**Protein polysulfidation-dependent persulfide dioxygenase activity of ethylmalonic
encephalopathy protein 1**

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ABSTRACT

Reactive persulfide species such as glutathione persulfide (GSSH) are highly abundant biomolecules. Persulfide dioxygenase (also called ethylmalonic encephalopathy protein 1, ETHE1) reportedly metabolizes GSSH to GSH with simultaneous oxygen consumption. How ETHE1 activity is regulated is still unclear, however. In this study, we describe the possible role of protein polysulfidation in the catalytic activity of ETHE1. We first found that ETHE1 catalyzed the persulfide dioxygenase reaction mostly for glutathione polysulfides, GS-(S)_n-H, as well as for GSSH, but not for other endogenous persulfides such as cysteine and homocysteine persulfides/polysulfides. We then developed a novel method to detect protein polysulfidation and named it the polyethylene glycol-conjugated maleimide-labeling gel shift assay (PMSA). PMSA analysis indicated that most cysteine residues in ETHE1 were polysulfidated. Site-directed mutagenesis of cysteine residues in ETHE1 combined with liquid chromatography tandem mass spectrometry for polysulfidation determination surprisingly indicated that the Cys247 residue was important for polysulfidation of other Cys residues and that the C247S mutant possessed no persulfide dioxygenase activity. These results suggested

that ETHE1 is a major enzyme regulating endogenous GSSH/GS-(S)_n-H and that its activity is controlled by polysulfidation of the Cys247 residue.

Keywords:

Persulfide dioxygenase

Ethylmalonic encephalopathy protein 1

Protein polysulfidation

Reactive persulfide

1. Introduction

Ethylmalonic encephalopathy protein 1 (ETHE1) is a protein encoded by the gene responsible for the inborn autosomal recessive disorder ethylmalonic encephalopathy [1].

ETHE1 is also a persulfide dioxygenase and may play an important role in hydrogen sulfide detoxification in the mitochondrial matrix [2,3]. Several researchers [3-5] proposed that ETHE1 may catalyze the oxygen-dependent conversion of glutathione persulfide (GSSH) to glutathione (GSH) and sulfite (Eq. 1):

Pettinati et al. reported that ETHE1 contains an Fe(II) cofactor at its active site that is liganded by aspartate (Asp154) and two histidine residues (His79 and His135) (Supplementary Fig. S1) [6]. Several clinical studies identified certain mutations in the *ETHE1* gene of patients with ethylmalonic encephalopathy [1,2,7]. The missense and nonsense mutations, occurring at highly conserved residues, caused reduction in protein stability, protein expression, iron content, and enzymatic activity (Supplementary Fig. S1) [1,2,7,8]. Moreover, oxidative modification of the Cys247 residue, which is located close to the enzyme active site iron, was found in human and *Arabidopsis thaliana*

ETHE1 protein structures [6,9]. The catalytic mechanism of this enzyme reaction of ETHE1 remains unclear, however.

Reactive persulfides and polysulfides such as GSSH and cysteine persulfide (CysSSH) are endogenously produced in mammalian cells and tissues by cystathionine- γ -lyase and cystathionine- β -synthase with the substrate cystine [10]. Although several researchers proposed that reactive persulfides/polysulfides may function as redox signaling molecules in cellular processes via their potent antioxidant ability [10-16], details of the signaling mechanism remain elusive. Furthermore, formation of persulfides/polysulfides (-SSH or -S-(S)_n-H) on cysteine (Cys) residues in proteins, a process also called protein polysulfidation [10-12,17-19], was proposed to occur through reaction of protein thiols with various endogenous low-molecular-weight (LMW) persulfides such as GSSH [10,12]. Although protein polysulfidation is widely suggested as an important pathway in redox signaling and biology, the exact function and biological relevance of protein polysulfidation are not fully understood.

In this study, we found that the ETHE1 protein was polysulfidated at almost all Cys residues *in vitro* and *in vivo*. We also showed that the Cys247 residue was essential for

ETHE1 persulfide dioxygenase activity and maintenance of polysulfidation levels on the rest of Cys residues. To our best knowledge, this is the first report revealing a critical role of protein polysulfidation in the enzyme reaction of ETHE1 persulfide dioxygenase.

2. Experimental procedures

2.1. Materials

Iodoacetamide (IAM), monobromobimane (MBB), 2,2'-dithiodipyridine (DTP), 5'-dithiobis(2-nitrobenzoic acid) (DTNB), tris-(2-carboxyethyl)phosphine (TCEP), protease inhibitor cocktail, and sodium hydrogen sulfide were obtained from Nacalai Tesque (Kyoto, Japan). *N*-Ethylmaleimide (NEM) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Methyl methanethiosulfonate (MMTS), *p*-chloromercuribenzoic acid (PCMB), and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and salts used were of analytical grade. 8-Nitroguanosine 3',5'-cyclic monophosphate (8NcG) was prepared according to the method previously reported [20]. 2-Aminosulfonyl benzothiazole (ASBT), 2-methylsulfonyl benzothiazole (MSBT), and biotinylated PEG [polyethylene glycol (PEG) 36-mer]-maleimide (Biotin-PEG₃₆-MAL, BPM) were provided by Dojindo Laboratories (Kumamoto, Japan). The polysulfide-sensitive probe SSP2 was kindly provided by Ming Xian (Washington State University) [21]. Various authentic polysulfide compounds and their MBB adducts were synthesized according to our previous report [10].

2.2. Preparation of recombinant human ETHE1

To construct a plasmid for ETHE1 expression (pQE70-hETHE1), we obtained a DNA fragment corresponding to human ETHE1 missing 11 N-terminal codons via polymerase chain reaction and inserted it between the *Sph*I and *Bam*HI sites of pQE-70.

Site-specific mutants of ETHE1 were constructed from pQE70-hETHE1.

Supplementary Table S1 shows primer sequences.

To obtain recombinant proteins, BL21 (DE3) transformed with pQE70-hETHE1/mutants was grown at 37 °C in LB medium containing 0.25 mM FeSO₄.

Recombinant ETHE1 proteins were purified with Ni-NTA agarose (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Protein concentration was determined by using Protein Assay CBB Solution (Nacalai Tesque), and protein sample purity was analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Cell culture

A549 cells were cultured in high-glucose Dulbecco's Modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (MP Biomedicals, Solon, OH, USA) and 1% penicillin-streptomycin. For the PEG-maleimide-labeling gel shift assay (PMSA) (see below), cells lysate was prepared with ice-cold RIPA buffer (10 mM Tris-HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl, pH 7.4) plus 1 mM TCEP and 1% protease inhibitor cocktail, which was homogenized by passing them 20 times through a 26-gauge needle on ice. After centrifugation at 20,000 g (4 °C, 25 min), the supernatant was applied to a PD SpinTrap G25 column (GE Healthcare, Buckinghamshire, UK) to remove LMW compounds. After the samples were incubated with 1 mM TCEP at 37 °C for 1 h, they were stored at -80 °C until use.

2.4. Analyses for activity and substrate specificity of ETHE1 persulfide dioxygenase

We investigated the activity and substrate specificity by means of liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

ETHE1 proteins were incubated with GSH/Cys/homocysteine (HCys) polysulfides at 37 °C for 15 min in 100 mM Tris-HCl buffer (pH 7.5). After incubation, the remaining polysulfides were reacted with 5 mM MBB for 15 min to form bimane adducts,

followed by quantitative LC-ESI-MS/MS (6430; Agilent Technologies, Inc., Santa Clara, CA) with known amounts of stable isotope-labeled polysulfide derivatives as internal standards [10]. For negative controls, ETHE1 proteins were boiled at 95 °C for 5 min and used in this analysis.

2.5. Measurement of persulfide dioxygenase ETHE1 via the polysulfide-sensitive fluorescence probe SSP2

We also determined the persulfide dioxygenase activity of ETHE1 via measurement of polysulfide consumption with the polysulfide-sensitive probe SSP2. Recombinant proteins were incubated with polysulfides at 37 °C for 15 min in 100 mM Tris-HCl buffer (pH 7.5) and were then reacted with 100 μ M SSP2 in the presence of 1 mM CTAB for 10 min at room temperature in the dark. Methoxyfluorescein (SSP2 reaction product) fluorescence intensity was determined at $\lambda_{\text{EX}} = 482$ nm and $\lambda_{\text{EM}} = 518$ nm with a microplate reader (SH-9000; Corona Electric Co., Ltd., Ibaraki, Japan). Polysulfide concentrations were calculated from the standard curve of methoxyfluorescein. One unit of activity was defined as consumption of 1 μ mol polysulfide in 15 min.

2.6. Detection of protein polysulfidation via PMSA

Protein polysulfidation was detected by using PMSA, which we developed. The samples (0.3 mg/ml recombinant protein, 3 mg/ml cell lysate) were buffer exchanged to RIPA buffer by using the PD SpinTrap G-25 column and then reacted with various electrophiles (3 mM) at 37 °C for 1 h, diluted 3-fold with RIPA buffer, followed by the reaction with 2.5 mM BMP at 37 °C for 1 h. After heat denaturation, samples were loaded onto SDS-PAGE and detected by means of Coomassie brilliant blue (CBB) staining or Western blotting with anti-ETHE1 antibody (sc-161566; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.7. Determination of protein polysulfidation levels by LC-ESI-MS/MS

Wild-type ETHE1 and C247S mutant polysulfidation was analyzed by using LC-ESI-MS/MS according to our previous method but slightly modified [10]. Briefly, alkylated ETHE1 proteins (wild type and C247S) were digested by Pronase (Merck Millipore, Darmstadt, Germany) in the presence of known amounts of isotope-labeled

internal standards. After addition of 0.1% formic acid, Pronase digests were centrifuged and supernatants were subjected to LC-ESI-MS/MS with known amounts of internal standards as just described, for accurate quantification of both Cys and CysSSH residues in each ETHE1 protein.

2.8. *Statistical analysis*

Data are means $\pm$ SD of at least three independent experiments unless otherwise specified. For statistical comparisons, we utilized unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Newman-Keuls post-hoc test via GraphPad Prism software. We considered $P < 0.05$ as statistically significant.

3. Results

3.1. *ETHE1* activity measurement

ETHE1 persulfide dioxygenase activity was previously detected by measuring oxygen consumption [3,4,6,8,22]. We recently developed a selective and quantitative detection method, LC-ESI-MS/MS with stable isotope-labeled standards, for reactive persulfides such as CysSSH, GSSH, and HCys persulfide and reported their endogenous production in cells and tissues [10]. Here, to directly determine enzymatic activity based on consumption of reactive persulfides and investigate whether these reactive persulfides can serve as substrates of ETHE1, we prepared recombinant human ETHE1 (Supplementary Fig. S2), which allowed us to perform substrate specificity analysis via our LC-ESI-MS/MS approach (Fig. 1A). Our results indicated that GSSH is the best substrate for this dioxygenase reaction, a finding that agrees with results in previous reports [3,5]. Our current study also revealed that the enzyme effectively decomposed not only persulfide (GSSH) but also GSSSH (trisulfide) and GSSSSH (tetrasulfide) by means of its unique catalytic activity. These activities were completely abolished by boiling at 95 °C for 5 min, without significant reduction of Cys/HCys polysulfides.

We also developed a convenient method for enzyme activity measurement by using the polysulfide-sensitive probe SSP2. With this technique, we found that ETHE1 catalyzed decay of GSH polysulfides (GS-(S)_n-H), including the well-known substrate GSSH, but not Cys and HCys polysulfides (Fig. 1B), findings that agreed with the MS-based results described above. This report is the first to provide direct evidence for ETHE1-mediated metabolism of reactive polysulfides.

3.2. Contribution of Cys247 residue to ETHE1 enzymatic activity

Crystallographic analysis revealed that human ETHE1 has a mononuclear iron ion in its active site and suggested that the Cys247 residue, which is highly conserved in many organisms (Supplementary Fig. S1), is located close to the catalytic site pocket [6]. In addition, substitution at the Cys161 residue was found in a cohort study of patients with ethylmalonic encephalopathy [2]. However, no reports demonstrated the functional involvement of Cys residues in ETHE1 enzymatic activity. To investigate highly conserved Cys residues (Cys80, Cys139, Cys161, Cys247), we prepared a series of Cys-to-serine mutants (Supplementary Fig. S2). As Fig. 2 shows, a fluorescent assay

with SSP2 indicated that mutation of Cys247 caused activity loss. Cys80 and Cys161 mutations produced no such inhibitory effects. These results suggested that Cys247 is essential for ETHE1 persulfide dioxygenase activity.

3.3. PMSA detection of protein polysulfidation of human ETHE1

The sulfide transfer from LMW polysulfides such as GSSH to high-MW protein-bound Cys residues may be a mechanism of protein polysulfidation [12]. We hypothesized that ETHE1 may be polysulfidated, because ETHE1 interacts with GSSH in the reaction shown in Eq. 1. We developed a novel method for determination of protein polysulfidation, i.e., PMSA (Fig. 3A). In the initial step, both protein Cys thiol (-SH) and polysulfide $-(S)_n-H$ groups are alkylated by using different blocking reagents such as 8NcG [20] and other electrophiles, e.g., IAM, NEM, and MBB (Fig. 3A, Reaction 1). We used BPM in the second reaction (Fig. 3A, Reaction 2), in which the pegylation probe reacted with polysulfidated Cys residues that still contained additional sulfur atoms, which resulted in bands being shifted up on SDS-PAGE. Efficiency of destruction of protein polysulfides during the blocking procedure varied

depending on electrophilicity and the molecular structure of individual alkylating reagents. That is, treatment with weak electrophiles, e.g., 8NcG, may leave protein polysulfides intact as in the original, whereas treatment with strong electrophiles, e.g., MMTS and DTP, caused disruption of the polysulfide structure. PMSA analysis therefore clarified the degree of protein polysulfidation, as a change in SDS-PAGE band mobility. Also, we determined the number of polysulfidated Cys residues from the apparent molecular weight of the protein calculated on the basis of a standard curve drawn for the protein markers. For example, as seen in Fig. 3B, the IAM-treated lane indicated that 6-8 residues were polysulfidated. Because human ETHE1 protein has 9 Cys residues, almost all Cys residues on the protein were polysulfidated. PMSA with cell lysates of the human lung cancer cell line A549 showed a profile similar to that for the recombinant protein prepared by the *E. coli* expression system (Fig. 3C), which suggests that ETHE1 is endogenously polysulfidated at almost all Cys residues.

3.4. Potential role of Cys247 residue in ETHE1 polysulfidation

To correlate enzymatic activity and protein polysulfidation, we studied protein

polysulfidation of several Cys mutants including C247S. As Fig. 4A illustrates, PMSA indicated that the C247S mutant produced a marked decrease in protein polysulfidation. In contrast, C80S and C161S showed profiles similar to the wild-type profile. We also analyzed protein polysulfidation of wild-type ETHE1 and Cys247S mutant quantitatively by using LC-ESI-MS/MS. Protein polysulfidation of Cys247S was attenuated by 54% compared with that of wild-type ETHE1 (Fig. 4B). These results suggested that Cys247 is essential for ETHE1 persulfide dioxygenase activity and is critically involved in maintenance of protein polysulfidation during the enzymatic reaction.

4. Discussion

Reactive persulfides and polysulfides, which include GSSH (a well-known substrate for ETHE1) and CysSSH, are endogenously produced in large amounts in mammalian cells and tissues [10]. We investigated the substrate specificity of ETHE1 persulfide dioxygenase by using our own MS-based technique. The results showed that ETHE1 metabolized GS-(S)_n-H as well as GSSH but did not do so with other polysulfide derivatives of Cys and HCys (Fig. 1A). We also successfully developed a fluorometric method, with the polysulfide-sensitive probe SSP2, to detect enzymatic activity based on consumption of a reactive persulfide substrate instead of consumption of oxygen. The results obtained by our fluorescent assay correlate well with those obtained by LC-ESI-MS/MS (Fig. 1B). Moreover, these findings are consistent with those in the report by Kabil and Banerjee [3]. Pettinati et al. suggested a possible oxidative modification (i.e., cysteinyl sulfinic acid, RSO₂H) at the Cys247 residue in ETHE1, with a location vicinal to the active site iron ion, which is likely either of catalytic relevance or representative of nonproductive or damaging protein oxidation [6], as is well documented with other oxygenases and metalloproteins [23]. These authors also proposed that the Cys247 residue may play an important role in ETHE1 persulfide

dioxygenase activity by unknown mechanisms [6]. Our present study revealed that the Cys247S mutant had no persulfide dioxygenase activity (Fig. 2) and that protein polysulfidation in the Cys247S mutant was markedly reduced compared with that in wild-type ETHE1 (Fig. 4). These findings indicated that ETHE1 may obtain additional sulfide residues from the substrate GS-(S)_n-H during its catalytic reaction, which would result in extensive protein polysulfidation at the Cys247 residue (Eq. 2):

The reactive sulfide residues occurring via site-specific polysulfidation may be transferred to other Cys residues in the same protein, this process thereby functioning as a sulfide transfer system catalyzed by the ETHE1 protein.

ETHE1 gene mutations correlate well with the autosomal recessive metabolic disease ethylmalonic encephalopathy, which leads to death in infancy [1,4,7]. In addition, ETHE1 was overexpressed in hepatocellular carcinoma cells and was proposed to enhance cancer progression by suppression of p53 activity [24,25]. The Cys247 residue, which was essential for persulfide dioxygenase activity of ETHE1 as revealed herein, is exposed to the protein surface [6], so the Cys247 residue in the ETHE1

protein may become a new target for therapeutic drugs for ethylmalonic encephalopathy and hepatocellular carcinoma.

In the past decade, several studies reported protein polysulfidation for specific Cys residues by different experimental techniques including modified tag-switch assays, MS-based methods, and a pull-down procedure [10,11,18,19]. These methods are based on the nucleophilicity of protein polysulfides, but our preliminary experiments showed that the structure and chemical reactivity of protein polysulfides are more complicated than previously thought (unpublished data). Therefore, we developed a convenient method for detection of polysulfidated proteins, which we described herein, by using BPM and several alkylating reagents (Fig. 3A). With this technique, we can quantify protein polysulfide levels as a difference in band mobility on SDS-PAGE. As Fig. 3B shows for the recombinant protein prepared by using an *Escherichia coli* cell expression system, the lane for the BPM alone treatment showed a band at the top (band fully shifted up), which was affected only moderately by blocking with mild electrophilic reagents such as 8NcG, ASBT, and IAM, with almost the same or slightly lower mobility. The groups blocked by strong electrophilic reagents such as DTP,

DTNB, and PCMB, however, had bands at the bottom of the gel, which suggested that these strong electrophiles caused decomposition of the polysulfide structure, thus resulting in complete inhibition of the second reaction of BPM labeling. PMSA demonstrated that the endogenous ETHE1 protein in A549 cells was also extensively polysulfidated at almost all Cys residues (Fig. 3C). These findings strongly suggested that protein polysulfidation commonly occurs in *E. coli* and humans.

Although our method developed herein is quite convenient and simple, false-negative reactions may occur. The bands that were shifted up by BPM treatment in the group with mild electrophilic reagents may result from an incomplete blocking reaction. Therefore, confirmation with MS is required to unequivocally identify protein polysulfidation.

In conclusion, we developed two novel methods for measurement of ETHE1 activity and detection of polysulfidated proteins. With these techniques, we discovered that the Cys247 residue in ETHE1 plays essential roles in both the enzymatic activity of ETHE1 and maintenance of polysulfidation of the whole protein. Additional experiments to clarify the molecular mechanism of such extensive protein polysulfidation will provide

insights and knowledge about the true physiological function of ETHE1 and ethylmalonic encephalopathy and even reactive polysulfide formation occurring ubiquitously in cells and tissues in various organisms.

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Conflicts of Interest

The authors declare that no conflicts of interest are associated with this study.

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Figure legends

Fig. 1. Enzymatic activity of human ETHE1. (A) Substrate specificity of ETHE1 dioxygenase detected by quantitative LC-ESI-MS/MS. Enzymatic reactions were performed in the presence of GSH polysulfides with or without recombinant ETHE1 protein for 15 min. After incubation, the polysulfide content in the reaction mixture was measured by using LC-ESI-MS/MS. (B) SSP2 assay for measurement of ETHE1 activity. ETHE1 (0.2, 2, 10, 20 µg/ml) was incubated with GSH/Cys/HCys polysulfides as described above, and polysulfide levels in the mixture were detected by using the reactive persulfide-sensitive fluorescent probe SSP2. Results are means ± SD (n = 3). * $P < 0.05$, ** $P < 0.01$ (unpaired Student's t -test).

Fig. 2. Enzymatic activities of wild-type and Cys mutants of human ETHE1. After 15-min incubation with wild-type ETHE1 or Cys mutants in the presence of GSH polysulfide, polysulfide levels in the reaction mixture were measured via the SSP2 assay. We defined 1 unit of activity as consumption of 1 µmol polysulfide in 15 min. Results are means ± SD (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (vs. wild type, one-way

ANOVA with the Newman-Keuls post-hoc test).

Fig. 3. Detection of ETHE1 polysulfidation. (A) Schematic diagram of PMSA for identification of polysulfidated proteins. In the initial step, both protein Cys thiol (-SH) and polysulfide $-(S)_n-H$ functional groups are alkylated by blocking reagent (Reaction 1). In the second reaction with Biotin-PEG₃₆-MAL (BPM), the pegylation probe reacted with polysulfidated Cys residues that contained additional sulfur atoms (Reaction 2). (B, C) Protein polysulfidation of recombinant ETHE1 protein and endogenous ETHE1 protein expressed in A549 cells. After PMSA treatment, signals were detected by using CBB staining (B) or Western blotting with anti-ETHE1 antibody (C). Numbers on the gels indicate the number of BPM labels in the protein, indicative of the number of polysulfidated Cys residues in the protein. 8NcG: 8-nitroguanosine 3',5'-cyclic monophosphate; ASBT: 2-aminosulfonyl benzothiazole; MSBT: 2-methylsulfonyl benzothiazole; IAM: iodoacetamide; MBB: monobromobimane; MMTS: methyl methanethiosulfonate; NEM: *N*-ethylmaleimide; DTP: 2,2'-dithiopyridine; DTNB: 5'-dithiobis(2-nitrobenzoic acid); PCMB:

p-chloromercuribenzoic acid.

Fig. 4. Protein polysulfidation of Cys-to-serine mutants. (A) Detection of protein polysulfidation in Cys-to-serine mutants. Protein polysulfidation of wild-type and Cys mutants of human ETHE1 was identified by using PMSA with ASBT and IAM. (B) Polysulfidation of ETHE1 proteins (wild type and C247S mutant) was identified via LC-ESI-MS/MS. Representative LC-ESI-MS/MS chromatograms of endogenous Cys and CysSSH residues of ETHE1, with isotope-labeled internal standards (left panel). The ratio CysSSH/Cys residue (% of wild type) is shown for each ETHE1 protein (right panel). Results are means $\pm$ SD (n = 3). * P < 0.01 (unpaired Student's *t*-test).

Figure

Figure 1

Figure 2

Figure 3

Figure 4