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# Irreversible oxidation of protein cysteine residues

by

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# GENERAL INTRODUCTION

It has been appreciated for some time that reactive oxygen species represent a necessary but destructive consequence of oxidative respiration. However, current experimental evidence also supports the hypothesis that reactive oxygen plays a role in cellular signaling, and may be important for cellular homeostasis. Protein cysteine residues reflect the dual character of cellular oxidation, since cysteine residues have been implicated in both the transmission of oxidant-derived signals and as sites that are sensitive to oxidative damage. In this regard, a better understanding of the oxidative modifications that constitute permanent damage to protein cysteine may provide a clear demarcation of cellular oxidative injury.

## Reactive Oxygen: Oxidative Damage and Oxidative Signaling

The "classical" understanding of reactive oxygen species in biological systems holds that these molecules are destructive to cells. Reactive oxygen species such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals damage cells through lipid peroxidation (1), DNA strand breaks and base modification (2), and protein oxidative modifications (3-5). They are derived from several sources. For instance, aerobic cells produce energy by generating a proton gradient from the progressive transport of electrons along mitochondrial protein complexes. The redox-acitve metal centers of the complexes can pass electrons to oxygen, generating superoxide anion and potentially other reactive oxygen species (6,7). Certain compounds such as paraquat and menadione are extremely effective at uncoupling electrons from metal centers and continually generate reactive

oxygen through a process called redox cycling (8,9). In some instances, oxygen is used as an electron acceptor to re-oxidize metal centers. Xanthine oxidase contains a molybdenum complex that catalyzes the oxidation of xanthine to uric acid. Reduced molybdenum is re-oxidized by oxygen in order to regenerate the active enzyme, producing superoxide anion and hydrogen peroxide as a result (10,11). Finally, immune cells such as neutrophils and macrophages produce reactive oxygen through NADPH oxidase, myeloperoxidase, and nitric oxide synthase (12, 13) in order to destroy invading microorganisms; however, by-stander cells are often affected by the respiratory burst, potentially leading to chronic inflammation and complications in atherosclerosis and rheumatoid arthritis (14,15).

With this paradigm in mind, the role of biological antioxidants is clear. Antioxidants are a defense against damage produced by reactive oxygen species, and act antagonistically to cellular oxidation by intercepting radicals and reactive oxygen or by terminating radical chain reactions. Common cellular antioxidants include ascorbate, α-tocopherols, selenium, and low molecular weight thiols such as glutathione (16, 17). Certain proteins such as superoxide dismutase, catalase, and glutathione peroxidase have activities that remove reactive oxygen species from cells (18). Cells with depleted antioxidant capacity will experience periods of oxidative stress by effectively increasing the concentration of reactive oxygen impinging upon cellular systems. Thus, a plausible explanation for the increased levels cellular oxidative damage detected in Alzheimer's disease, Parkinson's disease, and aging is that concentrations of glutathione, a critical cellular antioxidant, are typically depleted in these diseases (19-21).

In 1987 nitric oxide was identified as the endothelium-derived relaxing factor (22,23), and with this discovery reactive forms of oxygen and nitrogen were recognized as potential

signaling molecules. Nitric oxide is a relatively stable, uncharged radical species that readily diffuses across membranes (24). It can be considered a reactive oxygen species, but is also commonly classified as a reactive nitrogen species. Nitric oxide is endogenously produced by three different isoforms of nitric oxide synthase. The endothelial and neuronal isoforms are stimulated by calcium. Nitric oxide stimulates vasodilation by binding to the heme group of guanylate cyclase, resulting in the production of cyclic guanosine monophosphate (cGMP), and consequent activity of cGMP dependent protein kinases (25). Thus, a clear signal cascade is produced through the action of a reactive oxygen species. Oxidants such as hydrogen peroxide and superoxide anion have been reconsidered as possible signaling molecules (26). Indeed, hydrogen peroxide has been shown to activate the mitogen-activated protein kinase pathway in cell culture (27), although it is unknown whether this represents a physiological signaling mechanism.

While experimental evidence supports the hypothesis that signal transduction can occur through reactive oxygen, the potential for reactive oxygen to act as a destructive oxidizing agent is always present. Nitric oxide clearly illustrates this notion since it not only functions as a cellular signal, it also reacts rapidly with superoxide anion to produce peroxynitrite, an extremely strong oxidizing agent capable of damaging a variety of cellular targets (28). Several criteria determine the role of an oxidant in a cell, including its concentration, the overall redox status of the cell, and the sub-cellular location of oxidant production. Certainly the cellular substrate "hit" by the oxidant is a critical consideration as well, and in this regard protein cysteine residues provide one of the best targets for oxidation. In fact, protein cysteines are the most sensitive amino acid to oxidative modification (29,30).

They react rapidly with a variety of oxidants, and are present in millimolar concentrations in cells, thus providing ample substrate for reactive oxygen species (5).

## Protein Cysteine Oxidative Modification

Cysteine residues are not only the most rapidly oxidized amino acid, they also demonstrate a functional versatility that is unrivaled by other amino acid residues. In fact, these two characteristics are somewhat antagonistic, since cysteine oxidation typically impairs proteins that require reduced sulfhydryls for activity. For example, cysteines act as a catalytic nucleophile in enzymes such as glyceraldehydes 3-phosphate dehydrogenase, protein tyrosine phosphatase, and cysteine proteases such as the caspases. Oxidation of these cysteines inhibits the proteins' activity (31-33). DNA-binding proteins with zinc finger motifs coordinate zinc with either four cysteine residues or a combination of two cysteines and two histidines. Oxidation of these cysteines prevents the binding of zinc (34). Finally, the C-terminal cysteine residues in proteins such as H-ras become post-translationally lipidated through thioether and thioester linkages, localizing this protein to cellular membranes. Oxidation of these cysteine residues could prevent lipidation and membrane association of H-ras (35).

The extent to which proteins are affected by cysteine oxidation *in vivo* depends on the type of oxidation that occurs. Protein thiols can undergo a variety of oxidative modifications, but a simple classification is to describe them as either reversible or irreversible (36-38). Reversible modifications can be restored to a reduced thiol through thiol-disulfide exchange reactions and include modifications such as protein S-thiolation, S-nitrosylation, and intramolecular disulfide bonds (35). The transient, switch-like nature of reversible

modifications has made them appealing candidates for cellular signaling in proteins such as protein tyrosine phosphatase, AP-1, p53, and NF-κB (32, 39-41). It is obvious that not all cysteines are involved in redox signaling, and it has been suggested that reversible oxidation of some protein cysteine residues also plays a role in the detoxification of reactive oxygen (5, 42). The critical feature of reversible cysteine oxidation, however, is that the modifications are temporary, and will only exhibit an affect until an oxidized cysteine is reduced.

On the other hand, irreversible cysteine oxidation clearly represents permanent oxidative damage. These modifications include sulfinic and sulfonic acids and cannot be reduced by thiol-disulfide exchange (37, 38). While it has been hypothesized that cells prevent irreversible cysteine oxidation through protein S-thiolation *in vivo*, periods of oxidative stress could promote the formation of these modifications (5). It is remarkable that irreversible oxidation of protein cysteine has remained relatively unstudied, since cysteine residues have prominent roles in protein functions. In order to understand how protein cysteine irreversible oxidation could occur *in vivo*, it is useful to first examine the chemistry of reversible modifications since the initial steps in both types of sulfhydryl oxidation are the same.

## Reversible Oxidation

Oxidation of a cysteine residue begins when an oxidant (Ox) reacts with a protein sulfhydryl group (PrSH), generating an unstable intermediate such as sulfenic acid (PrSOH) or thiyl radical (PrS•).

$$PrSH + Ox^{2+} \rightarrow PrSOH + Ox^{0}$$

$$PrSH + Ox^{2+} \rightarrow PrS\bullet + Ox^{1+}$$

Sulfenic acids represent a two-electron oxidation of the protein thiol group and are generated by non-radical reactive oxygen species such as hydrogen peroxide (43) and hypochlorous acid (44). Thiyl radicals are produced from one-electron oxidations of the thiol group and can be generated by superoxide anion (45), hydroxyl radicals, and peroxyl radicals (46). In the presence of a low molecular weight thiol (RSH), these intermediates promote the formation of a disulfide bond between the low molecular weight thiol and the protein:

$$PrSOH + RSH \rightarrow PrS\text{-}SR + H_2O$$

$$PrS\bullet + RSH \rightarrow PrS\text{-}SR\bullet^- + O_2 \rightarrow PrS\text{-}SR + O_2\bullet^-$$

Sulfenic acids will generate a disulfide with a release of water, while thiyl radicals will require an electron acceptor such as molecular oxygen to remove one electron in order to form a stable disulfide (5). This mechanism has been termed protein S-thiolation. Alternatively, another protein sulfhydryl group can react with the sulfenic acid or thiyl radical to generate an intra- or inter-molecular disulfide bond. Proteins such as peroxyredoxins reduce hydrogen peroxide through this kind of mechanism (42).

While any low molecular weight thiol potentially S-thiolates protein cysteine, S-glutathiolation is the most prevalent form of S-thiolation *in vivo*, due to the low millimolar concentrations of glutathione in most cell types. Evidence for protein S-thiolation *in vivo* has been well documented in both cell culture and animal models. In cell models, it has been shown that the sulfhydryl groups of creatine kinase (47) and carbonic anhydrase III (48) are rapidly S-thiolated in response to reactive oxygen generators and thiol specific oxidants. S-thiolated cysteine residues can be restored to protein sulfhydryls by the action of glutaredoxin through thiol-disulfide exchange reactions (49):

$$PrS\text{-}SR + R'SH \rightarrow PrSH + R'S\text{-}SR$$

Thus, S-thiolation and dethiolation represents a cyclic mechanism that restores protein sulfhydryls to a reduced state through the formation of a reversible disulfide bond.

Protein S-nitrosylation also is a reversible modification, and it occurs by a mechanism that is somewhat analogous to the development of a disulfide from a thiyl radical. Protein sulfhydryl groups react directly with nitric oxide (NO•), and oxygen removes the extra electron in order to generate a stable nitrosothiol bond (50).

$$PrSH + NO\bullet \rightarrow PrS\text{-}NO\bullet^{-} + O_2 \rightarrow PrS\text{-}NO + O_2\bullet^{-}$$

It has also been suggested that oxygen first removes an electron from nitric oxide, followed by the formation of the nitrosothiol bond. The nitric oxide moiety can be transferred between thiols by transnitrosylation, a mechanism similar to thiol-disulfide exchange (51). Thus, S-nitrosylated proteins can be restored to a protein thiol by through transnitrosylation, a reaction that may be accelerated by nitrosothiol reductase (52). Nitrosothiols are surprisingly stable and have been suggested to exert biological effects *in vivo* (53). For instance, H-ras GTPase activity is believed to be stimulated by the S-nitrosylation of cysteine 118 (54). Other proteins with reactive thiols such as glyceraldehyde 3-phosphate dehydrogenase, creatine kinase, and glycogen phosphorylase have also been shown to be susceptible to S-nitrosylated in mouse models after stimulation of nitric oxide synthase (55).

## Irreversible Oxidation

Irreversible oxidation results from the progressive oxidation of unstable cysteine intermediates such as sulfenic acids and thiyl radicals. For instance, sulfinic acids are produced when the sulfur atom of a sulfenic acid experiences a two-electron oxidation.

Another two-electon oxidation of the sulfinic acid produces a sulfonic acid (also called cysteic acid) (52).

$$PrSOH + Ox^{2+} \rightarrow PrSO_2H + Ox^0$$

$$PrSO_2H + Ox^{2+} \rightarrow PrSO_3H + Ox^0$$

Thiyl radicals may produce sulfinic acids by reacting with molecular oxygen to produce a thiyl peroxyl radical, which subsequently rearranges to produce a sulfinic acid with the concurrent loss of a single electron (56).

$$PrS\bullet + O_2 \rightarrow PrS\text{-}OO\bullet + O_2 \xrightarrow{H^+} PrSO_2H + O_2\bullet^-$$

Again, the sulfinic acid could be progressively oxidized to a sulfonic acid. Little is known about protein sulfinic and sulfonic acids. *In vitro* protein oxidation experiments have confirmed the presence of protein sulfinic and sulfonic acids by mass spectrometry (57), but very little is known about these modifications *in vivo*. Evidence for irreversibly oxidized forms of carbonic anhydrase III have been detected in rat liver tissue and isolated hepatocytes using isoelectric focusing and western blotting, but these experiments have not specifically identified the modifications as sulfinic or sulfonic acid. These experiments did suggest that an irreversibly oxidized form of the protein accounted for 10-20% of the total carbonic anhydrase III (38); however, demonstrating that irreversibly oxidized protein cysteine represents a major form of oxidative damage in cells.

It has been hypothesized that low molecular weight thiols prevent irreversible oxidation by reacting with sulfenic acids or thiyl radicals before they become further oxidized. When low molecular weight thiols such as glutathione become depleted or reactive oxygen species concentrations increase, S-thiolation may not occur rapidly enough to prevent unstable protein thiol intermediates from becoming irreversibly oxidized (58). Moreover, the

disulfide bond of an S-thiolated protein may protect the protein from irreversible oxidation since disulfides are oxidized relatively slowly in comparison to reduced thiols (59). Protein S-nitrosylation are relatively stable, although experiments with S-nitrosylated matrix metalloproteinase-9 suggest S-nitrosothiols may also oxidize to sulfinic acids over time (60).

A variety of amino acid modifications have been used as markers for protein oxidation, including hydroxylation of leucine, valine, and tyrosine; total protein carbonyls; and 3-nitrotyrosine. These amino acids have been shown to be elevated in aged individuals, atherosclerotic plaques, and neurodegenerative diseases (61). Cysteine sulfinic and sulfonic acids could also function as a marker for determining the overall oxidative status of cells, and are also likely to increase in an age-dependent manner. Certainly if cellular glutathione decreased in an age related fashion, S-glutathiolation will be less effective in preventing irreversible oxidation (74). Protein degradation mechanisms also slow with age and could contribute to an accumulation of irreversibly oxidized proteins (62).

Suitable methods are required in order to detect irreversibly oxidized proteins *in vivo*. The sulfonic acids can be readily quantitated by amino acid analysis (63), but the method has not been applied to the analysis of protein sulfinic acids. Using this method it would be possible to relate the amount of damage to either total cysteine in a sample or another amino acid. Irreversible damage to specific proteins could be examined using two-dimensional electorphoresis followed by mass spectrometry, but the method is cumbersome for multiple samples and will not necessarily provide quantitative results. Ideally, irreversible oxidation of individual proteins could be assessed using gel-base techniques coupled with western blotting. This approach has been used to detect S-glutathiolated and irreversibly oxidized forms of carbonic anhydrase III *in vivo* (38), but the method may not be useful for some

proteins, since other post-translational modifications such as phosphorylation potentially complicate the results.

## Dissertation Organization

This dissertation contains a general introduction, three chapters, a general summary and conclusions section, a references section for the general introduction and summary and conclusions section, and one appendix. The papers making up Chapters 1 and 2 have been published in scholarly journals. Chapter 3 will be formatted for publication in an appropriate journal. Materials presented in the appendix have also been published as part of a joint project with another laboratory.

Chapter 1 presents a novel method for quantitating sulfinic and sulfonic acids. All experiments were performed by the author, except for the data regarding irreversible oxidation of soluble liver protein extracts presented in Table II which was obtained by Tiequan Zhang. The majority of the writing was provided by the author, with additional writing and revision provided by Dr. James Thomas.

Chapter 2 demonstrates the relationship between protein S-glutathiolation and irreversible oxidation of carbonic anhydrase III and uses the methods presented in Chapter 1. Experiments presented in Figures 2, 6, 8, and 12 were conducted by the author. The author also wrote the result sections corresponding to these figures and Table 1, along with figure legends, and explanations relating to the methods. All other writing was provided by Dr. Robert Mallis. Final editing provided by Dr. Robert Mallis and Dr. James Thomas.

Chapter 3 further validates the hypothesis presented in Chapter 2 using creatine kinase as a second model protein. A relationship between protein cysteine irreversible

oxidation, aging, and glutathione concentrations is further developed. The author performed all experiments and writing, with suggestions for revision provided by Dr. James Thomas.

Finally, the appendix contains supplementary material relevant to Chapters 1-3, all experiments relating to the figures and table were conducted by the author. Dr. Jamil Momand contributed the largest body of writing in the published manuscript, but the writing in the appendix is by the author.

Advice, guidance, discussion, and editing were provided throughout by Dr. James Thomas.

# CHAPTER 1. QUANTITATION OF PROTEIN SULFINIC AND SULFONIC ACID, IRREVERSIBLY OXIDIZED PROTEIN CYSTEINE SITES IN CELLULAR PROTEINS.

A paper published in the series *Methods in Enzymology*[1]

Michael Hamann[2], Tiequan Zhang[3], Suzanne Hendrich[3], and James A. Thomas[2,4]

## Introduction

Irreversible oxidation of reactive protein sulfhydryls is potentially important in the normal cellular function of a myriad of proteins. Reactive sulfhydryls are found as catalytic centers in some enzymes (1,2), as metal-binding sites whose structural role is reminiscent of that for protein disulfides (3,4), in lipid attachment sites (5), and on protein surfaces where their function is not clearly delineated at present (6,7). In each case these protein sites may undergo reversible oxidation to either or both S-thiolated and S-nitrosylated forms. It has been suggested that these protein sites may be irreversibly oxidized to either a sulfinic or sulfonic acid when protective mechanisms fail. However, data on the extent of irreversible damage are missing for lack of a suitable method to quantitate this protein modification. The method described here provides a quantitative method for determination of both protein sulfinic acid and protein sulfonic acid and demonstrates that the sulfinic acid oxidation state

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is far more prevalent than previously thought. Further we suggest that normal tissues contain a significant amount of irreversibly damaged protein sulfhydryls.

Figure 1 shows an experiment that defines the need for methods to study protein sulfinic and sulfonic acids. The oxidation state of creatine kinase (Fig. 1, lane 1) was monitored by thin gel electrofocusing as reported earlier in this series (8). Oxidation by hydrogen peroxide in the presence of glutathione (Fig. 1, lanes 2 and 3) produced a dithiothreitol-sensitive oxidation (additional acidic protein bands in lane 2). The sensitivity of the protein modification to a sulfhydryl reductant, dithiothreitol (DTT), is compatible with the charge changes resulting from S-glutathiolation. When the protein was oxidized in the absence of glutathione (Fig. 1, lanes 4 and 3, DTT was without effect on the oxidation-generated charged forms of the protein. These new forms probably represent either protein sulfinic or sulfonic acid derivatives of reactive protein cysteine.

## Principles of Method

Irreversible oxidation of protein sulfhydryls (not reduced easily by thiol-based reagents) may produce either sulfinic or sulfonic forms of protein cysteine. The procedure presented here provides a method to determine both protein sulfinic and sulfonic acid. Protein sulfhydryls are first alkylated with iodoacetamide to prevent artifactual modification of exposed reactive cysteines during sample processing. Iodoacetamide is the reagent of choice for protection, as the S-acetylated forms of cysteine that are generated during acid hydrolysis are stable to subsequent quantification steps. The alkylated protein is then denatured with urea in the presence of a thiol reductant to expose buried protein sulfhydryls. Exposed sulfhydryls are again quickly alkylated with iodoacetamide. The completely

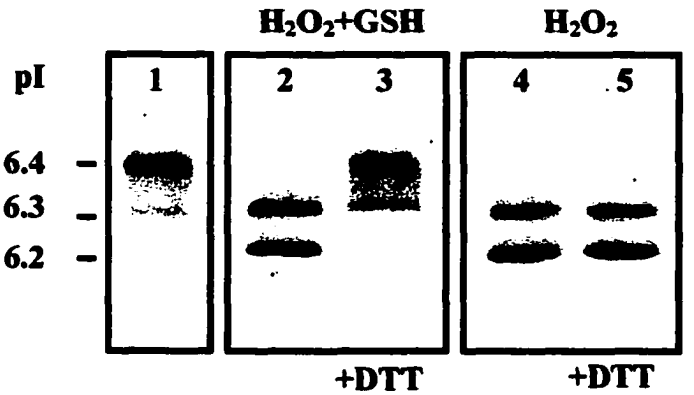



**Figure1. Reversible and irreversible oxidation of creatine kinase.**

Lane 1 shows untreated creatine kinase. In lanes 2-5, creatine kinase in 20 mM β-glycerophosphate, pH 7.4, was oxidized with 0.6 mM $H_2O_2$ for 15 min at 37° in the presence (lanes 2/3) and absence (lanes 4/5) of 0.3 mM GSH. After reaction, an aliquot was reduced with 10 mM dithiothreitol (DTT, lanes 3/5). All samples were alkylated with 40 mM iodoacetamide, and protein was stained with Coomassie blue.

alkylated protein sample is suitably prepared for acid hydrolysis and amino acid determination (Fig. 2A). However, cysteine sulfinic acid is not detectable after acid hydrolysis (Fig. 2D), necessitating a modification of the procedure. It is likely that a modification to the free amino group of the sulfinic acid prevents its detection. To overcome this difficulty, the protein sample is mildly oxidized with sodium hypochlorite to oxidize sulfinic acid residues to sulfonic acid. Figure 3 illustrates the use of hypochlorite on both a reduced and a partially oxidized form of creatine kinase. There is no cysteic acid in either protein unless the samples are oxidized with hypochlorite. The reduced creatine kinase has no cysteic acid even after hypochlorite addition. At least 10 mM sodium hypochlorite is required for complete oxidation of protein sulfinic acid in the oxidized creatine kinase sample. The protein cysteine content is not altered significantly by hypochlorite. By a suitable comparison of protein samples with and without added sodium hypochlorite, it is possible to determine both protein sulfinic and sulfonic acids quantitatively.

After all reactants are removed by extensive dialysis, protein samples are hydrolyzed by vapor phase techniques and dissolved in pure water for analysis. Dialysis is necessary to remove interfering materials that prevent quantitative reaction with the fluorescence reagent, o-phthalaldehyde. Dissolved protein hydrolyzates are derivatized with o-phthalaldehyde by a sample autoinjector just prior to separation on a reversed-phase high-performance liquid chromatography (HPLC) column.

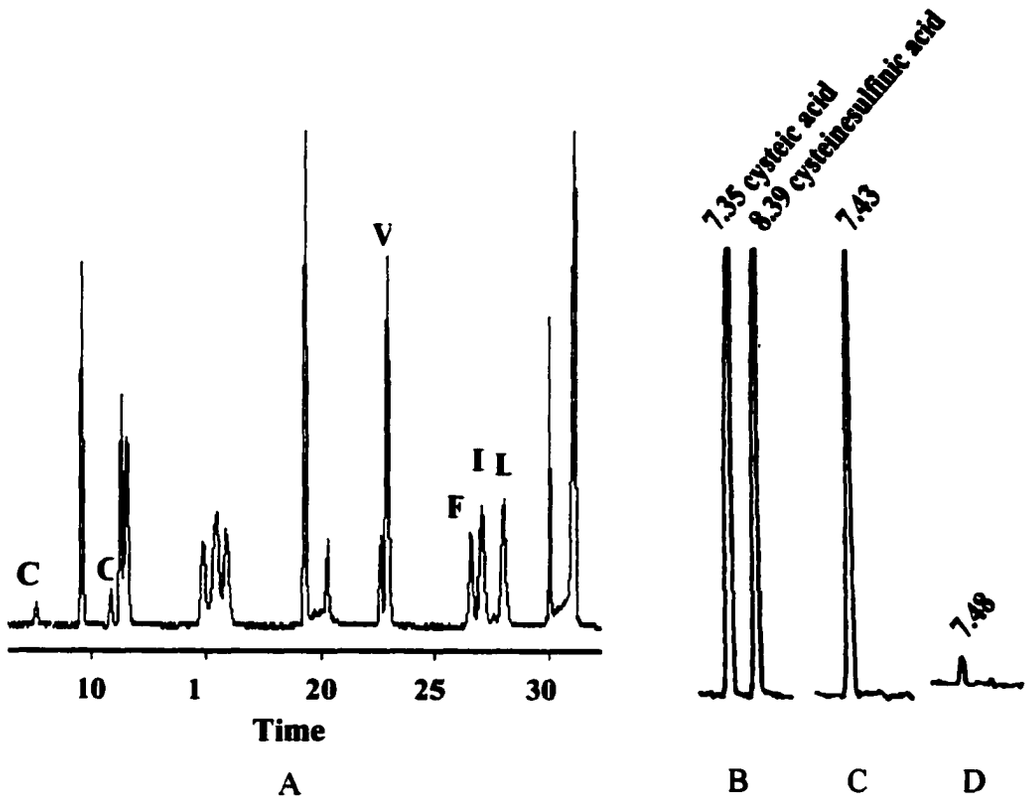



**Figure 2. Reversed-phase HPLC of o-phthalaldehyde-derivatized amino acids.**

(A) Fifty picomoles of cysteic acid was added to a hydrolyzate of creatine kinase, and the sample was analyzed by reversed-phase HPLC. The column was equilibrated with 50 mM acetate buffer, pH 5.4, at 1.0 ml/min, and amino acids were eluted with a gradient of acetonitrile (AN): 0 to 13% AN, first 6 min; 13% AN, next 8 min; 13 to 26% AN, next 2 min; and 26% AN, next 12 min. o-Phthalaldehyde-derivatized amino acids were quantified with a fluorescence detector (excitation $\lambda = 334$ nm, emission $\lambda = 425$ nm). CA, cysteic acid; C, carboxymethyl cysteine; V, valine; F, phenylalanine; I, isoleucine; L, leucine. (B) Separation of 80 pmol cysteic and cysteine sulfinic acid by reversed-phase HPLC. (C) Separation of 80 pmol of cysteic acid after the acid hydrolysis procedure. (D) Separation of 80 pmol of cysteine sulfinic acid after acid hydrolysis.

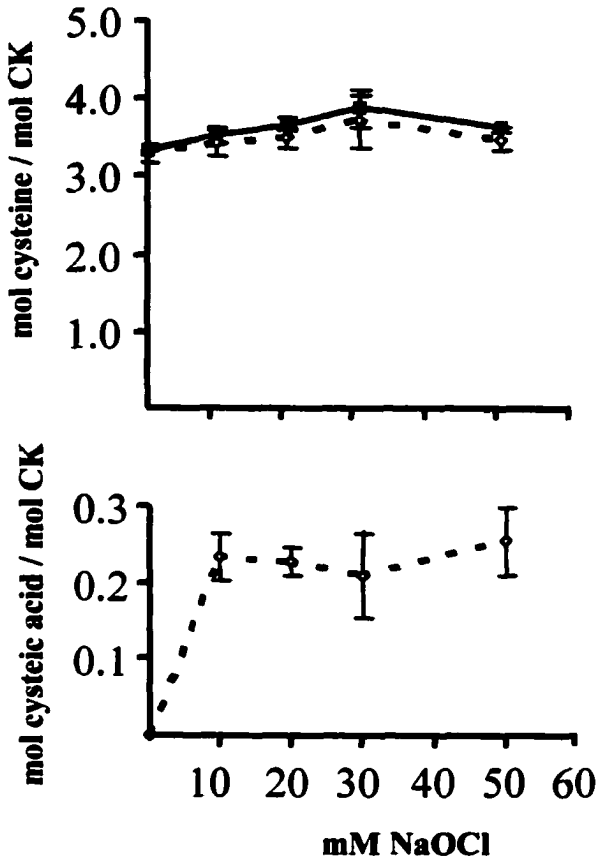



**Figure 3. Oxidation of protein sulfinic acid by sodium hypochlorite.**

Both reduced and hydrogen peroxide-oxidized creatine kinases were analyzed for cysteine and cysteic acid without hypochlorite and after oxidation with 0, 10, 20, 30, and 50 mM sodium hypochlorite. (Top) Cysteine in reduced (solid line) and oxidized (dashed line) CK. (Bottom) Cysteic acid from oxidized creatine kinase. Reduced creatine kinase contained undetectable amounts of cysteic acid (not shown).

## Detailed Procedure

### Sample preparation

Pure protein samples in 20 mM β-glycerophosphate are completely reduced with 10 mM DTT for 20-30 min at 37° before processing. Proteins are then alkylated with 40 mM iodoacetamide for 20 min at room temperature in the dark. (Iodoacetamide stock solutions are made just prior to use.) At this stage, it is convenient to analyze the oxidation state of a pure protein by gel electrofocusing as in Fig. 1. This analysis provides corroborative data (by densitometry) for comparison with sulfinic and sulfonic acid analysis. More complex mixtures of proteins are prepared in a similar manner as follows. This procedure describes the details for a mixture of soluble proteins from rat liver, but its general nature suggests that other biological samples may be processed with only minor changes. A protein extract from rat liver is homogenized in 20 mM β-glycerophosphate, pH 7.0, containing 5 mM EDTA and 5 mM EGTA (0.1 g liver/ml buffer). The homogenate is centrifuged at 16,000*g* for 15 min and then at 160,000*g* for 30 min to generate a particulate-free sample. Soluble proteins are alkylated with 40 mM iodoacetamide for 20 min at room temperature in the dark.

### Protein reduction and denaturation

Iodoacetamide-treated protein samples are suitably diluted with 100 mM phosphate buffer, pH 7.4, containing both 15 mM DTT and 9.5 M urea. The protein is incubated at 37° for 20 min to completely reduce and denature the sample. It is again alkylated with 40 mM iodoacetamide and is then divided into two aliquots. One aliquot is treated with 10 mM NaOCl for 5 min at room temperature, followed by 50 mM DTT. The second aliquot is

treated only with DTT. Oxidation with NaOCl may produce samples that have a yellow color, but dithiothreitol removes the color.

**Acid hydrolysis**

Acid hydrolysis requires that urea be removed from the sample. Samples are dialyzed for approximately 24 hr. The protein is efficiently hydrolyzed in small amounts with a gas-phase hydrolysis apparatus such as the Waters Accu-Tag. Sample tubes (5 x 60 mm) are precleaned with deionized water and heated at 500° for 8 hr. Each hydrolysis tube is loaded with 30-50 μl of sample containing 5-15 μg of protein. Samples are dried in a Speed-Vac and placed in a reaction vessel with 350 μl 6 N HCl and 10 μl of phenol. The vessel is evacuated and filled with nitrogen three times to eliminate unwanted air and is finally evacuated to ~80 milliTorr. Samples are hydrolyzed in a heat block at 150° for 65 min; after hydrolysis, excess HCl is removed by vacuum.

**HPLC analysis**

Samples are dissolved in a suitable volume of deionized water for HPLC separation. Because reaction with o-phthaladehyde (OPA) produces a somewhat unstable product, it is convenient to process samples with an autosampler with the capability to mix samples immediately before injection. A stock of OPA is prepared by dissolving 20 mg of OPA in 500 μl HPLC grade methanol, followed by 20 μl of 3-mercaptopropionic acid and 4.5 ml of 400 mM borate buffer, pH 9.5. The autosampler mixes one part OPA solution with four parts protein hydrolyzate, waits for 1 min, and then injects the sample onto a column (150 x 4.6 mm, 5 μm 125 Å). Conditions for elution of the column are described in Fig. 2.

## Analysis of Data

When analyzing pure proteins with a known amino acid composition, the amount of cysteic acid or cysteine in the sample can be normalized to the expected amount of leucine, valine, phenylalanine, or isoleucine. These peaks all gave quantitatively similar results for the protein samples tested. Data are reported as mole fraction cysteic acid or cysteine.

If the amino acid composition of the sample is unknown, it is necessary to compare the cysteic acid or cysteine peak to the total amino acid concentration of the sample injected onto the column. Thus, a small aliquot of the aqueous sample is analyzed in comparison to an amino acid standard with a similar o-phthalaldehyde-based method (9). The total amino acid concentration is calculated from the total fluorescence of the sample. Data are reported as the percentage of the total amino acid content of the sample.

Data in Table I show the analysis of two pure proteins with reactive cysteines. The samples are analyzed by gel electrofocusing prior to amino acid analysis (Fig. 4). The quantitative densitometry results for these samples, summarized in Table I, suggest that the oxidized creatine kinase contains 0.3 irreversibly oxidized cysteines and the oxidized carbonic anhydrase III contains nearly 0.4. The amino acid analysis of creatine kinase shows that it contains no cysteic acid (Table I, column C) and 0.23 cysteine sulfinic acid (Table I, column E). Thus, the single reactive cysteine in creatine kinase is stable as a sulfinic acid. Analysis of carbonic anhydrase III shows it to contain 0.13 mol fraction cysteic acid and 0.13 sulfinic acid. Because this protein contains two reactive cysteines, it is tempting to suggest that one is more easily oxidized to a cysteic acid than the other. Normalization of the data to leucine, valine, phenylalanine, or isoleucine gave comparable results.

**Table I. Sulfinic and cysteic acid content of creatine kinase and carbonic anhydrase III.**

| Protein | Total irreversibly oxidized protein[a] | Cysteine (mol/mol protein) | | Cysteic acid (mol/mol protein) | | Calculated sulfinic acid (column D – column C) |
|---|---|---|---|---|---|---|
| | | A without NaOCl | B with NaOCl | C without NaOCl | D with NaOCl | |
| Creatine kinase[b] | | | | | | |
| Reduced[a] | 0.04 | 3.3±0.01 | 3.5±0.1 | ND[d] | ND | |
| Oxidized[a] | 0.33 | 3.3±0.1 | 3.4±0.2 | ND | 0.23±0.03 | 0.23±0.03 |
| Carbonic anhydrase III[c,d] | | | | | | |
| Reduced[a] | ND | 3.9±0.1 | 4.3±0.1 | ND | ND | |
| Oxidized[a] | 0.38 | 3.7±0.1 | 3.8±0.1 | 0.13±0.01 | 0.26±0.02 | 0.13±0.01 |

[a] Protein samples illustrated in Fig. 4. The amount of irreversibly oxidized creatine kinase and carbonic anhydrase III was determined by densitometry of the isoelectric-focusing gel.

[b] Containing a total of 4 cys.

[c] Containing a total of 5 cys.

[d] Not detectable, i.e., detection limit >0.03 mol/mol

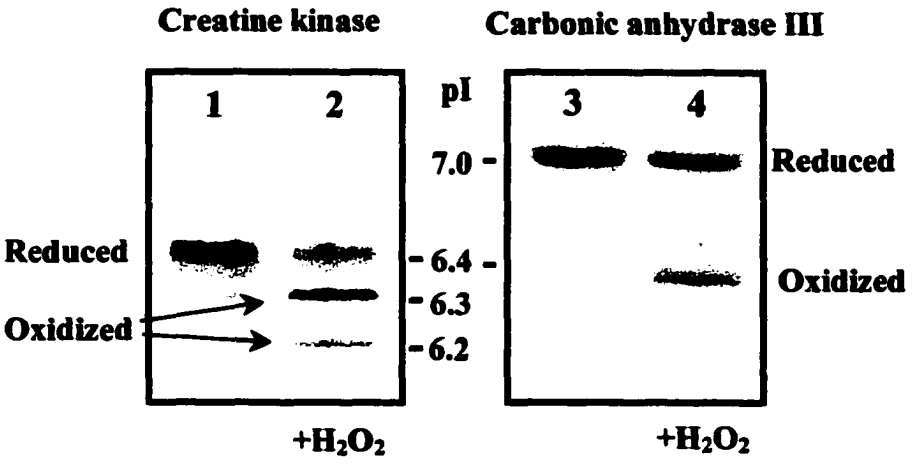



**Figure 4. Irreversibly oxidized creatine kinase and carbonic anhydrase III.**

Creatine kinase and carbonic anhydrase III were treated with hydrogen peroxide (0.2 and 2 mM, respectively) for 10 min at 37° in 20 mM β-glycerophosphate (pH 7.4). Samples were reduced with dithiothreitol and analyzed by gel electrofocusing as described in Fig. 1.

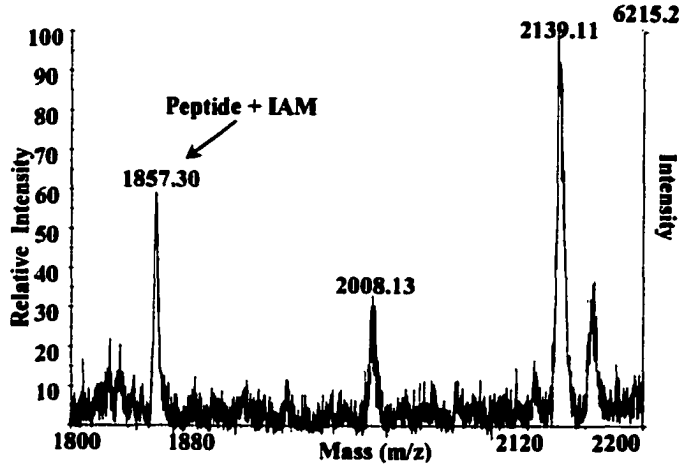
In order to confirm that oxidized creatine kinase contains a cysteine in the sulfinic acid form, a sample of the protein is analyzed by MALDI mass spectrometry. For this analysis the reduced protein is compared with the oxidized and NaOCl-treated oxidized creatine kinase. Peptides are generated as described in Fig. 5. Analysis focuses on the peptide containing Cys-283 (residue 276 to 292), the most reactive cysteine in creatine kinase. Figure 5A shows that the reduced protein contains a peptide with a mass consistent with the reduced form of Cys-283 and no other appropriate peaks of a similar mass. Figure 5B shows that the oxidized protein contains a peptide with a mass consistent with the sulfinic acid form of Cys-283, and Fig. 5C shows that NaOCl converts that peptide to one with a mass consistent with a cysteic acid. Thus, mass spectral data confirm the presence of a sulfinic acid derivative in creatine kinase.

The applicability of this method to other proteins is shown in Table II. The cysteine, cysteine sulfinic acid, and cysteic acid content of five different pure proteins and the protein mixture from a soluble extract of rat liver are determined. Data for cysteine content reflect some variability, but still a good consistency for the analysis of this amino acid with different proteins. The samples of BSA, hemoglobin, and hexokinase all contain significant amounts of sulfinic acid and less sulfonic acid. The analysis of soluble rat liver proteins suggests that the mixture contains a rather typical amount of cysteine (approximately 3.5%), little cysteic acid, and 0.05% sulfinic acid. A simple calculation shows that between 1 and 2% of the cysteine is irreversibly oxidized in this sample. This number may have more significance if one considers that most protein cysteines are buried and not exposed to oxidizing conditions. A generous estimate might suggest that one in four cysteines is "reactive" to oxidants. Based on that assumption, the protein contains about 0.9% reactive cysteines, of which more than

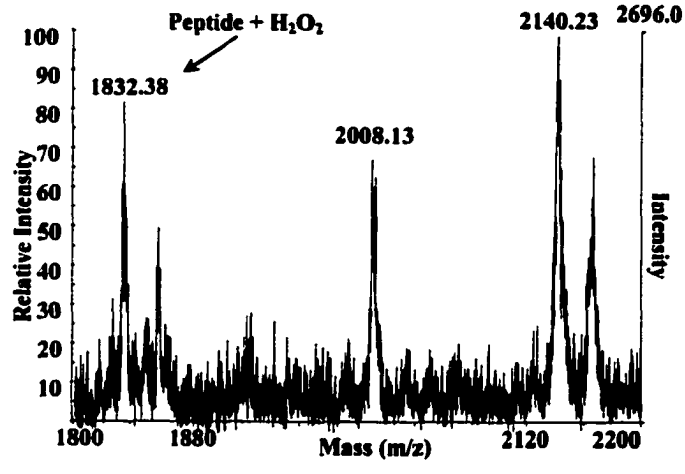
**Figure 5. Identification of cysteine sulfinic acid in oxidized creatine kinase.**

Peptides from reduced, highly oxidized, and NaOCl-treated oxidized creatine kinase (oxidized with 0.6 mM $H_2O_2$ for 10 min at 37° in 20 mM β-glycerophosphate, pH 7.4) were obtained by digestion with trypsin (0.03 mg/ml) for 5 hr at 37° followed by V8 protease (0.02 mg/ml, 5 hr, 37°). Digests were applied to MALDI sample plates with α-cyano-4-hydroxycinnamic acid for mass spectral analysis, and spectra were collected in the negative ion mode ([M-H]). (A) Peptides from reduced, IAM-treated creatine kinase. The calculated average mass of the peptide containing Cys-283 (plus the acetamide modification) was 1857.2, and the unmodified peptide mass was 1800.1. (B) Peptides from hydrogen peroxide treated creatine kinase. The calculated average mass of the peptide with a Cys-283 converted to a sulfinic acid was 1832.1. (C) Peptides from sodium hypochlorite and hydrogen peroxide-treated creatine kinase. The calculated mass of the peptide containing Cys-283 as a sulfonic acid was 1848.1. Peaks at ~2008 and ~2140 are shown to compare the ionization efficiency of each sample.

A


Peptide + IAM
1857.30
2008.13
2139.11
6215.2
Relative Intensity
Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
1800
1880
2120
2200

B


Peptide + $H_2O_2$
1832.38
2008.13
2140.23
2696.0
Relative Intensity
Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
1800
1880
2120
2200

C

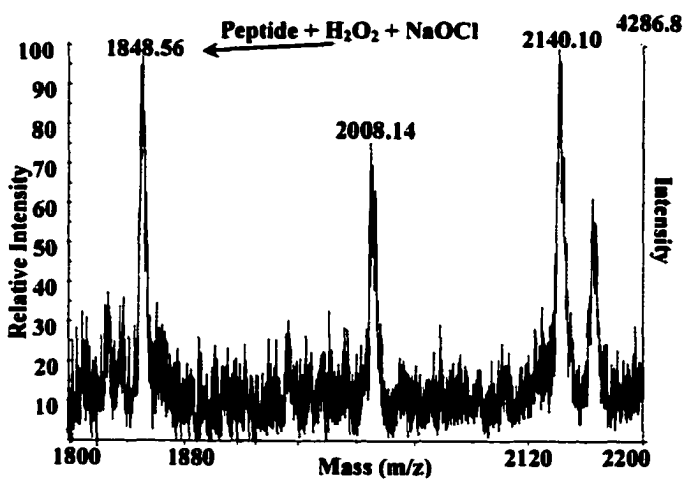

Peptide + $H_2O_2$ + NaOCl
1848.56
2008.14
2140.10
4286.8
Relative Intensity
Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
1800
1880
2120
2200

**Table II. Sulfinic and cysteic acid content of several proteins and rat liver soluble protein extract.**

| Protein[a] | Cysteine from sequence | Cysteine (mol/mol protein) A without NaOCl | Cysteine (mol/mol protein) B with NaOCl | Cysteic acid (mol/mol protein) C without NaOCl | Cysteic acid (mol/mol protein) D with NaOCl | Calculated sulfinic acid (column D - column C) |
|---|---|---|---|---|---|---|
| BSA | 35 | 35.6±1.0 | 33.7±0.8 | ND[b] | 0.07±0.004 | 0.07±0.004 |
| Hemoglobin | 3 | 2.4±0.01 | 2.3±0.1 | ND | 0.07±0.01 | 0.07±0.01 |
| Hexokinase | 4 | 4.9±0.2 | 4.7±0.2 | 0.09±0.03 | 0.21±0.02 | 0.12±0.04 |
| Fructose-1,6-bisphosphatase | 6 | 6.2±0.2 | 6.3±0.5 | ND | ND | ND |
| GAPDH | 4 | 4.2±0.1 | 4.0±0.2 | ND | ND | ND |
| | | mol % total amino acids | | mol % total amino acids | | |
| Soluble liver protein extract | | 3.5±0.4 | 3.6±0.4 | ND[c] | 0.05±0.007 | 0.05±0.007 |

[a] Bovine serum albumin was purchased from Sigma. Human hemoglobin containing both α and β chains was a gift from M. Hargrove, Iowa State University. Yeast hexokinase was purchased from Fluka (Ronkonkoma, NY). Porcine fructose-1,6-bisphosphatase was a gift from H. Fromm, Iowa State University. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was purchased from Sigma. The soluble liver protein extract was prepared from male rat liver as described in the text.

[b] Not detectable: detection limit >0.03 mol/mol.

[c] Not detectable: detection limit >0.02% total amino acids.

5% are sulfinic acid. Thus, protein sulfinic acid is an oxidized form of cysteine that constitutes a significant fraction of the reactive protein sulfhydryls in normal rat liver tissue.

## Application

The method described here requires only small amounts of protein (10-15 μg) for analysis of the stable forms of protein cysteine. Because it is possible to obtain this amount of protein from many samples, it should now be possible to gather data on the amount and distribution of this protein damage in biological samples. The amount of protein obtained by immunoprecipitation techniques would probably be adequate for this analysis. Immunoprecipitation may make it possible to evaluate a number of individual proteins in mixed protein samples.

## Acknowledgment

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## References

1. S. P. Colowick, J. vanEys, and J. H. Park, *Comp. Biochem.* **14**, 1 (1966).
2. D. C. Watts, *in* "The Enzymes," Vol. VIII, p. 383. Academic Press, New York, NY, 1973.
3. A. Klug and J. W. R. Schwabe, *FASEB J.* **9**, 597 (1995).
4. S. Wu, N. H. Bishopric, D. J. Discher, B. J. Murphy, and K. A. Webster, *Mol. Cell. Biol.* **16**, 1035 (1996).

5. D. R. Lowy and B. M. Willumsen, *Annu. Rev. Biochem.* **62,** 851 (1993).

6. R. J. Mallis, B. W. Poland, T. K. Chatterjee, R. A. Fisher, S. Darmawan, R. B. Honzatko, and J. A. Thomas, *FEBS Lett.* **482,** 237 (2000).

7. A. M. Gold and D. Blackman, *Biochemistry* **9,** 4480 (1970).

8. Y. Ji, R. P. M. Akerboom, H. Sies, and J. A. Thomas, *Methods Enzymol.* **301,** 145 (1999).

9. H. Godel, T. Graser, P. Földi, P. Pfaender, and P. Fürst, *J. Chromatogr.* **297,** 49 (1984).

# CHAPTER 2. IRREVERSIBLE THIOL OXIDATION IN CARBONIC ANHYDRASE III: PROTECTION BY S-GLUTATHIOLATION AND DETECTION IN AGING RATS

A paper published in the journal *Biological Chemistry*[1]

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## Abstract

Proteins with reactive sulfhydryls are central to many important metabolic reactions and also contribute to a variety of signal transduction systems. In this report, we examine the mechanisms of oxidative damage to the two reactive sulfhydryls of carbonic anhydrase III. Hydrogen peroxide ($H_2O_2$), peroxy radicals, or hypochlorous acid (HOCl) produced irreversibly oxidized forms, primarily cysteine sulfinic acid or cysteic acid, of carbonic anhydrase III if glutathione (GSH) was not present. When GSH was approximately equimolar to protein thiols, irreversible oxidation was prevented. $H_2O_2$ and peroxyl radicals both generated S-glutathiolated carbonic anhydrase III *via* partially oxidized protein sulfhydryl intermediates, while HOCl did not cause S-glutathiolation. Thus, oxidative damage from $H_2O_2$ or AAPH was prevented by protein S-glutathiolation, while a direct reaction between GSH and oxidant likely prevents HOCl-mediated protein damage. In

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cultured rat hepatocytes, carbonic anhydrase III was rapidly S-glutathiolated by menadione. When hepatocyte glutathione was depleted, menadione instead caused irreversible oxidation. We hypothesized that normal depletion of glutathione in aged animals might also lead to an increase in irreversible oxidation. Indeed, both total protein extracts and carbonic anhydrase III contained significantly more cysteine sulfinic acid in older rats compared to young animals. These experiments show that, in the absence of sufficient GSH, oxidation reactions lead to irreversible protein sulfhydryl damage in purified proteins, cellular systems, and whole animals.

## Introduction

Glutathione (GSH) is an essential component in the protection of cells from oxidants. It is thought to prevent oxidation of proteins by two major mechanisms. First, it may function as a direct scavenger of reactive oxygen species (Wefers and Sies, 1983; Winterbourn, 1993; Winterbourn and Metodiewa, 1994; Pichorner *et al.*, 1995). This first function allows proteins to avoid modification during oxidative events. Second, it can form mixed disulfides with proteins, termed S-glutathiolation of proteins (Park and Thomas, 1988; Mallis *et al.*, 2000). S-glutathiolation is a reversible oxidation of protein cysteine residues that may prevent further oxidation (Park and Thomas, 1988; Lii *et al.*, 1994, Hamann *et al.*, 2001). Both the oxidation of GSH to glutathione disulfide (GSSG) and the S-glutathiolation of proteins occur within seconds of addition of oxidants to cell cultures and are among the earliest measurable reactions to occur during an oxidative event (Chai *et al.*, 1994; Lii *et al.*, 1994; Ravichandran *et al.*, 1994; Schuppe-Koistinen *et al.*, 1994; Dafré and Reischl, 1998). It is important to understand the relationship between direct scavenging and protein-mediated

scavenging of reactive oxygen species by GSH because both mechanisms may affect the functioning of a wide range of proteins in cells during oxidative events.

Carbonic anhydrase III is a cytosolic protein that can be used as a model to study S glutathiolation. It has two cysteine residues that are reactive to 1-chloro-2,4-dinitrobenzene (DTNB), alkylating agents, and oxidants (Engberg *et al.*, 1985; Chai *et al.*, 1991). It has been shown *in vitro* to be S-glutathiolated by hydrogen peroxide ($H_2O_2$), diamide, GSSG and the xanthine/xanthine oxidase $H_2O_2$/superoxide-generating system (Chai *et al.*, 1991; Lii *et al.*, 1994; Jung and Thomas, 1996). When GSH is not present to protect protein cysteines, oxidants will also cause irreversible oxidation of carbonic anhydrase III *in vitro* (Lii *et al.*, 1994; Hamann *et al.*, 2001; Thomas and Mallis, 2001). Irreversible oxidation is the formation of oxidized protein cysteine residues that are not reducible by thiol-disulfide exchange. These products may be either cysteine sulfinic acid (cysteine$SO_2H$) or cysteine sulfonic acid (cysteine$SO_3H$) (Wefers and Sies, 1983; Miller and Claiborne, 1991; Yeh *et al.*, 1996; Becker *et al.*, 1999; Hamann *et al.*, 2001). $H_2O_2$ has been shown to produce cysteine sulfinic acid in creatine kinase and both sulfinic and sulfonic acid in carbonic anhydrase III (Hamann *et al.*, 2001). Cysteine sulfinic and sulfonic acid are present in a wide range of purified proteins and cysteine sulfinic acid is found in total protein extracts of rat liver (Hamann *et al.*, 2001), and so the formation of cysteine sulfinic or sulfonic acid may be a general mechanism for damage to proteins by oxidants.

Differences in protein cysteine oxidation (reversible and irreversible) which occur with changing GSH levels may be responsible for redox regulation of cellular processes (Storz *et al.*, 1990; Sen and Packer, 1996; Cotgreave and Gerdes, 1998), although the mechanism for oxidant-mediated regulation of individual proteins is not well-characterized

on a molecular level. Cellular glutathione can affect such diverse processes as transcription (Storz *et al.*, 1990; Sen and Packer, 1996), apoptosis (Cotgreave and Gerdes, 1998), damage to DNA (Park *et al.*, 1998), cell division (Park *et al.*, 1998), proliferation of cancer cells (Terradez *et al.*, 1993), susceptibility to diseases (Terradez *et al.*, 1993; Herzenberg *et al.*, 1997; Müller and Gebel, 1998), and enzyme activity (Thomas *et al.*, 1995b). GSH was presumed to regulate these processes either by acting as an antioxidant, preventing oxidation of critical cysteines (Winterbourn and Metodiewa, 1999), or through formation of GSSG and subsequent S-thiolation of the proteins involved (Dröge *et al.*, 1994). Because GSSG levels in cells rarely reach levels necessary for S-glutathiolation, other mechanisms are necessary to explain S-glutathiolation *in vivo*. In fact, available evidence suggests that proteins are better scavengers of oxidants than low molecular weight thiols such as GSH (Mallis *et al.*, 2001). There is no reason to suspect that glutathione is a required intermediary between oxidant and protein; instead, it most likely functions to provide a free reactive thiol where steric factors prevent formation of a protein-protein disulfide. Carbonic anhydrase III is in fact S-glutathiolated in hepatocytes without corresponding increases in GSSG levels (Chai *et al.*, 1994). When GSH is depleted from hepatocytes using the γ-glutamylcysteine synthase inhibitor buthionine sulfoximine (BSO), the radical generator menadione causes irreversible oxidation of carbonic anhydrase III (Lii *et al.*, 1994). Thus, at normal GSH concentrations, reduced and S-glutathiolated forms of protein should predominate after oxidation. Irreversibly oxidized proteins may predominate when GSH concentrations are low. For example, it has been proposed that tyrosine phosphatase is reversibly regulated by S-glutathiolation, and that S-glutathiolation prevents permanent inactivation of this protein by reactive oxygen species (Barrett *et al.*, 1999a, b).

The effects of three different oxidants, $H_2O_2$, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and hypochlorous acid (HOCl) on carbonic anhydrase III are studied here. This study attempts to determine the role of GSH concentration in the protection of protein cysteine residues from irreversible oxidation. In particular it defines at what concentrations GSH reacts directly with oxidants to prevent protein oxidation, and at what concentrations it participates in S-thiolation reactions. Differences between three oxidants, $H_2O_2$, AAPH, and HOCl, are explored in this context. Experiments are also performed in intact hepatocytes to demonstrate the validity of the *in vitro* model system. Evidence shows that proteins are good scavengers of oxidants relative to GSH, and that a major role of GSH in protection of proteins is in S glutathiolation reactions. Furthermore, irreversibly oxidized forms of carbonic anhydrase III are more abundant in normal aged rat tissue as well as in oxidant treated hepatocytes when GSH is depleted.

## Results

### GSH protects carbonic anhydrase III from irreversible oxidation by $H_2O_2$

Purified carbonic anhydrase III is modified specifically on up to two cysteine residues following addition of 1-chloro-2,4-dinitrobenzene (DTNB), GSSG, diamide, *t*-butyl hydroperoxide, and xanthine/xanthine oxidase (Engberg *et al.*, 1985; Chai *et al.*, 1991; Lii *et al.*, 1994; Jung and Thomas, 1996). It is modified in cells treated with diamide, menadione, and *t*-butyl hydroperoxide (Lii *et al.*, 1994; Jung and Thomas, 1996).

$H_2O_2$ reacts readily with thiols to form sulfenic acid which reacts readily with available thiols to form disulfides (Figure 1). In the absence of available thiols, sulfenic acid will continue to react with $H_2O_2$ to form sulfinic acid and sulfonic acid (Miller and

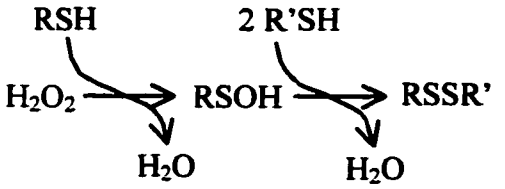



**Figure 1. The reaction mechanism of $H_2O_2$ with sulfhydryls.**

$H_2O_2$ reacts directly with sulfhydryls to form sulfenic acid (Winterbourn and Metodiewa, 1999). Sulfenic acid will then react rapidly with free thiols to form a disulfide.

Claiborne, 1991; Yeh *et al.*, 1996; Hamann *et al.*, 2001). Carbonic anhydrase III reaction with oxidants was analyzed by isoelectric focusing (IEF). In IEF gels, addition of negative charge to the protein by either S glutathiolation or oxidation to sulfinic and sulfonic acids, results in a more acidic protein band. Reversible modifications are defined by the loss of the acidic protein band on reduction with DTT. Irreversible modifications are resistant to DTT reduction. IAM, NEM and DTT were equally effective for preventing any oxidation of carbonic anhydrase III.

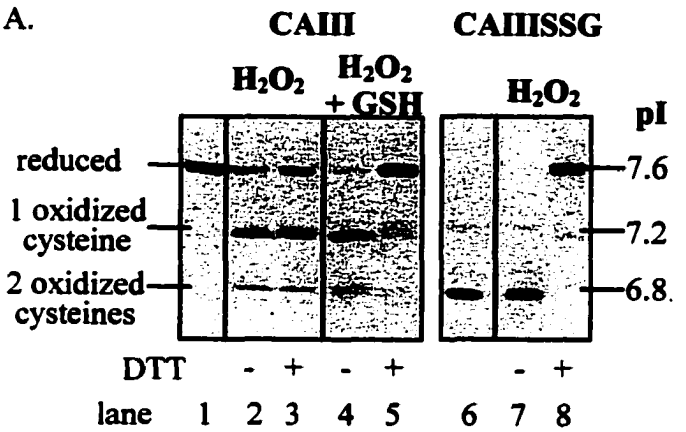
When carbonic anhydrase III was incubated with $H_2O_2$ for 15 minutes (Figure 2A), acidic bands with pI's of 7.2 and 6.8 appeared. These bands were mostly irreversible to DTT reduction (indicated by the 5% change between lanes 2 and 3), suggesting the cysteines were almost entirely irreversibly oxidized. When oxidized with $H_2O_2$ in the presence of GSH (lanes 4 and 5), carbonic anhydrase III was modified to a greater extent and the acidic forms were mostly sensitive to DTT. A minor amount of the reactive protein cysteine (~10%) remained insensitive to DTT. Fully S-glutathiolated carbonic anhydrase III migrates with a pI of 6.8 (lane 6). When this thiolated form of carbonic anhydrase III was treated with H2O2, no additional bands appeared on the gel, and all modification was completely reversible (lanes 7 and 8). These results indicated that a disulfide bond between carbonic anhydrase III and GSH prevented oxidation of the protein cysteine residues to sulfinic and sulfonic acids.

When $H_2O_2$-treated carbonic anhydrase III was analyzed for protein sulfinic and sulfonic acid, the amount of sulfinic acid was comparable to the amounts of irreversibly oxidized carbonic anhydrase III detected by IEF (Figure 2B). Sulfonic acid was not detected in these samples, indicating it was not a major product of $H_2O_2$-mediated oxidation.

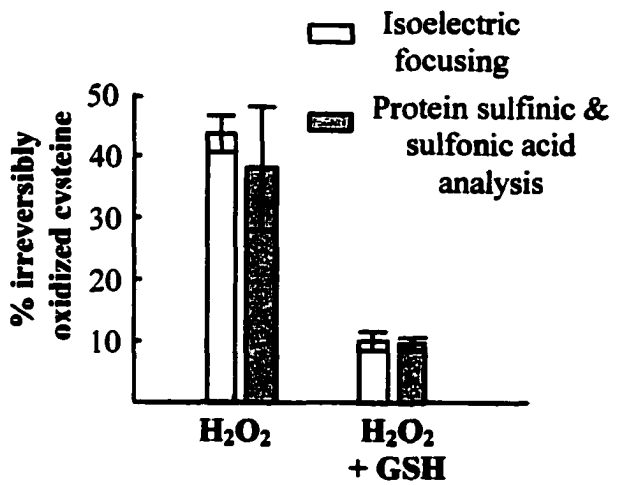
**Figure 2. GSH prevents irreversible oxidation of carbonic anhydrase III by $H_2O_2$.**

(A) Carbonic anhydrase III (30 μM) or S-glutathiolated carbonic anhydrase III was incubated with 2 mM $H_2O_2$ for 15 min at 37 °C in 20 mM β-glycerophosphate buffer pH 7.4. Carbonic anhydrase III was treated with $H_2O_2$ in either the absence or presence of 0.3 mM GSH. The reactions were stopped by treating the samples with 40 mM IAM. A portion of each was reduced with 10 mM DTT for 30 min at 37 °C before addition of IAM. (B) Carbonic anhydrase III was analyzed for irreversibly oxidized cysteine by densitometry of IEF gels or protein sulfinic and sulfonic acid analysis as described in Materials and Methods. Irreversibly oxidized cysteine was found to be entirely sulfinic acid; sulfonic acid was not detected in these samples. (Detection limit=2.5%).

A.


CAIII
CAIIISSG
H2O2
H2O2 + GSH
H2O2
pI
reduced
1 oxidized cysteine
2 oxidized cysteines
7.6
7.2
6.8
DTT - + - + - +
lane 1 2 3 4 5 6 7 8

B.


Isoelectric focusing
Protein sulfinic & sulfonic acid analysis
% irreversibly oxidized cysteine
50
40
30
20
10
H2O2
H2O2 + GSH

Untreated carbonic anhydrase III did not contain a measurable amount of sulfinic or sulfonic acid (data not shown).

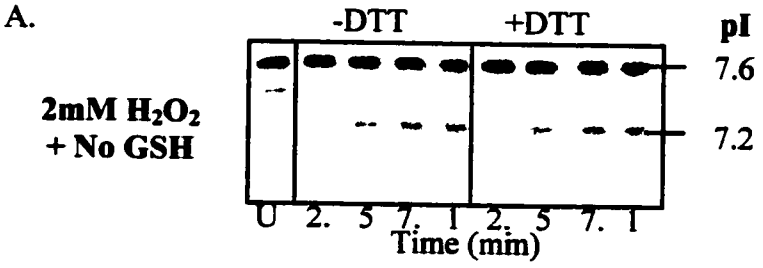
Oxidation of carbonic anhydrase III was progressive with time and reversibility depended on GSH concentration (Figure 3). In the absence of GSH (Figure 3A), the modification of carbonic anhydrase III was not reversible by DTT (compare the right and left half of the gel). When GSH was added to the reaction (Figure 3B), the oxidative modification was more pronounced and reversible (compare right and left half of each gel). Figure 3C quantifies the oxidatively modified carbonic anhydrase III. As the GSH concentration increased, reversible modification (open symbols) increased and irreversible modification (closed symbols) decreased. This indicates that reversible modification of carbonic anhydrase III is a facile reaction in comparison to irreversible modification and is favored when GSH is available.

The concentration of GSH needed to protect carbonic anhydrase III from $H_2O_2$ mediated damage was determined at two concentrations (10 μM and 30 μM) of carbonic anhydrase III. Figure 4A shows that reversible modification (open symbols) reached nearly maximal levels, and the protein was protected from irreversible oxidation (closed symbols) by 50 μM GSH at both protein concentrations. At GSH concentrations below 50 μM, irreversible oxidation of carbonic anhydrase III was dependent on carbonic anhydrase III concentration. $H_2O_2$ caused more irreversible oxidation at 5 μM and 15 μM GSH at the higher concentration of carbonic anhydrase III. Complete protection of 30 μM carbonic anhydrase III from irreversible oxidation required 50 μM GSH, while 10 μM carbonic anhydrase III was maximally protected at 15 μM GSH. Thus, protection of proteins by GSH was efficient when GSH:protein molar ratios approached one. When 5 μM GSH was

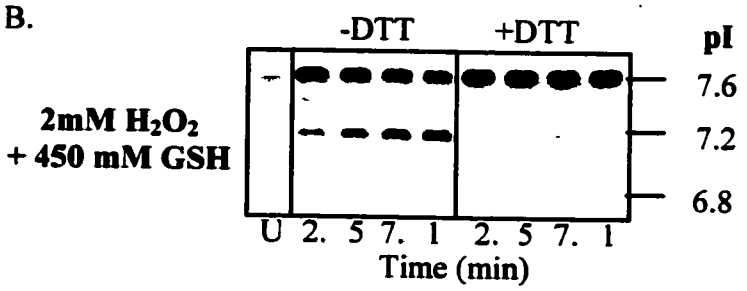
**Figure 3. Oxidation of carbonic anhydrase III with $H_2O_2$ and GSH.**

Carbonic anhydrase III (10 μM) with the indicated concentration of GSH was incubated with 2 mM $H_2O_2$ at 37 °C in 20 mM sodium phosphate buffer pH 7.4. Reactions were stopped at the indicated time by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced (right half of each gel) by incubating the reaction mixtures with 10 mM DTT for 20 minutes followed by addition of 20 mM NEM. IEF separations were performed as described in Materials and Methods. (A) Carbonic anhydrase III+$H_2O_2$ without addition of GSH. (B) Carbonic anhydrase III+450 μM GSH+$H_2O_2$. (C) Analysis of the reaction between carbonic anhydrase III, GSH and $H_2O_2$. Modified cysteines of carbonic anhydrase III were determined by analysis of the IEF separations in panels (A) and (B) of this Figure as well as a similar experiment with 150 μM GSH (not shown) as described in Materials and Methods.

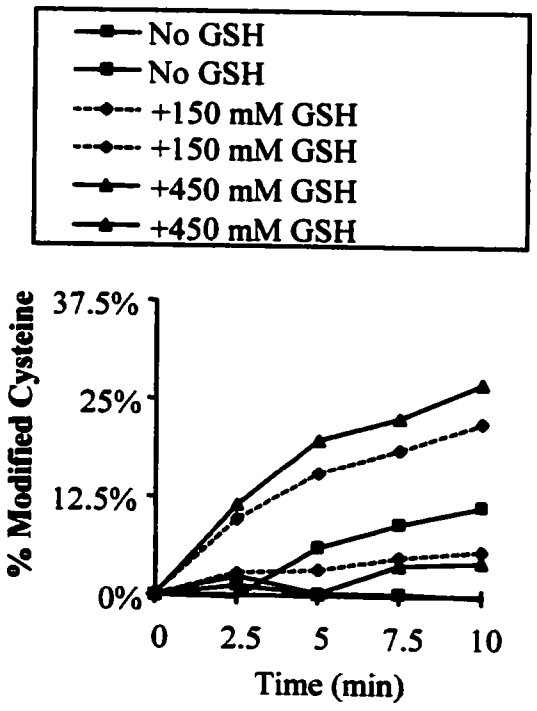
A.


-DTT
+DTT
pI
7.6
7.2
2mM $H_2O_2$
+ No GSH
U 2. 5 7. 1 2. 5 7. 1
Time (min)

B.


-DTT
+DTT
pI
7.6
7.2
6.8
2mM $H_2O_2$
+ 450 mM GSH
U 2. 5 7. 1 2. 5 7. 1
Time (min)

C.


No GSH
No GSH
+150 mM GSH
+150 mM GSH
+450 mM GSH
+450 mM GSH
% Modified Cysteine
37.5%
25%
12.5%
0%
0
2.5
5
7.5
10
Time (min)

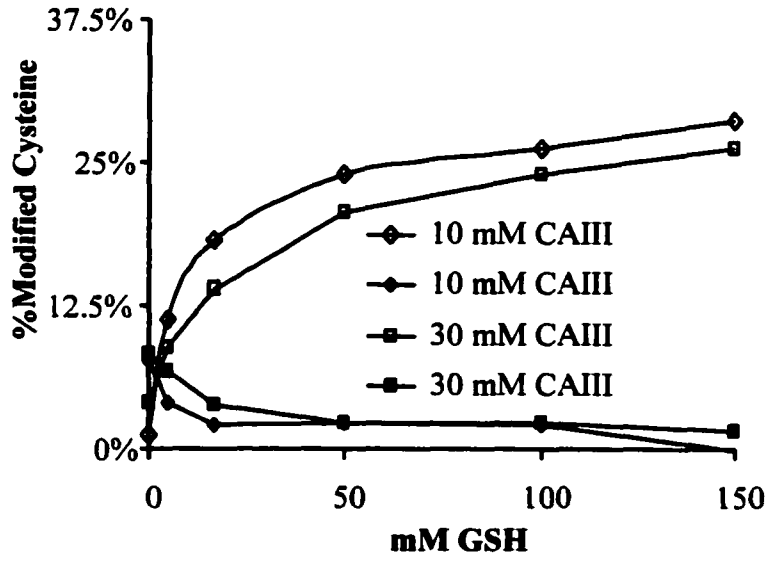



**Figure 4. Effect of GSH concentration on carbonic anhydrase III modification by $H_2O_2$.**

Carbonic anhydrase III (10 μM or 30 μM) with the indicated concentrations of GSH was incubated with 2 mM $H_2O_2$ for 10 minutes at 37 °C and in 20 mM sodium phosphate buffer at pH 7.4. Reactions were stopped, carbonic anhydrase III was reduced, and results were analyzed as in Figure 3.

present, S-glutathiolated protein accounted for 50% and 100% of the GSH available in reactions of $H_2O_2$ with 10 μM and 30 μM carbonic anhydrase III, respectively. Thus, when GSH is limiting, S-glutathiolation is likely to be the primary function of GSH in protection of carbonic anhydrase III from irreversible oxidation by $H_2O_2$. At 1.3 mM GSH, 50% of the carbonic anhydrase III was S-glutathiolated, and none was irreversibly oxidized (data not shown). Carbonic anhydrase III was modified by $H_2O_2$ even when GSH concentrations were 130 times greater than the protein concentration.

The reaction between carbonic anhydrase III and 75 μM GSSG produces little protein modification in comparison to that of carbonic anhydrase III, 150 μM GSH, and $H_2O_2$ (data not shown). GSSG clearly is not an intermediate in the S glutathiolation of carbonic anhydrase III by $H_2O_2$.

**GSH protects carbonic anhydrase III from irreversible oxidation by the peroxyradical generator AAPH**

AAPH is a compound that generates alkyl peroxyradicals by thermal decomposition (Figure 5). It has been used as a model for peroxyradical-mediated oxidation both *in vitro* and *in vivo* (Thomas *et al.*, 1995a; Gesquiere *et al.*, 1999; Marangon *et al.*, 1999; Wang and Joseph, 1999).

AAPH-mediated oxidation produced protein bands with the same acidic pI's as those produced by $H_2O_2$ (Figure 6A, lanes 2 and 3) and an additional band with a more alkaline pI. These bands were largely insensitive to DTT reduction. A protein band with a more alkaline pI could result from the radical addition of a positively charged amidinopropane group from AAPH, or by the loss of a negative charge by decarboxylation of an acidic amino acid. It is

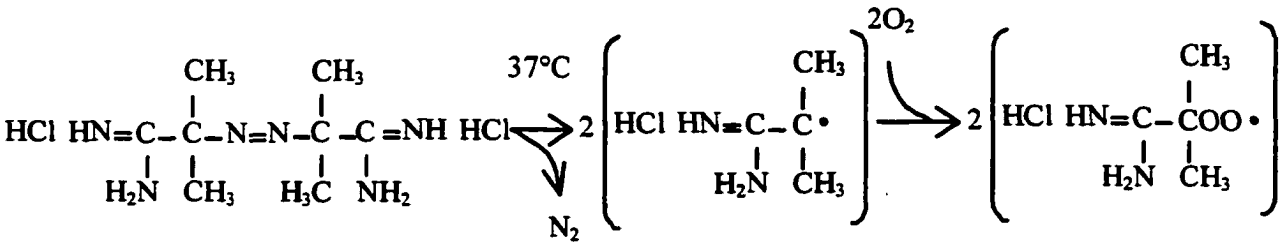


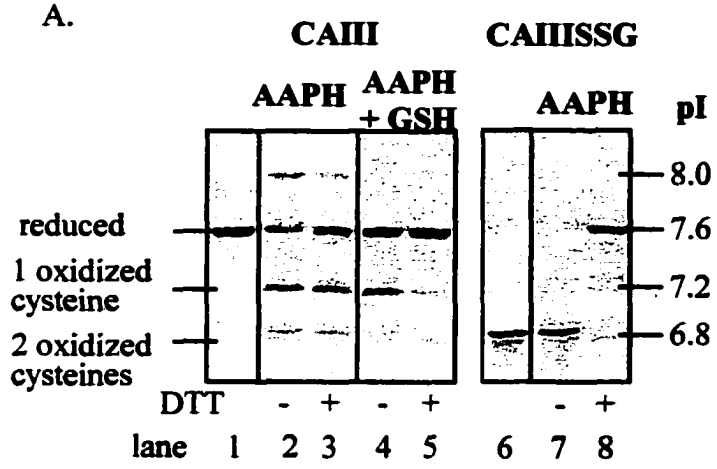
**Figure 5. The reaction mechanism of AAPH.**

AAPH splits homolytically at 37 °C to form 2 mol of alkyl radical per mol of AAPH (Niki, 1990), which then react quickly with molecular oxygen to form peroxyradicals.

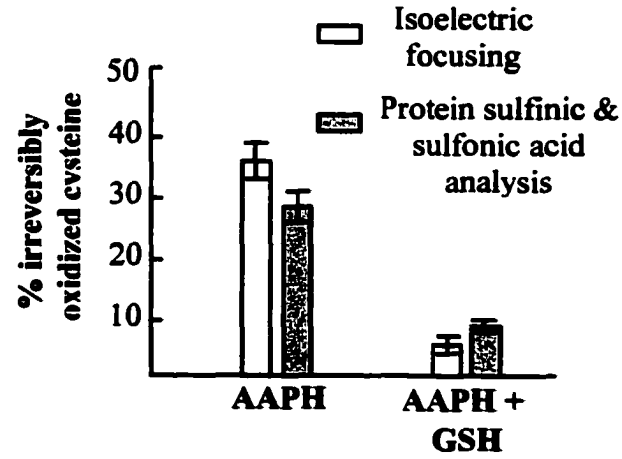
**Figure 6. Oxidation of carbonic anhydrase III by AAPH.**

(A) Carbonic anhydrase III or S-glutathiolated carbonic anhydrase III (30 μM) was incubated with 50 mM AAPH for 20 min at 37 °C in 50 mM sodium phosphate buffer pH 7.4. All other conditions were the same as described in Figure 2A, except that reactions were stopped with 20 mM NEM. (B) The amount of irreversibly oxidized cysteine found in AAPH oxidized carbonic anhydrase III was determined by densitometry of IEF gels or protein sulfinic and sulfonic acid analysis. Irreversibly oxidized cysteine was determined to be 25% sulfinic acid, 75% sulfonic acid in samples treated with AAPH alone; 37% sulfinic acid, 63% sulfonic acid in samples treated with AAPH in the presence of GSH.

A.


CAIII
CAIIISSG
AAPH
AAPH + GSH
AAPH
pI
8.0
7.6
7.2
6.8
reduced
1 oxidized cysteine
2 oxidized cysteines
DTT - + - + - +
lane 1 2 3 4 5 6 7 8

B.


Isoelectric focusing
Protein sulfinic & sulfonic acid analysis
% irreversibly oxidized cysteine
50
40
30
20
10
AAPH
AAPH + GSH

interesting to note that either S-glutathiolation (Figure 6A) or NEM pretreatment (not shown) of carbonic anhydrase III prevented formation of the positively charged band, suggesting that protein cysteine residues are involved. The modification affected less than 10% of the protein.

In the presence of GSH, AAPH produced reversible oxidation (lanes 4,5) while irreversible oxidation was almost entirely prevented. When fully S-glutathiolated carbonic anhydrase III was treated with AAPH, no additional modification of the protein occurred (lanes 7, 8). Figure 6B compares the amount of sulfinic and sulfonic acid in AAPH-oxidized carbonic anhydrase III to the amount of oxidized protein determined by IEF. Unlike oxidation with $H_2O_2$, AAPH-treated carbonic anhydrase III produced a considerable amount of cysteine sulfonic acid. Seventy-five percent of the irreversibly oxidized cysteine detected from AAPH-treated carbonic anhydrase III was recovered as sulfonic acid. The presence of GSH decreased the total amount of irreversibly oxidized cysteine detected, and 63% of this was recovered as sulfonic acid. Sulfinic and sulfonic acid were not detected in untreated carbonic anhydrase III or in AAPH treated S-glutathiolated carbonic anhydrase III (data not shown).

Since the ratio of GSH to protein is a critical factor in preventing oxidative damage to proteins, the effect of GSH on AAPH oxidation was examined in detail. When GSH was varied from 0 to 150 μM (a five-fold molar excess of GSH), the amount of irreversible damage decreased to less than 5%, while the amount of reversible oxidation was nearly a mirror image of that effect (Figure 7A). Total modification of carbonic anhydrase III remained quite constant (approximately 30% of the reactive cysteines were modified). When GSH was raised to 1.3 mM, total protein modification decreased as a result of the scavenging

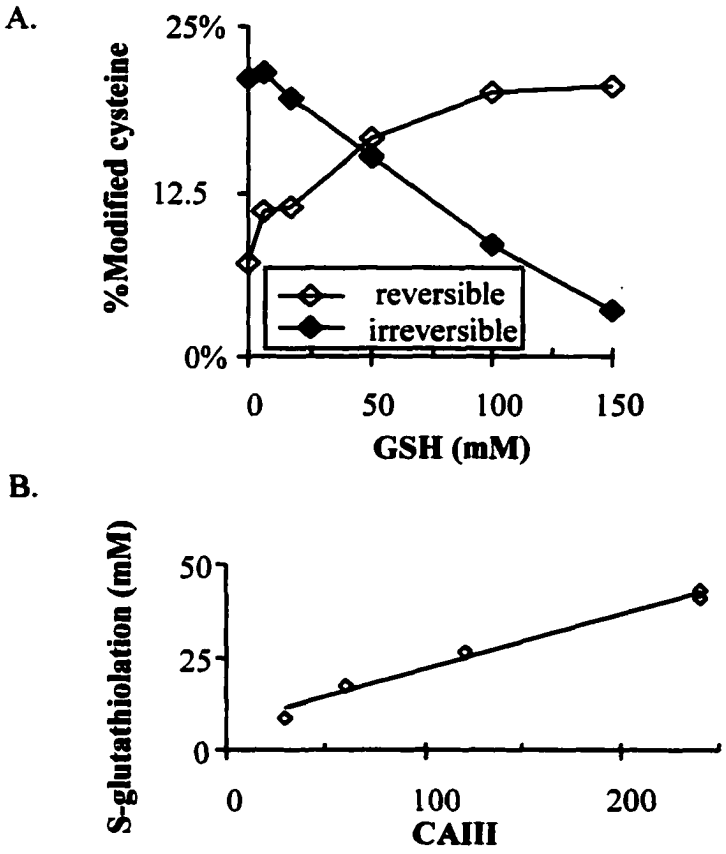



**Figure 7. Effect of GSH concentration on carbonic anhydrase IH modification by peroxyradicals.**

Carbonic anhydrase III was incubated with 50 mM AAPH for 20 minutes at 37 °C in sodium phosphate buffer pH 7.4. Reactions were stopped, carbonic anhydrase III was reduced, and results were analyzed as in Figure 3. (A) Carbonic anhydrase III (30 μM) was incubated with varying concentrations of GSH in the presence of AAPH. (B) AAPH was incubated with 30 μM to 240 μM carbonic anhydrase III in the presence of 150 μM GSH.

effect of the free glutathione pool on peroxyradicals (data not shown). The amount of modification caused by AAPH in the presence of GSH could not be accounted for by the concentration of GSSG produced by direct oxidation of that molecule (not shown).

When carbonic anhydrase III concentration was varied from 30 μM to 240 μM during AAPH oxidation with 150 μM GSH, reversible modification increased in proportion to carbonic anhydrase III concentration (figure 7B). Irreversible modification was negligible in this experiment (data not shown). S-glutathiolation was about 30% at 30 μM carbonic anhydrase III and decreased only slightly to about 20% modification when carbonic anhydrase concentration was 240 μM. Since the amount of S-glutathiolated carbonic anhydrase III increased with carbonic anhydrase III concentration, it appears that carbonic anhydrase III trapped peroxyradicals very efficiently. When the concentration of carbonic anhydrase III was 240 μM, there was 40 μM of protein bound glutathione, accounting for 25% of the total GSH available. S-glutathiolated protein therefore accounts for a significant fraction of oxidized glutathione in this experiment.

**GSH does not protect HOCl-treated carbonic anhydrase III by S-glutathiolation**

Although HOCl oxidizes GSH to the sulfonamide (Winterbourn and Brennan, 1997), less is known about its effect on protein thiols. The sulfenyl chloride has been proposed as a possible intermediate in the oxidation of GSH (Winterbourn and Brennan, 1997; Pullar *et al.*, 2001). It seems likely that such an intermediate would occur with protein thiols, as well. In the presence of excess GSH, this intermediate might react to produce an S-glutathiolated protein.

When carbonic anhydrase III was oxidized with HOCl (Figure 8A, lanes 2 and 3) the protein migrated to the lower pI's indicative of irreversibly oxidized carbonic anhydrase III. The extent of irreversible oxidation determined by IEF was in agreement with values determined by analysis of protein sulfinic and sulfonic acids (Figure 8B), of which protein sulfonic acid represented 41% of the total. However, in the presence of excess GSH, about 8% of the protein reactive cysteine was reversibly modified (lanes 4 and 5), suggesting that protein S-glutathiolation is not a major mechanism occurring with HOCl. When these samples were analyzed for protein sulfinic and sulfonic acid, 20% of the reactive cysteine was irreversibly oxidized, mostly to the sulfonic acid (75% of the total irreversibly oxidized cysteine). The discrepancy between the IEF analysis and the protein sulfinic and sulfonic acid analysis could result from the formation of a charge neutral adduct, possibly a form of sulfinamide (Raftery *et al.*, 2001), which was converted to a sulfonic acid during acid hydrolysis of the protein sample. If this hypothesis is correct, GSH may simply trap HOCl, thus delaying oxidation of a reactive intermediate to the sulfinic or sulfonic acid and allowing the putative sulfinamide to form. Since this modification apparently results in the formation of sulfonic acid in protein samples with no apparent charge differences, it might in fact be detectable by these characteristics in any protein sample under study. This concept is of interest with respect to the potential oxidative modifications that might occur *in vivo* (Pullar *et al.*, 2001).

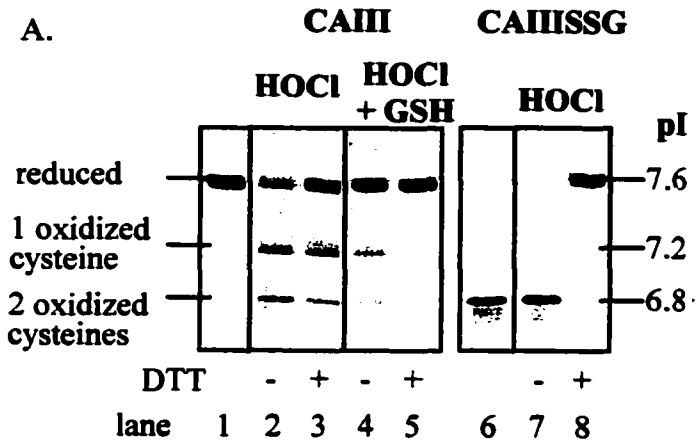
**Cysteine acts *via* S-thiolation to protect carbonic anhydrase III thiols from oxidative damage.**

GSH has been shown to be the major thiol participating in S-thiolation reactions in

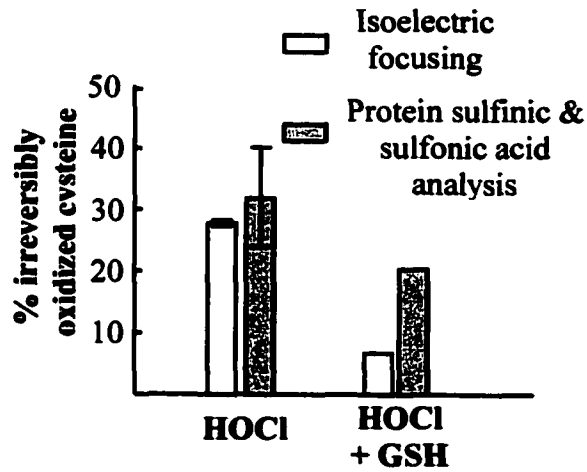
**Figure 8. HOCl causes irreversible oxidation but not S-glutathiolation of carbonic anhydrase III.**

(A) Carbonic anhydrase III or S-glutathiolated carbonic anhydrase III (30 μM) was incubated with 150 μM HOCl for 20 min at 37 °C in 50 mM sodium phosphate buffer pH 7.4. All other conditions were the same as described in Figure 6. (B) The amount of irreversibly oxidized cysteine found in HOCl oxidized carbonic anhydrase III was determined by densitometry of IEF gels or protein sulfinic and sulfonic acid analysis. Irreversibly oxidized cysteine was determined to be 59% sulfinic acid, 41% sulfonic acid in samples treated with HOCl alone; 25% sulfinic acid, 75% sulfonic acid in samples treated with HOCl in the presence of GSH. The error in the irreversible oxidation analysis by IEF is smaller than the linewidth in this illustration.

A.


CAIII
CAIIISSG
HOCl
HOCl + GSH
HOCl
pI
reduced
1 oxidized cysteine
2 oxidized cysteines
7.6
7.2
6.8
DTT - + - + - +
lane 1 2 3 4 5 6 7 8

B.


Isoelectric focusing
Protein sulfinic & sulfonic acid analysis
% irreversibly oxidized cysteine
50
40
30
20
10
HOCl
HOCl + GSH

cells (Chai *et al.*, 1994). This may be because of a special affinity of protein S-thiolation sites for GSH, or because of the relatively high concentration of GSH compared to other low molecular weight thiols in cells (Mallis and Thomas, 2000). We therefore studied whether cysteine would also protect carbonic anhydrase III from oxidative damage by $H_2O_2$. When carbonic anhydrase III was incubated with $H_2O_2$, 15% of the reactive cysteines were modified by a single negative charge (Figure 9, second bar). Cysteine prevented this modification by $H_2O_2$ as shown by its nearly complete inhibition of negative charge modification (third bar). In order to be able to detect the neutrally charged S-cysteylated carbonic anhydrase III, the protein was reacted with the negatively charged alkylating agent iodoacetic acid (IAA). S-cysteylation appears in this assay as an inhibition of IAA derivitization, while irreversible oxidation and IAA derivitization are indistinguishable (Thomas *et al.*, 1995a). When carbonic anhydrase III reacts with IAA, it is modified with a negative charge on 50% of the reactive sites (fourth bar), which agrees with previously published reports of rat liver carbonic anhydrase III (Thomas *et al.*, 1995a). If carbonic anhydrase III is incubated with $H_2O_2$ before IAA derivitization, there is a modest increase in negatively charged modification (fifth bar). This small increase indicates that little of the modification caused by $H_2O_2$ occurred on the thiol which is does not react with IAA. Only 15% of the reactive cysteines are modified with a negative charge when cysteine is present during the reaction of $H_2O_2$ with carbonic anhydrase III (sixth bar), indicating that more than 35% of the sites are S-cysteylated. Therefore cysteine also acts to protect proteins *via* S-thiolation.

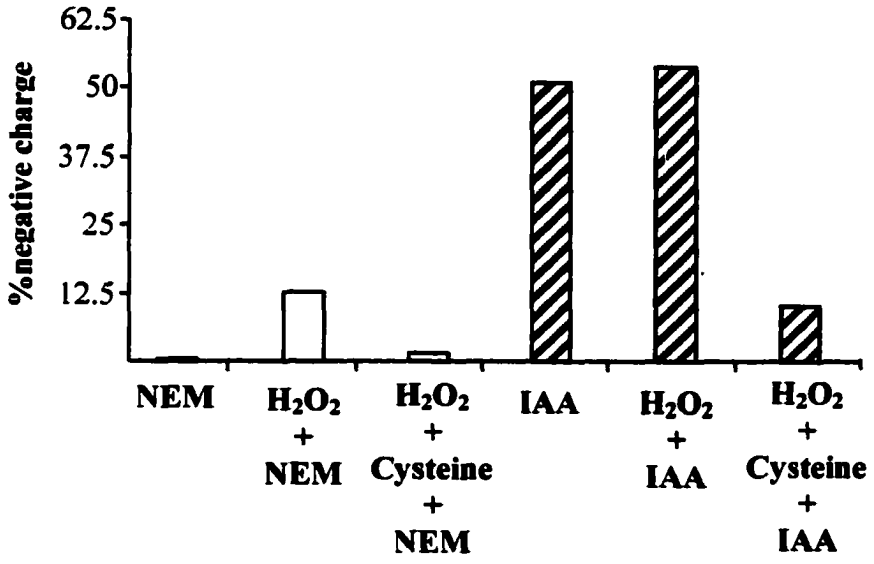



**Figure 9. Protection of carbonic anhydrase III from $H_2O_2$ oxidation by cysteine.**

Carbonic anhydrase III (10 μM) was incubated with 2 mM $H_2O_2$ in the absence and presence of 150 μM cysteine at 37 °C and at pH 7.4 for 10 minutes. The reactions were stopped with either 10 mM NEM (open bars) or 10 mM IAA (crosshatched bars) as indicated. IEF was performed as described in Materials and Methods. Negative charge modification was then calculated as the % modification as described in Materials and Methods.

**GSH protects carbonic anhydrase III from irreversible oxidation in hepatocytes**

Since relatively small changes in the molar ratio of GSH to protein sulfhydryls can have significant effects on the protection of reactive protein sulfhydryls, the protein modifications occurring in oxidant-treated rat hepatocytes were studied. Because hepatocytes contain a large amount of endogenous carbonic anhydrase III, they provide a suitable model to examine modification of that protein *in vivo*.

Cellular glutathione pools of primary hepatocytes were depleted with DEM, a substrate for cellular glutathione S-transferase, and oxidative stress was initiated with menadione, a redox cycling compound that generates reactive oxygen species. It has previously been shown that menadione promotes S-glutathiolation of carbonic anhydrase III as well as other hepatocyte proteins (Lii *et al.*, 1994). Figure 10 describes the experimental conditions for depletion of total glutathione by DEM. After 20 minutes, 0.03 mM DEM depleted total glutathione to 80% of normal, while 2 mM DEM depleted total glutathione to 15% of normal. After a medium change, the cells were incubated with 2 mM menadione for 15 minutes and then the medium was again changed. Total glutathione remained at approximately the same level or increased slightly after DEM-containing medium was removed. Menadione had no measurable effect on total glutathione. DEM caused no increase in cell lysis as measured by lactate dehydrogenase activity found in the culture medium and menadione caused only a slight (5%) increase even two hours after its addition to cells (not shown). Thus, the initial loss in total glutathione with DEM treatment was not the result of a loss of cellular integrity.

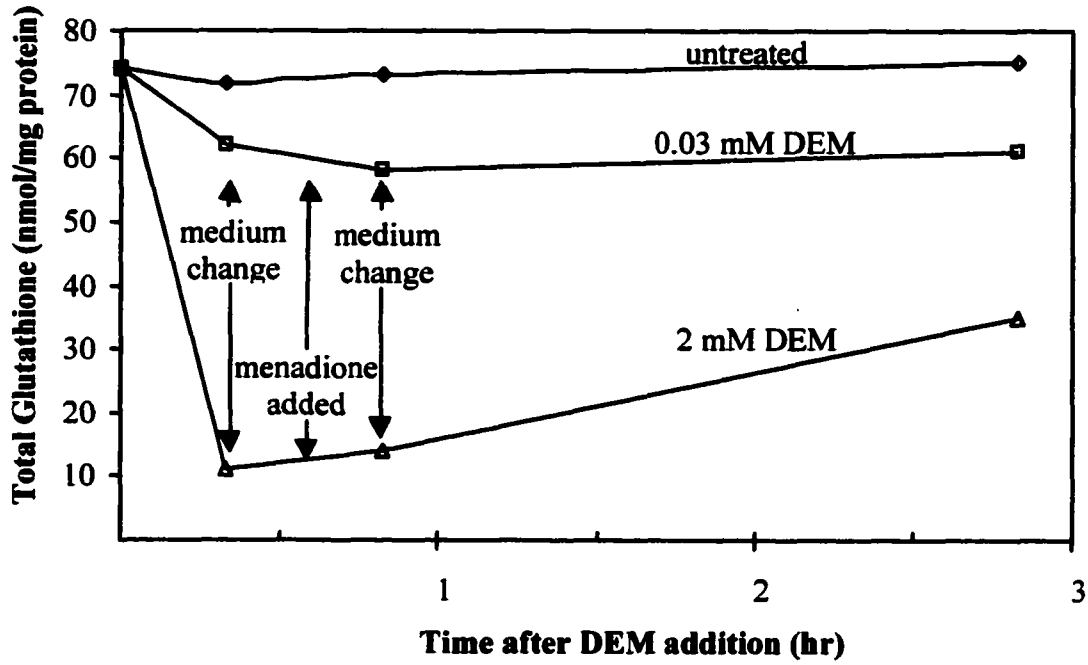


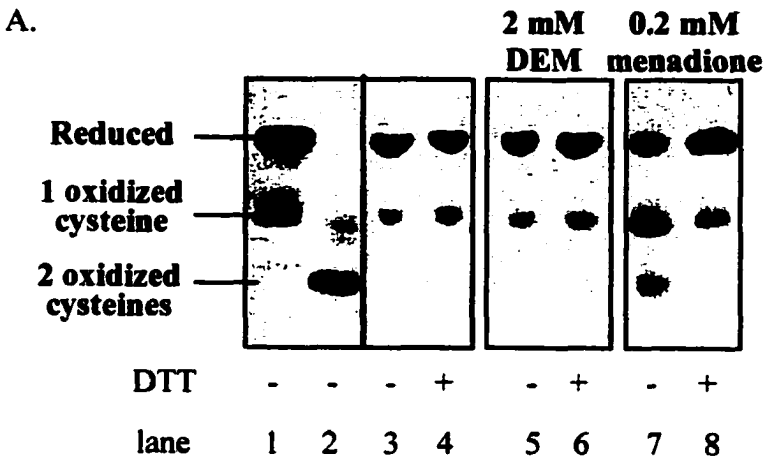

**Figure 10. Effects of DEM and menadione on hepatocyte glutathione.**

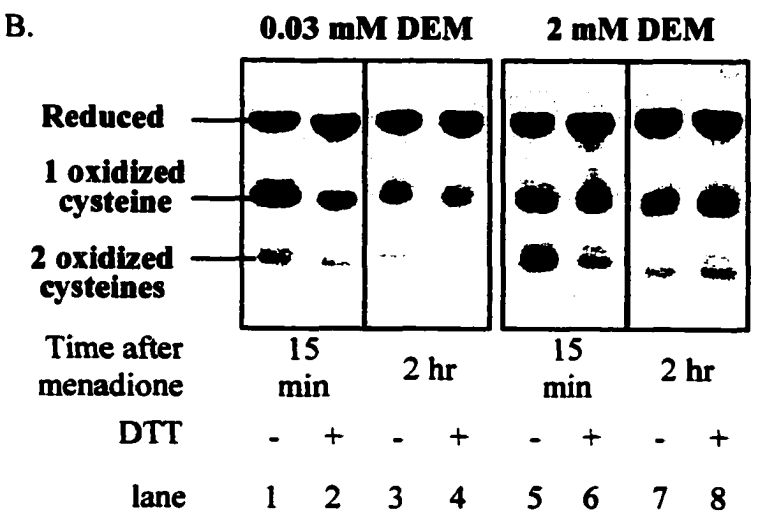
Hepatocytes were first incubated in medium B for 1 h, and DEM was added to a final concentration of 0.03 mM or 2 mM. After 20 min the medium was replaced with fresh medium B and 0.2 mM menadione was added 15 min later. After 15 min medium was replaced with fresh medium A. At the times indicated, cells were rinsed twice with cold buffered saline and lysed as described in Materials and Methods. (A) Glutathione is expressed as the mean of duplicate cultures. Duplicates varied by less than 6%.

Modified forms of carbonic anhydrase III in cells were monitored by IEF coupled with Western blot analysis (Lii *et al.*, 1994; Thomas *et al.*, 1995a). Figure 11A shows an analysis of carbonic anhydrase III after DEM and menadione treatment of hepatocytes. Lanes 1 and 2 are samples of pure carbonic anhydrase III which show the positions of the reduced, singly and doubly S-glutathiolated forms of the protein. A single irreversibly oxidized form of carbonic anhydrase III resistant to reduction by DTT was found in untreated hepatocytes (lanes 3,4). Serial dilution of these samples shows that nearly one third of the protein contains a single irreversibly oxidized cysteine (data not shown). DEM (lanes 5,6) had no effect on carbonic anhydrase III, while menadione caused a reduction-sensitive oxidation on up to two cysteine residues in hepatocytes (lane 7, 8) as reported previously (Lii *et al.*, 1994). DEM and menadione were used in combination in the experiment shown in Figure 11B. Menadione potentiated a rapid oxidation within 15 minutes after 0.03 mM DEM treatment (lane 1) that was not completely reversible with DTT treatment (lane 2, compare to panel A, lane 8). Two hours after the addition of menadione, S-glutathiolation had disappeared (lane 3) while irreversible oxidation appears unchanged from that at the initial observation (compare lane 2 and 4). In a similar experiment where glutathione was more extensively depleted by 2 mM DEM, menadione produced less S-glutathiolation (lane 5) and considerably more irreversible oxidation (lane 6, compare to panel A, lane 8). Two hours later, there was no S-glutathiolated protein and again the irreversibly oxidized forms persist (lanes 7,8).

**Figure 11. Effects of menadione on hepatocyte carbonic anhydrase III in DEM-treated cultures.**

Rat hepatocytes were treated with DEM followed by menadione as described in Figure 10. Carbonic anhydrase III was separated by electrofocusing and detected by Western blot analysis. A portion of each sample was treated with 50 mM dithiothreitol before separation by electrofocusing. Each pair of lanes shows the carbonic anhydrase III forms in an untreated and a reduced sample (50 mM dithiothreitol) of the hepatocyte protein mixtures indicated below. Each lane contained 0.5 μg total proteins. In panel (A), lanes 1 and 2 show the reduced, partially S-glutathiolated, and fully S-glutathiolated forms of carbonic anhydrase III. Lanes 3,4 contain the protein mixture from untreated hepatocytes. Lanes 5,6 show proteins from cells treated with 2 mM DEM for 20 min. Lanes 7 and 8 are proteins from untreated cells exposed to 0.2 mM menadione for 15 min. In panel (B) hepatocytes were pretreated with either 0.03 mM DEM (lanes 1 – 4) or 2 mM DEM (lanes 5 – 8) followed by 0.2 mM menadione for 15 min. At the times indicated (0 time is the time when menadione was added to the culture) hepatocytes were washed and protein extracts were prepared.


A.
2 mM DEM
0.2 mM menadione
Reduced
1 oxidized cysteine
2 oxidized cysteines
DTT - - - + - + - +
lane 1 2 3 4 5 6 7 8


B.
0.03 mM DEM
2 mM DEM
Reduced
1 oxidized cysteine
2 oxidized cysteines
Time after menadione
15 min
2 hr
15 min
2 hr
DTT - + - + - + - +
lane 1 2 3 4 5 6 7 8

**Irreversible oxidation of protein cysteines occur increasingly with age**

One of the hallmarks of the aging process is a decrease in overall GSH levels (Hagen *et al.*, 1999). Since glutathione was necessary to protect carbonic anhydrase III *via* S-glutathiolation in hepatocytes, it was thought that aged animals, containing less glutathione than normal, might have increased protein sulfinic and sulfonic acid. Table 1 shows that the soluble protein fraction from young rat livers (1 month old male and female) contained cysteine sulfinic acid equivalent to approximately 0.8 mol % of the total protein cysteine content. The livers of older male animals (16 months) contained approximately 1.1 mol % cysteine sulfinic acid, *i.e.*, a 38% increase, while livers of older female animals (23 months) contained approximately 1.3 mol % cysteine sulfinic acid, *i.e.*, a 62% increase. The difference between young and old female rats was significantly different by the student's *t*-test. Protein sulfonic acid was below detection limits in these samples (<0.15%).

When the carbonic anhydrase III of old rats was compared to that in young animals, increased irreversible oxidation was observed (Figure 12). Two gel-based separation methods gave similar results in this experiment. First, MalPEG (Wu *et al.*, 2000) was used to derivatize the protein sample since it increases the overall molecular weight of a protein by 5 kDa per reactive thiol (Figure 12A). The apparent molecular weight of the MalPEG-tagged protein appears higher than expected when analyzed by SDS-PAGE. The addition of the large, uncharged polyethylene glycol group probably retards the migration of a protein through the gel since electrophoretic mobility is a function of both protein charge and size. The reagent does not react with cysteine residues that are irreversibly oxidized, and irreversibly oxidized forms of carbonic anhydrase can be separated from undamaged protein by SDS-PAGE Western blots. As described previously (Cabiscol and Levine, 1995), the

**Table 1: The effect of aging on rat liver sulfinic acid content[a]**

| Soluble liver protein extract | Cysteic acid (% of total cysteine) | | Calculated sulfinic acid (column B - column A) |
|---|---|---|---|
| | A without NaOCl | B with NaOCl | |
| Male | | | |
| 1 month | N.D.[b] | 0.80±0.14 | 0.80±0.14 |
| 16 month | N.D. | 1.11±0.23 | 1.11±0.23 |
| Female | | | |
| 1 month | N.D. | 0.83±0.17 | 0.83±0.17 |
| 23 month | N.D. | 1.29±0.20 | 1.29±0.20[c] |

[a] Liver extract was prepared and analyzed for protein sulfinic and sulfonic acid as described in Materials and Methods. Protein extracts were made from 3 rats for each sex and age group.

[b] N.D. = not detected (< 0.15% of total cysteine)

[c] There was a significant difference between young and old female rats ($t<0.05$)

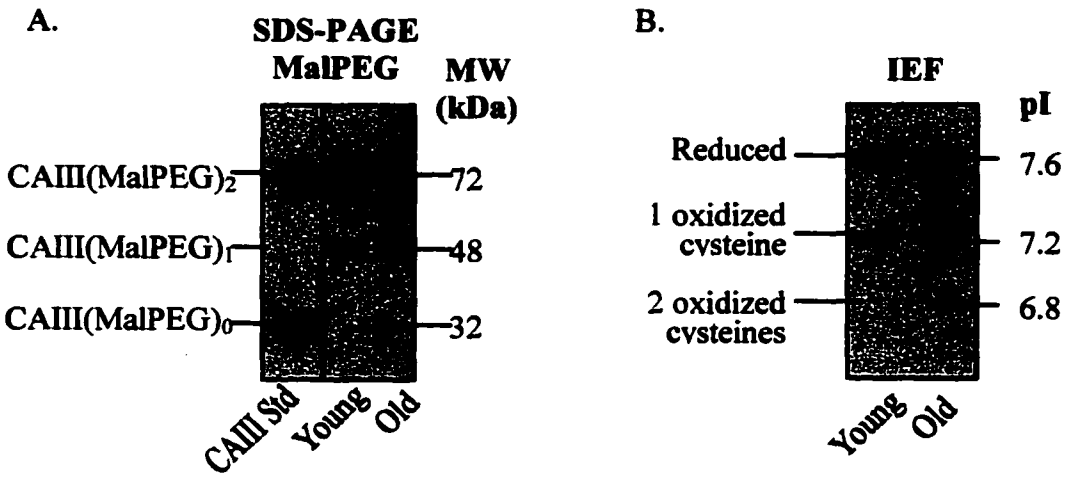



**Figure 12. Irreversible oxidation of rat liver carbonic anhydrase III and cytosolic proteins increases with age in rats.**

(A) Irreversible oxidation of carbonic anhydrase III in both one and 16 month old male rats was determined from western blots of MalPEG-treated samples. The first lane indicates the migration of carbonic anhydrase III when unmodified (lower band) and modified by MalPEG on two sites (upper band). Lanes 2 and 3 indicate the migration of MalPEG-treated samples from young and old rats, respectively. (B) IEF separation of DTT-treated soluble extracts of young and old rats, detected for carbonic anhydrase III. The positions of reduced, singly and doubly modified proteins are indicated.

carbonic anhydrase III level was five-fold higher in young rats than in old rats (data not shown). It was necessary to normalize the samples from young and old rat for this difference in carbonic anhydrase III. In a control experiment, pure carbonic anhydrase III, added to young and old rat liver extracts, migrated as a single band on SDS-PAGE gels with a molecular size of 30 kDa, while MalPEG-treated carbonic anhydrase III was approximately 72 kDa (not shown). The left lane in Figure 12A shows a sample in which equal amounts of reduced carbonic anhydrase III were mixed with MalPEG-derivatized carbonic anhydrase III before gel separation. The two lanes on the right were obtained from MalPEG-treated protein extracts from young and old animals. The carbonic anhydrase III from older rats contained an increased amount of carbonic anhydrase III that did not react with MalPEG (two bands with lower molecular size). An analysis of these protein samples by IEF gels gave similar results (Figure 12B). The sample from old rats showed a significant increase in the amount of protein focusing with a pI of 7.2, *i.e.*, protein with one irreversibly oxidized reactive sulfhydryl, and even a faint band with a pI of 6.8 that corresponds to protein with damage to both reactive sulfhydryls.

Gel-based methods of analysis can be quantified by densitometry, but since MalPEG-derivitization produces a significant broadening of the derivatized band it is not as amenable to such evaluation as the electrofocusing gels. Quantification of the IEF gel results showed that the carbonic anhydrase III in young rats contained 1.5% of the total cysteine as irreversibly oxidized forms while the old rats contain approximately 2.5% oxidized. Since only two of the cysteines in carbonic anhydrase III are reactive and the protein contains 5 cysteines, the observed oxidation represents 3.7% of the reactive cysteine in young animals and 6.2% in old animals. When these data are compared to the total protein cysteine pool

(data from Table 1), it appears that the oxidation state of carbonic anhydrase III is similar to or slightly higher than the entire protein pool.

## Discussion

The experiments presented here suggest that three distinct mechanisms for protection of protein cysteines may be important during oxidative stress (Figure 13). First, an oxidant can react directly with a protein molecule (probably by oxidation of a variety of different exposed protein structural components), resulting in the formation of a partially oxidized protein cysteine residue that is activated for further reaction with other cellular constituents. A protein thiyl radical, protein cysteine thioperoxide, protein cysteine thioperoxyradical, or protein cysteine sulfenic acid (Stortz *et al.*, 1990; Thomas *et al.*, 1995b; Denu and Tanner, 1998; Winterbourn and Metodiewa, 1999) might represent the partially oxidized protein cysteine. This modified cysteine most frequently reacts with GSH to form the S-glutathiolated protein because of the abundance of GSH as a reactant. This mechanism suggests an antioxidant role of GSH that has not previously been appreciated. Thus, if the concentration of GSH is decreased, the activated protein sulfhydryl intermediate might instead react with molecular oxygen or some other oxidant which can result in further oxidation of the cysteine to forms that seem to be metabolically inert, *i.e.*, irreversibly oxidized. Protein cysteine sulfinic or cysteine sulfonic acid (Figures 2 and 6) are possibly the major highly oxidized forms of cysteine present in normal cells. Second, the oxidant may react first with GSH to form an activated form of glutathione, which then reacts with protein cysteine to form S-glutathiolated protein. Finally, the oxidant might react with glutathione exclusively and thereby form significant amounts of GSSG. Since GSSG can potentially

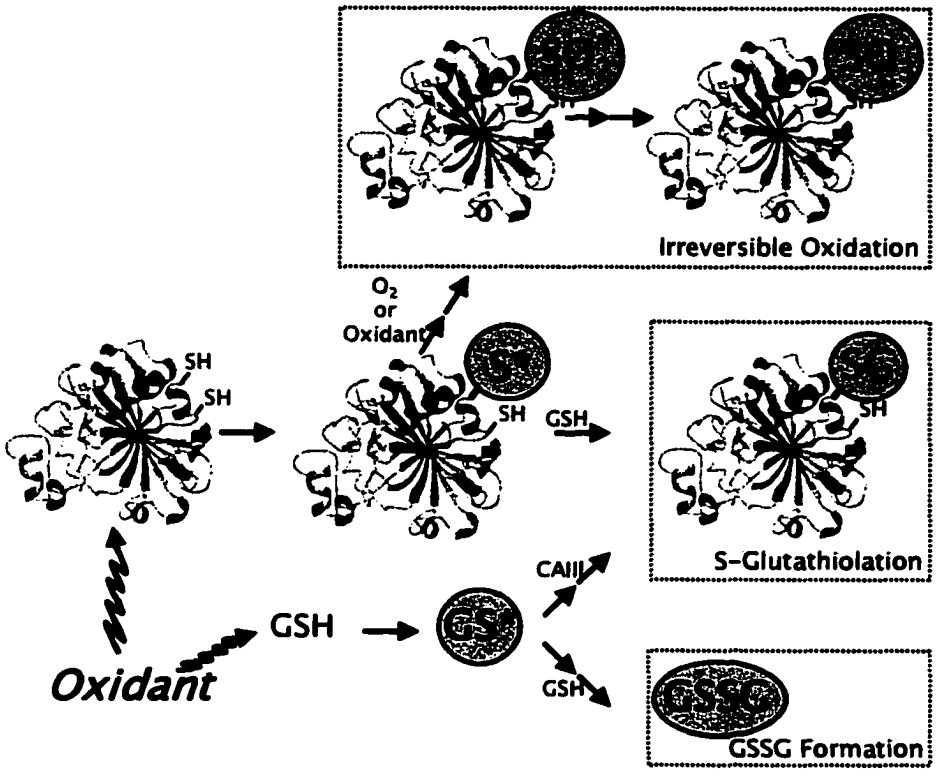



**Figure 13. Mechanisms for protection of protein cysteine from irreversible oxidation.** Oxidants (lower left corner of Figure) react with the protein to form an activated intermediate, (denoted S*, *e. g.* thiyl radical or cysteine sulfenic acid), which then reacts with GSH to form S-glutathiolated protein or may react with oxidants or O2 to form protein cysteine sulfinic (SO2) or sulfonic acid (SO3). Oxidants can also react with GSH to form an activated intermediate, GS*, which then reacts with a protein sulfhydryl to form S-glutathiolated protein or with GSH to form GSSG. While GSSG may S-glutathiolate proteins, the rate of this reaction is slow in comparison to the direct oxidation mechanisms discussed above.

react with protein sulfhydryls to form S-glutathiolated species, the possibility of protein modification by this mechanism is dependent on formation of significant amounts of GSSG. However, the reaction of GSSG with carbonic anhydrase III (and other proteins as well) appears to be too slow to generate significant S-thiolated protein in cells (Mallis *et al.*, 2001). Although these mechanisms are all of potential importance, most data suggest that the first mechanism is of primary importance *in vivo*. Since this mechanism also provides a unique hypothesis for the potential role of glutathione as a trapping antioxidant for damaged protein sulfhydryls, it is well suited to explain the results of the experiments reported in this manuscript.

Oxidation of carbonic anhydrase III with HOCl, AAPH or $H_2O_2$ produced considerable irreversible oxidation in the absence of GSH. Multiple acidic bands are generated on the IEF separation which are not reducible by DTT treatment. These bands also occur in oxidation of carbonic anhydrase III by a variety of other mechanisms (Lii *et al.*, 1994; Thomas *et al.*, 1995a; Thomas and Mallis, 2001). Reactions between thiols and reactive oxygen species often result in the formation of sulfinic acid or sulfonic acid (Wefers and Sies, 1983; Miller and Claiborne, 1991; Yeh *et al.*, 1996; Becker *et al.*, 1999; Hamann, *et al.*, 2001), both of which are negatively charged sulfur compounds. Indeed, HOCl, AAPH and $H_2O_2$ treatment of carbonic anhydrase III all result in the formation of protein cysteine sulfinic acid (Figure 2) or both sulfinic and sulfonic acid (Figures 6 and 8). GSH prevented irreversible oxidation by AAPH or $H_2O_2$ and simultaneously participated in S-glutathiolation of carbonic anhydrase III (Figures 2 and 6). The protection of carbonic anhydrase III and formation of S-glutathiolated protein was dependent on the concentration of GSH relative to the concentration of the protein. Additionally, as S-glutathiolation increased, irreversible

oxidation decreased in a GSH-dependent manner. This suggests that S-glutathiolation is linked to the prevention of irreversible oxidation (Park and Thomas, 1988; Lii *et al.*, 1994; Thomas *et al.*, 1995b). Indeed, when S-glutathiolated carbonic anhydrase III was treated with the oxidants used in this study, formation of irreversibly oxidized carbonic anhydrase III was not observed (Figures 1, 4, and 6). While it is not unreasonable to expect that a disulfide bond between protein cysteine and glutathione could be oxidized to forms such as thiosulfinate esters, it is probable that these reactions are significantly less favorable than oxidation of a protein thiol to a disulfide.

At lower GSH concentrations, initial oxidation of both proteins and glutathione are possible, since S-glutathiolated protein is a measured endpoint in both reactions. The increase in total protein modification at low GSH concentrations suggests either that initial formation of oxidized glutathione is an important mechanism, or that formation of irreversible oxidation products is less favored than the S-glutathiolation reaction. Because formation of cysteine sulfinic acid and cysteine sulfonic acid require multiple oxidation events, this supposition is reasonable. S-glutathiolation of carbonic anhydrase III remained extensive when GSH was several-fold more concentrated than the protein. This indicates that interaction of oxidant with the protein is important. The fact that irreversible oxidation occurs and that levels of irreversible oxidation in the absence of GSH are comparable to levels of total oxidation in the presence of GSH is further evidence that the protein reacts directly with the oxidant.

GSSG formation is not an important mechanism of S-glutathiolation in HOCl, AAPH or $H_2O_2$-mediated oxidation, and so GSSG formation may be important only at very high GSH:protein ratios. This corroborates studies with purified H-Ras in reactions with $H_2O_2$

and GSH (Mallis *et al.*, 2001), suggesting that this is a general phenomenon for proteins and not limited to carbonic anhydrase III. In reactions using rat liver carbonic anhydrase III (Thomas and Mallis, 2001), identical molar ratios of GSH:protein (approximately 10:1) produced a wide variety of results depending on the oxidant. This shows that carbonic anhydrase III is an efficient scavenger of reactive oxygen species, but that its scavenging ability is heavily dependent on the nature of the oxidant.

The reaction of $H_2O_2$ with carbonic anhydrase III and cysteine shows that cysteine is as effective as an S-thiolating agent as GSH when carbonic anhydrase III is the protein substrate. If carbonic anhydrase III is typical of cytosolic proteins with reactive cysteines, then the predominance of S-glutathiolated proteins in oxidized cells is likely the result of the greater concentration of GSH in cells (Chai *et al.*, 1994). Since carbonic anhydrase III does not appear to have a binding site for GSH (Mallis *et al.*, 2000), this result should be relevant to many surface-exposed cysteine sites on proteins.

A previous study using BSO to deplete glutathione in hepatocytes had shown formation of irreversibly oxidized carbonic anhydrase III with menadione (Lii *et al.*, 1994). The present experiments suggests that a minimal depletion of cellular glutathione may allow menadione to cause irreversible oxidation of carbonic anhydrase III. Further depletion results in extensive irreversible oxidation. Using DEM to deplete cellular glutathione has the advantage over using BSO in that the depletion occurs within 20 minutes. Thus, the irreversible oxidation does not seem to be a result of long-term secondary effects of glutathione depletion, but rather is likely to be the direct result of GSH deficiency. The results obtained with the purified protein show that decreasing the amount of available GSH is sufficient to result in irreversibly oxidized protein instead of S-glutathiolated protein and

thus, the same is likely to be true *in vivo*. Because irreversibly oxidized carbonic anhydrase III increased slightly with menadione treatment after a minimal depletion of GSH, the concentration of GSH in hepatocytes may be just sufficient to protect the proteins in these cells from oxidative damage. This implies that in disease states where glutathione is depressed, there may be a significant impact on the protein pool even in situations when the depression is minimal. The persistence of irreversibly oxidized forms for over two hours after menadione challenge suggests that turnover of irreversible oxidation products is quite slow and buildup of damaged proteins can occur over time. This is borne out by the observed increase in irreversible modification of carbonic anhydrase III and in cysteine sulfinic acid content overall in rat livers of older animals *versus* younger animals. The fact that a variety of oxidants produced either cysteine sulfinic or sulfonic acid *in vitro* and that sulfinic acid is found in the proteins of young and old animals suggests that these irreversible modifications are important cellular modifications warranting further study.

Finally, the reaction mechanisms postulated here strongly suggest that regulation of proteins can occur through S-glutathiolation without the necessity of forming GSSG as an intermediate. This makes S-glutathiolation a plausible initial event in the activation of signaling cascades by oxidants. While the prevention of irreversible oxidation by S-glutathiolation has obvious value in preserving the function of cysteine-containing proteins, progressive irreversible oxidation of cysteines may contribute to degeneration of signaling seen in aging, cancer and other disease states (Liu *et al.*, 1996; Rattan and Clark, 1996; Barrett *et al.*, 1999a, b; Navarro *et al.*, 1999).

## Materials and Methods

### Materials

L-cysteine, dithiothreitol (DTT), reduced glutathione (GSH), glutathione disulfide (GSSG), and N-ethylmaleimide (NEM), galactose, dexamethasone, collagen (type VII, from rat tail), bovine serum albumin, diethyl maleate, buthionine sulfoximine (BSO), sodium selenite, 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), NADPH, glutathione reductase (type III, from baker's yeast), iodoacetamide, iodoacetic acid (IAA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), phenylmethylsulfonyl fluoride (PMSF), and leupeptin were from Sigma Chemical Company (St. Louis, USA). 2,2'-Azobis(2amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, USA). Sulfosalicylic acid dihydrate and sodium hypochlorite was from Aldrich Chemical Company, Inc. (Milwaukee, USA). Leibovitz's L-15 medium with L-glutamine, bovine insulin, human transferrin, penicillin G, and streptomycin sulfate were from Gibco-BRL (Grand Island, USA). Collagenase (type I) was obtained from Worthington Biochemical Corp. (Freehold, USA). Ampholytes (pH 5 – 8 and pH 4 – 6) and Percoll were obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, USA). PVDF membrane was from Millipore Corporation (Bedford, USA). Net-Fix for PAG was from Serva Biochem. Inc.(Westbury, USA). O-(2-maleimidoethyl)-O'-methylpolyethylene glycol 5000 (MalPEG) was obtained from Shearwater Corp. (Huntsville, USA). Purified recombinant human erythrocyte carbonic anhydrase III was the generous gift of D.L. Silverman (University of Florida, Gainesville, USA).

**Protein assay**

Protein concentrations were determined as described by Lowry *et al.* (1951).

**Isoelectric focusing (IEF) of carbonic anhydrase III**

Purified carbonic anhydrase III was separated on horizontal slab gels [5.0% (acrylamide/2.7% bisacrylamide)/0.3% ampholyte pH 4.0 – 6.0/1.7% ampholyte pH 5.0 – 8.0] at 1500 V and 1.1 W/cm for 50 minutes as previously described for rat liver carbonic anhydrase III (Chai *et al.*, 1991). The reduced form of the human erythrocyte enzyme separated at pI 7.6, a slightly more basic pI than the rat liver enzyme (reduced form pI=7.0) used previously in this laboratory (Chai *et al.*, 1991). Gels were stained with Coomassie Brilliant Blue R-250 and air-dried.

**Quantification of IEF gels**

Gels were scanned and bands were quantified using Image-Quant v3.3 (Molecular Dynamics Inc.). The extent of modification of carbonic anhydrase III was calculated by determining relative band densities within individual lanes. The percent modification of carbonic anhydrase III reactive cysteines was calculated from the following relationship:

%modification=(density of band with 1 oxidized cysteine + 2 × density of bands with 2 oxidized cysteines)/(2 × density of all bands) × 100%

Reversible modification is the difference in % modification between DTT-untreated and DTT-treated lanes. Irreversible modification is the % modification in DTT-treated lanes.

**Protein sulfinic and sulfonic acid analysis**

Samples were analyzed for irreversibly oxidized forms of cysteine (Hamann *et al.*, 2001). Briefly, samples were denatured by incubation at 37 °C for 15 min in 120 mM phosphate buffer, pH 7.4 containing 7 M urea and 10 mM DTT. The samples were then treated with 40 mM IAM and incubated at room temperature for 20 min to block any remaining reduced cysteine residues. Each sample was split into two fractions, one of which was treated with 10 mM HOCl for 5 min at room temperature in order to oxidize sulfinic acids to sulfonic acids. The reactions were terminated after five minutes with the addition of 30 mM DTT. The samples were extensively dialyzed and hydrolyzed by vapor phase acid. Amino acid analysis was conducted using precolumn derivatization with OPA, and the amino acids were separated by reverse phase HPLC. Individual samples of hydrolyzed carbonic anhydrase III were compared by determining the moles of leucine in each sample. Since carbonic anyhydrase III contains 22 leucine residues, the total leucine was divided by 22 to determine the moles of carbonic anyhydrase III. The percent of reactive cysteine converted to cysteic acid was calculated according to the following relationship:

%cysteic acid=(mol of cysteic acid/2 reactive cysteine residues)/(mol of carbonic anyhydrase III) ×100%

HOCl converts sulfinic to sulfonic acid; therefore, the percent of cysteic acid found in a sample treated with HOCl represents the percent of total irreversibly oxidized reactive cysteine. Cysteic acid found in samples not treated with HOCl represents the percent of reactive cysteine detected as protein sulfonic acid. The percent of protein sulfinic acid can be

calculated by subtracting the total protein sulfonic acid from the total irreversibly oxidized cysteine.

Liver protein extracts were compared on the basis of total cysteine content. The molar amount of cysteic acid detected was divided by the total mol of cysteine detected.

**Hepatocyte isolation and culture**

Male Sprague-Dawley rats (200 – 250 grams) were from Sasco Co. (Omaha, USA). Water and a crude cereal-based diet were offered freely. Hepatocytes were isolated by perfusion with 0.05% collagenase as described (Thomas *et al.*, 1995a). A male rat was anesthetized with secobarbital sodium (100 mg/kg body weight), and the liver was perfused at 37°C through the portal vein with 0.05% Type I collagenase. After approximately 15 min of perfusion, the liver was removed, sieved through nylon mesh, and cells were washed with L-15 medium. The cells were suspended in a 10% Percoll solution and centrifuged to separate parenchymal cells from dead cells. Isolated hepatocytes (0.5□106 cells/ml) were plated on collagen precoated culture plates in medium A (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, 5 ng/ml sodium selenite, 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate). Cell viability was greater than 90% by Trypan blue exclusion. The culture medium was changed after 5 h to remove dead and unattached cells and again after 24 h. Hepatocytes were cultured for 48 h before starting experiments.

Hepatocytes that would be treated with diethyl maleate (DEM) and menadione were first incubated in medium B (Leibovitz' Medium without BSA, insulin, transferrin and dexamethasone) for 1 h. DEM was added to this culture medium and the medium was

replaced with fresh medium B for subsequent cell treatment. After a 15 min equilibration, menadione (in a DMSO vehicle) was added to a final concentration of 0.2 mM. The final concentration of DMSO was 0.2% in the medium. Since DMSO did not cause protein S-thiolation, alter cellular glutathione, or alter the cellular response to menadione, it was not directly added to control cultures in the experiments described here. Menadione was left on the cultures for 15 min, then the medium was removed, and cells were incubated in medium A for up to 24 h.

**Hepatocyte extract preparation**

If glutathione was to be determined, experiments were terminated by rinsing the cells twice with cold phosphate-buffered saline (PBS). Cells were immediately placed in 250 µl cold buffer containing 20 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, and 5 µg/ml leupeptin, pH 7.4. Cells were completely disrupted with a small cell homogenizer, and homogenates were centrifuged at 10 000 *g* at 4° for 30 min. An aliquot of each culture extract was treated with sulfosalicylic acid (final concentration was 10%) and the acid-soluble material was used to measure total glutathione by the DTNB-glutathione reductase recycling method (Tietze, 1969).

For preparation of protein extracts of hepatocytes, washed cell cultures were lysed as above in 20 mM HEPES, 5 mM EDTA, and 5 mM EGTA, pH 7.4, containing 50 mM N-ethyl maleimide. N-ethyl maleimide reacts with protein and non-protein sulfhydryls rapidly to prevent artifactual modification of protein sulfhydryls during sample preparation. Two aliquots of each sample were prepared, one of which was treated with 50 mM dithiothreitol at 37° for 20 min before separation on electrofocusing gels.

**Electrofocusing/western blot analysis of protein extracts**

The molecular forms of carbonic anhydrase III in protein extracts were determined by separation on IEF gels as described above. This separation was combined with antibody detection after transfer to PVDF membranes as previously described (Lii *et al.*, 1994; Thomas *et al.*, 1995). The electrofocusing gel containing NetFix was separated from the Gelbond, equilibrated with cold 0.7% acetic acid for 15 min, and transferred at constant voltage, 12 V or 15 V (3 mA/cm2) for 30 min in a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, USA). Because proteins have a net positive charge in acetic acid, they were transferred to the membrane placed on the cathodic side of the gel. The PVDF membrane was washed with 150 mM NaCl, 15 mM Tris-HCl, pH 7.4, containing 0.3% Tween-20, and incubated with a rabbit anti-carbonic anhydrase III antiserum (supplied by S. Hendrich, Iowa State University, USA). Anti-rabbit IgG alkaline phosphatase conjugate from Sigma Chemical Company, along with *p*-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate, were used to visualize the bound carbonic anhydrase III antibody.

**Lactate dehydrogenase activity**

Medium was collected from hepatocytes and lactate dehydrogenase activity was measured directly with LDH lactate-dehydrogenase (EC 1.1.1.27) UV-test from Sigma. Total cellular lactate dehydrogenase was determined by lysing washed cells in 250 µl 20 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, and 5 µg/ml leupeptin, pH 7.4 buffer. The particle-free cellular supernatant was prepared by centrifugation and used to determine lactate dehydrogenase activity.

**Preparation of soluble liver protein extracts from young and old rats**

One month and 16 month old male, and one month and 23 month old female Fisher 344 rats were used. Food (Teklad Diet #7002) and water were provided *ad libitum*. Rats were sacrificed by $CO_2$ inhalation, the liver was removed, rinsed with ice cold saline, and frozen immediately using a liquid nitrogen cooled clamp. Livers were powdered using a liquid nitrogen cooled, stainless steel mortar and pestle and stored at – 80° until use. Soluble liver protein extracts were prepared by homogenizing liver tissue in ice-cold homogenization buffer (20 mM β-glycerophosphate, 10 mM DTT, 5 mM EDTA, and 5 mM EGTA, pH 7.4) The homogenate was centrifuged at 16 000*g* for 10 min at 4 °C, the supernatant was recovered, and centrifuged in a Beckman Airfuge Ultracentrifuge at 160,000 *g* for 30 min (twice). The soluble protein extract produced by this procedure was incubated at 37 °C for 30 min to remove any DTT reversible modifications prior to protein sulfinic and sulfonic acid analysis or MalPEG analysis.

**MalPEG analysis of protein extracts**

Soluble liver protein extracts were diluted ten-fold in 20 mM β-glycerophosphate buffer, pH 7.4, containing 5 mM MalPEG. Samples were applied to a 10% SDS-PAGE gel. Proteins were transferred to PVDF membrane, and carbonic anhydrase III was detected as described above, with the exception that the gel was equilibrated in buffer containing 48 mM Tris, 39 mMv glycine, 1.3 mM SDS, and 20% methanol prior to transfer. Anti-rabbit IgG horseradish peroxidase conjugate with ECL chemiluminescent detection from Amersham Pharmacia Biotech (Piscataway, USA), was used to visualize the bound carbonic anhydrase III antibody.

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**References**

Barrett, W.C., DeGnore, J.P., Keng, Y.-F., Zhang, Z.-Y., Yim, M.B., and Chock, P.B. (1999a). Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. J. Biol. Chem. *274*, 34543 – 34546.

Barrett, W.C., DeGnore, J.P., König, S., Fales, H.M., Keng, Y.-F., Zhang, Z.-Y., Yim, M.B., and Chock, P.B. (1999b). Regulation of PTP1B *via* glutathionylation of the active site cysteine 215. Biochemistry *38*, 6699 – 6705.

Becker, K., Savvides, S.N., Keese, M., Schirmer, R.H., and Karplus, P.A. (1999). Enzyme inactivation through sulfhydryl oxidation by physiologic NO-carriers. Nature Struct. Biol. *5*, 267 – 271.

Berlett, B.S., and Stadtman, E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. *272*, 20313 – 20316.

Cabiscol, E., and Levine, R. (1995). Carbonic anhydrase III: oxidative modification *in vivo* and loss of phosphatase activity during aging. J. Biol. Chem. *270*, 14742 – 14747.

Chai, Y.-C., Jung, C.-H., Lii, C.-K., Ashraf, S.S., Hendrich, S., Wolf, B., Sies, H., and Thomas, J.A. (1991). Identification of an abundant S-thiolated rat liver protein as carbonic anhydrase III; characterization of S-thiolation and dethiolation reactions. Arch. Biochem. Biophys. *284*, 270 – 278.

Chai, Y.-C., Ashraf, S.S., Rokuton, K., Johnston Jr., R.B., and Thomas, J.A. (1994). S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. Arch. Biochem. Biophys. *310*, 273 – 281.

Cotgreave, I.A., and Gerdes, R.G. (1998). Recent trends in glutathione biochemistry – glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation? Biochem. Biophys. Res. Commun. *242*, 1 – 9.

Dafré, A.L., and Reischl, E. (1998). Oxidative stress causes intracellular reversible S-thiolation of chicken hemoglobin under diamide and xanthine oxidase treatment. Arch. Biochem. Biophys. *358*, 291 – 296.

Dröge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, H.-P., Roth, S., and Gmünder, H. (1994). Functions of glutathione and glutathione disulfide in immunology and immunopathology. FASEB J. *8*, 1131 – 1138.

Engberg, P., Millquist, E., Pohl, G., and Lindskog, S. (1985). Purification and some properties of carbonic anhydrase from bovine skeletal muscle. Arch. Biochem. Biophys. *241*, 628 – 638.

Gesquière, L., Loreau, N., Minnich, A., Davignon, J., and Blache, D. (1999). Oxidative stress leads to cholesterol accumulation in vascular smooth muscle cells. Free Radic. Biol. Med. *27*, 134 – 145.

Denu, J.M., and Tanner, K.G. (1998). Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry *37*, 5633 – 5642.

Hagen, T.M., Ingersoll, R.T., Lykkesfeldt, J., Liu, J., Wehr, C.M., Vinarsky, V., Bartholomew, J.C., and Ames, B.N. (1999). (R)-α- Lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increase metabolic rate. FASEB J. *13*, 411 – 418.

Hamann, M., Zhang, T., Hendrich, S., and Thomas, J.A. (2001). A method for quantitation of protein sulfinic and sulfonic acid, irreversibly oxidized protein cysteine sites in cellular proteins. Methods Enzymol., in press.

Herzenberg, L.A., De Rosa, S.C., Dubs, J.G., Roederer, M., Anderson, M.T., Ela, S.W., Deresinski, S.C., and Herzenberg, L.A. (1997). Glutathione deficiency is associated with impaired survival in HIV disease. Proc. Natl. Acad. Sci. USA *94*, 1967 – 1972.

Jung, C.-H., and Thomas, J.A. (1996). S-glutathiolated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione. Arch. Biochem. Biophys. *335*, 61 – 72.

Lii, C-K., Chai, Y.-C., Zhao, W., Thomas, J.A., and Hendrich, S. (1994). S-thiolation and irreversible oxidation of sulfhydryls on carbonic anhydrase III during oxidative stress: a method for studying protein modification in intact cells and tissues. Arch. Biochem. Biophys. *308*, 231 – 239.

Liu, Y., Guyton, K.Z., Gorospe, M., Xu, Q., Kokkonen, G.C., Mock, Y.D., Roth, G.S., and Holbrook, N.J. (1996). Age-related decline in mitogen-activated protein kinase activity in epidermal growth factor-stimulated rat hepatocytes. J. Biol. Chem. *271*, 3604 – 3607.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265 – 275.

Mallis, R.J., and Thomas, J.A. (2000). Effect of S-nitrosothiols on cellular glutathione and reactive protein sulfhydryls. Arch. Biochem. Biophys. *383*, 60 – 69.

Mallis, R.J., Poland, B.W., Chatterjee, T.K., Fisher, R.A., Darmawan, S., Honzatko, R.B., and Thomas, J.A. (2000). Crystal structure of S-glutathiolated carbonic anhydrase III. FEBS Lett. *482*, 237 – 241.

Mallis, R.J., Buss, J.E., and Thomas, J.A. (2001). Oxidative modification of H-Ras: S-thiolation and S-nitrosylation of reactive cysteines. Biochem J. *355*, 145 – 153.

Marangon, K., Devaraj, S., Tirosh, O., Packer, L., and Jialal, I. (1999). Comparison of the effect of α-lipoic acid and α-tocopherol supplementation on measures of oxidative stress. Free Radic. Biol. Med. *27*, 1114 – 1121.

Miller, H., and Claiborne, A. (1991). Peroxide modification of monoalkylated glutathione reductase. Stabilization of an active-site cysteine-sulfenic acid. J. Biol. Chem. *266*, 19342 – 19350.

Müller, T., and Gebel, S. (1998). The cellular stress response induced by aqueous extracts of cigarette smoke is critically dependent on the intracellular glutathione concentration. Carcinogenesis *19*, 797 – 801.

Navarro, J., Obrador, E., Carretero, J., Petschen, I., Aviñó, J., Perez, P., and Estrela, J.M. (1999). Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth *in vivo*. Free Radic. Biol. Med. *26*, 410 – 418.

Niki, E. (1990). Free radical initiators as source of water- or lipidsoluble peroxyl radicals. Methods Enzymol. *186*, 100 – 108.

Park, E.-M., and Thomas, J.A. (1988). S-thiolation of creatine kinase and glycogen phosphorylase b initiated by partially reduced oxygen species. Biochem. Biophys. Acta *964*, 151 – 160.

Park, E.-M., Park, Y.-M., and Gwak, Y.-S. (1998). Oxidative damage in tissues of rats exposed to cigarette smoke. Free Radic. Biol. Med. *25*, 79 – 86.

Pichorner, H., Metodiewa, D., and Winterbourn, C.C. (1995). Generation of superoxide and tyrosine peroxide as a result of tyrosyl radical scavenging by glutathione. Arch. Biochem. Biophys. *323*, 429 – 437.

Pullar, J.M., Vissers, M.C.M., and Winterbourn, C.C. (2001). Glutathione oxidation by hypochlorous acid in endothelial cells produces glutathione sulfonamide as a major product but not glutathione disulfide. J. Biol. Chem. *276*, 22120 – 22125.

Raftery, M.J., Yang, Z., Valenzuela, S.M., and Geczy, C.I. (2001). Novel intra- and inter-molecular sulfinamide bonds in S100A8 produced by hypochlorite oxidation. J. Biol. Chem. *276*, 33393 – 33401.

Rattan, S.I.S., and Clark, B.F.C. (1996). Intracellular protein synthesis, modifications and aging. Biochem. Soc. Trans. *24*, 1043 – 1049.

Ravichandran, V., Seres, T., Moriguchi, T., Thomas, J.A., and Johnston Jr., R.B. (1994). S-thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes. J. Biol. Chem. *269*, 25010 – 25015.

Schuppe-Koistinen, I., Gerdes, R., Moldéus, P., and Cotgreave, I.A. (1994). Studies on the reversibility of protein S-thiolation in human endothelial cells. Arch. Biochem. Biophys. *315*, 226 – 234.

Sen, C.K., and Packer, L. (1996). Antioxidant and redox regulation of gene transcription. FASEB J. *10*, 709 – 720.

Storz, G., Tartaglia, L.A., and Ames, B.N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science *248*, 189 – 194.

Terradez, P., Asensi, M., Lasso de la Vega, M.C., Puertes, I.R., Viña, J., and Estrela, J.M. (1993). Depletion of tumour glutathione *in vivo* by buthionine sulphoximine: modulation by the rate of cellular proliferation and inhibition of cancer growth. Biochem. J. *292*, 477 – 483.

Thomas, J.A., and Mallis, R.J. (2001). Aging and oxidation of reactive protein sulfhydryls. Exp. Gerontol. *36*, 1519 – 1526.

Thomas, J.A., Zhao, W., Hendrich, S., and Haddock, P. (1995a). Analysis of cells and tissues for S-thiolation of specific proteins. Methods Enzymol. *251*, 423 – 429.

Thomas, J.A., Poland, B., and Honzatko, R. (1995b). Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch. Biochem. Biophys. *319*, 1 – 9.

Tietze, F. (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. *27*, 502 – 507.

Wang, H., and Joseph, J.A., (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic. Biol. Med. *27*, 612 – 616.

Wefers, H., and Sies, H. (1983). Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen. Eur. J. Biochem. *137*, 29 – 36.

Winterbourn, C.C. (1993). Superoxide as an intracellular radical sink. Free Radic. Biol. Med. *14*, 85 – 90.

Winterbourn, C.C., and Metodiewa, D. (1994). The reaction of superoxide with reduced glutathione. Arch. Biochem. Biophys. *314*, 284 – 290.

Winterbourn, C.C., and Brennan, S.O. (1997). Characterization of the oxidation products of the reaction between reduced glutathione and hypochlorous acid. Biochem. J. *326*, 87 – 92.

Winterbourn, C.C., and Metodiewa, D. (1999). Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. Free Radic. Biol. Med. *27*, 322 – 328.

Wu, H.H., Thomas, J.A., and Momand, J., (2000). p53 Protein oxidation in cultured cells in response to PDTC – a novel method for relating the amount of p53 oxidation *in vivo* to the regulation of p53-responsive genes. Biochem. J. *351*, 87 – 93.

Yeh, J.I., Claiborne, A., and Hol, W.G.J. (1996). Structure of the native cysteine-sulfenic acid redox center of enterococcal NADH peroxidase refined at 2.8 Å resolution. Biochemistry *35*, 9951 – 9957.

# CHAPTER 3. CYSTEINE OXIDATIVE DAMAGE IN SKELETAL MUSCLE AND HEART CREATINE KINASE INCREASES WITH AGE

A paper to be submitted to *The Journal of Biological Chemistry.*

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## Summary

Sulfinic and sulfonic acids are oxidized forms of protein cysteine that result in irreversible damage to cellular proteins. This paper reports on the possible role that protein S-glutathiolation plays in protecting protein sulfhydryls from irreversible oxidation, and demonstrates an increase in these damaged protein constituents in aged rat skeletal and heart muscle.

Creatine kinase was used as a model protein to examine the relationship between protein cysteine irreversible oxidation and S-glutathiolation. With pure creatine kinase it was possible to establish that hydrogen peroxide ($H_2O_2$) irreversibly oxidizes reactive protein cysteines to sulfinic acid, with little or no detectable sulfonic acid. In contrast, peroxyl radicals (generated with AAPH) oxidized creatine kinase to both sulfinic and sulfonic acid, demonstrating that the oxidation state of the cysteine was partially dependent on the oxidant used. Irreversible oxidation by either $H_2O_2$ or peroxyl radicals was prevented by glutathione through a mechanism that involved protein S-glutathiolation.

Protein extracts from the skeletal and heart muscle of one, three, and sixteen month old rats contained surprisingly large amounts of protein sulfinic acid, and the most aged

animals also contained approximately 30-40% less glutathione than the one month old rats. An age-dependent increase in the amount of total protein cysteine sulfinic acids associated with these tissues was reflected in the creatine kinase enzyme. It was approximately 2-fold more irreversibly oxidized in 16 month old rat than in younger animals. Thus, irreversible oxidation of protein sulfhydryls represents a major form of oxidative damage to proteins *in vivo*. The status of the glutathione pool could play a unique role in determining the extent of protein irreversible oxidation, and conditions which promote the depletion of cellular glutathione would be expected to accelerate the formation of protein sulfinic and sulfonic acids.

## Introduction.

Protein cysteine residues are sites of dynamic oxidative chemistry. Cysteine residues can undergo a large variety of oxidative modifications , but a useful classification is to describe the modifications as protein S-thiolation, S-nitrosylation, and irreversible oxidation (1). Protein S-thiolation and S-nitrosylation are reversible modifications in the context of a cellular environment, and can be reduced by thiol-disulfide exchange reactions involving glutaredoxin (2), thioredoxin (3), nitrosothiol reductase (4), or even simply the cellular glutathione pool (5). The reversible nature of protein S-thiolation and S-nitrosylation has made these modifications an appealing "vehicle" for the transmission of oxidant-derived cellular signals (6). Thus, cysteine residues have been suggested to act as redox sensors in signaling proteins such as p53 (7,8), AP-1 (9,10), and NF-κB(11), allowing cellular redox changes to be detected and translated into changes in DNA binding potential and protein expression.

Clearly a problem exists when cysteine residues become irreversibly oxidized. These modifications are not reduced by thiol-disulfide exchange, because the cysteine residues have been oxidized to sulfinic or sulfonic acids (12). If a particular cysteine plays an important functional role in a protein, irreversible oxidation of the residue results in permanent dysregulation. For example, cysteine residues in protein tyrosine phosphatases and glyceraldehyde 3-phosphate dehydrogenase are directly involved in catalytic mechanisms of these proteins; and irreversible oxidation will essentially knock-out the activity of these proteins (13,14,15). Alternatively, irreversible cysteine oxidation may increase the activity of certain proteins, as has been shown to be the case for matrix metalloproteinases 7 and 9 (16,17). Other functions such as metal binding (18), intramolecular disulfide bond formation (19), and protein lipidation (20) would be adversely affected by irreversible oxidation as well. In the cellular context, irreversible oxidation would permanently abrogate the normal functions of cysteine residues until a particular protein is degraded; therefore, the modifications could accumulate and exacerbate pathological states, such as aging, where cells are under oxidative stress (21).

It has been suggested that S-thiolation, and more specifically S-glutathiolation, can prevent the irreversible oxidation of surface-exposed cysteine residues (22). This hypothesis has been supported by experiments conducted with carbonic anhydrase III (23). When carbonic anhydrase III was treated with hydrogen peroxide or peroxyl radicals in the presence of excess glutathione *in vitro*, the cysteine residues were rapidly S-glutathiolated. Lower concentrations of glutathione resulted in only partial protection and increased irreversible oxidation. Experiments with hepatocytes demonstrated that diminishing the cellular glutathione concentrations with diethylmaleate and exposing them to menadione had

similar consequences. Despite the protective role of glutathione, an age-dependent increase in the amount of irreversibly oxidized carbonic anhydrase III was detected in rat liver tissues, and it was suggested to relate to age-dependent decreases in cellular glutathione (24).

This paper reports on experiments conducted with the muscle isoform of creatine kinase. Like carbonic anhydrase III, muscle creatine kinase is a cytosolic protein. It is a homodimeric protein with four total cysteine residues per monomer. Cysteine 283 is the most exposed and reactive cysteine residue and is sensitive to oxidative modification (25-27). The results presented here explore the role of S-glutathiolation in protecting creatine kinase from irreversible oxidation caused by hydrogen peroxide and peroxyl radicals. Novel methods are used to detect creatine kinase cysteine irreversible oxidation. One technique involves modifying creatine kinase thiols with MalPEG and detecting irreversibly oxidized forms by SDS-PAGE (28). A second technique allows specific quantitation of protein sulfinic and sulfonic acids by protein hydrolysis and HPLC analysis (27). It was found that irreversible oxidation of creatine kinase increases with age in rat heart and skeletal muscle tissues and may relate to a measured decrease total glutathione in these tissues.

## Materials and Methods.

### Materials:

Rabbit muscle creatine kinase, dithiothreitol (DTT), reduced glutathione (GSH), glutathione disulfide (GSSG), and N-ethylmaleimide (NEM), iodoacetamide, iodoacetic acid (IAA), and β-glycerophosphate were from Sigma Chemical Company (St. Louis, USA). 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, USA). Sodium hypochlorite was from Aldrich Chemical Company, Inc.

(Milwaukee, USA). O-(2-maleimidoethyl)-O'-methylpolyethylene glycol 5000 (MalPEG) was obtained from Shearwater Corp. (Huntsville, USA). Ampholytes (pH 5 – 8 and pH 4 – 6) for isoelectric focusing were obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, USA). PVDF membrane was from Millipore Corporation (Bedford, USA). Goat anti-human MM creatine kinase antibody was obtained from Research Diagnostics Incorporated (Flanders, NJ). Rabbit anti-goat horseradish peroxidase conjugate antibody was from Pierce (Rockford, IL)

**Creatine kinase purification:**

Commercially available rabbit skeletal muscle creatine kinase was purified using anion exchange chromatography in order to remove irreversibly oxidized creatine kinase and other contaminating proteins as previously described (28). Prior to experiments, creatine kinase was incubated at 37°C with 10 mM DTT for 30 min and dialyzed overnight against 20 mM β-glycerophosphate, pH 7.4. S-glutathiolated creatine kinase was generated by incubating the protein with 10 mM GSSG at 37°C for 15 min. GSSG was removed by dialysis.

**Isoelectric Focusing (IEF) of creatine kinase.**

Oxidized forms of creatine kinase were separated on horizontal slab gel (4% acrylamide, 1.7% pH 5-8 ampholytes, 0.3% pH 4-6 ampholytes) at 1500 V and 1.1 W/cm for 50 minutes as previously described (29). Gels were stained with Coomassie Brilliant Blue R-250 and air-dried.

**MalPEG tagging assay**

MalPEG has been used to detect reversible cysteine modifications (8,30). Figure 1 describes how the MalPEG tagging procedure was modified in order to detect irreversible oxidation of creatine kinase. Samples of purified creatine kinase or tissue extracts were incubated at 37°C with 10 mM DTT for 30 min. (This step was omitted for detection of S-glutathiolated creatine kinase.) Proteins were tagged by diluting samples ten-fold in 20 mM β-glycerophosphate, pH 7.4 containing 5 mM MalPEG, and incubated at room temperature for 30 min before teminating with four volumes of SDS-PAGE sample buffer containing NEM (0.125M Tris-Cl buffer, pH 6.8, containing 4% sodium dodecyl sulfate, 20% glycerol, 0.008% bromophenol blue, and 65 mM NEM). Samples were separated on 10% gels, and proteins were transferred to PVDF membrane after equilibrating the gel in buffer containing 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20% methanol. The membrane was blocked with 6% nonfat dry milk in wash buffer (15 mM Tris, 150 mM NaCl and 3% Tween-20, pH 7.4). Creatine kinase was detected using a goat polyclonal antibody diluted 1/7500 in the blocking buffer, followed by an anti-goat horseradish peroxidase conjugate (diluted 1/5000) and chemiluminescent detection.

**Densitometry:**

Gels and blots were scanned and bands quantitated using ImageQuant v3.3 (Molecular Dynamics Inc.). Creatine kinase runs on isoelectric focusing gels as a homodimer, with one reactive cysteine per subunit. The amount of irreversible cysteine oxidation per monomer of creatine kinase was reported by dividing the percentage of band density corresponding to a pI of 6.3 (one subunit oxidized and one subunit reduced) by two

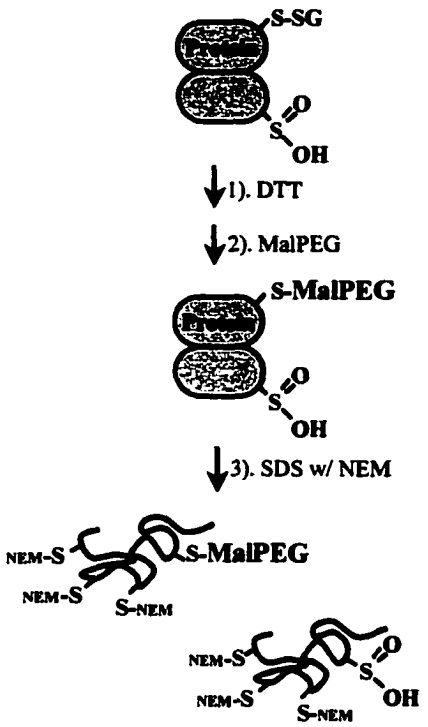



**Figure 1. Protocol for SDS-page analysis of irreversible protein sulfhydryl damage.**

Proteins are treated with dithiothreitol (DTT) to remove reversible sulfhydryl modifications such as S-glutathiolation or S-nitrosylation. Both reversible and irreversible modifications would be detected by this method if this step was omitted. Reactive (i.e., exposed) cysteine residues are then tagged with O-(2-maleimidoethyl)-O'-methylpolyethylene glycol 5,000 (MalPEG) and samples are finally denatured in non-reducing SDS-PAGE running buffer containing excess N-ethylmaleimide (NEM). All non-reactive protein cysteine residues are covalently blocked by NEM, preventing artifacts that might occur during SDS-PAGE electrophoresis.

and added to the percentage of protein detected in the band at 6.2 (both subunits oxidized). Creatine kinase is detected as a monomer by MalPEG analysis; therefore the amount of irreversibly oxidized cysteine per monomer was simply the density of creatine kinase detected in the oxidized band divided by the density of the total amount of creatine kinase. For quantitation of irreversibly oxidized creatine kinase from tissues, it was necessary to serially dilute NEM-treated extracts to produce band densities for total creatine kinase that were of similar magnitude to the densities corresponding to irreversibly oxidized creatine kinase.

**Protein sulfinic and sulfonic acid analysis**

Samples were analyzed for irreversibly oxidized forms of cysteine as described (27). Briefly, samples were incubated at 37 °C for 15 min in buffer containing 120 mM phosphate buffer, pH 7.4, 7 M urea and 10 mM DTT. Cysteine residues were then blocked with 40 mM IAM. Each sample was split into two fractions, one of which was treated with 10 mM HOCl for 5 min at room temperature in order to oxidize sulfinic acids to sulfonic acids. The reactions were terminated after five minutes with the addition of 30 mM DTT. The samples were extensively dialyzed and hydrolyzed by vapor phase acid. Amino acid analysis was conducted using precolumn derivatization with OPA and reverse-phase HPLC.

HOCl is used to convert protein sulfinic to sulfonic acid, which would otherwise be lost during acid hydrolysis. Therefore, the amount of cysteic acid found in a sample treated with HOCl represents the total protein irreversibly oxidized cysteine. Cysteic acid found in samples not treated with HOCl represents the amount of cysteine detected as protein sulfonic acid. Protein sulfinic acid can be calculated by subtracting the total protein sulfonic acid from

the total irreversibly oxidized cysteine. The total moles of creatine kinase in a hydrolysate was calculated on the basis of total leucine in a sample (37 mol leucine / mol creatine kinase) Heart and skeletal muscle protein extracts were compared by dividing moles of cysteic acid by the total moles of cysteine detected.

**Preparation of soluble protein extracts from young and old rats**

One, three, and sixteen month old male Fisher 344 rats were used. Food (Teklad Diet #7002) and water were provided *ad libitum*. Rats were sacrificed by $CO_2$ inhalation, the heart and hind leg skeletal muscle was removed, rinsed with ice cold saline, and frozen immediately using a liquid nitrogen cooled clamp. Tissues were powdered using a liquid nitrogen cooled, stainless steel mortar and pestle and stored at – 80° until use. Soluble protein extracts were prepared by homogenizing tissue in ice-cold homogenization buffer (20 mM β-glycerophosphate, 10 mM DTT, 5 mM EDTA, and 5 mM EGTA, pH 7.4) The homogenate was centrifuged at 16,000 *g* for 10 min at 4 °C, the supernatant was recovered, and centrifuged in a Beckman Airfuge Ultracentrifuge at 160,000 *g* for 30 min. The soluble protein extract produced by this procedure was incubated at 37 °C for 30 min to remove any DTT reversible modifications prior to analysis.

**Glutathione determination:**

The amount of reduced glutathione in protein extracts was determined using the method by Fariss and Reed (31), with the following modifications: Briefly, the amount of glutathione was quantitated by precipitating protein from extracts with 10% perchoric acid, followed by derivatizing the acid-soluble portion with 100 mM iodoacetic acid. The pH was

made alkaline by adding powderized potassium bicarbonate, and the sample were incubated in the dark for 20 min at room temperature. Finally, samples were were labeled for HPLC analysis by diluting them 2-fold with 1% dinitroflourobenzene in ethanol. Samples were normalized to milligrams of soluble protein.

**Protein assay**

Protein concentrations were determined as described by Lowry *et al.* (32).

## Results

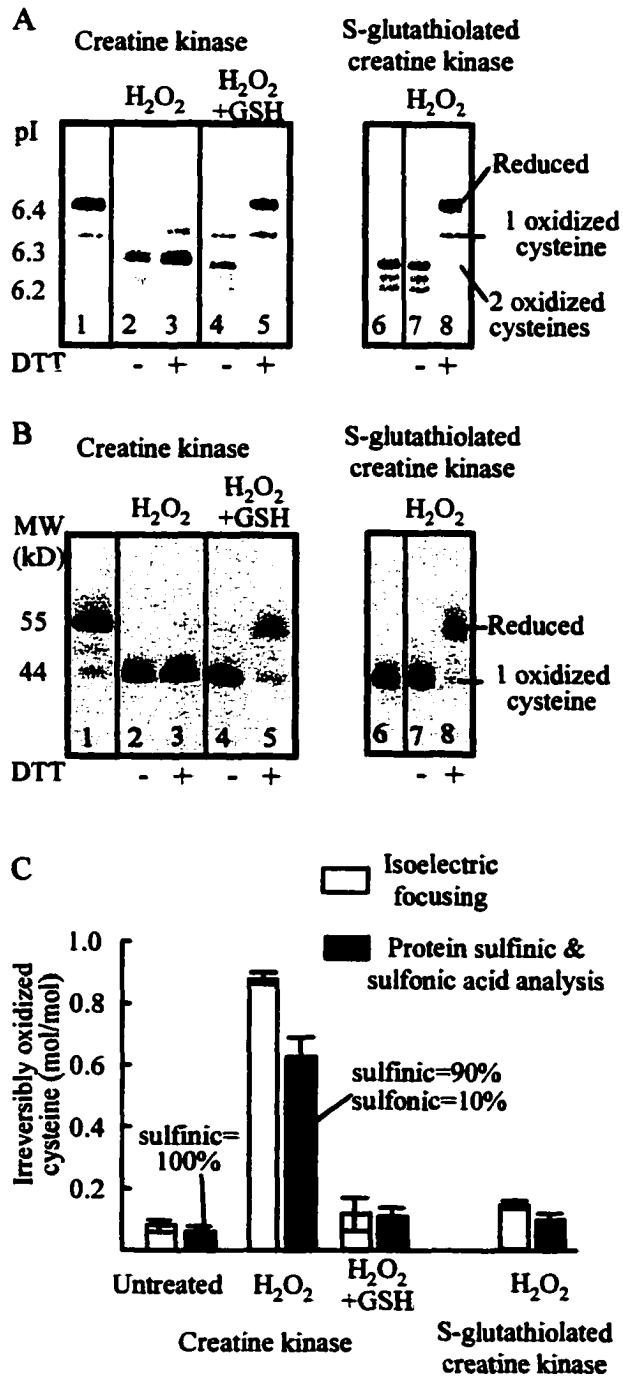
**Hydrogen peroxide oxidizes creatine kinase cysteine residues to sulfinic acid in the absence of glutathione.**

Sulfinic and sulfonic acids are irreversible sulfhydryl oxidation states that bear a negative charge and are unreactive towards neutrally charged alkylating reagents such as NEM or IAM. Isoelectric focusing gels (IEF) can be used to rapidly identify these modifications, since a protein with sulfinic and sulfonic acids will have more a acidic pI compared to the reduced, alkylated form. Reversible sulfhydryl modifications such as protein S-glutathiolation or protein sulfenic acids also result in a more acidic protein pI, but these are easily distinguished from irreversible oxidation by treating the protein with DTT prior to alkylation (27,29).

When creatine kinase is alkylated with NEM, the protein focuses with a pI of 6.4 on an IEF gel (Fig. 2A, lane 1). Bands corresponding to a pI of 6.3 or 6.2 appear when either one or two thiols per dimer of creatine kinase carry a negative charge. Approximately 90% of the protein migrated with a pI of 6.2 (lane 2) after incubating creatine kinase with

**Figure 2. Irreversible and reversible modification of creatine kinase with hydrogen peroxide.**

Creatine kinase or S-glutathiolated creatine kinase (25 μM) was incubated with 0.3 mM $H_2O_2$ for 30 min at 37°C in 20 mM β-glycerophosphate, pH 7.4. Creatine kinase was also treated with $H_2O_2$ in the presence of 0.3 mM glutathione (GSH). The reactions were split into two aliquots, and one was reduced with 10 mM DTT for 30 min at 37°C. Each aliquot was derivatized by ten-fold dilution into either 5 mM NEM or 5 mM MalPEG. A) An isoelectric focusing (IEF) gel of NEM treated samples. B) SDS-PAGE/western blot analysis of MalPEG treated samples using the methods described in Figure 1. C) Quantitation of irreversibly oxidized creatine kinase by densitometry of the IEF gels and by protein sulfinic and sulfonic acid analysis as described in Materials and Methods. The relative amount of sulfinic and sulfonic acids are indicated on the figure (detection limit=0.04 mol/mol).


A
Creatine kinase
S-glutathiolated creatine kinase
$H_2O_2$
$H_2O_2$ +GSH
pI
6.4
6.3
6.2
Reduced
1 oxidized cysteine
2 oxidized cysteines
1 2 3 4 5
6 7 8
DTT - + - +
- +
B
Creatine kinase
S-glutathiolated creatine kinase
MW (kD)
55
44
Reduced
1 oxidized cysteine
C
Isoelectric focusing
Protein sulfinic & sulfonic acid analysis
Irreversibly oxidized cysteine (mol/mol)
1.0
0.8
0.6
0.4
0.2
sulfinic=90%
sulfonic=10%
sulfinic=100%
Untreated
$H_2O_2$
$H_2O_2$ +GSH
Creatine kinase
S-glutathiolated creatine kinase

hydrogen peroxide for 30 min. Since only a minor amount of the total modification (< 10%) was sensitive to DTT reduction (compare lanes 2 and 3), almost all of the cysteine oxidation detected can be described as irreversible. Increasing the incubation time with DTT (up to 24 hours) or changing the reductant to 10 mM β-mercaptoethanol, glutathione, or sodium borohydride did not shift the pI of the oxidized protein, demonstrating that the oxidative modifications were unaffected by common reducing agents.

Protein S-glutathiolation prevents protein cysteine irreversible oxidation (23), and the mechanism can be demonstrated by incubating creatine kinase with hydrogen peroxide in the presence of 300 μM glutathione. Under these conditions, the protein's pI shifts to almost the same extent as samples treated in the absence of glutathione (compare lane 2 and lane 4), but the modifications are entirely DTT reversible (lane 5). The appearance of two minor bands with pI's lower than 6.2 indicate that a cysteine residue other than 283 was modified in this experiment, possibly cysteine 74 (33). These bands disappeared after treating with DTT, and therefore do not play a role in creatine kinase irreversible oxidation. The protective role of S-glutathiolation was further examined by specifically generating the S-glutathiolated form of creatine kinase and treating it with hydrogen peroxide. The hydrogen peroxide treated sample (lane 7) maintained the same pI as the untreated control (lane 6), showing that the protein was not further oxidized. Moreover, the cysteine residues remained reversibly modified, since DTT reduction restored the protein pI to 6.4. Clearly the disulfide bond between glutathione and creatine kinase cysteine residues inhibits hydrogen peroxide oxidation to sulfinic and sulfonic acids.

Theoretically irreversibly oxidized creatine kinase could be detected *in vivo* using IEF in conjunction with western blotting techniques. This approach has been used to detect

irreversibly oxidized forms of carbonic anhydrase III in both hepatocytes and rat liver protein extracts (34). Unfortunately, creatine kinase was not detected with high enough sensitivity to permit the identification of irreversibly oxidized forms of the protein, despite several changes to the transfer and blotting protocol (35). An alternative procedure was developed; creatine kinase was alkylated with MalPEG, and cysteine oxidation was detected by SDS-PAGE followed by western blotting. MalPEG is simply a maleimide group linked to a polyethylene glycol polymer of defined length. MalPEG-5000 was used in these experiments, which has about 113 repeating ethylene units. Like all maleimides, the reagent couples selectively with sulfhydryl groups at neutral pH, resulting in a stable covalent linkage. Oxidation of the sulfhydryl group prevents the coupling reaction. Reduced protein thiols "tagged" with MalPEG experience an increase in molecular weight in comparison to the oxidized protein, and the different forms can be readily separated by SDS-PAGE. The number bands detected on the SDS-PAGE gel depends on the number of reactive cysteines potentially modified by MalPEG (28).

Only two bands are detected when creatine kinase is treated with MalPEG; a 55 kD band corresponds to creatine kinase with a reduced cysteine, and a 43 kD band corresponds to protein with an oxidized cysteine (Figure 2, Panel B). Although MalPEG should only increase the molecular weight of reduced creatine kinase by 5 kD, the migration through the gel is retarded by the low charge to mass ratio of the polyethyleneglycol tag (lane 1). Unlike IEF, there are only two bands in these experiments, because creatine kinase dimers are dissociated by the presence of SDS in the sample loading buffer. Despite the differences in methodology, a comparison of panels A and B of Figure 1 demonstrates that either IEF or MalPEG tagging can determine similar information about protein cysteine oxidation. All

lanes in panel B represent an aliquot of the same sample that was applied to the IEF gel in Panel A, however, samples were alkylated with MalPEG instead of NEM. These results show that MalPEG could be used to detect irreversibly oxidized creatine kinase *in vivo*.

Irreversible cysteine oxidation detected by gel electrophoresis was related to protein sulfinic and sulfonic acids using procedures described in the Materials and Methods section (Fig. 2, panel C). Irreversibly oxidized creatine kinase represented in lanes 1, 3, 5, and 8 of panel A was quantitated using gel densitometry and compared to the results from sulfinic and sulfonic acid analysis. Typically, results from protein sulfinic and sulfonic acid analysis were ≥80% of the values determined by IEF, clearly establishing that the irreversible oxidation detected by IEF were occurring at protein cysteine residues. Protein sulfinic and sulfonic acid analysis also revealed that sulfinic acids constituted ~ 90% of the irreversible cysteine oxidation produced by hydrogen peroxide. Interestingly, hydrogen peroxide has previously been demonstrated to oxidize carbonic anhydrase III cysteines selectively to sulfinic acids (23). The simplest explanation for these results is that the oxidation of the sulfinic acid to a sulfonic acid represents a relatively slow reaction step, and is evidenced by the fact that sulfonic acids began to accumulate when creatine kinase was incubated with higher concentrations of hydrogen peroxide.

Hydrogen peroxide has been shown to inactivate creatine kinase (36), but the relationship to cysteine irreversible oxidation has not been determined. Figure 3 shows that when creatine kinase was incubated with hydrogen peroxide in a time-course experiment, the activity of the protein progressively diminishes while total sulfinic and sulfonic acids accumulate. The loss in activity correlates with the oxidation of a single cysteine residue. Since cysteine 283 is the most reactive cysteine residue and can also affect enzymatic activity

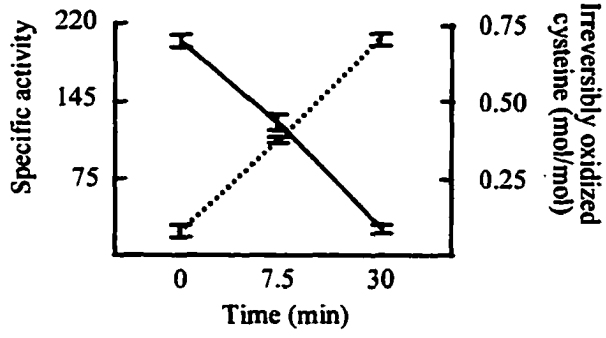



**Figure 3. Effects of irreversible oxidation on creatine kinase activity.**

Creatine kinase was incubated with $H_2O_2$ using the same conditions described in the legend to Figure 2. Aliquots of the reaction were removed at the indicated times and reduced with 10 mM DTT. When DTT was added prior to hydrogen peroxide, the activity was the same as the untreated sample and the amount of protein sulfinic acids did not increase. Creatine kinase specific activity was assayed using a commercially available kit (Sigma). Sulfinic acid analysis was performed as described in Materials and Methods. Sulfonic acids were not detected in these samples.

(26), it is reasonable to suggest that hydrogen peroxide permanently inactivates the protein by specifically oxidizing cysteine 283 to a sulfinic acid. IEF, MalPEG, and protein sulfinic and sulfonic acid analysis detected similar amounts of irreversibly oxidized creatine kinase in this experiment, indicating that all of these methods can detect the inactive form of the protein.

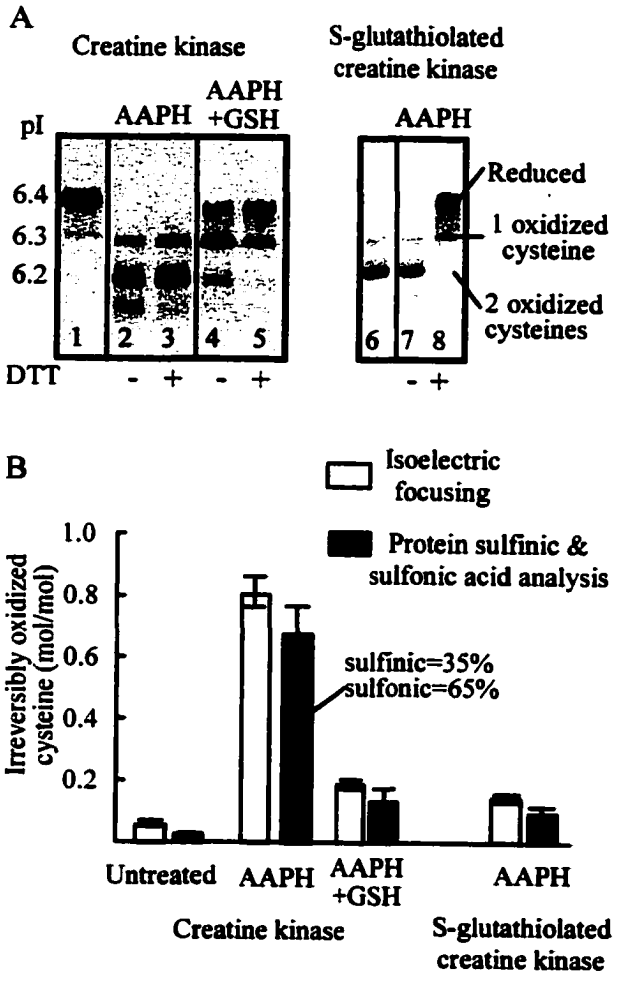
**Irreversible oxidation of creatine kinase by peroxyl radicals**

Hydrogen peroxide oxidizes thiols through a two-electron process involving a sulfenic acid intermediate (37). Radical-mediated thiol oxidation was investigated using peroxyl radicals generated with AAPH. AAPH is a thermal labile, azo-containing compound which decays to give two moles of carbon centered radicals per mole of AAPH. Molecular oxygen combines rapidly with the carbon-centered radicals to produce peroxyl radicals. The rate of AAPH decomposition is highly temperature dependent, and the conditions described in Figure 4 would have produced approximately 25 μM peroxyl radical by the end of the experiment (38).

Peroxyl radicals oxidized 95 % of the creatine kinase reactive cysteine residues after a 30 min incubation at 37°C (Fig. 4, panel A, lane 2), with only ~13 % of the total modification remaining sensitive to DTT reduction (compare lanes 2 and 3). It is interesting to note that the stoichiometry between the total peroxyl radicals produced and the amount of irreversible cysteine oxidation detected by IEF is almost one to one. This relationship underscores the reactivity of peroxyl radicals in comparison to hydrogen peroxide, as well as the oxidant's selectivity for protein thiol groups.

**Figure 4. Oxidation of creatine kinase with peroxyl radicals.**

Creatine kinase or S-glutathiolated creatine kinase (25 μM) was incubated with 10 mM AAPH for 30 min at 37°C in 50 mM phosphate buffer, pH 7.4. Incubations were also conducted in the presence of 50 μM GSH. The reactions were split into two aliquots, and one was reduced with 10 mM DTT for 30 min at 37°C. Each aliquot was derivatized with 40 mM iodoacetamide (IAM). DTT treated samples were reduced for 30 min at 37°C before alkylating with IAM. A) IEF analysis of IAM treated samples. B) Quantitation of irreversibly oxidized creatine kinase by densitometry of the IEF gels and by protein sulfinic and sulfonic acid analysis. The ratio of sulfinic and sulfonic acids detected in oxidized samples are indicated.


A
Creatine kinase
S-glutathiolated creatine kinase
AAPH
AAPH +GSH
pI
6.4
6.3
6.2
1 2 3 4 5
DTT - + - +
AAPH
Reduced
1 oxidized cysteine
2 oxidized cysteines
6 7 8
- +
B
Isoelectric focusing
Protein sulfinic & sulfonic acid analysis
Irreversibly oxidized cysteine (mol/mol)
1.0
0.8
0.6
0.4
0.2
sulfinic=35%
sulfonic=65%
Untreated
AAPH
AAPH +GSH
AAPH
Creatine kinase
S-glutathiolated creatine kinase

Incubating creatine kinase in the presence of 50 μM glutathione diminished the total amount of cysteine modification produced by peroxyl radicals to 52 % (lane 4). Concentrations of glutathione greater than 150 μM completely prevented S-glutathiolation of creatine kinase, suggesting that glutathione is reacting directly with peroxyl radicals and partially preventing the oxidation of creatine kinase. The protein was mostly S-glutathiolated, however, as about 65% the modifications remained sensitive to DTT reduction (lane 5). As with hydrogen peroxide, S-glutathiolation of creatine kinase protected protein cysteine residues from further oxidation.

Sulfinic and sulfonic acid analysis confirmed that irreversible oxidation detected by IEF was specifically due to cysteine oxidation, and that irreversible oxidation was both prevented and protected by S-glutathiolation (Fig. 4, panel B). Unlike oxidations with hydrogen peroxide, the irreversible cysteine oxidation generated by peroxyl radicals proved to be roughly 65% sulfonic acid, demonstrating that peroxyl radicals rapidly oxidized sulfinic to sulfonic acids.

**Protein cysteine irreversible oxidation and age**

Rat liver soluble protein extracts have been shown to contain measurable amounts of protein sulfinic acid that increase with age. These results were reflected in liver carbonic anhydrase III, where cysteine irreversible oxidation was demonstrated using IEF and MalPEG analysis (23). A similar approach was taken with soluble protein extracts prepared from the heart and skeletal muscle of one, three, and sixteen month old male rats. Total protein sulfinic and sulfonic acid content was measured and related to irreversibly oxidized creatine kinase detected by MalPEG analysis. Creatine kinase represented about 5 % of the

soluble protein in heart and 15 % in skeletal muscle, and were well suited for MalPEG analysis.

Soluble protein extracts from heart and skeletal muscle generally exhibited an age-dependent increase in the amount of protein sulfinic acid (Fig. 5, panel A). An exception was heart extracts from one and three month old rats, where the protein sulfinic acid content decreased from 3.1 to 2.5 mol % cysteine. However, the amount of sulfinic acid detected in heart extracts from 16 month old rats was greater than either of these age groups at 4.4 mol % cysteine. Skeletal muscle sulfinic acid content gradually increased from 1.1 mol % cysteine in one month old rats, to 1.3 mol % in three month old rats, and 1.9 mol % in the sixteen month old rats. The 1.7-fold increase in sulfinic acid measured in heart extracts from three and sixteen old rats and skeletal muscle extracts from one and sixteen month old rats was significant as determined by the Student's *t*-test. As previously reported for liver extracts, sulfonic acid was not detectable in any of the heart or skeletal muscle extracts (detection limit = 0.2%). Overall, the sulfinic acid content of heart was roughly two-fold greater than in skeletal muscle, regardless of age, and could reflect tissue-specific differences in reactive oxygen species concentrations or protein turnover rates.

Since glutathione clearly inhibits protein cysteine irreversible oxidation *in vitro*, it is reasonable to speculate that age-related decreases in cellular glutathione accelerate protein cysteine irreversible oxidation (21). In heart, glutathione levels diminished from 33.3 nmol/mg in the one month old rats, to 29.1 nmol/mg in three month old rats, and finally to 23.8 nmol/mg in sixteen month old rats. The trend was the same in skeletal muscle, although the amounts of glutathione detected were about half the amount detected in heart.

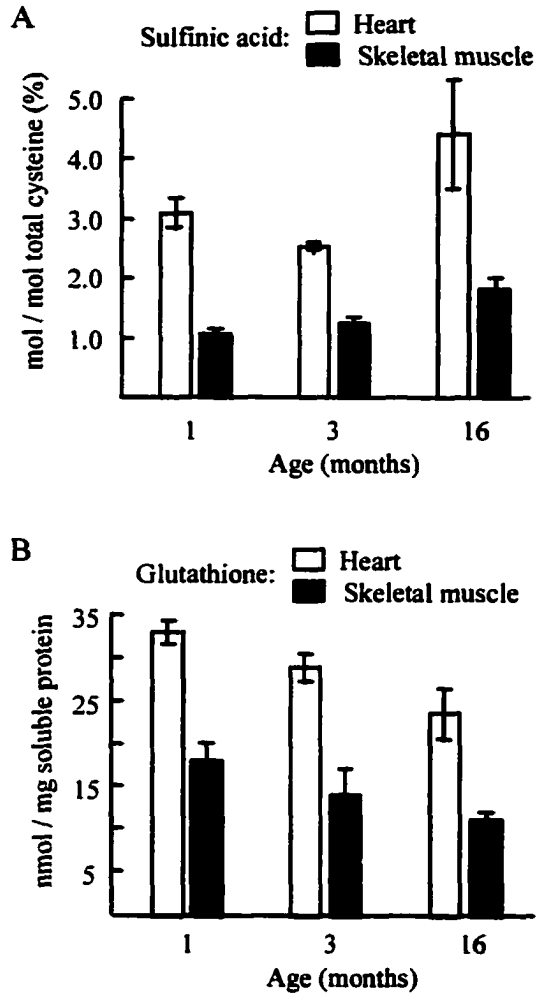



**Figure 5. The effect of aging on protein sulfinic acid and glutathione in rat heart and skeletal muscle extracts.**

Protein extracts were prepared from the heart and skeletal muscle of one, three, and sixteen month old Fisher 344 rats as described in Materials and Methods. A) Total protein sulfinic acids; sulfonic acids were not detected (detection limit = 0.2%). B) Total gluatathione was measured as described in Materials and Methods. Results are the mean and standard deviation for three rats per age group.

Glutathione progressively diminished with increased age from 17.8 to 13.9 to 11.1 nmol/mg. One month old rats had about 1.5-fold more glutathione than sixteen month old rats in both heart and skeletal muscle.

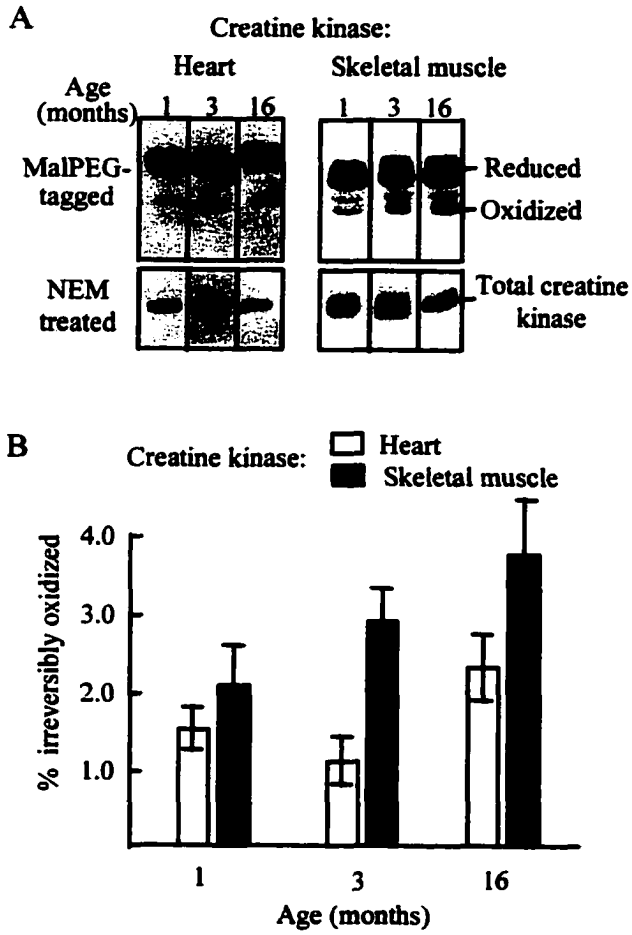
Figure 6, panel A shows representative blots of creatine kinase detected in rat heart and skeletal muscle. The upper portion demonstrates the results of MalPEG-tagging, and indicates that an irreversibly oxidized form of creatine kinase was present in all the samples, regardless of age, with a detectable increase in the 16 month old rats. The lower portion compares the total creatine kinase detected in the different extracts, and represents a 25-fold dilution of NEM-alkylated extracts. The same amount of total protein was loaded in each lane. Concentrations of creatine kinase remained constant over the different aged rats in skeletal muscle, while the heart tissue from 3 month old rats consistently showed a 1.4-fold increase compared to 1 or 16 month old rats. Panel B more clearly delineates the differences between ages by comparing the percentages of irreversibly oxidized creatine kinase. This percentage was determined as described in the Materials and Methods and represents a duplicate analysis of each of the three rats per age group. The same trends that were detected by analysis of the total sulfinic acids in heart and skeletal muscle are reflected in the creatine kinase pool; in this case however, heart creatine kinase was less irreversibly oxidized overall compared to skeletal muscle creatine kinase.

## Discussion

Experiments presented here support the hypothesis that S-glutathiolation protects protein cysteine residues from oxidation to sulfinic and sulfonic acids. Irreversible oxidation of creatine kinase by hydrogen peroxide and peroxyl radical occurred rapidly *in vitro*, but

**Figure 6. Comparison of the amount of irreversibly oxidized creatine kinase found in the heart and skeletal muscle of rats of different ages.**

Rat heart and skeletal muscle extracts were prepared as described in Materials and Methods. A) An aliquot of each extract was either tagged with 5 mM MalPEG or 5 mM NEM or and prepared for SDS-PAGE. MalPEG tagged samples were 25-fold more concentrated than NEM treated samples in order to effectively compare the amount of irreversibly oxidized creatine kinase with the total pool of creatine kinase found in tissues. Creatine kinase was detected using western blotting with an antibody specific for the muscle isoform of the protein. B) The relative amounts of irreversibly oxidized creatine kinase detected in different aged rats was determined by densitometry of western blots. Irreversibly oxidized creatine kinase was quantitated by detecting the amount of oxidized protein detected in MalPEG treated samples and comparing it to the total amount of creatine kinase detected in NEM treated extracts. Results are the mean and standard deviation for three different rats per age group.


A
Creatine kinase:
Heart
Skeletal muscle
Age (months)
1 3 16
1 3 16
MalPEG-tagged
Reduced
Oxidized
NEM treated
Total creatine kinase
B
Creatine kinase:
Heart
Skeletal muscle
% irreversibly oxidized
4.0
3.0
2.0
1.0
1
3
16
Age (months)

was prevented by glutathione through protein S-glutathiolation. Moreover, treating S-glutathiolated creatine kinase with hydrogen peroxide and peroxyl radicals completely prevented irreversible oxidation, demonstrating that the disulfide bond between protein cysteine and glutathione is not as rapidly oxidized as the free thiol (39). It is conceivable that the disulfide bond between glutathione and creatine kinase is oxidized to a disulfide S-oxide in these experiments and not detected with the methods used in this paper (40). However, the important consideration is that DTT was capable of reducing the oxidation by thiol-disulfide exchange; therefore, the modifications would be reducible *in vivo*. As with carbonic anhydrase III and H-ras, S-glutathiolation of creatine kinase by hydrogen peroxide or peroxyl radicals did not occur through the intermediary action of glutathione disulfide (19, 22). If it is assumed that all of the glutathione incubated with hydrogen peroxide (Figure 2) was oxidized to glutathione disulfide, the concentration could be no more than 150 μM. Incubating creatine kinase with this concentration of glutathione disulfide; however, provides no evidence of cysteine modification (data not shown). Thus, S-glutathiolation of creatine kinase requires the direct interaction of protein sulfhydryls with an oxidant, but it generates a disulfide bond that protects the protein from non-reducible damage.

It is well established that protein S-glutathiolation is a minor form of protein sulfhydryl modification in cells under basal conditions, but becomes more pronounced after exposure to an oxidant (41,42); consequently, protein thiols should be effectively protected from irreversibly oxidation *in vivo*. Even so, sulfinic acids were detected in rat heart and skeletal muscle regardless of age; although a definite age-related increase is evident (Fig. 5). In fact, when sulfinic acid content was calculated on the basis of total mgs protein in a sample, the amount of oxidation detected (i.e. about 0.5 nmol/mg) approximated levels

typical for protein carbonyls; currently the most robust measure of protein oxidation (43). It should be noted that results presented in Figure 5 were in terms of total protein cysteine. This may understate the magnitude of the oxidative damage occurring to proteins, as only a fraction of the total cysteines are surface-exposed and sensitive to oxidative modification. Since surface-exposed cysteines are often vital in protein functions, damage to these sites would be particularly detrimental. A generous estimate would suggest that surface-exposed cysteine residues account for one of every four cysteines; therefore, damage to surface-exposed cysteine could be 4-fold higher than the amounts displayed in Figure 5. Interestingly, heart and skeletal muscle were both devoid of sulfonic acids. Previous results from liver demonstrated a similar phenomenon (23). The tissues used in the analyses reported here were from rats that were not challenged with an oxidative stress, and the cellular redox potential may prevent sulfonic acids from rising to detectable levels (44). Sulfonic acids could appear during periods of more extreme or protracted oxidative stress, such as ischemia-reperfusion injury, and may be an effective marker of extreme damage to protein cysteine.

It has been suggested that relatively small fluctuations in cellular glutathione can significantly influence irreversible cysteine oxidation (23). Indeed, the relationship between total glutathione and protein sulfinic acids in skeletal muscle and heart provides compelling evidence in support of this notion. Diminished cellular glutathione concentrations are symptomatic of aging and may be associated with an age-dependent down-regulation of γ-glutamylcysteine synthetase (45); therefore, protein cysteine irreversible oxidation is unlikely to be confined only to heart and skeletal muscle. Of note, Alzheimer's and Parkinson's disease exhibit depleted glutathione levels (46,47), and irreversible oxidation of cellular

protein cysteines may significantly contribute to the pathologies of these diseases. Curiously, heart tissues contained both the highest sulfinic acid and glutathione content in comparison to skeletal muscle, but it could be speculated that glutathione levels are attenuated in a tissue-specific manner in order to compensate for potential differences in oxidant concentrations.

Age-related changes in total protein sulfinic acid in heart and skeletal muscle are reflected in the creatine kinase pool, although skeletal muscle creatine kinase was more irreversibly oxidized than in heart. This indicates that irreversible cysteine oxidation detected for an individual protein can vary from the amount of total protein sulfinic acid, and could simply relate to the 3-4 fold difference in creatine kinase concentration between heart and skeletal muscle. Other proteins in heart could be oxidized to a greater extent in order to account for the greater total sulfinic acid content in this tissue. The age-related increase in irreversibly oxidized creatine kinase reported here would have little effect on total creatine kinase activity in heart or skeletal muscle. Instead, these results demonstrate that MalPEG analysis of creatine kinase is an effective marker of protein cysteine irreversible oxidation *in vivo*. It is likely that the method would work equally well with either the mitochondrial or brain isoforms of creatine kinase, since both have a single reactive cysteine per polypeptide chain (48,49). Other proteins with reactive cysteines may also be appropriate for this analysis. The amount of irreversibly oxidized creatine kinase detected *in vivo* implies that the damaging aspect of sulfinic and sulfonic acid accumulation is probably realized through overall oxidation of the protein thiol pool rather than specific proteins. Protein activities which are up-regulated by cysteine irreversible oxidation (such as matrix metaloproteinases) are more likely to exhibit a profound cellular impact in comparison to protein which are rendered inactive, since doubling or tripling cysteine irreversible oxidation potentially

doubles or triples the protein's activity. H-ras may be good candidate in this regard. The GTPase activity of this protein has been shown to increase upon nitrosylation of cysteine 118 (50), and oxidizing the cysteine to a sulfinic acid may permanently activate the protein, leading to a constant stimulation of the MEK1 pathway.

Figure 7 summarizes the cellular relationship of protein S-thiolation and irreversible oxidation. Oxidants impinge upon the cellular protein pool from a variety of sources, including radicals formed from oxidative respiration, inflammatory responses, and exposure to radical-generating toxicants (51). Some oxidants react with the cellular protein thiol pool and generate intermediates such as sulfenic acids and thiyl radicals. While a small number of proteins may be able to structurally shield sulfenic acids and thiyl radicals from the cytosolic environment (52), these intermediates are unstable in most proteins. Low molecular weight thiols such as glutathione react with the intermediate and generate a disulfide bond that can be reduced within minutes in cells (2,42) . Occasionally, sulfenic acids or thiyl radicals will be further oxidized to generate irreversible cysteine oxidation, which will ultimately persist in cells until the protein is degraded. Cellular conditions that promote oxidant generation, deplete antioxidants and low molecular weight thiols, or inhibit protein degradation are expected to promote the accumulation of proteins with irreversibly oxidized cysteines, and this may provide causal explanations to the pathologies associated with aging.

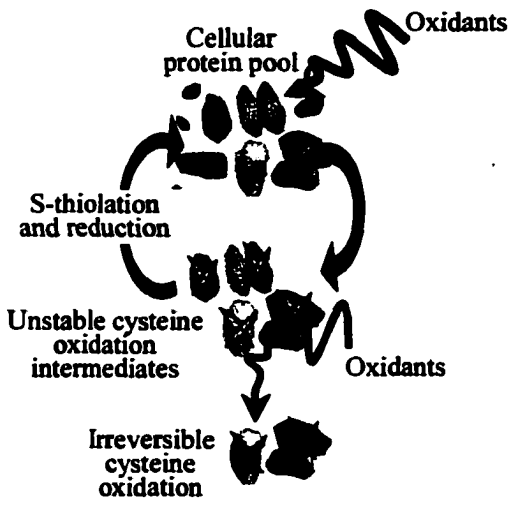



**Figure 7. A model for irreversible oxidation of protein cysteine *in vivo*.**

The cellular protein pool is exposed to oxidants in the form of reactive oxygen and nitrogen species, leading to the formation of unstable intermediates such as thiyl radicals and sulfenic acids. S-thiolation prevents further oxidation of the unstable intermediates and allows the protein sulfhydryl to be restored after subsequent reduction. Irreversible cysteine oxidation occurs if sulfenic acids and thiyl radicals are further oxidized to sulfinic and sulfonic acids.

**References:**

1. Thomas, J. A., Mallis, R. J., and Sies, H. (2002) in *Redox Regulation and Protein Thiols.* In Press.

2. Jung, C. H., and Thomas, J. A. (1996) *Arch. Biochem. Biophys.* **335,** 61-72.

3. Patel, J. M., Zhang, J., and Block, E. R. (1996) *Am. J. Respir. Cell. Mol. Biol.* **15,** 410-419

4. Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J., and Stamler, J. S. (2001) *Nature* **410,** 490-494.

5.Isaacs, J and Binkley, F. (1977) *Biochim. Biophys. Acta* **497**, 192-204.

6. Klatt, P., and Lamas, S. (2000) *Eur. J. Biochem.* **267,** 4928-4944

7. Rainwater, R., Parks, D., Anderson, M. E., Tegtmeyer, P., and Mann, K. (1995) *Mol. Cell. Biol.* **15,** 3892-3903

8. Wu, H. H., Thomas, J. A., and Momand, J. (2000) *Biochem. J.* **351,** 87-93

9. Klatt, P., Molina, E. P., De Lacoba, M. G., Padilla, C. A., Martinez-Galesteo, E., Barcena, J. A., and Lamas, S. (1999) *FASEB J.* **13,** 1481-1490.

10. Nikitovic, D., Holmgren, A., and Spyrou, G. (1998) *Biochem. Biophys. Res. Commun.* **242,** 109-112

11. Matthews, J. R., Kaszubska, W., Turcatti, G., Wells, T. N., and Hay, R. T. (1993) *Nucleic Acids Res.* **21,** 1727-1734

12. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *J. Biol. Chem.* **266,** 4244-4250

13. Barrett, W. C., DeGnore, J. P., Konig, S., Fales, H. M., Keng, Y. F., Zhang, Z. Y., Yim, M. B., and Chock, P. B., (1999) *Biochemistry* 1999 **38,** 6699-6705.

14 Skorey, K., Ly, H. D., Kelly, J., Hammond, M., Ramachandran, C., Huang, Z., Gresser, M. J., and Wang, Q. (1997) *J. Biol. Chem.* **272,** 22472-22480

15. Souza, J. M., and Radi, R. (1998) *Arch. Biochem. Biophys.* **360,** 187-194

16. Fu, X., Kassim, S. Y., Parks, W. C., and Heinecke, J. W. (2001) *J. Biol. Chem.* **276,** 41279-41287

17. Gu, Z., Kaul, M., Yan, B., Kridel, S. J., Cui, J., Strongin, A., Smith, J.W., Liddington, R.C., and Lipton, S. A. (2002) *Science* **297,** 1186-90.

18. Wilcox D. E., Schenk, A., Feldman, B. M., and Xu, Y. (2001) *Antioxid. Redox Signal.* **3,** 549-564.

19. Savitsky, P. A., and Finkel, T. (2002) *J. Biol. Chem.* **277**, 20535-20540

20. Mallis, R. J., Buss, J. E., and Thomas, J. A. (2001) *Biochem. J.* **355,** 145-153.

21. Thomas, J. A., and Mallis, R. J. (2001) *Exp. Gerontol.* **36,** 1519-26.

22. Thomas, J. A., Poland, B., Honzatko, R. (1995) *Arch. Biochem. Biophys.* **319**, 1-9

23. Mallis, R. J., Hamann, M., Zhao, W., Zhang, T., Hendrich, S., and Thomas, J. A. (2002) *Biol. Chem.* **383,** 649-662.

24. Hagen, T. M., Ingersoll, R. T., Lykkesfeldt, J., Liu, J., Wehr, C. M., Vinarsky, V., Bartholomew, J. C., and Ames, A. B. (1999) *FASEB J.* **13,** 411-8

25. Rao, J. K., Bujacz, G., and Wlodawer, A. (1998) *FEBS Lett.* **439,** 133-137.

26. Reddy, S., Jones, A. D., Cross, C. E., Wong, P. S., and Van Der Vliet, A. (2000) *Biochem. J.* **347**, 821-827.

27. Hamann, M., Zhang, T., Hendrich, S., and Thomas, J. A. (2002) *Methods Enzymol.* **348,** 146-156.

28. Makmura, L., Hamann, M., Areopagita, A., Furuta, S., Munoz, A., and Momand, J. (2001) *Antioxid. Redox Signal.* **3,** 1105-1118.

29. Miller, R. M., Sies, H., Park, E., and Thomas, J. A. (1990) *Arch. Biochem. Biophys.* **276,** 355-366.

30. Furuta, S., Ortiz, F., Zhu, Sun X., Wu, H. H., Mason, A., and Momand, J. (2002) *Biochem. J.* **365,** 639-648

31. Fariss, M. W. and Reed, D. J. (1987) *Methods Enzymol.* **143,** 101-109.

32. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265-275.

33. Hurne, A. M., Chai, C. L., and Waring, P. (2000) *J. Biol. Chem.* **275,** 25202-25206

34. Lii, C. K., Chai, Y. C., Zhao, W., Thomas, J. A., and Hendrich, S. (1994) *Arch. Biochem. Biophys.* **308,** 231-239.

35. Reddy, S. G., Cochran, B. J., Worth, L. L., Knutson, V. P., and Haddox, M. K. (1994) *Anal. Biochem.* **218,** 149-156.

36. Suzuki, Y. J., Edmondson, J. D., and Ford, G. D. (1992) *Free Rad. Res. Commun.* **16,** 131-136.

37. Winterbourn, C. C., and Metodiewa, D. (1994) *Arch. Biochem. Biophys.* **295,** 369-378.

38. Niki, E. (2002) *Methods Enzymol.* **186,** 100-108.

39. Coan, C., Ji, J. Y., Hideg, K., and Mehlhorn, R. J. (1992) *Arch. Biochem. Biophys.* **295,** 369-378.

40. Okamoto, T., Akaike, T., Sawa, T., Miyamoto, Y., van der Vliet, A., and Maeda, H. (2001) *J. Biol. Chem.* **276,** 29596-29602

41. Collison, M. W., and Thomas, J. A. (1987) *Biochim. Biophys. Acta* **928,** 121-129.

42. Seres, T., Ravichandran, V., Moriguchi, T., Rokutan, K., Thomas, J. A., and Johnston, R. B. Jr. (1996) *J. Immunol.* **156,** 1973-1980

43. Davies, M. J., Fu, S., Wang, H., and Dean, R. T. (1999) *Free Radic. Biol. Med.* **27,** 1151-1163.

44. Schafer, F. Q., Buettner, G. R., (2001) *Free Radic Biol Med* **30,** 1191-1212

45. Liu, R. M. (2002) *J. Neurosci. Res.* **68,** 344-351

46. Schulz, J. B., Lindenau, J., Seyfried, J., and Dichgans, J. (2000) *Eur. J. Biochem.* **267,** 4904-4911

47. Bharath, S., Hsu, M., Kaur, D., Rajagopalan, S., and Andersen, J. (2002) *Biochem. Pharmacol.* **64,** 1037-1048

48. Fritz-Wolf, K., Schnyder, T., Wallimann, T., and Kabsch, W. (1996) *Nature* **381,** 341-345

49. Bais, R. and Edwards, J. B. (1982) *Crit. Rev. Clin. Lab. Sci.* **16,** 291-335

50. Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., Quilliam, L. A. (1997) *J. Biol. Chem.* **272,** 4323-4326.

51. Betteridge, D. J. (2000) *Metabolism* 49, 3-8

52. Claiborne, A., Yeh, J. I., Mallett, T. C., Luba, J., Crane, E. J. 3rd, Charrier, V., and Parsonage, D. (1999) *Biochemistry* **38,** 15407-15416

# GENERAL SUMMARY AND CONCLUSIONS

Sulfinic and sulfonic acid analysis provides a method for specifically determining the amounts of irreversibly oxidized cysteine in protein samples. Irreversibly oxidized forms of creatine kinase and carbonic anhydrase III were used to establish a method that quantitates protein sulfinic and sulfonic acids through a unique protein hydrolysis and HPLC analysis protocol. When the technique was applied to rat liver protein extracts, measurable amounts of sulfinic acid detected which would have represented approximately 5% of the reactive cysteine residues, demonstrating that a significant fraction of the exposed protein pool is irreversibly damaged.

Irreversible oxidation of the reactive cysteines of creatine kinase and carbonic anhydrase III occurred *in vitro* in the presence of hydrogen peroxide and peroxyl radicals, and was prevented by glutathione through protein S-glutathiolation. Proteins that were specifically S-glutathiolated prior to incubation with an oxidant completely prevented irreversible oxidation, demonstrating that the disulfide bond between protein cysteine and glutathione protects the cysteine sulfur from higher-order oxidation states. Neither carbonic anhydrase III or creatine kinase was S-glutathiolated in vitro by oxidation of glutathione to glutathione disulfide, followed by subsequent thiol-disulfide exchange. It is unlikely that glutathione disulfide is an important S-thiolating agent *in vivo*, since it is rapidly exported from cells (64) and unlikely to reach sufficient concentrations for thiol disulfide exchange reactions. Thus, oxidants and protein sulfhydryls directly interact to produce either irreversible oxidation or S-glutathiolation, depending upon the concentration of glutathione present.

Irreversibly oxidized forms of creatine kinase and carbonic anhydrase III are present *in vivo* and show age-related increases. It is reasonable to expect that any proteins with exposed cysteine residues are targets for this kind of deleterious modification. Thus, proteins with thiols critical for function mentioned in the General Introduction, such as glyceraldehyde-3-phosphate dehydrogenase, Ras, or protein tyrosine phosphatases, are likely to all contain a modest level of cysteine irreversible oxidation, *in vivo*. Damaged forms of these proteins accumulate with age, either due to slower protein degradation, increased levels of reactive oxygen and nitrogen species, or insufficient cellular protective mechanisms. Skeletal muscle and heart tissue were demonstrated show age-related decreases in glutathione. While the results presented here do not expressly demonstrate that a diminished capacity for S-thiolation reactions is responsible for the increase in irreversible oxidation, it is a reasonable explanation of the data. The results also indicate that sulfinic acids represent the predominant form of cysteine irreversible oxidation found *in vivo* in liver, heart, and skeletal muscle. Protein sulfonic acids were not detected in either liver or skeletal muscle protein extracts. It is possible the protein sulfonic acids occur *in vivo*, however, but at levels well below sulfinic acids. Oxidation of a sulfinic acid to a sulfonic acid may represent a slow reaction step *in vivo*, and competition with other oxidizable substrates could inhibit oxidation to sulfonic acids.

## REFERENCES


1. Girotti, A. W. (1998) *J. Lipid. Res.* **39,** 1529-1542.

2. Kennedy, L. J., Moore, K. Jr., Caulfield, J. L., Tannenbaum, S. R., and Dedon, P. C. (1997) *Chem. Res. Toxicol.* **10,** 386-392.

3. Ischiropoulos, H. and al-Mehdi, A. B. (1995) *FEBS Lett.* **364,** 279-282.

4. Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., and Stadtman, E. R. (1990) *Methods Enzymol.* **186,** 464-478.

5. Thomas, J. A., Poland, B., Honzatko, R. (1995) *Arch. Biochem. Biophys.* **319,** 1-9.

6. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) *Arch Biochem Biophys* **237,** 408-414.

7. Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998) *Science* **281,** 64-71.

8. Di Monte, D., Sandy, M. S., Ekstrom, G., Smith, M. T. (1986) *Biochem. Biophys. Res. Commun.* **137,** 303-309.

9. Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) *J. Biol. Chem.* **257,** 12419-12425.

10. Fridovich, I. (1970) *J. Biol. Chem.* **245,** 4053-4057.

14. Stewart, R. C., Hille, R., and Massey, V. (1985) *J. Biol. Chem.* **260,** 8892-8904.

12. Roos, D., and Winterbourn, C. C. (2002) *Science* **296,** 669-671.

13. Bogdan, C. (2001) *Nat. Immunol.* **2,** 907-916.

14. Lusis, A. J. (2000) *Nature* **407,** 233–241.

15. **Bauerova K, Bezek A. (1999)** ***Gen. Physiol. Biophys.*** **18, 15-20.**

16. Chaudiere, J, and Ferrari-Iliou, R. (1999) *Food Chem. Toxicol.* **37,** 949-962.

17. Wefers, H., and Sies, H. (1988) *Eur. J. Biochem.* **174,** 353-357.

18. Michiels, C., Raes, M., Toussaint, O., and Remacle, (1994) *J. Free Radic. Biol. Med.* **17,** 235-248.

19. Bains, J. S., and Shaw, C. A. (1997) *Brain Res. Brain Res. Rev.* **25,** 335-358.

20. Schulz, J. B., Lindenau, J., Seyfried, J., and Dichgans, J. (2000) *Eur. J. Biochem.* **267,** 4904-4911

21. Bharath, S., Hsu, M., Kaur, D., Rajagopalan, S., and Andersen, J. (2002) *Biochem. Pharmacol.* **64,** 1037-1048

22. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. (1987) *Proc. Natl. Acad. Sci.* **84,** 9265-9269.

23. Palmer RM, Ferrige AG, Moncada S. (1987) *Nature* **327,** 524-526.

24. Wink, D., and Mitchell, J. (1998) *Free Radic. Biol Med.* **25,** 434-456.

25. Alderton, W. K., Cooper, C. E., Knowles, R. G. (2001) *Biochem. J.* **357,** 593-615.

26. Schulze-Osthoff, K., Bauer, M. K., Vogt, M., and Wesselborg, S. (1997) *Int. J. Vitam. Nutr. Res.* **67,** 336-342.

27. Abe, M. K., Kartha, S., Karpova, A. Y., Li, J., Liu, P. T., Kuo, W. L., and Hershenson, M. B. (1998) *Am. J. Respir. Cell. Mol. Biol.* **18,** 562-569.

28. Koppenol, W., Moreno, J., Pryor, W., Ishiropoulos, H., and Beckman, J. (1992) *Chem. Res. Toxicol.* **5,** 834-842.

29. Alvarez, B., Ferrer-Sueta, G., Freeman, B., and Radi, R. (1999) *J. Biol. Chem.* **274,** 842-848.

30. Stadtman, E. (1993) *Annu. Rev. Biochem.* **62,** 797-821.

31. Souza, J. and Radi, R. (1998) *Arch. Biochem. Biophys.* **360**, 187-194.

32. Barrett, W., DeGnore, J., König, S., Fales, H., Keng, Y., Zhang, Z., Yim, M., and Chock, P. (1999) **38,** 6699-6705.

33. Mohr, S., Zech, B., Lapertina, E., and Brüne, B. (1997) *Biochem. Biophys. Res. Commun.* **238,** 387-391.

34. Wilcox D. E., Schenk, A., Feldman, B. M., and Xu, Y. (2001) *Antioxid. Redox Signal.* **3,** 549-564.

35. Mallis, R. J., Buss, J. E., and Thomas, J. A. (2001) *Biochem. J.* **355,** 145-153.

36. Radi, R., Beckman, J., Bush, K., and Freeman, B. (1991) *J. Biol. Chem.* **266**, 4244-4250.

37. Takakura, K., Beckman, J., MacMillan-Crow, L., and Crow, J. (1999) *Arch. Biochem. Biophys.* **323,** 197-207.

38. Lii, C. K., Chai, Y. C., Zhao, W., Thomas, J. A., and Hendrich, S. (1994) *Arch. Biochem. Biophys.* **308,** 231-239.

39. Nikitovic, D., Holmgren, A., and Spyrou, G. (1998) *Biochem. Biophys. Res. Commun.* **242,** 109-112.

40. Rainwater, R., Parks, D., Anderson, M. E., Tegtmeyer, P., and Mann, K. (1995) *Mol. Cell. Biol.* **15,** 3892-3903.

41. Matthews, J. R., Kaszubska, W., Turcatti, G., Wells, T. N., and Hay, R. T. (1993) *Nucleic Acids Res.* **21,** 1727-1734

42. . Hirotsu, S., Abe, Y., Okada, K., Nagahara, N., Hori, H., Nishino, T., and Hakoshima, T. (1999) *Proc. Natl. Acad. Sci.* **96,** 12333-12338.

43. Winterbourn, C and Metodiewa, D. (1999) *Free Radic. Biol. Med.* **27,** 322-328.

44. Folkes, L., Candeias, L., and Wardman, P. (1995) *Arch. Biochem. Biophys.* **323,** 120-126.

45. Armstrong, D., and Buchanan, J. (1978) *Photochem. Photobiol.* **28,** 743-755.

46. Simic, M. and Hunter, E. (1986) *J. Free Radic. Biol Med.* **2,** 227-230.

47. Collison, M. and Thomas, J. (1987) *Biochim. Biohys. Acta* **928,** 121-129.

48. Chai, Y., Hendrich, S., and Thomas, J. (1994) *Arch. Biochem. Biophys.* **310,** 264-272.

49. Jung, C. and Thomas, J. (1996) *Arch. Biochem. Biophys.* **335,** 61-72.

50. Gow, A. J., Buerk, D. G., and Ischiropoulos, H. (1997) *J. Biol. Chem.* **272,** 2841-2845.

51. Tsikas, D., Sandmann, J., Rossa, S., Gutzki, F., and Frölich, J. (1999) *Anal. Biochem.* **270,** 231-241.

52. Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J., and Stamler, J. S. (2001) *Nature* **410,** 490-494.

53. Mallis, R., and Thomas, J. (2000) *Arch. Biochem. Biophys.* **383,** 60-69.

54. Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., Quilliam, L. A. (1997) *J. Biol. Chem.* **272,** 4323-4326.

55. Jaffrey S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., and Snyder, S. H. (2001) *Nat. Cell Biol.* **3,** 193-197.

56. Oae, S., Takata, T., and Kim, Y. H. (1981) *Chem. Soc. Jpn.* **54,** 2712-2723.

57. DeGnore, J. P., Konig, S., Barrett, W. C., Chock, P. B., and Fales, H. M. (1998) *Rapid Commun. Mass Spectrom.* **12,** 1457-1462.

58. Thomas, J. A., and Mallis, R. J. (2001) *Exp. Gerontol.* **36,** 1519-1526.

59. Coan, C., Ji, J. Y., Hideg, K., and Mehlhorn, R. J. (1992) *Arch. Biochem. Biophys.* **295,** 369-378.

60. Gu, Z., Kaul, M., Yan, B., Kridel, S. J., Cui, J., Strongin, A., Smith, J.W., Liddington, R.C., and Lipton, S. A. (2002) *Science* **297,** 1186-1190.

61. Davies, M. J., Fu, S., Wang, H., and Dean, R. T. (1999) *Free Radic. Biol. Med.* **27,** 1151-1163.

62. Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A. and Markesbery, W. R. (1991) *Proc. Natl. Acad. Sci.* **88,** 10540–10543.

63. Ozols, J. (1990) Methods Enzymol. **182,** 587-601.

64. Eklow, L., Moldeus, P., and Orrenius, S. (1984) *Eur. J. Biochem.* **138,** 459-463.

# APPENDIX. A SENSITIVE ASSAY FOR REVERSIBLY OXIDIZED PROTEIN SULFHYDRYL GROUPS

Edited portions of a paper published in the journal *Antioxidants and Redox Signaling* [1]

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## Introduction

Reversible oxidation of protein sulfhydryl groups is typically detected by blocking free cysteines with alkylating reagents, reducing the reversible modifications with dithiothreitol (DTT), and labeling released sulfhydryl groups with a fluorophore or chromophore. These techniques have detection limits within the 10–300 pmol range, often requiring purification of a significant amount of protein for the analysis (1). The speed, utility, and sensitivity of detecting thiol oxidation could be greatly enhanced if techniques could be coupled with western blotting (2 - 4). In the assay developed here, sulfhydryl oxidation is identified using MalPEG; a maleimide-containing compound that is attached to a long polyethylene glycol tail of defined length. The maleimide group selectively forms a stable covalent linkage to free thiols, while the long tail increases the overall mass of a tagged thiol by 5,000 Daltons. Thus, protein cysteine oxidation can be distinguished as a band shift on an SDS-PAGE gel, with the number of bands detected depending on the number of modified thiols per polypeptide.

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This method has been used in conjunction with western blotting to detect reversibly oxidized p53 in MCF7 breast cancer cells (4); however, it was not determined if MalPEG reacted efficiently and completely with protein sulfhydryl groups. Creatine kinase, a protein with a single exposed thiol group on the surface, was used as a model protein to address this critical issue. Commercial creatine kinase preparations were found to contain a significant fraction of irreversibly oxidized protein by isoelectric focusing gel electrophoresis, and these oxidized forms of creatine kinase were removed using a novel purification procedure in order to clearly establish MalPEG tagging efficiency. The results demonstrate that MalPEG was able to react with 100% of the available thiol groups of creatine kinase.

## Experimental Procedures

### Reagents

Rabbit muscle creatine kinase was purchased from CalBiochem–NovaBiochem (San Diego, CA, U.S.A.) or Sigma–Aldrich (St. Louis, MO, U.S.A.). Concentrations were determined using a calculated extinction coefficient of 40,152 $M^{-1}cm^{-1}$ at 280 nm. Ampholytes (pH 5 – 8 and pH 4 – 6) for isoelectric focusing were obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, USA). Methoxypolyethylene glycol-maleimide, MW 5,000 (MalPEG), was purchased from Shearwater Polymers, Inc. (Huntsville, AL, U.S.A.)., 2-hydroxyethyldisulfide (HED), β-glycerophosphate, iodoacetamide (IAM), dithiothreitol (DTT), and Coomassie Brilliant Blue were purchased from Sigma–Aldrich.

**Isoelectric gel electrophoresis**

Oxidized forms of creatine kinase were separated on horizontal slab gel (4% acrylamide, 1.7% pH 5-8 ampholytes, 0.3% pH 4-6 ampholytes) at 1500 V and 1.1 W/cm for 50 minutes as previously described (5). Gels were stained with Coomassie Brilliant Blue R-250 and air-dried.

**Isolation of creatine kinase with HED-blocked cysteine residues**

Creatine kinase was dissolved to 8 mg/ml in 20 mM Tris, pH 7.6 containing 10 mM DTT, and the solution was incubated at 37°C for 30 min to remove DTT-reversible modifications. DTT was removed by overnight dialysis against 20 mM Tris, pH 7.6. The protein was treated with 20 mM hydroxyethyldisulfide (HED) at 37°C for 30 min to reversibly block reactive cysteine residues. Irreversibly oxidized cysteine residues do not react with HED (data not shown). HED-treated creatine kinase was centrifuged at 15,000 g for 5 min and filtered through a 0.22-mm cellulose acetate spin-filter (Spin-X column, Costar, Acton, MA, U.S.A.). The reduced form of creatine kinase was separated using anion-exchange HPLC. The protein was loaded on a 10 × 64 mm Bioscale DEAE-5 anion-exchange column (Bio-Rad, Hercules, CA, U.S.A.). and eluted with an isocratic gradient of 20 mM Tris, pH 7.6, at a flow rate of 1.5 ml/min. Fractions were analyzed by IEF and those containing fully blocked creatine kinase were pooled, concentrated to 2–3 mg/ml, and stored at -80°C. Reduced protein was prepared by incubating HED-blocked creatine kinase with 10 mM DTT for 30 min at 37°C followed by overnight dialysis against 20 mM β-glycerophosphate, pH 7.4.

**MalPEG tagging of creatine kinase**

Creatine kinase samples were diluted four-fold into 20 mM β-glycerophosphate, pH 7.4 containing 4 mM MalPEG. The tagging reaction was incubated at room temperature for 30 min and terminated by adding four volumes of SDS-PAGE sample buffer containing NEM (0.125M Tris-Cl buffer, pH 6.8, containing 4% sodium dodecyl sulfate, 20% glycerol, 0.008% bromophenol blue, and 65 mM NEM). Samples were separated on 10% gels and stained with Coomassie Blue.

## Results

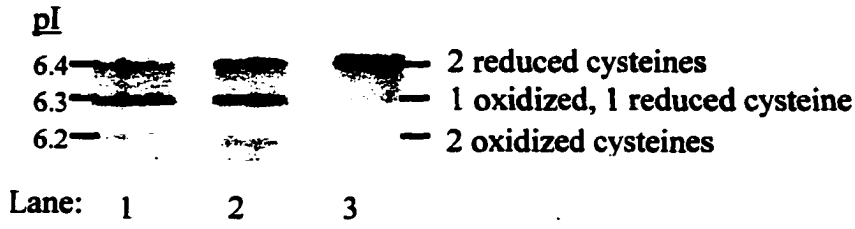
Creatine kinase is a homodimeric protein, with one reactive cysteine per subunit (6). The oxidation state of the cysteine residues can be rapidly assessed by IEF. When the cysteine residues are blocked with a charge-neutral alkylating reagent such as IAM, creatine kinase migrates to a pI of 6.4 on the gel. Irreversible oxidation will produce sulfinic and sulfonic acids that are resistant to DTT reduction, unreactive towards IAM, and also bear a negative charge. The increase in negative charge causes protein bands to shift towards more acidic pI's on IEF gels. If a single cysteine is irreversible oxidized, the protein migrates to a pI of 6.3. If both cysteines are irreversibly oxidized, the protein pI is 6.2 (8).

IEF analysis indicated that creatine kinase from two commercial sources was 40-50% irreversibly oxidized (Figure 1A, lanes 1 & 2). In order to have a standard preparation of creatine kinase that was not subject to further oxidative damage, cysteine residues were reversibly blocked using hydroxyethyldisulfide (HED), and the reduced form of the protein was purified by

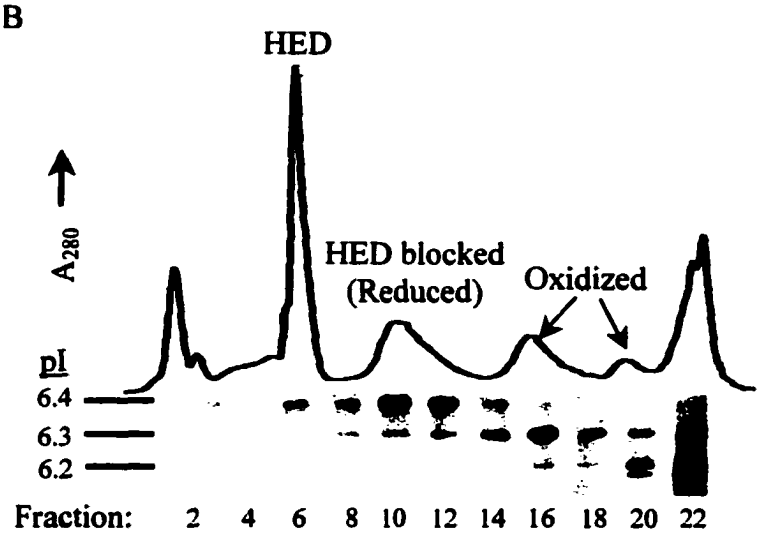
**Figure 1. Purification of the reduced form of creatine kinase.**

A. Two micrograms of creatine kinase were applied to IEF gels and analyzed for the presence of irreversible cysteine oxidation. Creatine kinase was incubated with 10 mM DTT for 30 min at 37°C in 20 mM β-glycerophosphate, pH 7.4, then alkylated with 40 mM IAM for 20 min at room temperature. Lane 1, creatine kinase received from CalBiochem–Novabiochem; lane 2, creatine kinase received from Sigma; lane 3, purified creatine kinase. B. Purification of reduced creatine kinase from irreversibly oxidized forms. Approximately 2–3 mg of HED treated creatine kinase was separated by anion–exchange chromatography. Elution positions of HED-blocked (reduced) creatine kinase and irreversibly oxidized forms are shown. Column fractions were analyzed by IEF after chromatographic separation.

A


pI
6.4
6.3
6.2
2 reduced cysteines
1 oxidized, 1 reduced cysteine
2 oxidized cysteines
Lane: 1 2 3

B


HED
A280
HED blocked
(Reduced)
Oxidized
pI
6.4
6.3
6.2
Fraction: 2 4 6 8 10 12 14 16 18 20 22

anion-exchange chromatography. HED is the disulfide form of mercaptoethanol and selectively blocks cysteine residues through thiol-disulfide exchange. Like IAM, the blocking reaction generates a charge-neutral modification. Figure 1B demonstrates that HED-blocked creatine kinase was readily separated from the more negatively charged, irreversibly oxidized forms by anion exchange chromatography. Purification was assessed by IEF, and it was found that was 95 % migrated with a pI of 6.4 (Figure 1A, lane 3). The protein was concentrated to 2 mgs/ml and aliquots were stored at -80°C, with no changes in the amount irreversibly oxidized creatine kinase detected after several months of storage. Prior to experiments the mercaptoethanol blocking group was removed by treating the protein with DTT, and excess DTT was removed by dialysis.

Figure 2 and Table 1 demonstrates the efficiency of the MalPEG tagging reaction. The 43 kD band shown in Figure 2B, lane 1 is consistent the normal molecular weight of creatine kinase. Lane 2 shows that the band shifts to 55 kD when the protein is treated with MalPEG, and corresponds to the tagging of a single reactive cysteine located on a single monomer of creatine kinase. Unlike IEF, only two possible band positions appear because creatine kinase dimers are dissociated by the presence of SDS in the sample-loading buffer. Furthermore, only a single, reactive cysteine is tagged. The three cysteines in the interior of creatine kinase only reacted with MalPEG in the presence of denaturants such as guanidine hydrochloride and generate three additional bands with molecular weights greater than 55 kD (data not shown). MalPEG should only increase the molecular weight of a single thiol by 5 kD, but MalPEG- tagging probably retarded the migration by significantly lowering the protein's charge to mass ratio.

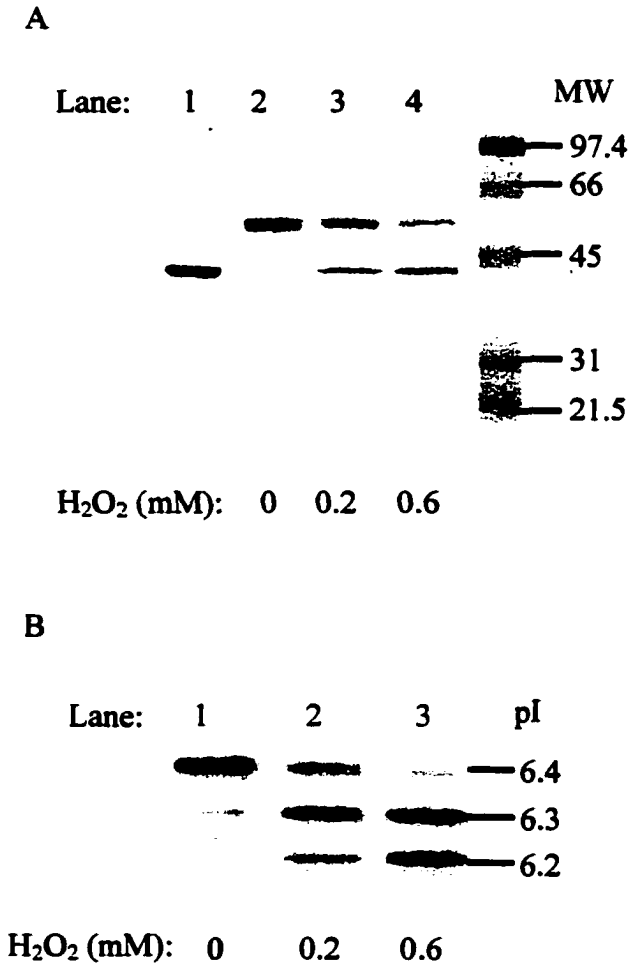



**Figure 2. MalPEG and IEF analysis of hydrogen peroxide oxidized creatine kinase.**

Creatine kinase (25 μM) in 20 mMβ-glycerophosphate buffer, pH 7.4 was treated with 0, 0.2, or 0.6 mM hydrogen peroxide for 10 min at 37°C. The reactions were quenched with 2 mM DTT and incubated for an additional 15 min to remove any reversible modifications. A. The reactions were treated with MalPEG and analyzed by SDS PAGE as described in the Experimental Procedures section. B. Samples were alkylated with 40 mM IAM and analyzed by IEF.

**Table 1: Comparison of IEF and MalPEG analysis of reduced and oxidized creatine kinase[a]**

| Method | Protein cysteine | Percent of creatine kinase monomer | | |
|---|---|---|---|---|
| | | Hydrogen peroxide (mM) | | |
| | | 0 | 0.2 | 0.6 |
| MalPEG | Reduced | 94 ± 1 | 62 ± 2 | 40 ± 4 |
| | Oxidized | 6 ± 1 | 38 ± 2 | 60 ± 4 |
| IEF | Reduced | 94 ± 1 | 55 ± 3 | 27 ± 4 |
| | Oxidized | 6 ± 1 | 45 ± 3 | 73 ± 4 |

[a]Duplicates of the samples illustrated in Figure 5 were used for gel densitometry. For MalPEG analysis, the percent of density associated with the 55 kD band was used to determine the amount creatine kinase with reduced cysteine, and the 43 kD band was used for oxidized cysteine. For IEF analysis, the percentage of band density corresponding to a pI of 6.3 (one subunit oxidized and one subunit reduced) was divided by two and added to the percentage of protein detected in the band at 6.4 (both subunits reduced) or 6.2 (both subunits oxidized) in order to determine the percent of reduced or oxidized creatine kinase.

Gel densitometry demonstrated that 94% of the creatine kinase reacted with MalPEG (Table 1), while IEF analysis of the same sample showed that 94 % of the protein cysteine was reduced (Fig. 2B, lane 1; Table 1). Together these results indicate that MalPEG forms a covalent linkage with 100% of the available thiol groups in creatine kinase. MalPEG tagging and IEF were also compared using irreversibly oxidized forms of creatine kinase generated with hydrogen peroxide. When creatine kinase was incubated with 0.2 mM hydrogen peroxide, only 62% of the creatine kinase reacted with MalPEG (Fig. 5A, lane 3; Table 1), while treating with 0.6 mM hydrogen peroxide resulted in only 39% of the protein remaining reactive with MAL-PEG (Fig. 5A, lane 4; Table 1). The amount of oxidized creatine kinase detected by IEF analysis was about 10–20% higher than MalPEG analysis. As creatine kinase contains three other sulfhydryls, the higher level of oxidized cysteine detected by IEF may be due to a low level of irreversible oxidation at these sites. Regardless, the data clearly show that MalPEG analysis can be a useful tool to detect sulfhydryl oxidation.

## Discussion

The results presented here indicate that MalPEG effectively detects protein cysteine oxidation. MalPEG reacted completely with the reduced cysteines of creatine kinase, demonstrating that the reagent is useful in quantitative estimates of protein thiol oxidation. Experiments with creatine kinase demonstrate that irreversible protein cysteine oxidation can be detected with MalPEG. Alternatively, reversible oxidation (such as protein S-thiolation) can be determined by first capping protein sulfhydryl groups with either IAM or NEM, reducing the protein with DTT, and tagging the liberated sulfhydryl groups with MalPEG.

Using this approach, higher molecular weight bands will correspond to protein with oxidized cysteine residues (4).

There are several caveats to consider regarding the use of MalPEG. For instance, the method does not identify the chemical composition of the cysteine adduct. Specific identification of a cysteine modification would require the use of a second technique such as mass spectrometry. Efficient western analysis may be inhibited if the epitope recognized by a primary antibody contains the MalPEG tagged cysteine. Finally, two sulfhydryl groups in close proximity (i.e. vicinal dithiols) may prevent both sites from reacting efficiently with MalPEG, because a single MalPEG adduct may occlude access to the neighboring thiol.

Despite these potential limitations, the method opens the possibility of detecting endogenous cysteine oxidation on low-abundance proteins in tissues and biological fluids. With a 10-fold molar excess of MalPEG, the reaction was complete in less than 15 min at 27°C within a pH range of 6.2–8.0 (data not shown); therefore, the reagent could be readily incorporated in most experiments involving biological extracts. Previous attempts at detecting sulfhydryl oxidiation through molecular weight tags have used reagents with small molecular massed (~500 Da), limiting the assay to the analysis of very small molecular mass proteins (7). MalPEG greatly extends the utility of the assay by detecting oxidation on larger molecular weight proteins. Finally, when used in combination with western analysis, as little as 0.23 pmol of reversibly oxidized p53 has been detected (4). Clearly MalPEG analysis represents a versatile, sensitive assay for detecting protein cysteine oxidation.

**References:**

1. Wright, S. K., and Viola, R. E. Evaluation of methods for the quantitation of cysteines in proteins. *Anal. Biochem.* 265: 8–14, 1998.

2. Kim, J. R., Yoon, H. W., Kwon, K. S., Lee, S. R., and Rhee, S. G. Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal. Biochem.* 283: 214–221, 2000.

3. Wu, Y., Kwon, K. S., and Rhee, S. G. Probing cellular protein targets of $H_2O_2$ with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. *FEBS Lett.* 440: 111–115, 1998.

4. Wu, H. H., Thomas, J. A., and Momand, J. p53 protein oxidation in cultured cells in response to pyrrolidine dithiocarbamate: a novel method for relating the amount of p53 oxidation *in vivo* to the regulation of p53-responsive genes. *Biochem. J.* 351: 87–93, 2000.

5. Miller, R. M., Sies, H., Park, E. M., and Thomas, J. A. Phosphorylase and creatine kinase modification by thiol disulfide exchange and by xanthine oxidase-initiated S-thiolation. *Arch. Biochem. Biophys.* 276: 355–363, 1990.

6. Rao, J. K., Bujacz, G., and Wlodawer, A. Crystal structure of rabbit muscle creatine kinase. *FEBS Lett.* 439: 133–137, 1998.

7. Jakob, U., Muse, W., Eser, M., and Bardwell, J. C. Chaperone activity with a redox switch. *Cell* 96: 341–352, 1999.