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Fluorescein-labeled glutathione to study protein S-glutathionylation

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Abstract

Numerous studies of S-glutathionylation of cysteine thiols indicate that this protein modification plays a key role in redox regulation of proteins. To facilitate the study of protein S-glutathionylation, we developed a synthesis and purification to produce milligram quantities of fluorescein-labeled glutathione. The amino terminus of the glutathione tripeptide reacted with fluorescein isothiocyanate readily in ammonium bicarbonate. Purification by solid phase extraction on C8 and C18 columns separated excess reactants from desired products. Both oxidized and reduced fluorescein-labeled glutathione reacted with a variety of thiol containing proteins to yield fluorescent proteins.

Keywords

S-glutathionylation; glutathione; cysteine; fluorescein; biotinylation; thiol; disulfide

S-glutathionylation is the covalent modification of protein cysteine thiols by glutathione (GSH), an abundant cytosolic tripeptide containing a reduced cysteine thiol[1-3]. This protein modification is of interest because it is reversible, it serves as a means to regulate a protein's function and it is sensitive to the redox environment of a cell.

Protein thiols undergo thiol-disulfide exchange with GSSG, the oxidized form of glutathione [4-6]. We have studied the kinetics of this exchange reaction with tubulin, an abundant cytoskeletal protein with 20 reduced cysteines [5]. Under normal cellular conditions, GSH protects proteins from oxidation by reactive oxygen species. GSH concentrations in most cells are in the 1-10 mM range with a typical GSH:GSSG ratio of 100 to 400 [4]. However, under conditions of oxidative stress, GSH will not be present in sufficient quantities to prevent protein oxidation.

Current methods to study S-glutathionylation include using 1) ^{35}S-labeled glutathione, 2) an anti-GSH antibody and 3) biotinylated glutathione [2,7,8]. Our fluorescein-labeled molecules are easy to synthesize and purify, and allow for the rapid detection of S-glutathionylated proteins. Herein we describe the synthesis and purification of fluorescein-labeled glutathione, both GSH and GSSG, and their use to detect S-glutathionylation. Figure 1 shows the structure of GSH and the modifications introduced.

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GSH, GSSG, ninhydrin, and 5,5′dithiobis(2-nitrobenzoic acid) were from Sigma. Fluorescein isothiocyanate (FITC) was from Sigma or Fluka. Dithiothreitol (DTT), C8 and C18 Hypersep solid phase extraction columns (3 ml, 500 mg) and silica gel thin-layer chromatography plates (5 × 10 cm) were from Fisher. Tris(2-carboxyethyl)phosphine (TCEP), avidin-HRP, West Pico chemiluminescent substrate and the bicinchoninic acid (BCA) reagent were from Pierce.

For a 5 ml reaction, FITC (50 mM in DMF, 650 μl) was added dropwise to 4.35 ml 10.4 mM GSSG in 0.1 M ammonium bicarbonate pH 8.3. The final concentrations of FITC and GSSG were 6.5 mM and 9 mM, respectively. Reactions were also performed successfully on a 1 or 2 ml scale. Reactions proceeded at 25 °C for 12-18 hours on a rotating shaker.

The FITC/GSSG reaction progress was monitored by TLC on silica gel plates. To detect unreacted FITC, the solvent system used was acetonitrile, methylene chloride, and glacial acetic acid (90:10:1). FITC was detected near the solvent front. Because unreacted FITC interfered with subsequent purification steps, it was important that it all be consumed. Because each GSSG contains two amino termini, the concentration of amine in the reaction is 18 mM; thus it is nearly 3-fold higher than that of FITC. For the reaction conditions presented above, all FITC reacted within 12 hours. The reaction of 5 mM FITC with 5 mM GSSG (10 mM amine) was also successful on a 1-5 ml scale. At all FITC/GSSG ratios tested, reactions proceeded to completion faster in ammonium bicarbonate vs. sodium phosphate pH 8.0. Because GSSG is easy to remove by chromatography, it was routinely used in excess.

The FITC/GSSG reaction (2 of 5 ml) was loaded onto a C8 column (3 ml bed, 500 mg) conditioned with 2 ml methanol, followed by 2 ml water. The load eluent was collected as fraction 1. The C8 column was then washed with 2 × 1 ml water (fractions 2,3) and then by with 2 × 1 ml methanol (fractions 4,5).

Column fractions were analyzed by TLC using acetonitrile, water and glacial acetic acid (80:20:1) as the solvent system. Yellow spots indicated the presence of the fluorescein labeled glutathiones with either one (F-GSSG) or both amino termini (F-GSSG-F) labeled. GSSG and F-GSSG were detected by spraying the plate with 1% ninhydrin (w/v) in 90% methanol and heating on a hot plate. For F-GSSG with one free amine and one fluorescein, the ninhydrin-reactive spot was a mixture of purple and yellow. In all cases, the yield of F-GSSG was greater than that of F-GSSG-F.

Fraction 1, the load eluent, contained GSSG and F-GSSG whereas fractions 4 and 5 contained F-GSSG-F. FITC would have eluted in fractions 4 and 5 with a similar R_f value and would have complicated protein labeling studies using F-GSSG-F because FITC reacts with protein amines. The methanol fractions containing F-GSSG-F were dried in a Speed-Vac concentrator and stored at 4 °C until use.

A summary of the reaction components and products with R_f values is given in Table 1. HPLC retention times for fluorescein-labeled molecules are also presented in Table 1. A C18 reverse phase column and a gradient of 25-60% acetonitrile with 0.1% TFA were used for optimal separation. Detection was at 440 nm which is the absorbance maximum of fluorescein under acidic conditions. Quantitation of product yields by HPLC was complicated because the molar absorptivity of fluorescein decreases by approximately 50% as the solvent composition changes from 25-60% acetonitrile. This was confirmed by measuring fluorescein absorbance in varying solvent mixtures by UV-visible spectroscopy. Based on TLC and HPLC estimates, the FITC/GSSG reaction produced 60% F-GSSG and 40% F-GSSG-F.

To generate F-GSH, F-GSSG-F was resuspended in 0.1 M phosphate buffer pH 7.4 and treated with varying concentrations of either DTT or TCEP for 30-60 min at room temperature. Under these reaction conditions, F-GSSG-F did not reduce completely to F-GSH. However, fraction

1, containing GSSG and F-GSSG, was successfully treated with 50 mM TCEP for 30 min at room temperature to yield GSH and F-GSH.

A subsequent chromatographic step was required to separate GSH from F-GSH and to remove excess TCEP. The entire fraction (1 mL) was loaded onto a C18 column (3 ml, 500 mg) and GSH was detected in the load eluent. The column was washed with water until TCEP was undetectable. TCEP was detected by combining 10 μl of each water wash with 100 μl of either 10 mM DTNB, which yielded the yellow TNB product, or with BCA reagent, commonly used to determine protein concentration. The BCA reagent reacts with TCEP within seconds to yield a brown color.

F-GSH was eluted from the C18 column with 1 ml methanol. TLC analysis of F-GSH showed no contamination with GSH, GSSG or F-GSSG. To determine either F-GSH or F-GSSG-F concentration, absorbance values were compared to those of a fluorescein standard in 0.1 M phosphate buffer pH 8.0 at 490 nm. F-GSSG-F and F-GSH were stored dried at 4 °C with little change for up to 12 months.

Stability studies by TLC and HPLC showed that F-GSH was stable in neutral buffers at room temperature and air oxidized to F-GSSG-F very slowly with only 10% oxidation after 24 hours. F-GSH was analyzed by electrospray ionization mass spectrometry. Negative ion mode peaks at 695 and 347 m/z correspond to deprotonated F-GSH and the doubly deprotonated species, respectively.

To estimate cellular uptake, we determined a partition coefficient for F-GSSG-F. F-GSSG-F (200 μl, 1 mM) was mixed with 200 μl 1-octanol and the amount of F-GSSG-F in the organic layer was measured by UV-visible spectroscopy. Given that both fluorescein and GSSG have multiple negative charges at neutral pH, it was not unexpected that little F-GSSG-F partitioned into the organic layer. Only 2-3% of F-GSSG-F was detected in the octanol layer at pH 7.4 whereas 65% was detected at pH 6.0. Esterification of glutathione carboxyl groups would be expected to enhance uptake.

The reactions of both F-GSSG-F and F-GSH with proteins known to contain reactive cysteines including tubulin and GAPDH were investigated. GAPDH has been studied as a target for S-glutathionylation by several groups and we have detected S-glutathionylation and thiol-disulfide exchange using tubulin. Protein thiols undergo thiol-disulfide exchange with F-GSSG-F to yield oxidized protein and F-GSH. During this process, some mixed disulfide between protein and F-GSH, PSSG-F, was detected by dot blot and by SDS-PAGE under nonreducing conditions. Addition of DTT removed the fluorescein label from all protein samples tested. This exchange experiment yielded only low levels of glutathionylated proteins. This is not unexpected because PSSG-F is merely an intermediate in the thiol-disulfide exchange process [4,9].

More significant labeling of protein thiols with F-GSH was accomplished by combining reduced protein, F-GSH and an oxidant such as H_2O_2. Labeling was especially pronounced for GAPDH and tubulin. A cysteine, either of protein or GSH is first oxidized by H_2O_2 to an intermediate sulfenic acid, RSOH. That intermediate then reacts with a second thiol to form a disulfide.

$$\text{Protein}-\text{SH}+\text{F}-\text{GSH}+\text{H}_2\text{O}_2 \rightarrow \text{Protein}-\text{SSG}-\text{F}+2\text{H}_2\text{O}$$

The synthesis, purification and use of biotinylated glutathione have been described and biotinylated glutathione ethyl ester is commercially available [2,7,8,10,11]. Fluorescein-

labeled glutathiones, both F-GSH and F-GSSG-F, have some advantages over their biotinylated counterparts. To detect biotinylated proteins, an avidin conjugate is required and then either colorimetric or chemiluminescent detection. These steps can be time-consuming and expensive. Using 0.1- 1 mM concentrations of either F-GSH or F-GSSG-F, a protein (10-30 μM, 40-120 μM cys for GAPDH) is labeled in as little as 2-5 min. For a dot blot, a small portion of a reaction (2-4 μl) is spotted onto a nitrocellulose membrane, allowed to air dry and then washed with PBS. After 2-3 washes, one can observe a yellow protein spot on the membrane. A negative control with a reducing agent rules out nonspecific binding. For increased sensitivity, the membrane can be viewed under UV light. In total, no more than 30 min is required from start to finish.

The use of glutathiones modified at the tripeptide amino terminus requires that one establish that the modification does not alter the reactivity of the glutathione thiol. In early studies of biotinylated glutathiones, researchers compared the biotinylation of proteins with reactivity observed with an anti-GSH antibody [12]. To compare the reactivity of biotin and fluorescein labeled GSH/GSSG, we also synthesized and purified B-GSSG-B and B-GSH. Both biotinylated and fluorescein-labeled glutathiones reacted with model proteins in a similar fashion in terms of concentration and time-dependence.

To rule out gross modifications that alter glutathione reactivity, we performed a series of competition experiments using either biotin or fluorescein labeled glutathiones and unlabeled GSH/GSSG. As the concentration of GSH/GSSG increased, we observed a corresponding decrease in protein labeling with either biotin or fluorescein. The synthesis, purification and protein studies using our fluorescein-labeled glutathiones are simple and inexpensive. Protein S-glutathionylation can be assessed in minutes using items routinely found in any biochemistry laboratory.

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Abbreviations

BCA	bicinchoninic acid
B-GSH	biotinylated GSH
DTNB	5,5′dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
F-GSH	fluorescein-labeled GSH
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GSSG	oxidized glutathione
PBS	phosphate-buffered saline
TCEP	Tris(2-carboxyethyl)phosphine
TLC	thin-layer chromatography

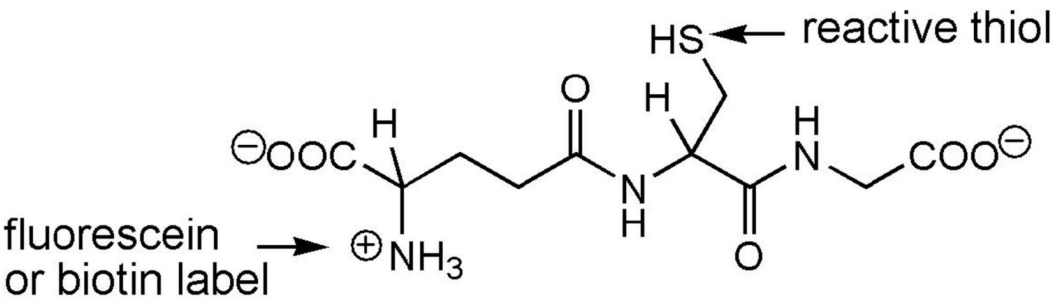

Figure 1.
Structure of GSH; Modification of the amino terminus by fluorescein or biotin does not alter the reactivity of the thiol.

Table 1

Summary of TLC and HPLC analysis of reactants and products

Molecule	R_f value	Color	ninhydrin reactivity	HPLC retention time (min)
GSSG	0.03	-	yes	
GSH	0.15	-	yes	
F-GSSG	0.25	yellow	yes	6.8
F-GSH	0.34	yellow	no	12.8
F-GSSG-F	0.88	yellow	no	15.7
FITC	0.91	yellow	no	19.1

The TLC solvent system used was 80:20:1 acetonitrile, water, glacial acetic acid. Silica gel plates were 5 × 10 cm. For HPLC separation, a C18 reverse phase column (Zorbax, 5 μm, 4.6 × 150 mm) was used with a linear gradient of 25 to 60% acetonitrile + 0.1% TFA over 20 min. The flow rate was 0.5 min and detection was at 440 nm.