Dethiolation of protein mixed-disulfides

Che-Hun Jung
Iowa State University

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Dethiolation of protein mixed-disulfides

Jung, Che-Hun, Ph.D.

Iowa State University, 1994
Dethiolation of protein mixed-disulfides

by

Che-Hun Jung

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirement for the Degree of

DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:
Signature was redacted for privacy.

In Charge of Major Work
Signature was redacted for privacy.

For the Major Department
Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1994
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GENERAL INTRODUCTION

Oxidative Stress

Dioxygen (O\textsubscript{2}) serves as the eventual oxidant in aerobic respiration and is therefore essential for aerobic organisms. It also has been known for a long time that oxygen is toxic. It has been suggested that the oxygen toxicity is due to partially reduced oxygen species, such as the superoxide radical (O\textsuperscript{2-}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and the hydroxyl radical (OH\textsuperscript{-}), generated through incomplete reduction of O\textsubscript{2} or by the activity of various enzymes (1). The reactive oxygen species (ROS) have been shown to damage the cellular components, such as proteins, DNA, lipids, and carbohydrates (2). They are an inescapable consequence of aerobic life.

The reactive oxygen species are also utilized in normal cellular processes. For example, fertilized sea urchin eggs produce H\textsubscript{2}O\textsubscript{2} through the "respiratory burst of fertilization", and the produced H\textsubscript{2}O\textsubscript{2} is used by ovoperoxidase to harden the egg envelope by cross-linking (3). During phagocytosis, macrophages produces large amounts of the reactive oxygen species. This phagocytosis-associated oxidative metabolism is important for microbicidal activity of macrophages (4). Activated neutrophils, eosinophils, and monocytes also release the reactive oxygen species as a defense against pathogens (5). It is an inescapable consequence that the ROS released by those cells cause damage to the neighboring cells.

Since ROS are deleterious, cells have evolved mechanisms to scavenge the ROS and to repair damage (6). Various enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, are important for eliminating the reactive oxygen species. Vitamin C and E, and glutathione can also neutralize ROS directly. It has been shown in our laboratory that protein S-thiolation (i.e., formation of mixed-disulfides between proteins and cellular glutathione) is a primary response to the reactive oxygen species. Moreover, the mixed disulfides produced during oxidative stress are transient and readily reversed by reduction reactions. Since protein S-thiolation results in activity changes of numerous enzymes (7, 8), metabolic changes due to protein S-thiolation should be considered as a primary result of oxidative stress. Since protein sulfhydryls are important for
catalytic and structural integrity of numerous proteins, reversible protein S-thiolation and dethiolation may be an important cellular mechanism to protect the important protein thiols against oxidative stress.

Protein S-thiolation

Protein S-thiolation is the formation of protein mixed-disulfides with low molecular mass thiols. Although the low molecular mass thiols may be glutathione, cysteine, homocysteine, or others, most of the thiols found in protein mixed-disulfides in vivo has been identified as glutathione. Therefore, protein S-thiolation in vivo is mainly S-glutathiolation. Protein S-thiolation has been studied in various cells such as hepatocytes (9, 10), heart cells (11, 12), and human endothelial cells (13), utilizing various model oxidants such as t-butyl hydroperoxide, menadione, diquat, diamide, and hydrogen peroxide. Recently, stimulated murine macrophages and human neutrophils were also studied, in an effort to examine a biologically more relevant system for oxidative stress (14, 15). These studies, with various cells and oxidants, showed that each cell type contained a unique set of S-thiolated proteins. Therefore, the effect of oxidative stress may also be unique for each cell type.

Protein S-thiolation can occur by a thiol-disulfide exchange between protein thiols and low molecular mass disulfides such as glutathione disulfide in vitro. Many researchers consider that thiol-disulfide exchange reactions may be the major mechanism for the formation of S-thiolated proteins because oxidative stress causes formation of glutathione disulfide (16). In many cases, the stimulation of protein S-thiolation accompanies an increase of glutathione disulfide. However, thiol-disulfide exchange is not the only mechanism for protein S-thiolation. The following observations suggest a second mechanism of S-thiolation. First, the thiol-disulfide exchange reaction is relatively slow to explain the rapid S-thiolation in cells (17). To overcome this problem, it has been suggested that S-thiolation might be more rapid if catalyzed by an enzyme such as thioltransferase (18). However, not a single substrate protein for this reaction has been described. Second, the increase in glutathione disulfide is not a prerequisite for S-thiolation, and S-thiolation is observed even under the conditions in which a significant amount of glutathione disulfide is not formed (15). Therefore, the formation of
glutathione disulfide should be considered as an unavoidable side reaction of oxidative stress, and a reaction which may not be directly tied to S-thiolation.

Protein S-thiolation via oxyradical-initiated intermediates has been proposed to explain rapid S-thiolation (19, 20). This mechanism suggests that protein thiol radicals and/or sulfenic acids are generated by ROS. Those "activated protein thiols", in turn, react with the reduced glutathione (GSH) directly to result in the formation of mixed disulfides. In this case, the formation of glutathione disulfide is not required for S-thiolation. A thiol radical of hemoglobin was found in erythrocytes from rats fed with hydroperoxides (21), indicating that protein thiol radicals were present in intact cells.

Several S-thiolated proteins has been identified from various cells. In rat hepatocytes, the 30 kDa protein is the most extensively S-thiolated protein by l-butyl hydroperoxide and diamide. It was identified as carbonic anhydrase III (22). The most extensively S-thiolated in PMA (phorbol 12-myristate 13-acetate)-stimulated neutrophils, a 42 kDa protein, was identified as actin (15). Recently, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was identified as a major S-thiolated protein in human endothelial cells (23) and monocytes (24). Since each cell type contains a unique set of S-thiolated proteins, the effect of oxidative stress may also be unique for each cell type. Therefore, identification of the S-thiolated proteins in various cells may be an essential step in understanding the effect of oxidative stress. Unfortunately, the complexity of protein S-thiolation has not been appreciated, partly because few S-thiolated proteins have been identified.

Carbonic anhydrase III (CA III) is the major carbonic anhydrase isozyme in the male rat liver and soleus muscle (25). A crystal structure of bovine muscle CA III has been solved at high resolution (26), and human muscle CA III has been cloned and well characterized (27). According to those studies, CA III is the least active form of carbonic anhydrase, showing only 0.3% of the activity of carbonic anhydrase II, the major isoform in erythrocytes. Rat liver CA III has been purified in our laboratory, and the enzymatic activity compared with carbonic anhydrases from other sources. Rat liver CA III showed unexpectedly high activity (100 times more active than bovine and human muscle CA III), and the catalytic properties
were quite different from other CA III's. Recently, the partial amino acid sequence of rat liver carbonic anhydrase III was deduced from the nucleotide sequence of cloned cDNA. The amino acid sequence of rat liver CA III was almost identical to that of rat muscle CA III. One interesting feature of the partial sequence was that rat liver CA III contained the conserved phenylalanine residue that has been associated with the lower activity of bovine and human muscle CA III. With the human enzyme, the CA III activity increased when the phenylalanine was replaced with smaller amino acids (28-30). A recent X-ray crystallography study showed that the active site structures of bovine muscle and rat liver CA III were superimposed on each other. Therefore, the structural basis for the lower activity of human and bovine muscle CA III, and for the higher activity of rat liver CA III must be addressed. In order to determine the complete sequence of rat liver CA III, the cloning of the CA III cDNA is currently under way.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been identified as a major S-thiolated protein in hydrogen peroxide-treated monocytes, endothelial cells, and lung carcinoma cells. When the cells containing $^{35}$S-labeled glutathione were treated with hydrogen peroxide, GAPDH was labeled with the radioactive glutathione and inactivation of the enzyme activity was directly correlated with the S-thiolation. Diamide, which caused S-thiolation of the same set of hepatocyte proteins as t-butyl hydroperoxide, did not S-thiolated human endothelial GAPDH (23). Since diamide caused extensive oxidation of cellular glutathione and the same concentration of diamide did not stimulate S-thiolation of GAPDH, thiol-disulfide exchange could not be the mechanism of GAPDH S-thiolation. Oxyradical-initiated S-thiolation was suggested. Since pure GAPDH was also inactivated by hydrogen peroxide alone, intramolecular disulfide bond formation or sulfenic acid formation instead of S-thiolation have been suggested as an inactivation mechanism. Overall, it will be very interesting to examine the structural basis of GAPDH inactivation by S-thiolation, including the competition between glutathione and a substrate, NADH, because the S-thiolation site overlaps with the NADH-binding site.

Recently, it has been suggested that transcription factors such as Fos/Jun, NF-kB, and
OxyR are regulated by oxidation/reduction of sulfhydryls (31, 32). Oxidation of sulfhydryl(s) by hydrogen peroxide activates the transcription factor, OxyR, and the activation is reversed by a reducing agent, dithiothreitol. Storz et al. suggested that OxyR may be regulated by sulfenic acid formation (32), but no experimental evidence was presented to support sulfenic acid formation. They reported that OxyR is regulated by oxidation/reduction even after changing 5 out of 6 cysteines to serines. Therefore, it is still possible that S-thiolation of the remaining sulfhydryl activates OxyR. The suggestion that Fos/Jun and NF-kB are regulated by oxidation/reduction is based on the OxyR experiment, and the molecular nature of the oxidation/reduction has not been adequately addressed.

It has been reported that many enzymes, such as glutamine synthetase, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, and pyruvate kinase are inactivated by mixed-function oxidation systems (33). The oxidative inactivation accompanies the conversion of some amino acid side chains to carbonyl derivatives (34) and the carbonyls are accumulated in cells during ageing (35, 36). The inactivation of E. coli glutamine synthetase also correlates with modification of a single histidine residue in each enzyme subunit and makes the enzyme susceptible to proteolytic degradation (37).

Recently, it was shown in our lab that oxyradicals oxidized carbonic anhydrase III irreversibly in the absence of glutathione. Protein S-thiolation occurred in the presence of glutathione (GSH) and the oxidation was reversed by reduction. Therefore, one possible function of protein S-thiolation may be the protection of functionally important protein sulfhydryls during oxidative stress through reversible S-thiolation/dethiolation of the potentially damaged sulfhydryls.

Gilbert (16) and Ziegler (7) suggested that biological disulfides such as glutathione disulfide (GSSG) might serve as messengers in metabolic regulation and that reversible S-thiolation/dethiolation of various enzymes may regulate metabolic pathways in cells. Although protein S-thiolation will certainly result in dramatic changes in cellular metabolism due to inactivation or activation of many enzymes, it is still controversial whether the intracellular redox state of cellular glutathione is significantly perturbed in response to normal...
physiological signals. The inflammatory response of macrophages and neutrophils may be an example of this perturbation. Chai et al. (9, 15) showed that some protein in hepatocytes were significantly S-thiolated under the culture conditions, even without adding exogenous oxidants. Therefore, some proteins may be more susceptible to the changes in the GSH/GSSG redox potential and the regulation of those proteins may be very important. S-thiolation of carbonic anhydrase III and phosphorylase b did not change the enzyme activities, although glyceraldehyde-3-phosphate dehydrogenase and creatine kinase were inactivated by S-thiolation. Therefore, not all the S-thiolation is "regulatory" and the function of protein S-thiolation may be to scavenge oxyradicals in some cases.

**Dethiolation**

Dethiolation is the reduction of protein mixed-disulfides. As discussed above, this reaction has received attention for some time as a means of regulating enzyme activities.

When hepatocytes and heart cells were exposed to t-butyl hydroperoxide, S-thiolation of proteins was quite transient, that is, the maximum S-thiolation occurred within 5 min and the extent of S-thiolation decreased rapidly thereafter. Since approximately 50 nmol/mg protein of total soluble protein thiols were estimated to be available for S-thiolation, the molar concentration of the thiols was approximately 10 mM in cells. S-thiolation in 0.5 mM t-butyl hydroperoxide-treated hepatocytes was less than 5 nmol/mg protein, less than 10% of the total available protein thiols. Rapid dethiolation in these cells and the limited amount of S-thiolation strongly suggested that the mechanisms for dethiolation are of major importance in the metabolism of S-thiolated proteins in intact cells.

Dethiolation can occur non-enzymatically by thiol-disulfide exchange reaction between S-thiolated proteins and reduced glutathione (GSH). This mechanism is supported by the fact that most cells contain high concentrations (1 to 10 mM) of glutathione. However, thiol-disulfide exchange may not be the only important mechanism for dethiolation, because the thiol-disulfide exchange reaction alone is relatively slow to explain the rapid dethiolation in cells (22).

Partially purified thioredoxin-like and glutaredoxin-like activities from various tissues, and
*E. coli* thioredoxin, greatly enhance the rate of dethiolation (38). The effective dethiolation by *E. coli* thioredoxin, the thioredoxin-like and glutaredoxin-like activities, and relatively slow dethiolation by reduced glutathione (GSH) alone, suggest that "enzyme"-catalyzed dethiolation may be a major mechanism for dethiolation in cells.

**Thioredoxin**

Thioredoxin is a small protein with the molecular mass of 12 kDa originally isolated from *E. coli* as a hydrogen donor for ribonucleotide reduction (39). *E. coli* thioredoxin contains two cysteines at the active site with the conserved amino acid sequence, -Trp-Cys-Gly-Pro-Cys- (40). The "spatially close" cysteine thiols undergo oxidation/reduction cycles during the reduction of ribonucleoside 5'-diphosphate to deoxyribonucleoside 5'-diphosphate. The disulfide in the oxidized form of thioredoxin [Trx-S\(^2\)] is reduced to a dithiol [Trx-(SH)\(_2\)] by NADPH in a reaction catalyzed by thioredoxin reductase (41). The reduced form of thioredoxin [Trx-(SH)\(_2\)] is then oxidized in a reaction by ribonucleotide reductase.

In addition to deoxyribonucleotide synthesis, thioredoxin has been implicated in a wide variety of reactions, including methionine sulfoxide reduction and sulfate reduction (42-45). Trx-(SH)\(_2\) is also an essential subunit of phage T7 DNA polymerase in the virus-infected cells (46) and it is required for the assembly of filamentous phages (47). *In vitro* the dithiol form of thioredoxin has been known as a protein disulfide reductant. The protein disulfides reduced by thioredoxin include apocytochrome c, chymotrypsin, trypsin, insulin, proinsulin, fibrinogen, choriogonadotropin, and α-macroglobulin (48-51). The reduction of insulin and DTNB is often used in the assay of thioredoxin activity (39, 52, 53).

Thioredoxin is a ubiquitous protein also found in yeast, plants, and animal cells. The genes encoding two different thioredoxins were cloned from yeast, *Saccharomyces cerevisiae* (54, 55) and the thioredoxins showed about 80% amino acid sequence identity. TRX2 gene, one of the yeast thioredoxin genes, was later identified essential for YAP1 transcription factor-dependent resistance to oxidative stress (56). Overall, the molecular nature and the cellular functions of yeast thioredoxins have not been extensively studied. Thioredoxins have been also studied in plants. A unique feature of the plant thioredoxin system is that plant...
thioredoxin is reduced by ferredoxin-thioredoxin reductase instead of NADPH-thioredoxin reductase (57). It has been suggested that the plant thioredoxin system mediates photoactivation of various enzymes such as fructose 1,6-bisphosphatase, NADP-glyceraldehyde 3-phosphate dehydrogenase, and NADP-malate dehydrogenase (58), and that thioredoxin is essential for photosynthetic growth (59).

Thioredoxins have been characterized from various animal sources such as bovine liver (52), rat liver (60), rabbit bone marrow (61), and human lymphocytes (62). The amino acid sequence of thioredoxin from rabbit bone marrow was determined by tandem mass spectroscopy (63), and that of human lymphoid cells and chicken embryo fibroblasts were derived from cloned cDNA sequences (62, 64). The animal thioredoxins have the same active site sequence, -Trp-Cys-Gly-Pro-Cys-, as *E. coli* thioredoxin, and also contain 3 additional cysteines apart from the active site. Two of the cysteines, Cys 68 and Cys 72, form an additional "vicinal" cysteine pair with the conserved sequence, -Cys-Glu-Val-Lys-Cys-. Those cysteines are apparently oxidized to disulfides during storage, resulting in aggregation and inactivation of the protein (65). The mutation of Cys 72 to serine prevented from both aggregation of human thioredoxin and inactivation by DTNB (66). The functional importance of the Cys 72 has also been described by another report, which suggests that thioredoxin is involved in the "early pregnancy factor (EPF)" system (67). The EPF system is assayed by a lymphocyte-modifying activity in pregnancy sera, that inhibits rosette formation. The mutation of cysteine 72 to serine completely eliminates the thioredoxin activity in the EPF assay, and mutation of the active site cysteines to serines does not have any effect (67). This indicates that the "extra cysteines" are also biologically important.

The mammalian thioredoxins have a variety of activities. Thioredoxins from rat liver and calf thymus facilitate reduction of insulin disulfides (60, 68). It also has been suggested that thioredoxin serves as glucocorticoid receptor-activating factor (69). Recently, It has also been showed that overexpression of thioredoxin inhibits the transcription factor, NF-kB, and activates another transcription factor, AP-1 (70), implicating that thioredoxin may be involved in regulation of those transcription factors.
It has been shown that the adult T cell leukemia-derived factor (ADF) which induces interleukin 2 receptors on human B lymphocytes is thioredoxin (71). This suggests that thioredoxin acts as a interleukin 1 species (72) and as an autocrine growth factor (73). Thioredoxin may act as an interleukin species through the protein disulfide reductase activity. Rubartelli et al. pointed out that thioredoxin is a protein secreted from fibroblasts, airway epithelial cells, and activated B and T cells without a signal sequence, similar to the secretion of interleukin 1β (74). Rozell et al. showed that rat thioredoxin was localized in the cytoplasm of cells, and was enriched at secretory granules in both endocrine and exocrine cells (75). This localization of thioredoxin in cells may be related with the secretion of thioredoxin from those cells. Recently, induction of human thioredoxin in glial cells was reported after transient global ischemia, suggesting a role of thioredoxin in neuroprotection during ischemic injury (76).

Although thioredoxin was first isolated as a hydrogen donor for ribonucleotide reductase, it had a variety of activities including reduction of protein disulfides, action as an interleukin species, and activation of glucocorticoid receptor. The list of the functions of this versatile protein is long and further investigation is required to reveal its cellular functions.

**Glutaredoxin**

Glutaredoxin is also a small protein (10 kDa) first isolated as a hydrogen donor for ribonucleotide reductase from a mutant of *E.coli* lacking thioredoxin activity (77). *E. coli* glutaredoxin contains two cysteines at the active site with the amino acid sequence, -Cys-Pro-Tyr-Cys- (78, 79), and the cysteines undergo oxidation/reduction cycle, similar to that of thioredoxin. Glutaredoxin is distinguished from thioredoxin is by the reduction of the oxidized form of glutaredoxin (Grx-S$_2^+$) with glutathione (GSH) (80, 81). Glutaredoxin is less abundant in *E. coli* than thioredoxin, and it has a lower K$_m$ for ribonucleotide reductase. Glutaredoxin is also involved in the reduction of disulfides and sulfate (81, 82), but it is not able to substitute for thioredoxin in methionine sulfoxide reduction (83). A null mutation of *E. coli*, lacking both thioredoxin and glutaredoxin, suggests that thioredoxin and glutaredoxin may not be essential for ribonucleotide reduction or for sulfate reduction, and that a new GSH-dependent
hydrogen donor for ribonucleotide reductase may exist in *E. coli* (84, 85).

Glutaredoxin has also been called thioltransferase. This name was first introduced to describe an enzyme activity which enhances the reduction of homocysteine, glutathione-coenzyme A mixed-disulfide, and cysteine-glutathione mixed-disulfide by glutathione (86). Mannervik and Axelsson introduced the term, suggesting that it catalyzed thiol-disulfide interchange (87). It was suggested that thioltransferase increased both the formation (S-thiolation) and reduction (dethiolation) of protein mixed-disulfides (88). It should be pointed out that, as discussed above in protein S-thiolation, not a single substrate protein has been found to be S-thiolated by this enzymatic mechanism.

Glutaredoxin has also been characterized from calf thymus (89), pig liver (90), rabbit bone marrow (91), and human erythrocytes (92). The mammalian glutaredoxins have disulfide reductase activities and the activity assay often uses the reduction of disulfides such as S-sulfocysteine and hydroxyethyl disulfide (89). Gravina and Mieyal reported that glutaredoxin of from human red blood cells significantly enhanced the dethiolation rate of S-glutathiolated hemoglobin (93).

Although the name "glutaredoxin" was suggested for a protein that acted as a hydrogen donor for ribonucleotide reductase, glutaredoxin from rabbit bone marrows was not active in the homologous ribonucleotide reductase assay in the presence of GSH, glutathione reductase, and NADPH (94). Pig liver glutaredoxin was also inactive in the rabbit and *E. coli* ribonucleotide reductase system, whereas calf thymus glutaredoxin was active (86, 95). Therefore, the role of mammalian glutaredoxins in ribonucleotide reduction remains for further investigation.

The mammalian glutaredoxins also contain an additional cysteine pair in the conserved sequence, -Cys-Ile-Gly-Gly-Cys-, although the function of those conserved cysteines is unclear. Unlike the variety of functions of thioredoxin, there have only a few reports of additional functions for glutaredoxin. The mammalian glutaredoxins show the dehydroascorbate reductase (96), and glutaredoxin-like protein in rat liver and kidney promotes iodothyronine $5^\prime$-deiodinase activities (97).
Protein disulfide isomerase

Protein disulfide isomerase (PDI) is an abundant protein present in the lumen of the endoplasmic reticulum (98). It is a homodimeric protein with a subunit molecular mass of 57 kDa. PDI has been so named because it catalyzes disulfide formation in secretory proteins and isomerization of intramolecular disulfide bonds. PDI has two distinct but identical regions similar to the active site of thioredoxin, containing the conserved amino acid sequence, -Cys-His-Gly-Cys- (99). Therefore, PDI is a member of the protein family which have two "spatially close" cysteines at the active site.

Protein disulfide isomerase was first isolated from rat liver microsomes as an enzyme catalyzing the conversion of inactive, reduced bovine pancreatic ribonuclease to the active form (100). It was shown later that the same enzyme preparation also activated randomly oxidized ("scrambled") ribonuclease (101).

Protein disulfide isomerase (PDI) is a multifunctional protein. It serves as the β-subunit of prolyl hydroxylase which catalyzes hydroxylation of proline residues within α-chains of procollagen (102, 103). It has been shown that PDI has dehydroascorbate reductase activity (96). Since ascorbic acid is a cofactor in the prolyl hydroxylase reaction, PDI may function to regenerate ascorbic acid.

An enzyme which reacted with antibodies against guinea pig uterus phosphoinositide-specific phospholipase C (PI-PLC) was cloned from a rat cDNA library (104). The derived amino acid sequence showed that it was not the same as rat liver PDI, but it had two separate regions containing a cysteine pair with the sequence, -Cys-Gly-His-Cys-. It is not clear at this point whether the cloned protein has the PDI activity, and what the function of the PI-PLC isoenzyme is.

Other proteins

The sequence (-Cys-X-X-Cys-) common to the redox active dithiols in thioredoxin, glutaredoxin, and protein disulfide isomerase has been found in many other proteins such as the β-subunit of ovine follitropin and bovine lutropin (105), and DsbA (106). DsbA is a periplasmic protein in E. coli with a molecular mass of 21 kDa. It has been suggested that the
function of DsbA is a bacterial protein disulfide isomerase, and that it is involved in the formation of protein disulfides. Bacteriophage T4 also contains a unique protein which contains both thioredoxin and glutaredoxin properties, with the active site sequence -Cys-Val-Tyr-Cys- (107). That is, it can be reduced by either thioredoxin reductase and NADPH or GSH alone (108).

Although the number of proteins containing "spatially vicinal" dithiols is increasing and they often show the disulfide reducing activity, they have generally been considered as nonspecific reductants. The specificity with protein disulfides have been little studied. In the first part of this dissertation, the relative specificities of glutaredoxin, thioredoxin, and protein disulfide isomerase for dethiolation of protein mixed-disulfides are compared. Glutaredoxin is the most effective among those tested. The structural basis of the dethiolation by glutaredoxin is discussed. The second part of this dissertation will present evidence for noncovalent glutathione-binding to S-thiolated proteins during dethiolation. The implication of glutathione-binding to protein will also be discussed.

**Dissertation Organization**

This dissertation contains two papers that will be submitted for publication with minor modifications to meet the journal's requirements. Following the papers, a general conclusion of this dissertation is presented. References cited in the general introduction follow the general conclusion.
DETHIOLATION OF $^{35}$S-GLUTATHIOLATED HEPATOCYTE PROTEINS BY GLUTAREDOXIN, THIOREDOXIN, AND PROTEIN DISULFIDE ISOMERASE

A paper to be submitted to the Journal of Biological Chemistry

Che-Hun Jung and James A. Thomas

ABSTRACT

The activity of glutaredoxin, thioredoxin, protein disulfide isomerase as dethiolases (i.e., enzymes catalyzing reduction of protein mixed-disulfides) was studied utilizing a mixture of hepatocyte $^{35}$S-glutathiolated proteins. The $^{35}$S-glutathiolated protein mixture was prepared from $^{35}$S-labeled rat hepatocytes, by stimulating S-glutathiolation with diamide. Dethiolation (i.e., reduction of protein mixed-disulfides) of individual $^{35}$S-labeled protein bands was analyzed by combining SDS-PAGE and autoradiography. This mixture had no contaminating glutaredoxin- or thioredoxin-like activity and it was suitable for studying the effects of the proteins as dethiolating agents.

All of the protein bands without exception could be completely dethiolated by pure glutaredoxin, thioredoxin, or protein disulfide isomerase. Glutaredoxin was the most effective of the dethiolases. For example, the 30 kDa protein, which has been identified as carbonic anhydrase III, was dethiolated 300 times faster by glutaredoxin than thioredoxin, and 200 times faster than protein disulfide isomerase. Dethiolation rates of individual hepatocyte proteins varied in minor ways from protein to protein. For example, the 15, 30, and 48 kDa proteins were quickly dethiolated by glutaredoxin, and the 27, 28, and 77 kDa proteins were dethiolated more slowly. The specificities of glutaredoxins from pig liver and bovine heart were compared, and were similar to each other. Thioredoxin from human and E. coli also have a similar reactivity and specificity.

Insulin reduction by glutaredoxin, thioredoxin, and protein disulfide isomerase was also compared. On the contrary to protein disulfide isomerase and thioredoxin, glutaredoxin did
not facilitate insulin reduction, suggesting that glutaredoxin might be specific for S-
glutathiolated proteins. Protein disulfide isomerase was more effective than thioredoxin for
insulin reduction. The standard redox potential of pig liver glutaredoxin was found to be
- 0.159 ± 0.004 V, indicating that glutaredoxin was thermodynamically a weaker reductant
than *E. coli* thioredoxin (redox potential of - 0.260 V). The experiments described in this
report strongly suggest that glutaredoxin is the major dethiolase for S-glutathiolated proteins
in cells. Glutathione-binding at the active site of glutaredoxin is suggested as a structural
factor for the effective dethiolation.

**INTRODUCTION**

An increase in the S-thiolation state of many proteins occurs rapidly in cells under
oxidative stress (1-6). Since the dethiolation process counteracts this increase, it is important
to understand its mechanism and the proteins responsible for dethiolation. All of the S-
thiolated proteins in hepatocytes and heart cells were quickly dethiolated in intact cells and the
dethiolation rates were somewhat different from protein to protein (1, 5, 7). For example, a
30 kDa protein band was readily dethiolated while a 45 kDa protein band remained S-
thiolated in t-butyl hydroperoxide-treated hepatocytes (7).

Enzyme-catalyzed dethiolation has been studied (8-11), and partially purified thioredoxin
and glutaredoxin from cardiac tissue and pure *E. coli* thioredoxin facilitated the reduction of
protein mixed-disulfides (8-11).

Thioredoxin, originally thought to function as a hydrogen donor for ribonucleotide
reduction, has also been known to cleave protein disulfide bonds (12-15). In fact, the assay of
thioredoxin activity often utilizes the reduction of insulin (16, 17). Glutaredoxin, also known
as thioltransferase, has been reported to reduce protein disulfides (18), and commonly used
activity assays are based on the reduction of various disulfides such as S-sulfocysteine and
hydroxyethyl disulfide (19-21). Protein disulfide isomerase, a major protein in the endoplasmic
reticulum, can also facilitate the reduction of insulin disulfide (22, 23), and many other
proteins (24). These three proteins have an active site composed of "spatially vicinal" dithiols
separated by two amino acids. The dithiol proteins have been considered to be nonspecific reductants and their activities have not been compared in direct experiments involving specific reactions.

In this report, we compared the relative reactivities of glutaredoxin, thioredoxin, and protein disulfide isomerase as dethiolases of protein mixed-disulfides. The dethiolation rates of the mixture and of individual proteins were studied. Dethiolation rates differed somewhat from protein to protein and glutaredoxin was clearly the most effective on a molar basis among those tested.

MATERIALS AND METHODS

Human recombinant thioredoxin was a generous gift from Dr. Angela M. Gronenborn at National Institute of Diabetes and Digestive and Kidney Diseases, NIH, and pig liver glutaredoxin was from Dr. Wells at Michigan State University. E. coli thioredoxin was purchased from Calbiochem (La Jolla, CA). Glutathione, glutathione disulfide, dithiothreitol, bovine serum albumin, collagen, N-ethylmaleimide, galactose, and dexamethasone were from Sigma chemical Co. (St. Louis, MO). Insulin (bovine), transferrin (human), penicillin G, streptomycin sulfate, Leibovitz's L-15 medium with L-glutamine were from Gibco BRL (Grand Island, NY). Collagenase was from Worthington biochemical Corp. (Freehold, NJ). SDS-PAGE molecular mass standards were from Bio-Rad laboratories (Richmond, CA). Tran$^{35}$S-label was from ICN biochemical, Inc. (Irvine, CA). Precasted 5-20 % gradient SDS-PAGE gels were purchased from Jule Inc. (New Haven, CT).

Preparation of $^{35}$S-glutathiolated Hepatocyte Proteins

Sprague-Dawley male rats (200-250 g) (Sasco Co., Omaha, NE) were used to prepare hepatocytes by collagenase digestion, as described by Bonney et al. (25). Cell viability was greater than 90% by trypan blue exclusion. The isolated hepatocytes were diluted to 0.5x10$^6$ cells/ml in Leibovitz's L-15 medium, pH 7.6, supplemented with 18 mM Hepes, 0.2% bovine serum albumin, 5 μg/ml insulin/transferrin, 1 μM dexamethasone, 5 mg/ml galactose, 50 Units/ml penicillin G, and 50 μg/ml streptomycin sulfate and plated on collagen-coated dishes.
The hepatocytes were incubated at 37 °C for 48 hours before [35S]-labeling.

Cells were incubated in L-15 medium containing 15 μg/ml cycloheximide for 30 minutes prior to labeling. Cycloheximide prevented protein synthesis during 35S-labeling. Cycloheximide-treated cells were then incubated for 4 hours with serum-free CMRL-1415 medium containing 40 μM methionine, 10 μM cystine, and 15 μg/ml cycloheximide with 3x10⁹ dpm/μmole. The low concentration of methionine and cystine in CMRL-1415 medium was convenient for labeling the cells with a higher specific activity. The specific activity of glutathione in cells was analyzed by HPLC as described by Fariss and Reed (26) and modified by Chai et al. (5). The acid-soluble materials in 35S-labeled hepatocytes were separated by a HPLC column and fractions were counted by liquid scintillation counter.

35S-labeled hepatocytes were treated with 2 mM diamide for 5 minutes to S-thiolate proteins. After incubation, the medium was removed and cells were rinsed twice with cold phosphate buffered saline. Cells were lysed and soluble proteins were extracted within a minute by scraping cells in 20 mM Hepes pH 7.4 buffer containing 5 mM EDTA, 5 mM EGTA, and 50 mM N-ethylmaleimide. N-ethylmaleimide reacted with all reactive free sulfhydryls in the extract. The extract was centrifuged at 10,000 x g for 30 minutes to obtain a particulate-free cell extract. The supernatant was dialyzed to remove low molecular mass radioactivity using 12 kDa molecular mass cut-off dialysis tubing. Glutaredoxin and thioredoxin activity in the extract were measured as described by Thomas et al. (27), neither glutaredoxin nor thioredoxin activity was detected in the extract.

Assay of dethiolation

Glutaredoxin and protein disulfide isomerase assay Pig liver glutaredoxin, bovine heart glutaredoxin, and bovine liver protein disulfide isomerase were preincubated at 30 °C with glutathione in 20 mM β-glycerophosphate buffer, pH 7. The dethiolation reactions were started by adding the 35S-thiolated hepatocyte proteins (final concentration, 1 mg/ml). The final concentration of glutathione was 0.3 mM. After an appropriate incubation time, proteins were precipitated by adding ice-cold bovine serum albumin (final concentration, 6 mg/ml) and trichloroacetic acid (final concentration, 10 %) in turn. The TCA-soluble radioactivity in the
supernatant after centrifugation, released by dethiolation, was counted by liquid scintillation.

**Thioredoxin assay** Human and E. coli thioredoxin were preincubated at 30 °C with NADPH and purified bovine heart thioredoxin reductase (see the next section) in 20 mM β-glycerophosphate buffer, pH 7. The final concentration of NADPH was 0.4 mM and that of thioredoxin reductase was 0.3 mg/ml. The extent of dethiolation was measured as described above for glutaredoxin and protein disulfide isomerase.

**Gel analysis of the dethiolation of individual protein bands** Dethiolation of individual protein bands was assessed by combining SDS-PAGE and autoradiography. Reactions were stopped by adding an equal volume of 0.125 M Tris-Cl buffer, pH 6.8 containing 4 % sodium dodecyl sulfate, 20 % glycerol, 0.008 % bromophenol blue, and 80 mM N-ethylmaleimide. N-ethylmaleimide stopped the reaction and blocked free sulfhydryls. Proteins were separated by 5-20 % gradient SDS-PAGE and the radioactivity associated with individual bands was quantitated by autoradiography and densitometry.

**Purification of Thioredoxin Reductase from Bovine Heart**

Thioredoxin reductase activity was measured during purification by a coupled reaction in which thioredoxin reductase reduces E. coli thioredoxin and the reduced thioredoxin in turn reduces the mixed-disulfide of S-[³H]glutathiolated phosphorylase b (27). The mixture of 0.5 mM NADPH, 2 μM E. coli thioredoxin, 6 mg/ml bovine serum albumin, and 0.3 μM S-[³H] glutathiolated phosphorylase b in 20 mM β-glycerophosphate buffer, pH 7, was incubated with a fraction containing thioredoxin reductase at 30 °C for 10 min. The protein-associated radioactivity was then precipitated by 10 % trichloroacetic acid (TCA) and TCA-soluble radioactivity in the supernatant was determined. One unit of thioredoxin activity corresponded to the release of 1 μmol of [³H]GSH/min. The thioredoxin reductase activity was linearly proportional up to 2 μunits of thioredoxin reductase, under the assay condition.

Thioredoxin reductase was purified from bovine heart as described by Luthman and Holmgren (17) with slight modifications. Heart tissue was homogenized in a Waring Blender with 3 volumes of 20 mM Tris-Cl buffer, pH 7.4. The homogenate was centrifuged at 100,000 x g for 1 hour at 4 °C. The supernatant was filtered through glass wool, and then applied to a
DEAE-Trisacryl column pre-equilibrated with 20 mM Tris-Cl buffer, pH 7.4. Thioredoxin reductase was eluted with 0-0.3 M NaCl gradient in the same buffer, and the active fractions were combined and concentrated in an Amicon concentrator with a YM 10 membrane. The concentrated fraction was applied to a Sephadex G-150 column pre-equilibrated with 20 mM β-glycerophosphate buffer, pH 7.0, and the active fractions were concentrated in an Amicon concentrator. The concentrated fractions were applied to a Cibacron Blue agarose column pre-equilibrated with 20 mM Tris-Cl buffer, pH 8.0. Thioredoxin reductase was eluted with 0-1 M KCl gradient. The purified thioredoxin reductase migrated as a single major band on SDS-PAGE with a molecular mass of 35 kDa. It has been reported that thioredoxin reductase from E. coli and other prokaryotes is a dimeric protein with the subunit molecular mass of 35 kDa (28, 29). It has been reported that thioredoxin reductase from human liver (30), bovine liver (31), and cultured HeLa cells (32) have a subunit molecular mass of 58 kDa. Therefore, the molecular mass (35 kDa) of purified thioredoxin reductase suggests that bovine heart thioredoxin reductase is more similar to prokaryotic isoenzymes. The specific activity of the purified thioredoxin reductase was 0.3 mU/mg protein. The protein concentration was determined, as described by Lowry et al. (33), using bovine serum albumin as standard.

**Purification of Bovine Heart Glutaredoxin**

Glutaredoxin activity was determined during purification, as described by Thomas et al. (27). The mixture of 0.3 mM glutathione, 6 mg/ml bovine serum albumin, and 0.3 μM S-[3H]glutathiolated phosphorylase b in 20 mM β-glycerophosphate buffer, pH 7, was incubated with a fraction containing glutaredoxin at 30 °C for 5 min. Ten percent trichloroacetic acid (TCA) was added to precipitate protein-associated radioactivity and TCA-soluble radioactivity was determined. One unit of the glutaredoxin activity corresponded to the release of 1 μmol of [3H]GSH/min under these assay condition.

Bovine heart was homogenized in a Waring Blender with 5 volumes of 50 mM Tris-Cl buffer, pH 7.8 containing 5 mM EDTA and 0.2 mM PMSF. The homogenate was centrifuged at 10,000 x g for 1 hour at 4 °C, and the supernatant was filtered through glass wool. The filtered supernatant was precipitated with 70 % ammonium sulfate, and the pellet was
resuspended in a minimal volume of 20 mM sodium phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer using dialysis tubing with 3500 molecular mass cutoff. After centrifugation to remove any insoluble material, the supernatant was applied to a Sephadex G-75 column pre-equilibrated with 20 mM sodium phosphate buffer, pH 7.0 containing 100 μM dithiothreitol. The active fractions were combined and concentrated in a Amicon concentrator. The pH of the pooled fraction was adjusted to 5.1 by adding 1 M acetic acid dropwise, and it was applied to a CM-Sepharose column pre-equilibrated with 10 mM sodium acetate buffer, pH 5.1. Glutaredoxin was then eluted with a 0-0.1 M NaCl gradient in the same buffer. The active fractions were combined and concentrated in an Amicon concentrator with a YM 3 membrane. The concentrated fraction was focused for 4 hours in a Rotofor preparative isoelectric focusing chamber containing 2 % Ampholine, pH range 5 to 8. The active fractions were concentrated in a Amicon concentrator with YM 3 membrane, and dialyzed to remove Ampholines. The specific activity of the purified glutaredoxin was 28 mU/mg protein. The specific activity of pig liver recombinant glutaredoxin was 75 mU/mg protein. Therefore, pig liver glutaredoxin showed about 3 times higher activity than bovine heart glutaredoxin.

RESULTS

35S-thiolated hepatocyte proteins

S-thiolation of the same group hepatocyte protein bands is stimulated by either t-butyl hydroperoxide or menadione (5). Since diamide could produce more extensive S-thiolation of hepatocyte proteins, we first compared the proteins that were S-thiolated by diamide to those in menadione-treated cells. Figure 1 (A) shows this comparison. Both lanes show an identical pattern of radioactivity suggesting that the same set of proteins might be S-thiolated with both agents and the modification by diamide was about 2 times greater than by menadione.

The proteins S-thiolated in diamide-treated hepatocytes were examined by SDS-PAGE [Figure 1 (B)] and the relative amount of radioactivity on each protein band was determined by densitometry [Figure 1 (C)]. The 30 kDa protein, which has been identified as carbonic
anhydrase III (6), was the most abundant S-thiolated protein, containing 20% of total radioactivity. Approximately 30 percent of the readily S-thiolated sites in hepatocyte proteins. All the protein bands were dethiolated by dithiothreitol, indicating that the radioactivity on proteins were incorporated by S-thiolation not by protein synthesis. The protein mixture contained less than 0.2% TCA-soluble radioactivity before reduction with dithiothreitol.

**Identification of low molecular mass thiols bound to the S-thiolated proteins**

The $^{35}$S-labeled thiols in the hepatocytes used to prepare $^{35}$S-thiolated proteins were analyzed by ion-exchange HPLC chromatography, as described by Chai et al (5). Figure 2 (A) showed that about 80% of the trichloroacetic acid-soluble radioactivity in the cells had the same retention time as glutathione.

The radioactivity was released by dithiothreitol from the isolated, S-thiolated proteins and identified by the same HPLC method. About 84% of protein-bound radioactivity was identified as glutathione, as shown in Figure 2 (B). Therefore, the mixed hepatocyte proteins contained primarily $[^{35}\text{S}]$-glutathione (i.e., S-glutathiolated). Using the specific activity of the cellular glutathione pool, we calculated that fourteen nmoles of glutathione were bound to 1 mg hepatocyte proteins. This represents approximately 30 percent of the readily S-thiolated sites in hepatocyte proteins.

**Dethiolation of mixed hepatocyte proteins**

Since glutaredoxin, thioredoxin, and protein disulfide isomerase (PDI) have been reported to facilitate protein disulfide reduction, they potentially catalyze reduction of protein mixed-disulfides (i.e., dethiolation). In order to compare the effectiveness of glutaredoxin, thioredoxin, and protein disulfide isomerase in dethiolation of the mixed $^{35}$S-glutathiolated hepatocyte proteins, various concentrations of each dethiolating protein were incubated with the hepatocyte proteins for 5 minutes. Dethiolation occurred linearly up to 40% dethiolation. As shown in Figure 3, each protein could catalyze the dethiolation of hepatocyte proteins. Glutaredoxin was far more effective than PDI and thioredoxin on a molar basis. Incubation of the mixed proteins alone without any reducing agent did not affect the protein-bound radioactivity. Therefore, the loss of the radioactivity by the reducing agents was indeed due to
reduction of protein mixed-disulfides. Five % of radioactivity was released by 0.3 mM glutathione alone. In the absence of thioredoxin there was no dethiolation of the proteins by NADPH and thioredoxin reductase. With 0.4 μM of each protein, 40 % of protein-bound radioactivity was released by glutaredoxin, and less than 10 % by PDI. Therefore, 0.4 μM of glutaredoxin enhanced the effectiveness of glutathione 8 times. Both human and E. coli thioredoxin dethiolated 3 % of total radioactivity.

Specificity of glutaredoxin, thioredoxin, and protein disulfide isomerase in dethiolation of hepatocyte proteins

Miller and Thomas reported that the dethiolation rate of phosphorylase b by glutaredoxin were 1.5 times faster than that of carbonic anhydrase III. (11). We speculated that glutaredoxin, thioredoxin, and protein disulfide isomerase might have unique substrate specificities. In other words, some proteins might be specifically dethiolated by glutaredoxin, and others by thioredoxin or protein disulfide isomerase. In order to examine the specificities for the mixture of hepatocyte proteins, the total mixture was dethiolated by 30 % with each dethiolating protein, and individual protein bands were assessed by autoradiography and densitometry.

Figure 4 shows the extent of dethiolation of 11 representative hepatocyte proteins when the amount of each dethiolating protein is adjusted to give 30 % dethiolation in 5 min. On a molar basis, glutaredoxin was always more effective than any other dethiolating protein, because less glutaredoxin than any other protein was used for this experiment (see the legend of Figure 4). For example, glutaredoxin, thioredoxin, and protein disulfide isomerase dethiolated the 100 kDa protein to a similar extent, but since 25 times less glutaredoxin was used, it dethiolated the 100 kDa protein 25 times more effectively than thioredoxin. It was 60 times more effective than protein disulfide isomerase. Table 1 compares the effectiveness of thioredoxin and protein disulfide isomerase to that of glutaredoxin for 11 selected protein bands. Glutaredoxin dethiolated the 30 kDa protein (carbonic anhydrase III) 300 times more effectively than thioredoxin, and 200 times more efficiently than protein disulfide isomerase.

It was possible to study the reduction state of thioredoxin under the reaction conditions
used in this experiment by isoelectric focusing. Figure 5 shows that most thioredoxin is in the reduced state under the reaction conditions, indicating that thioredoxin was sufficiently reduced during the course of reaction. Therefore, thioredoxin is intrinsically less active than glutaredoxin as a dethiolase. The concentration of glutaredoxin in hepatocytes is about 6 \mu M (20, 36) and the concentration of thioredoxin in the liver has been reported to be 5-10 \mu M (37). At those concentrations, glutaredoxin probably accounts for most of the dethiolation in these cells. Protein disulfide isomerase is approximately 10 \mu M (38) and it is also localized to the endoplasmic reticulum. Therefore, the contributions of thioredoxin and protein disulfide isomerase to dethiolation may not be significant under physiological conditions.

**Specificity comparison of glutaredoxin and thioredoxin from different sources**

Figure 6 (A) compares two different mammalian glutaredoxin from pig liver and bovine heart. The data show no obvious difference between the two proteins. Human recombinant thioredoxin and E. coli thioredoxin were also compared, as shown in Figure 6 (B). The thioredoxins were also very similar to each other.

**Insulin reduction by glutaredoxin, thioredoxin, and protein disulfide isomerase**

In order to examine the specificity of the proteins for a different reaction, each protein was tested as a reductant of the disulfide bonds of insulin. Insulin reduction has been used to determine the activities of both thioredoxin and protein disulfide isomerase (17, 22). As shown in Figure 7, glutaredoxin was a poor reductant for insulin disulfides, while both thioredoxin and protein disulfide isomerase were quite efficient. Protein disulfide isomerase was the most effective. On a molar basis, protein disulfide isomerase was 10 times more effective than *E. coli* thioredoxin, and human and *E. coli* thioredoxins had a similar activity.

**Redox potential of pig liver glutaredoxin**

Gan and Wells reported that reduced pig liver glutaredoxin focused at a pI of 6.4 and it can be converted to a form with a pI of 8.0 by oxidation with 2-hydroxyethyl disulfide (36). In this paper, the redox state of pig liver glutaredoxin was assessed by isoelectric focusing by using this pI shift. As shown in Figure 8 (A), pig liver glutaredoxin, oxidized during storage,
was incubated in various mixture of GSH/GSSG for 24 hrs at 25 °C and the free sulfhydryls were blocked with iodoacetic acid (IAA) to stop the reaction. The reduced form of pig liver glutaredoxin focused as one major and one minor band with pI's of 5.6 and 5.5, respectively. The major one was used to calculate the amount of the reduced of glutaredoxin in our experiment. Unfortunately, the oxidized forms of the protein after iodoacetic acid reaction did not focus distinctly and could not be quantitated by densitometry. Therefore, changes in the amount of the reduced glutaredoxin were used to assess the redox state of glutaredoxin.

The fraction of glutaredoxin in the reduced state (R) can be calculated from equation 2, when $K_{eq}$ is the equilibrium constant for reaction 1.

\[
\text{GRX}^{\text{ox}} + 2 \text{GSH} \rightleftharpoons \text{GRX}^{\text{red}} + \text{GSSG} \quad \text{(reaction 1)}
\]

\[
K_{eq} = \frac{[\text{GRX}^{\text{red}}][\text{GSSG}]}{[\text{GRX}^{\text{ox}}][\text{GSH}]^2} \quad \text{(equation 1)}
\]

\[
R = \frac{[\text{GSH}]^2/[\text{GSSG}]}{K_{eq} + [\text{GSH}]^2/[\text{GSSG}]} \quad \text{(equation 2)}
\]

Figure 8 (B) shows the relationship between $[\text{GSH}]^2/[\text{GSSG}]$ and the fraction of glutaredoxin in the reduced state (R). The solid line fits the equilibrium constant of $K_{eq} = 1020 \, (\text{M}^{-1})$. The equilibrium constant for each concentration of GSH and GSSG gave an average redox potential of $-0.159 \pm 0.004 \, \text{V}$. A redox potential of $-0.248 \, \text{V}$ was used for the GSH/GSSG couple in this calculation (44, 45). That is,

\[
E_{\text{GRX}}^\circ = E_{\text{GSH/GSSG}}^\circ - 0.02958 \times \log K_{eq}
\]

\[
= -0.248 + 0.02958 \times \log(1020) = -0.159 \, \text{V}
\]

**DISCUSSION**

When hepatocytes and heart cells were exposed to t-butyl hydroperoxide, S-thiolation of proteins was quite transient, that is, the maximal S-thiolation occurred within 5 min and the S-thiolation was rapidly reversed thereafter. This transient S-thiolation can be explained by rapid metabolism of the hydroperoxide (39) and by rapid dethiolation of the S-thiolated proteins. The rapid dethiolation suggests that there must be a mechanism which actively dethiolates the
S-thiolated proteins and therefore maintains protein thiols as the reduced state in cells. Enzyme-catalyzed dethiolation has been suggested as a mechanism to explain the rapid dethiolation in hepatocytes, because of the relatively slow dethiolation by glutathione alone.

Glutaredoxin, thioredoxin, and protein disulfide isomerase share a dithiol separated by two amino acids in their active sites. They have been suggested as reductants for protein disulfides but there has been no systematic comparison of the effectiveness of each protein in the reduction. In this report, we show that glutaredoxin is the most effective in dethiolation of hepatocyte proteins on a molar basis.

The interaction between glutathione and the amino acids at the active site of glutaredoxin has been described from X-ray crystallography of S-glutathiolated glutaredoxin (41). The results strongly suggest that glutaredoxin has a glutathione-binding site near the active thiol pair. Thus, glutathione on S-glutathiolated proteins may also bind to this site resulting in the facilitated dethiolation. Gravina and Mieyal reported that glutaredoxin did not facilitate dethiolation of S-cysteinylated hemoglobin but that of S-glutathiolated hemoglobin (18). Yoshitake et al. also reported that bovine liver glutaredoxin reactivated GSSG-inactivated phosphofructokinase more efficiently than bovine liver thioredoxin (42). The specific enhancement of dethiolation only for S-glutathiolated hemoglobin and the more efficient activation for GSSG-inactivated phosphofructokinase may also be due to the glutathione-binding at the active site of glutaredoxin.

The redox potential of E. coli thioredoxin has been reported as -0.26 V at pH 7 and 25 °C by Moore et al. (40) and by Krause et al. (23). The redox potential of pig liver glutaredoxin is determined as -0.16 V in this report. This high redox potential of glutaredoxin clearly indicates that E. coli thioredoxin is much stronger reductant than glutaredoxin thermodynamically, and that the more effective dethiolation by glutaredoxin than thioredoxin is not determined thermodynamically, but kinetically.

The standard redox potential of glutaredoxin implies that glutaredoxin may be in the fully reduced state in the liver under the reported concentrations of GSH and GSSG (10 mM GSH, GSH/GSSG = 300). Chai et al. reported that S-thiolation by t-butyl hydroperoxide was
transient (5), suggesting that there would be an efficient dethiolating activities. Under these conditions, about 80 % of glutathione became GSSG in 3 min, decreasing to 30 % in 30 min. With 2 mM GSH and 4 mM GSSG (80 % of the total GSH as GSSG), using the redox potential of - 0.16 V as determined in our work one can calculate that about 50 % of glutaredoxin would be in the reduced state. This implies that glutaredoxin can be quite active when the glutathione pool is highly oxidized. Menadione caused a gradual increase in protein S-thiolation with no apparent net dethiolation for up to 30 minutes. Approximately 97 % of glutathione was oxidized in 15 min and the redox state of glutathione remained up to 30 min. With 0.3 mM GSH and 4.85 mM GSSG (assuming that 97 % of the total GSH is oxidized), only 2 % of glutaredoxin would be in the reduced state. Therefore, S-thiolation by menadione may continually increase because glutaredoxin may not be significantly reduced.

The redox potentials of other dethiol proteins such as DsbA and protein disulfide isomerase (PDI) has been reported. The standard redox potential of DsbA was reported as - 0.089 V using a reference redox potential, - 0.205 V, for GSSG/GSH system at pH 7 at 30 °C, by Wunderlich and Glockhuber (43). This potential can be corrected to - 0.139 V by the suggested standard redox potential for GSSG/GSH, - 0.248 V, when that of NADP⁺/NADPH is - 0.310 V at pH 7.0 at 25 °C (44, 45). The redox potentials for bovine liver protein disulfide isomerase were also determined, but there was a significant disagreement between papers. Hawkins et al. reported the redox potential of bovine liver PDI as - 0.11 V (46). Lundström and Holmgren reported the redox potential of calf liver PDI as - 0.190 ± 0.010 V and - 0.175 ± 0.015 V, depending on the reference used in the determination (47). It has been suggested that the endoplasmic reticulum is an oxidizing environment (48). PDI and DsbA acts as oxidants in the oxidizing condition. The redox potential determination described in this paper is the first one for glutaredoxin from any sources. The high redox potential of glutaredoxin suggests that glutaredoxin itself can act as an oxidant depending on the redox condition in which it is present. Therefore, it will be very interesting to examine whether glutaredoxin is present in the cellular organelles other than the cytosol and whether it catalyzes disulfide bond formation.
It was an unexpected result that glutaredoxin was the most effective dethiolating protein among the dithiol proteins tested. This systematic comparison may be possible because a mixture of substrate was used. The method used in this report can be adapted in various protein modifications such as phosphorylation/dephosphorylation and sulfation/desulfation. By combining radioisotopes (or immunostaining methods) with SDS-PAGE (or isoelectric focusing), a mixture of proteins can be used to directly compare the specificity of certain enzymes.

REFERENCES


193, 265-275
Table 1. A comparison of the effectiveness of thioredoxin and PDI to glutaredoxin.

<table>
<thead>
<tr>
<th>Molecular Mass (kDa)</th>
<th>TRX/GRX</th>
<th>PDI/GRX</th>
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<td>15</td>
<td>0.013</td>
<td>0.0012</td>
</tr>
<tr>
<td>27</td>
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<td>28</td>
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<td>0.012</td>
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<td>30 (carbonic anhydrase III)</td>
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The dethiolation rates of protein bands in Figure 4 were compared on a molar basis. The rates of dethiolation for each dethiolase were normalized for the amount of dethiolase used. These values were divided to give the data shown above. N.D., not detected. The 27 kDa protein was not dethiolated by protein disulfide isomerase.
Figure 1. S-thiolated hepatocyte proteins

Hepatocyte proteins were S-thiolated and isolated as described under Materials and Methods. The proteins were separated on the basis of their mobilities on a SDS-polyacrylamide gel. The radioactivity associated with individual bands was visualized by autoradiography.

(A) A comparison of the S-thiolated proteins by diamide and by menadione. Hepatocytes were incubated with either 0.5 mM diamide for 5 min or 0.2 mM menadione for 30 min to stimulate protein S-thiolation. The isolated proteins were separated on a 10 % SDS-polyacrylamide gel.

(B) S-thiolated hepatocyte proteins by 2 mM diamide for 5 min. The isolated proteins were separated on a 5-20 % gradient SDS-polyacrylamide gel. In order to test whether the radioactivity associated with the proteins was due to S-thiolation the mixed proteins were reduced with 20 mM dithiothreitol for 1 hour at 30 °C.

(C) Relative radioactivity on individual protein bands. The relative radioactivity on each band was determined by scanning the autoradiogram, as described under Materials and Methods.
AUTORADIOGRAM

(A) DIAMIDE MENADIONE

(B) DITHIOHREITOL

(C) CA III (30)

radioactivity (%)
Figure 2. Identification of $^{35}$S-labeled materials in hepatocytes, and $^{35}$S-labeled thiols released from the hepatocyte protein mixture

(A) Perchloric acid (PCA)-soluble materials in hepatocytes. PCA-soluble materials were extracted from hepatocytes incubated with Tran$^{35}$S-label and were separated by a HPLC column, as described under Materials and Methods. The HPLC fractions were counted by a liquid scintillation.

(B) Identification of the radioactive thiols released by dithiothreitol. The $^{35}$S-thiolated hepatocyte proteins were incubated with 20 mM dithiothreitol at 30 °C for 1 hour. The radioactive thiols released by reduction were analyzed by the same HPLC method.
(A) $^{\gamma}$GSH

(B) GSH

$^{\gamma}$GSSG

time (min)
Figure 3. Dethiolation of hepatocyte proteins by glutaredoxin, thioredoxin, and protein disulfide isomerase

The hepatocyte proteins (1 mg/ml, 14 μM of $^{35}$S-thiolated proteins) were dethiolated with pig liver glutaredoxin (GRX) and bovine liver protein disulfide isomerase (PDI) in the presence of 0.3 mM glutathione. Human and E. coli thioredoxin (TRX) were incubated with 0.4 mM NADPH and bovine heart thioredoxin reductase (0.3 mg/ml) before adding the protein substrates. After 5 min incubation, the proteins were precipitated by adding bovine serum albumin (6 mg/ml) and 10 % trichloroacetic acid (TCA). The TCA-soluble radioactivity in the supernatant released by dethiolation was then counted.
Figure 4. Specificity of glutaredoxin, thioredoxin, and protein disulfide isomerase for individual proteins

The hepatocyte proteins (1 mg/ml) were dethiolated with 0.37 μM pig liver glutaredoxin and 25 μM bovine liver protein disulfide isomerase in the presence of 0.3 mM glutathione. Dethiolation by 10 μM human recombinant thioredoxin was done in the presence of 0.4 mM NADPH and bovine heart thioredoxin reductase (0.3 mg/ml). After 5 minutes, the reaction mixture was treated with an equal volume of 0.125 M Tris-Cl buffer, pH 6.8 containing 4 % sodium dodecyl sulfate, 20 % glycerol, 0.008 % bromophenol blue, and 80 mM N-ethylmaleimide. The individual proteins were separated on a 5-20 % gradient SDS-PAGE. Dethiolation of individual proteins was determined by autoradiography and densitometry, as described under Materials and Methods. Pig liver glutaredoxin and protein disulfide isomerase dethiolated 33 % of total protein-bound radioactivity, and human thioredoxin dethiolated 29 %. Open bar, glutaredoxin; Hatched bar, thioredoxin; Closed bar, protein disulfide isomerase.
Figure 5. Reduction state of thioredoxin

E. coli thioredoxin and human recombinant thioredoxin (10 μM) were pre-incubated in the presence of 0.4 mM NADPH and bovine heart thioredoxin reductase (0.3 mg/ml) for 30 min at 30 °C, as in Figure 4. After incubation, an equal volume of 40 mM iodoacetic acid (IAA) in 50 mM Tris-Cl buffer, pH 8.0 was added to block free sulphydryls. Thioredoxins were focused at the lower pIs when they were reduced and reacted with IAA. Lane 1, oxidized E. coli thioredoxin; Lane 2, E. coli thioredoxin reduced by thioredoxin reductase and NADPH; Lane 3, thioredoxin reductase alone incubated with NADPH; Lane 4, oxidized human thioredoxin; Lane 5, human thioredoxin reduced by thioredoxin reductase and NADPH.
Figure 6. Comparison of glutaredoxins and thioredoxins from different sources

(A) Comparison of dethiolation by pig liver glutaredoxin and bovine heart glutaredoxin. The hepatocyte proteins (1 mg/ml) were dethiolated to a similar extent (33 %) by 0.37 μM pig liver glutaredoxin or 1 μM bovine heart glutaredoxin. Dethiolation of individual proteins were determined as described in Figure 4. Open bar, pig liver glutaredoxin; Hatched bar, bovine heart glutaredoxin.

(B) Comparison of dethiolation by human recombinant thioredoxin and E. coli thioredoxin. The hepatocyte proteins (1 mg/ml) were dethiolated to a similar extent (29 %) by 10 μM human recombinant thioredoxin or 10 μM E. coli thioredoxin. Dethiolation of individual proteins were measured as described in Figure 4. Open bar, human thioredoxin; Hatched bar, E. coli thioredoxin
Figure 7. Insulin reduction by glutaredoxin, thioredoxin, and protein disulfide isomerase

Reduction of insulin disulfides by dithiothreitol was measured in the presence of glutaredoxin, thioredoxin, and protein disulfide isomerase, as described by Holmgren (34) and Bardwell et al. (35). The reaction mixtures contained 0.33 mM dithiothreitol, 0.13 mM insulin, 2 mM EDTA, and a dethiolating protein, all in 0.1 M sodium phosphate buffer, pH 7.0. The reactions were started by adding insulin. The reduction of insulin disulfide was measured at 650 nm at 25 °C. hTRX, human thioredoxin; eTRX, E. coli thioredoxin; PDI, bovine liver protein disulfide isomerase; GRX, pig liver glutaredoxin.
Figure 8. Redox equilibrium of pig liver glutaredoxin with glutathione

Pig liver glutaredoxin (0.5 mg/ml) was incubated with GSH/GSSG mixtures in 20 mM β-glycerophosphate buffer, pH 7.0, for 24 hr at 25 °C. After incubation, an equal volume of 40 mM iodoacetic acid in 50 mM Tris-Cl buffer, pH 8.0 was added to block free sulphhydryls stopping all reactions. (A) Isoelectric focusing of glutaredoxin. Oxidized and reduced forms of glutaredoxin were separated by isoelectric focusing and the amount of reduced glutaredoxin (the major band) was determined by densitometry. The GSH and GSSG concentrations were also measured by HPLC. The concentrations of glutathione were; Lane 1, 2.90 mM GSH and 0.226 mM GSSG; Lane 2, 0.965 mM GSH and 0.190 mM GSSG; Lane 3, 0.272 mM GSH and 0.147 mM GSSG; Lane 4, 0.081 mM GSH and 0.114 mM GSSG.

(B) Redox state of pig liver glutaredoxin compared to \([\text{GSH}]^2/\text{[GSSG]}\). The theoretical curve plotted in a solid line was drawn from equation 2 using an equilibrium constant of 1020 M⁻¹.
R (fraction of the reduced GRX)

\[
\text{[gSH]}^2/\text{[gSS]} \quad (B)
\]
DETHIOLATION OF S-GLUTATHIOLATED PROTEINS
BY GLUTATHIONE (GSH)

A paper to be submitted to the Journal of Biological Chemistry

Che-hun Jung and James A. Thomas

ABSTRACT

Direct dethiolation of a mixture \(^{35}\)S-glutathiolated proteins by glutathione was studied. The \(^{35}\)S-glutathiolated protein mixture was isolated from \(^{35}\)S-labeled rat hepatocytes after stimulation with diamide. The protein mixture was treated with N-ethylmaleimide to block reactive sulfhydryls. It contained no thioredoxin or glutaredoxin-like activities. Dethiolation of individual protein bands was analyzed by combining SDS-PAGE and autoradiography. All of the hepatocyte proteins were dethiolated by glutathione, but the dethiolation rates of individual proteins varied somewhat from protein to protein. The most rapidly dethiolated proteins, the 45 and 140 kDa proteins, were dethiolated 2.5 times faster than the least reactive proteins, the 74, 77, and 88 kDa proteins.

Dethiolation of the mixed hepatocyte proteins was saturable with respect to the concentration of glutathione. The half maximal rate of dethiolation occurred at 0.16 mM glutathione. Dethiolation of pure S-glutathiolated phosphorylase b also showed the same saturation kinetics with the half maximal rate at 0.16 mM glutathione. This confirmed the data with hepatocyte proteins and eliminated any potential contamination by GSH-dependent dethiolase such as glutaredoxin. Dethiolation rate was inhibited by denaturing agents such as guanidinium chloride and sodium dodecyl sulfate, and glutathione analogs, S-methyl glutathione and glutathione sulfonate. The saturation kinetics, inhibition by glutathione analogs and protein denaturants, strongly suggest that glutathione forms a noncovalent complex with the mixed hepatocyte S-glutathiolated proteins and with S-glutathiolated phosphorylase b, as a prerequisite to the reduction of the disulfides of those proteins. It is
suggested that the S-glutathiolation site on all these proteins contains a glutathione-binding site that is responsible for proper orientation of glutathione during its reduction of the protein-glutathione mixed disulfide bonds.

**INTRODUCTION**

Glutathione is the most abundant nonprotein thiol in most cells with a concentration as high as 10 mM in the liver (1). It has been suggested that glutathione may be an important component in various cellular activities such as the synthesis of precursors of DNA (2), detoxification of compounds by glutathione S-transferase system (3), and the removal of hydrogen peroxide and organic peroxides by glutathione peroxidase (4). It has also been suggested that protein thiols may act as an important antioxidant system and that protein thiols are protected from irreversible oxidations through protein S-thiolation and dethiolation (5, 6). Glutathione is of central importance in those processes.

Protein S-thiolation is the formation of mixed-disulfides between protein thiols and nonprotein thiols. In this process, glutathione is the major thiol forming the mixed-disulfides in vivo, even though other thiols such as cysteine, homocysteine, or cysteamine can also form mixed-desulfides. Protein S-thiolation is a dynamic process in which protein S-thiolation is stimulated rapidly in cells under oxidative stress and the S-thiolated proteins were in turn readily reduced by dethiolation reaction (7-10).

Dethiolation is a reverse reaction of S-thiolation (11). In the previous paper, we examined the role of dithiol proteins such as glutaredoxin, thioredoxin, and protein disulfide isomerase in dethiolation. All the dithiol proteins facilitated dethiolation of S-thiolated hepatocyte proteins and glutaredoxin was the most effective among those tested. It was then suggested that glutaredoxin probably accounted for most of the dethiolation in cells.

Dethiolation can also occur by thiol-disulfide exchange reaction between S-thiolated proteins and thiols. Since the cellular concentration of glutathione is as high as 10 mM in the liver, direct dethiolation by glutathione through thiol-disulfide exchange reaction may be an important mechanism in dethiolation. In this report, we examined direct dethiolation of mixed
hepatocyte proteins by glutathione. The results presented here suggest that glutathione forms noncovalent complexes with S-thiolated proteins during dethiolation. This binding greatly enhances the dethiolation rate at the lower concentration of glutathione. This is the first report on noncovalent glutathione-binding to proteins which undergo S-thiolation and dethiolation under oxidative stress.

MATERIALS AND METHODS

Glutathione, dithiothreitol, bovine serum albumin, collagen, N-ethylmaleimide, galactose, and dexamethasone were purchased from Sigma chemical Co. (St. Louis, MO). Insulin (bovine), transferrin (human), penicillin G, streptomycin sulfate, Leibovitz's L-15 medium with L-glutamine were from Gibco BRL (Grand Island, NY). Collagenase was from Worthington biochemical Corp. (Freehold, NJ). SDS-PAGE molecular mass standards were from Bio-Rad laboratories (Richmond, CA). Trans-35S-label was from ICN biochemical, Inc. (Irvine, CA). Precasted 5-20 % gradient SDS-PAGE gels were from Jule Inc. (New Haven, CT).

Preparation of 35S-glutathiolated Hepatocyte Proteins

Sprague-Dawley male rats (200-250 g) (Sasco Co., Omaha, NE) were used to prepare hepatocytes by collagenase digestion, as described by Bonney et al. (12). Cell viability was greater than 90% by trypan blue exclusion. The isolated hepatocytes were diluted to 0.5x10^6 cells/ml in Leibovitz's L-15 medium, pH 7.6, supplemented with 18 mM Hepes, 0.2% bovine serum albumin, 5 μg/ml insulin/transferrin, 1 μM dexamethasone, 5 mg/ml galactose, 50 Units/ml penicillin G, and 50 μg/ml streptomycin sulfate and plated on collagen-coated dishes. The hepatocytes were incubated at 37 °C for 48 hours before 35S-labeling.

Cells were incubated in L-15 medium containing 15 μg/ml cycloheximide for 30 minutes prior to labeling. Cycloheximide prevented protein synthesis during 35S-labeling. Cycloheximide-treated cells were then incubated for 4 hours with serum-free CMRL-1415 medium containing 40 μM methionine, 10 μM cystine, and 15 μg/ml cycloheximide with 3x10^9 dpm/μmole. The low concentration of methionine and cystine in CMRL-1415 medium
was convenient for labeling the cells with a higher specific activity. The specific activity of glutathione in cells was analyzed by HPLC as described by Fariss and Reed (13) and modified by Chai et al. (8). The acid-soluble materials in $^{35}$S-labeled hepatocytes were separated by a HPLC column and fractions were counted by a liquid scintillation counter.

$^{35}$S-labeled hepatocytes were treated with 2 mM diamide for 5 minutes to S-thiolate proteins. After incubation, the medium was removed and cells were rinsed twice with cold phosphate buffered saline. Cells were extracted within a minute by scraping cells in 20 mM Hepes pH 7.4 buffer containing 5 mM EDTA, 5 mM EGTA and 50 mM N-ethylmaleimide. N-ethylmaleimide was prepared fresh for each experiment. The extract was centrifuged at 10,000 x g for 30 minutes to obtain a particulate-free cell extract. The supernatant was dialyzed to remove low molecular mass radioactivity using 12-14 kDa molecular mass cut-off dialysis tubing.

Glutaredoxin and thioredoxin activity in the extract were measured as described by Thomas et al. (14), using $[^3H]$-phosphorylase b as a substrate. Neither glutaredoxin nor thioredoxin activity was detected in the extract. The protein concentration was determined as described by Lowry et al. (15) using bovine serum albumin as standard.

**Determination of the thiol anion concentrations**

The ultraviolet absorption was utilized to determine the concentrations of thiol anions at pH 7.0 (16). Ionization of the glutathione -SH group gives a unique absorption at 232 nm with a molar extinction coefficient of 5690 (M$^{-1}$cm$^{-1}$) and cysteine at 236 nm with a molar extinction coefficient of 5250 (M$^{-1}$cm$^{-1}$). The extent of ionization of glutathione and cysteine was 0.85 % and 3.44 %, respectively.

**Assay of dethiolation reaction**

In order to measure the overall dethiolation rate, either protein-bound radioactivity or the released radioactivity from proteins was determined. In the former case, aliquots of the reaction mixture were spotted on filter paper squares (17). The TCA-precipitable radioactivity bound to the paper was measured by liquid scintillation. In the latter case, the hepatocyte proteins, after reaction, were precipitated with 10 % trichloroacetic acid (TCA). The TCA-
soluble radioactivity in the supernatant, which represents the extent of dethiolation, was counted by liquid scintillation (14).

In order to measure dethiolation of individual protein bands, the reaction was stopped by adding an equal volume of 0.125 M Tris-Cl buffer, pH 6.8 containing 4% sodium dodecyl sulfate, 20% glycerol, 0.008% bromophenol blue, and 80 mM N-ethylmaleimide. N-ethylmaleimide stopped the reaction and blocked free sulfhydryls. Proteins were separated by 5-20% gradient SDS-PAGE. The radioactivity associated with individual bands was visualized by autoradiography, and quantitated by densitometry.

RESULTS

35S-thiolated hepatocyte proteins

In order to study dethiolation, S-thiolated proteins were prepared from hepatocytes stimulated with diamide. The mixture of proteins isolated from diamide-treated hepatocytes were characterized by separation on a SDS-PAGE gel [Figure 1 (A)]. The relative radioactivity on each protein band was determined by combining autoradiography and densitometry [Figure 1 (B)]. The 30 kDa protein, which has been identified as carbonic anhydrase III (18), was the most abundant S-thiolated protein, containing 20% of the total protein-bound radioactivity. All the protein bands were completely dethiolated by dithiothreitol, indicating that the radioactivity on proteins was derived from bound 35S-glutathione not from the protein itself. The protein mixture contained less than 0.2% TCA-soluble radioactivity when no reductant was added.

The radioactive materials incorporated into S-thiolated proteins were released by dithiothreitol, and identified by a HPLC method (8, 13). More than 84% of protein-bound radioactivity was identified as glutathione. Therefore, the mixed hepatocyte proteins contained mainly [35S]-glutathione (i.e. S-glutathiolated), with fourteen nmole of glutathione bound to one mg hepatocyte proteins. The extent of S-thiolation represents approximately 30 percent of the readily S-thiolated sites in hepatocyte proteins. N-ethylmaleimide (NEM) was added during isolation, so all the reactive sulfhydryls were either S-glutathiolated or NEM-modified.
NADPH-dependent and GSH-dependent dethiolases (i.e., reductases for protein mixed-disulfides) greatly enhanced dethiolation of hepatocyte proteins (19). Therefore, any contaminating dethiolase activities in the protein preparation could interfere the analysis of the dethiolation by glutathione. Although the dethiolase activities has been measured utilizing S-[^3H]glutathiolated phosphorylase b (14), it was not practical to directly measure dethiolase activities in the hepatocyte protein mixture because the much higher radioactivity on [^35S]-labeled hepatocyte proteins interfered the dethiolase assay. In order to measure dethiolase activities in the protein preparation, a S-glutathiolated protein mixture was prepared from the cells with no[^35S] label. Neither glutaredoxin nor thioredoxin activity was detected in the mixture of S-glutathiolated proteins, suggesting that the mixture of[^35S]-labeled hepatocyte proteins used for this paper may also be dethiolase-free. The dethiolases may be inactivated by N-ethylmaleimide during cell lysis if they were in the reduced state, and residual activity may have been eliminated during the extensive dialysis with a membrane that does not completely retain 12 kDa proteins.

Dethiolation of the hepatocyte proteins by various thiols

The time dependent dethiolation of hepatocyte proteins by reduced glutathione (GSH), cysteine, and dithiothreitol is shown in Figure 2. In the absence of any thiol, there was no detectable loss of the radioactivity on proteins. This indicated that the decrease of radioactivity by the thiols was solely due to dethiolation (i.e., reduction of mixed-disulfides). The reduction was not a 1st-order reaction. This non-1st order kinetics may partly be a result of various dethiolation rates of individual proteins consisting the mixture. The redox states of glutathione were determined by HPLC before and after dethiolation. After 80 minute incubation, the concentrations of reduced glutathione (GSH) in the reaction mixtures initially containing 5, 1, and 0.2 mM GSH were 4.9, 0.90, and 0.14 mM, respectively. Therefore, dethiolation by 0.2 mM GSH after 80 min was probably slower as a result of the decrease GSH and the decreased GSH may also contribute to the non-1st order dethiolation. Dethiolation by 1 mM cysteine and 5 mM dithiothreitol also showed similar non-1st order kinetics. One mM cysteine was as effective as one mM glutathione, and five mM dithiothreitol
was considerably more effective than the same concentration of glutathione.

In Figure 2, cysteine and glutathione gives equal rates of dethiolation at 1 mM. Since the pKa of the glutathione sulfhydryl has been reported to be 9.2 and that of cysteine to be 8.48 (15, 20), the concentration of cysteine thiol anion at pH 7.0 should be 5 times higher than glutathione if they follow simple dissociation of mono-ionic species. The thiolate ion concentrations in 1 mM glutathione and 1 mM cysteine at pH 7 were determined to be 8.5 μM and 34.4 μM, respectively, as described under Materials and Methods. Thus, the concentration of thiolate ion, the reactive species for dethiolation reaction, of 1 mM cysteine at pH 7.0 is 4 times higher than that of 1 mM glutathione and yet each gave an equal rate of dethiolation.

**Dethiolation by glutathione shows saturation kinetics**

The dethiolation rate is not directly proportional to the amount of glutathione, and Figure 3 shows that it is saturable with respect to the glutathione. The dethiolation rates followed Michaelis-Menten kinetics in which the half maximal rate of dethiolation occurred at 1.4 μM of glutathione thiolate, equivalent to 0.16 mM GSH. These saturation kinetics suggest that glutathione forms a noncovalent complex with S-glutathiolated proteins during dethiolation. Dethiolation by cysteine did not show saturation at this concentration.

Since the dethiolation rate of each protein in the mixture could be different, the relatively fast reaction at the lower concentration of glutathione might represent the dethiolation of the fast reacting proteins that were specific for glutathione. The slower increase in dethiolation rates at higher concentrations of glutathione might result from the dethiolation of the slower reacting proteins.

In order to examine this possibility, the dethiolation of pure, [³H]S-glutathiolated phosphorylase b was examined, as shown in Figure 4. [³H]S-glutathiolated phosphorylase b was prepared by diamide reaction as described by Thomas et al. (14). The dethiolation rate was again saturable with respect to glutathione and the half maximal dethiolation occurred at 1.4 μM of glutathione thiolate. This suggested that glutathione formed a noncovalent complex with S-glutathiolated phosphorylase b during dethiolation and that the saturation kinetics of
the hepatocyte protein mixture were also due to noncovalent GSH-binding to the proteins, analogous to enzyme-substrate complexes.

**Dethiolation of individual proteins by glutathione**

In order to examine specificity of dethiolation for individual proteins, the mixture of hepatocyte proteins was separated by gel electrophoresis and the extent of dethiolation by glutathione was determined by autoradiography and densitometry. Figure 5 shows the extent of dethiolation of the 12 major proteins in the mixture by 0.5 mM glutathione (5A) and by 5 mM glutathione (5B). Overall, the relative dethiolation of individual proteins by 0.5 mM GSH was similar to 5 mM GSH. The 45 and 170 kDa proteins were dethiolated most rapidly, approximately 2.5 times faster than the 74, 77, and 88 kDa proteins. **Inhibition by glutathione analogs and denaturing agents**

Figure 6 shows that both 4 M guanidinium chloride and 0.1 % sodium dodecyl sulfate decrease the rate of dethiolation. This decreased dethiolation may result from the loss of glutathione interaction with the S-glutathiolation site in the presence of denaturing agents. If glutathione binds to the site during dethiolation, glutathione analogs may compete for the same binding sites. Table 1 shows that either glutathione sulfonate or S-methyl glutathione inhibits the dethiolation, suggesting again that glutathione binds to proteins during dethiolation.

**DISCUSSION**

The experiments presented in this report suggest that glutathione is capable of forming a noncovalent complex with S-glutathiolated proteins, and this binding increases the effectiveness of low glutathione concentrations as a dethiolating agent. The dethiolation of both mixed hepatocyte proteins and pure phosphorylase b comply with the Michaelis-Menten model with the half maximal rate at 0.16 mM glutathione.

Since glutathione is such an abundant thiol in most cells, it has been suggested that thiol-disulfide exchange with reduced glutathione may be important for dethiolation. On the contrary, from the following observations, it has also been suggested that glutathione itself
may not be an important dethiolating agent (9, 21, 22). First, dethiolation is greatly facilitated by dithiol proteins, especially by glutaredoxin. In the previous paper, it was suggested that glutaredoxin might account for most of the dethiolation in cells by the reactivity and the cellular concentration of glutaredoxin. Second, glutathione showed a relatively lower reactivity on a molar basis and the low activity is probably due to the higher pKa of glutathione.

Noncovalent glutathione-binding to several glutathione-utilizing enzymes such as glutathione S-transferase (23, 24), glyoxalase I (25), glutathione peroxidase (26), and glutathione reductase (27) has been described in detail. Glutathione-binding to the glutathione-utilizing enzymes may not be surprising because enzyme catalysis includes enzyme-substrate complex. On the other hand, there have been reports of noncovalent interaction of glutathione with hemoglobin at the S-thiolation site (28). A putative glutathione-binding site in CdZn-metallothionein has also been suggested (29). Since hemoglobin and metallothionein has not been related with glutathione metabolism, the role of glutathione binding to those proteins is not understood.

Glutathione-binding to the S-glutathiolated proteins suggests that S-glutathiolated proteins may contain a GSH-binding site even in the reduced state. In addition, since the hepatocyte protein thiols available for S-thiolation has been estimated to be 50 nmol/mg protein, approximately 10 mM thiols in the liver, the concentration of glutathione-binding sites may also be as high as 10 mM in cells.

Reactive oxygen species (ROS)-initiated S-thiolation has been suggested as a major S-thiolation mechanism in which protein thiols are activated by ROS, and in turn a reduced glutathione reacts with the "activated" protein thiols to form a disulfide bond. The experiments presented in this report suggest that GSH-binding sites may be closely associated with the reactive sulfhydryls that participate in S-glutathiolation. That is, the binding sites provide glutathione ready for S-thiolation under oxidative stress.
REFERENCES


Table 1. Inhibition of dethiolation by glutathione sulfonate and S-methyl glutathione

<table>
<thead>
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<th>dethiolation (%)</th>
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<tr>
<td>0.14 mM GSH alone</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>+ glutathione sulfonate</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>(0.6 mM)</td>
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<tr>
<td>+ glutathione sulfonate</td>
<td>5.4 ± 0.5</td>
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<tr>
<td>(1.2 mM)</td>
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<tr>
<td>+ S-methyl glutathione</td>
<td>6.8 ± 0.4</td>
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<td>(0.6 mM)</td>
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S-glutathiolated hepatocyte proteins were dethiolated with 0.14 mM GSH in 20 mM β-glycerophosphate buffer, pH 7.0, at 30 °C. After 5 min incubation, the protein mixture was precipitated by adding bovine serum albumin (6 mg/ml) and 10 % trichloroacetic acid. The radioactivity in the supernatant was counted by a liquid scintillation. The same experiments were repeated in the presence of glutathione sulfonate and S-methyl glutathione.
Figure 1. S-glutathiolated hepatocyte proteins

Hepatocyte proteins were S-glutathiolated and isolated, as described under Materials and Methods. The proteins were separated on a SDS-polyacrylamide gel and the radioactivity associated with individual bands was visualized by autoradiography. (A) $^{35}$S-glutathiolated proteins from hepatocytes treated with 2 mM diamide for 5 min. The proteins were separated on a 5-20 % gradient SDS-polyacrylamide gel. The radioactivity associated with the proteins was released by reduction as shown in the right lane (20 mM dithiothreitol for 1 hour at 30 °C). Dithiothreitol did not cause any detectable change in the protein bands. (B) Fraction of the total radioactivity associated with separate protein bands were determined by scanning the autoradiogram.
Figure 2. Dethiolation of hepatocyte proteins by glutathione, cysteine, and dithiothreitol

Hepatocyte proteins (2 mg/ml) were dethiolated with various concentrations of glutathione in 20 mM β-glycerophosphate buffer, pH 7.0, at 30 °C. During the reactions, aliquots of the reaction mixture were spotted on squares of filter paper, and the radioactivity precipitated on the papers by 10 % trichloroacetic acid was measured by liquid scintillation counting.
Figure 3. Glutathione concentration dependence of dethiolation

The $^{35}$S-thiolated proteins were dethiolated with various concentrations of glutathione and cysteine for 5 min at 30 °C in 20 mM β-glycerophosphate buffer, pH 7.0. The proteins in the reaction mixture were then precipitated with 10% trichloroacetic acid (TCA). The TCA-soluble radioactivity in the supernatant, released by dethiolation, was counted.
Figure 4. Dethiolation of $[^3H]S$-glutathiolated phosphorylase b

Dethiolation of $[^3H]S$-thiolated phosphorylase b (0.3 μM) was measured, as described in the legend of Figure 3. After incubation, phosphorylase b was precipitated with 10 % trichloroacetic acid (TCA) and the TCA-soluble radioactivity in the supernatant was counted. The small graph inserted inside the figure is a double-reciprocal plot.
Figure 5. Dethiolation of individual proteins by glutathione

The protein mixture (2 mg/ml) were incubated with 0.5 and 5 mM GSH under the condition described in the legend of Figure 2. After 10 min incubation, dethiolation reaction was stopped by adding an equal volume of 0.125 M Tris-Cl buffer, pH 6.8 containing 4 % sodium dodecyl sulfate, 20 % glycerol, 0.008 % bromophenol blue, and 80 mM N-ethylmaleimide. N-ethylmaleimide stops the reaction and blocks free sulfhydryls. Proteins were separated by a 5-20 % gradient SDS-PAGE. The radioactivity associated with individual bands was quantitated by autoradiography and densitometry. (A) The extent of dethiolation by 0.5 mM glutathione. (B) Dethiolation by 5 mM glutathione.
Figure 6. Dethiolation of hepatocyte proteins in the presence of guanidinium chloride and sodium dodecyl sulfate

The protein mixture (2 mg/ml) were incubated at 30 °C with 0.5 mM GSH in the presence of 0.1 % sodium dodecyl sulfate and 4 M guanidinium chloride. During the reactions, aliquots of the reaction mixture were spotted on filter paper squares and the radioactivity precipitated on the papers by trichloroacetic acid was measured, as described in the legend in Figure 2.
SUMMARY AND CONCLUSION

The experiments described in this dissertation explored dethiolation of $^{35}$S-glutathiolated hepatocyte proteins. In the first paper, the redox-active dithiol proteins, glutaredoxin, thioredoxin, and protein disulfide isomerase, were examined as dethiolases (i.e., reductases for protein mixed-disulfides) by studying their specificities in dethiolation. Non-enzymatic dethiolation by glutathione (GSH) was studied in the second paper. These experiments gave a clue to understand the role of dethiolation in response to oxidative stress, and the mechanism of dethiolation to maintain the protein thiols in the reduced state in cells.

In order to study the specificities of those redox-active dithiol proteins, a mixture of $^{35}$S-glutathiolated hepatocyte proteins was used. Each of the dithiol proteins greatly enhanced the dethiolation rate and could completely dethiolate all of the S-glutathiolated proteins. When the dethiolation rates of individual proteins were compared, glutaredoxin was the most effective. For example, carbonic anhydrase III, a major S-thiolated protein in hepatocytes (22), was dethiolated 300 times faster by glutaredoxin than thioredoxin, and 200 times faster than by protein disulfide isomerase. Glutaredoxin from pig liver and bovine heart showed a similar specificity. Dethiolation rates of individual hepatocyte proteins by glutaredoxin varied in minor ways from protein to protein. For example, the 15, 30, and 48 kDa proteins were quickly dethiolated by glutaredoxin, and the 27, 28, and 77 kDa proteins were dethiolated more slowly.

Although glutaredoxin was the most effective enzyme for dethiolation, it did not catalyze the reduction of insulin disulfides. On the other hand, protein disulfide isomerase and thioredoxin were effective with insulin disulfides. This indicates that glutaredoxin may be specific for S-glutathiolated proteins. The cellular concentration of glutaredoxin and the reactivity of purified pig liver glutaredoxin strongly suggest that glutaredoxin probably accounts for most of the dethiolation in cells.

The standard redox potential of pig liver glutaredoxin was -0.16 V at pH 7.0 and 25°C, surprisingly higher than that (-0.26 V) of E. coli thioredoxin (41). This high redox potential indicates that glutaredoxin itself is a weaker reductant than thioredoxin. Glutathione-binding
at the active sites of glutaredoxin is suggested as a structural factor for the effective dethiolation. The high redox potential of glutaredoxin also implies that glutaredoxin may be in the fully reduced state in the liver under the reported concentrations of GSH and GSSG (10 mM GSH, 30 μM GSSG), and may be fairly active even under the highly oxidized conditions.

Since the cellular glutathione concentration may be as high as 10 mM, thiol-disulfide reaction of S-glutathiolated proteins by glutathione (GSH) may be an important dethiolation mechanism. Therefore, the nonenzymatic dethiolation of 35S-glutathiolated hepatocyte proteins by glutathione alone was also studied in this report. All of the S-thiolated proteins were dethiolated by glutathione. The dethiolation rates of individual proteins varied only in minor ways from protein to protein.

The dethiolation of the S-glutathiolated proteins was saturable with respect to the concentration of glutathione. The dethiolation rates follow the Michaelis-Menten kinetics with the half maximal rate at 0.16 mM glutathione, suggesting that dethiolation of S-glutathiolated proteins by reduced glutathione (GSH) may occur at the maximal rate in cells. This implies that thiol-disulfide exchange reaction by glutathione may also be significant in dethiolation.

The dethiolation rate decreased in the presence of denaturing agents such as guanidinium chloride and sodium dodecylsulfate. The glutathione analogs, S-methyl glutathione and glutathionesulfonate, also inhibited the dethiolation by glutathione. The saturation kinetics, and inhibition by glutathione analogs and denaturants, strongly suggested that glutathione forms a noncovalent complex with S-glutathiolated proteins during dethiolation.

The noncovalent glutathione-binding to the S-glutathiolated proteins suggests that those proteins may bind GSH even in the reduced state, unless S-glutathiolation induces additional GSH-binding to the proteins. It is suggested in this report that those GSH-binding sites in the reduced proteins may be filled with glutathione (GSH), and thereby the proteins are ready for S-thiolation under oxidative stress.

The experiments presented in this dissertation suggests that glutaredoxin may be the major dethiolases in most cells, and nonenzymatic dethiolation by glutathione may also be a significant mechanism under certain conditions. The glutathione-binding site on glutaredoxin
may be a structural factor for the effective dethiolation by glutaredoxin. Many proteins with reactive sulfhydryls may bind glutathione (GSH) noncovalently. The bound glutathione then forms a mixed-disulfide with the "reactive" protein thiols near the binding site under oxidative stress.
REFERENCES


ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. James A. Thomas for his support and patient guidance throughout my graduate studies. I would also like to thank Drs. Herbert J. Fromm, Agustin Kintanar, Michael McCloskey, and Alan Myers for serving on my advisory committee. I am greatly indebted to Dr. William W. Wells and Dr. Angela M. Gronenborn for their generous provision of the recombinant glutaredoxin and thioredoxin.