Detection and Affinity Purification of Oxidant-Sensitive Proteins Using Biotinylated Glutathione Ethyl Ester

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[10] Detection and Affinity Purification of Oxidant-Sensitive Proteins Using Biotinylated Glutathione Ethyl Ester

By DANIEL M. SULLIVAN, RODNEY L. LEVINE, and TOREN FINKEL

Introduction

Effects of oxidative stress in biological systems have long been the subject of intense scientific scrutiny. This work has established at least an ancillary role for oxidants in an astonishingly wide range of human diseases and, more recently, a role in normal signal transduction. Many of these studies have focused on toxicological effects of highly reactive species such as peroxyl, alkoxyl, and hydroxyl radicals, which are capable of modifying a wide range of cellular constituents. In proteins, potent oxidants such as the ones listed above can attack a variety of amino acid side chains and the polypeptide backbone,[1,2] inflicting irreversible damage. Such modifications are likely important in pathological processes, such as reperfusion injury and inflammation, in which cells are exposed to high levels of oxidative stress. However, a variety of antioxidant systems are employed by cells to prevent accumulation of highly reactive species in association with normal redox metabolism. Therefore processes associated with lower levels of oxidative stress, such as redox-dependent signal transduction, are more likely mediated by the less reactive species nitric oxide (NO), hydrogen peroxide (H_2O_2), and superoxide (O_2^-). The cellular constituents susceptible to attack by these radicals are more limited, and in proteins the sulfur-containing amino acids cysteine and methionine are likely to be the predominant sites of modification.

Among the most oxidant-sensitive proteins are those containing ionized cysteine thiols.[3] These thiolate anions, also referred to as reactive cysteines, are a feature of a variety of proteins, including a number of proteins involved in signal transduction. For example, thiolates have been described in transcription factors,[4,5] kinases,[6,7]

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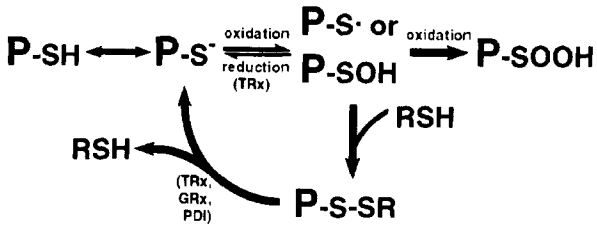

FIG. 1. Scheme for reversible redox modification of reactive cysteines. A reactive cysteine is a protein thiol (P–SH) that is ionized to the relatively nucleophilic thiolate anion ($P–S^-$) at physiological pH. The reactive cysteine can be readily oxidized to a thiyl radical ($P–S^·$) or sulfenic acid (P–SOH) under mild oxidative stress. These oxidized species are frequently unstable under physiological conditions and, if not enzymatically reduced, will react to form more stable intermediates. Further oxidation yields a sulfinic acid (P–SOOH), which is thought to be irreversible in biological systems. Alternatively, the thiyl radical or sulfenic acid can react with a low molecular weight thiol (RSH) to form a stable mixed disulfide (P–S–SR). The mixed disulfide can then be reduced by one of three known enzymes systems [thioredoxin (TRx), glutaredoxin (GRx), or protein disulfide-isomerase (PDI)] to restore the cysteine to its fully reduced state.

phosphatases[8–10] and small GTP-binding proteins.[11] In most, if not all cases, thiolates play an important role in the normal functioning of the protein. Therefore their modification would be expected to have functional consequences. Oxidation of a thiolate by superoxide or hydrogen peroxide produces a thiyl radical or sulfenic acid (Fig. 1). Under physiological conditions, these oxidation products are unstable and, unless enzymatically reduced back to the thiol, will rapidly react with other molecules to form more stable products. Further oxidation of a thiyl radical or sulfenic acid results in irreversible oxidation of the cysteine. Alternatively, these species can react with other thiols to form stable disulfides. In some instances the second thiol is contributed by another cysteine contained within the same protein or an associated protein, resulting in the formation of an intra- or intermolecular protein disulfide. If there are no protein cysteinyl thiols in the vicinity of the thiyl radical or sulfenic acid, they might react with one of several low molecular weight thiols that are present in the cell to form a mixed disulfide. Because of the relatively high concentration of glutathione (GSH) in the cell (1 to 10 m*M*),[12] it is expected that the majority of protein mixed disulfides formed as a consequence of oxidative stress contain GSH. Indeed, the transient incorporation of glutathione into cellular protein is a well-established

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response to oxidative challenge of intact tissues or cells in culture (reviewed in Refs. 13–15).

This process of protein thiolate oxidation and glutathiolation is intriguing in that it represents the reversible covalent modification of a protein attribute with functional importance. As such, the glutathiolation state of cellular proteins could serve to gauge the redox status of the intracellular environment, with the altered functional state of some modified proteins serving to transduce the oxidative stress into a biological response. Therefore a thorough accounting of proteins modified in this way and the functional consequences of modification could provide insight into the ways in which cells sense and respond to oxidative stress.

A number of techniques have been developed to study protein glutathiolation[16–18] or redox-dependent modification of reactive cysteines.[19,20] However, although these protocols can provide information regarding changes in the global glutathiolation or oxidation status of protein thiols, or can be used to study the glutathiolation state of individual proteins, it remains difficult to identify new glutathiolated proteins by these technologies. In addition, some of the available technologies are prone to artifacts arising from the need to inhibit protein synthesis while labeling the intracellular GSH pool, or the need to label proteins after cell lysis. We have described a novel reagent, biotinylated glutathione ethyl ester (BioGEE), and protocols that allows for the rapid purification of proteins that are oxidatively modified at reactive cysteine thiols *in situ*. This approach has a number of advantages over existing technologies. Because the tracer molecule can be incorporated into protein only as a consequence of cysteinyl thiol oxidation, the cell can be loaded without the need to inhibit protein synthesis. In addition, excess label is scavenged from the system before and during cell lysis so that incorporation of the label accurately reflects the glutathiolation state of the proteins before cell lysis. Finally, the use of biotin as a label allows sensitive nonradioactive detection and rapid affinity purification of labeled proteins with streptavidin conjugates.

By using BioGEE, we were able to demonstrate oxidative modification of several proteins in conjunction with tumor necrosis factor α (TNF-α)-stimulated apoptosis, and identified two proteins that had not previously been shown to be

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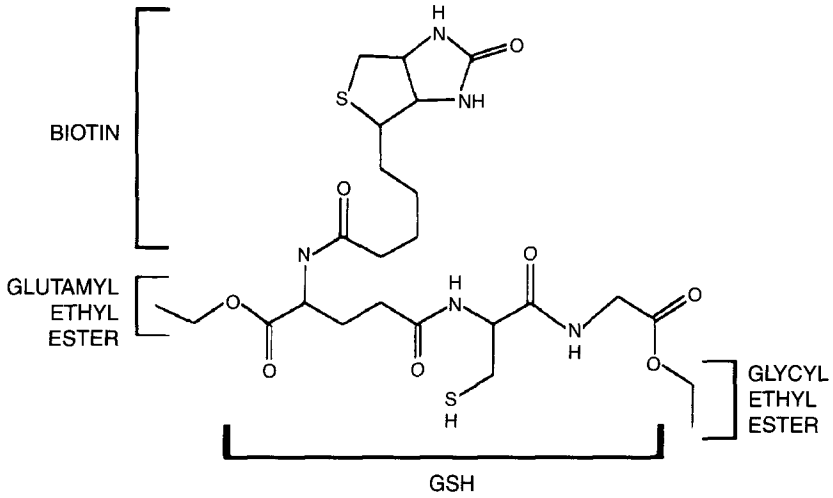

FIG. 2. Structure of BioGEE. The reagent BioGEE is composed of the glutathione tripeptide with a molecule of biotin incorporated at the γ-glutamyl amine and ethyl esters at one (glycyl) or both of the carboxyl groups.

glutathiolated.[21] In this chapter we present detailed protocols for the synthesis, purification, and use of BioGEE.

Preparation of Biotinylated Glutathione Ethyl Ester

BioGEE (Fig. 2) consists of the glutathione tripeptide modified to include ethyl esters at one or both of the carboxylates, and a molecule of biotin attached at the γ-glutamyl amine. The use of ethyl ester derivatives of glutathione is based on data indicating that the GSH mono(glycyl) or diethyl esters cross the plasma membrane much more efficiently than does GSH.[22–24] Incorporation of biotin into the molecule allows for simple and highly specific streptavidin-based detection and purification of proteins into which BioGEE is incorporated.

In our initial publication, establishing the utility of BioGEE, synthesis was accomplished by simply biotinylating a commercially available preparation of glutathione ethyl ester.[21] The crude biotinylation reaction was then added directly to cells in culture. Although this approach is simple and ultimately proved effective,

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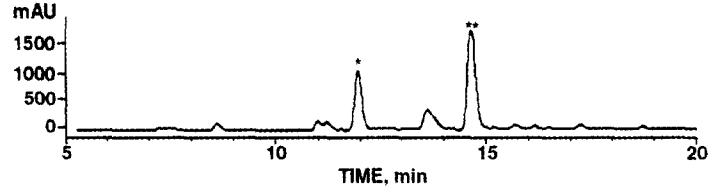

FIG. 3. BioGEE synthesis product. An aliquot of BioGEE prepared as described in text was solubilized in 0.05% (w/v) TFA and separated by reversed phase HPLC with both spectrophotometric and mass spectrometric detection (model 1100; Hewlett-Packard, Palo Alto, CA), using a Vydac narrow-bore C_{18} column (218TP5205; Vydac, Hesperia, CA). The initial solvent was 0.05% (w/v) trifluoroacetic acid and the other solvent was acetonitrile–0.05% (w/v) trifluoroacetic acid. The acetonitrile gradient was developed at 2%/min, with a flow rate of 0.2 ml/min. Mass analysis of the major peaks indicates that they are monoethyl (*) and diethyl (**) esters of biotinylated glutathione, with measured masses of 561.24 and 589.29, respectively. The expected masses were 561.6 and 589.6.

it has a number of drawbacks. First, it is necessary to limit both the concentration of the biotinylating reagent and the length of the biotinylation reaction in order to prevent incorporation of biotin at the glutathione –SH group. Second, only the monoethyl ester derivative of glutathione, which is taken up more slowly by cells than is the diethyl ester form,[25] is presently commercially available. Finally, the presence of unlabeled GSH ethyl ester in the crude reaction mixture might decrease the overall sensitivity of the protocol by competing with the biotinylated reagent.

To address these concerns, we devised the synthesis and purification scheme described below. First, the biotinylation reaction is carried out with glutathione disulfide. Because the sulfur moiety is protected in the oxidized form, the biotinylation reaction can go to completion without concern for incorporation of biotin at the sulfur. The biotinylated product is then reduced and purified by ion-exchange chromatography. The first purification step, using a strong cation exchanger, separates biotinylated from unbiotinylated GSH on the basis of the conversion of the GSH primary amine to a secondary amine in the biotinylated GSH. The second purification step uses a strong anion exchanger to separate biotinylated glutathione from excess reducing agent. The eluate from the anion-exchange column is then incubated in acidic ethanol to esterify the carboxylic acids. The products of this scheme consist primarily of mono- and diethyl esters of biotinylated glutathione (Fig. 3). As mentioned above, both the mono- and diethyl esters of glutathione are capable of crossing the plasma membrane, although the diethyl ester appears to be taken up more quickly. If desired, the yield of diethyl ester can be increased by extending the incubation time in acidic ethanol.

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Materials

NHS–biotin, Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid)], *N*,*N*-dimethylformamide, GSH, oxidized GSH (GSSG), and HCl in anhydrous ethanol can be obtained from Sigma (St. Louis, MO), and the ion-exchange resins AG-50 (hydrogen form) and AG-1 (chloride form) can be obtained from Bio-Rad (Hercules, CA). The AG-50 resin should be swelled in water and stored as a slurry. AG-1 should be equilibrated with 50 m*M* sodium acetate (pH 5.2) and can be stored as a slurry or in columns. The necessary equipment is a spectrophotometer or plate reader equipped for absorbance measurements at 405 nm and a vacuum centrifuge.

Method for Determination of Free –SH Using Ellman's Reagent

At several points in the BioGEE synthesis and purification scheme it is advantageous to determine the concentration of GSH, or other free thiols, in solution. This can be accomplished quickly and easily with Ellman's reagent and the protocol below.

1. Set up a GSH standard curve. We do a 2-fold serial dilution starting at 10 m*M* and going down to 0.312 m*M*.
2. Dissolve Ellman's reagent at 200 μM in 0.1 m*M* $NaPO_4$, pH 8.0.
3. Mix the standards and unknowns with the Ellman's reagent solution at 1 : 40 (v/v). Incubate the reactions for 5 min at room temperature and determine the absorbance of the solutions at 405 nm.

Method for Synthesis and Purification of Biotinylated Glutathione Ethyl Ester

1. Make up a solution containing 40 m*M* GSSG in 50 m*M* Na_2HPO_4 and adjust the pH to between pH 7.5 and 8.0 with NaOH.
2. Dissolve NHS–biotin to 0.1 *M* in *N*,*N*-dimethylformamide and add it, dropwise, to the GSSG solution with gentle mixing to a final concentration of 20 m*M*. Incubate the biotinylation reaction overnight at room temperature with mild agitation (shaking too vigorously will cause precipitation of the NHS–biotin).
3. Add dithiothreitol (DTT) to the biotinylation reaction to a final concentration of 80 m*M* and incubate for 1 hr at 60°.
4. Add the reaction mixture to a bed of AG-50 resin (0.5 volume of AG-50 per volume reaction mixture) and check that the pH of the slurry is pH 5.0 or less, using pH paper. Incubate the slurry for 1 hr at room temperature with shaking.
5. Centrifuge briefly to sediment the resin and transfer the supernatant to a fresh tube.
6. Adjust the pH of the supernatant to approximately pH 5.0 and load it onto an AG-1 column equilibrated with sodium acetate (pH 5.2). We typically use a 5-ml bed volume of AG-1 for 40 ml of AG-50-extracted biotinylation reaction.

7. Wash the column with 6 bed volumes of sodium acetate (pH 5.2), and then with 6 volumes of 90% (v/v) ethanol. If desired, the concentration of free thiol in the washes can be monitored with Ellman's reagent and the protocol described above. A large thiol peak, representing excess DTT, should come off the column in the wash. The thiol concentration should be nearly 0 by the end of the ethanol wash.

8. Elute glutathione from the anion-exchange column with 0.5 *N* HCl in 90% (v/v) ethanol. Collect the eluate in 1-ml fractions and determine the pH and thiol content of each. Glutathione should elute when the pH of the effluent falls below pH 3.

9. Combine the thiol-containing fractions, neutralize them with NaOH, and remove the precipitate by either centrifuging or filtering the sample.

10. Dry the eluate in a vacuum centrifuge and resuspend the pellet in an approximately one-half elution volume of 1.25 *M* HCl in anhydrous ethanol and incubate for at least 16 hr at room temperature with mild agitation.

11. Determine the thiol content of the esterification reaction with Ellman's reagent as described above. By 16 hr, free thiol in the reaction mixture represents predominantly mono- or diethyl esters of biotinylated glutathione.

12. On the basis of the thiol content and intended use, divide the reaction into single-use aliquots and dry the aliquots in a vacuum centrifuge. Store the aliquots at −80°.

Assay of Biotinylated Glutathione Ethyl Ester Incorporation into Cellular Protein

The experiments shown in Fig. 4 demonstrate oxidant-induced incorporation of BioGEE into cellular proteins and, consistent with the mechanism described above, the apparent selectivity of the reagent for reactive cysteines. These data along with our previously published work indicate that BioGEE is a sensitive and selective marker for redox-dependent modification of thiolates. Determination of BioGEE incorporation into total cellular protein or into selected proteins is quite simple and requires only standard western-blotting equipment and reagents.

Materials

The sulfhydryl-alkylating reagents *N*-ethylmaleimide (NEM) and iodoacetamide (IAM) can be obtained from Sigma. We use the Complete Mini protease inhibitor cocktail from Roche Molecular Biochemicals (Indianapolis, IN), nonfat dry milk from Bio-Rad, and the bicinchoninic acid (BCA) protein assay reagent, HRP–streptavidin, and Supersignal chemiluminescent substrate from Pierce (Rockville, IL). The following solutions are needed: phosphate-buffered saline (PBS; 150 m*M* NaCl, 6 m*M* $NaPO_4$; pH 7.4), PBS-T [PBS, 0.1% (v/v) Tween 20], radioimmunoprecipitation assay (RIPA) buffer [1% (v/v) Nonidet P-40

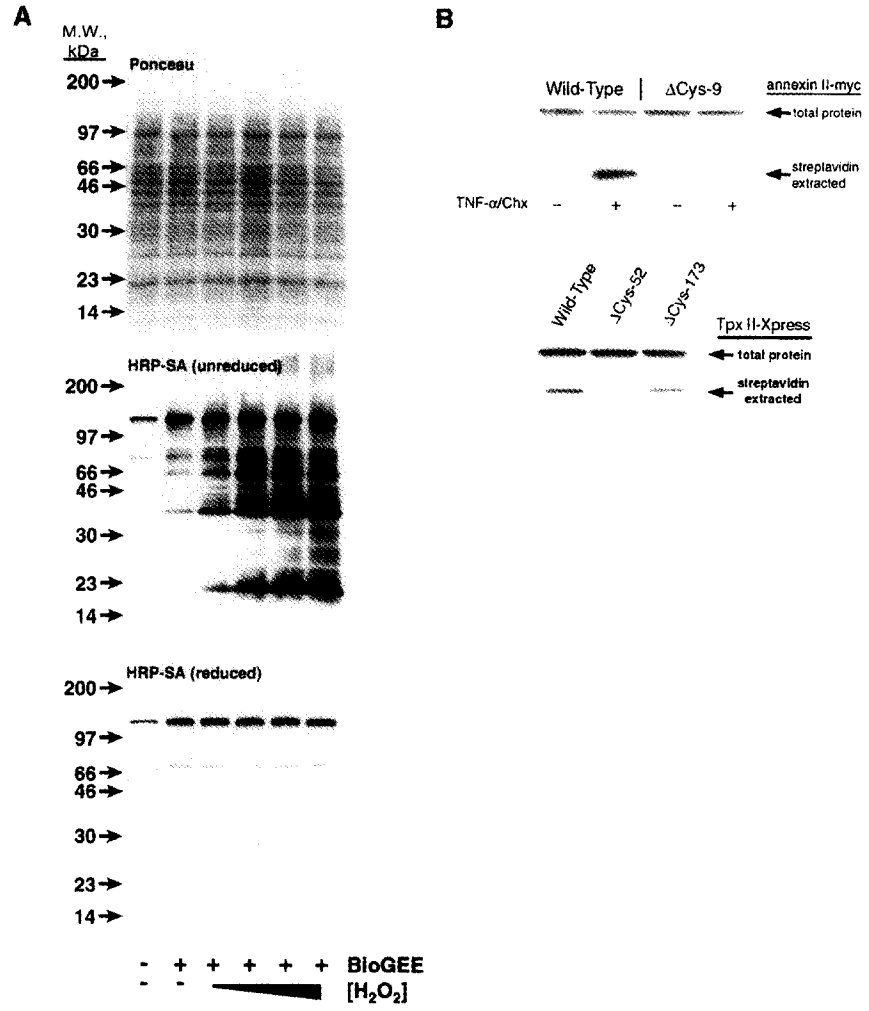

FIG. 4. Detection of BioGEE incorporation into protein, and selectivity for reactive cysteines. (A) HeLa cells were loaded with 0.5 m*M* BioGEE for 1 hr and then exposed to H_2O_2 (0, 0.12, 0.25, 0.5, and 1 m*M*) for 15 min. A soluble protein extract was obtained as described in text and 40 μg of protein from each extract was resolved by SDS–PAGE under either nonreducing or reducing conditions. The proteins were transferred to nitrocellulose and the blots were stained with Ponceau S. The blots were then blocked with milk and probed with horseradish peroxidase-conjugated streptavidin (HRP–SA). (B) HeLa cells were transfected with cDNAs encoding epitope-tagged wild-type or mutant annexin II and thioredoxin peroxidase II (Tpx II). The annexin II mutant contained a glycine substitution for the reactive cysteine at amino acid 9 (see Ref. 21), and the Tpx II mutants contained serine substitutions at the active site cysteines Cys-52 (reactive, see Ref. 26) and Cys-173 (not reactive). The cells were exposed to the indicated oxidative stress (TNF-α/cycloheximide 3-hr stimulation; H_2O_2, 15-min exposure), and a soluble protein extract was prepared as described in text. Biotin-containing proteins were affinity purified, blotted to nitrocellulose, and probed with antibodies against the indicated epitope tags.

(NP-40), 0.1% (w/v) sodium dodecyl sulfate (SDS), sodium deoxycholate (0.5 mg/ml), 150 m*M* NaCl, and 50 m*M* Tris-HCl (pH 7.5)], and hypotonic lysis buffer [1 m*M* EDTA, 1 m*M* EGTA, 50 m*M* Tris-HCl (pH 7.0)]. Standard equipment, solutions, and materials for SDS–polyacrylamide gel electrophoresis (PAGE) and transfer of proteins to nitrocellulose are also needed.

Method for Loading Cells with Biotinylated Glutathione Ethyl Ester

To load cells in culture we typically resolubilize BioGEE in culture medium at a concentration of approximately 0.5 m*M*, filter the medium, and then add it directly to cells. To estimate the concentration of BioGEE, one pellet from each synthesis lot should be dissolved in PBS and the concentration determined with Ellman's reagent as described above. In our experience, good results can be obtained by loading cells from 1 hr to overnight and in the absence or presence of up to 10% (v/v) fetal bovine serum (FBS). Although there is some basal incorporation of BioGEE into protein in unchallenged cells, the effect of oxidative stress on incorporation is obvious even after 16 to 18 hr of loading. Basal incorporation of BioGEE is probably a steady state phenomenon as oxidative stress appears to increase labeling of many of the same bands detected in the basal state. In addition, the extent of BioGEE incorporation in the basal state appears to be protein specific as we cannot detect basal incorporation of BioGEE into glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[21] a protein for which incorporation is readily detectable after oxidative stress.

Because BioGEE is likely to enter the cell by passive diffusion, we anticipate that it will be possible to load a wide variety of cell types and tissues with the reagent. The efficiency of loading might vary, however, depending on how BioGEE is metabolized by the individual cell type. For example, results of one study indicate that the diethyl ester of glutathione crosses the plasma membrane in both directions more rapidly than the monoethyl ester.[25] In some human cell types the diethyl ester is rapidly metabolized to the monoester, resulting in a partial trapping and concentration of the reagent inside of the cell. However, the rate of conversion of the monoethyl ester to GSH is slow in these same cells. In our experience oxidant-induced incorporation of BioGEE declines rapidly once the reagent is washed out of the culture medium, probably because of efflux of incompletely deesterified label from the cell. Therefore we typically leave the reagent in the culture medium during stimulation. The need to do this might, however, vary from one cell type to the next, depending on how rapidly the cell deesterifies the second carboxylate.

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Method for Harvesting Proteins: For Both Blotting and Affinity Purification

1. Wash the cells twice with ice-cold PBS and once with ice-cold PBS containing 50 m*M* *N*-ethylmaleimide (NEM) to scavenge any remaining BioGEE.
2. Harvest cells by scraping in RIPA or hypotonic lysis buffer containing NEM or iodoacetamide (IAM) at 50 m*M* concentration and protease inhibitors. We have obtained good results with both NEM and IAM. However, it is important to note that we do not know of a peptide-mapping resource that includes NEM-modified cysteines, and therefore IAM should be used if the goal of the experiment is to identify a protein by peptide mapping.
3. For cells harvested in RIPA buffer, homogenize by passing the lysate several times through a 22-gauge needle. For cells harvested in hypotonic buffer, lyse by freezing in a dry ice–ethanol bath and thawing at room temperature.
4. Centrifuge the lysates for 10 min at 12,000*g* and 4° to pellet insoluble material and transfer the supernatant to a fresh tube.
5. Determine the protein concentration in the supernatant, using BCA or other standard methods.

Method for Detection of Biotinylated Glutathione Ethyl Ester Incorporation into Protein

Standard laboratory Western blotting and immunoprecipitation protocols can be easily adapted for assaying BioGEE incorporation into total or selected proteins. It is, of course, important to keep in mind that BioGEE is incorporated via a disulfide bond and therefore reducing agents must be omitted from buffers and solutions unless the intention is to remove the label. We have obtained good results with both freshly prepared and frozen (−80°) lysates, and from as little as 5 μg of soluble protein. For detection of biotin in proteins blotted to nitrocellulose membranes, we typically block the membranes with 5% (w/v) nonfat dry milk in PBS-T for 1 hr at room temperature. The membranes are then probed with HRP-conjugated streptavidin at 1 μg/ml for 1 hr at room temperature. The blots are washed five times with PBS-T and developed with HRP chemiluminescent substrate according to the manufacturer protocol. It is important to be aware that biotin-containing proteins such as carboxyltransferases might also be detected by this protocol. However, because incorporation of biotin via a disulfide is unique to BioGEE, labeling with BioGEE can be confirmed by running a duplicate blot under reducing conditions (Fig. 4A).

BioGEE incorporation into individual proteins can be determined by probing blots of immunoprecipitates with streptavidin, or by probing blots of affinity-purified proteins (described below) with antibodies. In our experience the former approach is both more sensitive and more reliable than the latter.

Affinity Purification of Biotinylated Glutathione Ethyl Ester-Labeled Proteins

Perhaps the greatest strength of the method described here is that it provides a means to affinity purify proteins on the basis of their propensity to undergo redox modification at select amino acid residues. We have used the simple two-step scheme described below to successfully identify, by mass spectrometric peptide mapping and peptide sequencing, proteins purified from as little as 5 mg of soluble protein (Fig. 5). In the protocol described below proteins covalently bound to biotin are extracted in batch. The protocol can be readily adapted to a column if so desired.

Materials

Streptavidin–agarose can be obtained from Sigma and Centricon-10 ultrafiltration units can be obtained from Amicon (Beverly, MA). Biotin-blocked streptavidin is prepared by suspending 1 ml of streptavidin–agarose in 10 ml of (D-biotin 3 mg/ml in PBS), incubating the mixture for at least 1 hr at room temperature, and then washing the beads extensively with PBS. For in-gel protein detection we use the SilverXpress silver staining kit (Invitrogen, Carlsbad, CA) and GelCode Blue Coomassie staining reagent (Pierce).

Method

1. Obtain protein extracts as described above.
2. Add biotin-blocked streptavidin–agarose (50 μl/mg of soluble protein) to the extract and incubate for 30 min at 4° with gentle shaking.
3. Pellet the beads by brief centrifugation and transfer the supernatant to a fresh tube.
4. Add 100 μl of agarose-conjugated streptavidin per milligram of protein and incubate for 2 hr at 4° with gentle shaking.
5. Pellet the beads by brief centrifugation and remove the supernatant.
6. Wash the beads five times with approximately 10 volumes of ice-cold RIPA buffer.
7. Wash the beads two times with 10 volumes of room temperature PBS containing 0.1% (w/v) SDS.
8. Resuspend the agarose pellet in 1 volume of PBS–0.1% (w/v) SDS and incubate for 30 min at room temperature with gentle shaking.
9. Pellet the agarose and save the supernatant to be run in parallel as a −DTT control.
10. Resuspend the agarose pellet in 1 volume of PBS–0.1% (w/v) SDS containing 10 m*M* DTT and incubate for 30 min at room temperature with gentle shaking.

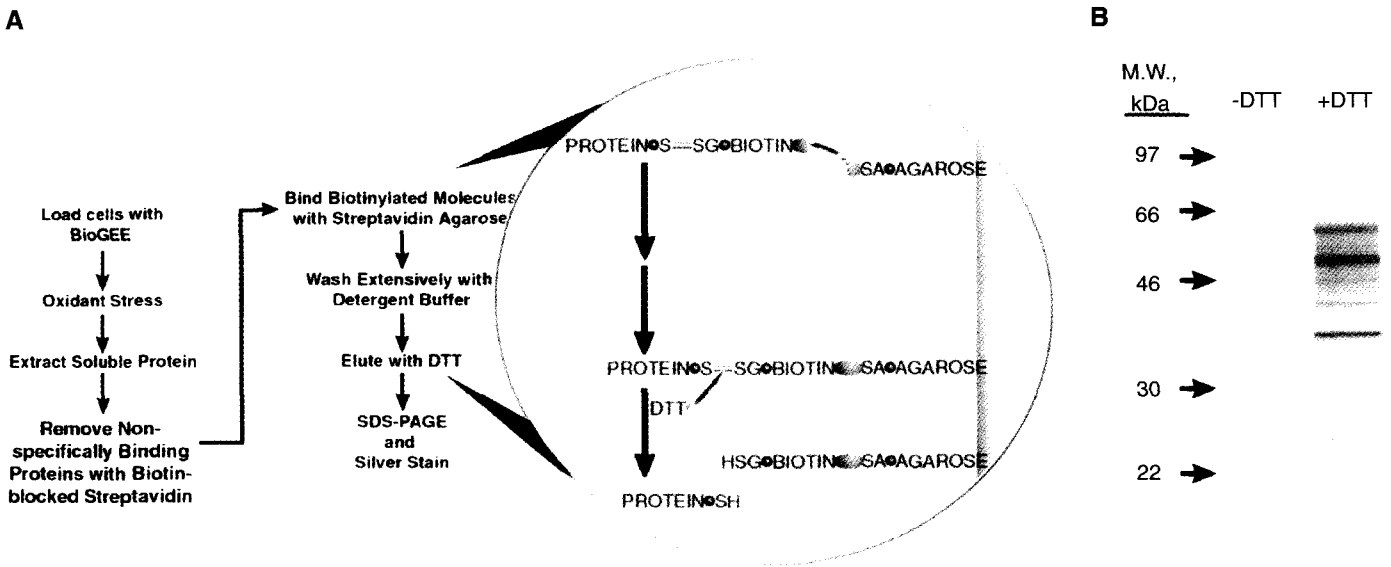

FIG. 5. Affinity purification of proteins labeled with BioGEE. (A) Diagram of the protocol devised for labeling and purification of S-glutathiolated proteins. Abbreviations: S, Sulfur; SG, glutathione; SA, streptavidin; DTT, dithiothreitol. (B) A soluble protein extract was obtained, as outlined in (A) and text, from HeLa cells treated with H_2O_2. Biotin-containing proteins were extracted from 0.5 mg of soluble protein with streptavidin–agarose. The beads were washed and eluted sequentially, first with PBS containing 0.1% (w/v) SDS (−DTT eluate) and then with PBS containing 0.1% (w/v) SDS and 10 m*M* DTT (+DTT eluate). The eluates were resolved by SDS–PAGE and proteins were detected by silver staining the gel.

11. Pellet the agarose and transfer the supernatant to a fresh tube.

12. Wash the beads with 1 volume of PBS–0.1% (w/v) SDS and combine the wash with the DTT eluate.

13. Add DTT to the −DTT eluate to a final concentration of 10 m*M* and transfer both the −DTT and DTT eluates to Centricon-10 ultrafiltration units. Concentrate proteins according to the manufacturer protocol.

14. Add SDS–PAGE sample buffer to the concentrated proteins and separate by SDS–PAGE.

15. Detect proteins by Coomassie or silver staining the gel, using standard protocols.

Interpretation of Data Obtained with Biotinylated Glutathione Ethyl Ester

The dynamic incorporation of BioGEE into protein in association with oxidative stress, its structural similarity to glutathione and apparent selectivity for reactive cysteines make BioGEE an excellent marker for reversible redox modification of proteins. However, as mentioned above, many details regarding the metabolism of BioGEE remain to be established. In particular, it is not known whether the enzyme systems involved in removing GSH from protein are effective at removing BioGEE. Even if BioGEE is completely deesterified to biotinylated GSH, it is conceivable that the remaining biotinyl moiety could affect the ability of enzymes, such as glutaredoxin, to use biotinylated GSH as a substrate. If that were the case, BioGEE might be expected to have a slower off rate and therefore accumulate in protein to a greater extent than endogenous glutathione. Likewise, the extent of BioGEE incorporation into protein is dependent on the unknown ratio of BioGEE to endogenous glutathione in the cell. It should therefore be stressed that the extent to which BioGEE is incorporated into a given protein cannot at this point be directly correlated with the extent to which that protein is glutathiolated. A finding that BioGEE is incorporated into a protein should therefore be taken as evidence of the existence of a redox-active cysteine that is sufficiently exposed to accept a molecule of glutathione. Such information is a crucial first step in understanding the effects of oxidants on the modified protein and ultimately on the cell.