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Biochimica et Biophysica Acta 1774 (2007) 813–821
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbapap

Pressure effects on the structure and function of human thioredoxin

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Received 20 November 2006; received in revised form 13 March 2007; accepted 14 March 2007
Available online 31 March 2007

Abstract

Thioredoxin is one of the major proteins that catalyze disulfide reduction and defines the thioredoxin superfamily bearing the CXXC structural motif. Human thioredoxin contains only 1 Trp residue proximal to the active site (WCGPC). We are interested in thioredoxin structure–function relationships, in particular, active site hydration and flexibility. Hence, in this study, we used hydrostatic pressure as a perturbation and monitored the conformational changes around the active site of thioredoxin by analyzing Trp fluorescence. The structure of thioredoxin was drastically altered by increasing pressure and did not completely refold after pressure release. The conformation in the active site vicinity was modified at low pressure (less than 100 MPa) and the Trp residue was completely exposed to aqueous medium at pressures above 350 MPa. Upon pressure release, thioredoxin showed no activity, although it folded 80% of the α-helical content relative to the native state. According to these results, pressure denaturation induces critical damage for the activity of thioredoxin, indicating extreme fragility of the active site with respect to pressure. This result is in contrast to the pressure effect on protein disulfide isomerase (PDI) which is organized by four thioredoxin-like domains including two WCGHC motifs.

Keywords: Thioredoxin; High pressure; Tryptophan fluorescence; Reduction activity

1. Introduction

It is important for nascent polypeptides to form the correct disulfide bonds. In the process of nascent protein folding, the disulfide bonds are formed in the endoplasmic reticulum, which harbors the glutathione redox system [1] and the unique enzymes for appropriate arrangement of disulfide bonds. Thioredoxin (Trx; MW 12000) is one of the major proteins that catalyze disulfide reactions. It represents the thioredoxin superfamily, which includes the PDI family and Dsb proteins and is characterized by the CXXC structural motif [2]. The CXXC motif functions as the active site in the reduction, oxidation and isomerization of disulfide bonds.

At present, thioredoxin has been thoroughly investigated in terms of its function, stability, interaction with other proteins, and its mechanism of action in the cell. The functions of thioredoxin include acting as the hydrogen donor for reductive enzymes such as ribonucleotide reductase [3,4], general reductant for disulfide bonds in proteins such as insulin [5], and a regulatory factor for enzymes or receptors in photosynthetic systems [6]. The principal function of thioredoxin is to reduce disulfide bonds in substrate proteins [5]. Reduced thioredoxin, Trx-$(SH)_2$ is produced by the reduction of oxidized thioredoxin, Trx-S_2, in connection with thioredoxin reductase (TrxR) and NADPH (β-nicotinamide adenine dinucleotide phosphate). Trx-$(SH)_2$ is a powerful thiol-disulfide oxidoreductase which served as a catalyst in the reduction of many exposed disulfides in proteins [7,8].

In mammals, the thioredoxin system can also be of primarily important for the supply of DNA precursors through its support of ribonucleotide reductase activity with glutathione depletion [9,10]. Thioredoxin and glutathione redox systems act as electron donors for human ribonucleotide reductase and prompt ribonucleotide reduction as well as other protein reactions supplying electrons from NADPH via their specific reduction mechanism [11–13]. Glutathione redox system and the Trx–TrxR reaction utilize NADPH in the cell. The investigation of

Abbreviations: PDI, Protein disulfide isomerase; Trx, Thioredoxin; TrxR, Thioredoxin reductase; CD, Circular dichroism; GR, Glutathione reductase; GSH, Glutathione reduced form; GSSG, Glutathione oxidized form; NADPH, β-Nicotinamide adenine dinucleotide phosphate; GdnHCl, Guanidine hydrochloride; CSM, Center of spectral mass

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doi:10.1016/j.bbapap.2007.03.013

thioredoxin in the cell recently focused on the electron transfer pathway and mechanism [14].

In vitro, the interaction between thioredoxin and other proteins such as cyclophilin (PPIase) has also been investigated using mutant Trx (CGPS) immobilized resin, which produces a stable Trx–protein complex [15,16]. Furthermore, the guanidine hydrochloride, urea and pH dependence of the structure stability for thioredoxin have been reported [17–20].

In this study, we focused on the effect of pressure on the stability of human thioredoxin, by monitoring the fluorescence of its single Trp residue located adjacent to the active site (WCGPC). Many recent high pressure studies on small proteins (~20 kDa) such as ribonuclease A [21], chymotrypsinogen [22], and staphylococcal nuclease [23] have revealed apparent two-state pressure-induced unfolding transitions (N→D) with excellent reversibility. Lysozyme [24] and carboxypeptidase Y [25] are denatured under high pressure in a multi-step manner with less reversibility. For pressure-denatured proteins, the penetration of water molecules induces the rupture of side-chain interactions, which disrupts the tertiary structure in the protein. However, pressure-denatured states appear to be more compact than the more random coil conformations observed with temperature and chemical denaturation at atmospheric pressure [26–28]. Pressure denaturation clarifies the original stability of each protein's structure because penetration of water occurred around flexible conformation with compression of cavities in protein. With the application of pressure, it is possible to investigate and trace proteins' structural weak point.

The effect of pressure on the structure and function of thioredoxin has not yet been investigated, although we have previously reported the results of the application of pressure to protein disulfide isomerase (PDI) [29]. PDI is composed of four thioredoxin-like domains including two WCGHC motifs [30]. PDI exhibited remarkable structural and functional reversibility to pressure-induced denaturation. The objective of the present work is to investigate the stability and the reversibility of thioredoxin against high pressure. The study focuses on the function of thioredoxin after pressure release, in particular, the study aims to clarify the pressure behavior of Trp residue adjacent to the active site.

2. Materials and methods

2.1. Materials

Human thioredoxin, glutathione reductase (GR), glutathione reduced form (GSH), and citrate synthetase were purchased from Sigma Chemical Co., USA. Thioredoxin reductase was obtained from Oriental Yeast Co., Ltd., Japan and glutathione oxidized form (GSSG) was purchased from Kohjin Co., Ltd., Japan. Insulin from bovine pancrease, β-nicotinamide adenine dinucleotide phosphate (NADPH), and guanidine hydrochloride (GdnHCl) were obtained from Nacalai tesque, Inc., Japan.

2.2. Fluorescence measurements of thioredoxin under high pressure

The concentration of thioredoxin was set to 20 μM in 50 mM Tris buffer (pH 7.5) for high pressure experiments. The sample of thioredoxin was incubated to equilibrate for 30 min at every 25 MPa up to 400 MPa. The fluorescence spectra of thioredoxin were monitored using Jasco FP-6500 spectrofluorometer equipped with Teramecs PCI400 high pressure cell (volume 2 ml). The excitation was performed at 290 nm with 3 nm slits width to exclude contributions from the tyrosine residues. The slit of emission was set to 3 nm width. This condition of fluorescence spectroscopy was set similar to the previous methods [29]. With the same condition, we also checked through light scattering method whether or not the aggregation appeared at high pressure and after releasing pressure. The spectral shifts of intrinsic Trp fluorescence spectra were calculated using the center of spectral mass (CSM) [31,32] which is described as the center of gravity in the spectrum. The CSM is defined by the equation

$$< \lambda >_j = \sum_j F_j \lambda_j \Big/ \sum_j F_j, \quad j = 300,\ 300.1,\ 300.2, \ldots, 500\text{nm}.$$

2.3. Quench effect on the Trp fluorescence in thioredoxin with GSSG and under high pressure

The fluorescence spectra were set to excitation wavelength of 290 nm with Ex 3 nm and Em 5 nm slits width using 2 mm × 3 mm high pressure quartz cell (200 μl). The concentration of thioredoxin was prepared at 20 μM with 1.0 mM, 50 mM, and 100 mM in 25 mM Tris buffer (pH 7.5). High pressure was applied to thioredoxin with 100 mM GSSG up to 400 MPa. The samples were incubated for 15 min at each condition.

2.4. Fluorescence measurements of thioredoxin in the present of GdnHCl

The concentrations of GdnHCl for the fluorescence spectra were set to 0.5, 1.0, 2.0, 3.0, 4.5, and 6.0 M. The measurement concentration of thioredoxin was 20 μM in 25 mM Tris buffer (pH 7.5) in each condition. The samples were incubated for 15 min. The fluorescence spectra were monitored by Jasco FP-6500 spectrofluorometer using 2 mm × 3 mm quartz cell. The performance of fluorescence spectroscopy was set at 290 nm excitation wavelength and Ex 3 nm, Em 5 nm slits width.

In the refolding measurement, 100 μM thioredoxin was denatured with 6.0 M GdnHCl in 12.5 mM Tris buffer (pH 7.5) for 30 min. The sample was then diluted 20 fold to give 5 μM thioredoxin including 0.3 M GdnHCl in 12.5 mM Tris buffer (pH 7.5) and allowed to stand for 1 h at room temperature. Native thioredoxin and thioredoxin with 0.3 M GdnHCl were also set at 5 μM in 12.5 mM Tris buffer (pH 7.5) to compare the refolding sample. The measurement was performed with 10 mm × 10 mm standard quartz cell. The performance of fluorescence spectroscopy was set at 290 nm excitation wavelength and Ex 3 nm, Em 5 nm slits width by Jasco FP-6500 spectrofluorometer. The analysis of all fluorescence spectra used the above CSM equation.

2.5. Circular dichroism measurements

Refolding approach in secondary structure of thioredoxin was performed with the dilution of GdnHCl concentration. The sample (100 μM thioredoxin) was prepared using 6.0 M GdnHCl in 12.5 mM Tris buffer (pH 7.5), and then, diluted 20 fold with 12.5 mM Tris buffer (pH 7.5) to give 5 μM thioredoxin. The CD spectra of thioredoxin were also measured in the presence of 0.3 M and 6.0 M GdnHCl and in the absence of GdnHCl.

In the refolding method after releasing pressure, the samples of fluorescence measurement (native and pressure-treated thioredoxin) were diluted 4-fold and set to 5 μM in 12.5 mM Tris buffer (pH 7.5) as a final concentration. 6.0 M GdnHCl denatured thioredoxin was also measured in the same condition. The CD measurements of thioredoxin were carried out at 20 °C getting the average of five scans from 190 to 270 nm range using Jasco J-805 spectropolarimeter.

At 0.3 M and 6.0 M GdnHCl, CD spectra were not detected below 205 nm and 215 nm, respectively, due to the strong UV absorption of GdnHCl.

2.6. Assay of thioredoxin activity

The thioredoxin activity was determined by the coexistence of glutathione redox system [33] and thioredoxin–thioredoxin reductase reaction system [34]. The reaction proceeded in both glutathione redox system and thioredoxin–thioredoxin reductase reaction system in conjunction with the transformation from NADPH to $NADP^+$ (Fig. 1). The reaction solution contained 30 μM insulin,