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Activity assays of mammalian thioredoxin and thioredoxin reductase: Fluorescent disulfide substrates, mechanisms, and use with tissue samples

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ABSTRACT

Thioredoxin (Trx) is a protein disulfide reductase that, together with nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (TrxR), controls oxidative stress or redox signaling via thiol redox control. Human cytosolic Trx1 has Cys32 and Cys35 as the active site and three additional cysteine residues (Cys62, Cys69, and Cys73), which by oxidation generates inactive Cys62 to Cys69 two-disulfide Trx. This, combined with TrxR with a broad substrate specificity, complicates assays of mammalian Trx and TrxR. We sought to understand the autoregulation of Trx and TrxR and to generate new methods for quantification of Trx and TrxR. We optimized the synthesis of two fluorescent substrates, di-eosin–glutathione disulfide (Di-E–GSSG) and fluorescein isothiocyanate-labeled insulin (FiTC–insulin), which displayed higher fluorescence on disulfide reduction. Di-E–GSSG showed a very large increase in fluorescence quantum yield but had a relatively low affinity for Trx and was also a weak direct substrate for TrxR, in contrast to GSSG. FiTC–insulin was used to develop highly sensitive assays for TrxR and Trx. Reproducible conditions were developed for reactivation of modified Trx, commonly present in frozen or oxidized samples. Trx in cell extracts and tissue samples, including plasma and serum, were subsequently analyzed, showing highly reproducible results and allowing measurement of trace amounts of Trx.

Protein disulfide reduction is a key factor for the catalytic mechanism of several cellular enzymes and is of major importance for controlling the activity of a large number of proteins [1,2]. Thioredoxin (Trx)[1] is the prototype protein disulfide reductase involved in reduction of ribonucleotide reductase [3,4], the essential enzyme for the synthesis of deoxyribonucleotides and DNA [5], methionine sulfoxide reductases [6], and peroxiredoxins [7] that are very important in the defense against oxidative stress. Trx proteins contain an active site WCGPC motif acting as disulfide reductases through thiol–disulfide exchange [8]. Electrons for the reduction come from nicotinamide adenine dinucleotide phosphate (NADPH) via thioredoxin reductase (TrxR), which is a selenoprotein in mammalian cells [9].

There is a fundamental difference between bacterial and mammalian thioredoxin systems. The former can be assayed using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as a substrate that is reduced by Trx–$(SH)_2$ and the reduction is coupled to NADPH and TrxR [10–12]. However, because mammalian TrxR uses DTNB directly as a substrate [13] for determination of Trx or TrxR activity, insulin is commonly used as a substrate [14,15] in the coupled reactions:

$$\mathrm{Trx{-}S_2 + NADPH + H^+ \xrightarrow{TrxR} Trx{-}(SH)_2 + NADP^+} \quad (1)$$

$$\mathrm{Trx{-}(SH)_2 + insulin{-}S_2 \longrightarrow TrxS_2 + insulin{-}(SH)_2} \quad (2)$$

By using an excess of NADPH and TrxR, Trx can be measured via the oxidation of NADPH or the generation of free SH groups in reduced insulin by DTNB after stopping the reaction with 6 M guanidine–HCl [15]. This method is very useful, but it has a drawback in that background blanks need to be subtracted to obtain correct results. Consequently, these methods are not very sensitive for measurements of low amounts of TrxR and Trx such as those found in serum.

Another complication in assaying mammalian Trx is the presence of three additional cysteine residues (Cys62, Cys69, and Cys73) in human Trx1 apart from Cys32 and Cys35, forming the

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[1] *Abbreviations used:* Trx, thioredoxin; NADPH, nicotinamide adenine dinucleotide phosphate; TrxR, thioredoxin reductase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FiTC, fluorescein isothiocyanate; GSSG, glutathione disulfide; EITC, eosin 5-isothiocyanate; BSA, bovine serum albumin; GR, glutathione reductase; Trx1, thioredoxin 1; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; PBMC, peripheral blood mononuclear cell; U937, human histiocytic lymphoma cells; Ramos, human Burkitt's lymphoma cells; K562, human chronic myelogenous leukemia cells; PDS, plasma-derived serum; PDI, protein disulfide isomerase.

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active site disulfide [16]. It is known that oxidation of Cys62 and Cys69 to a disulfide results in inactivation of Trx by formation of two disulfide forms lacking activity with TrxR1 in reaction 1 [17]. Furthermore, Trx can be nitrosylated and glutathionylated, also leading to inactive molecules [18–20]. To overcome this, preincubation with dithiothreitol (DTT) has been used in some protocols [12], but this gives rise to background problems.

The thioredoxin system is central for regulation of cell redox signaling and redox environment and has a close link with many human diseases such as cancer [21], diabetes [22], and neurodegenerative diseases [23,24]. There is currently a need to have better methods to detect Trx and TrxR activity in human patient samples, and there is a good indication that Trx and TrxR are possible biomarkers for the development of these diseases. Here we have the used two fluorescent substrates, fluorescein isothiocyanate-labeled insulin (FiTC–insulin) [25,26] and di-eosin–glutathione disulfide (Di-E–GSSG) [27], to characterize Trx-dependent disulfide reduction and to develop methods for activity determinations in samples that are commonly used in biomedicine.

Our results showed that FiTC–insulin was best suited for measurements of Trx and TrxR activity in the sub-picomole range and could be applied without using background subtraction. Measurements also took advantage of the capability of Trx1 to autoactivate itself by disulfide reduction following preincubation with NADPH and TrxR.

Materials and methods

FiTC, eosin 5-isothiocyanate (EITC), bovine or human insulin (henceforth insulin), bovine serum albumin (BSA), DTT, DTNB, GSSG, yeast glutathione reductase (GR), and NADPH were purchased from Sigma–Aldrich. Ultrafiltration spin tubes were purchased from Millipore. Human thioredoxin 1 (Trx1) was prepared as described previously [28], and rat recombinant TrxR with approximately 14 U/mg specific activity was prepared as described previously [29] or purchased from IMCO (Stockholm, Sweden; http://www.imcocorp.se).

Preparation of Di-E–GSSG

Labeling of GSSG with EITC [27] was carried out with minor modifications. Solid GSSG (4 mg) and EITC (14 mg) were mixed in 10 ml of 0.1 M potassium phosphate buffer (pH 9.0) in a molar ratio of 1:3 and incubated at 37 °C overnight with stirring. The separation procedure previously involving Sephadex G25 [27] was modified because the yield of purified Di-E–GSSG was 10% or less. Instead, precipitation using acetonitrile containing 0.1% trifluoroacetic acid was carried out with a 1:3 volume ratio of Di-E–GSSG/acetonitrile (v/v). A pellet composed of Di-E–GSSG formed, and the supernatant containing unreacted EITC was discarded. The pellet was washed once more with acetonitrile containing 0.1% trifluoroacetic acid and lyophilized after removal of the supernatant. After lyophilization, the sample was dissolved in 5 ml of 0.1 M potassium phosphate buffer (pH 7.5). The final yield of pure Di-E–GSSG was calculated to be approximately 80%.

Analysis of Di-E–GSSG by reverse phase HPLC

Analysis of the Di-E–GSSG purity was carried out by high-performance liquid chromatography (HPLC, Pharmacia, SMART system) using a reverse phase C_{18} column (Sephasil SC 2.1/10) equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Di-E–GSSG (10 nmol) was loaded and washed for 4 min with 0.1% trifluoroacetic acid in water. A gradient elution was performed starting by 50% (v/v) acetonitrile/water containing 0.1% (v/v) trifluoroacetic acid and ending at 100% (v/v) acetonitrile containing 0.1% trifluoroacetic acid in 30 min at a flow rate of 0.1 ml/min.

Preparation of FiTC–insulin

Labeling of insulin with FiTC was as described by Heuck and Wolosiuk [25,26] with some modifications. Insulin (50 mg) was suspended in 2.5 ml of 50 mM $NaHCO_3$ (pH 9.0) buffer. To dissolve the protein, the pH in the solution was titrated to 2.0 using 1 M HCl and then adjusted back to 9.0 with 1 M NaOH. The volume was adjusted to 5 ml with 50 mM $NaHCO_3$ (pH 9.0) buffer to yield a final concentration of 1.6 mM (10 mg/ml) and then was dialyzed extensively at 4 °C against 50 mM $NaHCO_3$ (pH 9.0) and 1 mM ethylenediaminetetraacetic acid (EDTA). Solid FiTC was dissolved in the dialysate at a molar ration of 1:2, and the mixture was incubated at 28 °C for 3.5 h with stirring. Nonreacted FiTC was separated from labeled insulin by centrifugation using ultrafiltration spin tubes with 5000 NMWL cutoff at 5000*g* for 30 min and was dialyzed extensively against TE buffer (50 mM Tris–Cl [pH 7.5] and 1 mM EDTA) at 4 °C.

Concentration determination of Di-E–GSSG and FiTC–insulin

The protein concentration of derivatized insulin was determined using the method of Bradford calibrated with BSA. Alternatively, the FiTC–insulin concentration was determined using a molar absorption coefficient of 94,000 M^{-1} cm^{-1} at 495 nm in PE buffer (0.1 M potassium phosphate [pH 7.5] and 1 mM EDTA) [25].

The concentration of purified Di-E–GSSG was determined by defining the concentration of the disulfides by reducing Di-E–GSSG to E–GSH (eosin–glutathione) using the thioredoxin system (Trx, TrxR, and NADPH). Di-E–GSSG was mixed with 6 μM Trx1, 10 nM recombinant rat TrxR, and 0.1 mM NADPH in PE buffer, and the absorbance at 340 nm was recorded over time. The decrease of A_{340} represents the consumption of NADPH. In the reaction, 1 mol of NADPH consumed is equivalent to 1 mol of Di-E–GSSG reduced, and the concentration of disulfides was calculated using the extinction coefficient of NADPH (6200 M^{-1} cm^{-1}) at A_{340} as follows:

$$\frac{\Delta A_{340}}{6200\ M^{-1}\ cm^{-1}} \times \text{dilution factor} = \text{Di} - \text{E} - \text{GSSG(M)}.$$

Alternatively, the concentration of Di-E–GSSG was determined by using the molar absorption coefficient for EITC (ε = 56,000 M^{-1} cm^{-1}) at 525 nm in 0.1 M PE buffer.

Skin tissue, PBMCs, and isolation of lymphocytes

Skin tissue from 3 healthy subjects, obtained from the Institute of Metabolic Science at the University of Cambridge (UK), were homogenized in TE buffer containing protease inhibitor cocktail (Roche Diagnostics), frozen and thawed, and then centrifuged to generate supernatants.

Peripheral blood mononuclear cells (PBMCs) were purified from 2 healthy blood donors (Blood Bank, Karolinska University Hospital, Stockholm, Sweden) by standard Ficoll–Paque centrifugation. The purified PBMCs were resuspended at 5×10^7 cells per milliliter in 200 μl of TE buffer and were sonicated. The lysates were cleared by centrifugation at 10,000*g* for 20 min. Lymphocytes were depleted by plastic adherence and iron phagocytosis. The protein content from the lysates was determined using Bradford modified assay (Bio-Rad) according to the manufacturer's manual.