ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 5 & 6, 2006

Forum Review

Global Methods to Monitor the Thiol–Disulfide State of Proteins *In Vivo*

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ABSTRACT

Cysteines play an important role in protein biochemistry. The unique chemical property and high reactivity of the free thiol group makes reduced cysteine a versatile component of catalytic centers and metal binding sites in many cytosolic proteins and oxidized cystine a stabilizing component in many secreted proteins. Moreover, cysteines readily react with reactive oxygen and nitrogen species to form reversible oxidative thiol modifications. As a result, these reversible thiol modifications have found a use as regulatory nano-switches in an increasing number of redox sensitive proteins. These redox-regulated proteins are able to adjust their activity quickly in response to changes in their redox environment. Over the past few years, a number of techniques have been developed that give insight into the global thiol–disulfide state of proteins in the cell. They have been successfully used to find substrates of thiol–disulfide oxidoreductases and to discover novel redox-regulated proteins. This review will provide an overview of the current techniques, focus on approaches to quantitatively describe the extent of thiol modification *in vivo*, and summarize their applications. *Antioxid. Redox Signal.* 8, 763–772.

INTRODUCTION

The thiol disulfide state of cellular proteins

Cysteines are one of the most rarely used amino acids in proteins of all organisms studied so far (76). When conserved, they often play crucial roles in the structure or function of proteins. This is due to their ability to stabilize protein structures by forming disulfide bonds or by coordinating transition metal ions. Moreover, the unique chemical properties and high reactivity of its thiol group often make cysteine also the amino acid of choice for active sites or to form redox centers in proteins (37, 62).

Proteins in the extracellular space and in oxidizing cell compartments, such as the endoplasmic reticulum or the bacterial periplasm, often contain disulfide bonds. These disulfide bonds provide significant structural stability and maintain the correct fold (24). Specialized thiol–oxidase systems have evolved to introduce and rearrange structural disulfide bonds in these proteins. The bacterial protein DsbA catalyzes disulfide bond formation in periplasmic proteins by transferring its disulfide bond to newly translocated proteins. In aerobiosis, the active site of DsbA then gets reoxidized by the membrane protein DsbB, which in turn transfers electrons to the quinone system and ultimately to molecular oxygen (6). Any nonnative disulfide bonds that might form in periplasmic proteins are resolved by the isomerase DsbC, which is maintained in a reduced state by the membrane protein DsbD (72). In the endoplasmic reticulum of eukaryotes, protein disulfide isomerase PDI introduces disulfide bonds into folding proteins and gets in turn reoxidized by the FAD containing oxidoreductases Ero1p or Erv2p (33, 38, 86, 92).

In the cytoplasm of pro- and eukaryotes, on the other hand, the majority of cysteines are found in their reduced thiol form, where they are located in binding pockets of substrates, coenzymes, or metal cofactors, or take part directly in catalytic re-

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actions (13, 29). Because of the redox sensitivity of cysteines, these cytoplasmic proteins are highly susceptible to inactivation by oxidation (45, 87). Therefore, several cellular systems have evolved to keep thiol groups of cytoplasmic proteins reduced and to maintain the thiol–disulfide homeostasis of the cytoplasm. Among those systems are the thioredoxin/thioredoxin reductase system and the glutaredoxin/ glutathione system (4, 32). Thioredoxins are small proteins containing a highly conserved redox active Cys–Gly– Pro–Cys motif in their active site. Because of the highly reducing redox potential of this motif, the free cysteines in the active site of reduced thioredoxin readily donate electrons to disulfide bonds in proteins, thus reducing the protein (59). During this process, thioredoxin becomes oxidized and forms a disulfide bond in its active site. This disulfide bond is subsequently reduced by the NADPH-dependent thioredoxin reductase in another thiol–disulfide exchange reaction (39, 44). Many enzymes that use thiol–disulfide exchange reactions in their catalytic cycle such as ribonucleotide reductase, methionine sulfoxide reductase, and phosphoadenylyl sulfate reductase, have been shown to depend on the presence of thioredoxin activity in the cell (83). Glutaredoxins are members of the second redox balancing system in the cytosol of many pro- and eukaryotes. They also reduce disulfide bonds in proteins via a thiol–disulfide exchange reaction but are kept in the reduced state by the small cysteine-containing tripeptide glutathione (32).

Oxidative stress

Although the thioredoxin and glutaredoxin systems are highly effective in maintaining the redox equilibrium of the cell under normal conditions, presence of elevated levels of reactive oxygen species (ROS) often overwhelms these systems. This can lead to the increased oxidation of thiol groups and to the accumulation of oxidatively modified proteins in the cytosol of cells (23, 34, 57, 60). Many physiological and pathological stress conditions such as heat shock (e.g., fever) and aging are related to elevated levels of ROS (42, 46, 47, 58, 80). Diseases associated with oxidative stress include the three most common causes of death in the United States, heart disease, cancer, and stroke, as well as many neurological disorders such as Alzheimer's and Parkinson's disease (1, 3, 8, 16, 17, 77). While the potentially damaging effect of ROS has long been recognized (36), more recent findings suggest that ROS and reactive nitrogen species (RNS) can also serve as potent cellular messengers (25, 70, 84). This and the clinical relevance of oxidative stress has led to the development of numerous methods to monitor the effect of ROS and RNS on biological molecules, such as lipids, nucleic acids, and proteins (12, 31, 64, 65).

From all the possible oxidative modifications that occur in proteins upon exposure of cells to ROS and RNS, oxidative modifications of the thiol group in cysteines are clearly exceptional: in contrast to side chain carbonylation or backbone fragmentation, which have primarily deleterious effects on protein activity, oxidative thiol modifications such as disulfide bonds have actually found a use in other proteins to provide structural stability (9, 22, 90). Moreover, while many other oxidative protein modifications are irreversible, most thiol modifications that involve sulfur in low oxidation states, such as disulfide bonds, mixed disulfides with glutathione (glutathionylation), and sulfenic acids can be either directly or indirectly reduced by the thioredoxin and glutathione/ glutaredoxin system and are therefore reversible *in vivo* (28, 32). This makes cysteine the amino acid of evolutionary choice as "nano-switch" in an emerging group of redox-regulated proteins including oxidative stress transcription factors such as OxyR, RsrA, Yap1p, and p53 (54–56, 79, 99), molecular chaperones such as Hsp33 (50), and metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase, FolE, and MetE (21, 45, 57). What all these proteins have in common is the presence of redox-sensitive cysteines in their regulatory center or active site, which are oxidized upon a change in the redox environment. Thiol oxidation usually changes the conformation and most importantly the activity of these proteins (11). The heat shock protein Hsp33 is a paradigm for redox-regulated proteins (50). Its cysteine containing zinc center is reduced under normal conditions. Under combined oxidative and heat stress, however, the cysteines become readily oxidized and Hsp33 forms a dimer that assumes the chaperoning task of the DnaK system, which is inactive under those conditions (93). When the redox environment returns to pre-stress levels, Hsp33 gets reduced and inactivated (43).

Chemistry of thiols

Thiols are readily oxidized by oxidizing agents such as air oxygen (10, 40). This is especially pronounced in the presence of metal catalysts. These reactions lead to a variety of products including disulfide bonds, sulfenic, sulfinic, and sulfonic acids (35, 51). Of those, only disulfide bonds and sulfenic acids are reversible by means of thiol-mediated reduction reactions catalyzed by the thioredoxin and glutaredoxin systems (81). Recently, sulfiredoxin, an ATP-dependent enzyme that reduces sulfinic acids and is conserved in higher eukaryotes including humans, has been discovered in yeast (14). This enzyme, however, seems to be limited to the reduction of overoxidized cysteines in members of the 2-Cys-Peroxiredoxin family (94).

The reactive entities in thiol–disulfide exchange reactions are the deprotonated thiolate anions (35, 51). Therefore, the nature of the microenvironment greatly affects the pK_a and therefore the reactivity of the thiol. Steric factors as well as the hydrophobic and electrostatic character of the environment influence the pK_a of the thiol group (18). Protonation of the thiol group dramatically decreases its reactivity. Thus, a rapid shift to low pH can be used to quickly stop thiol–disulfide exchange reactions in experimental settings (98).

Thiolates are also nucleophiles and therefore easily alkylated by electrophiles (35, 51). A wide range of thiol reactive agents are commercially available. They can be used to block and label thiols with colored or fluorescent dyes, radioactive labels, or immunodetectable antigens. Most commonly used alkylation agents are derivatives of iodoacetamide (IAM) and maleimides. While iodoacetamides undergo nucleophilic substitutions with the halogen as the leaving group, maleimides undergo nucleophilic additions at the double bond (35). Both reagents require the presence of the thiolate anion and are fairly specific at neutral pH values. At higher pH, some side reactions with other amino acids might occur (19, 52, 71).

MONITORING THE CELLULAR THIOL–DISULFIDE STATE

Over the past few years, several strategies have been designed to gain insight into the global thiol–disulfide state of the cell and especially of individual proteins. Obtaining a snapshot of the *in vivo* thiol state provides the unique opportunity to analyze the complex network of redox reactions in the cell. It is an excellent strategy to identify substrate proteins of individual thiol–disulfide oxidoreductases and to discover novel redox-regulated proteins. Most importantly, it allows the identification of proteins, whose thiol groups become targets of reactive oxygen and nitrogen species, which are present during numerous physiological and pathological conditions.

Analysis of the GSH/GSSG level in vivo

Measurement of the glutathione system, the redox buffer in the cytosol of most eu- and prokaryotic cells, can be used to determine the overall "redox-potential" of the cell (85). In its reduced form, glutathione (GSH) is a cysteine containing tripeptide (γ-Glu–Cys–Gly). Upon oxidation, an intermolecular disulfide bond forms between two glutathione molecules, yielding oxidized glutathione (GSSG). In the cytoplasm, the equilibrium is usually heavily shifted towards the reduced form (e.g., in wild-type *Escherichia coli* GSH: GSSG = 223:1) (5) and the steady state is maintained by the NADPH-dependent glutathione reductase (20). The GSH: GSSG ratio can be determined using chromatographic or combined enzymatic/spectrophotometric methods that allow the quantitative differentiation between oxidized and reduced glutathione (2). If the absolute concentrations of reduced and oxidized glutathione are known, the half cell potential of the glutathione system can be calculated using the Nernst equation:

$$E = E^0 - \frac{RT}{nF} \cdot \ln \frac{[GSH]^2}{[GSSG]}$$

The ratio of reduced and oxidized glutathione and more specifically the half cell potential provides a general overview of the thiol disulfide redox state of the cell, which changes dramatically upon oxidative insults or upon deficiencies in the cellular redox-balancing systems (5, 85).

Reporter proteins

To analyze the redox conditions of a specific cellular environment, the activity of disulfide-dependent reporter proteins such as alkaline phosphatase can be used (27). Alkaline phosphatase is a periplasmic *E. coli* protein that requires two disulfide bonds for conformational stability and full enzymatic activity (89). The activity of alkaline phosphatase can be easily tested in a colorimetric assay using *p*-nitrophenylphosphate (PNPP) as substrate (15). An engineered variant of alkaline phosphatase (PhoAΔ2-22), which lacks its targeting sequence, remains in the strongly reducing environment of the cytosol and is inactive. Only if the redox environment of the cytoplasm shifts to more oxidizing conditions, PhoAΔ2-22 can acquire its disulfide bonds and gains enzymatic activity. This makes PhoAΔ2-22 an excellent probe to screen for mutations that allow disulfide bond formation in the cytoplasm in *E. coli* (27). In combination with genetic studies, this approach has successfully been used to dissect the roles of the major disulfide oxidoreductases in maintaining the reducing character of the cytoplasm (78).

In a similar way, green and yellow fluorescent protein variants containing an engineered pair of redox active cysteines have been used as fluorescent probes to monitor redox changes *in situ* (74). These proteins change their emission properties upon formation of an intramolecular disulfide bond. This allows measurements of the redox potential of the cytoplasm and mitochondria and its change in response to membrane permeable reductants and oxidants (30, 41, 75).

Nonreducing/reducing "diagonal" PAGE

While the GSH:GSSG ratio or the use of specific reporter proteins gives an overview of the general redox conditions within a biological system, no direct conclusions can be drawn about the redox states of individual proteins in the cell. Over the past few years, a number of different proteomic methods have been developed that allow a global analysis of the redox state of cellular proteins. One of these methods is the nonreducing/reducing "diagonal" two-dimensional PAGE, which was first developed to identify artificially disulfide-crosslinked ribosomal proteins (88). Later this method was used to analyze cotranslational disulfide bond formation and folding of nascent protein chains in the endoplasmatic reticulum of mammalian cells (66–68). It has only recently been established to identify cytoplasmic proteins that undergo disulfide bond formation upon exposure of cells to oxidative stress (23).

In a nonreducing/reducing 2D PAGE experiment, as in all experiments that investigate the *in vivo* thiol/disulfide state of proteins, the first step involves the rapid blocking of all free thiol groups to prevent unwanted thiol–disulfide exchange reactions. This is accomplished by the addition of membrane permeable thiol trapping reagents such as *N*-ethyl maleimide (NEM) or iodoacetamide (IAM) either to the intact cells shortly before lysis or to the protein extract immediately after cell lysis (98). The complex mixture of thiol-trapped proteins is then separated in the first dimension on a regular SDS PAGE using nonreducing SDS sample buffer. The lane containing the separated proteins is excised from the gel, incubated in reducing SDS-buffer and placed horizontally on the second dimension gel. The proteins are then separated under reducing SDS PAGE conditions (Fig. 1). After staining the gel, three groups of proteins can be distinguished. The first group includes all those proteins that do not contain any disulfide bonds. These proteins form a perfect diagonal line, because their migration behavior is identical in both dimensions. The second group includes protein oligomers that are covalently linked by an intermolecular disulfide bond. These proteins are found below the diagonal line. They migrate in the nonreducing SDS PAGE to a position corresponding to their combined mass while in the reducing SDS PAGE, they migrate according to their respective monomeric masses. The third group of proteins that can be distinguished are those

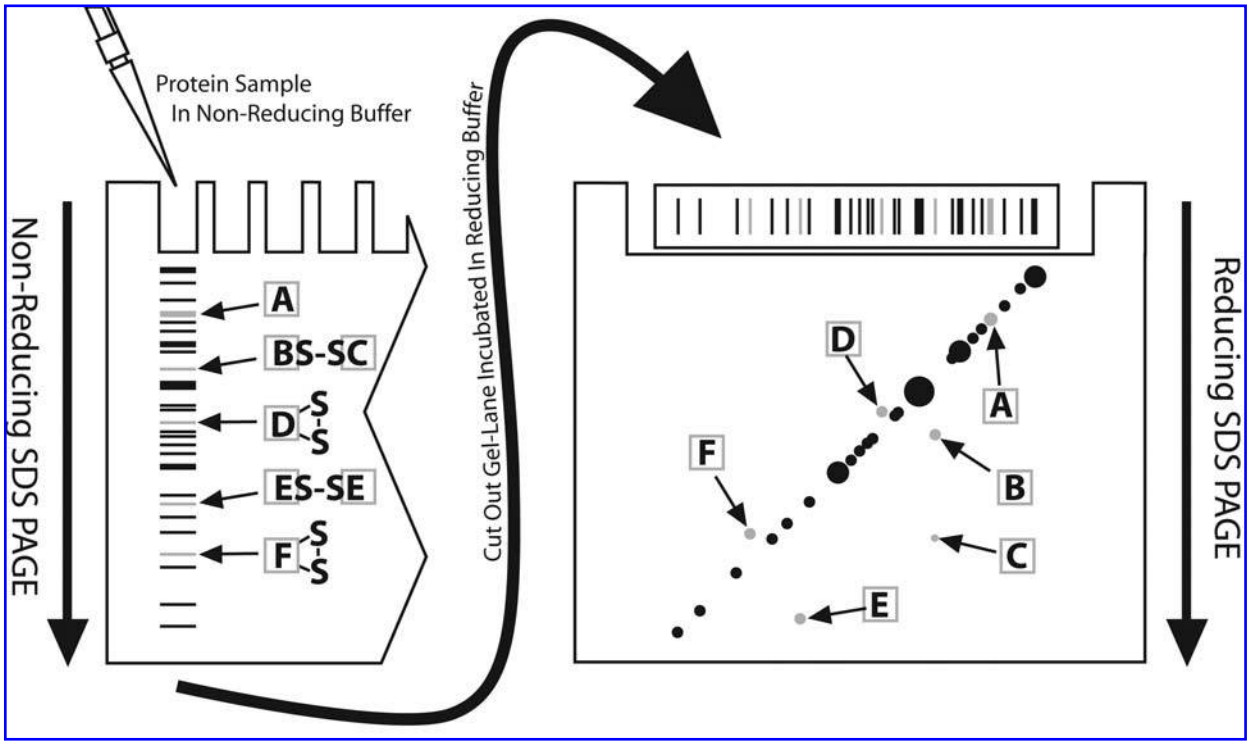

FIG. 1. Nonreducing/reducing "diagonal" 2D PAGE. Proteins are separated in the first dimension in a nonreducing SDS PAGE. The gel lane is excised from the gel, incubated in reducing Laemmli buffer and placed horizontally onto a reducing SDS PAGE. In this second dimension, the proteins are now separated under reducing conditions. Proteins containing no disulfide bonds (**A**) will migrate on a perfect diagonal line. Proteins, which are present as disulfide linked heterodimers (B–S–S–C) will migrate below the diagonal line as two distinct protein species **B** and **C**. Disulfide linked homodimers (E–S–S–E) appear as a single protein species **E** below the diagonal in the second dimension. Proteins that contain intramolecular disulfide bonds (D–SS or F–SS) appear as defined protein spots **D** or **F** slightly above the diagonal line in the second dimension.

that form intramolecular disulfide bonds. These proteins are found slightly above the diagonal line formed by the unmodified proteins. They migrate faster under nonreducing conditions than under reducing conditions, presumably because they form a more compact structure with their disulfide bonds still intact. To identify the individual proteins, the protein spots are excised from the gel and analyzed using mass spectrometry.

This method is a simple and direct approach that can be performed with basic equipment found in most laboratories. It is especially well suited to find interaction partners of specific proteins that are connected by intermolecular disulfide bonds. The epitope tagged protein of interest is expressed in cells and immunoprecipitated under nonreducing conditions. The immunoprecipitate can then be resolved by nonreducing/reducing 2D PAGE and proteins that form disulfide bonds with the bait protein will appear below the diagonal on the gel. By using this approach, proteins that form disulfide bonds with Hsp70 under oxidative stress conditions (23) as well as substrate proteins of the disulfide-bond forming periplasmic protein DsbA in *E. coli* have been identified (53).

Labeling of oxidatively modified thiols

Overview. A powerful technique to globally monitor the thiol–disulfide state of proteins *in vivo* combines the selective labeling of oxidized thiol groups in cellular proteins with proteomic analysis. It involves the blocking of all free thiols with thiol-reactive reagents in the first step. In the second step, *in vivo* modified thiol groups are reduced and subsequently labeled with a detectable thiol-reactive chemical (see Fig. 2 for schematic overview). Depending on the specificity of the thiol-reductant, this method can be used to get an overview of all reversible *in vivo* thiol modifications (57) or can be applied to probe for distinct thiol modifications such as nitrosylations and glutathionylations (49, 60). A number of thiol-selective chemicals are available to label the newly reduced thiols. The differentially labeled protein extract can then be separated on 1D or 2D PAGE and proteins are identified using mass spectrometric analysis. Here we describe the individual steps that are involved, and present an overview of commonly used reducing agents and thiol-reactive reagents.

The first step: freezing the in vivo *thiol–disulfide status.* To obtain an *in vivo* snapshot of the thiol state of proteins, it is absolutely essential to prevent nonspecific air oxidation during sample preparation. Therefore, it is necessary to block all free thiols rapidly upon cell lysis. One approach to prevent nonspecific thiol–disulfide exchange reactions is to block thiol groups while the proteins are still within the cells. This can be achieved by adding a membrane-permeable thiol-reactive reagent such as NEM and IAM to the cell culture (98). Although both reagents react reasonably fast with accessible

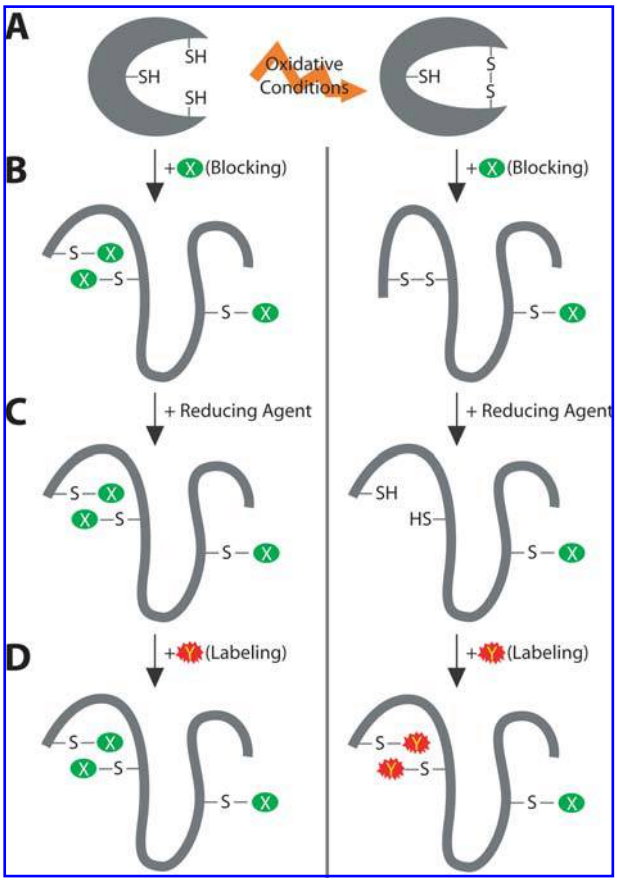

FIG. 2. Labeling of oxidatively modified thiols–a schematic overview. (**A**) A putative redox-regulated protein with three cysteines exists under (*left*) control conditions in a fully reduced form. Under (*right*) oxidative conditions, however, two of the cysteines form a disulfide bond. (**B**) In the blocking step, all free cysteines are rapidly and irreversibly blocked under denaturing conditions using a thiol reactive chemical. (**C**) In the reduction step, a reducing agent is added to the proteins. This will not alter the (*left*) reduced protein, but will reduce the thiol modification in the (*right*) oxidized protein, resulting in the formation of two new thiol groups. (**D**) In the labeling step, a thiol-specific chemical is added, which carries a detectable label such as a fluorescent group, an immunoreactive group, or a radioactive chemical. This will interact with the newly accessible thiol groups, and specifically label all *in vivo* thiol modified proteins.

free thiol groups, a block of free cysteines in the cell might lead to a perturbation in the thiol–disulfide state, which could trigger a physiological response. Additionally, buried thiol groups might not be accessible for these reagents (24, 82). A way to circumvent these problems is to first "acid trap" the proteins and then block all free thiol groups with NEM and IAM in a strongly denaturing buffer *in vitro*. In the "acid trap", trichloroacetic acid (TCA) is added to the growing cell culture. This not only lyses the cells and denatures the proteins but rapidly protonates all thiolates (59, 98). This significantly slows all thiol–disulfide exchange reactions, which as outlined above are strongly dependent on the presence of deprotonated thiolate anions. Moreover, protons also rapidly access any thiolates that are buried in the structure because of their small size and because of the denaturing effect of the low pH conditions. After pelleting the TCA precipitated proteins, the thiol-blocking reagent is added in a highly concentrated denaturing buffer. This shifts the pH into the neutral range and at the same time keeps all free thiol groups accessible to the alkylating agent.

The second step: defining the specificity of the method. The reduction step defines the nature of the thiol modifications that will subsequently be detected. To detect all reversible thiol modifications, unspecific reductants such as dithiothreitol (DTT) or Tris(2-carboxyethyl)phosphine (TCEP) can be used (57). This provides a "general overview" over all reversible thiol modifications *in vivo*. To detect specific modifications, specialized reductants need to be used. These include ascorbic acid to reduce nitrosothiols (49), sodium arsenite to reduce sulfenic acids (91), or glutaredoxins to reduce glutathionylated cysteines (60).

The third step: visualizing the previously modified thiols. A wide variety of detectable thiol reactive agents can be used to label the previously modified cysteines, and the choice depends largely on the detection method that is available. Biotin linked to a thiol reactive group such as IAM or NEM can be used to immunopurify thiol modified proteins using streptavidin columns (49, 60) or to detect the thiol modified proteins using Western blot analysis (61). Several thiol-reactive fluorescent dyes have been used including monobromobimane (97) and Cy5 maleimide (63). Alternatively, radioactive derivatives of standard thiol reactive reagent such as ^{14}C-NEM and ^{14}C-IAM can be used to specifically label *in vivo* oxidized thiols (57).

The thiol-trapped proteins can then be separated either by traditional 2D PAGE or, if the sample complexity is reasonably low, by standard 1D PAGE. The thiol-reactive reagent is then used to detect proteins with oxidative thiol modifications. Proteins of interest can be identified from gels using mass spectrometric techniques.

QUANTIFYING *IN VIVO* THIOL MODIFICATIONS: THE DIFFERENTIAL THIOL TRAPPING TECHNIQUE

In our lab, we have recently developed a differential thiol trapping technique, which is to our knowledge the first quantitative method to describe *in vivo* thiol modifications on a global scale. In this method, nonmodified thiol groups are labeled with nonradioactive IAM while *in vivo* modified thiol groups are labeled with radioactive ^{14}C-IAM (57). This technique has several advantages over earlier fluorescent/immunological and diagonal PAGE approaches. With our method, all modified proteins are chemically identical, regardless of their original oxidation state *in vivo*. Thus, *in vivo* oxidized and reduced forms of the same protein migrate to the identical spot in a 2D PAGE gel and the extent of thiol modification in a given protein can be quantitatively expressed as the ratio of radioactivity to protein. This approach avoids problems of pI and mass shifts, which are commonly found in fluorescent la-

beling approaches and which makes it nearly impossible to find the corresponding nonlabeled protein (63, 96). Similarly, labeling oxidized thiol groups with biotin allows for the affinity-purification and subsequent identification of thiol modified proteins but eliminates the possibility to determine what percentage of that protein is thiol modified *in vivo*. This knowledge, however, is crucial to evaluate whether the observed thiol modifications are physiologically relevant.

The ratio approach

In the differential thiol trapping technique, all *in vivo* thiol-reduced proteins are labeled with cold IAM, while the thiol modified proteins are labeled with ^{14}C-IAM. Once the proteins are separated on 2D gels, the extent of oxidative modification in each protein spot can be then expressed as the ratio of radioactivity in the respective spot on the autoradiographs and the protein on the stained 2D gel (Fig. 3). This "ratio approach" is especially useful when the oxidation state of proteins under different growth conditions is compared, because any change in protein amount due to differential protein expression or proteolysis is being taken into account. Therefore, this approach allows conclusions about changes in the thiol status of proteins *in vivo*, even when gene expression is altered. Additionally, the compatibility of IAM with mass spectrometry facilitates protein identification by peptide mass fingerprinting.

The full picture: reversing the differential labeling

In the differential thiol trapping technique, a detectable label is attached to *in vivo* oxidatively modified thiol residues. This, however, provides only half of the information that is necessary to describe what fraction of a protein is oxidized *in vivo*. To visualize the reduced fraction of the protein as well, a reversed trapping approach can be used, in which only the free cysteines are radioactively labeled (57). This, when performed in parallel to the "regular" trapping, provides a quantitative information about the ratio of reduced to oxidized protein in the cell. This knowledge allows determination of the *in vivo* half cell potential of cysteines or cysteine pairs if the specific mechanism of action is known. We estimated the *in vivo* half cell potential of the cysteine pair C44–C49 in the enzyme lipoamide dehydrogenase Lpd, whose mechanism of thiol–disulfide reactions is known (57).

APPLICATIONS

Snapshot of the cellular thiol state

Methods to monitor the thiol disulfide state of cellular proteins *in vivo* provide the unique opportunity to obtain a snapshot of the cellular redox state. Using the differential thiol trapping method on exponentially growing *E. coli* cells, we were able to divide the 100 most abundant proteins into

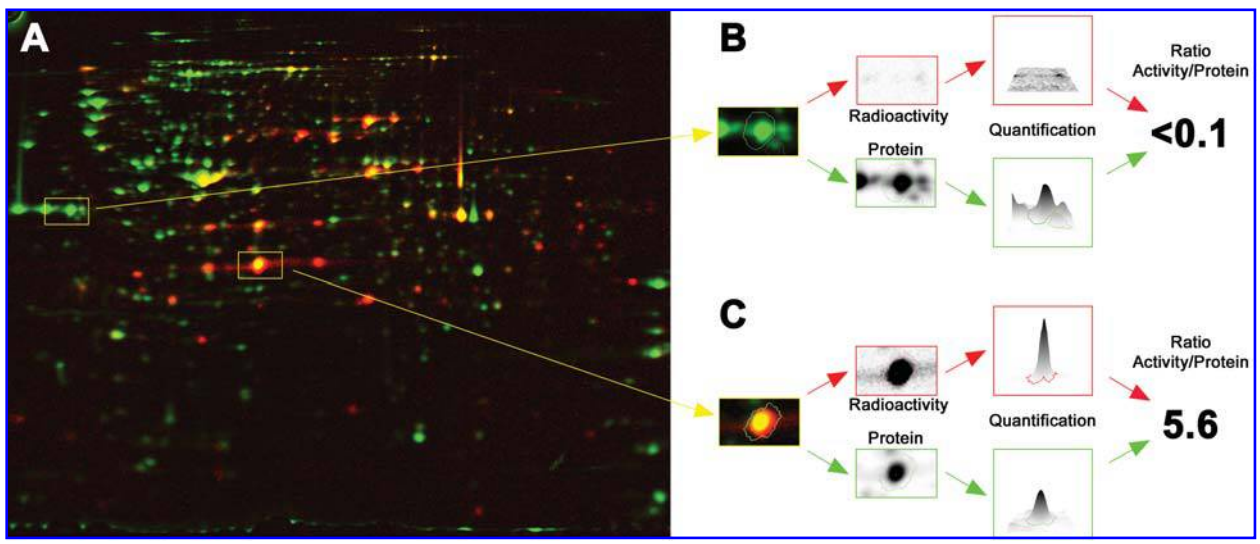

FIG. 3. Global quantification of *in vivo* thiol modifications. (**A**) False colored overlay of the Coomassie stained protein image (*green*) and autoradiograph (*red*) of a 2D gel from aerobically grown *E. coli* protein extracts. Cells were harvested in mid exponential phase and proteins were subjected to a differential thiol trapping method. This method involves the differential alkylation of *in vivo* reduced and oxidized thiols with nonradioactive and radioactive iodoacetamide, respectively. This leads to the complete alkylation of all thiol groups in a protein, independently its original thiol status and guarantees the identical migration of *in vivo* modified and unmodified proteins on a 2D gel. The color of a spot is a qualitative readout for the oxidation state of a protein. Proteins that appear *green* are reduced or do not contain cysteines, whereas proteins that appear *red* are highly oxidized. Mass spectrometric identification revealed that the most abundant reduced proteins are located in the reducing cytoplasm or do not contain cysteines, whereas most of the highly oxidized proteins are located in the oxidizing periplasm. (**B, C**) To quantify the extent of oxidation in each individual spot, the relative amount of radioactivity and protein amount for each protein is quantified and a ratio of radioactivity per protein is calculated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

two groups: proteins that incorporated low amounts of radioactive iodoacetamide and were mostly reduced *in vivo* (green spots in Fig. 3) and proteins that incorporated a substantial amount of ^{14}C-radioactivity and were significantly thiol modified *in vivo* (red spots in Fig. 3). Further analysis revealed that all of the proteins with low ^{14}C-activity to protein ratio were either localized in the cytoplasm or did not contain cysteines at all. Most of the proteins that we identified to be significantly thiol modified *in vivo*, on the other hand, were identified as periplasmic proteins, including some with known structural disulfide bonds (57). The only exceptions were the two cytoplasmic proteins Lpd and AceF, which are known to use disulfide bond formation during their catalytic cycle. Lpd and AceF are two subunits of the pyruvate dehydrogenase multienzyme complex, which links glycolysis to the citric acid cycle. During the oxidative decarboxylation of pyruvate, electrons are ultimately transferred to NAD^+. This electron transfer involves a disulfide transfer from Lpd to lipoic acid, the prosthetic group of AceF (26, 73). Thus, our method made it possible to see these enzymes "in action" (57).

Identification of substrates of oxidoreductases

Global methods to monitor the thiol–disulfide state of cellular proteins are particularly suited to find substrate proteins of oxidoreductases. A straightforward approach involves the analysis of the *in vivo* redox state of proteins in cells deficient for one particular oxidoreductase and its comparison to the redox-state of proteins in wild-type cells. In this way, numerous substrates of the periplasmic disulfide bond forming protein DsbA and of the major cytoplasmic disulfide reductase Thioredoxin 1 in *E. coli* have been identified (57). Alternatively, thioredoxin substrates in plants were discovered using diagonal and standard 2-dimensional PAGE. Here, purified thioredoxin was added to protein extracts to reduce all *in vivo* oxidized thiol groups. The newly reduced cysteines were then labeled with a fluorescent probe (97). A third approach that can be used requires detailed knowledge about the thiol–disulfide exchange mechanism of the oxidoreductase. It involves the use of oxidoreductase variants and has been successfully applied to identify substrate proteins of *E. coli* DsbA and several thioredoxins in plants (7, 53, 69, 95). Mutants of DsbA and thioredoxin were constructed that are still able to attack the cysteines of their substrate proteins and form intermolecular disulfide bonds. Due to their mutation, however, they are unable to resolve this bond or are able to resolve this bond but only very slowly and stay attached to their substrate proteins. The oxidoreductase can either be bound to a column as bait for potential substrates in cell extract (7, 69, 95) or can be expressed *in vivo* and immunopurified in complex with its substrates (53). To identify the substrate proteins, the intermolecular disulfide bond is reduced. This causes the dissociation of the substrate proteins, which can then be identified by 2D PAGE and mass spectrometry.

Monitoring the redox state of proteins in response to ROS and RNS

With the increasing interest in redox regulation of protein function and the finding that reactive oxygen species (ROS) and nitrogen species (RNS) function as potent second messengers (70, 84), it is not surprising that several global methods have been developed to identify redox-regulated proteins and proteins prone to oxidative damage. Treatment of mammalian cells with oxidants has been found to lead to the specific glutathionylation of a number of proteins. Some of these proteins have recently been identified (60). To reduce and specifically label only *in vivo* glutathionylated cysteine residues, cell extracts were incubated with purified glutaredoxin. Then, all newly reduced proteins were labeled with biotin-linked NEM, which allows for their purification using avidin–agarose columns. The affinity purified proteins were then separated on 2D PAGE and identified using mass spectrometry. In a separate approach, incorporation of radioactive glutathione was used to specifically label *in vivo* glutathionylated proteins. Subsequent analysis of the labeled proteins on 2D gels allowed the identification of glutathionylated proteins in human T lymphocytes (34).

To identify *S*-nitrosylated proteins, a technique termed "biotin switch method" was developed (48, 49). Here, ascorbic acid is used as the specific reductant for nitrosothiols and biotin is used for the labeling process. This method has been used to identify *S*-nitrosylated proteins in neurons and plants (48, 49, 61).

To gain a global overview of the general thiol–disulfide status of *E. coli* proteins upon oxidative stress treatment, we applied our differential thiol trapping technique to *E. coli* cells exposed to a nonlethal dose of H_2O_2. We found that the effects of H_2O_2 treatment on proteins were highly specific and thiol modifications were only observed in a distinct subset of proteins (57).

OUTLOOK

Thiol redox proteomics provides a global insight into the oxidation state of cysteines in proteins and has been successfully applied to identify target proteins of *in vivo* thiol modification and substrate proteins of oxidoreductases. The identification of proteins that change their oxidation state upon a change in the redox environment of the cell is the first step in understanding the complex connections between oxidative stress and disease, an area of research where causes and symptoms are not always easily distinguished. The future challenge will be to use these methods to identify possible small but potentially highly significant changes in the thiol–disulfide status of proteins that are associated with diseases. This should aid in finding new diagnostic methods and in comprehending the mechanisms of disease progression that will eventually lead to the development of cures.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Julia Bandow, Paul Graf, and the members of our lab for many helpful discussions and for critically reading the manuscript.

ABBREVIATIONS

2D, two dimensional; DTT, dithiothreitol; GSH, glutathione (reduced); GSSG, glutathione (oxidized); IAM, iodoactemide; NEM, *N*-ethylmaleimide; PAGE; polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl)phosphine.

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Date of first submission to ARS Central, October 25, 2005; date of acceptance, November 19, 2005.

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