIATIONS:

P4H	Prolyl 4-hydroxylase
PDI	Protein disulfide isomerase
AA	Ascorbic acid
DHA	Dehydroascorbic acid
sDHA	Semi-dehydroascorbate
α-KG	α-ketoglutarate
GSH	Glutathione
GSSG	Glutathione disulfide
GSO_3^-	Glutathione sulfonic acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
GR	Glutathione disulfide reductase

CHAPTER 1: Literature Review

INTRODUCTION:

Collagen is the most abundant extracellular protein and belongs to a rapidly growing family of 19 known proteins and more than 30 gene products (1). Mutations in collagen genes can produce defects of bones and related tissues that range from osteogenesis imperfecta, lethal chondrodisplasias to osteoporosis (2). Osteogenesis imperfecta occurs due to a mutation in Type I collagen gene and is characterized by brittle bones and weak tendons (2). Collagen is unusual in that it has an amino acid composition of one third glycine and one third proline (3). Proline residues, N- terminal of glycine residues in the procollagen molecule get hydroxylated to hydroxyproline. These hydroxyproline residues enhance the thermal stability of the collagen triple helix (4). The enzyme responsible for this hydroxylation reaction, prolyl-4-hydroxylase (P4H), is a resident protein of the endoplasmic reticulum. It belongs to a group of α-ketogluterate (α-KG) dependent dioxygenases (5). Other enzymes that belong to this family are:

γ-butyrobetaine hydroxylase, which converts γ-butyrobetaine to the lipid transporter carnitine (6). This enzyme has been characterized from a variety of sources, pseudomonas (7), calf liver (8) and human kidney (9). The enzyme from all the sources are dimers and the pseudomonas enzyme has a MW of 95 kDa (6).

Dopamine β-hydroxylase, is a copper containing mono-oxygenase that converts nor-epinephrine to the neurotransmitter epinephrine (10). This enzyme found in the chromaffin granules, has a MW of 290,000 and 4 copper atoms per tetramer enzyme (11).

Lysyl hydroxylase and prolyl 3-hydroxylase are other enzymes that are also involved in collagen metabolism and are responsible for the hydroxylation of lysyl residues and proline residues at the third position respectively. The hydroxyl groups of

hydroxy-lysine residues serve as carbohydrate attatchment sites and play an important role in stabilizing the intramolecualr linkages in collagen (12). A defective lysyl hydroxylase has been found in certain genetic defects like Ehlers-Danlos syndrome Type VI (13). All of the above mentioned enzymes utilize α-KG and molecular oxygen as cosubstrates and ferrous iron and ascorbic acid as cofactors (14). A general reaction catalyzed by these enzymes may be written as:

$$\text{substrate} + \alpha\text{-KG} + O_2 \xrightarrow[\text{Ascorbic acid}]{Fe^{2+}} \text{hydroxylated substrate} + \text{succinate} + CO_2$$

Perhaps the most studied among these enzymes is P4H. The central role played by prolyl 4-hydroxylase in the stability of the collagen triple helix makes the enzyme a potential target for anti-fibrotic drugs. These drugs are used in the treatment of fibrotic disorders in which there is excess collagen production. It has been demonstrated that fibrotic cells contain increased P4H levels and a good correlation has been observed between the amount of P4H activity and the rate of collagen synthesis in cultured cells and *in vivo* (15).

SOURCES OF P4H AND SUBUNIT COMPOSITION:

Prolyl 4-hydroxylase activity has been found in many tissues including chicken embryos (16); fetal rat skin (14); newborn rat and mouse liver,lung and skin; adult rat heart, kidney, lung, muscle, spleen and skin (17, 18); fibrotic rat liver (19); various cultured cells including mouse and human fibroblasts (20). Prolyl hydroxylases with similar properties have also been identified in plant tissues (21). Skin of new born rats and chicken embryos are rich sources of the enzyme which has been purified to

homogeniety from both these sources (16, 18).

The prolyl-4-hydroxylase isolated from chicken embryos is a hetero-tetramer with a molecular weight of approximately 240,000, the subunit composition being 2 α subunits and 2 β subunits. The α subunit has a molecular weight of 64,000 and the beta subunit has a molecular weight of 60,000 (22). The tetramer is the active form of the enzyme. Small amounts of activity can be detected in the dimer but the monomers had no activity (5). The rates of synthesis of the 2 subunits are regulated differently. The β subunit is synthesized in a large excess and enters a pool in the endoplasmic reticulum before it assembles into the tetramer. The α subunit however seems to be incorporated into the tetramer immediately after its synthesis (23). The α subunit when compared to the β subunit is also much more unstable and easily degraded. Therefore regulation of the amounts of active P4H in the cell seems to occur primarily through regulation of α subunit levels (24).

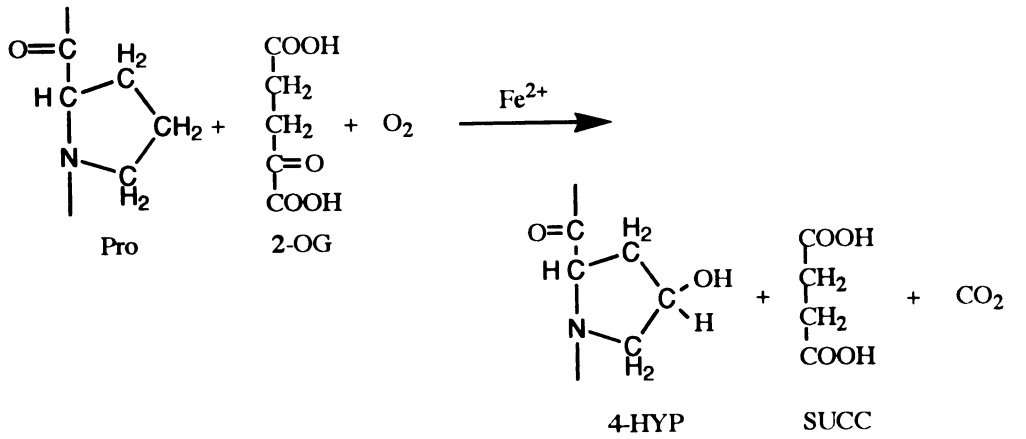
COFACTORS AND REACTION MECHANISM: (Fig 1)

Studies with $^{18}O_2$ by Fujimoto and Tamiya and also by Prockop, Kaplan and Udenfriend demonstrated that prolyl-4-hydroxylase is a di-oxygenase (25, 26). Prockop and Juva discovered that the enzyme activity was inhibited by EDTA and that the enzyme requires ferrous iron specifically for its function (27). Prolyl-4-hydroxylase also requires ascorbic acid as a reducing agent and this requirement for ascorbic acid is very specific.

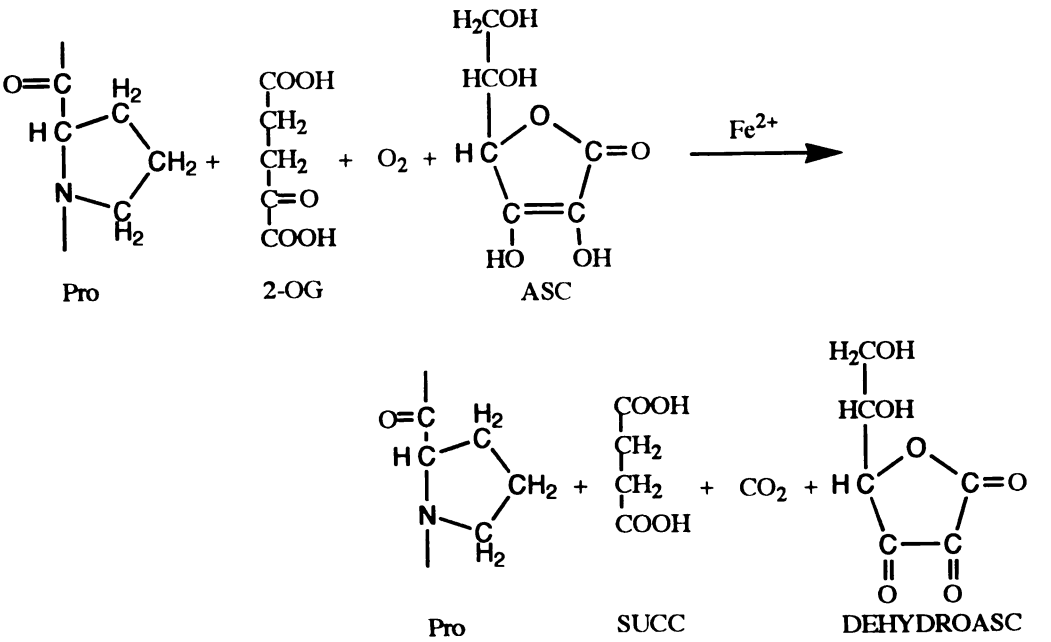
Figure 1: Schematic representation of the coupled and uncoupled reactions catalyzed by prolyl 4-hydroxylase. Pro= proline, 2-OG= a - keto glutarate, 4-Hyp= 4-hydroxyproline, SUCC= succinate, ASC= ascorbate, DEHYDROASC= dehydroascorbate. Reproduced with permission from reference 5.

Figure 1

A) Coupled Decarboxylation Hydroxylation Reaction



B) Uncoupled Decarboxylation Reaction:



It is thought that the role of ascorbic acid in this reaction is to maintain enzyme bound iron in the ferrous state (28). α-KG is a cosubstrate in the prolyl-4-hydroxylase reaction and one mole of α-KG is stoichiometrically decarboxylated to succinate for every mole of hydroxy-proline that is formed (22). Extensive kinetic studies suggest that the prolyl-4-hydroxylase reaction involves ordered binding of Fe^{2+}, αKG, oxygen and the peptide substrate to the enzyme and an ordered release of the products (29). The iron however does not dissociate from the enzyme in between catalytic cycles. Binding of moleclar oxygen and decarboxylation of α-KG leads to the formation of an active iron-oxygen complex called the ferryl ion which subsequently transfers the oxygen to a proline residue to form hydroxyproline (29). In some uncoupled reaction cycles, decarboxylation of αKG does not subsequently result in hydroxy-proline formation. In these uncoupled reaction cycles the active iron oxygen complex is probably converted intoa ferryl ion making the enzyme unavailable for further catalytic cycles unless reduced to the ferrous state by ascorbate (30).

ACTIVE SITE AND SUBUNIT CHARACTERISTICS OF P4H:

Prolyl 4-hydroxylase appears to contain two active sites per $\alpha_2\beta_2$ tetramer as suggested by the presence of two Fe^{2+} atoms (27) and two peptide binding sites in one tetramer (31). Studies involving suicide substrates such as Coumalic acid (32), a peptide containing oxa-proline and doxorubicin (33) and photo-affinity labelling (34, 35) suggest that both the αKG and the peptide binding sites are located in the α subunit.

Prolyl-4-hydroxylase present in unicellular and multicellular green algae like Chlamydomonas and Volvox appear to be monomers and the algal enzyme is very similar to the α subunit of the vertebrate enzyme (36). These pieces of information suggest that

the α subunit contributes to the major part of the vertebrate prolyl-4-hydroxylase. There are two different forms of the alpha subunit, α(I) and α(II) that are thought to be alternative splicing products of the same gene. $\alpha(I)_2\ \beta_2$ and $\alpha(II)_2\ \beta_2$ tetramers can form. Poly-L-Proline is an inhibitor of α(I) but not α(II) (37). Site directed mutagenesis studies of cysteine residues in the active site have shown that the α subunit is intramolecularly linked through disulfide linkages (38).

Characterization of cDNA clones for the beta subunit of prolyl-4-hydroxylase demonstrated that these were highly homologous to those determined for rat protein disulfide isomerase (39). It is now known that these two proteins are products of the same gene. PDI is a resident protein of the endoplasmic reticulum and catalyzes the rearrangement of disulfide bonds in proteins in vitro and is also considered to be an *in vivo* catalyst for isomerization of disulfide bonds and helps in the folding of nascent secretory proteins (40). During the assembly of P4H tetramer, PDI is derived from a pre-existing pool.

Two other proteins that are involved in thiol-disulfide exchange and maintainance of the thiol disulfide ratio in the cell are thioltransferase (glutaredoxin) and thioredoxin (41). These proteins have a molecular weight of about 11 KDa and are called thiol-disulfide oxidoreductases because the catalytic activity of the enzyme resides in an active pair of cysteine residues that can exist either in an oxidised or reduced form (42). The cysteines can undergo thiol-disulfide exchange by coupling to specific flavoproteins like thioredoxin reductase in the case of thioredoxin or GSH and glutathione disulfide (GSSG) reductase as in the case of thioltransferase. Although thioredoxin and thioltransferase have little sequence homology, their global conformations are considered

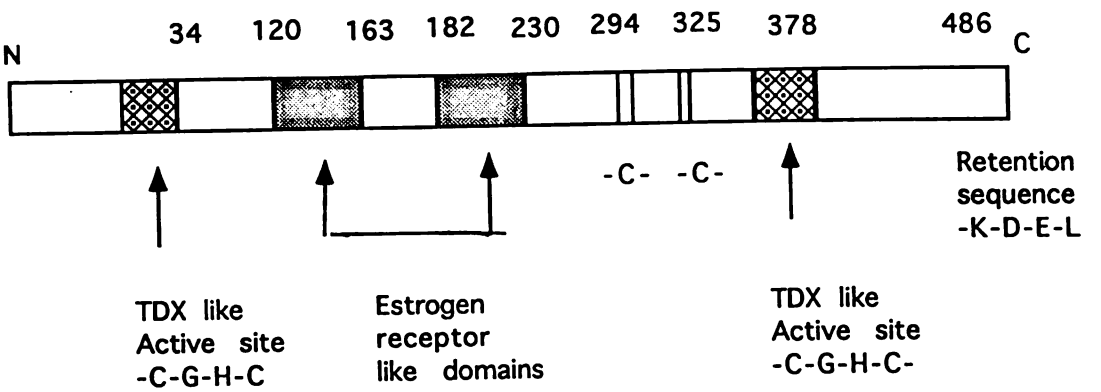
to be similar as determined by X-ray crystallography (43). Thioredoxin, thioltransferse and PDI have 2 C-X-X-C domains that are believed to be the catalytically active cysteins (44).

In addition to their thiol-disulfide exchange activities and disulfide isomerase activity, Wells, et al. showed that thioltransferase and PDI possessed the ability to regenerate ascorbic acid in vitro in a glutathione dependant manner (45). Although thioltransferase is a much more efficient DHA reductase than PDI, PDI may contribute significantly since it is estimated that 0.4% of total rat liver protein is PDI (46). Thioredoxin however did not exhibit DHA reductase activity. Fig 2 is a schematic representation of the primary structure of PDI showing the estrogen receptor like domains and the thioredoxin like domains.

Over the last few years, many functions have been attributed to PDI which has led to its recognition as a multi-functional protein. PDI has been shown to function as part of a triglyceride transfer complex (47), is homologous to a glycosylation site binding protein (48), and acts as a thyroid hormone binding protein (49) PDI has also been shown to exhibit chaperone anti-chaperone activity, *in vitro* (50). Although many functions have been attributed to this multifunctional protein, its function as the β subunit of prolyl-4-hydroxylase has yet not been defined. Many functions have been attributed to PDI as part of the prolyl-4-hydroxylase complex.

Figure 2: Schematic representation of the primary structure of PDI, showing the 2 thioredoxin like active site cysteines and the 2 estrogen receptor like domains.

Figure 2:


N
34
120
163
182
230
294
325
378
486
C
-C- -C-
Retention sequence -K-D-E-L
TDX like Active site -C-G-H-C
Estrogen receptor like domains
TDX like Active site -C-G-H-C-

It has been shown to help in preventing the misfolding and aggregation of the α subunit and in the absence of PDI the α subunit aggregates out of solution (51). PDI has also been shown to maintain the procollagen α subunit complex in its native conformation (52). The C-terminal KDEL sequence of PDI is responsible for the endoplasmic reticulum retention of prolyl-4-hydroxylase as demonstrated by site directed mutagenesis (53). Site directed mutagenesis studies of the active site cysteins responsible for the disulfide isomerase activity of PDI, have demonstrated that PDI's disulfide isomerase activity is not essential for the assembly or catalytic ability of P4H (54). Since PDI posseses glutathione dependent DHA reductase activity and since ascorbic acid is one of the cofactors used in the reaction, one could speculate that one of the functions of PDI as the β subunit of prolyl-4-hydroxylase is to regenerate the cofactor ascorbic acid.

EFFECT OF ASCORBIC ACID ON COLLAGEN SYNTHESIS AND PROLINE HYDROXYLATION IN CULTURED HUMAN FIBROBLASTS:

Ascorbic acid has been shown to stimulate collagen synthesis and increase processing of procollagen to collagen in cultured cells (55). This effect of ascorbic acid is thought to be mediated primarily through its role in the P4H reaction. Studies done on cardiac procollagen turnover in the presence of ascorbic acid and inhibitors of P4H like 3,4, dihydroxybenzoic acid and pyridine 2,4- dicarboxylic acid ethyl ester suggest that procollagen proline hydroxylation may be important for procollagen turnover (56). In these studies, ascorbic acid deficient fibroblasts showed decreased rates of prolyl hydroxylation and total collagen accumulation without significant reduction in α1(I) and α1(III) collagen m-RNA levels. The fraction of procollagen degraded was also substantially increased in ascorbate deficient cells. It is important to recall that human

cells donot synthesize ascorbic acid and hence cells grown in the absence of ascorbate are grown under scorbutic conditions. In other studies done with human skin fibroblasts, ascorbate increased the amount of [^{3}H]hydroxyproline synthesized but did not increase the amount of P4H (57). Studies carried out in cultured femurs from 15 day old chick embryos showed that these cells could be cultured for at least 5 days without ascorbate additions to the medium before hydroxylation of proline was significantly impaired (58). When the ascorbate concentration dropped to 6 μg/g wet tissue, hydroxyproline formation was reduced by 75-85%. Thus ascorbic acid appears to have one primary effect on the synthesis of procollagen: it is necessary for hydroxyproline formation and consequently for stability of the collagen triple helix.

INTERRELATIONSHIP BETWEEN ASCORBIC ACID AND GLUTATHIONE:

Ascorbic acid, in addition to functioning as a cofactor in hydroxylation reactions mentioned above, has long been known as an important antioxidant in the cell. Ascorbic acid acts as a free radical scavenger and in the process undergoes either one electron oxidation to give the free radical semi-dehydroascorbate (sDHA) or undergoes two electron oxidation to form dehydroascorbate (DHA) (59). Deficiency of this vitamin can lead to a condition called scurvy which is characterized by connective tissue disorders. Humans , guinea pigs and some other primates cannot synthesize ascorbic acid as they lack the key enzyme L-gulonolactone oxidase involved in the synthesis of this vitamin (60). Hence it is necessary that ascorbic acid is included in the diet of these animals. Unable to synthesize ascorbate, it is important that these animals possess the ability to regenerate cellular ascorbic acid from its oxidation products sDHA and DHA.

Glutathione (GSH) has long been known to regenerate ascorbic acid from DHA

chemically (61). In this reaction two molecules of GSH form glutathione disulfide (GSSG) while dehydroascorbate is reduced to ascorbate. Evidence for the involvement of GSH in maintaining tissue ascorbate levels comes from glutathione depletion studies done in Meister's laboratory (62). GSH levels in cells can be lowered through the administration of L-buthionine-SR-sulfoximine or BSO. BSO is potent inhibitor of gamma glutamyl cysteine synthetase, a key enzyme in the bio-synthesis of GSH. Severe GSH deficiency induced by the administration of BSO to adult mice lead to tissue and muscle damage. These effects can be reversed through the administration of GSH esters, which can be taken readily into cells and cleaved to GSH by intracellular esterases. The role played by GSH in maintaining ascorbate levels is also well substantiated by GSH depletion studies done in newborn rats. Newborn rats have a lower ascorbate synthesizing ability when compared with the adult animal. When newborn rats were made GSH deficient by administration of BSO, extensive tissue damage and marked decrease in ascorbate levels and increased DHA to ascorbate ratios were observed in liver, kidney, lung, brain and eye. This type of tissue damage is associated with mitochondrial swelling presumably induced by radical toxicity in the absence of GSH or ascorbate. Scorbutic guinea pigs showed decreased mitochondrial GSH levels. Administration of GSH esters to scorbutic guinea pigs, resulted in increased tissue ascorbate and GSH levels. This increase in ascorbate levels can be attributed to the recycling of DHA to ascorbate by GSH. When large doses of ascorbic acid were given to BSO treated animals, mortality was decreased significantly. One surprising effect of the simultaneous administration of ascorbate to BSO treated newborn mice was that increased levels of GSH were observed in tissues, i.e., ascorbate had a sparing effect on

GSH metabolism. GSH being an important anti-oxidant, is also essential for protection against oxidative and free radical damage and other types of toxicity (63).

Although much was known about the importance of GSH for the maintainance of tissue ascorbate, not much was known about enzymes that catalyze this reaction. Wells et al discovered that thioltransferase and PDI possessed DHA reductase activity (45). Prior to this discovery the existence of a mammalian DHA reductase remained in doubt. Plant DHA reducatases however were well known (64). It is now known that 4 enzymes, including thioltransferase and PDI possess DHA reductase activity. One is an NADPH-dependent DHA reductase, detected in rat liver and later identified to be 3α-hydroxysteroid dehydrogenase (65). The other is a 32 kDa, GSH dependent DHA reductase that was recently purified and characterized from human erythrocytes (66). Table 1 gives kinetic constants for the different enzymes.

Fig 3 is a schematic representation of the proposed redox reactions that ascorbic acid undergoes in a cell. As mentioned earlier, ascorbate can undergo either one electron or two electron oxidation to form sDHA or DHA respectively. Two sDHA free radicals can undergo disproportionation at a rapid rate of 10^5 M^{-1} S^{-1} to form ascorbate and DHA. sDHA can alternatively be reduced to ascorbate through the membrane bound NADH dependant sDHA reductase. DHA which is the first stable oxidation product of ascorbate, can be reduced chemically by GSH or enzymatically by any one of the DHA reductases. GSH, in the process is oxidized to its disulfide form which can then be reduced to GSH by GSSG reductase in an NADPH dependent manner. Ascorbic acid present outside the cell may be oxidized to DHA which can then be transported into the cell by a proposed DHA transporter. The DHA can then be reduced to ascorbate by GSH

catalyzed by any of the above mentioned DHA reductases. The proposed mechanism for the action of thioltransferase or PDI is through the formation of a thiohemiketal intermediate with one of the active site cysteines. The other cysteine may then displace the ascorbic acid, forming an intramolecular disulfide bond. Two molecules of GSH could then reduce the cysteines and the GSSG thus formed can be regenerated via GSSG reductase.

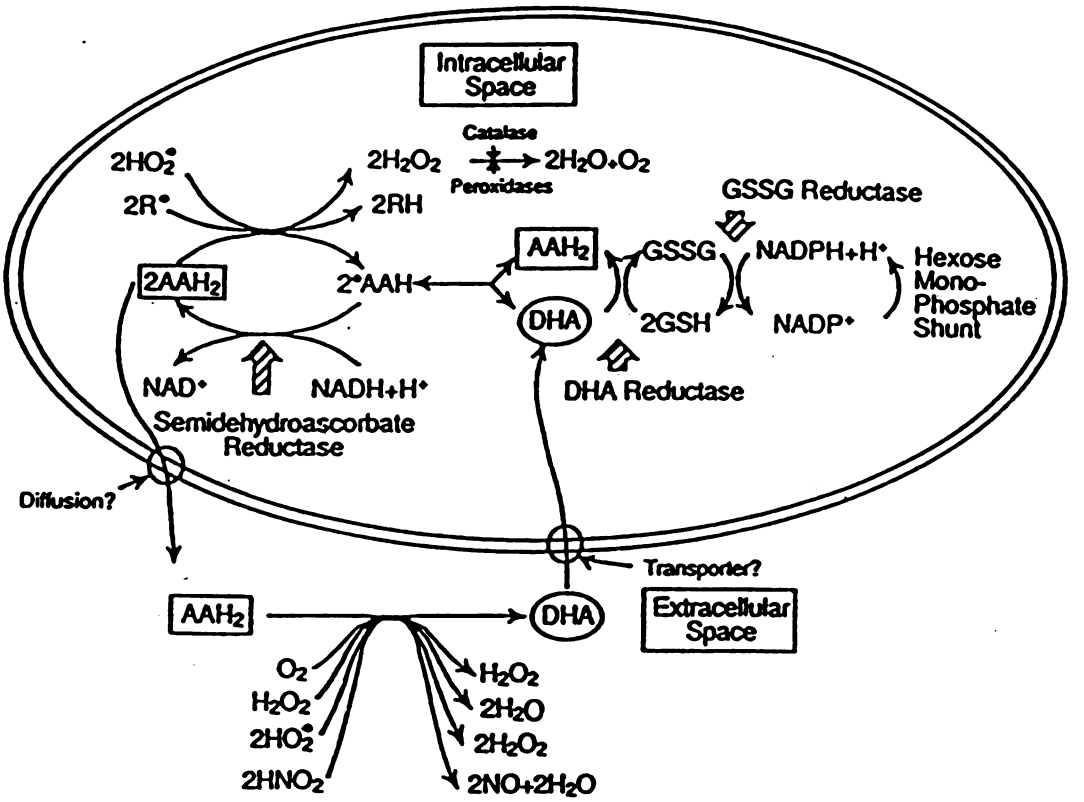
Since ascorbic acid is a cofactor in the P4H reaction and PDI, one of the subunits of P4H, we speculated that PDI might be involved in recycling asocrbate through its DHA reductase activity. In this study, the involvement of PDI's DHA reductase activity in recycling ascorbate in the P4H reaction was examined.

Table 1 : Kinetic Parameters for Mammalian Dehydroascorbate Reductases (66)

Parameter	Human RBC DHA Reductase	Rat Liver DHA Reductase	Pig Liver Thioltransferase	Bovine Protein Disulfide Isomerase
$k_{cat}(min^{-1})$	316 ± 1	140 ± 40	374 ± 20	16 ± 1
K_m(app) (mM)	DHA 0.21 ± 0.06	0.25 ± 0.06	0.26 ± 0.09	2.8 ± 0.4
	GSH 3.5 ± 0.3	2.8 ± 0.6	3.5 ± 0.3	2.9 ± 0.4
$k_{cat}/K_m(M^{-1}sec^{-1})$				
DHA	$2.47 \pm 0.64 \times 10^4$	$9.52 \pm 3.64 \times 10^3$	$2.43 \pm 0.85 \times 10^4$	93 ± 14
GSH	$1.51 \pm 0.11 \times 10^3$	$0.83 \pm 0.30 \times 10^3$	$1.81 \pm 0.2 \times 10^3$	91 ± 14

Figure 3: Schematic representation of the pathways by which ascorbic acid is regenerated in the cell. AAH2 = ascorbate, DHA = dehydroascorbate, GSH= glutahione,GSSG= glutathione disulfide, $AAH^{\cdot}$ = semidehydroascorbate. Reproduced with permission from reference 58.

Figure 3:


Intracellular Space
Catalase
Peroxidases
GSSG Reductase
Hexose Mono-Phosphate Shunt
Semidehydroascorbate Reductase
DHA Reductase
Diffusion?
Transporter?
Extracellular Space

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CHAPTER 2: Purification of prolyl 4-hydroxylase and protein disulfide isomerase.

SUMMARY:

Prolyl 4-hydroxylase and protein disulfide isomerase were purified from 15 day old chicken embryos and chicken liver respectively. Purification of P4H from chicken embryos involved 30-65% ammonium sulfate fractionation, Poly (L proline) affinity chromatography and a Biogel A 1.5 mm 200-400 mesh gel filtration column. The enzyme was homogeneous as analyzed by SDS-PAGE. The % yield and fold purification obtained were 87.2 and 3078 respectively. PDI was purified from chicken liver, by 55-85% ammonium sulfate fractionation followed by CM-sephadex C 50, DEAE sephacel and sephacryl S 300 columns. PDI was near homogeneous when analyzed by SDS-PAGE. A yield of 7.5% and a 435.1 fold purification was achieved using this procedure.

INTRODUCTON:

Collagen is a unique protein and undergoes many cotranslational and post-translational modifications. The post-translational modifications of these proteins follow the synthesis of the poly peptide and involve different hydroxylation processes that are essential for the formation of the stable triple helix (1). Three specific hydroxylases, prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase are involved in the process which occurs in the cisternae of the endoplasmic reticulum (2). Most of the hydroxylation occurs cotranslationally and some post-translationally, until formation of the collagen triple helix prevents further hydroxylation. Two glycosylases are involved in the glycosylation of hydroxylysine residues to form glucosyl or galactosylhydroxylysine (3). Prolyl 4-hydroxylase is a tetramer with a molecular weight of about 250,000. Both the α and the β subunit of the enzyme have been cloned and the β subunit has been identified as protein disulfide isomerase (4), (5). P4H activity has been identified in a variety of tissues including fetal rat skin ands guinea pig carageenan granuloma (6), new born rat (7) and newborn mouse lung and skin (8), various cultured cells including human and mouse fibroblasts (9). Skin of new born mammals and chicken embryos are rich sources of P4H and hence have been the major sources for purification of this enzyme (10). The β subunit of P4H, Protein disulfide isomerase, is a multifunctional enzyme, and has been previously purified from bovine (11), rat liver and from chicken embryos (12). PDI is an abundant protein forming 0.4% of total liver protein and 1.5-2% of microsomal liver protein (13). In this chapter, the purification of prolyl 4-hydroxylase from 15 day old chicken embryos and purification of PDI, the β subunit of P4H from chicken liver are discussed.

MATERIALS AND METHODS:

Poly(L-proline) of molecular weight 6000-8000 and 30,000 were purchased from Sigma. Sepharose4B, DEAE Sephacel, CM sephadex C50 were obtained from pharmacia. Biogel A 1.5, 200-400 mesh was obtained from Bio-Rad. White leghorn 15 day old chicken embryos were obtained from The Department of Animal Science, Michigan State University.

P4H Activity assay: P4H was assayed for activity by the method based on the hydroxylation coupled decarboxylation of α-KG (14). The assay system in a 1 ml assay volume contained 50 mM Tris-HCl, pH 7.4, 3-5 μg P4H, 100 μM -(Pro-Gly-Pro)-$_9$.H_2O, 5 μM ferrous sulfate, 1.5 mg BSA, 0.07 mg catalase, 0.5 mM ascorbic acid, and 1-[14C]α-KG, adjusted to a specific acitivity of 60,000 cpm / μ mol with unlabelled α-KG. The components were added to tubes kept on ice and the reaction was initiated with α-KG. The tubes were sealed with rubber stoppers with plastic cups containing filter paper soaked in 1 M NaOH. The tubes were then transfered from ice to a 37°C and incubated with gentle shaking for 10 min. The reaction was quenched with 250 μl of 50% TCA and [^{14}C]-CO_2 collection was allowed for 1 hour at 37°C. The cups were then transfered to scintillation vials and scintillation cocktail was added. The cups were allowed to soak for 1 hour in the cocktail before the samples were counted.

Definition of units of P4H activity: 1 unit of P4H activity is defined as the amount of enzyme required to synthesize 1 μmole of hydroxyproline per minute at 37°C, under the assay conditions described above.

PDI activity assay: PDI was assayed for activity using the insulin turbidity assay (15). The assay system in a 500μl volume contained 100mM sodium phosphate, pH 7.0, 15-20 μg PDI, 0.16 mM insulin, 1 mM dithiothreitol and 2 mM EDTA. Controls run simultaneously

received no enzyme but an equal volume of enzyme storage buffer. The insulin, being insoluble in water was first acidified to increase its solubility with 1 M HCl and then the pH was adjusted to 6.0 with 1 M NaOH. The reaction, i.e., light scattering of aggregating B chains of insulin, was monitored at 650 nm for 30 min at 30°C. Control values were subtracted from the enzyme catalyzed value and initial enzymatic rates were calculated as change in A_{650}/min.

Purification of P4H:

P4H was purified by the method of Tuderman et al (16). The term, enzyme buffer, is used for a solution containig 0.1 mM NaCl, 0.1 mM glycine, and 0.01 mM Tris-HCl buffer, pH 7.8. Dithiothreitol was omitted from P4H purification procedure, since the presence of DTT is believed to cause dissociation of the α and the β subunits of P4H.

Preparation of Poly(L-Proline) agarose: Poly(L-Proline) was coupled to agarose by a cyanogen bromide activation technique (16). About 100ml of 4% agarose (sepharose4B) was washed with distilled water and pH adjusted to 11. Cyanogen bromide (25 g)was then added and the mixture stirred continously for 20-30 min on ice, maintaining the pH at 11. The resin was then washed rapidly in a buchner funnel with a buffer containing a solution of 0.14 M NaCl and 0.1 M $NaHCO_3$, pH 9.3. Then, 1 g of Poly-L-Proline, molecular weight of 30,000 was dissolved in water and added to the resin and the reaction was allowed to proceed with continous stirring for about 20 hours at 4^0C. The resin was then equilibrated with enzyme buffer and then packed into a 9 X 23 cm column. Subsequent purification steps were carried out at 4°C.

About 400 g of 15 day old chicken embryos were washed with 0.9% saline solution. Twice the volume of enzyme buffer containing 0.1 % Triton-X 100 and 74 μg / ml PMSF

was added and the embryos were homogenized in a waring blender in three one min spurts. The homogenate was further blended using a Tekmar homogenizer for three more min. The homogenate was then centrifuged at 10,000 rpm for 30 min and the resultant supernatant was subjected to 30-65% ammonium sulfate fractionation. The pellet from the second fractionation was dissolved in enzyme buffer and dialyzed overnight against 2 litres of the same buffer. The buffer was changed twice, after an interval of 4 hours each.

The dialysate was centrifuged at 10,000 rpm for 30 min and the supernatant was mixed with the Poly(L-proline) affinity resin in roller bottles overnight. The column was packed, pouring the supernatent from the roller bottles first through the column and then the resin mixed with some buffer. This was done to prevent loss of the poly(L-proline) affinity resin during transfer from the roller bottles to the column. The column was washed with enzyme buffer until A 280 reached 0.05 absorbance units. Protein was eluted with 30 ml of poly(L-proline) molecular weight 6000-8000, at a concentration of 4mg / ml. The fractions were monitored at 230 nm since the protein gives higher absorbance at that wavelength.. The fractions showing absorbance were then pooled and concentrated using centricon 30 concentrator. The concentrated fractions were loaded onto a Biogel A 1.5 mm gel filtration column (5 X 75 cm), and 4 ml fractions were collected, concentrated and assayed for activity. This gel filtration step, was useful in removing the Poly(L-Proline) used to elute the enzyme from the affinity column.

Purification of protein disulfide isomerase:

PDI was purified from chicken liver, using a method described by Kivirikko and Myllyla (12), which is a modification of the Lambert and Freedman method for the purification of Bovine liver PDI (11).

About 400g of chicken liver was cleaned of fat. An equal volume of homogenization buffer containing 0.1 M sodium phosphate, pH 7.5, 0.1M NaCl, 5mM EDTA, and 1% (v/v) Trixton-X-100 was added to the tissue and homogenized in a waring blender for four bursts of 30 sec. The homogenate was allowed to stir at 0-4°C for an hour, and centrifuged at 10,000 rpm for 30 min. The resulting supernatent was subjected to 55-85% ammonium sulfate precipitation. Centrifugations were carried out at 10,000 rpm for 30 min. The precipitate obtained from the second ammonium sulfate precipitation was dissolved in a buffer containing 25mM sodium citrate, pH 5.3, 0.1M NaCl and 1mM EDTA. The resulting solution was then dialyzed overnight against the same buffer.

The dialyzed sample was applied to a CM-sephadex C-50 column equilibrated with the sodium citrate buffer, pH 5.3. The flow through was collected in an erlenmeyer flask and assayed for activity. PDI, did not bind to the column at this pH and was present in the flow through. The flow through was then adjusted to a pH of 6.2 and loaded on to a DEAE-sephacel column (9 X 23 cm), equlibrated with the sodium citrate buffer, pH 6.2. The column was washed with pH 6.2 buffer until absorbance reached baseline and PDI was eluted with a 900ml gradient of 0.1-0.6 M NaCl in the same buffer. Fractions of 5ml were collected and assayed for activity. The active fractions were pooled and concentrated using a centricon 30 concentrator to a 2ml volume. The concentrated enzyme was loaded onto a sephacryl S-300 column equilibrated with pH 6.2 buffer. Fractions of 2ml were collected and assayed for activity. Active fractions were pooled and concentrated. The enzyme was stored at -20°C.

SDS-PAGE ANALYSIS:

P4H and PDI were analyzed for purity using SDS-PAGE. A stacking gel of 6% acrylamide

and a separating gel of 10% was used. Low molecular weight markers were used as standards and the gel was stained using Coomassie Brilliant Blue R-250.

RESULTS:

P4H was purified to homogeniety from 15 day old chicken embryos using ammonium sulfate precipitation, Poly-L-proline affinity chromatography and gel filtration (Figure 1) . The elution profiles for the two columns are shown in Figures 3 and 4. P4H eluted between fraction numbers 20 and 48 for the affinity column. For the gel filtration column, P4H consistantly eluted as the second protein from the column, uually between fraction numbers 35-47. The enzyme obtained had a specific activity of 0.8-1.1 μmoles /min/mg protein under the above mentioned assay conditions. About 1.5mg of pure enzyme was obtained from 400 g of chicken embryos with a yield of 87.2% and a 3072 fold purity. (Table 1).

PDI was purified to near homogeniety from chicken liver (Figure 2). Elution profiles and PDI activity for the DEAE sephacel column and the sephacryl S-300 column are shown in figures 7 and 8. Yields and purification fold are shown in Table 2. Yield obtained was 7.5% and the procedure gave 435.1 fold purified enzyme calculated based on change in absorbance at 650nm/ min/ mg protein and compared with the activity obtained for the initial homogenate. About 2.4 mg of pure enzyme was obtained from 400g of chicken liver.

Figure 1: SDS-PAGE analysis of purified chicken embryo prolyl 4-hydroxylase:
Lane 1: Bio-Rad low molecular weight markers, Lane 2: chicken embryo P4H. The gel was stained with Coomassie Brilliant Blue R-250.

Figure 1

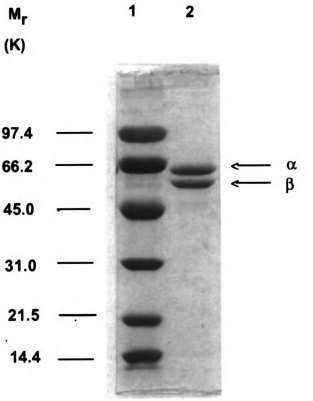

Figure 2: SDS-PAGE analysis of purified chicken liver protein disulfide isomerase: Lane 1: Bio-Rad low molecular weight markers, Lane 2: chicken liver PDI. The gel was stained with Coomassie Brilliant Blue R-250.

Figure 2

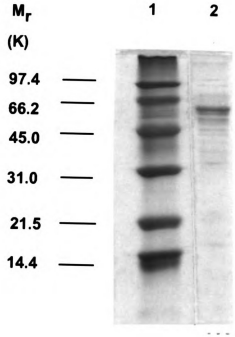

Fig 3: Poly(L-proline) affinity column elution profile :
P4H was eluted with poly(L-proline) and the fractions were monitored both at 230nm (---●---) and 280 nm (---■---). P4H eluted along with Poly (L-proline) between fraction numbers 20 and 48.

Figure 3:

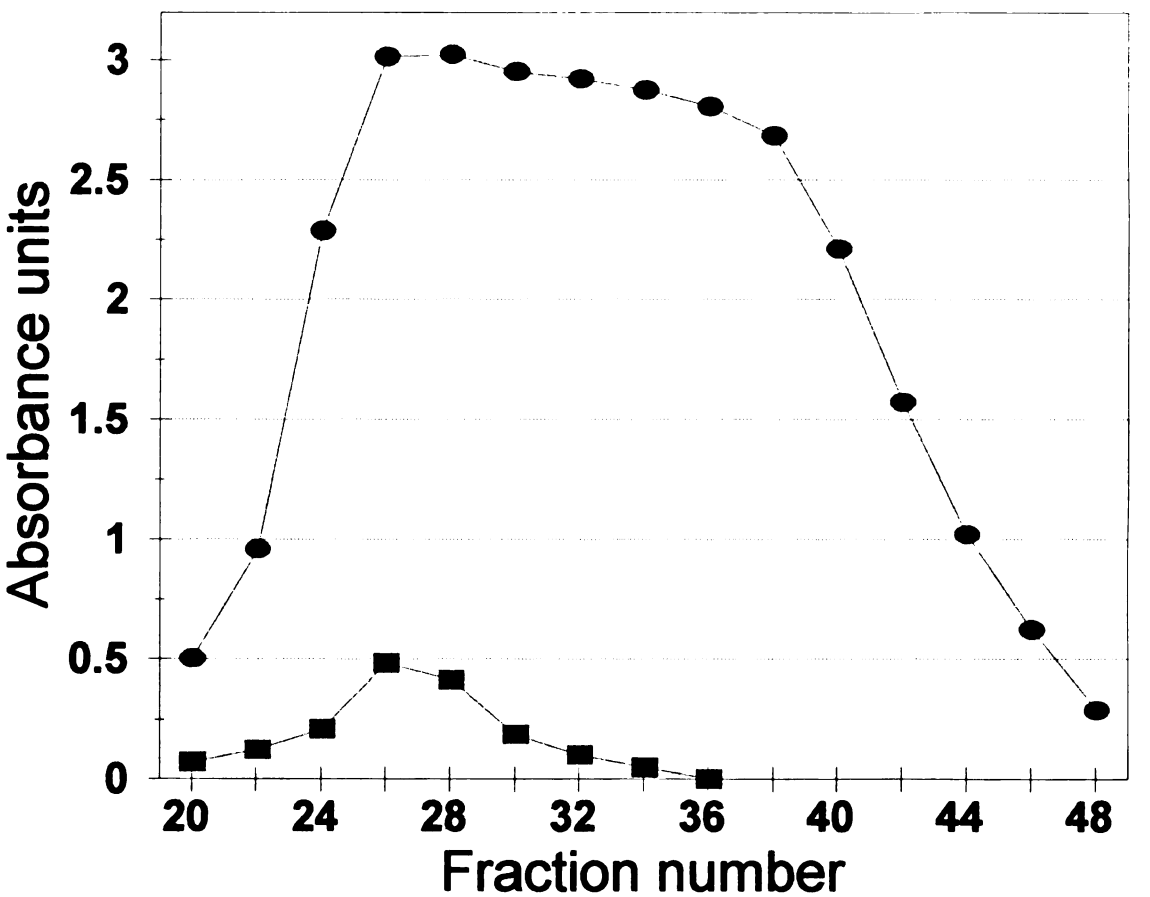
Absorbance units
3
2.5
2
1.5
1
0.5
0
20
24
28
32
36
40
44
48
Fraction number

Figure 4: Biogel A 1.5, 200-400 mesh gel filtration column elution profile:
P4H eluted between fractions 35-47. Poly (L-proline) eluted after P4H in fraction 49-65.

Figure 4:

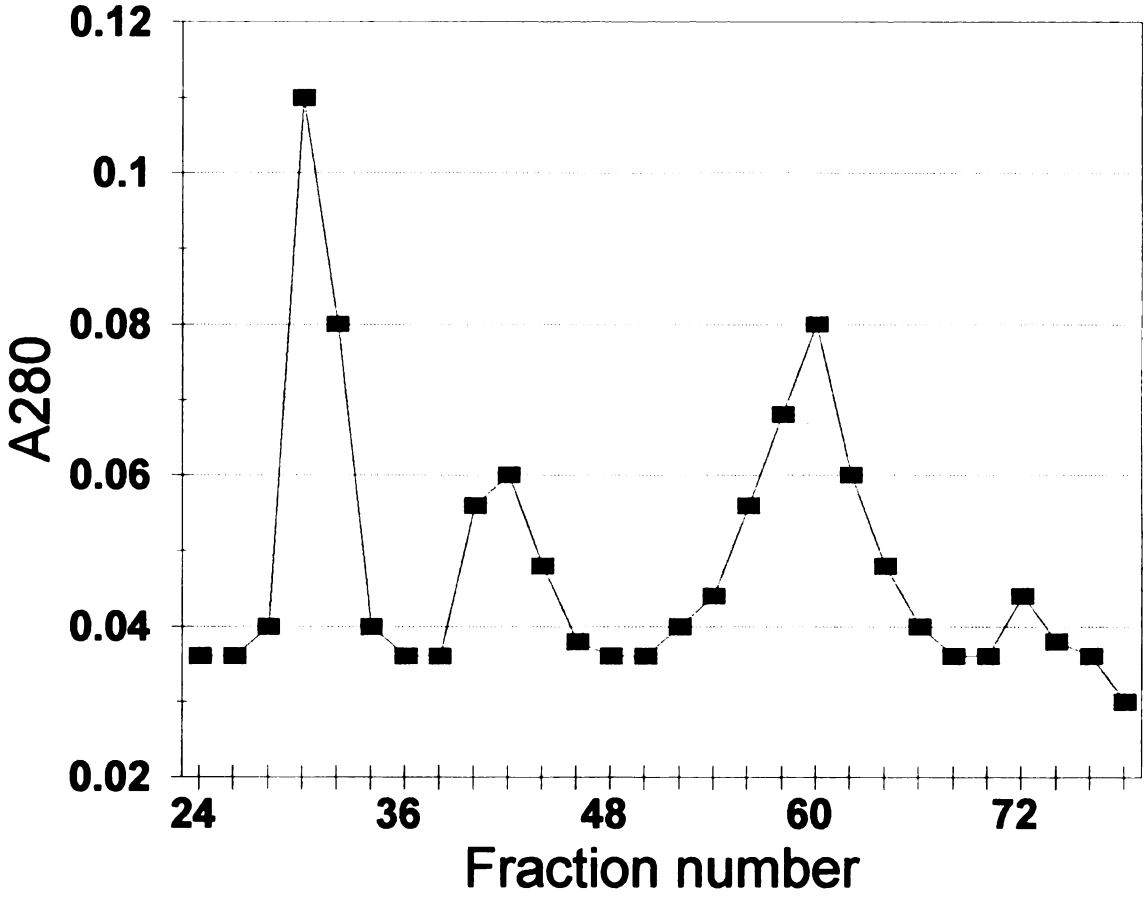

Table 1: Purification of P4H[a] from 15 day old chicken embryos:

Step in purification	Total protein (mg)[b]	Total activity (units)[c]	% recovery	Specific activity (units/ mg protein)	Purification (Fold)
$(NH_4)_2SO_4$ fractionation, 30-65%.	5000	1.44	100	.00028	1
After affinity chromatography and gel filtration	1.45	1.25	87.2	0.86	3078

a P4H was purified using the method of Tuderman et al.

b Protein estimations were done using the BCA protein assay.

c P4H was assayed for activity based on the decarboxylation of α-KG. 1 unit of P4H activity is defined as the amount of enzyme needed to synthesize 1μ mole of hydroxyproline per minute at 37°C under the assay conditions described in the text.

Figure 5: DEAE sephacel column elution profile for chicken liver PDI:
PDI was eluted from the column using a linear gradient of 0.1-0.6 M NaCl. The fractions were monitored at 280 nm (--●--) and assayed for activity using insulin turbidity assay. The initial rates were calculated based on the change in absorbance at 650 nm / min (--■--).

Figure 5 :

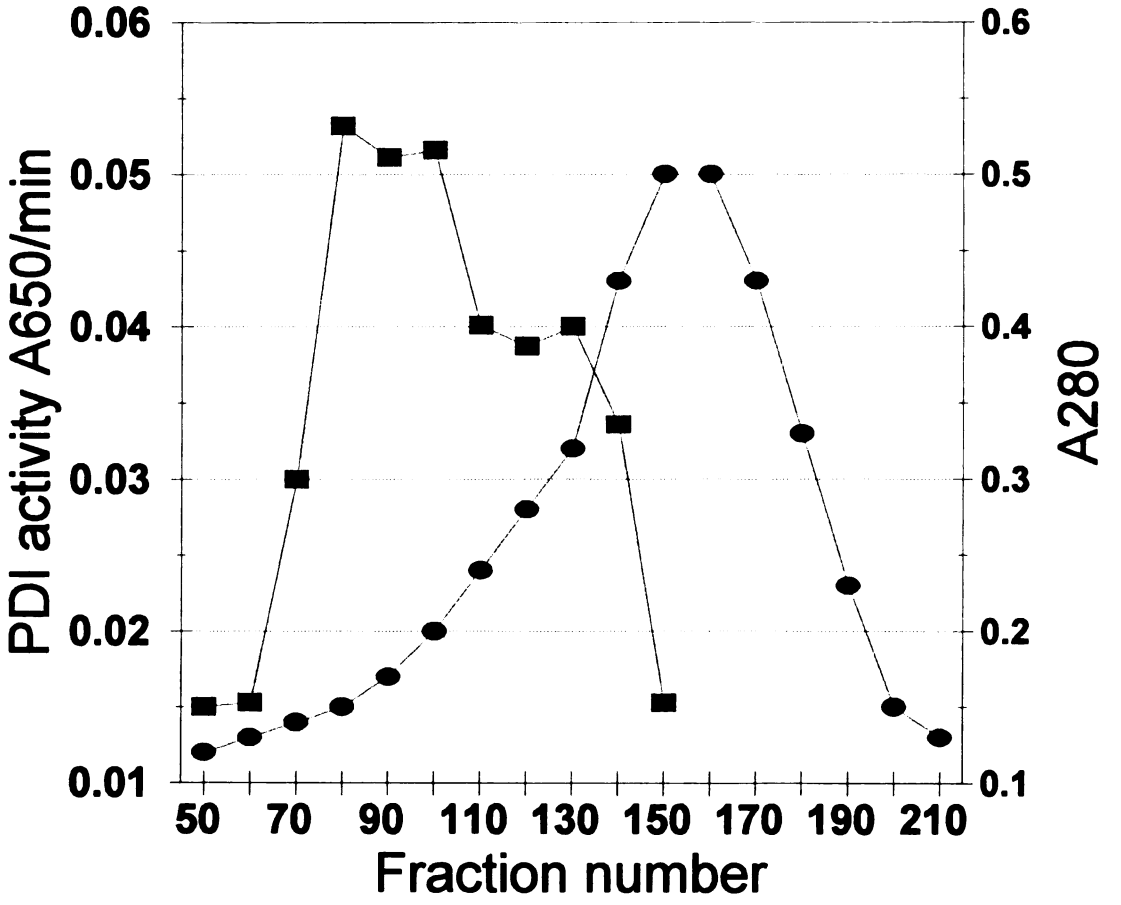
0.06
0.05
0.04
0.03
0.02
0.01
PDI activity A650/min
0.6
0.5
0.4
0.3
0.2
0.1
A280
50
70
90
110
130
150
170
190
210
Fraction number

Figure 6: Sephacryl S 300 gel filtration column elution profile for chicken liver PDI: Fractions were monitored at 280 nm (--●--) and assayed for activity using insulin turbidity assay. The initial velocities were calculated based on change in absorbance at 650 nm / min (--■--) .

Figure 6 :

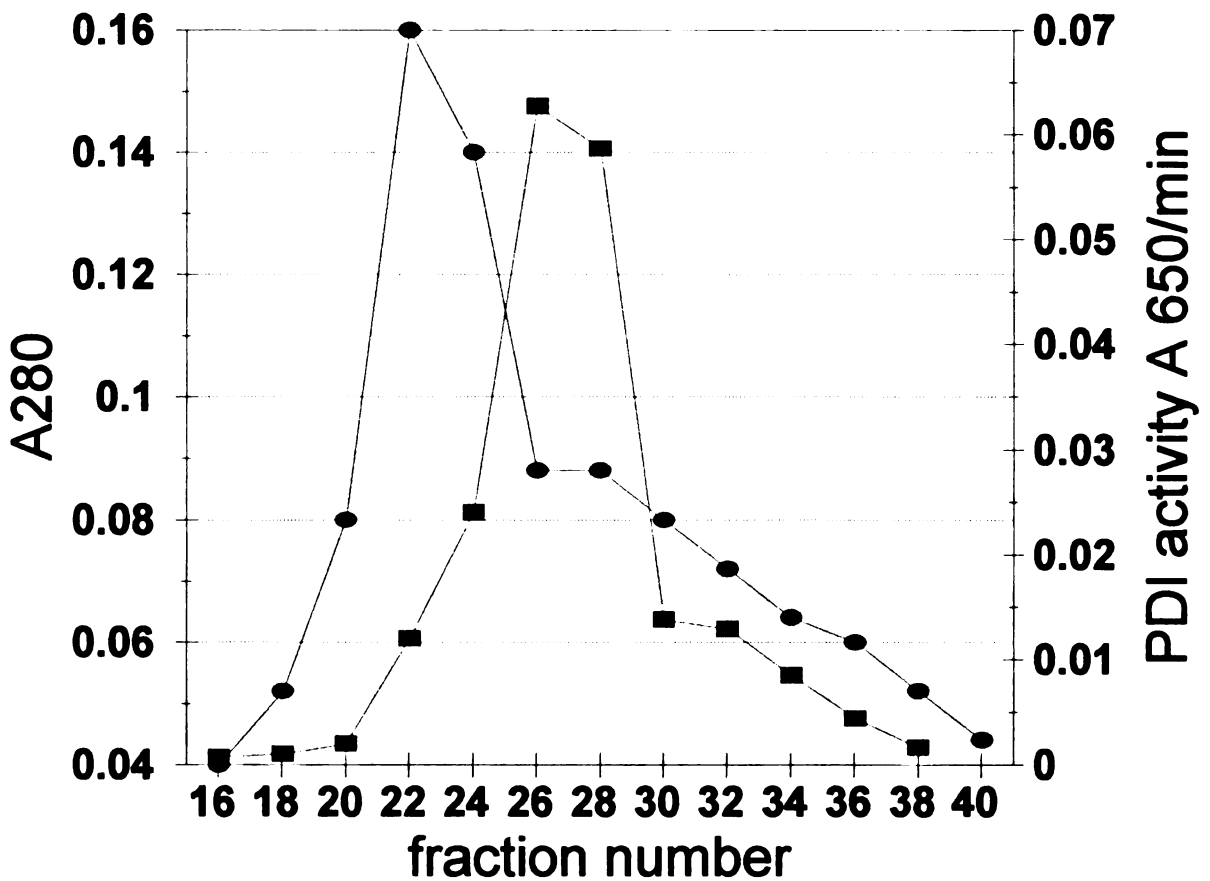
0.16
0.14
0.12
0.1
0.08
0.06
0.04
A280
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
PDI activity A 650/min
16 18 20 22 24 26 28 30 32 34 36 38 40
fraction number

Table 2: Purification of PDI [a] from chicken liver (400 g)

Purification step	Total protein (mg)[b]	PDI activity ΔA_{650}/ min	Specific activity (ΔA_{650}/min/mg)[c]	Yield (%)	Purification (fold)
Homogeinzation	10,507	390.4	0.037	100	1
55-85% Ammonium sulfate pellet	412.2	131.1	0.32	33.5	8.6
After CM-sephadexC50 and DEAE sephacel	11.2	69.2	6.17	17.7	192.8
Sephacryl S-300	2.4	38.7	16.1	7.5	435.1

a PDI was purified by the method of Kivirikko and Myllyla.

b Protein estimations were done using BCA method.

c PDI was assayed for activity based on insulin turbidity assay. Initial rates and specific activities were calculated based on change in absorbance at 650 nm / min .

Table 3: Apparent Km values for substrates and cofactors of chicken embryo P4H

Reactant	Apparent Km values for chicken embryo P4H (mM)
Fe^{2+}	0.005 (17)
Ascorbate	0.3 (18)
α- keto glutarate	0.01 (17)
-(Pro-Gly-Pro)n-	0.06 (18)
Oxygen	2.6 (18)

DISCUSSION:

P4H was purified from 15 day old chicken embryos and was homogeneous as observed by SDS-PAGE. This procedure, described by Tuderman, et al. (16) is a high yield procedure . The affinity column step makes use of the high affinity of P4H for Poly-L-Proline, Ki = 0.2 μ g / ml (19). Comparison of the gel filtration column elution profiles obtained from those obtained by Tuderman, et al. showed that in the Tuderman procedure, P4H eluted first, while in our preperations, an additional protein peak eluted from the column first and P4H eluted second (figure 4). Comparison of specific activities obtained here with those reported previously is difficult owing to the differences in the assay conditions and concentrations of substrates and cofactors. Ascorbate concentrations used in the present P4H assays were 0.5 mM while those used in the literature were 2 mM. Hence the specific activity (0.8-1.1 μ moles/ min/ mg protein) obtained by us was lower than that obtained previously (1.5 μ moles/ min/ mg protein) (14). Percentage recovery and fold purification obtained in this study were similar to those documented previously (14). The molecular weight of P4H has been previously determined to be 248,000, the α subunit being 64,000 and PDI (the β subunit) about 60,000 respectively by sedimentation equilibrium and SDS-PAGE (20). The chicken embryo P4H has an isoelectric point of 4.4 (21). Table 3 summarizes the apparent Km values for the various substrates and cofactors for chicken embryo P4H.

Chicken liver PDI is a homodimer and has a molecular weight of 60,000 as observed on SDS-PAGE (21) . The enzyme has an isoelectric point of 4-4.5 (21). The procedure used above, for the purification of PDI from chicken liver, was a modification of the Lambert and Freedman procedure for the purification of Bovine liver PDI (11). The chicken liver PDI purification, did not use the heat treatment step used in the purification of

bovine liver PDI. In addition this procedure used a Sephacryl S 300 gel filtration column in place of a G75 column.

PDI purified from chicken liver was used for inhibitor studies and potential inhibitors of PDI's DHA reductase activity like antibodies to PDI, estradiol and glutathione sulfonate were tested. The effect of antibodies to PDI and estradiol on PDI's DHA reductase activity will be discussed in chapter 3. The effect of gutathione sulfonate, a glutathione analog on PDI's DHA reducatase activity is discussed in chapter 4.

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CHAPTER 3: Potential inhibitors of PDI's DHA reductase acitvity: 17 β-estradiol and bovine PDI antiobodies

SUMMARY:

The effect of 17 β-estradiol and antibodies to bovine liver PDI were tested on chicken liver PDI's DHA reductase activity. 17 β-estradiol inhibited PDI's disulfide isomerase activity with an apparent K_i of 100 nM and this is in agreement with that obtained in the literature. Estradiol however had no inhibitory effect on PDI's DHA reductase activity. Bovine liver PDI antibodies cross reacted with chicken liver PDI, as determined by western blot analysis. The antibodies however did not inhibit the DHA reductase activity of PDI, under the assay conditions used.

INTRODUCTION:

Inhibitors of P4H have received much interest as potential anti-fibrotic drugs. P4H, by hydroxylating proline to hydroxy proline, plays a critical role in the stability of the collagen triple helix (1). Many substrate analogs that bear resemblance to α-ketoglutarate or ascorbate, inhibit P4H activity. The two most potent competitive inhibitors of P4H with respect to α-KG are pyridine 2,4 and pyridine 2,5 dicarboxylate with an apparent Ki of 0.8 μM and 2 μM respectively (2). Pyridine 2,5 dicarboxylate also inhibited P4H activity non-competitively with respect to iron and uncompetitively with respect to ascorbate. P4H activity was competitively inhibited by 3,4 dihydroxy benzoate with respect to both α-KG and ascorbate as variable substrates with an apparent Ki of 5 μM (3). Oxalyl amino acid derivatives like oxalyl glycine and oxalyl alanine inhibited P4H activity competitively with respect to α-KG with an apparent Ki of 1.9 μM and 40 μM respectively (4). In addition to these chemical compounds and the structural analogs that act as inhibitors of P4H, six monoclonal antibodies to human P4H have been isolated (5). Five out of the six antibodies caused negligible inhibition of P4H activity and one antibody caused strong inhibition and it is possible that it reacts with a site close to the active site of the enzyme. Out of the six monoclonal antibodies, five react with the β subunit and one with the α subunit as determined by western blots and immunofluorescent studies. This suggests that the β subunit of P4H is more immunogenic.

Inhibitors of PDI, the β subunit of P4H, have been reported. Bacitracin, a peptide antibiotic (6) and 17 β-estradiol have been reported to inhibit the disulfide isomerase activity of PDI (7). The primary sequence of PDI has two estradiol receprtor like domains. 17 β-estradiol has been shown to bind to PDI through these domains and non-competitively inhibit

the disulfide isomerase activity of PDI, the apparent Ki being 100 nM. Although some inhibitors of the disulfide isomerase activity of PDI are known, no inhibitors for the DHA reductase activity have been reported so far.

In an attempt to find an inhibitor for PDI's DHA reductase activity, the effect of antibodies to bovine liver PDI and 17 β-estradiol were tested on chicken liver PDI's DHA reducatse activity. The inhibitor would serve as a useful tool to distinguish the chemical regeneration of ascorbic acid by GSH from the proposed enzymatic regeneration by PDI, during the P4H catalytic process. The effect of bovine liver PDI antibodies and 17β-estradiol on PDI's DHA reductase activity are discussed in this chapter.

MATERIALS AND METHODS:

Glutathione, dithiothreitol were purchased from Boehringer-Mannheim. 17 β estradiol, bovine insulin and ascorbic acid were purchased from Sigma. Antibodies to bovine liver PDI were previously generated in the lab.

PURIFICATION OF CHICKEN LIVER PDI:

Chicken liver PDI was purified by the method of Kivirikko and Myllyla, described in chapter 2 (8).

PDI ACTIVITY ASSAY:

The disulfide isomerase activity of PDI was assayed using the insulin turbidity assay (9). The assay system in a 500 μl volume contained 100 mM sodium phosphate, pH 7.0, 15-20 μg PDI, varying concentrations of 17 β-estradiol in methanol, 0.16 mM insulin, 1mM dithiothreitol and 2mM EDTA. Controls received no enzyme but an equal volume of enzyme storage buffer. For estradiol inhibition studies, the controls received an equal volume of methanol. The concentration of methanol both in the controls and the enzyme catalyzed assays did not exceed 1%. The enzyme was preincubated with estradiol or antibodies for 10 min at 30°C. The insulin, being insoluble in water was first acidified to increase its solubility with 1 M Hcl and the pH adjusted to 6.0 with 1 M NaOH. The reaction, i.e., light scattering of aggregating B chains of insulin was monitored at 650 nm for 30 min at 30°C. Control values were subtracted from enzyme catalyzed values and initial enzymatic rates were calculated as change in A_{650}/min.

PREPARATION OF DHA: The required amount of ascorbic acid was weighed and dissolved in chelex water. Bromine (20 μl), was added to the solution and vortexed for 30 secs (10). Nitrogen gas was bubbled through the solution until the yellow color disappears

and for 10-15 min after that. The DHA prepared in this way is stable for upto 4 hours when kept on ice.

DHA REDUCTASE ACTIVITY ASSAY:

The assay system in a 500 µl assay volume contained 200 mM sodium phosphate, 1 mM EDTA, pH 6.85, 70 µg PDI, varying concentrations of 17 β estradiol dissolved in methanol or anti-bovine PDI antibody in 10 mM phosphate buffer pH 7.0, 1mM DHA and 2mM GSH (10). The components were added in the indicated order. The enzyme was preincubated for 10 min at 30°C with estradiol or the antibody . The reaction was initiated with GSH and monitored at 265.5 nm for 3 min at 30°C, in a Gilford Response II spectrophotometer. Controls run in the absence of enzyme or estradiol received the same volume of enzyme storage buffer, and different dilutions of pre-immune serum or methanol to account for possible non-specific interactions. The concentration of methanol in the cuvettes did not exceed 1 %. Control values were subtracted from enzyme catalyzed values and initial rates were calculated using the mM extinction coefficient of 14.7 for ascorbic acid (11).

WESTERN BLOT ANALYSIS:

Western blots were performed to assess interactions of bovine liver PDI antibodies with purified chicken liver PDI. The enzyme samples were separated on an SDS-PAGE (6% stacking gel and 10% separating gel). This was then transferred onto a nitrocellulose membrane at 70 V for 3 hours, in transfer buffer containing 10 mM Tris, 78 mM glycine and 20% methanol. After the transfer, the membranes were incubated in 1% BSA in TBST for 1 hour and then washed twice for 15 min each in TBST. The membrane was then incubated with 1: 10,000 dilution of rabbit anti-bovine PDI antibodies for 1 hour. After three 10 min washes in TBST, the membrane was incubated with secondary antibody- Anti rabbit IgG

alkaline phosphate conjugate for 1 hour. Color development was done with BCIP-NBT.

RESULTS:

Effect of 17 β-estradiol on PDI's disulfide isomerase activity:

PDI's disulfide isomerase activity was inhibited by 17β-estradiol (Figure 1). This effect of estradiol on the disulfide isomerase activity has been previously demonstrated (7). Half maximal inhibition was observed at 100 nM. Disulfide isomerase activity was inhibited by 33% and 56.5% at 500 nM and 1 μM estradiol respectively.

Effect of 17β-estradiol and rabbit anti-bovine PDI antibodies on PDI's DHA reductase activity:

17β-estradiol had no inhibitory effect on PDI's DHA reductase activity (Table 1). Six concentrations of estradiol were used. Initial velocities obtained, expressed as nmoles / min ascorbic acid formed, showed no significant difference in the absence or presence of estradiol. At some levels, initial velocities were slightly higher in the presence of estradiol.

Although bovine liver PDI antibodies, cross reacted with chicken liver PDI, as determined by western blot analysis (Figure 2), it had no inhibitory effect on PDI's DHA reductase activity. The effect of 4 dilutions of antibody were tested and controls run simultaneously received the same dilution of pre-immune serum. This was done to test for any non specific interactions. No significant difference in activity was observed in the presence or absence of antibody and as with the estradiol inhibition studies, a slight increase in initial velocity was observed in some samples in the presence of antibody.

Figure 1: Inhibition of PDI's disulfide isomerase activity by 17β estradiol. Chicken liver PDI's disulfide isomerase activity was measured by insulin turbidity assay. Initial rates are calculated as change in A_{650} / min and the values are averages of two separate experiments.

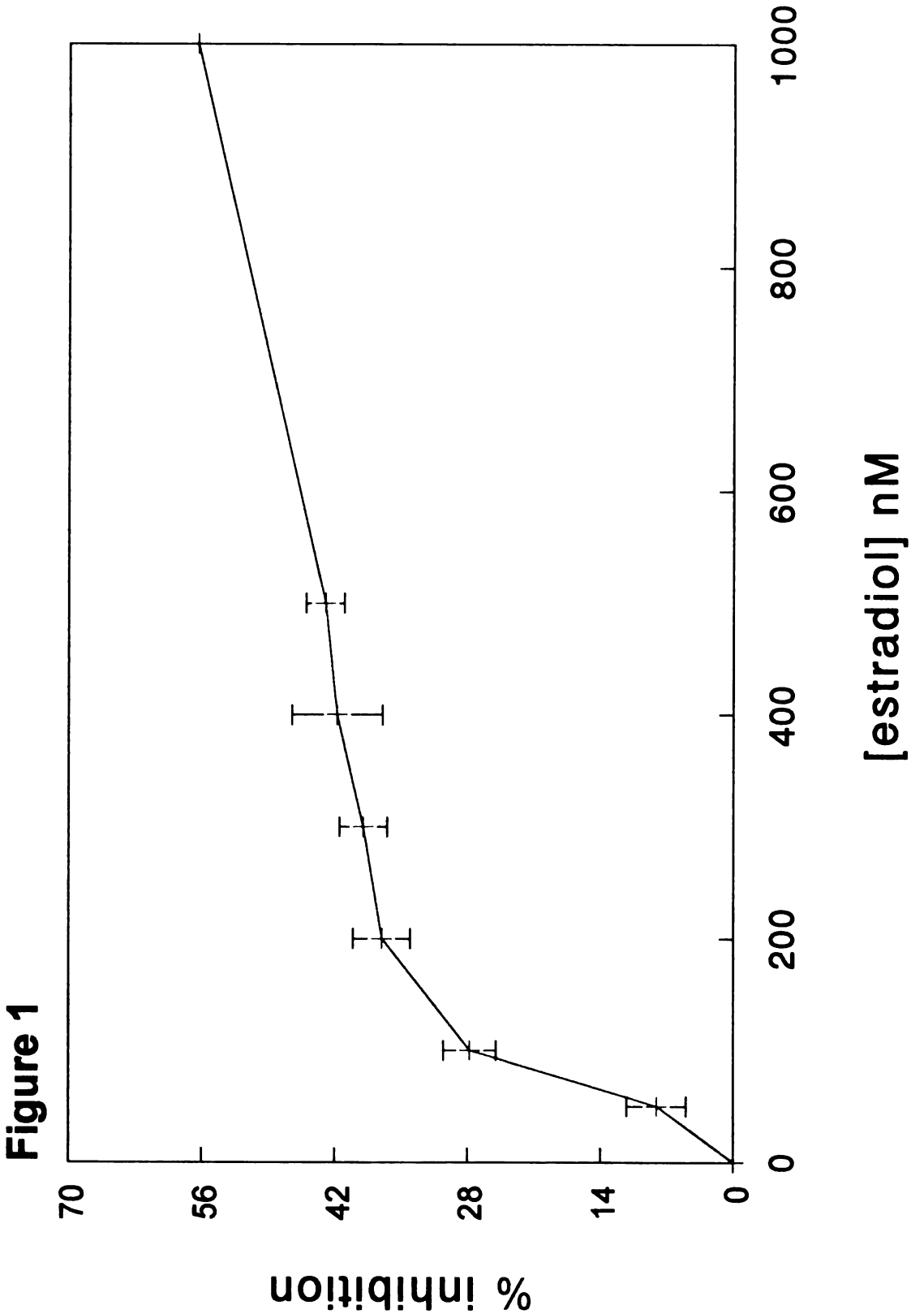

Figure 1

Figure 2: Western Blot analysis of purified chicken liver PDI.
Lane 1:Bio-Rad low molecular weight markers used as a negative control, Lane 2: Purified Bovine liver PDI used as a positive control, Lane 3: Purified chicken liver PDI.

Figure 2

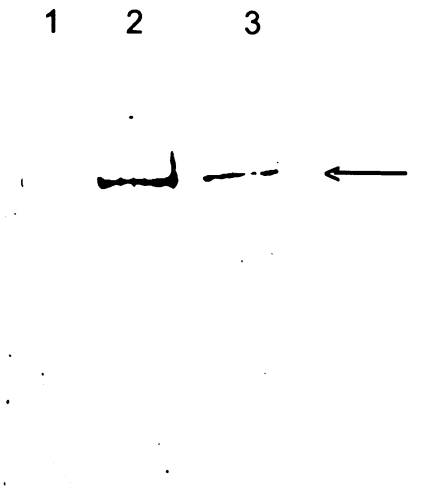

Table 1 : Effect of 17β estradiol on chicken liver PDI's DHA reductase activity [a]:

	Estradiol concentration (nM)	Initial Velocity[b]
1	0	3.8 ± 0.67
2	50	3.6 ± 0.31
3	100	4.7 ± 0.1
4	200	4.3 ± 0.35
5	400	4.4 ± 0.06
6	500	4.0 ± 0.8
7	1000	5.1 ± 0.3

a chicken liver PDI's DHA reductase activity was assayed spectrophotometrically by measuring increase in absorbance at 265.5 nm.

b Initial velocity is expressed as nmoles ascorbic acid/ min calculated using a mM extinction coefficient of 14.7. The values are expressed as averages ± S.D obtained from 2 separate experiments.

Table 2: Effect of bovine liver PDI antibodies on chicken liver PDI's DHA reductase activity [a]

	Antibody dilution	Initial velocity[b]
1	0	3.50 ± 0.04
2	1:10	4.02 ± 0.45
3	1:100	4.51 ± 0.5
4	1:1,000	4.18 ± 0.2
5	1:10,000	4.9 ± 0.03

a chicken liver PDI's DHA reductase activity was measured as the increase in absorbance at 265.5 nm.

b Initial velocity is expressed as nmoles ascorbate / min calculated using a mM extinction coefficient of 14.7. The values are expressed as averages ± S.D obtained from 2 separate experiments.

DISCUSSION: Disulfide isomerase activity of PDI was inhibited by 17β-estradiol with an apparent Ki of 100 nM. These results are in agreement with those obtained previously in literature (7). PDI actvity in literature, was measured by [^{125}I]insulin degradation or reactivation of randomly oxidized ribonuclease in the presence of reduced glutathione, while in this study PDI activity was monitored by the insulin turbidity assay. In the present study, the effect of estradiol on PDI's DHA reductase activity was investigated. 17 β-estradiol, did not inhibit chicken liver PDI's DHA reductase activity. Although the mechanism for PDI's DHA reductase activity is not known, it is thought that the active site cysteines that are responsible for the disulfide isomerase activity of PDI are also involved in the DHA reductase mechanism. It is interesting to note that estradiol inhibits one activity of the enzyme but not the other. It has also been demonstrated previously that this inhibition of the disulfide isomerase activity is mediated through the binding of estradiol to the estrogen receptor like domains on PDI and this binding results in decreased insulin (substrate) crosslinking (7). A 43% and a 30% decrease in [^{125}I] insulin crosslinking to PDI was observed at 1 μM estradiol in placental and liver samples respectively. It is possible that the different substrates, insulin and ascorbate interact differently with a common enzyme active site.

Antibodies to bovine liver PDI, cross reacted with chicken liver PDI, but did not inhibit its DHA reductase activity, under the assay conditions used. This may be due to a species difference between bovine and chicken liver PDI. The antibodies used in this study were polyclonal and it is possible that they may have reacted with an epitope not related with the active site or involved in the activity of the enzyme.

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Chapter 4: Glutathione stimulates Prolyl 4-hydroxylase activity and regenerates ascorbic acid

SUMMARY:

Purified chicken embryo prolyl 4-hydroxylase was assayed for activity using a method based on the decarboxylation of 1-[^{14}C] α-ketoglutarate. An ascorbic acid regeneration system consisting of glutathione, NADPH and glutathione disulfide reductase stimulated basal prolyl 4-hydroxylase activity by 150%. To differentiate the chemical regeneration of ascorbic acid from dehydroascorbic acid by glutathione from the proposed enzymatic regeneration by the β subunit of prolyl 4-hydroxylse, it was necessary to use an inhibitor of protein disulfide isomerase's dehydroascorbate reductase activity. Glutathione sulfonic acid , a glutathione analog, inhibited PDI's DHA reductase activity competitively.Chicken liver protein disulfide isomerase and chicken embryo prolyl 4-hydroxylase had an apparent K_i of 2.2 mM and 2.3 mM for glutathione sulfonate, respectively. The stimulation of prolyl 4-hydroxylase activity seen at 0.5 mM ascorbate, in the presence of glutathione and the regeneration system was inhibited 55% and 53%, respectively in the presence of 5 mM glutathione sulfonate. Basal prolyl 4-hydroxylase activity in the absence of glutathione was also inhibited by glutathione sulfonate, at 0.5 mM ascorbate but not at 1 mM and 2 mM ascorbate. These observations suggest that protein disulfide isomerase, the β subunit of prolyl 4-hydroxylase, may be involved in recycling the cofactor ascorbate at physiological ascorbate concentrations.

INTRODUCTION:

Prolyl 4- hydroxylase (EC 1.14.11.2) (P4H) , a dioxygenase that catalyzes the post-translational hydroxylation of proline residues in collagen, uses molecular O_2 and α-ketoglutarate as cosubstrates and ferrous iron and ascorbic acid (AA) as cofactors (1). Oxidative decarboxylation of α-ketoglutarate to succinate and carbon dioxide, leads to the formation of an active iron oxygen complex called the ferryl ion that subsequently transfers the oxygen to the proline to form hydroxy-proline (1). In some reaction cycles, the decarboxylation of α-ketoglutarate does not subsequently result in the hydroxylation of proline (2). AA acts as an alternate oxygen acceptor and helps maintain enzyme bound iron in the ferrous state (3). P4H is a heterotetramer with a molecular weight of 248,000, consisting of α_2 and β_2 subunits. The α subunit contains most of the catalytic site as deduced from studies using substrate analogues and photoaffinity labelling (4). The α-ketoglutarate binding site can be subdivided into 3 distinct subsites (5) and substrate analogue studies have also revealed a partial identity between the ascorbate binding site and the α-ketoglutarate binding site (6). Site directed mutagenesis studies have shown that histidine residues in the active site are responsible for the binding of iron (7). The β subunit of P4H has been identified as protein disulfide isomerase (PDI), an enzyme that catalyzes the isomerization of disulfide bonds in nascent secretory proteins (8). In addition to its disulfide isomerase activity, PDI has been shown to possess chaperone/antichaperone activity (9), act as a glycosylation site binding protein (10) and a thyroid hormone binding protein (11). In addition, PDI is capable of recycling ascorbic acid from its oxidation product, dehydroascorbate (DHA), in a glutathione (GSH) dependant manner (12). Although PDI is now recognized as a multifunctional protein, its

role as the β subunit of P4H is not clear. As the β subunit of P4H, it may be involved in preventing misfolding and aggregation of the α subunit (13), and retaining P4H in the lumen of the endoplasmic reticulum owing to its C terminal KDEL sequence (14). Site directed mutagenesis of the active site cysteines responsible for the disulfide isomerase activity of PDI did not affect the assembly or activity of P4H tetramer (14). Knowledge that PDI possesses glutathione dependent DHA reductase activity, led to the speculation that the β subunit of P4H may be involved in recycling ascorbate during the catalytic process. In this study we investigated PDI's involvement in recycling ascorbate, one of the cofactors in the P4H reaction.

MATERIALS AND METHODS:

Materials: α-KG, glutathione sulfonate (GSO_3^-), and ascorbate were purchased from Sigma. GSH, NADPH and glutathione disulfide reductase were purchased from Boehringer-Mannheim. 1-[^{14}C]α-KG was purchased from NEN and the peptide substrate -(pro-gly-pro)-$_9$.H_2O was obtained from the peptide research institute, Japan.

Purification of P4H: P4H was purified from 15 day old white leghorn chicken embryos (Department of Animal Science, Michigan State University) by the method of Tuderman, et al.(15).

Purification of PDI: PDI was purified from chicken liver by the method of Kivirikko and Myllyla (16) which is a modification of the Lambert and Freedman method used for purification of bovine liver PDI.

P4H activity assays: P4H was assayed for activity by the method based on hydroxylation coupled decarboxylation of 1-[^{14}C] α-KG (17). The basal assay system, in a 1 ml assay volume, consisted of 50 mM Tris.HCl, pH 7.4, 100 μM -(pro-gly-pro)-$_9$.H_2O, 3-5 μg P4H, 5 μM ferrous sulfate, 1.5 mg BSA, 0.07 mg catalase, 0.5 mM ascorbic acid unless specified otherwise and 1-[^{14}C]α-KG, adjusted to a specific activity of 60,000 cpm/μmole with unlabelled α-KG. In addition to these components, 2 mM GSH and a GSH regeneration system consisting of 0.1 mM NADPH and 10 μg GSSG reductase was included (Basal + GSH and Basal + GSH + regeneration system) in some assay mixtures. The GSSG reductase was diluted to the required concentration in triple distilled water. The inhibitor, GSO_3^-, was neutralized with 1 M NaOH before addition. All reagents were kept on ice until added. Fresh solutions of ascorbic acid, ferrous sulfate, α-KG, GSH and NADPH were prepared before each assay. Since ascorbic acid oxidizes rapidly in the

presence of transition metals, it was dissolved prior to use in chelex-treated water. Ferrous sulfate was also dissolved just before use to prevent any hydroxide formation. The peptide substrate, $(PGP)._9 H_2O$, was boiled and cooled on ice just before addition. All controls received P4H, but no peptide substrate to account for the uncoupled decarboxylation catalyzed by the enzyme (2). The above mentioned components were aliquoted into tubes kept on ice in the indicated order, and the reaction was initiated with the addition of 1-[^{14}C]α-KG. The tubes were sealed with rubber stoppers with cups containing filter paper soaked in 1M NaOH and transferred from ice to a 37°C water bath where they were incubated with gentle shaking for 10 min. The reaction was quenched with 250 µl of 50% TCA and [^{14}C]-CO_2 was trapped for 1 hour at 37°C and counted in a liquid scintillation counter. All assays were performed in duplicate and averages were calculated to obtain the final value.

DHA reductase activity assays: The assay system (500 µl) contained 200 mM sodium phosphate, 1 mM EDTA, pH 6.85, 70 µg PDI, varying concentrations of the inhibitor, GSO_3^-, 1 mM DHA and 2 mM GSH. The components were added in the indicated order. The enzyme was preincubated with the inhibitor for 5 min at 30°C, the reaction initiated with GSH and monitored at 265.5 nm at 30°C, using a Gilford response II spectrophotometer. Controls run in the absence of enzyme, received the same volume of enzyme storage buffer. Controls were run both in the absence and the presence of inhibitor, and the appropriate control values were subtracted from the enzyme catalyzed values. Initial velocities were calculated based on the mM extinction coefficient of 14.7 for ascorbic acid(18).

Ascorbate analysis:

Ascorbic acid analysis in the P4H assay system, was carried out on a C18 reversed phase HPLC column using an ESA coulochem II electrochemical detector. The assays were performed as described above, but without radioactive α-KG. At the end of 10 min, 250 µl of the assay mixture were quenched with 750 µl of a solution containing 10% m-phosphoric, 1 mM EDTA, 1 mM thiourea. The samples were centrifuged at 10,000 rpm for 3 min and the supernatant diluted with the m-phosphoric acid solution until the ascorbic acid concentration was between 1-2 µM. The samples were kept on ice until used. The ascorbate concentration was calculated from integrated area units, based on a standard ascorbate curve (1 µM-8 µM).

Statistical analysis: Averages and standard deviations were calculated using Quattro Pro spread sheet program. Specific statistical comparisons were done according to student's "t" test using the statistical program Graph Pad Instat.

RESULTS: Chicken embryo P4H was homogeneous as observed by SDS-PAGE . Chicken liver PDI was purified to near homogeniety. P4H activity was linear with respect to both time and enzyme concentration. The enzyme activity remained linear for up to 20 min at the substrate concentration used. The concentration of enzyme (3-5 μg) used for the studies was within the linear range of enzyme activity. PDI, as part of the P4H complex, exhibited DHA reductase activity, when assayed spectrophotometrically (data not shown).

AA dose response with and without AA regeneration system: In order to investigate the involvement of PDI's DHA reductase activity in the P4H reaction, the effects of an ascorbate regeneration system, comprised of GSH, GSSG reductase and NADPH were tested on P4H activity. In an attempt to optimize P4H assay conditions, an ascorbic acid dose response study was performed at five ascorbate concentrations, with and without the regeneration system (Figure 1). P4H activity was linear with ascorbate concentrations up to 1mM and then started to plateau. Incorporation of an ascorbate regeneration system resulted in a 120% stimulation over basal at 0.1 mM and 0.2 mM ascorbate. A 150% stimulation over basal (all components of the assay system) was seen at 0.5 mM ascorbate which was statistically significant, $p<0.05$. At 1 mM and 2 mM ascorbate (saturating concentration), the regeneration system had no stimulatory effect on enzyme activity. At 0.5 mM ascorbate, 2 mM GSH had no significant stimulatory effect on the uncoupled decarboxylation reaction carried out without the peptide substrate (data not shown). Keeping the ascorbate concentration constant at 0.5 mM, P4H activity was assayed at four GSH levels (Figure 2). Although augmentation of activity was observed at all GSH concentrations, statistically significant increases in activity were observed at 2 mM, 3

mM and 4 mM. The ascorbate and GSH levels were kept constant at 0.5 mM and 2 mM respectively for further assays.

The regeneration of ascorbate from its oxidation product DHA by GSH, is a well documented chemical reaction(19). In order to differentiate the chemical regeneration of ascorbate by GSH from the proposed enzymatic regeneration by PDI, the β subunit of P4H, an inhibitor for PDI's DHA reductase activity was used.

Inhibition of PDI'S DHA reductase activity by GSO_3^-: The effect of GSO_3^-, a GSH analog was tested on the DHA reductase activity of chicken liver PDI. GSO_3^- mimics GS^-, the ionized form of GSH, but does not possess the reducing properties of GSH since the sulfhydryl group is modified to a sulfonic acid group. DHA reductase activity of PDI was inhibited competitively by GSO_3^-, with an apparent K_i of 2.2 mM (Figure 3). Chemical regeneration of AA from DHA by GSH was unaffected even at high levels of GSO_3^- (data not shown).

Effect of GSO_3^- on P4H activity:

P4H activity was inhibited competitively by GSO_3^- with respect to GSH as the activator (Figure 4). The apparent K_i obtained was 2.3 mM, which agreed well with that obtained for inhibition of PDI's DHA reductase activity by GSO_3^-.

Figure 1: Effect of AA on P4H activity in the presence of GSH and the regeneration system. All assays were of 10 minute duration. The values are averages of 3 separate experiments done in duplicates. Basal assay sytem,--**O**--, contained 50 mM Tris-HCl pH 7.4, 100 μM -(pro-gly-pro)-$_9$.H_2O, 3 μg P4H, 5 μM ferrous sulfate, 1.5 mg BSA, 0.07 mg catalase, different ascorbate levels, 1-[^{14}C]α-KG with a specific activity of 60,000 cpm/μmole. Enzyme activity is expressed as μmoles [^{14}C]-CO_2 released/min/mg protein ± S.D. --◆-- Basal + 2 mM GSH, --■-- Basal + 2 mM GSH + reg. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. * = statistically significant difference from basal, $p < 0.05$

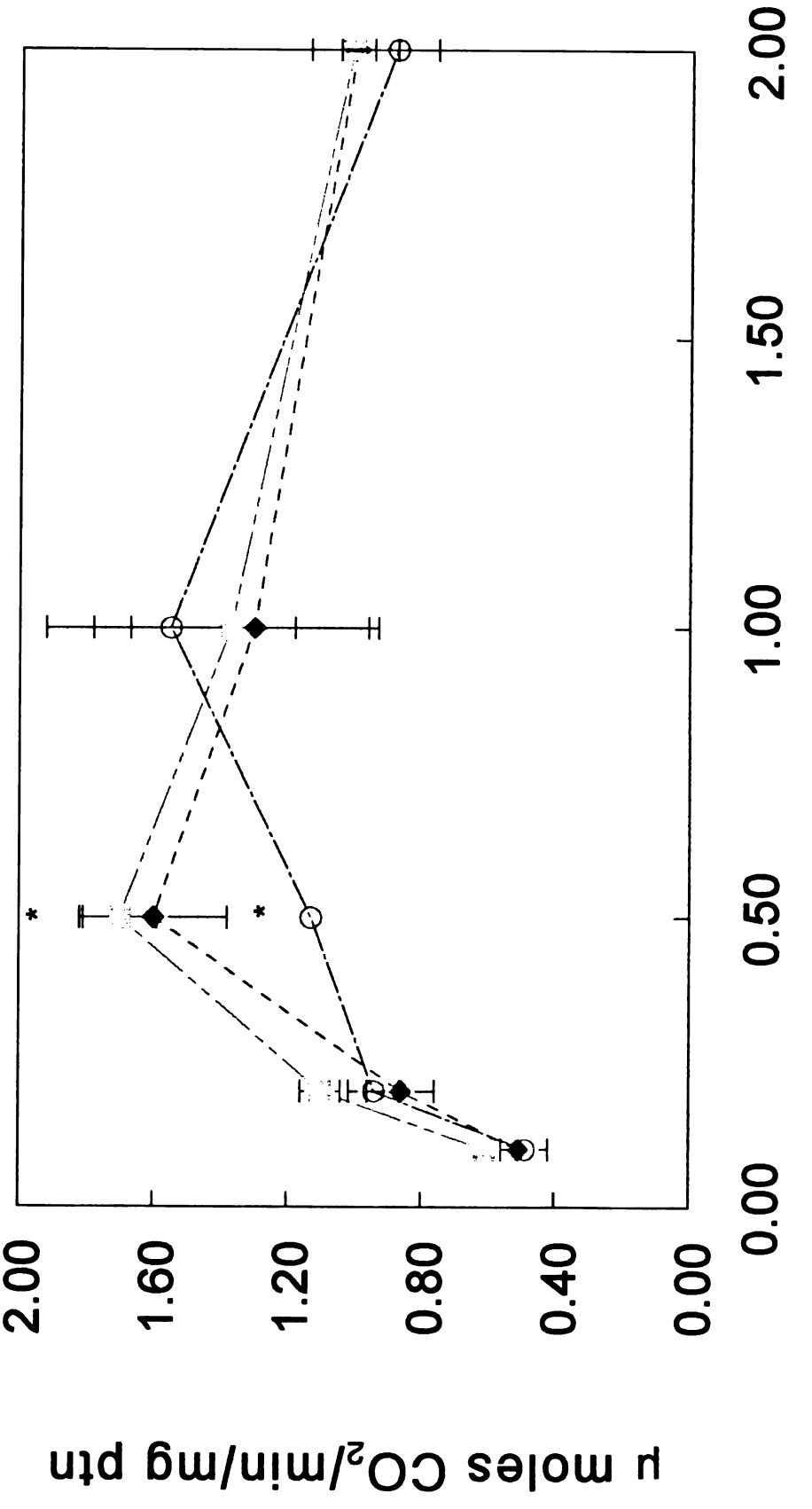


Figure 1
μ moles CO2/min/mg ptn
2.00
1.60
1.20
0.80
0.40
0.00
0.00
0.50
1.00
1.50
2.00
[AA] mM
*
*

Figure 2: Effect of GSH and the GSH regeneration system on P4H activity. All assays were of 10 minute duration. The values are averages of 3 separate experiments done in duplicates. Basal assay system,contained 50 mM Tris-HCl, pH 7.4, 100 μM -(-pro-gly-pro)-$_9$. H_2O, 3 μg P4H, 5 μM ferrous sulfate, 1.5 mg BSA, 0.07 mg catalase, 0.5 mM AA, different GSH levels, 1-[^{14}C] α-KG with a specific activity of 60,000 cpm/μmole. Enzyme activity is expressed as μmoles [^{14}C]-CO_2 released/min/mg protein ± S.D. --**O**-- Basal + GSH,--■-- Basal +GSH + reg. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. * = statistically significant difference from basal, $p < 0.05$.

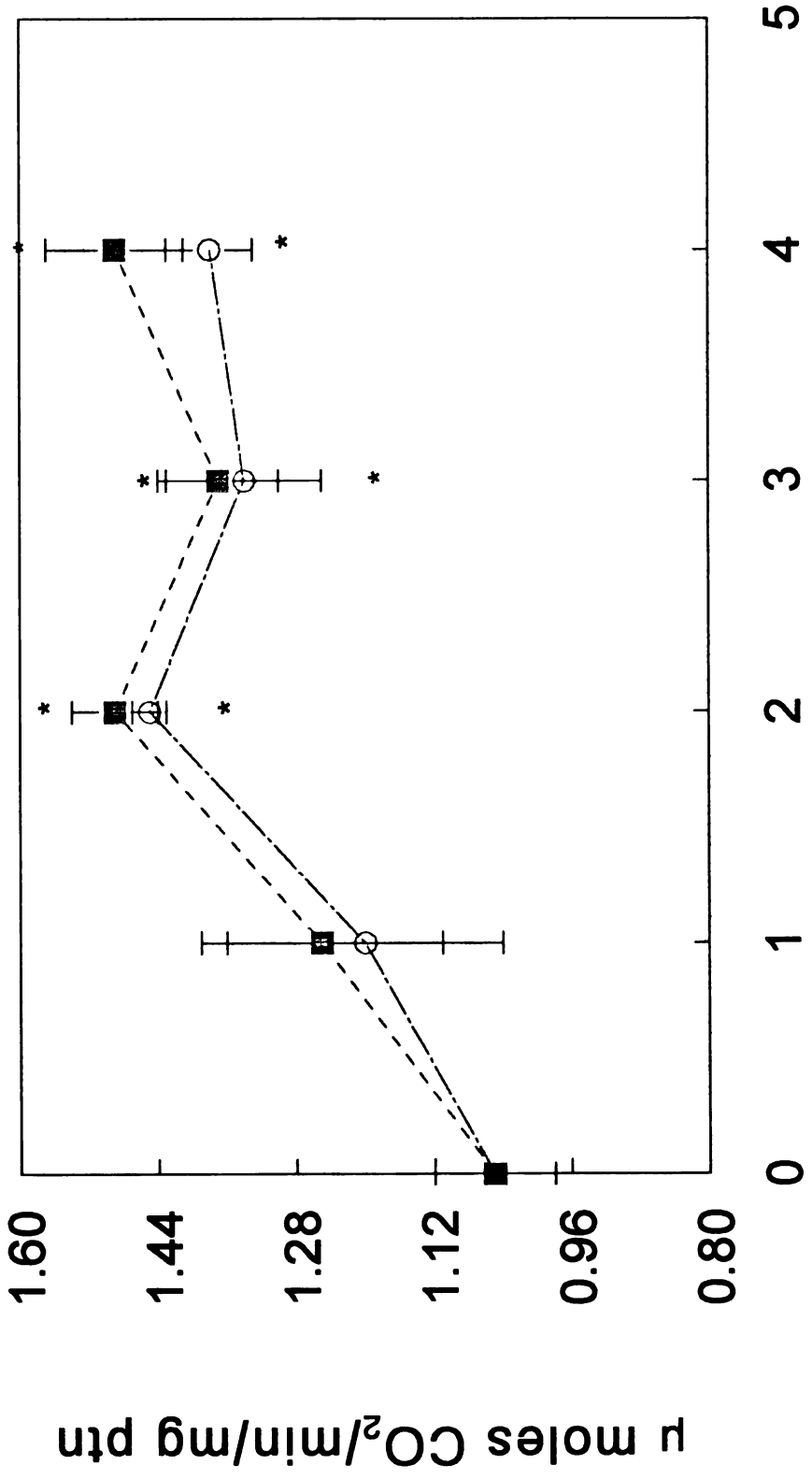


Figure 2

Figure 3: Dixon plot showing competitive inhibition of chicken liver PDI's DHA reductase activity by GSO_3^-. Assay systems contained 200 mM sodium phosphate, pH 7.4, 1 mM EDTA, 70 μg PDI, 1 mM DHA and varied concentrations of neutralized GSO_3^-. The GSH levels were kept constant at 1 mM --✚--, 2 mM --◆--, or 3 mM --■-- and GSO_3^- concentration varied. The reactions were monitored for 3 min at 265.5 nm and 30°C. Data represent the average of 3 separate experiments.

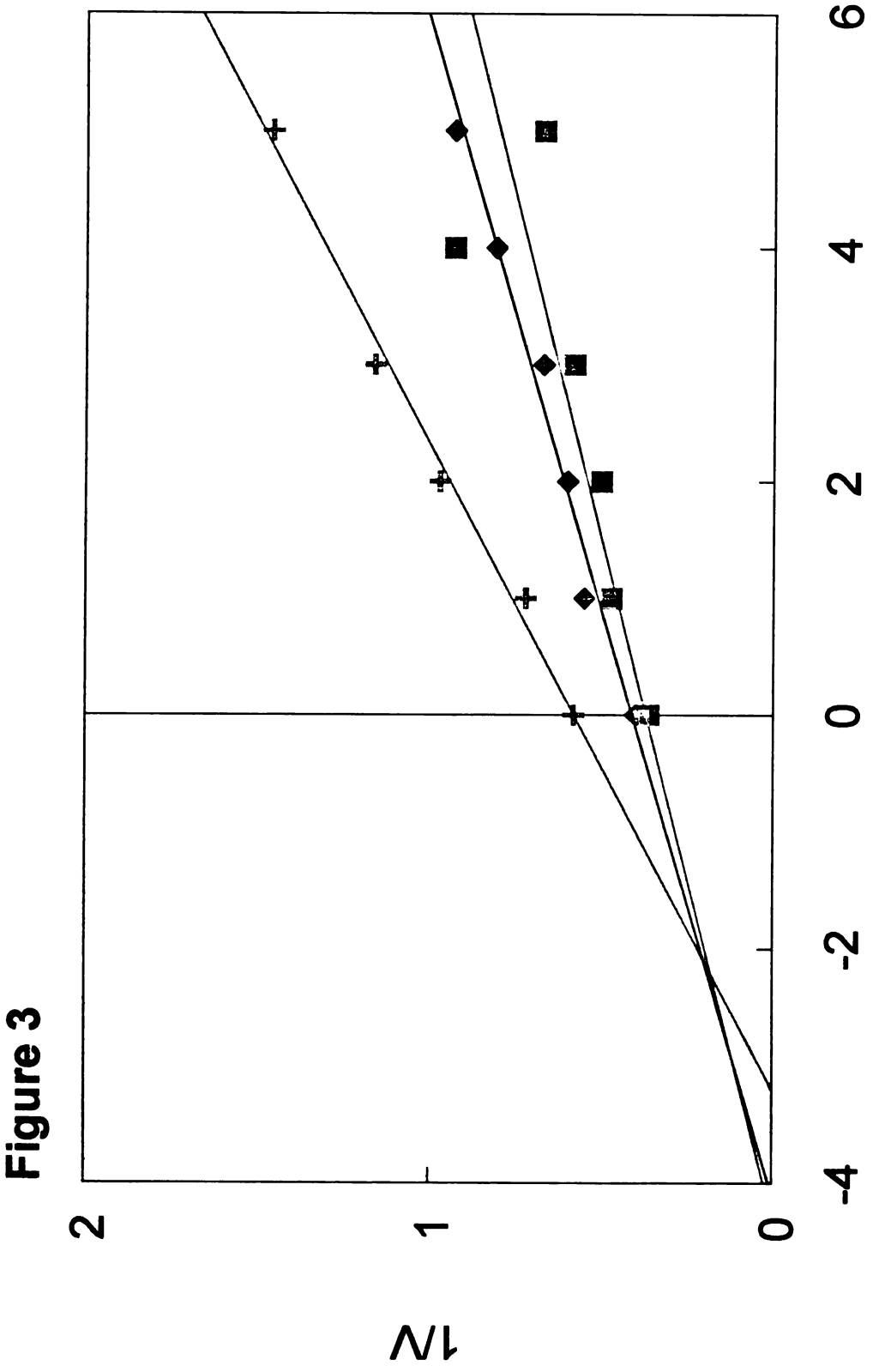
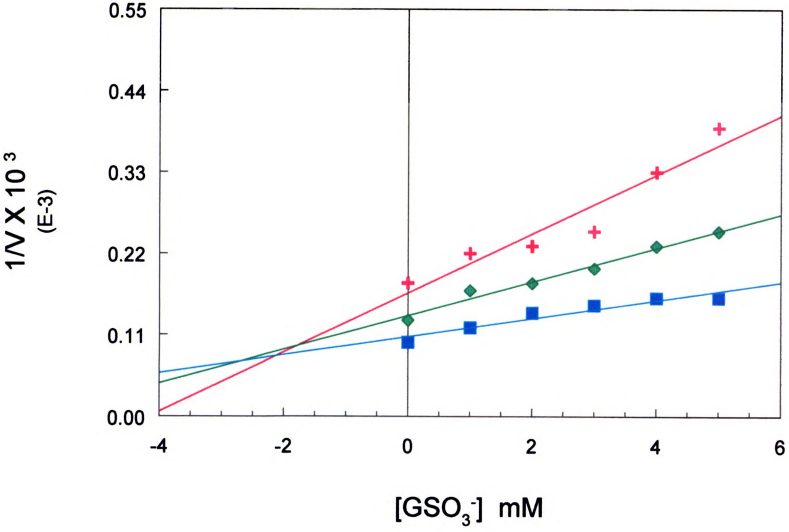


Figure 3

Figure 4: Dixon plot showing competitive inhibition of chicken embryo P4H activity with respect to GSH by GSO_3^-. All assays were of 10 min duration. Results were obtained from 3 separate experiments, done in duplicates. Assays contained 50 mM Tris-HCl, pH 7.4, 100 μM -(pro-gly-pro)-$_9$.H_2O, 5 μM ferrous sulfate, 1.5 mg BSA, 0.07 mg catalase, 0.5 mM ascorbate, 1-[^{14}C]α-KG with a specific activity of 60,000 cpm/μmol. 1/v is initial velocity or 1/cpm. GSH concentrations were held constant at 1 mM --✚--,2 mM --◆-- and 3 mM --■-- and assays were performed at five GSO_3^- concentrations.

Figure 4



The stimulation of P4H activity seen in the presence of 2 mM GSH and the regeneration system, was inhibited by GSO_3^-. A GSO_3^- dose response indicated that this stimulation was inhibited progressively with increasing GSO_3^- concentrations. Keeping enzyme activity in Basal + GSH samples at 100%, addition of 3 mM, 4 mM and 5 mM GSO_3^- inhibited P4H activity by 43%, 50% and 55% respectively (Figure 5A). These differences were statistically significant when compared with Basal + GSH samples in the absence of GSO_3^- . P4H activity in Basal + GSH + regeneration system samples was inhibited by 46%, 52% and 53% at 3 mM, 4 mM and 5 mM GSO_3^- respectively. These were statistically significant differences when compared with the appropriate control lacking GSO_3^-.

If PDI's DHA reductase activity is involved in recycling ascorbate in the P4H reaction, one would expect GSO_3^- to inhibit the stimulation seen in the presence of GSH and the regeneration system. Although we did see inhibition of P4H activity in the presence of GSH, basal P4H activity in the absence of GSH was also inhibited by GSO_3^-. Keeping basal P4H activity in the absence of inhibitor at 100 %, 41%, 46% and 50% inhibition was observed at 3 mM, 4 mM and 5 mM GSO_3^- respectively, and the differences were statistically significant (Figure 5A).

Effect of GSO_3^- on P4H activity at 1 mM and 2 mM ascorbate:

Since activity in both basal and basal + GSH samples were inhibited at 0.5 mM ascorbate, the effect of GSO_3^- on P4H activity was tested at higher ascorbate concentrations. No significant decrease in basal activity was observed at 1 mM and 2 mM ascorbate upon inclusion of 1, 3 and 5 mM GSO_3^- (Figures 5B and 5C).

As previously mentioned, in the ascorbate dose response experiment, no significant

increase in basal activity was observed at 1 mM and 2 mM ascorbate upon incorporation of GSH and the regeneration system (Figures 5B and 5C). P4H activity was inhibited in basal + GSH samples by 35% and 42% at 1 mM , but not at 2 mM ascorbate in the presence of 3 mM and 5 mM GSO_3^- respectively.

Ascorbate analysis: In order to investigate whether changes in enzyme activity, corresponded to changes in ascorbate levels, P4H assay samples were analyzed for their ascorbate content at the end of incubation. Three concentrations of ascorbate, 0.5 mM, 1 mM and 2 mM were used for this study.

At 0.5 mM ascorbate, the amount of ascorbate in basal samples remaining at the end of 10 min were about 350 μM in the chemical reaction (Figure 6) and about 400 μM in the enzymatic reaction (Figure 6). At 1 mM and 2 mM ascorbate, basal levels in the chemical (data not shown) and enzymatic reactions were not significantly different from each other (Figure 7).

At 0.5 mM ascorbate, the ascorbate levels maintained by GSH in the chemical reaction, were approximately 400 μM (Figure 6). In the enzymatic reaction, GSH and the regeneration system, maintained ascorbate levels close to 500 μM. The difference in the ascorbate levels in the presence of GSH and the regeneration system as a result of the enzymatic and the chemical reactions were significantly different from each other. Ascorbate levels remained unaffected in basal + GSH and basal + GSH + regeneration system in the chemical reaction in the presence of 1 mM, 3 mM and 5 mM GSO_3^- when compared to samples lacking GSO_3^- (data not shown). In the presence of 1 mM GSO_3^-, the ascorbate levels were lowered to 462.1 $\pm$ 14.2 μM in the enzymatic reaction. However, inclusion of 3 mM and 5 mM GSO_3^- to the enzymatic assay samples resulted in

a decrease in the ascorbate levels, to about 420 ± 8.7 μM and 415 ± 10.7 μM, respectively. These values were significantly lower than those lacking the inhibitor in which ascorbate concentrations were maintained near 500 μM ($p<0.0001$). At 1 mM AA, GSH maintained ascorbate concentrations at 850± 50 μM in the chemical reaction and at 946± 34 μM in the enzymatic reaction. In the presence of 5 mM GSO_3^-, the chemical reaction remained unaffected (data not shown), but ascorbate concentrations in the enzymatic reaction dropped to 891.3± 26.3, a statistically significant decrease with a p value < 0.05 (Figure 7). No statistically significant decrease was observed at 2 mM ascorbate and 5 mM GSO_3^- (Figure 7)

Fig 5 A: Effect of GSO_3^- on P4H activity at 0.5 mM AA. All assays were of 10 minute duration. Assays were performed as described in the methods section. Basal --●--, Basal+ 2 mM GSH --■--, Basal + 2 mM GSH + reg --◆--. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. The values are specific activities expressed as μmoles [^{14}C]-CO_2 released/min/mg protein ± S.D. These are averages of 3 separate experiments done in duplicates. + = statistically significant difference from Basal + GSH, # = statistically significant difference from Basal + GSH + reg and * = significant difference from Basal, $p<0.05$.

Figure 5A

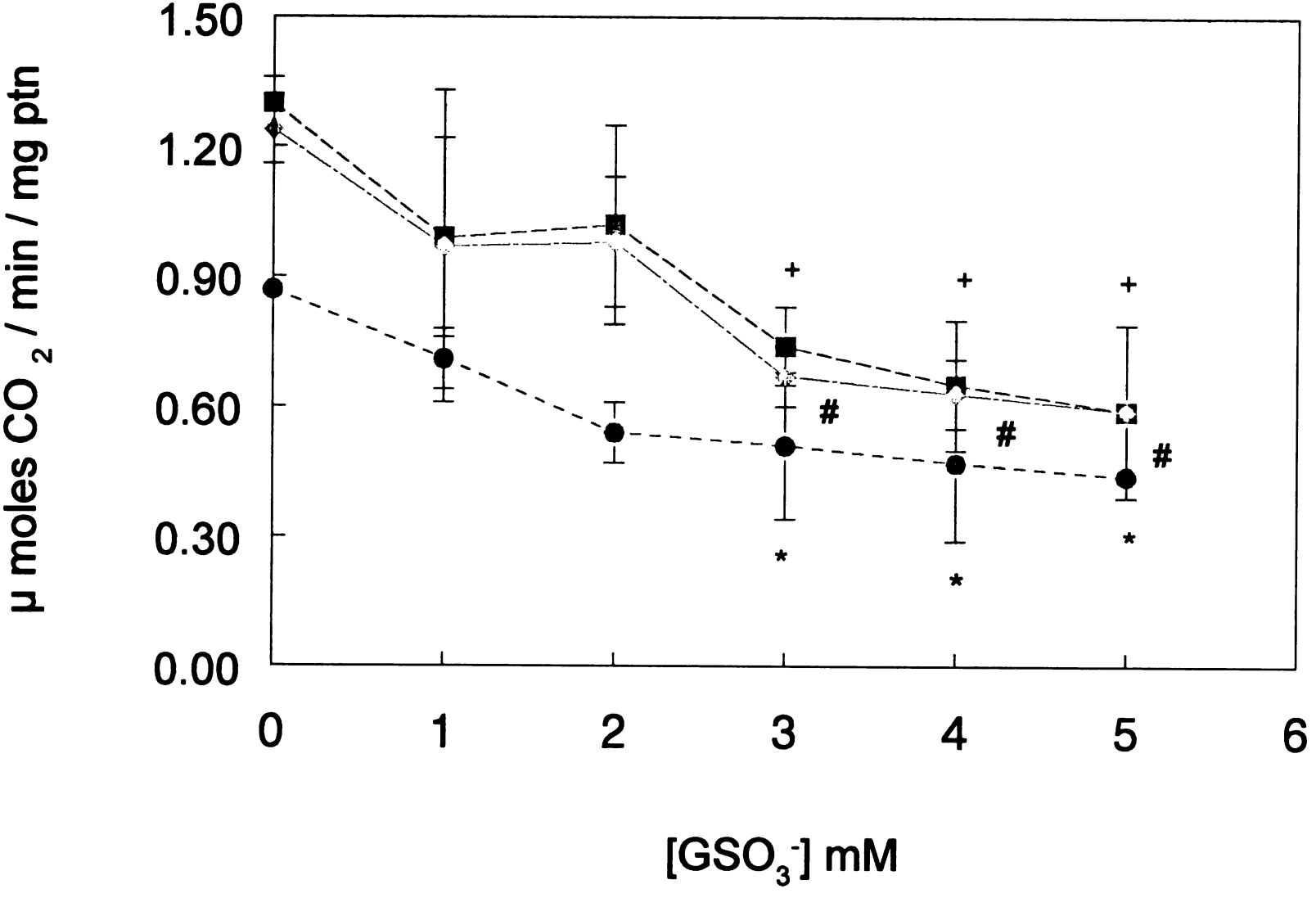


Fig 5 B: Effect of GSO_3^- on P4H activity 1 mM AA. All assays were of 10 minute duration. Assays were performed as described in the methods section. Basal --●--, Basal+ 2 mM GSH --■--, Basal + 2 mM GSH + reg --◆--. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. The values are specific activities expressed as μmoles [^{14}C]-CO_2 released/min/mg protein ± S.D. These are averages of 3 separate experiments done in duplicates. + = statistically significant difference from Basal + GSH, # = statistically significant difference from Basal + GSH + reg and * = significant difference from Basal, $p<0.05$.

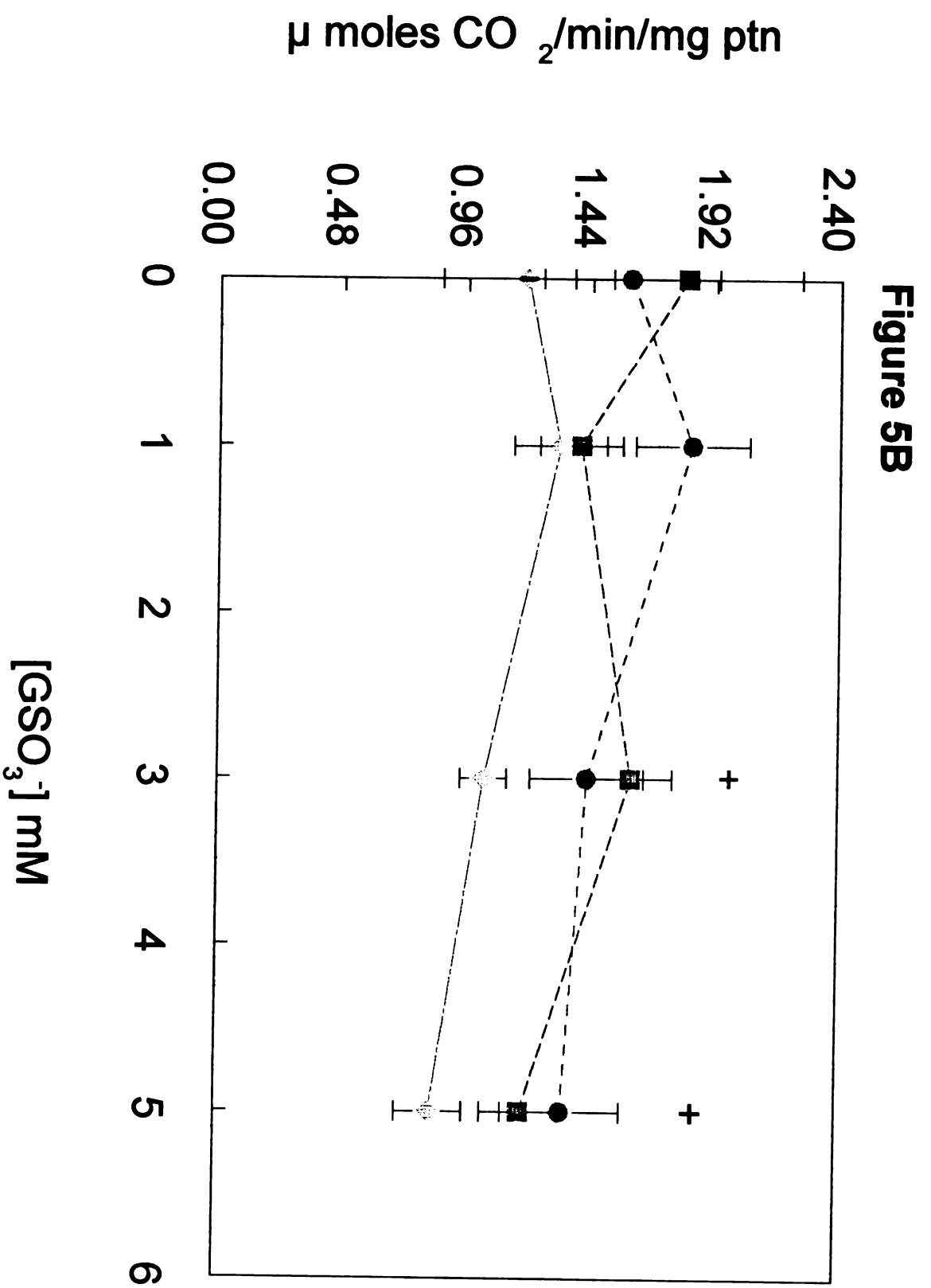


Figure 5B

Fig 5 C: Effect of GSO_3^- on P4H activity at 2 mM AA. All assays were of 10 minute duration. Assays were performed as described in the methods section. Basal --●--, Basal+ 2 mM GSH --■--, Basal + 2 mM GSH + reg --◆--. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. The values are specific activities expressed as μmoles [^{14}C]-CO_2 released/min/mg protein ± S.D. These are averages of 3 separate experiments done in duplicates. + = statistically significant difference from Basal + GSH, # = statistically significant difference from Basal + GSH + reg and * = significant difference from Basal, $p<0.05$.

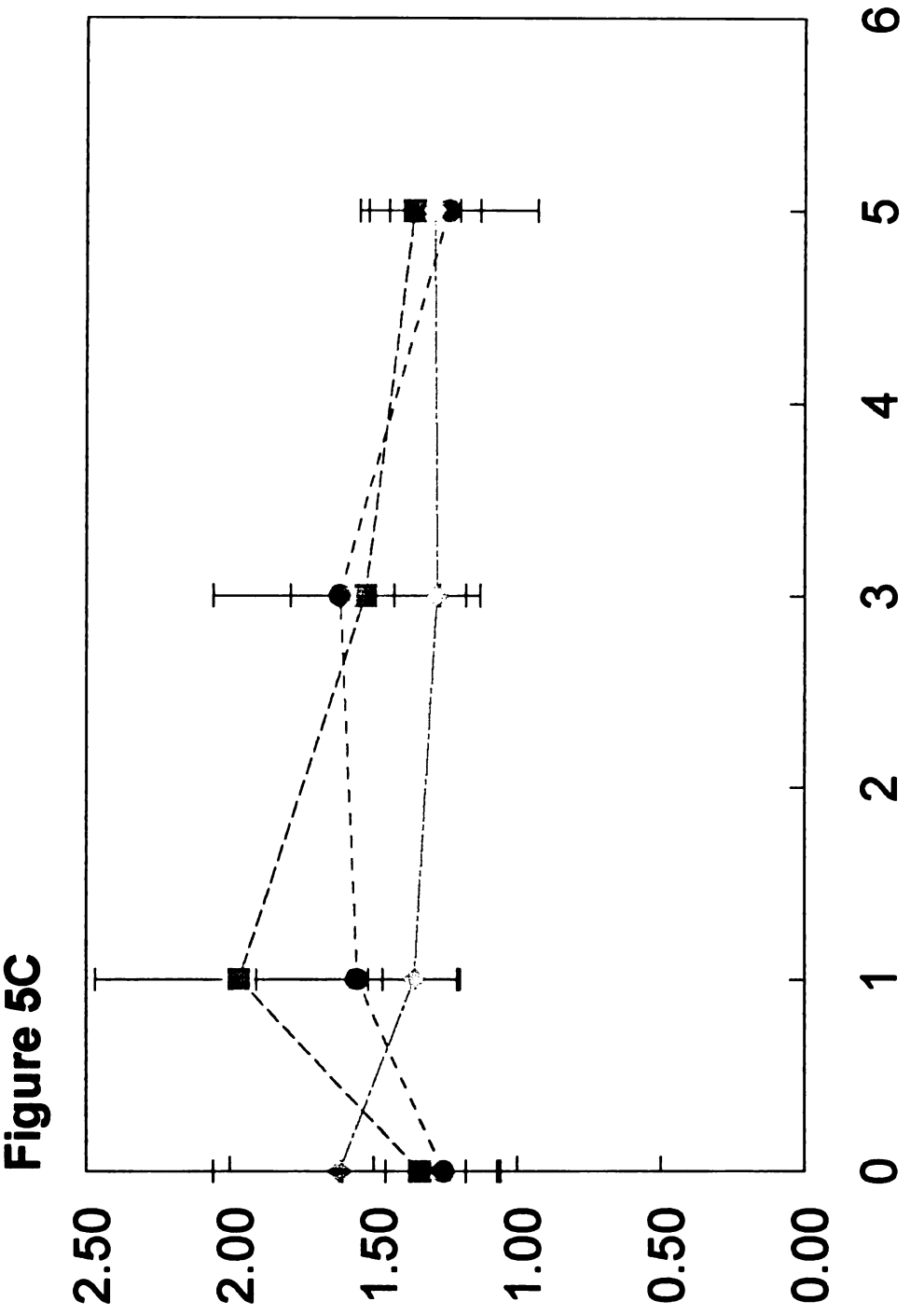


Figure 5C
μ moles CO 2/min/mg ptn
2.50
2.00
1.50
1.00
0.50
0.00
0
1
2
3
4
5
6
[GSO3-] mM

Fig 6 : Effect of GSO_3^- on AA levels in the P4H reaction. All assays were of 10 minute duration. Results were obtained from 3 separate experiments, done in duplicates. Assays contained 50 mM Tris-HCl, pH 7.4, 100 μM -(pro-gly-pro)-$_9$.H_2O, 5 μg P4H, 5 μM ferrous sulfate, 1.5 mg BSA, 0.07 mg catalase, 0.5 mM ascorbate, 0.1 mM α-KG. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. B = basal, reg. = GSH regeneration system. * = statistically significant difference from the appropriate control lacking GSO_3^-, $p<0.05$. ■, Chem; ▨, Enz; ▩, Enz + 1 mM GSO_3^-; ▥, Enz + 3 mM GSO_3^-; ▤ ,Enz + 5 mM GSO_3^- .

Figure 6

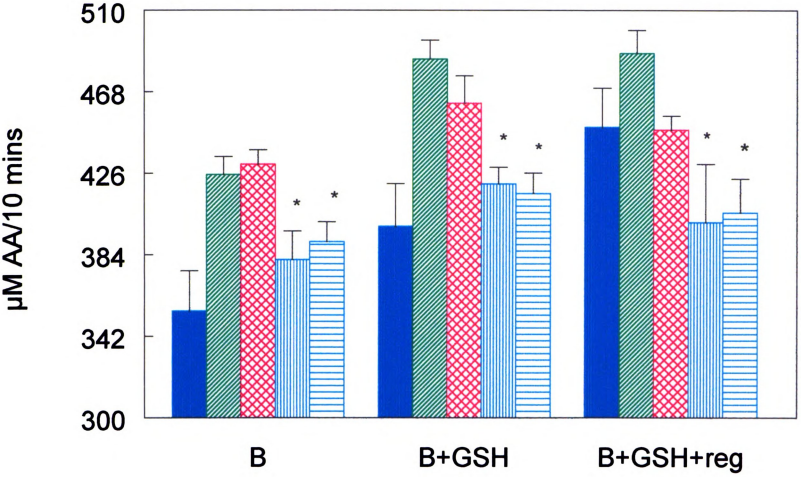
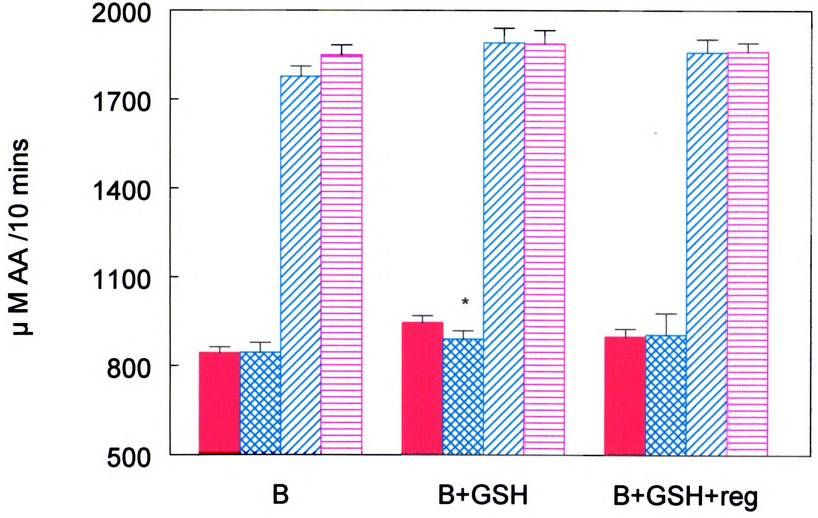


Fig 7: Effect of 5 mM GSO_3^- on 1 mM and 2 mM AA levels in the P4H reaction. All assays were of 10 minute duration. Results were obtained from 2 separate experiments done in duplicates. Assays contained 50 mM Tris-HCl, pH 7.4, 100 μM -(pro-gly-pro)-$_9$.H_2O, 5 μM ferrous sulfate, 5 μg P4H, 1.5 mg BSA, 0.07 mg catalase, 0.5 mM ascorbate, 0.1 mM α-KG, and 5 mM neutralized GSO_3^-. Reg. contained 0.1 mM NADPH and 10 μg GSSG reductase. B = Basal, reg. = GSH regeneration system. * = statistically significant difference from Basal + GSH $p < 0.05$. ■, Enz + 1 mM AA; ▩, Enz + 1 mM AA + 5 mM GSO_3^-; ▨, Enz + 2 mM AA; ▤, Enz + 2 mM AA + 5 mM GSO_3^-.

Figure 7



DISCUSSION: The role of PDI as the β subunit of P4H has long been under investigation, and is not yet defined. Reconstitution studies have suggested that it might act as a chaperone in preventing aggregation of the α subunit(13). Site directed mutagenesis studies showed that the disulfide isomerase activity of PDI was not required for P4H activity (14). Recently, published data indicate that, co-expression of the α subunit of P4H with ERp60, an isoform of PDI that has disulfide isomerase activity, does not form functional tetrameric enzyme (20). There is thus some speculation regarding the role of PDI in the P4H reaction. In this investigation, we tested the hypothesis that PDI's DHA reductase activity might be involved in recycling the cofactor, ascorbate. The results presented here support the suggestion that PDI's DHA reductase activity is involved in the P4H reaction.

Our studies with different ascorbate levels indicated that P4H activity was linear with ascorbate concentration and reached maximal activity by 1 mM ascorbate. Previous kinetic studies of P4H have shown the Km for ascorbate to be about 0.36 mM (21). In the presence of GSH, GSSG reductase and NADPH, P4H activity was stimulated at limiting ascorbate levels of 0.1-0.5 mM, but not at ascorbate concentrations of 1 mM and 2 mM. This may be due to increased availability of ascorbate in the presence of the regeneration system at limiting ascorbate levels, and more than sufficient ascorbate already present to carry on the reaction in the case of saturating levels. This view is supported by the ascorbate analysis data. A statistically significant increase in enzyme activity was observed at 0.5 mM ascorbate in the presence of 2 mM GSH and the regeneration system. This stimulation was consistent with ascorbate levels being maintained at 0.5 mM in the presence of P4H (enzymatic reaction) whereas in the

absence of P4H (chemical reaction) the ascorbate levels were significantly lower. Stimulation of P4H activity by dithiothreitol has been previously documented (22). This action was attributed to reduction of thiols by DTT at the enzyme active site. Based on our observation with GSH, it is also possible that DTT regenerated the ascorbic acid in the assay system, thereby resulting in increased availability of ascorbate to the enzyme and apparent increased enzyme activity.

In order to distinguish the chemical regeneration of ascorbate by GSH from the enzymatic regeneration by PDI, the β subunit of P4H, it was necessary to use an inhibitor for PDI's DHA reductase activity. We report here, for the first time that glutathione sulfonate is a competitive inhibitor of PDI's DHA reductase activity. In addition, GSO_3^-, inhibited the stimulation of P4H activity seen in the presence of GSH at 0.5 mM and 1 mM, but not 2 mM or saturating ascorbate concentrations. Ascorbate analyses of the assay samples at 0.5 mM ascorbate, indicated that statistically significant lowering of ascorbate levels was observed in the presence of GSO_3^- while in the absence of GSO_3^-, levels of AA were maintained at essentially 0.5 mM. These results are consistent with those expected if PDI's DHA reductase activity is involved in maintaining ascorbate in the assay system, and presumably, *in vivo*.

Previous studies with substrate analogs have shown that ascorbate acts as an "inner sphere" reductant and binds to a site in the enzyme that bears partial identity to the α-KG binding site (6), (23). These studies also suggested that the ascorbate binding site might be partially located in the β subunit of the enzyme. In our present investigation, basal P4H activity in the absence of GSH was inhibited by GSO_3^- at 0.5 mM ascorbate but not at 1 mM and 2 mM ascorbate. However, increasing α-KG and the peptide substrate

concentrations, did not block the effect of GSO_3^-, i.e., inhibition of basal P4H activity (data not shown). These results may be interpreted as specific interaction between GSO_3^- (GSH) and ascorbate, in the proximity between the ascorbate and GSH binding sites.

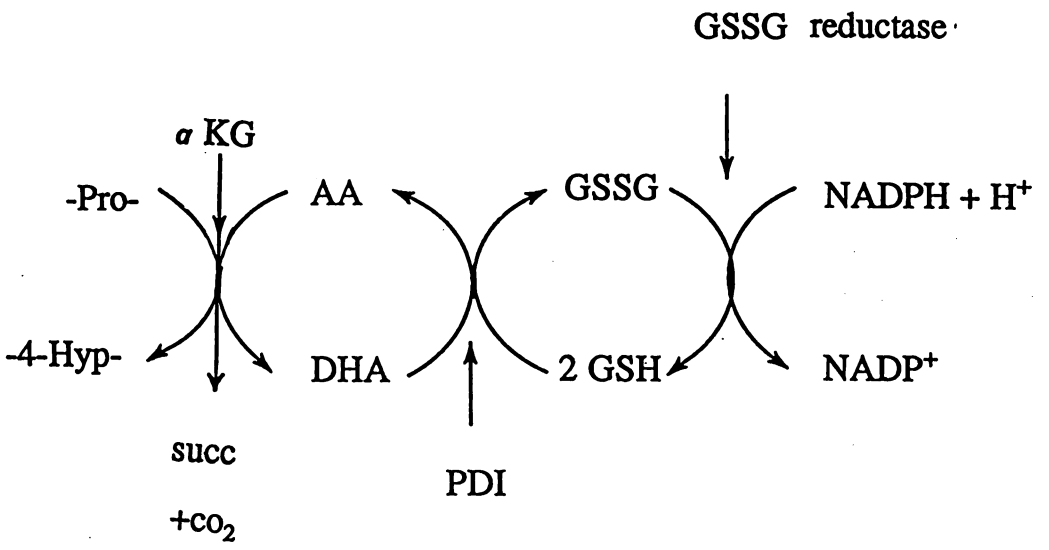
The catalytic mechanism of PDI's DHA reductase activity is not yet known. It is possible that cysteines in the active site that are responsible for the disulfide isomerase activity may also be responsible for the DHA reductase activity of PDI (12). Previous studies involving site directed mutagenesis of the active site cysteine residues of PDI have shown that PDI's disulfide isomerase activity is not necessary for the assembly or activity of P4H (14). The ascorbate levels used in this study was 2 mM. In our present investigation, neither the ascorbate regeneration system, nor GSO_3^- had any significant effect on enzyme activity when 2 mM ascorbate was used. However, at 0.5 mM ascorbate, significant stimulation in enzyme activity and significant decrease in activity was observed in the presence of the ascorbate regeneration system and GSO_3^-, respectively. As previously indicated, these increases and decreases in activity correlated well with increased and decreased ascorbate levels. These observations suggest that PDI's DHA reductase activity is essential for P4H activity when the ascorbate levels are limiting, i.e., physiological. Liver, heart and eye ascorbate levels in 14 day old chicken embryos have been estimated to be 3-11 mg /100 g tissue(0.17-0.62 mM) (24). It has been shown that ascorbate concentrations of 50 μg/gm tissue (0.3mM) is required for maximum collagen synthesis in granulomas in guinea pigs (25). Hence, 0.5 mM ascorbate is within the physiological range, for a variety of tissues.

A schematic representation of our proposed mechanism for the regeneration of ascorbate in the P4H reaction is presented in Figure 8. P4H is a member of a family of

dioxygenases, some other enzymes being lysyl hydroxylase and γ-butyrobetaine hydroxylase. These enzymes have similar reaction mechanisms and use ascorbate as a cofactor. It will be of interest to study the effect of PDI,thioltransferase or other DHA reductases (26) on these enzymes. Although dehydroascorbate reductases are not known to be subunits of these enzymes, they are likely candidates for recycling physiological levels of ascorbate in these reactions, within the appropriate cellular compartments.

Fig 8: Proposed mechanism for the regeneration of AA by PDI in the P4H reaction.
Hyp = hydroxyproline, AA = ascorbate, DHA = dehydroascorbate, GSH = glutathione, GSSG = glutathione disulfide, PDI = protein disulfide isomerase, succ = succinate

Figure 8


GSSG reductase
α KG
-Pro-
AA
GSSG
NADPH + H+
-4-Hyp-
DHA
2 GSH
NADP+
succ
+CO2
PDI

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Appendix: Directions for future research

Ascorbic acid has been shown to stimlate collagen synthesis in human fibroblasts (1). Chan et al have shown that stimulation of collagen synthesis by ascorbic acid is not at the transcriptional level nor is it due to decreased degradation of collagen but it may be post-translationally regulated. This may be through the activation of prolyl 4-hydroxylase (2). Human cells are unable to synthesize ascorbic acid since they lack the enzyme L-gulonolactone oxidase (3). It is thus important that these cells possess the ability to recycle ascorbic acid. Ascorbate has a very short half life of about 0.9 hours in culture and hence cultured cells are unintentionally grown under scorbutic conditions. It would thus be of interest to study the effect of ascorbic acid on collagen synthesis in normal, transformed and keloid (synthesizing excess collagen) human fibroblasts and the ability of these cells to recycle ascorbic acid.

As mentioned previously, there are now 4 known dehydroascorbate reductases that may be involved in maintaining the ascorbate levels in cells (4, 5). In addition to the levels and the relative activities of these enzymes, the ability of these cells to recycle ascorbic acid will depend on the levels of glutathione in the cell. Hence, it will be of interest to study the effect of lowered glutathione levels on collagen synthesis and ascorbic acid recycling in the above mentioned fibroblasts. Glutathione levels in cells can be lowered through the administration of buthionine sulfoximine or BSO, which is an inhibitor of γ- glutamyl cysteine synthetase, a key enzyme involved in glutathione synthesis (6). These studies will provide insight into the ability of these cells to recycle ascorbic acid in a glutathione dependant manner such as through thioltransferase and PDI.

The working hypothesis is that cells treated with BSO will show depressed collagen synthesis and depressed ascorbate levels in the presence of DHA in the medium,

but will show imcreased collagen synthesis and ascorbate levels in the presence of ascorabte. However cells that have not been subjected to BSO treatment will show increased collagen synthesis and increased cellular ascorbate irrespective of the presence of ascorbate or DHA in the medium.

It will also be interesting to study the effect of increased glutathione levels on collagen synthesis and ascorbate levels in fibroblasts. This may be achieved through the administration of cysteine delivery agents like N-acetyl cysteine and L-2-oxothiozolidine-4-carboxylate (7).

Important parameters to be measured in control and treated cells will be collagen synthesis and hydroxyproline formation, using the method of Philipps et al (8). Ascorbate levels can be mesured through C-18 reversed phase HPLC followed by electrochemical detection (9). GSH/GSSG levels and the relative levels of thioltransferase, PDI, prolyl 4- hydroxylase and their relative acitvities will serve as useful indices of ascorbic acid recycling by these fibroblasts. These studies will provide valuable information on the ability of transformed and keloid fibroblasts to recycle ascorbic acid when compared with normal fibroblasts and the implications of this in cancer or fibrotic disorders.

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