

REVIEW ARTICLE

Persulfide synthases that are functionally coupled with translation mediate sulfur respiration in mammalian cells

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Received 14 February 2018; **Revised** 16 April 2018; **Accepted** 26 April 2018

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Cysteine persulfide and polysulfide are produced in cells and exist in abundance in both low MW and protein fractions. However, the mechanism of regulation of the formation of cellular cysteine polysulfides and the physiological functions of cysteine persulfides/polysulfides produced in cells are not fully understood. We recently demonstrated that cysteinyl-tRNA synthetase (CARS) is a novel cysteine persulfide synthase. CARS is involved in protein polysulfidation that is coupled with translation. In particular, mitochondria function in biogenesis and bioenergetics is also supported and up-regulated by cysteine persulfide derived from mitochondrial CARS (also known as CARS2). Here, we provide an overview of recent advances in reactive persulfide research and our understanding of the mechanisms underlying the formation and the physiological roles of reactive persulfides, with a primary focus on the formation of cysteine persulfide by CARS and the most fundamental mitochondrial bioenergetics mediated by persulfides, that is, sulfur respiration.

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Abbreviations

8-nitro-cGMP, 8-nitroguanosine 3,5-cyclic monophosphate; CARS, cysteinyl-tRNA synthetase; CBS, cystathionine β -synthase; CPERS, CysSSH synthase; CSE, cystathionine γ -lyase; CysSSH, cysteine persulfide; Cys-tRNA, cysteinyl-tRNA; Drp1, dynamin-related protein 1; EcCARS, *Escherichia coli* CARS; ETC, electron transport chain; GSSH, glutathione persulfide; HPE-IAM, β -(4-hydroxyphenyl)ethyl iodoacetamide; K_{cat} , catalytic rate constant; KO, knockout; NRF2, nuclear factor-erythroid-2-related factor 2; PLP, pyridoxal phosphate; PUNCH-P, puromycin-associated nascent chain proteomics; PUNCH-PsP, PUNCH-P modified for polysulfides proteomics; RNS, reactive nitrogen oxide species; WT, wild type

Introduction

Reactive persulfide species including cysteine persulfide (CysSSH) are found in abundant quantities in both prokaryotic and eukaryotic cells (Toohey, 2011; Fukuto *et al.*, 2012; Ida *et al.*, 2014; Shimizu, *et al.*, 2017; Peng, *et al.*, 2017; Akaike *et al.*, 2017). Various forms of persulfide/polysulfide species exist in cells as cysteine-containing low MW compounds such as glutathione persulfide (GSSH) and CysSSHs in proteins. Persulfide/polysulfide species have a unique redox property that differs from that of simple thiols because of the additional sulfur atoms. CysSSH can act as a strong nucleophile and an antioxidant and may play an important role in regulating oxidative stress and redox signalling in cells (de Beus *et al.*, 2004; Mustafa *et al.*, 2009; Vandiver *et al.*, 2013; Ida *et al.*, 2014; Gao *et al.*, 2015; Yang *et al.*, 2015; Kasamatsu *et al.*, 2016). ROS are produced during inflammation and infection and induce oxidative stress. ROS and reactive nitrogen oxide species (RNS), which are produced by the reaction of ROS and NO, cause various chemical modifications such as oxidation and nitration of biomolecules, which results in cell damage (Beckman *et al.*, 1990; van der Vliet and Cross, 2000; Halliwell, 2007). Some electrophiles that depend on ROS and RNS for formation, however, are endogenous signalling molecules and mediate redox signalling (Forman *et al.*, 2004; Sies, 2014). These electrophilic signalling molecules include nitro derivatives and oxidized derivatives of fatty acids and 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) (Sawa *et al.*, 2007; Uchida and Shibata, 2008; Rudolph and Freeman, 2009; Schopfer *et al.*, 2011). We have studied the signalling mechanisms and physiological roles of 8-nitro-cGMP and found that the signalling by this compound is regulated by CysSSH in cells. CysSSH effectively reacted with 8-nitro-cGMP to form 8-SH-cGMP and thereby nullified the electrophilic signalling activity of 8-nitro-cGMP (Nishida *et al.*, 2012; Ida *et al.*, 2014; Ihara *et al.*, 2017). We developed a quantitative analytical system for the persulfide/polysulfide species of cysteine and related compounds that involved LC-MS-MS and found that different amounts of various persulfide/polysulfide species were produced in mammalian cells (Ida *et al.*, 2014). We identified two transsulfuration enzymes, **cystathionine β -synthase (CBS)** and **cystathionine γ -lyase (CSE)**, that participate in endogenous CysSSH formation (Nishida *et al.*, 2012; Ida *et al.*, 2014). CBS and CSE can catalyse CysSSH production via C-S cleavage of cysteine. It was also found that appreciable amounts of CysSSH exist in different cellular proteins. We recently demonstrated, for the first time, that cysteinyl-tRNA synthetase (CARS) can act as a novel CysSSH-producing enzyme (Akaike *et al.*, 2017). Biochemical analyses revealed that CARS can produce CysSSH from a substrate cysteine. CARS can also mediate direct incorporation of CysSSH into proteins during translation, which results in the formation of protein persulfides and polysulfides. Of great importance is our discovery that CysSSH-producing activity is critically involved in the regulation of mitochondrial function – a new physiological role of persulfide/polysulfide (Figure 1). This article provides an overview of recent advances in reactive persulfide research and discusses our understanding of the formation mechanism and physiological roles of reactive persulfides, with a primary focus on unique mechanisms of CARS-

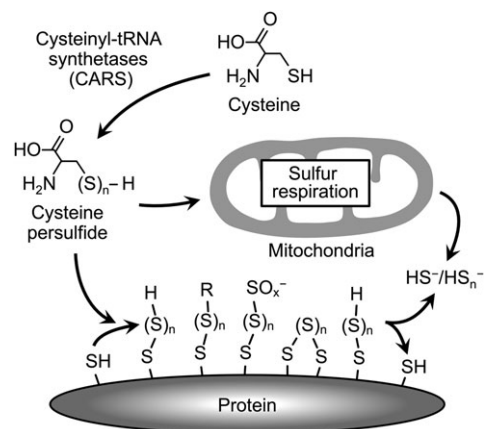


Figure 1

CysSSH formation, protein polysulfidation and regulation of mitochondrial function mediated by the novel CysSSH-producing enzyme CARS. CARS produces CysSSH from cysteine. CysSSH-producing activity of CARS is critically involved in translation-coupled protein polysulfidation. Protein polysulfidation may be involved in the regulation of protein functions, such as expression of enzyme activity and maintenance of protein structure. CysSSH produced by mitochondrial CARS2 mediates the sulfur-mediated bioenergetics in mitochondria (sulfur respiration). The molecular mechanisms underlying sulfur respiration are shown in Figure 5.

dependent CysSSH production and the fundamental versatile physiological functions of reactive persulfides in various organisms, for example, sulfur respiration in mitochondria.

Unique reactive properties of cysteine persulfide and protein polysulfides

CysSSH has unique reactive properties that distinguish it from parental **cysteine**. Persulfide/polysulfide species of cysteine possess both nucleophilic and electrophilic properties (Fletcher and Robson, 1963; Parker and Kharasch, 1959; Abdolrasulnia and Wood, 1980). On the basis of potential nucleophilicity of polysulfides as well as their nucleophilicity of thiol residues, we can detect various polysulfide species present in cells and differentiate them from parental thiols. We recently developed a convenient method to detect proteins bearing persulfide/polysulfide moieties that is based on the biotin-polyethylene glycol-conjugated maleimide (biotin-PEG-MAL) labelling gel shift assay (PMSA) (Jung *et al.*, 2016). With this PMSA, we demonstrated such proteins in *Escherichia coli* and mammalian cells. To establish a selective and quantitative detection method for low MW persulfides/polysulfides and protein persulfides/polysulfides, we developed a detection method that utilizes LC-electrospray ionization (ESI)-MS-MS and utilized β -(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) as a trapping agent (Figure 2A) (Numakura *et al.*, 2017; Akaike *et al.*, 2017). HPE-IAM has a mild electrophilicity and forms stable adducts with persulfides/polysulfides so that we can avoid artificial degradation of polysulfide structures during the labelling process. By using this selective and quantitative LC-ESI-MS-MS

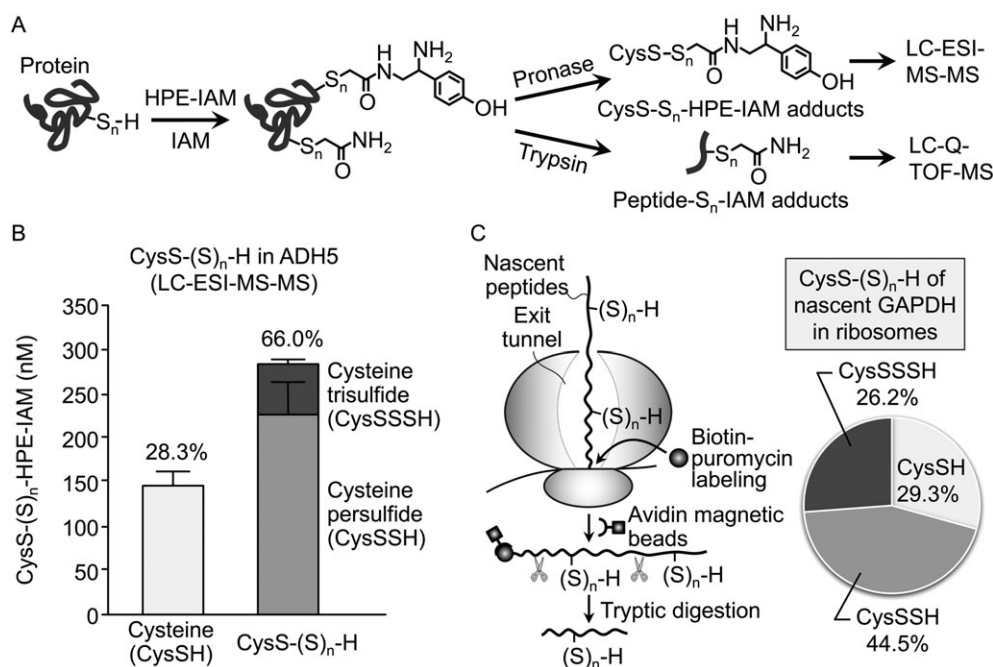


Figure 2

(A) Schematic illustration of specific and quantitative analytical systems for protein polysulfidation that used HPE-IAM labelling and MS. Polysulfidated cysteine residues in proteins were modified and stabilized with HPE-IAM or IAM. Amounts of cysteine polysulfides were quantified by LC-ESI-MS-MS analysis of proteinase-digested protein samples. The sites of polysulfidated cysteine residue in proteins were determined with LC-quadrupole-time-of-flight-MS using trypsin digests of various proteins. (B) Quantitative identification of cysteine polysulfide levels in recombinant ADH5 protein by using HPE-IAM labelling and LC-ESI-MS-MS. In ADH5 protein, the level of polysulfidated cysteine (right column) was much higher than that of non-polysulfidated cysteine (left column), which suggested extensive polysulfidation in ADH5 protein. (C) Schematic illustration of a new method for identification of protein polysulfidation in the nascent peptide PUNCH-PsP (left), and the level of cysteine polysulfides incorporated into the GAPDH nascent peptide as determined by PUNCH-PsP (right). In PUNCH-PsP, newly synthesized peptides were labelled with biotin-puromycin and collected with avidin-magnetic beads. Levels of cysteine polysulfides were determined, using the analytical systems shown in (A).

analysis, we found that various proteins, such as alcohol dehydrogenase 5 (ADH5) and GAPDH, are highly polysulfidated in cells. For example, LC-ESI-MS-MS analysis, after pronase digestion of HPE-IAM-labelled ADH5, demonstrated that 70% of cysteine residues in ADH5 were endogenously polysulfidated (Figure 2B). Additional LC-quadrupole-time-of-flight-MS analyses identified sites of polysulfide formation and the sulfur chain length in each protein. Recent studies suggested that protein polysulfidation may be involved in the regulation and maintenance of the activity of some enzymes (Jarosz *et al.*, 2015; Jung *et al.*, 2016; Millikin *et al.*, 2016). Further investigation using analytical systems we developed for persulfide/polysulfide stated above will contribute to clarify the role of protein polysulfidation in redox metabolism signalling (Akaike *et al.*, 2017).

Protein polysulfidation mediated by CARS

As mentioned above, our PMSA and LC-MS-MS analyses clearly showed that different types of proteins are highly polysulfidated endogenously (Ida *et al.*, 2014; Ono *et al.*, 2014; Doka *et al.*, 2016; Jung *et al.*, 2016; Akaike *et al.*,

2017). To understand how protein polysulfidation occurs in cells, we studied whether CARS can catalyse direct incorporation of CysSSH/Cys-(S)_n-H into tRNA. CARS is an enzyme that produces cysteinyl-tRNA (Cys-tRNA) via cysteine and aminoacyl-tRNA (Carter *et al.*, 1993; Woese *et al.*, 2000; Guo *et al.*, 2010). Our recent work revealed for the first time that CARS from *E. coli* (EcCARS) produces CysSSH-tRNA by using CysSSH as a substrate. CysSSH-tRNA can be a substrate for protein synthesis in ribosomes as CysS-tRNA and can lead to translation-coupled protein polysulfidation of newly synthesized proteins. This translation-coupled co-translational protein polysulfidation is clearly shown by puromycin-associated nascent chain proteomics (PUNCH-P), as recently reported (Aviner *et al.*, 2014), with PUNCH-P being modified for polysulfide proteomics (PUNCH-PsP) (Figure 2C) (Akaike *et al.*, 2017). This PUNCH-PsP approach allowed us to identify intact forms of CysS-(S)_n-H residues in nascent peptides of GAPDH present only within *E. coli* ribosomes. In fact, the PUNCH-PsP we conducted showed significant polysulfidation at the Cys²⁴⁷ residue of the nascent peptides of GAPDH protein immediately after the synthesis in the ribosomes in *E. coli* cells. All native forms of cysteine, CysSSH and CysSSH residues were also efficiently recovered from native whole GAPDH protein, and polysulfidation affected

more than 60% of the Cys²⁴⁷ residues of the mature protein. Thus, the extensive and prevalent cysteine polysulfidation is now found to co-translationally occur in ribosomes and continues to be physiologically maintained in mature proteins in cells.

In addition, we confirmed a novel function of EcCARS as a CysSSH synthase (CPERS) by showing its catalytic activity in generating CysS-(S)_n-H, for example, CysSSH and CysSSSH, from the substrate cysteine. The CPERS activity of EcCARS depends partly on **pyridoxal phosphate (PLP)** but not on **ATP** and tRNA, the latter two being required for Cys-tRNA biosynthesis by EcCARS. We also performed a stable isotope (³⁴S) tracer experiment combined with LC-MS-MS analysis in an attempt to understand the mechanism of cysteine polysulfidation catalysed by CARS. Our analyses clearly showed cleavage of a sulfur atom from one cysteine and its transfer to another cysteine to form CysSSH (Figure 3).

CARS as a CysSSH-producing enzyme

CARS is involved in the translation process as a canonical function by catalysing Cys-tRNA synthesis. Our rigorous investigation of the mechanism of protein polysulfidation demonstrated that CARSs also have CPERS activity and are critically involved in protein polysulfidation (Akaike *et al.*, 2017). Some aminoacyl-tRNA synthetases reportedly function in physiological processes beyond translation, known as moonlighting functions of aminoacyl-tRNA synthetases (Wakasugi and Schimmel, 1999; Guo *et al.*, 2010). Therefore, CPERS activity in CARS is regarded as a relatively new function of aminoacyltransferase beyond translation. In fact, CARS efficiently produces CysSSH by using cysteine as a substrate, in a manner that is independent of the aminoacyltransferase reaction. Figure 3 shows the reaction mechanism of transsulfidation catalysed by CARSs that our recent study clarified. CARS has a very low K_M and a high catalytic rate constant K_{cat} , which indicates that EcCARS is quite efficient in producing CysSSH (Ono *et al.*, 2014; Yadav *et al.*, 2016). The K_{cat}/K_M value of CARS is almost equal to

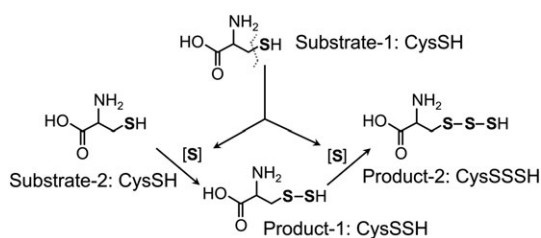


Figure 3

Diagram of the reaction mechanisms in the formation of CysSSH and polysulfides, as catalysed by CARSs. During the formation of cysteine persulfides, sulfur atoms are cleaved from donor cysteine (Substrate-1) and transferred to acceptor cysteine (Substrate-2) to form the cysteine persulfide (Product-1). Subsequent transfer of sulfur to Product-1 forms cysteine trisulfide (Product-2). CysSH, cysteine; CysSSSH, cysteine hydrotrisulfide.

the value of EcCARS. However, CSE and CBS utilize only **cystine** (not cysteine) as a substrate, which is quite distinct from CARSSs, which use cysteine (not cystine) for CysSSH production (Figure 4) (Ida *et al.*, 2014). Intracellular cystine concentrations are usually at low micromolar or submicromolar levels, which are much lower than the K_M value of CSE (more than 200 μ M). Thus, CSE may play a minor role in directly producing CysSSH under physiologically relevant conditions. Also, the cystine/CSE reaction may not outcompete reactions with other major enzymes that metabolize cystine and GSH, which can strongly interact with and consume cysteine under physiological baseline conditions in cells and in various organs. In fact, the intracellular cysteine concentration is reportedly 100–1000 μ M (Ida *et al.*, 2014), which is apparently much higher than the K_M of CARS. Also, intracellular levels of cysteine are regulated by the uptake of cystine from the extracellular space, which is mediated by **xCT, the cystine-glutamate transporter**. Expression of this xCT transporter is regulated by the transcriptional factor nuclear factor-erythroid-2-related factor 2 (NRF2) (Sasaki *et al.*, 2002). Thus, NRF2 may regulate CysSSH production by CARS through the regulation of intracellular cysteine levels, which is mediated by the xCT transporter. Therefore, it is quite reasonable to suggest that CARS may be a major enzyme responsible for endogenous generation of CysS-(S)_n-H (Figure 4).

CysSSH production by EcCARS depended on the addition of PLP. Proteomic analyses suggested that several preferential

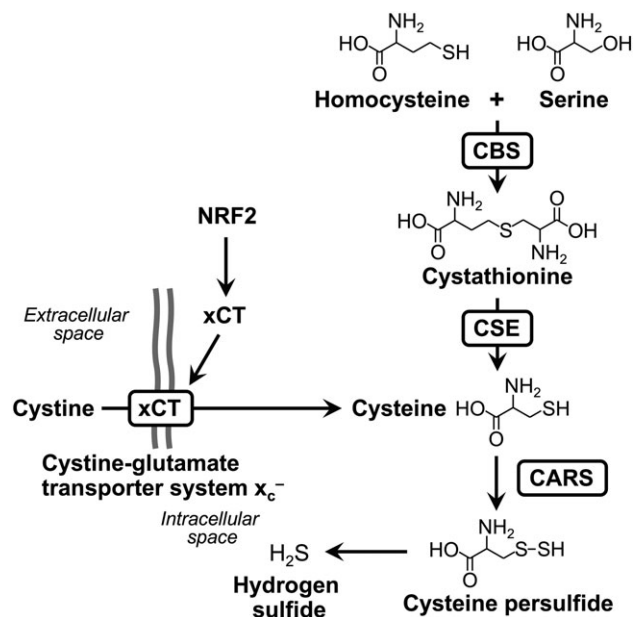


Figure 4

Diagram of the mechanisms involved in the cellular formation of CysSSH and H₂S and regulation of this formation by CARS, CBS and CSE. CBS and CSE are involved in cysteine biogenesis, though the canonical trans-sulfuration pathway. Cystine transport by cystine-glutamate transporter system, mediated by xCT, also contributes to the regulation of intracellular cysteine levels. Cysteine biosynthesis and cystine uptake affect cysteine persulfide production by CARS via modulation of cellular cysteine levels.

sites exist for PLP binding in EcCARS, including lysine residues at the ⁷³KIIK⁷⁶ and ²⁶⁶KMSK²⁶⁹ motifs (Akaike *et al.*, 2017). Sequence data obtained previously suggested that several lysine residues, at the KIIK and KMSK motifs, are highly conserved in EcCARS and other homologues in different organisms, including mammals (Akaike *et al.*, 2017). Two well-conserved cysteine residues are bound to Zn²⁺ at the active centre for tRNA aminoacylation (Zhang *et al.*, 2003). To determine the role of PLP bound to EcCARS, we generated several lysine mutants of this enzyme, with which we measured CysS-(S)_n-H formation and translation. We found greatly reduced CysSSH and CysSSSH synthesis in various lysine-to-alanine mutants, compared with the wild type (WT), at K73A, K76A, K266A, K269A, and double mutants K73/76A and K266/269A, of EcCARS. All these mutant enzymes had intact protein synthesis potential as determined in the PUREflex cell-free protein synthesis assay (Akaike *et al.*, 2017). The amounts of PLP bound to EcCARS were quantified by using LC-ESI-MS-MS with 2,4-dinitrophenylhydrazine. This 2,4-dinitrophenylhydrazine-labelling LC-MS-MS analysis revealed that the amounts of PLP bound to the enzymes decreased in lysine mutants compared with the WT and, more important, that PLP levels correlated well with persulfide-producing activities. Cysteine-to-aspartate mutants such as C28D (and C28S) and the double mutant C28/209D, however, still had high persulfide production, similar to WT persulfide production, although these mutants had greatly reduced protein synthesis and translational activity (Akaike *et al.*, 2017).

The three-dimensional EcCARS structure constructed by computer modelling supported the finding that PLP binds to specific lysine residues at the ⁷³KIIK⁷⁶ and ²⁶⁶KMSK²⁶⁹ motifs of EcCARS. The computer-based prediction suggested two possible PLP-binding sites at K73 and K269 of the EcCARS KIIK and KMSK motifs. In addition, this computer modelling indicated that PLP-bound motifs have a vicinal location within 10–20 Å but different from that of the ATP-binding HIGH motif and the Zn²⁺-binding active site of EcCARS for Cys-tRNA biosynthesis. A comparable change in PLP binding capacity and stability seems to exist, caused by mutation of any of the four lysine residues, because each lysine mutation at the KIIK and KMSK motifs affected EcCARS synthesis of all CysS-(S)_n-H to a great degree. One conceivable reason for the comparable effect is that PLP may need more than one lysine residue, rather than just one lysine binding, to realize the stable binding and full catalytic activity of CARS and thus act as CPERS during formation of CysS-(S)_n-H. That is, PLP may need to be stabilized by binding to multiple lysine residues to achieve stable binding and demonstrate potent catalytic activity. Otherwise, reactive persulfides derived from CARS, because of their nucleophilic nature, will quickly degrade an aldehyde group of PLP, which may block the catalytic activity of PLP even properly bound to these particular lysine residues of CARS. The computational structural analysis showing close locations (within 20 Å) of these lysine residues in the KIIK and KMSK motifs supports this interpretation. These data together indicate that EcCARS has a moonlighting function as a CPERS, which is independent of its original catalytic function as an aminoacyl-tRNA synthetase (Akaike *et al.*, 2017).

CPERS activity of mammalian CARSs

CPERS activity of CARSs can be observed not only in bacterial CARS (EcCARS) but also in mammalian CARSs (Akaike *et al.*, 2017). Two different mammalian CARSs have been found, one located in the cytosol, CARS1, and the other, CARS2, in the mitochondria (Hallmann *et al.*, 2014; Coughlin *et al.*, 2015). We now know that both CARSs (mouse CARS1 and human CARS2) show strong CysS-(S)_n-H-producing activities that are dependent on PLP. In fact, the CPERS activity of CARS2 correlates well with the cellular PLP levels and that CARS2 activity can be modulated by exposing the enzyme to different concentrations of PLP (Akaike *et al.*, 2017). We utilized CARS1-deficient and CARS2-deficient HEK293T cells generated via the CRISPR/Cas9 system to determine how much cellular CysS-(S)_n-H originated from CARS1 and CARS2. Although CARS1-knockout (KO) cells are not yet available, CARS2 KO cells have been successfully established. Activity of one of the clones, with a 30 bp deletion and a 8 bp insertion downstream of the translation-initiating codon in the CARS2 first exon, was analysed by using LC-MS-MS methods and the results showed that CysS-(S)_n-H and GSSH levels were significantly decreased in CARS2 KO cells, implying that CARS2 was a major producer of persulfide/polysulfide species. Although low but detectable levels of CARS2 were still seen in CARS2 KO cells, the cells were also treated with siRNA against CARS2, and this treatment caused decreases of 67 and 42% in CysSSH and GSSH levels respectively. After knockdown of CARS1 in CARS2 KO cells, CysSSH decreased only slightly, thus indicating a predominant role of CARS2 as a CPERS.

Adding back WT CARS2 in CARS2 KO cells significantly increased persulfide levels. The CARS2 C78/257D mutant restored persulfide production in CARS2 KO cells, but the K124/127A and K317/320A mutants (mutants of KIIK and KMSK motifs, respectively) did not have this effect. CARS2 KO cells had greatly reduced Cys-tRNA synthetase activity and adding back the C78/257D mutant did not increase the Cys-tRNA synthetase activity of the KO cells, as shown by expression of mitochondrial cytochrome *c* oxidase subunit 1 (MTCO1, encoded by mitochondrial DNA), although full CPERS activity was restored. These data confirm that CARS2 acts as a CPERS in mammals and that this activity is distinct from CARS activity.

We also investigated the contributions of CSE and CBS to endogenous persulfide production. Silencing CSE and CBS significantly decreased intracellular cysteine levels, which in turn reduced persulfide production. In CARS2 KO cells, CSE and CBS knockdown also led to lower cysteine levels but not to lower persulfide production. These findings show that the metabolic pathways mediated by CSE/CBS in each cell line provide cysteine, regardless of CARS2 expression. In other words, CBS and CSE are involved in the cysteine biosynthesis rather than CysSSH production under physiological conditions. Furthermore, almost two-thirds of CysSSH appears to be supplied by CARS2 in HEK293T cells, as shown by the corresponding decrease in CysSSH levels. The remaining CysSSH in CARS2 KO cells is not obtained via CSE/CBS expressed in HEK293T cells, because no additional reduction in CysSSH occurred even after CSE/CBS knockdown in CARS2 KO cells. CSE and CBS therefore most likely do not participate

directly in persulfide production but instead may support cysteine biosynthesis and the supply of cysteine to CARS, at least under physiological conditions (Figure 4).

Cars2-deficient mice revealing CPERS activity of CARSs *in vivo*

We investigated the CPERS actions of CARS2 *in vivo* by generating *Cars2*-deficient mice via CRISPR/Cas9 technology (Akaike *et al.*, 2017). We designed a guide RNA against exon 1 of *Cars2*. We also developed a mutant mouse line with a mutant *Cars2* allele (line 1) that had a 200 bp deletion with a translation-initiating codon in exon 1. Although the *Cars2*^{-/-} deletion was embryonically lethal, the heterozygous *Cars2*^{+/-} strain of mice are usually born without any abnormal macroscopic appearances or growth profiles, found during a 6 month observation period after birth. These mice expressed only half the mitochondrial CARS2 protein and a markedly decreased CysSSH production, relative to the WT mice. Because no substantial change in mitochondrial DNA-encoded MTCO1 was noted, we believe that *Cars2*^{+/-} mice had intact Cys-tRNA synthetase activity. Sulfide metabolites in the liver of *Cars2*^{+/-} mice and their WT littermates were quantified via LC-MS-MS analysis and HPE-IAM, as described earlier. Compared with their WT littermates, these *Cars2*^{+/-} mice exhibited a marked difference in persulfide production. In major organs of *Cars2*^{+/-} mice, compared with those of WT mice, endogenous levels of CysSSH and its derivatives – GSSH, sulfite, thiosulfate and hydropolysulfides – were decreased by almost 50%. We also developed another strain of *Cars2*^{+/-} mice (line 2) with an alternative guide RNA that targeted *Cars2* exon 3 to eliminate the possibility of off-target effects by the guide RNA that we used to produce line 1 *Cars2*^{+/-} mice. Line 2 *Cars2*^{+/-} mice had phenotypes, almost identical to those of line 1 mice.

The finding that *Cars2*^{+/-} mice demonstrated an approximate 50% reduction in CysSSH should be emphasized, inasmuch as it suggests that *Cars2* contributes almost all of the CysSSH production in mouse tissues under physiological conditions. This suggestion is confirmed by the fact that *Cars2* modification did not change the expression of other sulfide-metabolizing enzymes, such as CSE, CBS and **3-mercaptopyruvate sulfur transferase**. Our relatively long-term observation for less than 6 months with *Cars2*^{+/-} mice did not show any difference in their macroscopic appearance, compared with the WT mice.

To investigate whether the mitochondrial CARS isoform CARS2 can generate CysSSH and provide it to the cell, we isolated mitochondria from mouse liver and determined the release of *de novo*-synthesized CysSSH from these organelles. Mitochondria did indeed release a large amount of CysSSH, which lends support to the idea that CysSSH that is generated in mitochondria is discharged into the cytoplasm and sustains protein polysulfidation. CysSSH generated from whole-cell proteins was decreased in *Cars2*^{+/-} mice, but cysteine levels were affected. In particular, production of 20–30% of CysSSH in all cellular proteins (polysulfidation) depended on CARS2 expression, not only in an *in vivo* study using *Cars2* KO mice but also in an *in vitro* experiment with cell cultures,

as shown by HPE-IAM labelling LC-MS-MS analysis with isolation of whole cell and tissues proteins. These findings suggest that CysSSH generated from CARS2 contributes significantly to polysulfidation, which appears to be mediated by post-translational and co-translational processes, the former being controlled by the thioredoxin–thioredoxin reductase system, as recently reported (Doka *et al.*, 2016). On the basis of these data, we expect that CysSSH produced in mitochondria is released into the cytoplasm and provides sulfur to proteins for polysulfidation. This evidence was the first demonstration that positively confirmed CARS2 as the major CPERS in mammals.

Regulation of mitochondrial functions by reactive persulfide species

Importantly, our recent study also demonstrated novel physiological roles of CARS as a CPERS during regulation of mitochondrial functions (Figure 5) (Akaike *et al.*, 2017). *CARS2* KO cells showed greatly modified mitochondrial morphological features (i.e. shrunken or fragmented appearance), which improved considerably when CARS2 was added back, as shown by MitoTracker Red fluorescence staining, transmission electron microscopy and immunofluorescence staining for translocase of outer mitochondrial membrane 20 and CARS2. Unlike other lysine mutants tested, WT CARS2 and the C78/258D mutant exhibited a much improved mitochondrial morphology. Related to these results, deletion of the CARS2-activated dynamin-related protein 1 (Drp1), a primary mitochondrial fission mediator (Akhtar *et al.*, 2016), and adding back WT CARS2 and the C78/257D mutant, but not the K317/320A mutant, significantly attenuated Drp1 GTPase activity, thus generating CysSSH without CARS

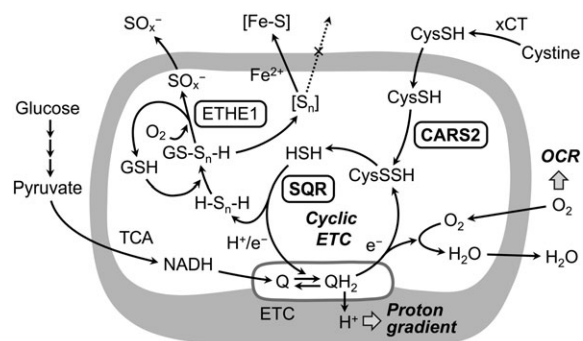


Figure 5

Regulation of the mitochondrial ETC mediated by CARS2 and CysSSH. CysSSH produced by CARS2 in mitochondria is reductively metabolized to CysSH and HS⁻, which may be further oxidized by the sulfide:quinone oxidoreductase (SQR), in a manner linked to ETC in mitochondria (cyclic ETC). The CysS-(S)_n-H-dependent HS⁻ metabolism may be coupled with formation of iron–sulfur clusters (Fe–S), that is controlled by the mitochondrial ETC. Ethylmalonic encephalopathy protein 1 reportedly catalyses oxidative degradation of glutathione polysulfide (GS-S_n-H), whose formation is catalysed by SQR and related to cysteine persulfide production by CARS and cyclic ETC. Q/QH₂, ubiquinone/ubiquinol; TCA, tricarboxylic acid.

activity. Under normal culture conditions, Drp1 in HEK293T cells was extensively polysulfidated, as demonstrated by our new biotin-PEG-MAL capture method. Both *CARS2* KO and *CARS1/2* double-knockdown cells had markedly suppressed Drp1 polysulfidation. Drp1 is probably activated by chemical depolysulfidation or by a post-translational process maintained physiologically by the thioredoxin–thioredoxin reductase system, for example. We therefore established that Drp1 is an important signal effector molecule that is regulated in a reversible manner via a unique process involving polysulfidation and depolysulfidation.

Evidence has clearly shown the contribution of *CARS2* to the biogenesis and functioning of mitochondria. Specifically, mitochondrial DNA that was normalized by nuclear DNA decreased in *CARS2* KO cells but was restored by adding back WT *CARS2* and the C78/257D mutant but not the lysine mutants, which implied that *CARS2*-derived persulfide improved mitochondrial biogenesis. The membrane potential of mitochondria decreased in *CARS2* KO cells, but it was restored or increased after adding back or overexpressing the WT and the C78/257D mutant but not after using lysine mutants. We utilized extracellular flux analysis to determine the oxygen consumption rate (OCR) in HEK293T *CARS2* KO cells. This OCR in *CARS2* KO cells was approximately 50% of that in WT cells, a result that agrees with the incomplete elimination of *CARS2* protein and thereby reduced MTCO1 expression in *CARS2* KO cells. Introducing WT *CARS2* and the C78/257D mutant, but not the lysine mutant, restored the decreased OCR in *CARS2* KO cells. These observations led to the novel concept that *CARS2*-derived CysSSHs play an important role in the mitochondrial electron transport chain (ETC), which postulates a completely new and fundamental role of persulfides in maintaining mitochondrial bioenergetics.

During our efforts to clarify how *CARS2*-derived CysSSH supports mitochondrial bioenergetics, we noted a profile of human *CARS2* products in a cell-free enzyme reaction that was quite different compared with cellular *CARS2* metabolism in HEK293T cells in culture. *CARS2* synthesized primarily CysSSH/CysSSH in cell-free solutions but, in HEK293T cells, there was preferential formation of the hydrosulfide ion HS⁻ (from H₂S) together with thiosulfate, rather than formation of CysSSH. We therefore hypothesized that the mitochondrial compartment serves as a unique metabolic environment for additional metabolism of *de novo* CysSSH synthesized by *CARS2*, perhaps being coupled with the mitochondrial ETC.

An investigation of the metabolic profile of CysSSH and its derivatives in HEK293T cells led to identification of a close relationship between *CARS2*-dependent CysSSH production and ETC function. We used two methods to inhibit the cellular ETC and thereby to induce loss of mitochondrial DNA. In one, the specific inhibitor of complex III antimycin A was used and in the other, the ETC disrupter ethidium bromide was used. Both ETC treatments resulted in significantly increased CysSSH production and simultaneously reduced HS⁻ production, as determined by HPE-IAM labelling LC-MS-MS analysis. These opposite, stoichiometric relationships between CysSSH and H₂S formation led to the belief that conversion of CysSSH to H₂S depends on ETC activity and is mediated by the ETC in the cells. It is conceivable, therefore,

that CysSSH generated from *CARS2* in mitochondria is reduced by accepting an electron from the ETC, thus generating H₂S.

These findings also provide strong support for the involvement of the *CARS2*-CysSSH pathway in mitochondrial function because *CARS2*-dependent CysSSH production is integrated into and tightly linked to the mitochondrial ETC, which is itself implicated in energy metabolism. Studies have found that low concentrations (nM range) of H₂S maintained ETC function that may have been mediated by the sulfide: quinone reductase and other enzymes that oxidize sulfides to thiosulfate (Grieshaber and Völkel, 1998; Griesbeck *et al.*, 2002; Gubern *et al.*, 2007; Ono *et al.*, 2014; Szabo *et al.*, 2014; Hine *et al.*, 2015). However, how H₂S was supplied endogenously in mitochondria remained unclear. Our various studies suggest that CSE, CBS and 3-mercapopyruvate sulfur transferase are not primary H₂S sources in mitochondria in different mammalian cell lines and *in vivo* in mice (Nishida *et al.*, 2012; Morikawa *et al.*, 2012; Ono, *et al.*, 2014; Shirozu *et al.*, 2014; Nakano *et al.*, 2015; Yadav *et al.*, 2016). In fact, our recent study was the first to confirm that HS⁻ (or H₂S) was formed indirectly from *CARS2* via CysSSH generation in the mitochondrial environment (Akaike *et al.*, 2017). In addition, another study found that CysSSH may be partly responsible for endogenous formation of iron–sulfur clusters (Takahashi *et al.*, 2017). Because iron–sulfur clusters are synthesized and utilized in complexes I–III of the ETC in mitochondria (Stehling and Lill, 2013) and are actively transported outside the mitochondria, CysSSH-dependent metabolism of HS⁻ may be tied to production of iron–sulfur centres of the mitochondrial ETC and cytosolic formation and maintenance of different iron–sulfur complex systems. Therefore, *CARS2* serves as a major CPERS, which aids mitochondrial biogenesis and bioenergetics.

As described above, the CysSSH metabolizing pathway is critically involved in mitochondrial bioenergetics in mammalian cells (Figure 5). This suggests bioenergetics mediated by sulfur, instead of being mediated by oxygen, which is a catabolic process known as sulfur respiration in bacteria. Some photosynthetic bacteria derive their energy from sulfur compounds such as H₂S as an electron donor (Shimizu *et al.*, 2017). Also, sulfur-oxidizing bacteria (e.g. *Acidithiobacillus thiooxidans*) obtain energy by oxidising H₂S, using sulfide: quinone oxidoreductase (Yin *et al.*, 2014). In *Staphylococcus aureus*, reactive persulfides have been reported not only in cysteine but also in CoA, as CoA-SSH (Peng *et al.*, 2017). In this context, our finding is the first demonstration of sulfur respiration in mammalian cells, as shown in Figure 5 (Akaike *et al.*, 2017). The discovery of sulfur respiration in mammals may have major implications not only to fundamental biology, but also to disease pathogenesis related to energy metabolism in cancer and stem cell biology.

In conclusion, this overview of recent advances in reactive persulfide research describes our development of a new analytical approach to CysSSH quantification and our discovery of a new CysSSH-producing enzyme, *CARS*. We have also detailed the novel role of CysSSH produced by *CARS2* in the regulation of mitochondrial functions, including sulfur respiration. Co-translational protein-bound CysSSH formation may have an important role in protecting proteins from oxidative stress and in redox signal regulation. Continued

elucidation of the physiological functions of reactive persulfides formed from new persulfide-producing enzymes that we discovered will promote the development of new therapeutic agents for various diseases involving oxidative stress.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Acknowledgements

We thank J.B. Gandy for her excellent editing of the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research and Grants-in-Aid for Scientific Research on Innovative Areas (Research in a Proposed Research Area), from the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

Conflicts of interest

The authors declare no conflicts of interest.

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