1H, 13C, and 15N resonance assignments of human glutathione peroxidase 4

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Abstract
Glutathione peroxidase 4 (GPx4) behaves as an antioxidant enzyme capable of directly reducing peroxidized phospholipids within cell membranes. Recently, GPx4 has attracted attention as a target molecule for cancer therapy because it induces the immortalization of cancer cells suppressing ferroptosis. In this study, to analyze the function and structure of GPx4 by solution NMR, we performed resonance assignments of GPx4 and assigned almost all backbone 1H, 13C, and 15N resonances and most of the side chain 1H and 13C resonances. Using these assignments, the secondary structure of GPx4 was analyzed by the TALOS + program. GPx4 has six helices and seven strands. Then, the backbone dynamics were examined by the {1H}–15N heteronuclear NOE experiment. GPx4 was found to be rigid except for a short loop region. These results will provide basis for functional analysis and the first solution structure determination of GPx4.

Keywords GPx4 · Redox homeostasis · Ferroptosis · FLYA

Biological context
Glutathione peroxidase (GPx) family proteins, including GPx1-8, have a crucial role in the antioxidative defense system in intracellular redox homeostasis. They catalytically recycle reactive oxygen species, mainly hydrogen peroxide, using glutathione as a reducing agent (Toppo et al. 2009; Brigelius-Flohé and Maiorino 2013; Deponte 2013). Among them, GPx4 is a unique antioxidant enzyme that directly reduces complex hydroperoxides such as peroxidized phospholipids produced in the cell membrane (Maiorino et al. 2012). GPx4 has recently attracted much attention as a key regulator of lipid peroxidation-dependent regulated cell death, termed ferroptosis (Yang et al. 2014). In addition to the antioxidative function described above, GPx4 is known to be involved in several biological events, i.e., it functions as a structural protein in spermatogenesis and a regulator of gene expression (Ursini et al. 1999). Recent studies have demonstrated that some drug-resistant cancer cells are dependent on the enzymatic activity of GPx4 to maintain the intracellular redox state to suppress ferroptosis (Bayır et al. 2020; Harris and DeNicola 2020; Jiang et al. 2021). Therefore, GPx4 is also attracting attention as a target molecule for cancer therapy.

The following GPx4 isoforms with different intracellular localizations were reported: 197-residue mitochondrial GPx4 (mGPx4), cytoplasmic GPx4 (cGPx4), which is the N-terminal 27-residue truncated isoform of mGPx4, and sperm nuclear GPx4 (snGPx4), which is the isoform in which the N-terminal 28 residues of mGPx4 are replaced by 84 residues with different sequences (Brigelius-Flohé and Maiorino 2013). The crystal structures of cGPx4 and mGPx4 have been reported for the wild type, various mutants, and complexes with inhibitors (Scheerer et al. 2007; Janowski et al. 2016; Sakamoto et al. 2017; Borchert et al. 2018; Moosmayer et al. 2021; Liu et al. 2022). These structural studies demonstrated that GPx4 forms a catalytic center with selenocysteine (Sec73 in the case of mGPx4) and the surrounding glutamine (Gln108), tryptophan (Trp163), and asparagine (Asn164) residues. However, the details of the functions, such as the interaction with the substrate and the regulation of the activity, are not yet fully understood.
(34–197, U73G) have been reported by Labrecque and Fuglestad (2021). In this study, we report the nearly complete backbone and side chain resonance assignments for human mGPx4 (Cys29−Phe197) containing eight mutations (Cys29Ser, Cys37Ala, Cys64Ser, Sec73Cys, Cys93Arg, Cys102Ser, Cys134Glu, and Cys175Val) (hereafter referred to as GPx4mu). The sequence of GPx4mu is identical to the sequence of GPx4, which was previously used by Sakamoto et al. to determine the crystal structure (Sakamoto et al. 2017). We also report the secondary structure and the dynamics of the main chain using the assignments. These results will provide an important basis for the determination of the first solution structure of GPx4 and will aid in obtaining detailed information on interactions with substrates, cofactors, and inhibitors.

Methods and experiments

Recombinant protein expression and purification

The cDNA of GPx4mu encoding human GPx4 (Cys29−Phe197) with eight mutations (Cys29Ser, Cys37Ala, Cys64Ser, Sec73Cys, Cys93Arg, Cys102Ser, Cys134Glu, Cys175Val) was constructed following a previous report (Sakamoto et al. 2017). The cDNA fragment was amplified by PCR and cloned into the pCold-GST vector for preparation as a glutathione S-transferase (GST) fusion protein (Hayashi and Kojima 2008). The plasmids were transformed into Escherichia coli BL21 (DE3) (Merck KGaA, Darmstadt, Germany). The uniformly 15N- or 13C/15N-labeled GPx4mu proteins were overexpressed by culturing the transformed cells in M9 medium containing 0.5 g L−1 [U-15N]-l-glucose or 1.0 g L−1 [U-13C6]-l-glucose or 0.5 g L−1 [U-15N] ammonium chloride, and either 4.0 g L−1 β-glucose or 1.0 g L−1 [U-13C6] β-glucose was used for uniform 15N- or 13C/15N-labeling of the GPx4mu proteins, respectively, with the procedures described below. The cells were cultured at 37 °C until the optical density at 600 nm reached 0.4–0.6, and then the cells were rapidly chilled to 15 °C in an ice-water bath. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside and continued cell cultivation at 15 °C overnight (approximately 20–24 h). Following cultivation, the cells were harvested by centrifugation and stored at −80 °C. The cell pellet was resuspended in lysis buffer (pH 8.0) composed of 50 mM tris(hydroxymethyl)aminomethane hydrochloride, 300 mM potassium chloride (KCl), 0.1 mM ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt dihydrate, and 1 mM dithiothreitol (DTT). The cell suspension was homogenized by sonication and subjected to centrifugation. GST-tagged GPx4mu was purified from the supernatant of the centrifuged lysate using Glutathione Sepharose™ 4B resin (Cytiva, Marlborough, MA). Then, the GST-tags were removed with human rhinovirus 3C protease, and the GPx4mu proteins were further purified using a HiLoad™ 26/600 Superdex™ 200 pg column (Cytiva, Marlborough, MA) equilibrated with 50 mM potassium phosphate buffer (pH 6.8) containing 50 mM KCl and 1 mM DTT on an ÄKTA™prime plus system (Cytiva).

NMR spectroscopy

For the NMR measurements, the GPx4mu proteins were dissolved at a concentration of 0.4 mM in 95/5% H2O/D2O containing 50 mM potassium phosphate (pH 6.8), 50 mM KCl and 1 mM DTT. All NMR measurements were performed using an AVANCE III HD 800 MHz NMR spectrometer equipped with TXI CryoProbe™ (Bruker) at 298 K. For the backbone resonance assignments, 1H−15N HSQC, nonuniform sampling (NUS)-(H)NCO, NUS-(H)N(CA)CO, NUS-HNCAB, and NUS-CBCA(CO)NH were measured. For the side chain assignments, NUS-HBHA(CACO)NH, HCCH-TOCSY (aliphatic and aromatic region), 15N-edited NOEY, and NUS-13C-edited NOESY (aliphatic and aromatic region) were measured. 1H−15N heteronuclear NOE and its reference spectra (Farrow et al. 1994) were measured in an interleaved fashion, with or without 2 s of 1H saturation. All the sampling schedules for NUS spectra were generated using the Poisson gap sampling method (Hyberts et al. 2010).

NUS spectra were processed using hmsIST (Hyberts et al. 2012) and NMRPipe (Delaglio et al. 1995), and uniform sampling spectra were processed using NMRPipe. Chemical shift assignments were performed initially by the combined use of MagRO-NMRView and the FLYA module of CYANA (Güntert et al. 1997; López-Méndez and Güntert 2006; Kobayashi et al. 2018). The automatically assigned chemical shifts were then verified by the author’s visual inspection, some of the assignments were corrected, and some unassigned atoms were assigned manually using MagRO-NMRView. The secondary structure was determined by the TALOS + program (Shen et al. 2010) using HN, N, C’, Cα, Cβ, and Hα chemical shifts. The peak intensities of the 1H−15N heteronuclear NOE and the reference spectra were measured using NMRFAM-Sparky software (Lee et al. 2015). The 1H−15N heteronuclear NOE and the error values were obtained by Monte Carlo simulation using a homemade Python program.

Extent of assignment and data deposition

Completeness of the assignment

Backbone amide 1H and 15N resonance assignments of GPx4mu were obtained for 163 out of 166 nonproline
residues (98%) (Fig. 1). Arg32, Gly137, and Asn159 could not be assigned. These residues were located on the loop or the N-terminal flexible region. In addition, 100% Cα and Hα resonance assignments were also achieved. For the side chain, 77% 1H, 76% 13C, and 13% 15N were also assigned.

Some side chain 1H resonances showed statistically unusual upfield shifts evaluated by the wwPDB Validation Service, namely, Hγ of Arg60, Hβ of Ser71, Hβ of Phe105, Hγ2 of Val135, Hβ of Lys145, Hγ of Gln150, and Hγ13 of Ile189. Referring to the crystal structure of GPx4mu (PDB ID, 5H5Q, 5H5R, and 5H5S), these atoms seem to be either involved in electrostatic interactions with acidic residues or ring current shifts induced by aromatic residues.

The assigned chemical shifts have been deposited in the Biological Magnetic Resonance Data Bank (BMRB) under the accession number 51379.

**Secondary structure analysis**

The secondary structure of GPx4mu determined by the TALOS + program, which consists of six helices and seven strands, is shown in Fig. 2. In the crystal structures of GPx4mu (PDBID: 5H5Q, 5H5R, and 5H5S) (Sakamoto et al. 2017), the residues Ser40 to Glu43 form a short helix, but this helix was not identified in the TALOS + analysis. The other secondary structures, including the four long helices with seven strands forming the thioredoxin motif (Scheerer et al. 2007) and the two short helices, were essentially the same as those of the crystal structures.

**Backbone dynamics**

Next, we measured {1H}–15N heteronuclear NOE to examine the backbone dynamics of GPx4mu (Fig. 3a). The hetero NOE values for most residues between Arg36 and the C-terminus were close to 1, indicating that GPx4 is basically rigid on the pico-nanosecond time scale. However, the hetero NOE value of Gly158 was 0.45, indicating that the main chain is mobile (Kay et al. 1989), and the hetero NOE value of Ile156 was 0.69, which is lower than that of other residues and indicates the relatively high mobility of the main chain. The signals of Leu157 in the {1H}–15N heteronuclear NOE spectra were weak, and thus the NOE value for Leu157 in Fig. 3 may not be accurate. These
residues are located in a region that is thought to be a truncated form of the surface-exposed loop present in the other GPx isoforms (Scheerer et al. 2007). Comparing the existing GPx4 crystal structures with each other, this region is poorly converged (Fig. 3b), which is consistent with the \textsuperscript{1}H–\textsuperscript{15}N heteronuclear NOE values.

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**Author contributions** KI, and TS carried out the experiments. KF analyzed the data with support from NK, TF, and CK. CK supervised the project. KF and KI wrote the original draft and KF prepared figures. KF, KI, TS, TF, and CK revised the draft. All authors reviewed the manuscript.

**Declarations**

**Conflict of interest** The authors declare that they have no conflicts of interest.
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