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Multiparametric protocol for the determination of thiol redox state in living matter

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ABSTRACT

Thiol redox state (TRS) evaluation is mostly restricted to the estimation of GSH and GSSG. However, these TRS parameters can estimate the GSSG/GSH potential, which might be useful for indicating abnormalities in redox metabolism. Nonetheless, evaluation of the multiparameric nature of TRS is required for a more accurate assessment of its physiological role. The present protocol extends the partial assessment of TRS by current methodologies. It measures 15 key parameters of TRS by two modular subprotocols: one for the glutathione (GSH)- and cysteine (CSH)-based nonprotein (NP) thiols/mixed disulfides (i.e., GSH, GSSG, GSSNP, CSH, CSSNP, NPSH, NPSSNP, NP_xSH_{NPSSNP}, NP_xSH_{NPSH}), and the other for their protein (P) thiols/mixed disulfides (i.e., PSH, PSSG, PSSC, PSSNP, PSSP, NP_xSH_{PSSNP}). The protocol eliminates autoxidation of GSH and CSH (and thus overestimation of GSSG and CSSNP). Its modularity allows the determination GSH and GSSG also by other published specific assays. The protocol uses three assays; two are based on the photometric reagents 4,4′-dithiopyridine (DTP) and ninhydrin (NHD), and the third on the fluorometric reagent *o*-phthaldialdehyde (OPT). The initial assays employing these reagents have been extensively modified and redesigned for increased specificity, sensitivity, and simplicity. TRS parameter values and their standard errors are estimated automatically by sets of Excel-adapted algebraic equations. Protocol sensitivity for NPSH, PSH, NPSSNP, PSSP, PSSNP, CSH, CSSNP, PSSC, NP_xSH_{NPSSNP}, and NP_xSH_{NPSH} is 1 nmol –SH/CSH, for GSSNP 0.2 nmol, for GSH and GSSG 0.4 nmol, and for PSSG 0.6 nmol. The protocol was applied on human plasma, a sample of high clinical value, and can be also applied in any organism.

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Introduction

Evaluating cellular thiol redox state (TRS) is very important in a wide variety of biological processes [1,2]. TRS comprises many protein (P) and nonprotein (NP) thiol parameters such as glutathione (GSH), cysteine (CSH), and reduced NP and P thiols (NPSH and PSH, respectively), as well as their symmetric and mixed disulfides. The commonly used indicators for the assessment of TRS are restricted to GSH and its symmetric oxidized disulfide (GSSG). The role of GSH in intracellular redox state signaling, enzymic antioxidant defense, and in response to environmental factors is well established [3,4]. Additionally, GSH regulation is related to many physiological processes, in which CSH and PSH and their mixed and symmetric protein disulfides (PSSNP and PSSP, respectively) are also involved [5]. Glutathione disulfide (GSSG) can activate many enzymes (e.g., glucose-6-phosphatase and acid phosphatase) and inhibit others (e.g., glycogen synthetase, pyruvate kinase, adenylate cyclase, phosphorylase/phosphatase, and ribonucleotide reductase) [4].

Aerobic organisms need a system to restore key sulfhydryl groups to their reduced state after exposure to oxidant stress. Without a process to reduce protein disulfides, vulnerable cysteinyl residues of essential enzymes might remain oxidized, leading to changes in catalytic activity. This function is fulfilled by the thiol-disulfide exchange catalyzed by thiol transferases in the

Abbreviations: TRS, thiol redox state; GSH, glutathione; GSSG, oxidized glutathione; GSSNP, mixed disulfides of glutathione with nonprotein (NP) thiols; CSH, cysteine; CSSNP, mixed disulfides of cysteine with nonprotein (NP) thiols; NPSH, reduced nonprotein (NP) thiols; NPSSNP, mixed disulfides of nonprotein thiols (NPS); NP_xSH_{NPSSNP}, the NP thiol component of NPSSNP, excluding the NPSH components GSH and CSH; NP_xSH_{NPSH}, the NP thiol component of NPSH, excluding the NPSH components GSH and CSH; PSH, reduced protein (P) thiols; PSSG, mixed disulfides of glutathione (GS) with protein thiols (PS); PSSC, mixed disulfides of cysteine (CS) with protein (PS) thiols; PSSP, mixed disulfides of protein thiols (PS); PSSNP, mixed disulfides of nonprotein (NP) with protein (PS) thiols; NP_xSH_{PSSNP}, the NP thiol component of PSSNP, excluding the NPSH components GSH and CSH; Note: abbreviations of the reagents used in the present study are listed in the section of Materials under "Reagents."

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presence of GSH. Many proteins are activated or inhibited in vitro by the disulfide exchange between the protein and the GSH or other small-molecule disulfides (R′SH + RSSR ↔ R′SSR + RSH). Whereas many proteins are active when the key sulfhydryls are in the thiol form, others require them to be in the oxidized, disulfide form. Because the thiol-transferase reaction is bidirectional, the equilibrium is determined by the redox state of the cell. Moreover, cells avoid CSH autooxidation to CSSC, producing potentially toxic oxygen radicals by storing most of the nonprotein CSH as GSH [4].

The role of NP thiols other than GSH and GSSG in mixed disulfides (NPSSNP), such as coenzyme A (CoASH), in the TRS of cells is usually underestimated, although they have significant contribution to the physiological consequences of oxidative stress [5,6]. Such examples are the increase of CoASSG mixed disulfide levels during hydroperoxide metabolism [7], its inhibitory effect on enzymes such as GSSG reductase (GR), phosphofructokinase and fatty acid synthase, and its activating action on fructose 16-bisphosphatase [4].

PSH, PSSP, and PSSNP are also important indicators of oxidative stress. Although PSSNP are often designated in the literature as PSSG [8] due to GSH contribution (glutathionylation), this designation is limiting since they may also contain CSH (designated as PSSC; cysteinylation)—a particular form of protein S-thiolation is cysteyl-glycylation [9]—and to a lesser degree other minor NP thiols (NP_xSH_{PSSNP}, e.g., CoASH). Interdisulfide bridges between different protein thiols as well as intradisulfide bridges within proteins undergoing oxidative tertiary structure modification can produce symmetric disulfides (PSSP) [10]. These can be significant thiol redox markers since they are involved in inactivation of enzymes, transporters, and transcriptional factors during oxidative stress conditions [10], and also in pathological processes associated with oxidative stress in animal and plant cells under stress [10–13].

Protein thiols/disulfides can be extracellular and part of membrane and subcellular structures, contributing to protein stability [14] and the regulation of redox homeostasis [1]. Of special metabolic interest is the involvement of the ROS-generated protein cysteine sulfenic acid in the formation of mixed GSH disulfides via thiol-disulfide exchange reactions [15–18]. Redox-dependent signaling events involving the posttranslational oxidative modification of proteins have now been accepted as an important regulatory process, controlled by a number of redox-dependent modifications of some protein cysteinyl thiols, including an interchange between the reduced thiol and several different oxidized disulfide states [19]. It has been proposed that catalytically important sulfhydryl groups in PSH are protected from oxidative stress by reacting reversibly with GSH to form PSSG [20]. PSSG can inactivate enzymes (e.g., fructose-1,6-biphosphate aldolase) and make proteins more or less susceptible to proteolysis [21]. Also, specific oxidation/reduction of particular protein thiols may represent an important event in cellular and oxidative signaling cascades [22,23]. In addition, protein thiols could react with nitric oxide radical to form *S*-nitrosothiols, which are involved in signal transduction and posttranslational protein modification [24]. All these render the simultaneous quantitative assessment of thiols in organisms very important, since cellular TRS is associated with normal as well as abnormal metabolic processes.

TRS evaluation: Methodologies and limitations

TRS is normally evaluated by the total cellular thiol content [25] or by GSH and GSSG [8,26,27]. GSH is estimated by photometric assays based on Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, or DTNB) [28], which are not GSH specific since they do not discriminate it from CSH [29]. GSSG is usually determined by enzymatic assays [8,30,31], while GSH has been also evaluated more specifically by HPLC after derivatization with *N*-ethylmaleimide (NEM) [30]. GSH and GSSG are also evaluated by the *o*-phthaldialdehyde (OPT)-based fluorometric assay [32]. However, if proteins in the sample are not effectively removed (e.g., by precipitation) they could cause interference because OPT, as with GSH, can bind covalently with closely spaced (~3 Å) sulfhydryl and ε-amino groups of cysteine and lysine residues [33,34]. Adopting this assay in the present protocol (see next section), we modified it to eliminate such protein interference and increase its specificity and sensitivity. Similarly, PSSG is underestimated by photometric assays because of oxidation of the measured GSH during the procedure [35,36]. There is also lack of assays for the simultaneous determination of CSH, nonprotein oxidized disulfides of CSH (cystine, CSSC, CSH-GSH disulfide, CSSG), and PSSC. The available methodologies, mainly HPLC based, measure the CSH component of the P/NP-CSH disulfides (via derivatized thiol detection by LC-MS after tri-n-butylphosphine, TBP, reduction [37], or by LC fluorescence following TBP [38] and tris(2-carboxyethyl)phosphine, TCEP [39], disulfide reduction).

Concerning protein thiols, there are many methods available for measuring changes in the reduced protein thiol status of cells and tissues [19]. In general, these methods make use of chemicals that react with thiol groups with a variety of tags or labels, which allow the extent of incorporation to be measured. There are chemical methods involving thiol derivatization by thiol-labeling agents based on maleimide, iodoacetamide, iodoacetate, and thiosulfates. These agents may take the form of an affinity label such as biotin, a fluorophore, a radionucleotide, or a label that changes the molecular weight of the protein. Other protein thiol-labeling agents include alkyl halides, arylating agents, thiosulfates, and disulfide compounds (such as DTNB). Antibodies to GSH have been used to investigate the thiol oxidation state of samples in enzyme-linked immunosorbent assays (ELISA). Moreover, cells can be metabolically labeled by incubation with [^{35}S]cysteine, and can then undergo a variety of treatments, and S-thiolation (normally interpreted as S-glutathiolation) can be measured [19].

Normally, the PSH component of PSSNP, PSSG, and PSSC has been determined by Ellman's reagent-based assays [40,41], which do not discriminate the NP thiol component of PSSG, PSSC, and PSSNP [41]. Other methodologies quantify protein thiols without correlating the PS thiol component of PSSG, PSSC, PSSNP, and PSSP to its corresponding NP thiol component, which they do not measure [42]. However, simultaneous determination of incomplete sets of P- and NP-TRS parameters based on GSH and CSH has been performed by GC-MS [37], laser-induced fluorescence capillary electrophoresis [43], and HPLC methodologies [38,39,42,44–46], as well as by fluorometric and photometric assays [47–52]. However, in a case where plasma total thiols were determined after reduction with TCEP, the involved HPLC methodology [39] does not discriminate P from NP thiols (e.g., CSH and GSH resulting from the reduction of PSSC and PSSG, respectively).

Advantages of a multiparametric TRS protocol

Restricting TRS evaluation to the estimation of GSH and GSSG can offer a calculation of the GSSG/GSH potential, which might be useful at best as an analytical tool to disclose disturbances in redox metabolism [53]. Even so, a new technique of noninvasively measuring thiol redox potentials has shown that the GSH/GSSG ratio is 50,000 rather than about 100 in the cytosol [54], and that the cytosolic GSSG concentration is much more tightly regulated than expected (GSSG is rapidly transported into the vacuole by the ABC-C transporter Ycf1 in yeast), providing also a mechanistic

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