Title: Dual avatars of *E. coli grxB* encoded Glutaredoxin 2 perform ascorbate recycling and ion channel activities

Authors

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43 Abstract

44 Glutaredoxins (Grxs) are single-domain redox enzymes of the thioredoxin superfamily,
 45 and primarily function as glutathione (GSH) dependent disulphide reductases. Whereas,
 46 the *E. coli* Glutaredoxin 2 (*EcGrx2*) encoded by *grxB* has two conserved GST-fold
 47 domains, it still lacks a classical Grx-like functions. In this study, we show for the first
 48 time, that *EcGrx2* exists in both soluble and membrane integrated forms. The soluble form
 49 associates with a previously unidentified GSH dependent dehydroascorbate (DHA)
 50 reductase, and the membrane integrated form possesses ion channel activities. Using
 51 enzyme kinetic data and structural data we unequivocally demonstrate that *EcGrx2*
 52 recycles ascorbate (AsA) from DHA. This ability to recycle AsA is inhibited by Zinc
 53 (Zn^{2+}). We also show that both wildtype and the *E. coli* *grxB* deletion mutant can be
 54 rescued from H_2O_2 -induced oxidative stress using ascorbate as an antioxidant, which
 55 otherwise is only known as a carbon source in bacteria. Moreover, the *grxB*⁻ mutant is
 56 susceptible to intracellular killing by ROS producing macrophages. We further discovered
 57 that *EcGrx2* integrates into the native *E. coli* membrane and show that the purified soluble
 58 protein readily inserts into artificial lipid bilayer membrane and conducts ions *in vitro*.
 59 Our data demonstrates a highly conserved functional similarity among *EcGrx2*-orthologs
 60 and highlights that the utilization and subsequent recycling of ascorbate as an antioxidant
 61 by *grxB* harbouring gram-negative bacteria, including human pathogens, may provide a
 62 survival advantage under hostile oxidative environments.

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Introduction

Omnipresent Grxs are involved in various biological functions such as reduction of ribonucleotide reductase (RNR), phosphoadenosine phosphosulfate (PAPS) reductase, and arsenate reductase, cell cycle control, signal transduction, and dehydroascorbate reductase activities¹⁻⁴. However, *EcGrx2* is atypical and has a two-domain architecture^{5,6} compared to the single-domain Grx1, 3 and 4 of *E. coli*^{7,8}. The biochemical role of this duplication is unclear. While *EcGrx2* lacks key RNR reduction activity, it reduces protein and mixed disulphides in ArsC⁴ and PAPS reductase. Notably, *EcGrx2* is abundantly expressed during stationary phase, upregulated under osmotic shock, and acidic conditions^{9,10}. Thus, predictably, null mutants (*grxB*⁻) were reported to show distorted morphology and an increased susceptibility under H₂O₂-induced oxidative conditions^{4,11}. Cumulatively, these reports strongly suggest *EcGrx2* as an antioxidant enzyme. Here, we employ a structure-based approach to decipher the precise biochemical functions associated with the three-dimensional structure of *EcGrx2*.

Results

Soluble *EcGrx2* is a GSH-dependent DHA reductase

To comprehensively elucidate its biochemical activities, we first compared the crystal structure of *EcGrx2* with that of its eukaryotic structural homologs. The structure of monomeric *EcGrx2*^{5,7} has a N-terminal domain (NTD; GST_N_3) containing βαβαβα thioredoxin-fold¹², and an all α-helical C-terminal domain (CTD; Glutaredoxin2_C)¹³ (Fig. 1a). Based on the structural similarity search, we retrieved the *Pennisetum glaucum* dehydroascorbate reductase (*PgDHAR*)¹⁴ and human chloride intracellular channel CLIC1 (*HsCLIC1*) with high Z-score of >14.4 in DALI searches, in spite of the low sequence identity (<20%). The overall structure of *EcGrx2*, *PgDHAR*, and *HsCLIC1* are highly conserved (RMSD ≤3.6 Å). It is noteworthy that both plant DHARs and *HsCLICs* are

redox enzymes that recycle AsA from DHA using GSH as a cofactor^{15,16}. Plant DHAR is part of the well-studied AsA-GSH based antioxidant system (Foyer-Halliwel-Asada pathway) that helps recover from environmental stress¹⁷. The enzymatic activity of *PgDHAR* and *HsCLIC1* is imparted by a catalytic cysteine present in the conserved glutaredoxin motif: Cxx[C/S] (Fig. 1b). Further, the active site in these structural orthologs is composed of canonical GSH binding ‘G-site’ at the NTD, and a hydrophobic substrate binding ‘H-site’ from the CTD¹⁴. A closer inspection of the structure-guided multiple sequence alignment and the electrostatic surface analysis reveals a distinct positively charged pocket conserved in *EcGrx2* with a CxxC motif (Cys9-Pro10-Tyr11-Cys12) (Fig. 1c). These structural and active site similarities lead us to hypothesize that soluble *EcGrx2* may possess the ability to recycle AsA from DHA like its eukaryotic structural orthologs.

Therefore, we then asked whether *EcGrx2* recycles AsA from DHA, like *PgDHAR* and *HsCLIC1*^{16,17}. As we hypothesized, the purified *EcGrx2* showed GSH-dependent DHA reductase activity at an optimal pH of 7.4, following Michaelis-Menten kinetics (Supplementary Fig. 1 a, b, c and Supplementary Table 1). The $K_m(\text{DHA})$, $K_{cat}(\text{DHA})$ and specific activity were $2879 \pm 640.4 \mu\text{M}$, 229.47 min^{-1} and $1.8845 \mu\text{M}^{-1}\text{min}^{-1}\text{mg}^{-1}$, respectively. We then probed to find the role of Cys9 and Cys12 in DHA reductase activity after chemical modification by carbamidomethylation¹⁸, which abrogated the enzyme activity completely (Fig. 1d). We then substantiated this observation by creating a double mutant (C9S/C12S) that resulted in loss of enzyme activity (Fig. 1d). The above data clearly shows that *EcGrx2* recycles AsA from DHA in a GSH-dependent manner. As thiol-reactive divalent cations are known to inhibit DHA reductase activity of plant DHAR¹⁵, we performed a thermal shift assay (TSA) to screen for divalent cations that inhibit *EcGrx2* enzyme activity. In contrast to other divalent metal ions that did not show

significant effect on melting temperature (T_m), except Cu^{2+} (Supplementary Fig. 1d), addition of Zn^{2+} ion resulted in a decrease in T_m by 7 °C, implying direct binding to *EcGrx2*. Further, we also observed that Zn^{2+} abrogates DHA reductase activity of *EcGrx2*, competitively (Fig. 1e). Then, in order to understand the molecular mechanism of how Zn^{2+} inhibits DHA reductase activity, we determined the first crystal structure of *EcGrx2* in complex with Zn^{2+} and GSH at 1.6 Å (Table 1, PDB:7D9L). The complex structure revealed a hitherto unknown mode of Zn^{2+} inhibition at the enzyme active site, wherein a highly conserved histidine (His8), two water molecules, and the cysteine of oxidised GSH (GSOO:GSF) form a tetrahedral coordination¹⁹ with Zn^{2+} (Fig. 1f,g and Supplementary Table 2). Interestingly, Zn^{2+} is coordinated by GSF, instead of canonical thiol (-SH) group of catalytic cysteine. More so, the bound GSH is oxidised to sulfinic acid form (GSOO) by the Zn^{2+} at the G-site (Supplementary Fig. 2a). Moreover, there is no significant change noticed in the overall structure of *EcGrx2* among native (2.38 Å), GSH bound (1.45 Å) and Zn^{2+} inhibited crystal structures reported here (Table 1, PDB: 7DKR, 7DKP), and by others (PDB:4KX4, 3IR4). Further, we performed 1 µs molecular dynamics simulation at 300K and found that the Zn^{2+} inhibited complex is very stable with an RMSD of GSF;1.36 ± 0.52 Å, Zn^{2+} ; 1.31 ± 0.42, water-1; (2.2 ± 0.03 Å) and water-2; 2.21 ± 0.02 Å (Fig. 1 h, i, Supplementary Fig. 2 b, Supplementary video 1). In addition to the active site bound Zn^{2+} , a second Zn^{2+} ion is present in the crystal structure which is tetrahedrally coordinated by symmetry-related *EcGrx2* monomer involved in crystal packing (Supplementary Fig. 2c, d). The above data unequivocally demonstrate that the AsA recycling DHA reductase activity of *EcGrx2* involves the conserved CxxC motif and the divalent cation Zn^{2+} inhibits the enzyme activity by binding at the G-site, prompting us to probe whether AsA is used as an antioxidant by *E. coli*.

Ascorbate rescues *E. coli* from H₂O₂ induced oxidative stress

AsA has been previously studied as a carbon source in bacteria²⁰⁻²² and there is no evidence for its use as an antioxidant by *E. coli*. Therefore, we performed AsA supplementation assay with wild type *E. coli* (BW25113) and *EcGrx2* deletion mutant (JW1051-3: Δ *grxB*734::kan) strains, using M9 minimal media-agar plates containing varying concentrations of AsA (0.5-15.1 mM) under H₂O₂ induced oxidative stress (Fig. 2a, b, Supplementary Table 3). When AsA was not supplemented, the zone of clearance was larger in *Grx2* mutant compared to that of wild type strain, suggesting that the *grxB* deletion mutation increases bacterial sensitivity to H₂O₂, in concurrence with the earlier observation⁴. We further observed a concentration dependent decrease in zone of clearance upon AsA supplementation in both wild type and mutant strains, indicative of an antioxidant role played by AsA in minimal media devoid of redox active chemicals.

***EcGrx2* provides bacteria survival advantage inside LPS activated RAW264.7 macrophages**

As the *grxB*⁻ mutant shows phenotypic changes only in the presence of oxidising environment⁹, we asked whether *EcGrx2* helps bacteria survive inside the immune cells that produce more ROS upon bacterial infection, as predicted before⁹. We then screened both wild type and *grxB* mutant strains for their ability to survive inside the host cell (RAW264.7 macrophages) upon infection. The *grxB* mutant *E. coli* showed a significant decrease in survival ($P < 0.001$) compared to the wild type strain (Fig. 2c, Supplementary Table 4), indicating a possible antioxidant role for *EcGrx2* inside the host macrophages. Having now demonstrated DHA reductase activity for the soluble form of *EcGrx2*, we further asked whether the protein is also integrated into the membrane, similar to its eukaryotic orthologs *HsCLICs*²³⁻²⁵ and plant DHAR²⁶.

***EcGrx2* moonlights as an ion channel**

To understand the dimorphic nature of *EcGrx2*, we probed the dual localization of *EcGrx2*-eGFP in wild type *E. coli* (BW25113) under stationary phase conditions during which maximal expression was expected. Confocal microscopy followed by TIRF-SIM analysis clearly show that *EcGrx2*-eGFP is localized into both cytoplasmic and membrane compartments whereas eGFP alone is confined to the cytoplasm (Fig. 3a (i-v)). Membrane integration of *EcGrx2*-eGFP was further assessed by isolating pure membrane fraction from *E. coli*, and subsequent extraction with various detergents with SDS as control. The membrane fraction was first washed with 2 M urea, before detergent extraction, to exclude the peripherally attached soluble *EcGrx2* from the membrane integrated form. We show that *EcGrx2* is present in the urea washed membrane and also in the detergent extracted supernatant (Fig. 3b), confirming that *EcGrx2* is integrated into the native *E. coli* membrane. Then, we sought to determine if the soluble form of *EcGrx2* inserts spontaneously into the planar lipid bilayer and forms a functional ion channel similar to that of *HsCLICs*^{27,28}.

The channel-forming properties such as ion conductance and gating of *EcGrx2* were examined using single-channel electrical recordings. Planar lipid bilayer were made by the Montal and Muller technique²⁹ using DPhPC (1, 2-diphytanoyl-sn-glycero-3-phosphocholine) lipids that provide highly stable membranes. Soluble *EcGrx2* inserts into the lipid bilayers at + 50 mV, forming channels that fluctuated between different conductance states, indicating the formation of non-uniform channels in the membrane (Fig. 3c). Notably, the channel formation is associated with noisy transient spikes of varying conductance states and the channel remained in an open conductance state for a longer duration at + 50 mV (Fig. 3c, d). Importantly, channel activity is evident from the short

ion current flickering events corresponding to the open and closed conductance states. At -50 mV, the channel showed numerous transient openings and fluctuating conductance states with increased noise leading to rapid closure of the channel (Fig. 3e). Further, we observed asymmetry in the channel conductance states and fluctuation with respect to the channel with the direction of the applied voltage. At higher voltages of +100 mV, *EcGrx2* exhibited voltage-dependent gating subsequently resulting in the complete channel closure (Fig. 3f). The frequency of gating events increased with the increase in the voltage and the time duration of open and closed states varied from several seconds to minutes. Our single-channel electrical recordings confirm the channel formation of *EcGrx2*, which exhibited consistent electrical properties such as ion conductance and gating in a membrane environment. The above data demonstrate that *EcGrx2* is membrane integrated and the soluble form readily inserts into artificial bilayer membrane and conducts ions.

Taken together, our data show that a) the atypical two domain containing *EcGrx2* is a GSH dependant DHA reductase that recycles AsA from DHA in its soluble monomeric form; b) Zn^{2+} ion inhibits *EcGrx2* enzyme activity; c) The complex crystal structure followed by molecular dynamics simulation analysis shows Zn^{2+} ion forms a stable inhibitory complex through tetrahedral coordination geometry at the G-site; d) AsA, as an antioxidant, rescues *E. coli* cells from H_2O_2 induced stress *in vitro*; e) *E. coli grxB* mutant is susceptible to increased killing by RAW macrophages upon infection; f) *EcGrx2* is dimorphic and has a moonlighting membrane integrated form (Fig. 3g); and g) The soluble form of *EcGrx2* inserts into artificial bilayer membrane and forms transient ion channels, similar to *HsCLICs*.

217 Discussion

218 Eukaryotes use AsA as a major antioxidant and thus recycling from either
 219 monodehydroascorbate (MDHA) radical and/or DHA is essential for cellular survival and
 220 alleviation of oxidative stress induced damage. Though AsA utilization and DHA
 221 reduction by *E. coli* has been studied for some time^{20,30,31}, the antioxidant role of AsA and
 222 its enzyme-mediated recycling remained unexplored till now. The identity of DHA
 223 reductase activity reported for partially purified fraction of *E. coli* extract³² remains
 224 unknown. Thus, GSH dependent AsA recycling activity reported here shows that *EcGrx2*
 225 may well be a part of the hitherto unknown bacterial antioxidant defence system.
 226 Interestingly *grxB* gene is widely distributed among Gram-negative proteobacteria
 227 (Supplementary Fig. 3 a, b). The extensive parallels; dimorphic nature (Fig. 3g), sub-
 228 cellular localization^{33,34}, and dual biochemical activities conserved among *EcGrx2*,
 229 *HsCLICs* and plant DHARs support the above hypothesis. Further studies are needed to
 230 assess whether the moonlighting membrane integrated ion channel form of *EcGrx2* also
 231 plays a protective role under oxidative stress. As *grxB* is required for late stages of
 232 bacterial growth and survival inside macrophages, *EcGrx2*-orthologs from pathogenic
 233 bacteria are potential drug targets for therapeutics that could function by disrupting the
 234 microbial antioxidant defense system.

235 Materials and Methods

236 **Materials:** Unless otherwise indicated all chemicals and reagents were purchased from
 237 Sigma–Aldrich (Merck). Restriction enzymes were from New England Biolabs.
 238 Oligonucleotides were from Macrogen. Bacterial culture media were from Himedia.
 239 Strains BW25113 (CGSC#:7636) and JW1051-3 (CGSC#9012)³⁵ were obtained from *E.*

coli Genetic Stock Center, Yale university. Cell culture materials and media are from Nunclon, Cell clone, Gibco and Merck.

Bacterial strains, plasmids, primers and culture conditions: Details are summarized in Supplementary Table 5. *E. coli* strains were grown with shaking at 130 rpm in Luria-Bertani (LB) or in LB auto induction (LB-AIM)³⁶ media without trace elements (Formedium) or in 1.5% LB agar plates. The following concentrations of antibiotics were used: Ampicillin 100 µgml⁻¹, Chloramphenicol 34 µgml⁻¹, Kanamycin 50 µgml⁻¹. *E. coli* DH5a was used as the host strain for all plasmid constructions.

Plasmid construction: pETM11-*grxB*, encoding His6-*EcGrx2*, was generated by PCR amplification of *E. coli grxB* from pET24a-*grxB* (gifted by H. Jane Dyson, The Scripps Research Institute) and cloned into pETM11 (EMBL). pETM11-*grxB*^{C9S/C12S} and pETM1-*grxB*^{C9S}, encoding His6-*EcGrx2*^{C9S/C12S} and His6-*EcGrx2*^{C9S}; double and single mutants, were made by PCR amplification using mutagenic primers and pETM11-*grxB* as template. pQE30-*grxB* encoding His6-*EcGrx2* was generated using pETM1-*grxB* as template for PCR amplification. pET16b-*grxB-eGFP*, encoding *EcGrx2-eGFP-His6*, was generated by amplifying *grxB* from pET24a-*grxB* and cloned into pET16b-eGFP (gifted by Aravind Penmatsa, MBU, IISc, Bangalore). pQE30-*grxB-eGFP*, encoding His6-*EcGrx2-eGFP*, was generated by PCR amplification of *grxB-eGFP* from pET16b-*grxB-eGFP*. Plasmid pQE30-*eGFP*, encoding His6-eGFP, was generated by PCR amplification of *eGFP* from pET16b-*eGFP*.

Protein expression and purification: *EcGrx2(native)*: pET24a encoding tagless native *EcGrx2* was transformed into *E. coli* Rosetta2 (DE3) pLysS and overexpression was optimized in LB-AIM. Briefly, cells from single colony were inoculated for primary culture in 25 ml LB broth. Secondary culture was raised from 1% of the primary inoculum at 37 °C at 130 rpm to an OD₆₀₀ of 0.6, supplemented with Kanamycin and Chloramphenicol. The culture was grown at 25 °C, 130 rpm for 24 hours. Cells were harvested and resuspended in ice-cold buffer-A (Supplementary Table 6) and were disrupted by sonication at room temperature (RT). Cell lysate was centrifuged at 15000 g for 30 minutes at 4 °C and the supernatant fraction was 0.45 µm filtered. The filtrate was dialyzed against 10 mM sodium acetate buffer pH 4.5-5.0 containing 10mM NaCl and centrifuged at 18900 x g for 10 minutes at 4 °C. Supernatant obtained after centrifugation was directly loaded onto 5 ml HiTrap-SP FF cation-exchange column (GE) equilibrated with buffer-B. Protein was eluted over a linear gradient of 10-100mM NaCl. Cleaner fractions were pooled and concentrated using ultracentrifugation device; Centriprep (3kDa, Amicon). Protein was passed through Superdex S75 16/60 column (GE) equilibrated with buffer-C. Peak fractions were pooled and dialyzed against buffer-D. Peak fractions were pooled and concentrated to 35 mg/ml. Protein purity was assessed using a 4-20% SDS-PAGE and concentration were estimated using OD_{280nm} measurement and theoretical molar extinction coefficient (22920 M⁻¹cm⁻¹). Prior to storage, dithiothreitol (DTT) was added to final concentration of 10 mM. 50 µl aliquots in PCR vials were flash frozen in liquid nitrogen (LN2) and stored at -80 °C till further use.

EcGrx2^{C9S/C12S} and *EcGrx2^{C9S}*: The following protocol was followed for overexpression and purification of both *EcGrx2^{C9S/C12S}* and *EcGrx2^{C9S}* mutants. Briefly, plasmids pETM11-*grxB^{C9S/C12S}* and pETM11-*grxB^{C9S}* were transformed into *E. coli* BL21(DE3). Overexpression and cell lysis were carried out similar to native *EcGrx2*. The 0.45 µm

filtered supernatant was passed through 5ml HisTrap FF column (GE) equilibrated with buffer-E. The column was washed with 10 cv of buffer-E followed by elution using a linear gradient of 10-300 mM Imidazole. Peak fractions were pooled and concentrated using centrprep and was passed through Superdex S75 16/60 column (GE) equilibrated with buffer-F. Peak fractions containing pure protein were pooled, concentrated to 4 mg/ml and 50 µl of aliquots were flash frozen in LN2 and stored at -80 °C. *EcGrx2-eGFP-His6*: Plasmid pQE30- *grxB-eGFP* was transformed into DH5α and tested for protein expression prior to microscopic studies. Briefly, cell lysis was carried out similar to native *EcGrx2* and the lysate was centrifuged at 15000 g for 30 minutes at 4 °C and then the supernatant was used for binding Ni-NTA beads, eluted with buffer-G and checked on SDS-PAGE. *eGFP-His6*: Plasmid pQE30-*eGFP* was transformed into DH5α and checked for expression, similar to *EcGrx2-eGFP-His6*, prior to microscopic studies.

DHA reductase assay: All enzymatic reactions were analysed using a UV-Spectrometer: 3100 (Amersham / Pharmacia). DHA reductase activity of *EcGrx2* and its mutants *EcGrx2*^{C9S} and *EcGrx2*^{C9S/C12S} was assayed as described earlier³⁷ with minor modifications. Prior to enzyme assay, protein aliquotes were thawed and exchanged to buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl to remove DTT and βME, using 3kDa Centrprep (GE). The reaction was initiated by adding 2 mM GSH into the 500 µl reaction mixture containing 0.08 µg protein in 50 mM sodium phosphate buffer pH 7.4 and 0.1 mM DHA. Absorbance of AsA was monitored at 265 nm for 5 minutes. Curve fitting and V_{max} and K_m were calculated using GraphPad Prism software. For inhibition assay, the reaction was initiated by adding 2 mM GSH and a fixed concentration of 45µM ZnSO₄ (IC₅₀) in 500 µl reaction mixture containing 0.08 µg of protein, with varying

concentrations of DHA (0.01-9 mM). Control experiments were carried out without ZnSO₄. Lineweaver-Burk plot was plotted using Excel (Microsoft).

Chemical modification: *EcGrx2* was incubated with 0.5 to 2 mM iodoacetamide (IAM) at 4 °C for 2 h, followed by buffer exchange into 20 mM Tris-HCl pH 8.0, 150 mM NaCl using centrprep (3 kDa) to remove excess reagents.

Thermal shift assay (TSA): 20 μM tagless *EcGrx2* was added to the buffer containing 20 mM Tris-HCl pH 7.5, 10 mM NaCl and 2X SYPRO orange dye (S6650, Invitrogen), and 20 μM of various metal ions (CuSO₄, FeSO₄, ZnSO₄, CaCl₂, NiSO₄, MgSO₄, CoCl₂, MnCl₂). The mixture was spun at 1000 x g prior to TSA. The samples were heated from 20 to 95 °C at the rate of 1 °C min⁻¹ and fluorescence signals were monitored using quantitative real-time PCR (CFX96 Real-Time System, Bio-Rad). Each experiment was done in triplicate and the average values were taken for analysis. Assay buffer with and without added protein were used as controls.

Crystallization and structure determination: *EcGrx2*-GSF- Zn²⁺: Prior to setting up crystallization plates, DTT was added to a final concentration of 5 mM to the tagless protein (25 mg/ml) and incubated for 2 hr at RT. GSH and DHA were added to the above mixture to a final concentration of 20 and 10 mM, respectively, and incubated overnight at 4 °C. The protein-ligand solutions were centrifuged at ~ 16000 x g for 15 minutes at 4 °C. Crystallization trials were done at 20 °C using sitting-drop vapor diffusion method using protein to precipitant ratio; 1:1, 1:2 and 2:1 to a final volume of 150 nl, using commercial crystallization screens (Hampton Research and Molecular Dimensions) and Mosquito robot (TTP Labtech). Plate-like crystals were obtained after 5 days in the condition

containing 10 mM 0.01 M ZnSO₄·7H₂O, 0.1M MES monohydrate pH 6.5, 25% PEGMME

550. Crystals were quickly washed and soaked in the reservoir condition containing 20%

(v/v) glycerol as a cryoprotectant and flash-cooled in LN₂. Diffraction data were

collected at ID29 beamline, ESRF, Grenoble, France and integrated and scaled using the

autoPROC³⁸. The crystal belongs to the space group P2₁2₁2 with one *EcGrx2*-Zn-GSF

complex per asymmetric unit. Initial phases were obtained using Molecular Replacement

method, using AutoMR module in PHENIX suite³⁹ with *EcGrx2*-GSH (PDB: 4KX4) as

the search model. Iterative model building and refinement were carried out in Coot⁴⁰ and

Refmac⁴¹. Coordinates and restraints for GSF were generated using through JLigand

(CCP4i suite). The final model was refined to R_{work}/R_{free} = 0.13/0.18 (Table 1).

Tetrahedral coordination of Zn²⁺ was validated using CheckMyMetal server¹⁹

(Supplementary Table 2). ***EcGrx2*-GSH:** Protein at 25 mg/ml was treated in a similar way

as in the case of *EcGrx2*-Zn-GSF complex, before setting up plates. GSH bound *EcGrx2*

crystals were obtained after 5-10 days in 0.2 M ammonium acetate, 0.1 M sodium citrate

tribasic dihydrate pH 5.6, and 30% PEG 4000, flash-cooled in LN₂ with 20% (v/v)

glycerol as cryoprotectant. Data were collected at ID30-A1 beamline (ESRF) and

integrated and scaled as mentioned before. Crystals diffracted to 1.45 Å and belonged to P

2₁ space group with four molecules of *EcGrx2*-GSH complex per asymmetric unit. Data

processing, molecular replacement, model building and refinement were performed as

above. The final model was refined to R_{work}/R_{free} = 0.138/0.160. ***EcGrx2*-apo:** Crystals

belonging to the space group P 2₁ 2₁ 2₁ were grown at 35 mg/ml in 0.1 M HEPES pH 7.5,

20% w/v polyethylene glycol 10,000 and diffracted beyond 2.38 Å and X-ray data was

collected at XRD2 beamline, Elettra, Trieste, Italy. Data processing and scaling were done

using iMosflm⁴². Molecular replacement, model building, refinement was done as above.

The final model was refined to Rwork/Rfree =0.195/0.249. Structure-based sequence alignment was made using Dali server. Figures were made using PyMol (Schrodinger).

Molecular dynamics stimulation: Atomistic simulations were performed using the *EcGrx2*-apo and *EcGrx2*-Zn²⁺-GSF complex structures using Schrodinger (licenced to ICGEB). First, individual structures were incorporated into Maestro. The protein preparation wizard was used to assign bond orders, addition of hydrogens, filling missing side chains, creating zero bond order to metals and creating disulphide bonds. Hydrogen bond network was optimized at pH 7.4 and crystallographic water molecules were retained during protein preparation. A final restrained minimization was performed using the OPLS3e force field. The system was built using Desmond. The orthorhombic box was solvated with TIP3P solvent model and further neutralized with 0.15 M NaCl as counter ions. Simulations were performed at 300 K and at 1.0325 bar pressure using NPT ensemble. Solvated system was relaxed with a series of energy minimization before production MD. The total simulation time for each system was set to 1000 ns and the coordinates were saved at an interval of 50 ps.

Ascorbate supplementation assay: The assay was performed using BW25113 and JW1051-3 (*grxB*⁻, Kan^R) strains using a plating protocol⁴³ with modifications. Briefly, cells were grown in LB broth till OD₆₀₀ of 0.3. Aliquots consisting of 0.5 ml culture and 4.5 ml of soft agar, containing 0.8% agar in M9 media with respective antibiotics, were mixed and poured immediately over hard agar (1.5% agar in M9 minimal media, with respective antibiotics). After the plates were solidified, a 0.8 cm diameter filter paper disc (Whatman 3mm Chr), saturated with 10 µl of 6.6 M H₂O₂, was placed in middle of the plate and incubated at 37 °C for 12 h. Each experiment was done in triplicates with

biological replicates (n=2). The zone of clearance (diameter) was calculated by taking the mean value of measurements taken from three different directions. Statistical analysis, one-way ANOVA followed by Tukey's test, was done using SPSS (V. 20). A p-value of ≤ 0.05 is considered significant for the test.

Macrophage killing assay: RAW 264.7 macrophages were grown in Dulbecco's modified Eagle's media (DMEM, cell clone) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO) and maintained at 37 °C in a humidified, 5% CO₂ atmosphere. LPS (20 ng/ml) treatment was given to RAW 264.7 cells for 24 h (for pre-activation of macrophages), washed with plain DMEM and infected with respective bacteria (BW25113 and JW1051-3) with a MOI of 10, for 30 min. Thereafter, the cells were washed with 1X PBS and maintained in complete DMEM with amikacin (200µg/ml) for 1 h to kill extracellular bacteria. Subsequently, cells were washed twice with 1X PBS and maintained in complete DMEM for monitoring at different experimental time points (0, 30, 60, 120 and 240 min). At every timepoint, cells were lysed using 1X PBS containing 0.06% SDS, 1/100 dilution of the lysate was spread on plain LB plates, and incubated at 37 °C for 12 h. Plates were collected after 12 h and colony count was taken for calculating the CFU/ml.

Confocal and TIRF-SIM microscopy: BW25113 WT cells transformed with pQE30-*grxB-eGFP* and pQE30-eGFP, expressing His6-*EcGrx2*-eGFP and His6-eGFP, were grown at 37 °C till stationary phase, 12 h. Cells were pelleted down at 2095 x g at RT and washed twice with 1X PBS, followed by M9 minimal media to remove the autofluorescence from LB media. Cells were then incubated for 10 minutes at RT with FM4-64 plasma membrane stain (#T13320, Invitrogen) and washed using M9 minimal media. Cells were layered onto slides coated with poly-L-lysine, mounted using

Fluoroshield (# F6057, Sigma) and sealed. Images were acquired using a Nikon A1R-A1 fluorescence microscope with a $\times 100$ objective with 4.1 X zoom factor. The same field of view was chosen for TIRF-SIM imaging. Membrane localization of *EcGrx2* in BW25113 was observed using 100X objective and a SIM based illumination using ± 1 order light with a fixed critical angle for TIRF Imaging. The light source was a 488 nm laser. Fluorescence was observed through a 488 nm band-pass filter using an electron-multiplying CCD (iXon 897, Andor). The beam was totally internally reflected at an angle of 63° from the surface normal. An elliptical area of $32 \times 32 \mu\text{m}$ was illuminated for TIRF-SIM imaging. The images were processed using NIS-Elements AR software.

Membrane isolation and detergent extraction: BL21 (DE3) cells transformed with pET16b-*grxB-eGFP*, expressing *EcGrx2*-eGFP-His6, were resuspended at 1:10 ratio (pellet:buffer) in lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM KCl, 10 mM MgCl_2 , 0.2 mg ml^{-1} Lysozyme, 3 $\text{units } \mu\text{l}^{-1}$ DNase-I, 1 mM Benzamidine, 1 mM PMSF were disrupted by sonication. Cell lysate was centrifuged at 15000 g for 20 min at 4°C . The supernatant was spun at 1,00,000 x g for 1 h at 4°C . The pellet containing pure membrane was resuspended at 1:10 ratio in wash buffer containing 50 mM Tris-HCl pH 8.0, 5 % Glycerol, 100 mM KCl, 1 mM PMSF and 2 M Urea, using a Dounce homogenizer, and left at 4°C for overnight. Sample was spun again at 1,00,000 g at 4°C for 1 h. The pellet was further washed with resuspension buffer containing 50 mM Tris-HCl pH 8.0, 5 % Glycerol, 100 mM KCl and 1 mM PMSF, at 1:10 ratio, and spun at 1,00,000 x g at 4°C for 1 h. Equal amount of washed membrane pellet was solubilized in the above buffer containing 1% DM, DDM, OG, LDAO and SDS, respectively, and incubated at RT for 1 h and spun at 1,00,000 x g for 1 h. Supernatant containing detergent extracted *EcGrx2* were subjected to reduced and denaturing SDS-PAGE and western blot analysis using anti-His

antibodies (A7058, sigma). Equal volume of ultra-supernatant was loaded on the gel. SDS: sodium dodecyl sulphate, OG: n-Octyl- β -D-glucopyranoside, DDM: n-dodecyl- β -D-maltopyranoside, DM: n-Decyl- β -D-Maltopyranoside, LDAO: Lauryl dimethylamine oxide.

Bilayer electrophysiology: Planar lipid bilayer bilayers were formed using 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids) lipids by employing Montal and Muller technique. Lipid bilayers were formed across $\sim 100 \mu\text{m}$ in diameter an aperture in a 25- μm thick polytetrafluoroethylene (Teflon) film (Goodfellow, Cambridge). Notably, Teflon film separated the bilayer cuvette made in Delrin into cis and trans chambers (500 μL each). Bilayers were formed by pre-painting on each side of the Teflon aperture with hexadecane in n-pentane (1 μL , 5 mg mL^{-1}). Then, both cis and trans chambers were filled with 1 M KCl, 10 mM MES, pH 6.0 that acts as the electrolyte. Finally, DPhPC lipids in n-pentane (2 μL , 5 mg mL^{-1}) were added to both sides of the chamber and a solvent-free lipid bilayer was formed by lowering and raising the electrolyte buffer subsequently bringing the two lipid surface monolayers at the aperture. The purified *EcGrx2* was added to the cis side of the bilayer chamber with 1 mM DTT and a voltage of + 100 mV was applied to facilitate the channel insertion. The cis chamber was connected to the grounded electrode and the trans chamber was attached to the working live electrode. A potential difference was applied through a pair of Ag/AgCl electrodes, set in 3% agarose containing 3.0 M KCl. The current was amplified by using an Axopatch 200B amplifier, digitized with a Digidata 1550B and recorded with the pClamp 10.6 acquisition software (Molecular Devices, CA) with a low-pass filter frequency of 2 kHz and a sampling frequency of 10 kHz. The data were analysed and prepared for presentation with pClamp (version 10.6, Molecular Devices, CA) and Origin 9.0.

Phylogenetic tree construction: HMMER 3.3.3 was used to retrieve *EcGrx2* like sequences with *E. coli* str. K-12 substr. MG1655 Grx2 as input from UniProtKB database. *grxB* gene encoded Grx2 sequences were manually selected (one per genus) with the highest E-value and having two domain architecture similar to *EcGrx2*. A total of 152 sequences was used for multiple sequence alignment using MUSCLE. The aligned sequences were then given as input to MEGA-X⁴⁴ to construct a phylogenetic tree using maximum likelihood method with default settings. The tree with the highest log likelihood (-40838.84) was used to make the circular phylogenetic tree using iTOL⁴⁵.

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622 **Author contributions:** SNS, BKD and RR generated constructs, expressed, purified
623 *EcGrx2*, performed enzyme assays. PR performed initial expression and purification. SNS
624 performed ascorbate supplementation and SK and AC performed statistical analysis. SNS,
625 AK, RR and AA solved the crystal structures and SS performed MD simulation. SU and
626 SNS performed macrophage killing assay, supervised by DK. NP and SNS carried out ion-

channel recordings, supervised by KRM. P. Ray and AC provided inputs to experimental plan and data analysis. SNS and AA wrote the manuscript with inputs from all the authors. AA conceived, planned and orchestrated the project.

Competing interests: Authors declare that they have no competing interests.

Data availability: The atomic coordinates and structure factors are available from Protein Data Bank under the accession codes 7DKR, 7DKP, 7D9L for native, GHS-bound and Zn²⁺ inhibited structures, respectively. The raw X-ray diffraction data is available from Integrated Resource for Reproducibility in Macromolecular Crystallography (IRRMCM) repository: <https://proteindiffraction.org/>

Fig. 1. *EcGrx2* is a DHA reductase and Zn²⁺ ion inhibits enzyme activity. (a) Structural superposition of *EcGrx2* (7DKP, green) and *PgDHAR* (5EVO, cyan) and *HsCLIC1* (1K0M, red). (b) Structure-based multiple sequence alignment showing conserved active site motif: CxxC(S). Pg: *Pennisetum glaucum*, Hs: *Homo sapiens*, Ec: *Escherichia coli*, St: *Salmonella typhimurium*. (c) Electrostatic potential surface maps of predicted active site region in *EcGrx2* in comparison to *PgDHAR* and *HsCLIC1* showing conserved residues. (d) Michaelis-Menten kinetics* of *EcGrx2*; native, double mutant and chemically modified protein is shown. *EcGrx2* (●), *EcGrx2*-reduced and treated with IAM (▲), *EcGrx2*^{C9S/C12S} (■). (e) Lineweaver-Burk plot* showing competitive inhibition by Zn²⁺. *EcGrx2* (◆), *EcGrx2* + 45 μM ZnSO₄ (■). (f) Crystal structure of Zn²⁺ inhibited *EcGrx2* with the inset showing omit difference (Fo-Fc) map contoured at 3σ (grey), wherein Zn²⁺ ion is shown at 15σ (green). (g) Tetrahedral coordination of Zn²⁺ with GSF, two waters (W1, W2) and His8 is shown. (h) RMSD plot of 1 μs MD simulation of *EcGrx2*-GSF-Zn²⁺ complex is shown for protein backbone (Blue), GSF (Green) and Zn²⁺ (Grey). (i) Distance plot representing interatomic distance between Zn²⁺ and the two coordinating waters are shown. *Kinetic values represent mean ± standard deviation of technical triplicates (n=3). AsA monitored (min⁻¹) at A₂₆₅ is plotted against different concentration of DHA (μM).

Figure-2

Fig. 2. AsA rescues *E. coli* from H_2O_2 induced stress and *EcGrx2* mutant is susceptible to macrophage mediated killing. (a, b) ascorbate supplementation assay using filter disc saturated with H_2O_2 is shown using wild type and mutant strains BW25113, JW1051-3 ($\Delta grxB$, Kan^R), respectively. Zone of clearance (cm) was calculated from triplicates (biological replicates, n=2), and statistical significance calculated using one-way ANOVA (*, $p < 0.05$). (c) Survival of wild type and mutant *E. coli* strains inside LPS activated RAW264.7 murine macrophages is represented as number of CFU/ml, plotted at different time points. Bars = mean $\pm$ s.e.m., values are from three independent experiments. *Significantly different from WT ($p < 0.001$) using one-way ANOVA.

Figure 3

Fig. 3. *EcGrx2* exists in membrane integrated form and soluble protein conducts ion after inserting into lipid bilayer. (a) Confocal and TIRF-SIM microscopic images showing membrane localization of Grx2 in BW25113 *E. coli* cells. left: cells transformed with eGFP alone, right: cells transformed with Grx2-eGFP. (i) eGFP channel, (ii) stained for plasma membrane, (iii) merged images of (i) and (ii), and the inset (iv) showing 4 X zoom and the corresponding region under TIRF-SIM (v) is shown with arrows pointing to the membrane localized protein. Scale bars=5µM. (b) 4-20% reduced SDS-PAGE (left) and corresponding Western blot (right) loaded with detergent extracted samples using pure membranes isolated from BL21 (DE3). L: standard markers, S: ultra-supernatant, M: membrane after 2M urea wash. (c-f) Ion current recordings of *EcGrx2* reconstituted into planar lipid bilayers at (c) +50 mV, (d) flickering events characteristic of channels at +50 mV, (e) -50 mV showing noisy current fluctuations, (f) channel gating at +100 mV. Buffer conditions: 1 M KCl, 10 mM MES, pH 6.0. The current signals were filtered at 2 kHz and sampled at 10 kHz. (g) Model depicting the dimorphic and bifunctional nature of *EcGrx2*.

Uncropped gel and blot are given in supplemental information. (c-f) Ion current recordings of *EcGrx2* reconstituted into planar lipid bilayers at (c) +50 mV, (d) flickering events characteristic of channels at +50 mV, (e) -50 mV showing noisy current fluctuations, (f) channel gating at +100 mV. Buffer conditions: 1 M KCl, 10 mM MES, pH 6.0. The current signals were filtered at 2 kHz and sampled at 10 kHz. (g) Model depicting the dimorphic and bifunctional nature of *EcGrx2*.

Table 1. X-ray data collection and refinement statistics

Data collection (PDB)	<i>EcGrx2-apo</i> (7DKR)	<i>EcGrx2-GSH</i> (7DKP)	<i>EcGrx2-Zn²⁺-GSF</i> (7D9L)
X ray wavelength (Å)	1.00	0.96770	0.98400
Space group	P 2 ₁ 2 ₁ 2 ₁	P 1 2 ₁ 1	P 2 ₁ 2 ₁ 2
Cell dimensions			
a, b, c (Å)	28.47, 78.95, 89.28	49.827, 169.408, 49.836	46.095, 106.758, 42.130
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 93.52, 90.0	90.0, 90.0, 90.0
Resolution (Å)	44.64 - 2.38 (2.47 - 2.38)	42.893 - 1.449 (1.476 - 1.449)	42.319 - 1.60 (1.628 - 1.60)
Refs. total/unique	95444/8647	576328/137631	176283/28045
R _{merge} (%)	0.111 (0.297)	0.099 (0.635)	0.19 (1.207)
I/σ (I)	15.2 (6.5)	8.7 (1.9)	11.7 (3.4)
Completeness (%)	100 (100)	94.7 (89.0)	99.3 (99.4)
Redundancy	11.0 (9.5)	4.2 (4.2)	6.3 (6.2)
CC _{1/2}	0.997 (0.973)	0.997 (0.759)	0.995 (0.555)
Refinement			
Resolution (Å)	2.38	1.45	1.61
R _{work} /R _{free} (%)	0.195/0.249	0.138/0.160	0.130/0.182
No. of atoms			
Protein (non-H atoms)	1682	6906	1694
L-gamma-glutamyl-3-sulfino-L-alanyl-glycine	-	-	22
γ-L-Glutamyl-L-cysteinylglycine	-	80	-
Oxygen molecule	-	-	2
Zinc ion	-	-	2
Chloride ion	-	-	1
Sulfate ion	-	-	5
Citrate anion	-	39	-
Water	104	1314	156
Average B factor (Å²)			
Protein	20.839		14.332
Water	24.487	25.962	27.212
r.m.s. deviations			
Bond lengths (Å)	0.43	0.91	0.82
Bond angles (°)	0.55	1.02	1.06
Ramachandran plot			
Favoured	205 (96%)	847 (98%)	211 (98%)
Allowed	7 (3%)	19 (2%)	5 (2%)
Outliers	1 (0%)	3 (0%)	0

r.m.s., root mean square. Each data set was collected from a single crystal. Values in parentheses are for high-resolution shell.

Supplementary Figure 1

Supplementary Figure 1. Purification, DHA reductase activity and thermal stability assays with *EcGrx2*. (a) Chromatogram from Superdex S-75 (16/60) column with inset showing peak fractions loaded onto 4-20 % SDS-PAGE showing purified *EcGrx2* at ~24kDa size. (b) Michaelis-Menten graphs of *EcGrx2* showing DHA reductase activity. AsA min⁻¹ monitored at A₂₆₅ plotted against different concentration of DHA (μM) and (c) GSH (μM). (d) Identification of divalent metal ion binding to *EcGrx2* by thermal shift assay. Inset table shows comparison of melting temperatures (T_m) from thermal shift assay. Data shown represent three technical replicates. Different divalent metal ions are indicated with different colours.

Supplementary Figure 2. Structural analysis of Zn^{2+} inhibited complex crystal structure. (a) Electrostatic surface map of the active site showing Zn^{2+} inhibited state. Positively charged region is shown in blue while negatively charged region is in red. (b) Backbone RMSD profile of *EcGrx2*-apo and *EcGrx2*-GSF- Zn^{2+} complex during 1 μs MD simulation is shown. (c) Second Zn^{2+} binding site formed at the interface of symmetry related monomers: A and B with inset showing the tetrahedral coordination formed by A-His67 (blue), A-Asp170 (pea), Chloride ion (teal) and B-His139 (sand). The omit difference ($\text{Fo}-\text{Fc}$) map is contoured at 3σ with Zn^{2+} and Cl^- ions shown at 15σ and 3σ , respectively. (d) Crystal packing showing the second Zn^{2+} ion.

Supplementary Figure 3. Structural homology and distribution of *grxB* gene in Gram-negative bacterial genomes. (a) Structural superposition of *EcGrx2* (7DKP, violet) with *StGrx2* (3IR4, orange) showing very low RMSD. (b) Phylogenetic tree of Gram-negative bacteria possessing *grxB* encoded two domain Grx2 is given. Bootstrap value=1000.

Supplementary Table 1. Dehydroascorbate reductase activity data of *EcGrx2* and its mutants.

	<i>EcGrx2</i> *	<i>EcGrx2</i> C9SC12S*
K _m DHA (μM)	2879 (±640.4)	968 (±462.3)
K _m GSH (μM)	1630 (±363)	ND
Specific activity (μM ⁻¹ min ⁻¹ mg)	1.8845 (±.01)	0.09705
K _{cat} /K _m (DHA) (M ⁻¹ min ⁻¹)	7.9704	1.22090
Reference	This study	This study

*Mean± standard deviation of three separate set of experiments

Supplementary Table 2. CheckMyMetal (CMM)-report for *EcGrx2*-GSF-Zn²⁺ complex

Warning: Coordinating ligands by symmetry operation are labeled with prefix 'sym-'												
ID	Res.	Met al	Occu panc y	B factor (env.) ¹	Ligan ds	Val enc e	nVE CSU M	Geomet ry	gRMSD (°) ¹	Vacanc y ¹	Biden tate	Alt. metal
A:216	ZN	ZN	1	13.1 (15.1)	O ₄ N ₁	<u>1.6</u>	<u>0.18</u>	Tetrahe dral	6.3°	0	1	Cu, Co
A:217	ZN	ZN	1	19 (21)	O ₁ N ₂	1.9	0.045	Tetrahe dral	3.8°	<u>25%</u>	0	
A:218	CL	CL	1	<u>26.8</u> (22.6)		N/A	N/A	<u>Free</u>	N/A	N/A	N/A	
Legend:			Not applicable	Outlier	Boderline	Acceptable						

Column	Description
Occupancy	Occupancy of ion under consideration
B factor (env.) ¹	Metal ion B factor, with valence-weighted environmental average B factor in parenthesis
Ligands	Elemental composition of the coordination sphere
Valence ²	Summation of bond valence values for an ion binding site. Valence accounts for metal-ligand distances
nVECSUM ³	Summation of ligand vectors, weighted by bond valence values and normalized by overall valence. Increase when the coordination sphere is not symmetrical due to incompleteness.
Geometry ^{1,4}	Arrangement of ligands around the ion, as defined by the NEIGHBORHOOD algorithm
gRMSD(°) ¹	R.M.S. Deviation of observed geometry angles (L-M-L angles) compared to ideal geometry, in degrees
Vacancy	Percentage of unoccupied sites in the coordination sphere for the given geometry
Bidentate	Number of residues that form a bidentate interaction instead of being considered as multiple ligands
Alt. metal	A list of alternative metal(s) is proposed in descending order of confidency, assuming metal environment is accurately determined. This feature is still experimental. It requires user discrimination and cannot be blindly accepted

Supplementary Table 3. Statistical analysis of organisms in ascorbate supplementation assay

TUKEY HSD (one way ANOVA)				
Dependent variable	(I) Groups	Dependent variable	(J) Groups	Sig.
JW1051-3	0% ascorbate	JW1051-3	0.3% ascorbate	0.000
BW25113	0% ascorbate	BW25113	0.3% ascorbate	0.000
BW25113	0% ascorbate	JW1051-3	0.3% ascorbate	0.000

*The mean difference is significant at 0.05 level.

Supplementary Table 4. Statistical analysis of bacterial survival in LPS activated RAW264.7

TUKEY HSD (one way ANOVA)			
Dependent variable	(I) Groups	(J) Groups	Sig.
0 min	JW1051-3	BW25113	0.92
	BW25113	JW1051-3	0.92
30 min	JW1051-3	BW25113	0.98
	BW25113	JW1051-3	0.98
60 min	JW1051-3	BW25113	0.18
	BW25113	JW1051-3	0.18
120 min	JW1051-3	BW25113	0.1
	BW25113	JW1051-3	0.1
240 min	JW1051-3	BW25113	<0.001
	BW25113	JW1051-3	0

*The mean difference is significant at 0.05 level.

Supplementary Table 5. Plasmids, *E. coli* strains, and oligos used in this study

Strains/Plasmids/Oligos	Expression host / oligo sequence	Description
<i>E. coli</i> BW25113 (WT)		$\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>
<i>E. coli</i> JW1051-3 ($\Delta grxB$)		$\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta grxB734::kan$, <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i> ; Kan ^R
pET24a- <i>grxB</i>	<i>E. coli</i> Rosetta2 (DE3) pLysS	Native <i>EcGrx2</i> without tag, H. Dyson Lab, Scripps Research Institute, La Jolla, USA) ⁵
pET16b-eGFP		Aravind Penmasta Lab, MBU, IISc, Bangalore
pETM11- <i>grxB</i>	<i>E. coli</i> Rosetta2 (DE3) pLysS	<i>grxB</i> cloned under NcoI and KpnI
pETM11- <i>grxB</i> ^{C9S/C12S}	"	<i>grxB</i> ^{C9S/C12S} cloned under NcoI and KpnI
pQE30- <i>grxB</i>	"	<i>grxB</i> cloned under NcoI and BamHI
pET16b- <i>grxB-eGFP</i>	"	<i>grxB</i> cloned under NcoI and KpnI
pQE30- <i>grxB-eGFP</i>	"	<i>grxB-eGFP</i> cloned under BamHI and HindIII.
pQE30-eGFP	"	<i>eGFP</i> cloned under BamHI and KpnI
EcG1FP	GTACTCCCATGGTGAAGCTA TACATTTACGAT	For PCR amplification of <i>grxB</i> from pET24a- <i>grxB</i> and clone into pETM11 under NcoI and KpnI.
EcG1RP	GTACTCGGTACCTTAAATCG CCATTGATGA	"
EcG2FP	TACATTTACGATCACAGCCC TTACAGCCTCAAAGCCCGCA TG	To generate <i>grxB</i> -C9S/C12S double mutant
EcG2RP	CATGCGGGCTTTGAGGCTGT AAGGGCTGTGATCGTAAATG TA	"
EcG3FP	GTACTCGGATCCATGAAGCT ATACATTTACGAT	For PCR amplification of <i>grxB</i> from pETM11- <i>grxB</i> and clone into pQE30 between BamHI and KpnI.
EcG3RP	GTACTCGGTACCTTAAATCG CCATTGATGA	"
EcG4FP	GTACTCCCATGGTGAAGCTA TACATTTACGAT	For PCR amplification of <i>grxB</i> from pET24a- <i>grxB</i> and clone into pET16b-eGFP under NcoI and KpnI.
EcG4RP	GTACTCGGTACCAATCGCCA TTGATGATAA	"
EcG5FP	GTACTCGGATCCATGAAGCT ATACATTTACGAT	For PCR amplification of <i>grxB-eGFP</i> from pET16b- <i>grxB-eGFP</i> and clone into pQE30 under BamHI and HindIII.
EcG5RP	GTACTCAAGCTTTTATCCGG ACTTGTACAGCTC	"
EcG6FP	GTACTCGGATCCATGGCGGC CGCCGCGGTGAGC	For PCR amplification of <i>eGFP</i> from pET16b-eGFP and clone into pQE30 under BamHI and KpnI.
EcG6RP	GTACTCGGTACCTTATCCGG ACTTGTACAGCTCGTC	"

Supplementary Table 6. Composition of buffers used for protein purification and membrane extraction.

Name of the buffer	Composition	pH	NaCl (mM)	KCl (mM)	Imidazole (mM)	β ME (mM)	DTT (mM)	Glycerol (%)	PMSF (mM)
A*	50 mM Tris-HCl	7.5	10			3			1
B	10 mM MES	6.5	"				3		
C	20 mM Tris-HCl	8.0	1000			3			
D	"	"	150			3			
E	25 mM Tris-HCl	"	"		10				
F	"	"	"		10				
G	"	"	"						
H	"	"	"		300				
I	20 mM Tris-HCl	"	"						
J#	50 mM Tris-HCl	"		150					1
K\$	"	"		100				5	1
L	"	"		100				5	1

*Contains 1 mM Benzamidine-HCl, 3 units μ l⁻¹ DNase I and 0.2 mg ml⁻¹ Lysozyme, #-contains 10mM MgCl₂, 1 mM Benzamidine-HCl, 3 units μ l⁻¹ DNase I and 0.2 mg ml⁻¹ Lysozyme, \$- contains 2M Urea.

Supplementary Video 1. MD simulation trajectory showing Zn²⁺, coordinated waters (W1, W2), GSF, and His8 of *EcGrx2*.

(a)

(b)

(c)

(a) Uncropped SDS-PAGE after membrane isolation and detergent extraction, (b) Corresponding western blot of (a), and (c) Scanned full images of petri plates used for making Figure 2a. Strains BW25113 and JW1051-3 are labelled, by hand, as BW2113 and JW1051, respectively.