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Determination of glutaredoxin enzyme activity and protein S-glutathionylation using fluorescent eosin-glutathione

Lucia Coppo [a, **, 1], Sergio J. Montano [a, 1], Alicia C. Padilla [b], Arne Holmgren [a, *]

[a] Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-17177, Stockholm, Sweden
[b] Department of Biochemistry and Molecular Biology, Campus de Rabanales, University of Córdoba, 14071, Córdoba, Spain

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ABSTRACT

Glutaredoxins catalyze glutathione-dependent disulfide oxidoreductions, particularly reduction of glutathione (GSH)-protein mixed disulfides. Mammalian glutaredoxins are present in the cytosol/nucleus as Grx1 or in mitochondria as Grx2a. Here we describe di-eosin-glutathione disulfide (Di-E-GSSG) as a new tool to study glutaredoxin (Grx) activity. Di-E-GSSG has almost no fluorescence in its disulfide form due to self-quenching, whereas the reduced form (E-GSH) has a large fluorescence emission at 545 nm after excitation at 520 nm. Di-E-GSSG was a very poor substrate for glutathione reductase, but we discovered that the molecule was an excellent substrate for glutaredoxin in a coupled assay system with GSH, nicotinamide adenine dinucleotide phosphate (NADPH), and glutathione reductase or with lipoamide, NADH, and lipoamide dehydrogenase. In addition, Di-E-GSSG was used to glutathionylate the free SH group of bovine serum albumin (BSA), yielding eosin-glutathionylated BSA (E-GS-BSA) readily observed in ultraviolet (UV) light. E-GS-BSA also displayed a quenched fluorescence, and its Grx-catalyzed reduction could be followed by the formation of E-GSH by fluorescence emission using microtiter plates. This way of measuring Grx activity provided an ultrasensitive method that detected Grx1 and Grx2 at picomolar levels. Human Grx1 was readily quantified in 40 μl of plasma and determined to be 680 ± 208 pM in healthy controls.

Glutaredoxin (Grx) was discovered as a glutathione (GSH)-dependent hydrogen donor for ribonucleotide reductase in *Escherichia coli* and is present in all organisms with GSH [1]. Glutaredoxins are a family of GSH-disulfide oxidoreductases that have a thioredoxin fold, a GSH-binding site, and the characteristic active site motif CXXC/S [2–4].

Protein glutathionylation is a regulatory mechanism with an increasing recognized importance, and the pivotal role of Grx in catalyzing deglutathionylation is well established in the literature [5].

The Grx system—nicotinamide adenine dinucleotide phosphate (NADPH), yeast glutathione reductase (GR), GSH, and Grx—plays important roles in many cellular mechanisms such as electron transport to ribonucleotide reductase [6], apoptosis regulation [7–9], cellular differentiation [10,11], and defense against oxidative stress [12–16]. For example, Grx1 enrichment in dopaminergic neurons attenuated the cell toxicity of paraquat, an environmental Parkinsonian toxin [17]. The best characterized glutaredoxins in mammalian cells are Grx1 [18] and Grx2 [19,20].

Grx1 is predominantly present in the cytoplasm but has been found in the nucleus [21,22], whereas different isoforms of Grx2 are localized in the mitochondria and in the nucleus [19]. Grx1, but not Grx2, has been detected in human plasma by Western blot under physiological and pathological conditions [23–25]. Grx1 is capable of reducing glutathione peroxidase 3 residing in plasma [26]. However, the secretion mechanism and function of extracellular Grx1 is still unknown.

Established ways to determine Grx activity consist in following the oxidation of NADPH or nicotinamide adenine dinucleotide

Abbreviations: Grx, glutaredoxin; GSH, glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; GR, yeast glutathione reductase; NADH, nicotinamide adenine dinucleotide; HED, hydroxyethyl disulfide; GSSG, glutathione disulfide; Di-E-GSSG, di-eosin-glutathione disulfide; E-GS-BSA, eosin-glutathionylated bovine serum albumin; E-GSH, eosin glutathione; UV, ultraviolet; IAM, iodoacetamide; DTT, dithiothreitol; MW, molecular weight; hGrx1, human glutaredoxin 1; hGrx2, human glutaredoxin 2; EGrx1C14S, *E. coli* glutaredoxin 1 C14S mutant; CPS, counts per second; SDS, sodium dodecyl sulfate; Trx, thioredoxin; PAGE, polyacrylamide gel electrophoresis.

* Corresponding author.
** Corresponding author.
E-mail addresses: lucia.coppo@ki.se (L. Coppo), arne.holmgren@ki.se (A. Holmgren).
[1] Equal first authorship.

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(NADH) at 340 nm coupled to the reduction of hydroxyethyl disulfide (HED) in the presence of GSH and GR [27] or of lipoamide and lipoamide dehydrogenase in the presence of glutathione disulfide (GSSG) [28]. Both assays, however, have limitations concerning the sensitivity of absorbance detection and specificity because NADPH and NADH are used as electron donors by many other different redox enzymes.

To overcome these limitations, we have developed new sensitive assays for activity measurement of glutaredoxins using eosin-labeled glutathione disulfide (Di-E-GSSG) and eosin-glutathionylated bovine serum albumin (E-GS-BSA) as key components. Both substrates have strongly quenched fluorescence in their disulfide forms but show increases of fluorescence up to 20-fold when eosin glutathione (E-GSH) is released. Furthermore, E-GS-BSA has turned out to be specific as a Grx substrate.

As a component of a new in vitro method, we used Di-E-GSSG to visualize proteins that can be glutathionylated using gel electrophoresis exposed to ultraviolet (UV) light. By adding Grx and a complete glutathione system, we could also observe deglutathionylation reactions.

Materials and methods

Reagents

BSA (99%, protease and γ-globulin free), iodoacetamide (IAM), dithiothreitol (DTT), GR, GSH and NADPH were purchased from Sigma–Aldrich. Di-E-GSSG was prepared as described previously [29]. Spectra/Por dialysis membranes were purchased from Spectrum Laboratories, and Amicon Ultra centrifuge filter tubes with 30,000 and 3000 MW (molecular weight) cutoff were purchased from Merck Millipore. All other chemicals were standard laboratory reagents. Recombinant human glutaredoxin 1 (hGrx1), human glutaredoxin 2 (hGrx2), and *E. coli* Grx 1 mutant C14S (EGrx1C14S) were purified as described previously [4,18,19,30].

Glutathionylation of BSA

BSA (0.3 mM) was incubated with Di-E-GSSG (0.9 mM) in 0.1 M potassium phosphate buffer (pH 7.5) for 60 min at 37 °C. E-GS-BSA was separated from E-GSH and unreacted Di-E-GSSG by centrifugation using Ultra centrifuge filter tubes with 30,000MW cutoff at 10,000 *g* for 10 min, followed by dialysis against 0.1 M potassium phosphate buffer (pH 7.5) using dialysis membranes with 14,000 MW cutoff. The concentration of purified E-GS-BSA was determined both by the method of Bradford calibrated with BSA and using the molar absorption extinction coefficient of E-GS-BSA (33,400 M^{-1} cm^{-1} at OD_{520} in 0.1 M potassium phosphate buffer, pH 7.5).

Preparation of alkylated BSA

BSA (0.3 mM) was incubated with IAM (1 mM) in 0.1 M potassium phosphate buffer (pH 7.5) for 60 min at 37 °C. Alkylated BSA was separated from the unreacted IAM by dialysis against 0.1 M potassium phosphate buffer (pH 7.5) using dialysis membranes with 14,000 MW cutoff. The concentration of alkylated BSA was determined both by the method of Bradford calibrated with BSA and measuring the absorbance at 280 nm using the molar absorption extinction coefficient (44,000 M^{-1} cm^{-1} in 0.1 M potassium phosphate buffer, pH 7.5).

Fluorescence measurements

The fluorescence emission spectra of E-GS-BSA were measured in a FluoroMax spectrofluorometer (Spex) using 1-cm quartz cuvettes. Fluorescence intensity was measured in counts per second (CPS).

The cleavage of Di-E-GSSG and E-GS-BSA and the release of E-GSH were measured by fluorescence in black 96-well microtiter plates in a 100-μl final volume, recording fluorescence emission at 545 nm after excitation at 520 nm using either a Victor^3 fluorescence plate reader or an EnSpire 2300 Multilabel Reader (both from PerkinElmer).

Grx activity assay using HED assay and Di-E-GSSG or E-GS-BSA as disulfide substrates

The activity of glutaredoxin was assessed using the established HED assay [27] and a modified protocol replacing HED with Di-E-GSSG or E-GS-BSA. Various concentrations of hGrx1 (0.3–4.5 nM) or hGrx2 (0.6–5.1 nM) were added to a master mix composed of GR (50 nM), GSH (1 mM), NADPH (0.25 mM), and alkylated BSA (0.1 mg/ml) (final concentrations) to avoid any background reaction with the free thiol of BSA in 0.1 M potassium phosphate buffer (pH 7.5). GSSG (20 μM), Di-E-GSSG (20 μM), or E-GS-BSA (20 μM) was added to start the reaction, and the absorbance at 340 nm or the fluorescence emission (for the eosin-labeled compound) was recorded.

Turnover was calculated using standard curves of Di-E-GSSG and E-GS-BSA generated with DTT (10 mM final concentration).

Lipoamide–lipoamide dehydrogenase coupled Grx activity determination

The activity of glutaredoxin was alternatively measured in an assay coupled to lipoamide–lipoamide dehydrogenase [28] now measuring the reduction of Di-E-GSSG. Either hGrx1 (0.8–4 nM) or hGrx2 (7–35 nM) was incubated with lipoamide (0.75 mM) and lipoamide dehydrogenase (1.8 μM) in the presence of NADH (0.5 mM), followed by adding Di-E-GSSG (50 μM) in a 100-μl final volume and recording the fluorescence emission at 545 nm after excitation at 520 nm.

Blood plasma

Blood plasma was obtained from healthy blood donors at the Karolinska Hospital (Stockholm, Sweden). These were aliquoted and stored at −20 °C until their use for further studies. Plasma (40 μl) was added to 50 μl of the master mix (50 nM GR, 1 mM GSH, 0.25 mM NADPH, and 0.1 mg/ml alkylated BSA), after which the reaction was started by adding 10 μl of 200 μM E-GS-BSA. Controls with no GR, GSH, and NADPH were included (sample background) as well as hGrx1 standards to quantify activity.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

All of the gels were run under nonreducing conditions in NuPAGE 4–12% Bis–Tris gels using NuPAGE MES SDS (sodium dodecyl sulfate) running buffer (Life Technologies).

To test whether the bright signal of eosin was proportional to the amount of labeled protein, we ran different amounts of E-GS-BSA and then exposed the gel to the UV light.

To visualize the deglutathionylation process exerted by Grx, hGrx1 or *E. coli* Grx1 C14S mutant were prereduced with 10 mM DTT and desalted using Ultra filtration spin tubes with 3000 MW cutoff at 5000 *g* for 30 min. Di-E-GSSG was incubated with both types of Grx, and in parallel *E. coli* Grx1 C14S was incubated with E-GS-BSA. After 30 min, the samples were dialyzed and then run in SDS gel, exposed to the UV light, and then stained with Coomassie brilliant blue.