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ORIGINAL PAPER

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Determination of the thiol redox state of organisms: new oxidative stress indicators

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Abstract This study describes a new methodology by which the concentrations of non-protein (NP) thiols glutathione (GSH), cysteine (CSH), *N*-acetylcysteine (AcCSH), and protein (P) thiols (PSH), as well as the contribution of these components to symmetric and mixed disulfides (NPSSR, NPSSC, NPSSCAc, PSSR, PSSC, PSSCAc, PSSP) can reliably be measured. The methodology consists of a strict sequence of methods which are applied to every sample. Free thiols at any given state of the procedure are measured by Ellman's assay, the CSH fraction is measured by its unique response in the ninhydrin assay, AcCSH is selectively measured with ninhydrin after enzymatic deacylation, proteins are separated from non-protein thiols/disulfides by precipitation with trichloroacetic or perchloric acid, disulfides are reduced into free thiols with borohydride, mixed disulfides between a protein and a non-protein component are measured by extracting the non-protein thiol from the protein pellet after borohydride treatment, and protein thiols/disulfides are measured after resolubilization of the protein pellet.

When this method was applied to animal and fungal tissue, new molecular indicators of the thiol redox state of living cells were identified. The findings of the present study clearly show that the new parameters are very sensitive indicators of redox state, while at the same time the traditional parameters GSH and GSSG often remain constant even upon dramatic changes in the overall redox state of biological tissue. Therefore, unbiased assessment of the redox state also requires explicit measurement of its most sensitive thiol indicators.

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Introduction

The thiol redox state is an essential parameter of prokaryotic and eukaryotic cells and it is associated with all major biological processes [1]. The thiol redox state of cells is characterized by the levels of certain components like glutathione (GSH), cysteine (CSH), and protein thiols (PSH), as well as their contribution to symmetric and mixed disulfides. Traditionally, GSH and its disulfide form (GSSG) are used as indicators of oxidative stress.

GSH is considered as the major regulator of the intracellular redox state and participates in redox reactions via the reversible oxidation of its active thiol [2]. The role of GSH is related to many physiological processes in which other non-protein (NP) thiols as well as their symmetric and mixed disulfides are involved. Glutathione is the only thiol emphasized in the literature, while the contribution of cysteine (CSH) and other minor thiols like γ-glutamylcysteine and CoA in the thiol redox state is largely ignored. Similarly, GSSG is the only reported disulfide, although it may represent only a small fraction of the total non-protein mixed disulfides (NPSSR). In the formation of NPSSR may participate GSH and CSH (designated as NPSSG and NPSSC, respectively) and many other minor non-protein thiols like γ-glutamylcysteine and CoA [3, 4]. NPSSR can exist also as disulfide-*S*-oxides, which have been reported to make significant contribution to the physiological consequences of oxidative stress [3, 5].

Similarly, protein thiols (PSH), protein disulfides (PSSP), and mixed disulfides (PSSR) are important indicators of oxidative stress. Protein/non-protein mixed disulfides (PSSR) are commonly designated in literature as PSSG [4] to emphasize the prominent participation of GSH (via GSSG disulfide exchange reactions) [6]. This may not be always the case, since PSSR could also contain CSH (designated as PSSC) and to a lesser degree other minor non-protein thiols like γ-glutamylcysteine, CoA etc. The

mechanism of formation of PSSR remains obscure. It is thought to be mediated via the rapid reaction of non-protein disulfide-*S*-oxides with protein thiols [3]. On the other hand, protein disulfides can result (a) from formation of inter-disulfide bridges between protein thiols (leading to the formation of protein aggregates) and (b) from formation of intra-disulfide bridges within proteins undergoing oxidative tertiary structure modification. Intra-PSSPs may be very important thiol redox indicators, since they are formed during activation/inactivation of receptors, in inactivation of enzymes, transporters and transcriptional factors during oxidative stress conditions [7], whereas inter-PSSPs are formed in animal cells during pathological processes associated with oxidative stress as well as in plants under stress [7, 8, 9, 10].

Protein thiols/disulfides can be extracellular, constitute part of membrane and sub-cellular structures, contribute to the regulation of redox homeostasis, or they can be involved in allosteric, enzymatic, and receptor-mediated responses [1]. It has been proposed that catalytically important sulfhydryl groups in PSH can be protected from oxidative stress by reacting reversibly with glutathione to form PSSG [11]. Furthermore, PSSG can be formed to activate certain enzymes (e.g., trypsin and collagenase), to inactivate enzymes (e.g., fructose-1,6-biphosphate aldolase), and to make proteins more or less susceptible to proteolysis [12]. Also, specific oxidation/reduction of particular protein thiols may represent an important event in cellular signaling cascades [13]. In addition, protein thiols can react with nitric oxide radical to form *S*-nitrosothiols, which are involved in signal transduction and post-translational protein modification [14].

All these interrelated roles of thiols raise the need for simultaneously monitoring their levels in organisms, since certain thiol redox state-associated patterns may identify normal/abnormal metabolic processes. So far, the thiol redox state has been partially measured either as total cellular thiol content [15] or by its components GSH and GSSG [4, 16, 17]. Usually, GSSG is determined by relatively cumbersome enzymatic assays [4]. Furthermore, GSH is overestimated by Ellman's reagent-based photometric assays [18], which are non-specific, since they do not discriminate it from CSH [19]. In addition, fluorometric assays for GSH/GSSG [20] are equally non-specific and sensitive to Ellman's-based assays, and are quite laborious in practice mainly due to fluorescence quenching and other interference problems. Similarly, PSSG is underestimated by photometric assays because of oxidation of the measured GSH during the procedure [6, 21]. Moreover, no general assays are available for the simultaneous determination of CSH, NPSSC, PSSR, NPSSR, and PSSC, although the protein thiol component (PSH) of the last three of these disulfides can be readily determined by the Ellman's reagent [22].

In the present study, for the first time a methodology is offered by which all the important thiol/disulfide classes of a particular sample are explicitly measured, providing detailed characterization of the general thiol redox state of organisms. The components of the thiol redox state were measured in various organs of mouse and in two fungi, one unicellular (yeast) and the other multicellular (the filamentous phytopathogen *Sclerotium rolfsii*). In particular, the method discriminates among NPSSR, NPSSC, PSSR, PSSC, PSSP, PSH, CSH, and GSH. The last of these is determined more accurately than by previous photometric assays, since the method distinguishes it from CSH. The method has incorporated the ninhydrin assay [23] for the determination of CSH (free or as mixed disulfides), after proper modification to improve its specificity for CSH. Moreover, this study introduces NPSSR as a new thiol redox indicator of high oxidative stress in place of GSSG, by comparing both indicators in the selected fungi grown under oxidative-stress-inducing conditions (carbon source exhaustion).

Finally, the method was extended to measure *N*-acetylcysteine (AcCSH) and its mixed disulfides NPSSCAc and PSSCAc. This meets the need for tracing AcCSH and its disulfidic derivatives, since this thiol, a less toxic and more easily cell-penetrating form of CSH [24], has multiple therapeutic applications: it is used as an adjunct to cancer chemotherapy and as cytoprotective drug and modulator of thiol levels affected by various pathological conditions or abnormal biological processes [25].

Materials and methods

Materials

Glutathione disulfide (GSSG, disodium salt), reduced glutathione (GSH), beta-NADPH (tetrasodium salt), tris(hydroxymethyl)aminomethane (Tris), 5,5′-dithiobis(nitrobenzoic acid) (DTNB), diethylenetriaminepentaacetic acid (DTPA), AcCSH, guanidine-HCl, ethylenediaminetetraacetic acid (EDTA, disodium salt), Coomassie Brilliant Blue G250 (CBB-G250), bovine serum albumin (BSA, fraction V), *N*-ethylmaleimide (NEM), *o*-phthalaldehyde, 1-chloro-2,4-dinitrobenzene (CDNB), yeast extract, peptone, GSSG reductase (from bakers yeast), acylase (from porcine kidney), GSH-*S*-transferase (from equine liver), butylated hydroxyanisole (BHA), 2-thiobarbituric acid (TBA), and glycine were obtained from Sigma Co (St. Louis, Missouri, USA). Sodium borohydride (BH), trichloroacetic acid (TCA), perchloric acid (PCA), acetone, diethylether, absolute ethanol, and methanol were obtained from Merck-Schuchardt (Hohenbrunn, Germany). Butanol-1 was from SDS (Peypin, France). L-Cysteine (CSH) was from Ferak (Berlin, Germany) and L-cystine, urea, and ninhydrin were obtained from Serva (Heidelberg, Germany). All other reagents used in this work were of the highest analytical grade.

Organisms and experimental conditions

The general thiol redox state was determined on microbial and animal organisms. Two fungi were used, a multicellular (filamentous) phytopathogen fungus *Sclerotium rolfsii*, and a unicellular fungus the yeast *Saccharomyces cerevisiae* (strain L1494, provided by Dr Dennis Synetos, School of Medicine, University of Patras, Greece). *Sclerotium rolfsii* was grown as stated previously [26], and thiol redox state was measured in young (3-day-old) and mature (6-day-old) fungal colonies. In addition, thiol redox state was measured in 6-day-old *S. rolfsii* grown in a medium supplemented either with 1.5 mM CSH or with 10 mM AcCSH. Yeast strain L1494 was grown in 1% yeast extract, 2% peptone, and 2% D-glucose. Thiol redox state was measured in this strain at early, mid, and late log growth stages (0.2, 0.7, and 1.2 absorbance units at 660 nm, respectively). Furthermore, thiol redox state was mea-

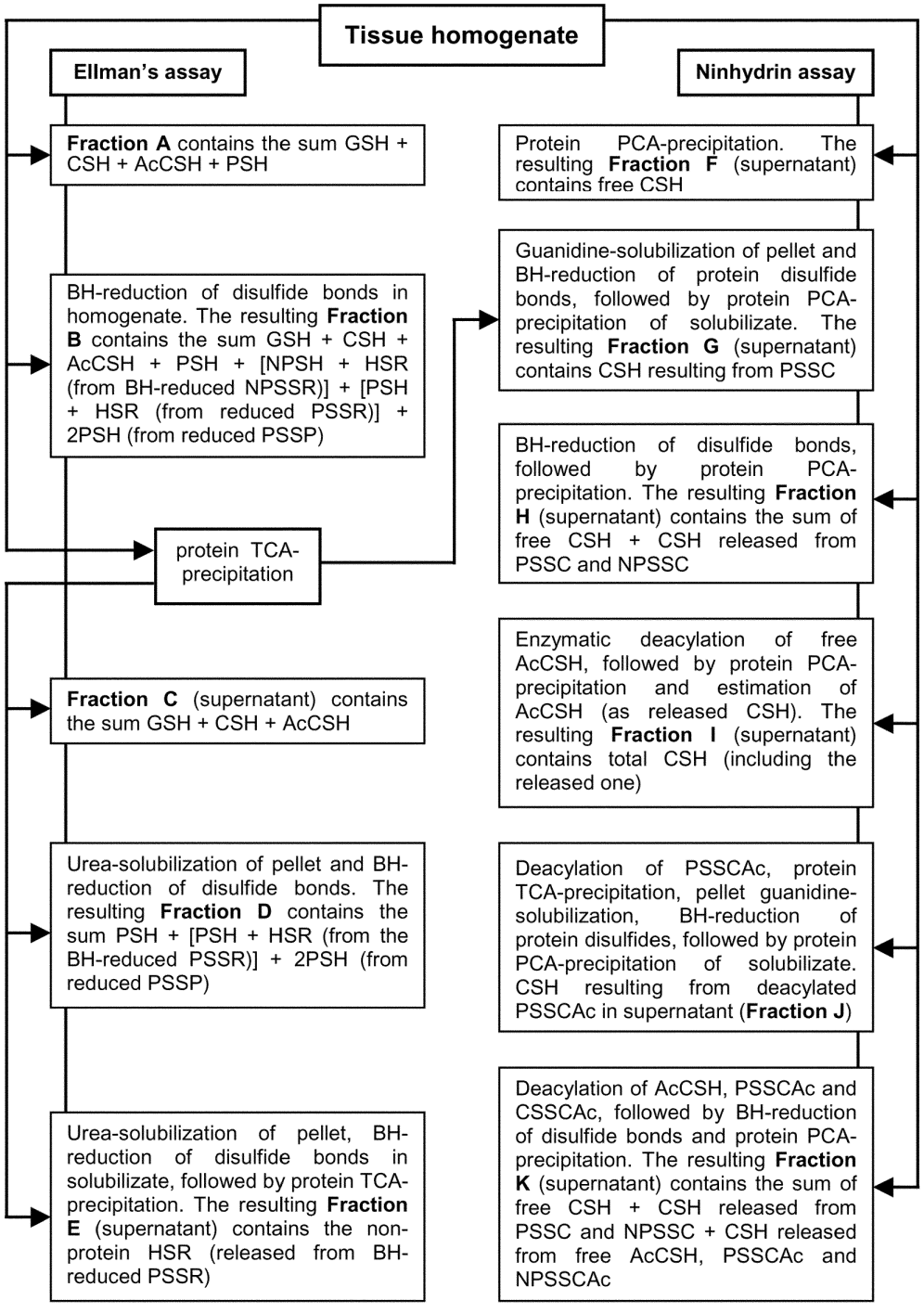

Fig. 1 Tissue fractionation flow chart with the methods for assaying the thiol redox components of the resulting fractions, as explained in detail in the "Materials and methods"

sured in various organs (heart, kidney, liver, and brain) of a 3-, 6-, and 12-month-old mouse, type BalbC (from Theagenion Anticancer Hospital of Thessaloniki). In addition, thiol redox state was measured in the same organs of a 6-month-old mouse 6 h after intraperitoneal injection of 7 mmol kg^{-1} body weight cysteine [21] or AcCSH [27].

Initial tissue treatment

Fungi were harvested from growth medium by centrifugation at 6,000 g, and the resulting pellets were washed (twice) in 2–3 volumes ice-cold phosphate–EDTA buffer [10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA and 1 mM of the antioxidant BHA (in final 0.15% ethanol)]. Similarly, mouse tissue was gently washed in the same buffer. Fungal pellets/mouse tissue were ground in a porcelain mortar in liquid nitrogen to prevent artificial oxidation of sulfhydryl groups during homogenization. The resulting sample powder was mixed with phosphate–EDTA buffer (5 volumes/sample wet wt) and the mixture was further homogenized by sonication on ice for 1 min using a sonicator UP-50 H (Dr Hielscher GmbH, Teltow, Germany) set at 350 W cm^{-2}. The sonicated homogenate was adjusted (with phosphate–EDTA buffer) to contain approximately 2 and 20–40 mg protein mL^{-1} for fungal and mouse tissue, respectively, and was subsequently used for the assays of the thiol redox state method as well as for other complementary assays.

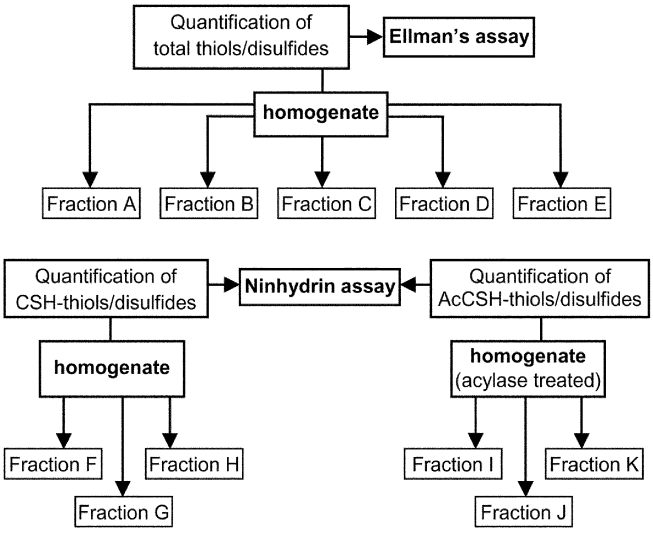

Fig. 2 Flow chart of fractions treated with Ellman's and ninhydrin assays. Detailed assay procedures for each fraction are described in the "Materials and methods" and in Fig. 1

Thiol redox state methodology

The essential elements of the methodology are (a) the strict sequence of tissue homogenate fractionation steps and their treatments, (b) spectroscopic assays, yielding raw data, and (c) the mathematical analysis of the raw data by which the concentrations of the individual components of the thiol redox state are estimated. A detailed description of the thiol redox state components measured in each fractionation step and the applied assays are shown in Figs. 1 and 2. In general, free thiols at any given state of the procedure are measured by Ellman's assay [18], the CSH component is measured by its unique response in the ninhydrin assay [23], AcCSH is selectively measured with ninhydrin after enzymatic deacylation [23], proteins are separated from non-protein thiols/disulfides by precipitation with trichloroacetic or perchloric acid, disulfides are reduced into free thiols with borohydride [6, 28], mixed disulfides between a protein and a non-protein component are measured by extracting the non-protein thiol from the protein pellet after borohydride reduction, and protein thiols/disulfides are measured after resolubilization of the protein pellet. Both Ellman's and ninhydrin assays were improved by facilitating access of the corresponding assay reagents to protein sulfhydryl/disulfide groups after protein denaturation by urea and guanidine, respectively. In addition, the ninhydrin assay was modified to improve its specificity for CSH.

Ellman's assay

This assay was applied to fractions A–E and requires approximately 10 mg total protein fungal/mouse tissue homogenate. For assaying fractions A–D, the homogenate was appropriately diluted (with phosphate-EDTA buffer) to contain approximately 2 mg protein mL^{-1}. For assaying fraction E, 5–8 mg total protein mouse/fungal homogenate was used. All fractions were assayed in at least three serial dilutions chosen to result in proportional thiol values. This would assure maximum solubilizing and reducing effectiveness of the urea and BH concentrations, respectively, used in the assay treatment of fungal/mouse tissue homogenates and protein precipitates. For the serial dilutions of fractions A, B, D, and E, cold phosphate–EDTA buffer was used; 5% TCA was used for fraction C. Fractionation procedures of tissue fractions A–E and their individual assays (Figs. 1 and 2) are described below.

Fraction A

A 0.1-mL aliquot of serially diluted fungal/mouse tissue homogenate (initially diluted to contain approximately 2 mg protein mL^{-1} stock) was added to a microcentrifuge tube, followed by 0.1 mL 0.3 M Tris-HCl, pH 8.2, 0.5 mL 10.2 M urea (containing 0.1 M Tris-HCl, 0.1 M glycine, 0.6 mM EDTA, pH 8.6), and 0.28 mL 100% methanol. The mixture was briefly sonicated (10 s). To that was added 0.02 mL 5 mM DTNB (in 100% methanol), and the mixture was incubated at room temperature (RT) for 30 min, followed by centrifugation for 5 min at 17,000 g. The absorbance at 412 nm was then measured against a corresponding sample blank (DTNB was substituted with methanol) and a reagent blank (homogenate was substituted with 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA).

Fraction B

Tris-HCl (0.015 mL 0.43 M, containing 0.46 M glycine, 2.7 mM EDTA, pH 8.6) and 0.2 mL 10.2 M urea (containing 0.1 M Tris-HCl, 0.1 M glycine, 0.6 mM EDTA, pH 8.6) were added to 0.085 mL of serially diluted fungal/mouse tissue homogenate (2 mg protein mL^{-1} stock). The mixture was placed in 7×1-cm glass tubes (minimum size used to account for foaming of sample due to subsequent treatment with BH) and solubilization was facilitated by brief sonication. Sample disulfide bonds were chemically reduced by the addition of 0.15 mL 1% (w/v) BH (prepared right before use in deionized distilled water), followed by incubation in a 40°C water bath for 40 min with intermittent vortexing. After reduction, excess BH was destroyed by the addition of 0.09 mL 1 N HCl and 0.3 mL acetone [6], followed by vigorous vortexing. Then, the pH of the resulting mixture was adjusted to 8.0 by the addition of 0.3 mL 1 M Tris-HCl in 1.3 mM EDTA, pH 8.5 for maximum color development after addition of 0.06 mL 5 mM DTNB and incubation at RT for 30 min. The mixture was transferred to a microcentrifuge tube, centrifuged for 5 min at 17,000 g, and absorbance of supernatant was measured at 412 nm against a corresponding sample blank (DTNB was substituted with methanol) and a reagent blank (homogenate was substituted with 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA).

Fraction C

A 0.2-mL aliquot of serially diluted fungal/mouse tissue homogenate (2 mg protein mL^{-1} stock) and 0.16 mL 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, were added to a microcentrifuge tube, and the mixture was brought to 5% (w/v) TCA by the addition of 0.04 mL 50% TCA stock. The mixture was then incubated on ice for 10 min, and the proteins were precipitated by centrifugation for 5 min at 17,000 g. Subsequently, 0.345 mL of the supernatant (appropriately diluted with 5% TCA) was transferred to a microcentrifuge tube and mixed with 0.69 mL 0.4 M Tris-HCl, pH 8.9, and 0.033 mL 5 mM DTNB. The resulting mixture was incubated at RT for 30 min and its absorbance was measured at 412 nm against a corresponding sample blank (DTNB was substituted with methanol) and a reagent blank (supernatant was substituted with 5% TCA).

Fraction D

A 0.1-mL aliquot of serially diluted fungal/mouse tissue homogenate (2 mg protein mL^{-1} stock) and 80 µL 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, were added to a microcentrifuge tube, and the mixture was brought to 5% (w/v) TCA by the addition of 20 µL 50% TCA. The mixture was then incubated on ice for 10 min, and the proteins were precipitated by centrifugation for 5 min at 17,000 g. The protein precipitate was washed with 1 mL 10% TCA, and the pellet was urea-solubilized by the addition of 42 µL 10 mM phosphate buffer, pH 7.2 (containing 1 mM EDTA),

8 µL 0.43 M Tris-HCl (containing 0.46 M glycine, 2.7 mM EDTA, pH 8.6), and 0.1 mL 10.2 M urea (containing 0.1 M Tris-HCl, 0.1 M glycine, 0.6 mM EDTA, pH 8.6), followed by brief sonication. The solubilizate was then transferred to 7×1-cm glass tubes, and the disulfide bonds were reduced by the addition of 75 µL 1% (w/v) BH (prepared right before use), followed by incubation in a 40°C water bath for 40 min (with intermittent vortexing) and by mixing with 45 µL 1 N HCl and 0.15 mL acetone. The pH of the resulting mixture was adjusted to 8.0 with 0.15 mL 1 M Tris-HCl (in 1.3 mM EDTA, pH 8.5) and to that was added 30 µL 5 mM DTNB. The mixture was then incubated at RT for 30 min, transferred to another microcentrifuge tube, and centrifuged for 5 min at 17,000 g. The absorbance of supernatant was measured at 412 nm against a corresponding sample precipitate blank (DTNB was substituted with methanol) and a reagent blank (homogenate was substituted with 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA).

Fraction E

A 0.1-mL aliquot of undiluted mouse tissue homogenate (20–40 mg protein mL^{-1}) and 80 µL 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, were added to a microcentrifuge tube, and the mixture was brought to 5% (w/v) TCA by the addition of 20 µL 50% TCA. The mixture was then incubated on ice for 10 min, and the proteins were precipitated by centrifugation for 5 min at 17,000 g. For assaying fungal tissue, 1 mL undiluted fungal tissue homogenate (2 mg protein mL^{-1}) was TCA-precipitated by mixing with 110 µL 50% TCA. The protein precipitate was washed with 1 mL 10% TCA, and the pellet was urea-solubilized by the addition of 42 µL 10 mM phosphate buffer, pH 7.2 (containing 1 mM EDTA), 8 µL 0.43 M Tris-HCl (containing 0.46 M glycine, 2.7 mM EDTA, pH 8.6), and 0.1 mL 10.2 M urea (containing 0.1 M Tris-HCl, 0.1 M glycine, 0.6 mM EDTA, pH 8.6), followed by brief sonication. The solubilizate was then transferred to 7×1-cm glass tubes, and the disulfide bonds were reduced by the addition of 75 µL 1% (w/v) BH (prepared right before use), followed by incubation in a 40°C water bath for 40 min (with intermittent vortexing). The resulting solubilizate was 15% TCA-precipitated (by addition of 55 µL 75% TCA) after 10 min incubation on ice and centrifugation at 17,000 g for 5 min. The resulting supernatant was mixed with 45 µL 1 N HCl and 0.15 mL acetone and its pH was adjusted to 8.0 by addition of 0.2 mL 2.5 M Tris-HCl, pH 11.2. Finally, 35 µL 5 mM DTNB was added to this mixture, and the absorbance at 412 nm was measured as in fraction D.

Absorbance values of all fractions were recorded with a UV-Visible Shimadzu spectrophotometer model UV-1200 (Shimadzu Co, Kyoto, Japan) and were converted to µmol SH groups (GSH equivalents) g^{-1} total tissue protein or wet wt by appropriate standard curves of pure GSH versus absorbance at 412 nm. These values were assigned with the same letters assigned to the corresponding fractions to be used for the derivation of mathematical equations for the determination of the individual thiols of the thiol redox state. Standard curves of pure GSH (0–20 µM) were used for fractions A, C, and E, treated according to the procedures used to assay these fractions. A single standard curve of GSH (0–40 µM) constructed following the procedure for assaying fraction B was used for fractions B and D. All standard curves were identical within 0–20 µM GSH concentration range (data not shown) and for simplicity reasons absorbances from fractions A–E were converted to concentrations of thiol groups by the standard curve for fraction A.

For the Ellman's assay the following controls were run: external controls were used to determine the effectiveness of disulfide bond splitting in protein and non-protein disulfides by BH in the presence of urea, and internal controls to determine the recovery of assaying protein and non-protein thiols and disulfides. Recovery in assaying GSH, CSH, and PSH was 100% and was determined after running internal controls with proportional known quantities of GSH and CSH added to fractions A and C, and known quantities of BSA added to fraction A. BSA contains one free SH group per protein molecule, and one mol BSA-SH was determined by the Ellman's assay to correspond to 0.35 mol GSH equivalents. This value is in agreement with data reported elsewhere [29].

A factor k was introduced to express the decimal percentage of effectiveness in reducing disulfide bonds. This factor was found to be 0.2 for reducing both protein and non-protein disulfide bonds, and was calculated after running external controls with known quantities of GSSG and CSSC (for non-protein disulfide bonds) and BSA (for protein disulfide bonds). GSSG and CSSC were added to fraction B, and BSA was added to fractions B and D. BSA was used since it contains 17 disulfide bonds [29]. Therefore, the effectiveness of splitting disulfide bonds in PSSP contained in fraction B was also 0.2, and was assumed to be the same for PSSR and NPSSR, which are contained in the same fraction. For fraction D, where thiol proteins were TCA-precipitated/urea-solubilized/BH-reduced, another effectiveness protein disulfide reduction factor, l, was introduced and determined to be 0.15 (after running external control with BSA). Having determined factors k and l, the recovery of NPSSR, PSSR, and PSSP was determined by adding known amounts of CSSC and GSSG (for non-protein mixed disulfides) to fraction B, and BSA (for protein disulfides) to fractions B and D. Thus, recovery factors r and m were introduced for the non-protein mixed disulfides and protein disulfides, and were determined to be 0.75 and 0.6, respectively. In addition, effectiveness of protein precipitation in the urea-solubilizate leading to fractions D and E (Fig. 1) was tested with tissue protein or BSA samples of known concentration and was 100%. All other TCA–protein precipitation steps were also 100% effective.

Ninhydrin assay

Assays for the unique reaction of CSH with acid–ninhydrin under acidic pH [30] and for AcCSH after its enzymatic deacylation to CSH [23] were used. Mouse/fungal tissue homogenate was treated in order to estimate the components CSH, NPSSC, PSSC, AcCSH, NPSSCAc, and PSSCAc alone and in sums in certain fractions (Figs. 1 and 2), after appropriate mathematical analysis of the data.

Fraction F

CSH was determined in this fraction: mouse/fungal tissue homogenate sample (20/80 µL) was brought to 90 µL with phosphate–EDTA buffer and was protein-precipitated by the addition of 10 µL 4 M PCA, incubation for 10 min in a water–ice bath, and centrifugation at 17,000 g for 5 min. Supernatant was saved for further treatment.

Fraction G

PSSC was determined in this fraction: mouse/fungal tissue homogenate sample (100/1,000 µL) was protein-precipitated by the addition of 11/110 µL 50% TCA, incubation for 10 min in a water–ice bath, and centrifugation at 17,000 g for 5 min. Protein precipitate was washed with 1 mL 10% TCA and it was solubilized (by brief sonication on ice) in 20 µL phosphate–EDTA buffer mixed with 5 µL Tris–glycine buffer (0.43 M Tris-HCl, containing 0.46 M glycine, 2.7 mM EDTA, pH 8.6) and 45 µL 4.5 M guanidine-HCl (in Tris–glycine buffer) (urea interferes with the assay). PSSC in the solubilizate was reduced by the addition of 30 µL 1% (w/v) BH (prepared right before use in phosphate–EDTA buffer), followed by incubation for 40 min in a water bath set at 40°C. The resulting BH-reduced solubilizate (100 µL) was protein-precipitated by the addition of 5 µL 70% (w/w) PCA, incubation for 10 min in a water–ice bath, and centrifugation at 17,000 g for 5 min. Supernatant was saved for further treatment.

Fraction H

The component sum CSH+PSSC+NPSSC was determined in this fraction: mouse/fungal tissue homogenate sample (15/70 µL) was brought to 80 µL with phosphate–EDTA buffer and to that was

added 10 µL 5.2% BH, followed by incubation at 40°C for 30 min. The resulting mixture was protein-precipitated by the addition of 10 µL 4 M PCA and the resulting supernatant was saved for further treatment.

Fraction I

In this fraction AcCSH was estimated: 20/80 µL mouse/fungal tissue homogenate was brought to 80 µL with phosphate–EDTA buffer and mixed with 10 µL acylase solution (5,000 enzyme units mL^{-1}, freshly prepared in phosphate–EDTA buffer). The mixture was incubated for 30 min at RT and was protein-precipitated by the addition of 10 µL 4 M PCA. Supernatant was saved for further treatment.

Fraction J

PSSCAc was estimated in this fraction: mouse/fungal tissue homogenate sample (100/1,000 µL) was mixed with 11/110 µL acylase solution. The mixture was incubated for 30 min at RT and was protein-precipitated by the addition of 12/120 µL 50% TCA. The precipitate was then treated (solubilized, BH-reduced, and protein-reprecipitated) as in fraction G and the resulting supernatant was saved for further treatment.

Fraction K

The component sum AcCSH+PSSCAc+NPSSCAc was estimated in this fraction: 15/70 µL mouse/fungal tissue homogenate was brought to 70 µL with phosphate–EDTA buffer and mixed with 10 µL acylase solution, the mixture was incubated for 30 min at RT and to that was added 10 µL 5.2% BH (prepared right before use), followed by incubation at 40°C for 30 min. The resulting mixture was protein-precipitated by the addition of 10 µL 4 M PCA and the resulting supernatant was saved for further treatment.

Ninhydrin assay procedure

The above resulting supernatants (65 µL) were treated with ninhydrin reagent as follows: supernatant sample (65 µL) was mixed with 65 µL glacial acetic acid and 65 µL freshly prepared acid–ninhydrin reagent (125 mg ninhydrin mixed with 3 mL glacial acetic acid and 2 mL concentrated HCl, and stirred slowly for 20 min). The resulting mixture was heated in a boiling water bath for 10 min and cooled to RT and was mixed with 435 µL 95% ethanol by vigorous vortexing. Absorbance intensity of the formed chromophore (quite stable for 20 min) was measured against reagent blank (omitting supernatant sample and replacing it with an equal volume of phosphate–EDTA buffer) after taking into account possible interference of unknown substances in the sample.

Sample interference with the ninhydrin assay

The chromophore formed due to the reaction of pure CSH with ninhydrin is pink, forming a major peak of absorbance at 560 nm with a shoulder between 522 and 534 nm and zeroing at 600 nm, the closest to the peak wavelength point in the horizontal baseline (Fig. 3A). This spectrum was also obtained with samples from *S. rolfsii* and mice at all experimental growth conditions, as well as with samples of yeast at the early log phase. This spectrum changed in samples from yeast at later growth stages. The peak at 560 nm decreased (forming an orange pink color) in samples at the mid log phase and disappeared (forming a brownish color) in samples at the late log phase (Fig. 3B), followed by an increase of absorbance of the spectrum portion between 522 and 400 nm and increase of the baseline slope (zero with pure CSH). When a certain amount of pure CSH was added to yeast sample from the late log phase a spectrum similar to that from the mid log yeast sample was obtained (Fig. 3C). Furthermore, its baseline slope was not changed and its $A_{560-600\,nm}$ after correction of the shifted baseline was identical to the $A_{560-600\,nm}$ of the spectrum obtained by equal amount of pure CSH (Fig. 3A). Based on these observations, the following mathematical equation was derived for correcting $A_{560\,nm}$ of endogenous CSH by subtracting from it the absorbance at 560 nm of the baseline due to the interfering substances. The latter was calculated by measuring the slope of the interfering baseline from absorbance values at 600 and 650 nm.

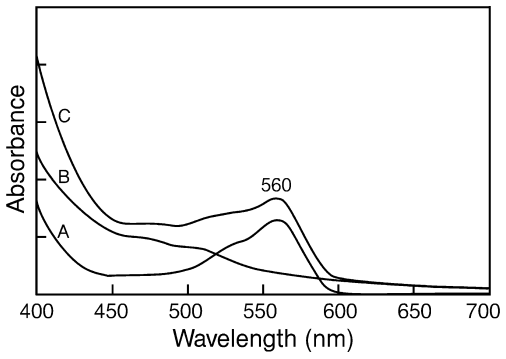

Fig. 3A–C Interference in the CSH-ninhydrin assay. **A** Spectrum of CSH-ninhydrin chromophore (using 20 nmol pure CSH). **B** Spectrum of chromophores formed by ninhydrin reaction with interfering substances in yeast (late log phase). **C** Spectrum of mixture of interfering substances and 20 nmol pure CSH

$$A_{c,560-600\,nm} = A_{m,560\,nm} - \left[A_{m,600\,nm} + 0.8\left(A_{m,600\,nm} - A_{m,650\,nm}\right)\right]$$

where subscripts c and m designate corrected and measured absorbance, respectively.

Raw data analysis

Data from Ellman's assay

Values *A*, *B*, *C*, *D*, and *E* (expressed in µmol glutathione-SH equivalents g^{-1} total tissue protein) designate various sums of certain thiol redox state components (each one also expressed in µmol glutathione-SH equivalents g^{-1} total tissue protein) found in the corresponding tissue homogenate fractions (see Fig. 1). Based on these sums and taking in account the disulfide bond splitting efficiency factors k=0.2 and l=0.15, and the recovery factors m=0.6 and r=0.75, the following equations are derived:

$$\mathrm{PSH} = A - C \tag{1}$$

$$\text{since } \mathrm{PSH} = (\mathrm{SH_{PSH}} + \mathrm{SH_{GSH+CSH+AcCSH}}) - \mathrm{SH_{GSH+CSH+AcCSH}} \text{ and GSH} + \mathrm{CSH} + \mathrm{AcCSH} = C \tag{2}$$

$$\mathrm{PSSR} = E/ml = E/0.09 \tag{3}$$

$$\text{since PSSR} = (\mathrm{PSSG} + \mathrm{PSSC} + \mathrm{PSSCAc}) = (\mathrm{SH_{GSH}} + \mathrm{SH_{CSH}} + \mathrm{SH_{AcCSH}}) \text{ and } E = lm(\mathrm{SH_{GSH}} + \mathrm{SH_{CSH}} + \mathrm{SH_{AcCSH}})$$

$$\mathrm{PSSP} = [D - m(A - C) - 2E]/2lm = [D - 0.6(A - C) - 2E]/0.18 \tag{4}$$

$$\text{since } D = m(A - C) + lm\mathrm{PSH_{PSSP}} + 2lm\mathrm{PSSR} \text{ [from Eqs. (1) and (3)]}$$

$$\mathrm{NPSSR} = [lmB - rlmA - rk[D - m(A - C)]]/2lmrk = [0.09B - 0.0675A - 0.15[D - 0.6(A - C)]]/0.027 \tag{5}$$

$$\text{since NPSSR} = (\mathrm{NPSSG} + \mathrm{GSSG} + \mathrm{NPSSC} + \mathrm{NPSSCAc}) \text{ and } B = rC + rk\mathrm{PSH_{PSSP}} + 2rk\mathrm{PSSR} + 2rk\mathrm{NPSSR}$$

PSH and PSSP are determined directly from Eqs. (1) and (4), PSSR and NPSSR from Eqs. (3) and (5), and GSH is determined mathematically after estimation of CSH and AcCSH by the following ninhydrin assay. NPSSC, NPSSCAc, PSSC, and PSSCAc are estimated by the same assay.

Data from ninhydrin assay

The ninhydrin assay determines directly whether the thiols CSH and AcCSH (after deacylation to CSH) exist in free form or are released from PSSC, PSSCAc, NPSSC, and NPSSCAc after BH reduction. Corrected absorbance values for supernatants resulted after no deacylation and deacylation of the samples were designated as Abs_1 and Abs_2, respectively, and were used as unknowns in a two-equation system, which was derived using known equimolar amounts of CSH and AcCSH. Derivation of this system of equations is necessary in experiments using AcCSH in order to account for the fact that when measuring CSH in mixture with AcCSH (before being deacylated), AcCSH interferes with the ninhydrin assay, since its absorption at 560 nm is approximately 10% that of equimolar CSH.

Specifically, for deriving the constants (P, Q, R, and W) of the following two-equation system [Eqs. (6) and (7)], four assays were performed: equimolar amounts (0–0.015 µmol in 65-µL sample volume) of CSH (assay 1) and AcCSH (assay 2) were assayed as in fraction F (in the absence of acylase) to derive the constants P and Q, respectively. Similarly, the same equimolar amounts of CSH (assay 3) and AcCSH (assay 4) were assayed (both in the presence of acylase) as in fraction I to derive constants R and W, respectively. The set of 2 equations (for n=0–0.015 µmol of equimolar CSH and AcCSH) is as follows:

$$\mu\text{mol CSH} = (n\text{Abs}_1/\text{P}_n) - (X\text{Q}_n/\text{P}_n) = 0.025\text{Abs}_1 - 0.1X \quad (6)$$

$$\mu\text{mol AcCSH} = X = [(n\text{Abs}_2/\text{W}_n) - (n\text{Abs}_1\text{R}_n/\text{P}_n\text{W}_n)]/ [1 - (\text{Q}_n\text{R}_n/\text{P}_n\text{W}_n)] = 0.028(\text{Abs}_2 - \text{Abs}_1) \quad (7)$$

Absorbances Abs_1 and Abs_2 (from assaying the resulting sample supernatants) are converted to µmol CSH and AcCSH by Eqs. (6) and (7), respectively. Specifically, Abs_1 and Abs_2 are converted to µmol free CSH and AcCSH (in sample supernatants from fractions F and I, respectively), µmol released CSH and AcCSH from PSSC and PSSCAc, respectively (in sample supernatants from fractions G and J), and µmol released CSH and AcCSH from thiol sums (CSH+PSSC+NPSSC) and (AcCSH+PSSCAc+NPSSCAc), respectively (in sample supernatants from fractions H and K).

In experiments where organisms are not administered with AcCSH, only fractions F, G, and H (Fig. 1) are assayed. In this case there is no need for derivation of the set of Eqs. (6) and (7), since µmol AcCSH=X=0. Instead, only Eq. (6) is used, which takes the following form (after substitution of the factor X with zero). This equation is actually converted to the equation of the standard curve of pure CSH:

$$\mu\text{mol CSH} = 0.025\text{Abs}_1$$

Equations (6) and (7) can be applied in fungal and mouse tissue as was shown by the 100% recovery of known concentrations of internal controls AcCSH and CSH (added separately or in certain proportions). These equations are used to calculate the CSH and ACSH content of the pairs of fractions F and I, G and J, and H and K (treated without and with acylase, respectively). The data derived from Eqs. (6) and (7) are mathematically manipulated as shown below to derive the final equations for CSH, AcCSH, PSSC, PSSCAc, NPSSC, and NPSSCAc.

Determination of CSH and AcCSH

These thiols are determined directly by Eqs. (6) and (7), and are expressed as µmol g^{-1} protein (designated as F and I for CSH and AcCSH, respectively):

$$\text{CSH} = F \quad (8)$$

$$\text{AcCSH} = I \quad (9)$$

Determination of PSSC and PSSCAc

These disulfides are also determined from Eqs. (6) and (7), and when converted to µmol g^{-1} protein are designated as G and J, respectively. PSSC and PSSCAc are estimated from Eqs. (10) and (11), taking into account the effectiveness (40%) of reduction of their disulfide bonds by BH (using as external control pure cystine).

$$\text{PSSC} = G/0.04 \quad (10)$$

$$\text{PSSCAc} = J/0.4 \quad (11)$$

Determination of NPSSC and NPSSCAc

NPSSC results after subtraction of CSH and PSSC from the sum (CSH+NPSSC+PSSC). This sum is designated as H and is expressed in µmol g^{-1} tissue protein. Similarly, NPSSCAc is estimated after subtraction of AcCSH and PSSCAc from the sum (AcCSH+NPSSCAc+PSSCAc), designated as K and also expressed in µmol g^{-1} tissue protein. Specifically, NPSSC is determined indirectly from the following general equation:

$$\mu\text{mol } 0.4\text{NPSSC} = \mu\text{mol sum } (\text{CSH} + 0.4\text{PSSC} + 0.4\text{NPSSC}) - \mu\text{mol CSH} - \mu\text{mol } 0.4\text{PSSC}$$

after taking in account (a) the effectiveness (40%) of BH reduction of NPSSC and PSSC disulfide bonds and (b) the recovery (100%) of CSH, NPSSC, and PSSC (using as external control pure cystine and internal control pure cysteine and cystine, respectively). NPSSCAc is similarly determined by the following general equation:

$$\mu\text{mol } 0.4\text{ NPSSCAc} = \mu\text{mol sum}(\text{AcCSH} + 0.4\text{ PSSCAc} + 0.4\text{ NPSSCAc}) - \mu\text{mol AcCSH} - \mu\text{mol } 0.4\text{ PSSCAc}$$

Finally, NPSSC and NPSSCAc are determined from Eqs. (12) and (13).

$$\text{NPSSC} = (H - F - G)/0.4 \quad (12)$$

$$\text{since } 0.4\text{ NPSSC} = H - F - G$$

$$\text{NPSSCAc} = (K - I - J)/0.4 \quad (13)$$

$$\text{since } 0.4\text{ NPSSCAc} = K - I - J$$

Estimation of the individual components of the thiol redox state

The following equations quantitate the individual components of the thiol redox state. They were derived by combination of the equation sets of the general thiol and ninhydrin assays. Equation thiol parameters with the minus and plus symbol in front, designate their omission and inclusion in corresponding experiments without and with use of AcCSH administration.

$$\text{NPSSR} = [0.09B - 0.0675A - 0.15[D - 0.6(A - C)]]/0.027$$
$$\text{NPSSC} = (H - F - G)/0.4$$
$$\text{PSSR} = E/0.09$$
$$\text{PSSC} = G/0.4$$
$$\text{PSH} = A - C$$
$$\text{PSSP} = [D - 0.6(A - C) - 2E]/0.18$$
$$\text{GSH} = C - F - I$$
$$\text{CSH} = F$$
$$\text{AcCSH} = I$$
$$\text{NPSSCAa} = (K - I - J)/0.4$$
$$\text{PSSCAc} = J/0.4$$

Sensitivity of the thiol redox state method

Thiol recovery and effectiveness factors (in BH-splitting protein/non-protein thiol disulfide bonds) that were determined in the individual assays of the thiol redox state method were used to establish its detection sensitivity limits for the individual thiol redox state components in fungal and mouse tissue.

Statistical treatment of raw data

Individual thiols (T) were calculated from the corresponding equations above, in which the mean values of *A* to *M* were applied. These equations were also used to estimate corresponding standard errors (SE_T) of individual thiols by applying to each of them the following general function of standard error, adapted from elsewhere [31], in which the corresponding SE of the mean values of *A* to *M* were applied:

$$SE_T = \pm\sqrt{\left\{\begin{array}{l}[(\partial T/\partial A)SE_A]^2 + [(\partial T/\partial B)SE_B]^2 \\ +[(\partial T/\partial C)SE_C]^2\ [(\partial T/\partial D)SE_D]^2 + \ldots\end{array}\right\}}$$

The final equations giving the corresponding SE of individual thiol values are the following:

$$SE_{NPSSR} = \pm\sqrt{(SE_A{}^2 + SE_B{}^2 + SE_C{}^2 + SE_D{}^2)}$$
$$SE_{NPSSC} = \pm\sqrt{(SE_F{}^2 + SE_G{}^2 + SE_H{}^2)}$$
$$SE_{PSSR} = \pm SE_E$$
$$SE_{PSSC} = \pm SE_G$$
$$SE_{PSH} = \pm\sqrt{(SE_A{}^2 + SE_C{}^2)}$$
$$SE_{PSSP} = \pm\sqrt{(SE_A{}^2 + SE_C{}^2 + SE_D{}^2 + SE_E{}^2)}$$
$$SE_{CSH} = \pm SE_F$$
$$SE_{AcCSH} = \pm SE_I$$
$$SE_{NPSSCAc} = \pm\sqrt{(SE_I{}^2 + SE_J{}^2 + SE_K{}^2)}$$
$$SE_{PSSCAc} = \pm SE_J$$

Other assays

For evaluating NPSSR as an indicator of high oxidative stress in comparison to GSSG, the latter was quantitated by an enzymatic assay [17]. These indicators were related with a common indicator of oxidative stress, lipid peroxidation [2], which was measured in the fungal organisms yeast and *S. rolfsii*. These organisms were selected for this test since they were grown under carbon-source-exhausting conditions expected to increase lipid peroxidation with fungal age. For evaluating the accuracy of the thiol redox state method in the determination of GSH, a fluorometric assay of similar sensitivity [4, 20, 25] and an enzymatic assay [32, 33] were used.

GSSG assay

Undiluted sample (0.5 mL) homogenates of fungal and mouse tissue were protein-precipitated by 5% TCA from a 50% TCA stock (prepared in 0.1 N HCl), after 10 min incubation in an ice–water bath followed by 5 min centrifugation at 17,000 g. Any remaining proteins were removed by five extractions with an equal volume of ice-cold ether. Reduced glutathione in the samples was prevented from interfering with the assay by complexing it with NEM [17]. Thus, samples were mixed with one-tenth the volume of 0.2 M NEM and were incubated for 1 h at 25°C. Unreacted NEM was removed by 10 extractions with 0.5 mL of ice-cold ether followed by rigorous shaking and water vacuum removal of traces of ether. The resulting supernatant was used for GSSG determination (if necessary, further diluted with 5% TCA in 0.01 N HCl).

Table 1 Recovery (R) of thiols determined by the thiol redox state method

	GSH[a]	R_{GSH} (%)	NPSSR[b]	R_{NPSSR} (%)	CSH[c]	R_{CSH} (%)	NPSSC[d]	R_{CSSC} (%)	AcCSH[e]	R_{AcCSH} (%)	PSSP[f]	R_{PSSP} (%)	PSH[g]	R_{PSH} (%)
Mouse kidney tissue	59.6 (10.4)	98.7	83.1 (11.05)	74.9	55.1 (5.12)	99.9	100.2 (1.23)	99.0	49.5 (0)	99.0	82.3 (37.2)	60.0	47.9 (44.3)	99.0
Fungal tissue	62.7 (13.0)	99.5	127.7 (70.0)	75.1	50 (0)	100	100 (0)	100	50 (0)	100	127.3 (11.1)	60.3	30.5 (26.6)	99.3

Thiol values in parentheses indicate µmol endogenous thiol g^{-1} total tissue protein (before addition of the stated amount of thiol). They were determined in a 3-month-old mouse and a 6-day-old fungal *S. rolfsii* colony
[a]µmol GSH g^{-1} total tissue protein after adding 50 µmol GSH g^{-1} total tissue protein
[b]µmol NPSSR g^{-1} total tissue protein after adding 100 µmol GSSG g^{-1} total tissue protein
[c]µmol CSH g^{-1} total tissue protein after adding 50 µmol CSH g^{-1} total tissue protein
[d]µmol NPSSC g^{-1} total tissue protein, assumed to have same recovery as CSSC. This was tested after adding 100 µmol CSSC g^{-1} total tissue protein
[e]µmol AcCSH g^{-1} total tissue protein after adding 50 µmol AcCSH g^{-1} total tissue protein
[f]µmol protein disulfide bonds g^{-1} total tissue protein after adding 200 µmol BSA disulfide bonds (or 11.7 µmol BSA) g^{-1} total tissue protein
[g]µmol protein-SH groups g^{-1} total tissue protein after adding 11.7 µmol BSA (or 11.7 µmol BSA-SH groups) g^{-1} total tissue protein. Free –SH groups in 11.7 µmol BSA were estimated to correspond to 4.1 µmol GSH equivalents, since only 35% of BSA–SH groups react with DTNB [29]

Table 2 Detection limits of the thiol redox state determination method

	NPSSR[b]	NPSSC[c]	PSSR[d]	PSSC[e]	PSH[f]	PSSP[g]	GSH	CSH	AcCSH	NPSSCAc[h]	PSSCAc[i]
nmol[a]	1	2.5	2	2.5	0.5	2	0.5	1	1	2.5	2.5
Mouse tissue (liver, heart, brain, and kidney)											
μmol g^{-1} protein	5	0.25	10	0.25	5	10	5	0.1	0.1	0.25	0.25
μmol g^{-1} wet wt	1.5	0.07	3	0.07	1.5	3	1.5	0.03	0.03	0.07	0.07
Fungal tissue (yeast and *S. rolfsii*)											
μmol g^{-1} protein	5	0.25	10	0.25	5	10	5	0.1	0.1	0.25	0.25
μmol g^{-1} wet wt	0.05	0.003	0.1	0.003	0.06	0.1	0.06	0.001	0.001	0.003	0.003
μmol g^{-1} dry wt	0.5	0.03	1	0.03	0.6	1	0.6	0.01	0.01	0.03	0.03

Minimum thiol concentrations were determined after running appropriate internal controls (see "Materials and methods") and taking into account corresponding recovery factors. Detection limits were determined in a 6-month-old mouse and a 6-day-old *S. rolfsii* colony and in yeast cells at mid log developmental growth stage

[a]nmol thiol per assay reaction final volume

[b]Expressed as contained GSH and determined using pure GSSG (quantitated non-enzymatically) as internal control

[c]Expressed as contained CSH and determined using pure CSSC as internal control

[d]Expressed as contained GSH and assuming same recovery as PSSP

[e]Expressed as contained CSH and assuming same recovery as CSSC

[f]PSH is expressed as in Table 1

[g]PSSP is expressed as in Table 1

[h]Expressed as contained AcCSH and assuming same detection limit as NPSSC

[i]Expressed as contained AcCSH and assuming same recovery as CSSC

The constituents of the assay mixture were added in the stated order as follows: 716 μL phosphate buffer (0.1 M sodium phosphate in 5 mM EDTA, pH 7.5), 24 μL 1 N NaOH, 100 μL sample, 100 μL 0.5 mM NADPH (in phosphate buffer) and 60 μL 10 mM DTNB (in phosphate buffer). The reaction was initiated by the addition of 1 unit GSSG reductase, and the reaction rate (absorbance change at 412 nm) was measured for 15 s. The reaction rate was corrected by running appropriate reagent (916 μL phosphate buffer+ 24 μL 1 N NaOH+60 μL 10 mM DTNB), sample (816 μL phosphate buffer+24 μL 1 N NaOH+100 μL 0.5 mM NADPH+60 μL 10 mM DTNB+1 unit GSSG reductase), and enzyme (716 μL phosphate buffer+24 μL 1 N NaOH+100 μL sample+100 μL 0.5 mM NADPH+ 60 μL 10 mM DTNB) blanks. The corrected reaction rate was converted to GSSG concentration from reaction rates corresponding to GSSG solutions of various known concentrations. Glutathione disulfide is expressed as μmol GSSG g^{-1} total tissue protein. Recovery of GSSG (100%) was determined by running internal controls with known quantities of pure GSSG added to homogenate (Table 1).

GSH assays

The following assays determined GSH in the homogenate. The fluorometric assay was performed according to a stated procedure [4, 20, 25], and fluorescence of the chromophore was recorded with a Shimadzu spectrofluorophotometer RF-1501 (Shimadzu Co, Kyoto, Japan). The enzymatic assay was based on the GSH-*S*-transferase reaction using CDNB as chromogenic reagent [32, 33].

Lipid peroxidation assay

It was assayed by a modified TBA-based method [34]. Specifically, 0.5 mL of homogenate from yeast and *S. rolfsii* was mixed with 0.5 mL TBA reagent [0.5% (w/v) TBA in 20% (w/v) TCA and 0.33 N HCl). To the resulting mixture was added 5 μL 2% (w/v) of the lipid antioxidant BHA (made in absolute ethanol) to prevent artificial lipid peroxidation during the assay. The mixture was incubated at 100°C for 15 min and brought to RT. To that was added 1 mL butanol-1, mixed by vigorous vortexing, centrifuged at 15,000 g for 3 min, and absorbance of the upper butanol layer was measured at 535 and 600 nm against butanol-treated sample and reagent blanks (0.5 mL sample plus 0.5 mL 20% TCA containing 0.33 N HCl and 0.02% w/v BHA, and 0.5 mL phosphate–EDTA buffer, plus 0.5 mL TBA reagent containing 0.02% w/v BHA, respectively). Absorbance difference $A_{535-600\,nm}$ was converted to malondialdehyde (MDA) equivalents using the extinction coefficient for MDA of $1.55\times10^5\,M^{-1}\,cm^{-1}$ [35]. Lipid peroxidation was expressed in nmol MDA mg^{-1} total protein.

Protein assay

Total protein concentration of the fungal and mouse homogenate was assayed by a modification of a CBB-G250-based method [36]. Homogenates were diluted (approximately 1:5 and 1:20 for fungal and mouse tissue, respectively) with phosphate–EDTA buffer, pH 7.2. To 81 μL of the homogenates were added 9 μL 12 N HCl and 10 μL 1% w/v Triton X-100, and the mixture was incubated at 100°C for 10 min. Then it was brought to room temperature, mixed with 0.9 mL 0.033% (w/v) CBB-G250 (in 0.5 N HCl) and incubated for 5 min (minimum time period for color development). Absorbance was measured at 620 nm against appropriate blanks (reagent and sample blanks) and was converted to protein concentration from a BSA (0–50 μg) standard curve.

Results and discussion

This study for the first time provides a methodology by which the main thiol and disulfide components (GSH, CSH, NPSSR, NPSSC, PSSR, PSSP, PSH, and PSSC) of a particular sample can explicitly be measured. This accomplishment is based upon the working scheme in Fig. 1, which represents a combination of different preparative steps, chemical treatments, and known spectrophotometric assays, properly modified to improve their specificity. Second, the method was also further extended to measure the disulfides NPSSC, PSSC, NPSSCAc and PSSCAc, PSSR, and NPSSR for the first time. Third, the method can be used to accurately determine PSSG (=PSSR–PSSC) and NPSSG (=NPSSR–NPSSC). An Excel program is available in the "Electronic Supplementary Material" for the automated calculation of the values of all thiol redox state components.

Thiol recovery and sensitivity of the method was determined by internal controls for both protein and non-protein thiols (Tables 1 and 2). The sensitivity of the method for the various thiols ranges from 0.5 to 2.5 nmol. Although the method is not specific for GSH, it determines this important thiol more accurately than other Ellman's reagent-based methods, since it discriminates GSH from CSH. Its accuracy for GSH in the tested organisms was similar to that measured by the enzymatic and fluorometric assays [4, 20, 25, 33]. The validity of the method was further verified by comparing the concentrations of GSH, PSSR, and PSSC with those previously reported in certain mouse organs (see data in the "Electronic Supplementary Material").

The method was also used in mouse and fungal tissue to investigate (a) the possible relationship between thiol redox state component patterns and oxidative-stress-associated aging, and (b) the possible variation of the traditional indicators GSH and GSSG upon CSH/AcCSH-mediated modulation of the overall thiol redox state (see data in the "Electronic Supplementary Material"). The measurement of the components of thiol redox state was accompanied by the quantification of lipid peroxidation, a common indicator of oxidative stress, in an attempt to identify non-protein disulfides, besides GSSG, that can serve as indicators of high oxidative stress. Hitherto ignored components of the redox state were found to be of more interest than the traditional indicators GSH and GSSG. Specifically, the data of this study clearly showed that these indicators are often insensitive even to pronounced changes in the overall redox state of biological tissue and that non-protein mixed disulfides are a much more critical indicator of oxidative stress. In particular, NPSSR and PSSP can be used as new indicators of high oxidative stress, since it was found that they were better related to lipid peroxidation than GSSG. Moreover, NPSSC and PSSC levels may be related with GSH and CSH levels by acting as a CSH pool to control the levels of free CSH and/or use it as precursor of GSH.

Conclusions

The method presented in this study is a valuable tool for simultaneously monitoring the redox state of interrelated thiol classes in microbes, animal and plant cells, tissues, and whole organisms under normal and abnormal metabolic conditions. Explicit measurements of all essential redox state components will be of great interest in the future, whether the measurements are done by this or by other more analytical methods that would also combine any kind of separation technique with mass spectrometry.

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