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Reversible inactivation of yeast mitochondrial phenylalanyl-tRNA synthetase under oxidative stress

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ABSTRACT

Background: Under oxidative stress cytoplasmic aminoacyl-tRNA synthetase (aaRSs) substrate specificity can be compromised, leading to tRNA mischarging and mistranslation of the proteome. Whether similar processes occur in mitochondria, which are major cellular sources of reactive oxygen species (ROS), is unknown. However, relaxed substrate specificity in yeast mitochondrial phenylalanyl-tRNA synthetase (*Sc*mitPheRS) has been reported to increase tRNA mischarging and blocks mitochondrial biogenesis.

Methods: Non-reducing denaturing PAGE, cysteine reactivity studies, MALDI-TOF mass spectrometry, enzyme assay, western blot, growth assay, circular dichroism, dynamic light scattering and fluorescence spectroscopy were used to study the effect of oxidative stress on *Sc*mitPheRS activity.

Results: *Sc*mitPheRS is reversibly inactivated under oxidative stress. The targets for oxidative inactivation are two conserved cysteine residues resulting in reversible intra-molecular disulfide bridge formation. Replacement of either conserved cysteine residue increased viability during growth under oxidative stress.

Conclusion: Formation of intra-molecular disulfide bridge under oxidative stress hinders the $tRNA^{Phe}$ binding of the enzyme, thus inactivating *Sc*mitPheRS reversibly.

General significance: The *Sc*mitPheRS activity is compromised under oxidative stress due to formation of intra-molecular disulfide bridge. The sensitivity of *Sc*mitPheRS to oxidation may provide a protective mechanism against error-prone translation under oxidative stress.

1. Introduction

Cells require a balanced redox environment to function normally. The redox environment is maintained by tight regulation between reactive oxygen species (ROS) generation and detoxification of ROS by antioxidants. When this regulation is perturbed, oxidative stress disrupts cellular homeostasis leading to many disease conditions, including diabetes, cardiovascular disease, neurodegenerative disease and cancer [1]. Several studies have shown that ROS can cause specific modifications to proteins leading to alterations in protein functions [2,3,4]. Elevated levels of protein oxidation have been reported due to exposure to oxidative stress [5,6]. The identified oxidative modifications of proteins are mainly carbonylation, nitrosylation, methionine oxidation and cysteine oxidation [7,8,9]. Cysteine is one of the most sensitive amino acids in the proteome towards cellular oxidation, and plays dual and somewhat antagonistic roles under oxidative stress by transmitting ROS-derived signals as well as impairing protein function by thiol modification [2,4,10]. Protein thiols can undergo an array of oxidative modifications due to the electronic structure of the sulfur atom which allows multiple oxidation states (−2 to +6). In cysteine the sulfur atom of the thiol group is in the lowest oxidation state. Thiol modification of cysteine can be classified as a reversible or an irreversible modification. Reversible modification can be restored by thiol-disulfide exchange. Glutathionylation, nitrosylation, and intra-molecular disulfide bridge formation are some of the examples of reversible modifications of thiols. In contrast, irreversible modifications of cysteine lead to permanent damage to proteins. Cysteine residues oxidized to sulfinic acid (SO_2H) and sulfonic acid (SO_3H) are examples of

Abbreviations: aaRS, aminoacyl-tRNA synthetase; β-ME, β-mercaptoethanol; DLS, dynamic light scattering; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; GluRS 1, glutamyl-tRNA synthetase 1; mtMetRS, mitochondrial methionyl-tRNA synthetase; *Hs*mitPheRS, human mitochondrial phenylalanyl-tRNA synthetase; IAM, iodoacetamide; L-Phe, L-phenylalanine; NEM, *N*-ethylmaleimide; *Sc*mitPheRS, yeast mitochondrial phenylalanyl-tRNA synthetase; SO_2H, sulfinic acid; SO_3H, sulfonic acid; ThrRS, threonyl-tRNA synthetase; YPD, yeast extract-peptone-dextrose

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irreversible changes [11]. Mitochondria are a major cellular source of ROS production; the pumping of protons out from the matrix to the inter-membrane space during ATP production creates an alkaline environment in the matrix that impacts redox potential and thiol ionization of the matrix. Mitochondria are rich in protein exposed thiols, about 60–90 mM, which are thought to play an important role in protecting cells from oxidative damage that would otherwise detrimentally impact fundamental processes such as gene expression and protein synthesis [12].

The aminoacyl-tRNA synthetases (aaRS) are central components of the cellular protein synthesis machinery. AaRSs play a major role in restricting the mRNA translation error rate to $\sim 10^{-4}$ by selectively charging cognate tRNAs with the appropriate amino acids. However, mischarging of tRNA by near-cognate amino acids may sometimes occur due to structural similarities between substrates. Many aaRSs can repair mischarged tRNAs using proofreading activities conferred by specific editing domains. It was previously demonstrated that impairment of editing function may lead to severe mistranslation resulting in growth inhibition in bacteria [13,14], mitochondrial dysfunction in yeast [15] and apoptosis in mammalian cells [16]. However, some aaRSs lack editing domains, such as for example the organelle-specific phenylalanyl-tRNA synthetase (PheRS). PheRS is the most structurally diverse enzyme among the members of the aaRSs family. Whereas the eukaryotic cytoplasmic PheRS exists as a heterotetramer $(\alpha\beta)_2$ the mitochondrial PheRS is a monomer. The mitochondrial PheRS is believed to have originated from eubacteria and it is basically a chimera of the catalytic domain of the α subunit and the B8 domain of the β subunit of bacterial PheRS. In eukaryotes, the cytoplasmic and the mitochondrial counterpart of PheRS are both sequentially and structurally distant from each other [17,18]. For the mammalian organelle-specific PheRS, the lack of an editing domain is compensated for by the high specificity for cognate amino acid *versus* near non-cognate amino acid, resulting in one Tyr-tRNA^{Phe} mischarging per ~7300 Phe-tRNA^{Phe} synthesized [15]. Oxidative stress has been found to be one of the major causes of mistranslation [19,20]. It has been reported that H_2O_2 impairs the editing function of threonyl-tRNA synthetase (ThrRS) leading to serine misincorporation at threonine codons in *E. coli* [19]. Glutamyl-tRNA synthetase 1 (GluRS1) is inactivated when *Acidithiobacillus ferrooxidans* cells are treated with H_2O_2 [21]. The sensitivity of aaRS function and quality control to oxidative damage raises the question of how enzymes such as the mitochondrial PheRS, which lacks an editing domain and resides in a potentially ROS-rich environment, respond to oxidative stress. Here we show that *Sc*mitPheRS exhibits impaired enzymatic activity due to formation of a reversible intra-molecular disulfide bridge under oxidative stress that negatively impacts tRNA^{Phe} binding. We propose that this may act as a protective mechanism to prevent translational infidelity under oxidative stress. To the best of our knowledge, this is the first report for any aaRSs where intra-molecular disulfide bridge formation under oxidative stress affects the enzyme activity.

2. Materials and methods

2.1. Materials

Malachite green hydrochloride, ammonium molybdate tetrahydrate, dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ammonium bicarbonate, iodoacetamide (IAM) and yeast tRNA^{Phe} were purchased from Sigma-Aldrich. *N*-ethylmaleimide (NEM) was purchased from Calbiochem. Sequencing grade trypsin was purchased from Promega. Inorganic pyrophosphatase was purchased from Roche. Amicon® ultra-0.5 ml 30 K centrifugal filter unit was purchased from Millipore. All other materials were of analytical grade from Sisco Research Laboratory Pvt. Ltd., India and Merck, India.

2.2. Protein expression and purification

E. coli BL21(DE3)/pET16b producing His6-tagged *Sc*mitPheRS encoded by the MSF1 gene was a gift from R.A. Zimmermann (University of Massachusetts, Amherst, MA). Point mutations were introduced by site-directed mutagenesis using the Quik-Change procedure (Stratagene). Primers were obtained from Integrated DNA Technologies (IDT). *E. coli* BL21(DE3) cells containing wild-type (WT) *Sc*mitPheRS plasmid were grown in media containing ampicillin at 37 °C to an optical density of 0.6. Cells were then induced by 0.5 mM IPTG for 4 h. After 4 h cells were harvested, sonicated at 75% output with a Sonics Vibra-Cell™. The cell lysate was centrifuged at 75,000 × *g* for 1 h and the supernatant was applied to a nickel-nitrilotriacetic acid-agarose column. Column was washed with 25 mM imidazole buffer, and eluted with 250 mM imidazole buffer. Fractions containing *Sc*mitPheRS were checked for purity in 10% SDS-PAGE, pooled and dialyzed in 50 mM Tris-HCl pH-7.5, 5 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, and 10% glycerol for 48 h at 4 °C with four buffer changes. Cysteine variants were purified following the same procedure.

2.3. Malachite Green assay for ScmitPheRS enzyme activity

Recombinant *Sc*mitPheRS was mixed with substrates L-phenylalanine (L-Phe), ATP, and yeast tRNA^{Phe} in presence of different concentrations of DTT to generate pyrophosphate (PP_i). PP_i was then hydrolyzed with inorganic pyrophosphatase to produce inorganic phosphate (P_i). Phosphate was detected using Malachite Green reagent. Assay was done in 96 well format and the following scheme was followed: 0.5 μM of enzyme was added to 50 μl of aminoacylation buffer with the following composition: 30 mM Na-Hepes (pH 7.2), 140 mM NaCl, 30 mM KCl, 40 mM $MgCl_2$, 200 μM ATP, 1 mM L-Phe, 2u/ml inorganic pyrophosphatase, and 8 μM tRNA^{Phe} and incubated for 30 min at 37 °C. Reactions were stopped by addition of 50 μl of Malachite Green reagent (0.7 N sulfuric acid containing 0.0876% Malachite Green hydrochloride, 0.05% Triton X-100 and 2.82% ammonium molybdate tetrahydrate), mixed and incubated at room temperature for 10 min and absorbances were measured at 620 nm using a Versamax microplate reader (Molecular Devices) [22,23].

2.4. Detection of disulfide bridge formation by SDS-PAGE

Protein samples (2 μM) were mixed with Laemmli sample buffer with or without reducing agent [30 mM β-mercaptoethanol (β-ME)] and boiled at 100 °C for 5 min. Samples were resolved onto 15% denaturing SDS-PAGE gel and stained with Coomassie brilliant blue solution to visualize the bands.

2.5. Sample preparation and MALDI-TOF MS analysis of ScmitPheRS for identification of disulfide-linked cysteines

Both oxidized and reduced form of *Sc*mitPheRS (5 μg) were added in 8 M urea followed by the addition of 10 mM of IAM in 50 mM ammonium bicarbonate and incubated for 30 min in dark at room temperature to modify the free thiols. The excess IAM was removed by buffer exchange with Amicon® ultra-0.5 ml 30 K centrifugal filter unit. Both the proteins were further reduced with 1 mM DTT for 30 min to free the thiol groups that were involved in disulfide bridge formation, followed by treatment with 10 mM NEM for another 20 min to modify the newly regenerated cysteines. After sequential alkylation protein samples were digested with sequencing grade trypsin. The resulting peptides were subjected to MALDI-TOF MS analysis.

2.6. Cysteine reactivity studies

A 40 mM stock solution of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was prepared in 50 mM Tris-HCl (pH 7.5) buffer containing

1 mM EDTA and 0.2% SDS. Molar concentration of the stock DTNB solution was determined by measuring the absorbance at 324 nm (molar extinction coefficient at 324 nm is 19,800 M^{-1} cm^{-1}). For reactive cysteine estimation, absorbance was recorded at 412 nm in Shimadzu UV-2450 Spectrophotometer. Protein samples (4 μM) were mixed with 2 mM of DTNB to ensure complete reaction of DTNB with free cysteine. DTT-treated samples were extensively dialyzed in 50 mM Tris–HCl (pH 7.5) buffer containing 1 mM EDTA before DTNB reaction to ensure the removal of any free DTT in the solution. The number of reactive cysteines was calculated using a molar extinction coefficient of 13,700 M^{-1} cm^{-1} in presence of 0.2% SDS.

2.7. Preparation of yeast cell extract and Western Blot analysis

Yeast (*Saccharomyces cerevisiae*) cells were grown in yeast extract-peptone-dextrose (YPD) media to mid-log phase. Then cells were treated with two different concentrations of H_2O_2 (2 mM and 5 mM) and incubated for additional 15 min. Cells were harvested by centrifugation and suspended in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol and 100 mM of IAM. Following glass bead lysis, it was centrifuged at 15,000 rpm for 30 min at 4 °C. 5 μl of 6× non-reducing SDS sample buffer was added to 10 μl of cell lysate and boiled at 100 °C for 5 min and the proteins were resolved on 12% Tris-glycine gel. Proteins were then transferred to PVDF membrane and blocked with 5% non-fat dry milk. Polyclonal antibody to *Sc*mitPheRS (BioBharati LifeScience Pvt. Ltd.) was used to detect the difference in band migration of *Sc*mitPheRS protein in H_2O_2 treated cells compared to control cells. The membrane was developed with goat anti rabbit IgG-HRP secondary antibody (BioBharati LifeScience Pvt. Ltd.) followed by detection using chemiluminescence (LumiGLO Reagent and Peroxide, Cell Signaling Technology).

2.8. Growth assays

E. coli NP37 cell was transformed with WT MSF1 gene cloned in pET16b and pRARE, which expresses six rare tRNAs (Novagene), and plated on LB plates supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol, 0.4 mM IPTG and incubated at 30 or 42 °C for 48 h. Similarly pET16b containing the cysteine mutants, namely, C306S and C432S, were also transformed with pRARE. For growth curve M9 minimal media was prepared with 0.2% glucose. Transformed *E. coli* NP37 cells were grown overnight in LB media, harvested by centrifugation, washed with M9 minimal media twice and used to inoculate the liquid media at an initial $OD_{600} \sim 0.04$, and were grown at 42 °C. Optical densities at 600 nm (OD_{600}) were recorded in Shimadzu UV-2450 Spectrophotometer after 12 h.

2.9. Substrate binding studies

Binding of L-Phe, ATP and $tRNA^{Phe}$ were determined from the quenching of tryptophan fluorescence. The fluorescence of 2 μM *Sc*mitPheRS was determined with excitation and emission at 295 nm and 340 nm respectively after incubation at 25 °C temperature. Immediately an aliquot of 2 μl of substrate was added and fluorescence values were taken after incubating the sample at the same temperature for 2 min. The ratio of two values was taken as a degree of quenching after correction for dilution and inner filter effect. The inner filter effect was corrected using the following formula:

$$F = F_{obs} \cdot \text{antilog}[(A_{ex} + A_{em})/2] \quad (1)$$

where A_{ex} is the absorbance at excitation wavelength and A_{em} is the absorbance at emission wavelength. This ratio at different substrate concentrations were determined and was fitted to a single-site binding equation using Kyplot (32 bit, version 2.0 beta 15; Koichi Yoshioka, 1997–2001) [24].

2.10. CD spectropolarimetry

CD spectra were measured in JASCO J-815 spectro-polarimeter at 25 °C. Oxidized and reduced samples of *Sc*mitPheRS were prepared by adding 15 μM of protein from the same purification batch in 50 mM Tris-HCl pH-7.5, 5 mM $MgCl_2$, 100 mM KCl, 5% glycerol without DTT and with 10 mM DTT respectively. Changes in the tertiary structure in *Sc*mitPheRS under oxidizing and reducing conditions were monitored by near-UV CD spectra in the region 250 nm to 350 nm in a 2 mm path length cuvette. Final spectra of the protein were obtained by subtracting the CD signals of buffers from that of corresponding protein samples.

2.11. Measurement of hydrodynamic diameter of ScmitPheRS under oxidized and reduced condition by dynamic light scattering (DLS)

Oxidized and reduced form of *Sc*mitPheRS was subjected to DLS experiments in Malvern Zetasizer Nano S (Malvern Instruments). A 4 mW He-Ne laser (633 nm) with a fixed detector angle of θ = 173° was used. *Sc*mitPheRS (4 μM) was incubated in 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl with and without 10 mM DTT at room temperature for 10 min, filtered using 0.22 μm filter device from Millipore and subjected to DLS measurements. Time-dependent fluctuation in the intensity of the scattered light due to Brownian motion of the particles is measured by DLS. Translational diffusion coefficient, D, can be determine by analyzing these fluctuations, which can be transformed into size distribution using the Stokes-Einstein equation:

$$D = \frac{kBT}{6\pi\eta rh} \quad (2)$$

where k_B is the Boltzmann's constant, T is the absolute temperature, η is the viscosity of the medium. The particle's diameter, which happens to be protein in this case, is related to D which depends on the size and conformation of the protein at a given temperature and viscosity of the medium.

3. Results

3.1. Cys306 and Cys432 are involved in intra-molecular disulfide bridge formation in oxidized ScmitPheRS in vitro

Purified recombinant *Sc*mitPheRS showed negligible activity in absence of reducing agent, but displayed gradual and significant increase in activity with increasing concentration of DTT in the aminoacylation assay mixture (Fig. 1A). Pre-treatment with H_2O_2 inactivated the aminoacylation activity of *Sc*mitPheRS in a dose-dependent manner, which was restored with the addition of DTT in the assay mixture (Fig. 1B). The above observation is most likely due to oxidation of one or more cysteines. *Sc*mitPheRS has 6 cysteine residues distributed throughout the protein (Cys 132, Cys 239, Cys 304, Cys 306, Cys 432 and Cys 457). A non-reducing SDS-polyacrylamide gel (15%) with or without β-ME pre-incubation was performed. Fig. 1C clearly indicated that under both oxidized and reduced condition, *Sc*mitPheRS exists as a monomer, discarding any possibility of inter-molecular disulfide bridge formation. However, the oxidized form migrated slightly faster than the reduced form of *Sc*mitPheRS indicating that the thiols of *Sc*mitPheRS may be involved in intra-molecular disulfide bridge formation.

In order to identify the cysteines that are involved in intra-molecular disulfide bridge formation, both the oxidized and the reduced forms were subjected to sequential alkylation followed by mass spectrometry analysis. One NEM-modified peptide ($SMC^{432}YR$) was observed for oxidized *Sc*mitPheRS, which was absent in reduced *Sc*mitPheRS (Fig. 2; Table 1). The peptide containing 2 cysteines, C304 and C306, was not detected. To identify the cysteines involved in intra-molecular disulfide bridge formation, C304, C306, and C432 were each

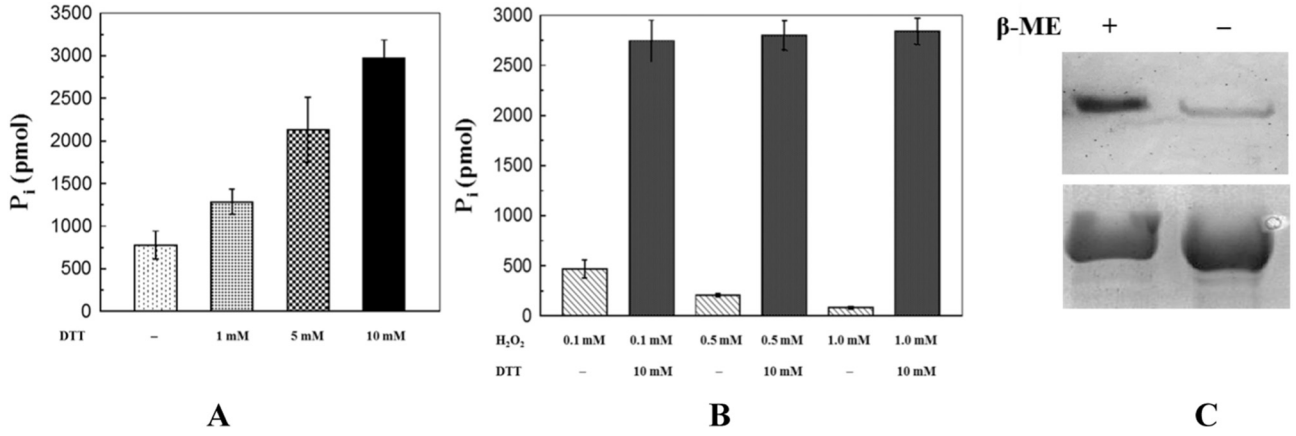


Fig. 1. The aminoacylation activity of *Sc*mitPheRS is sensitive to oxidation *in vitro*.
(A) Recombinant *Sc*mitPheRS was purified and dialyzed extensively in absence of any reducing agents. The activity of purified *Sc*mitPheRS was checked with Malachite Green assay in absence and in presence of DTT. (B) *Sc*mitPheRS was pre-treated with increasing concentrations of H_2O_2 for 10 min and aminoacylation activity was assayed in absence and in presence of 10 mM DTT. (C) Purified *Sc*mitPheRS was electrophoresed on 15% SDS-polyacrylamide gel with or without 30 mM β-ME pre-treatment and visualized after Coomassie brilliant blue staining.

replaced with serine generating three single cysteine mutants, C304S, C306S, and C432S, respectively. After purification of the mutants (C304S, C306S, and C432S), they were resolved on a 15% non-reducing SDS-PAGE with or without pre-incubation with β-ME. The results showed that the oxidized form of C304S migrated slightly faster than the reduced form as found in the case of WT *Sc*mitPheRS (Fig. 3). However, no difference in migration was observed for the oxidized and the reduced forms of C306S and C432S, indicating that C306 and C432 are involved in intra-molecular disulfide bridge formation.

3.2. Thiol reactivity of the oxidized and the reduced forms of WT and cysteine mutants of ScmitPheRS

To further ascertain the number of thiols involved in disulfide bridge formation, both oxidized and reduced forms of WT as well as cysteine mutants of *Sc*mitPheRS were subjected to DTNB analyses. The oxidized and reduced forms of the WT protein showed 4 and 6 reactive thiols, respectively, confirming that only two cysteines are involved in disulfide bridge formation (Table 2). In case of the cysteine mutants, C304S showed 3 and 5 reactive thiols under oxidized and reduced conditions, respectively, whereas C306S and C432S both have 5 reactive thiols for the oxidized as well as the reduced forms, confirming that these two cysteines (C306 and C432) are involved in disulfide bridge formation. C306S and C432S mutants of *Sc*mitPheRS showed comparable activity both in absence and presence of DTT, whereas C304S had negligible activity in absence of DTT (Fig. 4). The activity of C304S was fully restored in the presence of 10 mM DTT, as was also the case for WT *Sc*mitPheRS, indicating that C306 and C432 are involved in intra-molecular disulfide bridge formation under oxidative conditions.

To detect whether an intra-molecular disulfide bridge also exists endogenously under oxidative stress, yeast *S. cerevisiae* cells were treated with two different doses of H_2O_2 (2 mM and 5 mM). Western blot analysis using anti-*Sc*mitPheRS antibody showed a difference in migration of *Sc*mitPheRS protein from H_2O_2 treated cell lysate compared to non-treated control cell lysate. *Sc*mitPheRS protein migrated slightly faster for both 2 mM and 5 mM H_2O_2 treated cells compared to control cells (Fig. 5).

3.3. Inactivation of ScmitPheRS under oxidative stress restricts cell growth

The effect of oxidative stress on bacterial growth at a restrictive temperature was investigated in the *E. coli* NP37 strain, which encodes a temperature-sensitive PheRS, complemented with plasmids encoding WT *Sc*mitPheRS as well as the cysteine mutants C306S and C432S. WT *Sc*mitPheRS encoding cells showed a gradual decrease in growth with increasing concentrations of H_2O_2 whereas both the cysteine mutants showed only moderate decreases in growth compared to the WT. These data indicate that the WT *Sc*mitPheRS is much more sensitive to oxidative stress compared to the cysteine mutants *in vivo*. The slight decrease in growth of cells containing cysteine mutants in the presence of H_2O_2 is likely due to other toxic effects of oxidative stress (Fig. 6).

3.4. Intra-molecular disulfide bridge formation affects tRNA binding

In order to determine whether the intra-molecular disulfide bridge affects substrate binding, interactions of *Sc*mitPheRS with its substrates under oxidized and reduced conditions were studied by a fluorescence quenching method [25,26]. Binding data showed that except for $tRNA^{Phe}$, the other substrates, *i.e.*, L-Phe, and ATP, have comparable affinity for both oxidized and reduced forms of WT *Sc*mitPheRS. On the contrary, it was observed that the reduced form of WT *Sc*mitPheRS has ~7 fold increased binding affinity for $tRNA^{Phe}$ compared to the oxidized form (Table 3). However, the difference in binding affinity for $tRNA^{Phe}$ under oxidizing and reducing condition is insignificant for C306S and C432S *Sc*mitPheRS.

3.5. ScmitPheRS undergoes major conformational change under oxidative stress

A CD spectrum in the near-UV region (250–350 nm) is sensitive to small changes in the tertiary structure of protein where aromatic amino acids and disulfide bonds act as chromophores. Near-UV CD spectra of the reduced and the oxidized forms of WT, C306S and C432S *Sc*mitPheRS were examined which clearly suggested a conformational change due to oxidation in case of WT *Sc*mitPheRS (Fig. 7). Furthermore, it is well known from the literature that a broad absorption band around 260 nm is indicative of a disulfide bridge. In WT *Sc*mitPheRS, a major change was observed in this region under oxidative condition. In addition, dynamic light scattering data showed that the hydrodynamic diameter of the oxidized form significantly decreased to 5.6 nm compared to 6.5 nm in the reduced form of WT *Sc*mitPheRS, providing further support for a structural reorientation of *Sc*mitPheRS to form a more compact structure under oxidative stress (Fig. 8). In contrast, for C306S and C432S *Sc*mitPheRS the difference in hydrodynamic diameter under oxidizing and reducing condition is insignificant.

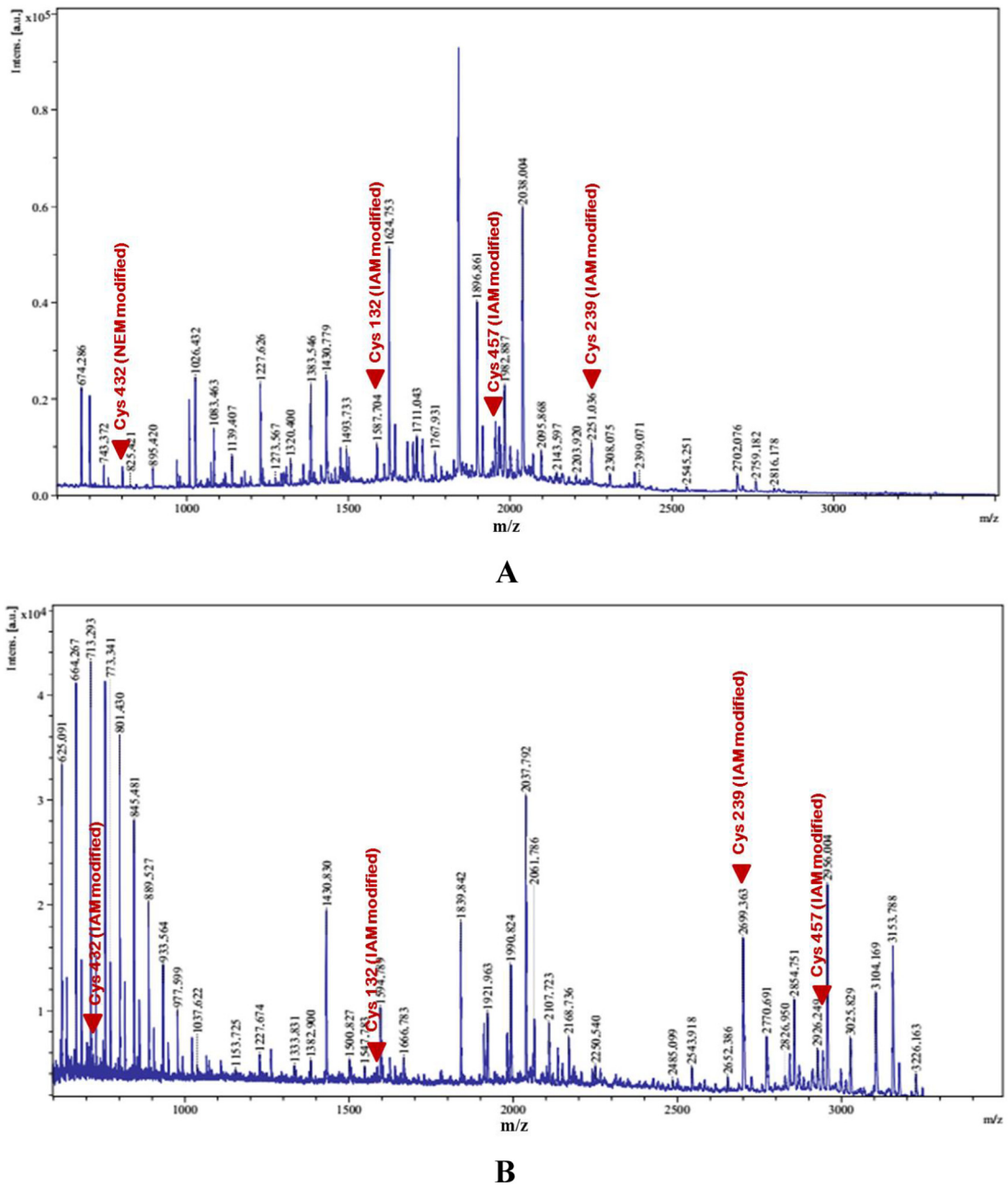


Fig. 2. MALDI-TOF MS analysis of *Sc*mitPheRS and assignment of the cysteine containing fragments. (A)MS spectra of the trypsin digested fragments of *Sc*mitPheRS oxidized *in vitro*. (B) MS spectra of the trypsin digested fragments of reduced *Sc*mitPheRS. Arrows show cysteine containing peptide fragments.

4. Discussion

Irreversible oxidation of cysteine thiols may lead to inactivation of enzymes and eventual cell death [27,28,29]. Thus, many enzymes adapt to form reversible disulfides as a protective measure under oxidative stress [30]. It has been demonstrated previously that exposure of yeast cells to oxidative stress not only leads to cell cycle arrest [31], but also inhibits protein synthesis to protect the cell from error-prone translation [3]. Similar to other eukaryotic organisms, yeasts also harbour several detoxification mechanisms including stalling protein synthesis as well as cell cycle arrest to allow the cell sufficient time to detoxify excess ROS [3,31]. Protein synthesis and cell division can resume once the cell returns to its normal redox state. In this context, it can be speculated that disulfide bridge formation in *Sc*mitPheRS under oxidative stress acts as a redox switch to protect the cell from error-prone translation, rather than simply protecting the thiol from irreversible oxidation. ROS mediated signalling has been thought to play an important role in cell proliferation and differentiation both in prokaryotic and eukaryotic cells [32]. ROS-induced reversible disulfide bond formation has emerged as a post translational modification that can transiently impact both protein structure and function [11]. These proteins are usually involved in oxidative stress defence where they use this reversible disulfide bond formation as a mechanism to modulate their activity [33]. *In situ* kinetic trapping revealed that an increase in respiratory chain derived oxidants leads to an increase in thiol oxidation level in human mitochondrial methionyl-tRNA synthetase (mtMetRS). However, how the mtMetRS function is regulated by thiol oxidation has not been determined so far [34]. *Sc*mitPheRS may be an important addition to a growing list of proteins which undergo reversible disulfide bridge formation and alteration of function and activity under oxidative stress [2,4,35].

Given that numerous redox-regulated pathways operate in

Table 1
Modifications of cysteines as determined by MALDI-MS. All *m/z* are protonated monoisotopic ions.

Peptide sequence	Cysteine residue	Modification	*m/z*
A: Cysteine containing fragments derived from oxidized *Sc*mitPheRS *in vitro*			
THTSAHELE**C**FQK	Cys 132	Cys_IAM	1587.7045
QEYMSDLEVDL**C**SQHLKR	Cys 239	Cys_IAM	2251.0359
SM**C**YR	Cys 432	Cys_NEM + Met oxidation	800.4321
NLTNAEVNTLQDMV**C**SK	Cys 457	Cys_IAM + Met oxidation	1953.0851
B: Cysteine containing fragments derived from reduced *Sc*mitPheRS *in vitro*			
THTSAHELE**C**FQK	Cys 132	Cys_IAM	1587.5762
ENNPKQEYMSHLEVDL**C**SQHLK	Cys 239	Cys_IAM	2699.3626
SM**C**YR	Cys 432	Cys_IAM	716.3128
INYQSMDRNLTNAEVNTLQDMV**C**SK	Cys 457	Cys_IAM	2944.5128

mitochondria, proper redox control is extremely important for their function [36]. Any imbalance in redox homeostasis leads to oxidation of the reactive thiols shifting the thiol-disulfide equilibrium towards disulfide formation [37]. The formation of disulfide bonds in proteins is dependent on the pK_a of cysteine side chain which is influenced by redox potential of the surrounding environment. The pH of yeast mitochondrial matrix (~pH 7.4) is higher than the cytosol (~pH 7.0) [38,39,40,41], making the thiols more susceptible to form thiolate [$-RS^-$] ions and thus rendering them apt for disulfide bridge formation [12]. C306 and C432 of *Sc*mitPheRS form an intra-molecular disulfide bond in the presence of oxidants and reversibly inhibit enzymatic activity. Our mechanistic studies revealed that the intra-molecular disulfide bond between C306 and C432 inactivates *Sc*mitPheRS by compromising its $tRNA^{Phe}$ binding capacity. Although the intra-molecular disulfide bond did not affect ATP and L-Phe binding, affinity towards $tRNA^{Phe}$ decreased substantially. One possible explanation is that the formation of a disulfide bridge may block the $tRNA^{Phe}$ binding pocket and hence access to the active-site of *Sc*mitPheRS is affected. Involvement of C432 in disulfide bridge formation, which is found to be present in the anticodon binding domain (ABD) of the enzyme, may be responsible for hindering $tRNA^{Phe}$ binding to the enzyme. Multiple sequence alignment of eukaryotic mitochondrial PheRS and eubacterial PheRS revealed that these two cysteines are conserved across different eukaryotic species, but are not conserved in eubacteria (Fig. S1) [42].

Modelling of the structure of *Sc*mitPheRS based on the *Hs*mitPheRS (human mitochondrial phenylalanyl-tRNA synthetase) structure suggests that the distance between C306 and C432 is ~35 Å and to form intra-molecular disulfide bond these two cysteines must come in close proximity which will compromise the conformation of the protein. However, it is not unusual and it has been seen in some instances two distantly located cysteines may come close to form intra-molecular disulfide bridge under oxidative stress. Motohashi et al. reported that under oxidative stress two distantly situated cysteines in Cyclophilin come in close proximity to form an intra-molecular disulfide bond which compromises the conformational integrity leading to loss in activity [43,44]. Choi et al. also established that the transcription factor OxyR undergoes significant structural change under oxidative stress to form a reversible intra-molecular disulfide bond between two distantly placed cysteines. The crystal structures of both oxidized and reduced

Table 2
DTNB assay of the WT and cysteine mutants of *Sc*mitPheRS to determine the number of reactive thiols.

The theoretical number of reactive thiols per *Sc*mitPheRS molecule and the number of reactive thiols obtained from the DTNB assay under oxidized and reduced condition are tabulated here. Release of the product 2-nitro-5-thiobenzoic acid was recorded at 412 nm in a Shimadzu UV-2450 spectrophotometer. The number of reactive thiols was calculated using a molar extinction coefficient of 13,700 M^{-1} cm^{-1} in presence of 0.2% SDS.

*Sc*mitPheRS	Theoretical −SH content	Measured −SH content	
		Oxidized	Reduced
WT	6	4.02 ± 0.16	5.93 ± 0.33
C304S	5	2.96 ± 0.08	5.09 ± 0.19
C306S	5	5.06 ± 0.25	4.93 ± 0.32
C432S	5	4.89 ± 0.14	5.08 ± 0.36

Values are the means of three independent experiments with standard errors indicated.

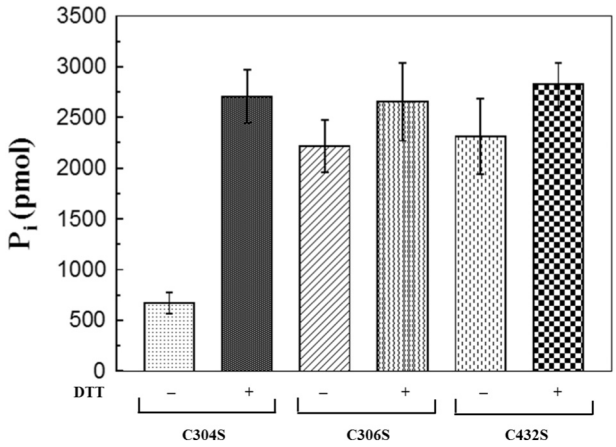


Fig. 4. The aminoacylation activity of cysteine mutants of *Sc*mitPheRS in absence and in presence of 10 mM DTT.
Phenylalanylation of yeast $tRNA^{Phe}$ by *Sc*mitPheRS was performed in aminoacylation buffer in absence and presence of 10 mM DTT. Protein and $tRNA^{Phe}$ concentrations were 0.5 μM and 8 μM respectively. The reaction was carried out at 37 °C for 30 min. P_i production was measured by Malachite Green reagent at 620 nm in a Versamax microplate reader (Molecular Devices).

forms of OxyR were determined which established that two cysteines 17 Å apart in space come in close proximity to form intra-molecular disulfide bond as a part of redox regulation [45]. In the case of WT *Sc*mitPheRS, the near-UV CD spectra showed significant conformational change, as did changes in the hydrodynamic diameter, under oxidative stress. The observations may only be explained if some distantly situated Cys residues can move significantly close under oxidative stress to form intra-molecular disulfide bridge. Our experimental observations clearly indicated a major conformational change occurs during the transition from reduced to oxidized conditions, leading to formation of the disulfide bridge between Cys306 and Cys432 which leads to the loss in the synthetase activity. It may be noted that conformational plasticity is also reported to be an inherent characteristic for the human analog of *Sc*mitPheRS, *Hs*mitPheRS. *Hs*mitPheRS undergoes large

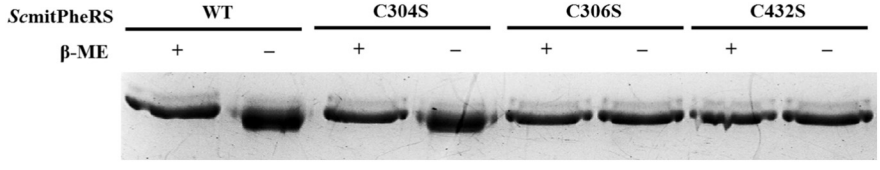


Fig. 3. Cys 306 and Cys 432 are involved in intra-molecular disulfide bridge formation in oxidized *Sc*mitPheRS *in vitro*.
Equal amounts of WT and cysteine mutants of *Sc*mitPheRS were electrophoresed on 15% SDS-polyacrylamide gel with (+) or without (−) 30 mM β-ME pre-treatment and visualized after Coomassie brilliant blue staining.

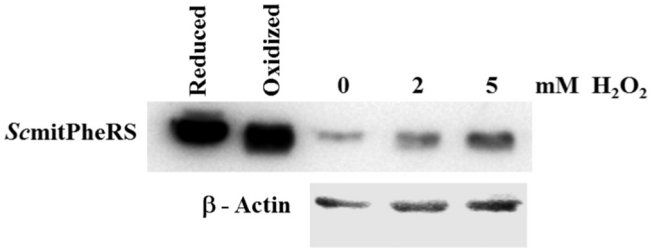


Fig. 5. Transient oxidation of *Sc*mitPheRS by H_2O_2 *in vivo*.
S. cerevisiae cells were grown up to mid-log phase and were treated with 0, 2, and 5 mM of H_2O_2 for 15 min. Cell lysis was done in the presence of 100 mM IAM and resolved by 12% SDS-PAGE with purified *Sc*mitPheRS (both reduced and oxidized) as a control. *Sc*mitPheRS was detected by immunoblot using anti-*Sc*mitPheRS antibody. 10 µl of each cell lysate was resolved on 12% SDS-PAGE gel. *β*-Actin was used as a loading control.

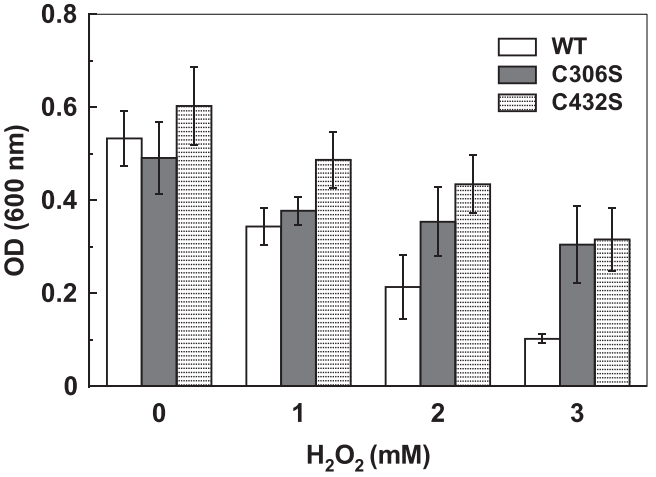


Fig. 6. Effect of H_2O_2 on growth of NP37 strain encoding WT *Sc*mitPheRS compared to cysteine mutants C306*S* and C432*S*.
Growth of *E. coli* NP37 (WT *Sc*mitPheRS) compared to *E. coli* NP37 (C306*S*) and *E. coli* NP37 (C432*S*) in M9 minimal media supplemented with H_2O_2. Data points are the mean of three independent cultures with error bars representing the ± SD.

Table 3
Dissociation constant (K_d) of *Sc*mitPheRS under oxidized and reduced condition.

Quenching of tryptophan fluorescence (λ_{ex} = 295 nm and λ_{em} = 340 nm) was measured to determine the dissociation constants (K_d) in presence of substrates (L-Phe, ATP, and yeast $tRNA^{Phe}$) with or without 10 mM DTT. The data were fitted to a single-site binding equation using Kyplot (32 bit, version 2.0 beta 15; Koichi Yoshioka, 1997–2001). Relative affinity for each substrate was calculated from the ratio of the association constant (K_a) of the reduced *Sc*mitPheRS to that of the oxidized *Sc*mitPheRS.

Ligands	Oxidized (M)	Reduced (M)	Relative affinity
ATP	$(6.9 \pm 0.94) \times 10^{-5}$	$(5.7 \pm 0.78) \times 10^{-5}$	1.21
L-Phe	$(6.6 \pm 1.17) \times 10^{-6}$	$(5.7 \pm 0.6) \times 10^{-6}$	1.15
$tRNA^{Phe}$ (WT)	$(2.4 \pm 0.42) \times 10^{-6}$	$(0.34 \pm 0.12) \times 10^{-6}$	7.15
$tRNA^{Phe}$ (C306S)	$(0.44 \pm 0.06) \times 10^{-6}$	$(0.35 \pm 0.05) \times 10^{-6}$	1.24
$tRNA^{Phe}$ (C432S)	$(0.43 \pm 0.09) \times 10^{-6}$	$(0.36 \pm 0.04) \times 10^{-6}$	1.18

Values are the means of at least three independent experiments with standard errors indicated. The concentration of *Sc*mitPheRS, both oxidized and reduced, was 2 µM.

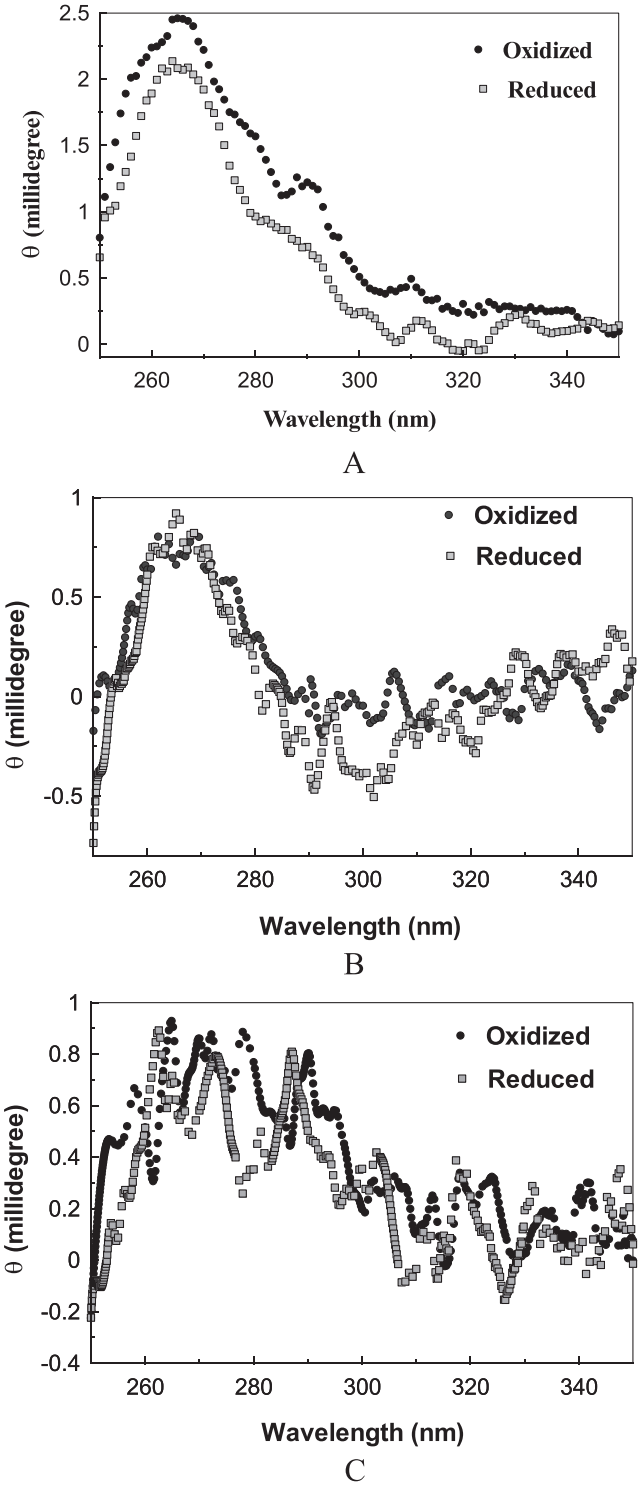
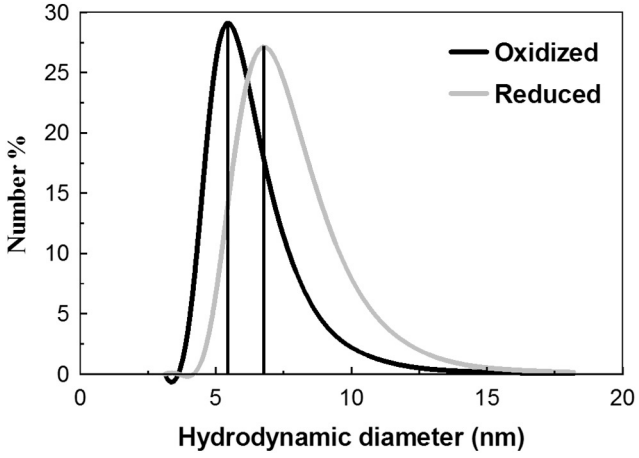
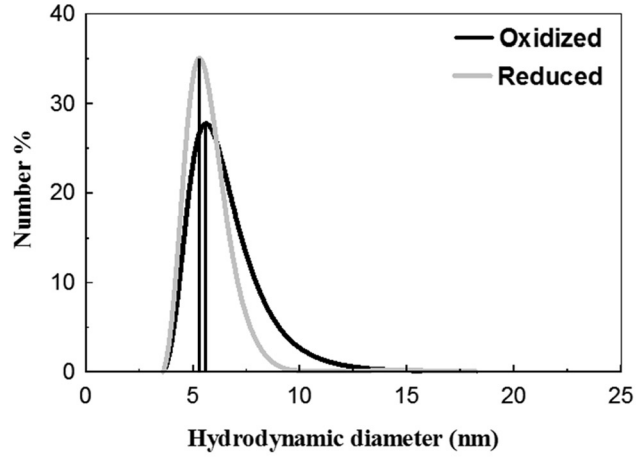


Fig. 7. Near-UV CD spectra of *Sc*mitPheRS under oxidizing and reducing condition. A. Near-UV CD spectra of WT *Sc*mitPheRS under oxidizing and reducing condition. B. Near-UV CD spectra of C306*S* *Sc*mitPheRS under oxidizing and reducing condition. C. Near-UV CD spectra of C432*S* *Sc*mitPheRS under oxidizing and reducing condition. *Sc*mitPheRS (15 µM) was incubated in 50 mM Tris-HCl (pH -7.5), 5 mM $MgCl_2$, 100 mM KCl, 5% glycerol without DTT and with 10 mM DTT. Spectra were recorded from 250 nm to 350 nm in a 2 mm path-length cuvette at 50 nm/min scan speed at 25 °C in JASCO J-815 spectropolarimeter.





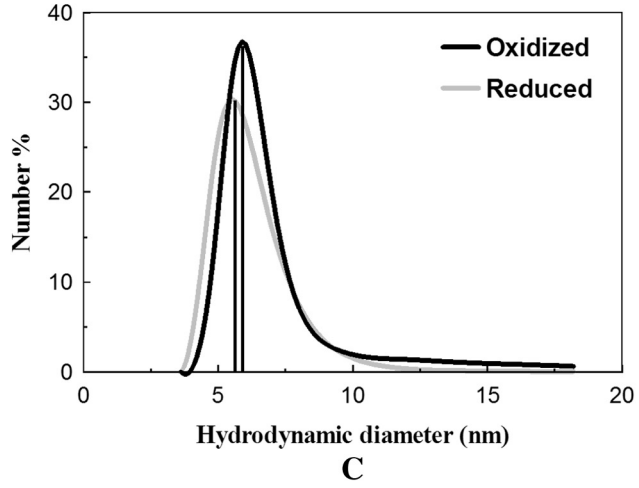


Fig. 8. Distribution of hydrodynamic diameter by number of the oxidized and the reduced form as obtained from DLS. A. Hydrodynamic diameter of WT *Sc*mitPheRS under oxidized and reduced condition. B. Hydrodynamic diameter of C306S *Sc*mitPheRS under oxidized and reduced condition. C. Hydrodynamic diameter of C432S *Sc*mitPheRS under oxidized and reduced condition. *Sc*mitPheRS (4 μM) was incubated in 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl with and without 10 mM DTT at room temperature. The data were plotted as number % *versus* hydrodynamic diameter (nm).

domain rearrangement during the transition from its inactive (closed) to active (open) form [46]. While interactions comparable to those we describe cannot be accommodated within either the open or closed forms of *Hs*mitPheRS, the ability to undergo such dramatic structural changes suggests that other conformations might also be accommodated by mitPheRSs, such as would be required to allow a C306/C342 disulfide bond.

Cellular amino acid pools are very susceptible to oxidation and L-Phe can readily be oxidized to *ortho*-Tyrosine (*o*-Tyr) and *meta*-Tyrosine (*m*-Tyr) as reported earlier [47,48]. *E. coli* cells with editing deficient PheRS have been shown to accumulate *m*-Tyr in the intracellular amino acid pool and incorporate *m*-Tyr in the proteome, slowing growth of the bacteria [47]. The human analog of *Sc*mitPheRS, *Hs*mitPheRS was reported to show only 5-fold lower affinity towards *m*-Tyr compared to cognate amino acid L-Phe, whereas L-Tyr shows 1000-fold less affinity [48]. Absence of any recognizable editing domain in *Sc*mitPheRS impels it to depend solely on the high specificity of L-Phe over L-Tyr to maintain translational fidelity, while the decreased specificity has been shown to hinder mitochondrial biogenesis [15]. Hence, it can be speculated from these findings that the presence of high levels of non-proteinogenic amino acids such as *m*-Tyr in the intracellular amino acid pool under oxidative stress may induce $tRNA^{Phe}$ mischarging. Thus, perturbation of optimal enzyme activity of *Sc*mitPheRS due to intramolecular disulfide bridge formation may be a protective measure to prevent the mischarging of $tRNA^{Phe}$ under oxidative conditions. The reversible switch between the active and inactive forms of the enzyme is thought to be maintained by the redox environment of the mitochondria. The inhibitory effect of H_2O_2 on growth of *E. coli* NP37 encoding WT *Sc*mitPheRS compared to the cysteine mutants suggested that disulfide bridge formation indeed inactivates the enzyme leading to inhibition of growth under oxidative stress. However, future studies are needed to establish whether reversible inhibition of *Sc*mitPheRS activity under oxidative stress plays a regulatory role to maintain translational fidelity rather than just protecting the protein thiols from irreversible oxidation.

5. Conclusions

In this study we have shown that *Sc*mitPheRS forms intra-molecular disulfide bridge under oxidative stress. The conformational rearrangement to form the disulfide bridge between two conserved cysteines leads to loss of aminoacylation activity of *Sc*mitPheRS. Monomeric PheRS lacks the editing domain required for proof-reading and it solely depends on its high substrate specificity compared to the tetrameric PheRS to maintain the translational fidelity. Decreased substrate specificity has also been seen to inhibit mitochondrial biogenesis. The reversible switch of *Sc*mitPheRS from active to inactive form might act as a rescue mechanism for cell to overcome the toxic effects of mistranslation under oxidative stress.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contributions

SC, SG, AC, MI and RB designed the experiments and analyzed the data. SC and SG performed the experiments. SC, MI and RB wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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