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The *E. coli* BolA protein IbaG forms a histidine-ligated [2Fe-2S] bridged complex with Grx4

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Abstract

Two ubiquitous protein families have emerged as key players in iron metabolism, the CGFS type monothiol glutaredoxins (Grxs) and the BolA proteins. Monothiol Grxs and BolA proteins form heterocomplexes that have been implicated in Fe-S cluster assembly and trafficking. The *E. coli* genome encodes members of both of these proteins families, namely the monothiol glutaredoxin Grx4, and two BolA family proteins, BolA and IbaG. Previous work has demonstrated that *E. coli* Grx4 and BolA interact as both apo and [2Fe-2S]-bridged heterodimers that are spectroscopically distinct from [2Fe-2S]-bridged Grx4 homodimers. However, the physical and functional interactions between Grx4 and IbaG are uncharacterized. Here we show that co-expression of Grx4 with IbaG yields a [2Fe-2S]-bridged Grx4-IbaG heterodimer. *In vitro* interaction studies indicate that IbaG binds the [2Fe-2S] Grx4 homodimer to form apo Grx4-IbaG heterodimers as well as the [2Fe-2S] Grx4-IbaG heterodimer, altering the cluster stability and coordination environment. Additionally, spectroscopic and mutagenesis studies provide evidence that IbaG ligates the Fe-S cluster via the conserved histidine that is present in all BolA proteins and by a second conserved histidine that is present in the H/C loop of two of the four classes of BolA proteins. These results suggest that IbaG may function in Fe-S cluster assembly and trafficking in *E. coli* as demonstrated for other BolA homologues that interact with monothiol Grxs.

Graphical Abstract

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Supporting Information Available

This supporting information is available free of charge via the internet at <http://pubs.acs.org>.

CGFS-type monothiol glutaredoxins (Grxs) are a subset of the thioredoxin (Trx) superfamily characterized by a CGFS active site motif rather than the classical CxxC motif found in dithiol Trxs and Grxs that catalyze thiol-disulfide exchange reactions. Numerous studies have linked CGFS Grxs to iron metabolism and demonstrated that the CGFS-type Grxs from both prokaryotes and eukaryotes form [2Fe-2S]-bridged homodimers with the active site cysteines and two GSH molecules ligating the Fe-S cluster.¹⁻³ A detailed picture of the specific functions of CGFS type Grxs has evolved primarily from work with yeast, plant, and vertebrate homologues of these proteins. Eukaryotes express multiple CGFS-type Grxs that are localized to different subcellular compartments. Mitochondrial versions (Grx5/GLRX5 in yeast and vertebrates) typically have a single Grx domain that interacts with the core iron-sulfur cluster (ISC) assembly machinery facilitating transfer of assembled [2Fe-2S] clusters from U-type scaffold to acceptor proteins, including the A-type carrier proteins.⁴⁻⁸ As such, mutation or deletion of Grx5 orthologs leads to phenotypes that suggest impairment of mitochondrial Fe-S cluster biogenesis and disruption of iron regulation. Cytosolic CGFS Grxs (Grx3/4/GLRX3 in yeast and mammalian cells) are multidomain proteins with one Trx-like domain and one or more Grx domains in tandem.^{1, 2} Studies in yeast and mammalian cells demonstrate that these Grx3/4 orthologs are critical for iron trafficking and signaling iron availability to iron regulatory proteins.⁹⁻¹³

The functions of CGFS monothiol Grxs in iron metabolism are closely linked with another widely conserved protein family, the BolA proteins. Prokaryote and eukaryote genome analyses demonstrated that CGFS Grxs and BolA proteins show strong gene co-occurrence and often cluster with Fe-S biogenesis operons, suggesting a shared role in Fe-S cluster metabolism.^{14, 15} This connection is supported by reports of human patients with mutations in the mitochondrial *bolA3* gene who exhibited mitochondrial phenotypes that overlapped with *glrx5* deficiencies, including defects in mitochondrial Fe-S cluster protein maturation impacting both respiration and lipoic acid synthesis.¹⁶⁻¹⁸ In addition, physical interactions between CGFS Grxs and BolA proteins have been reported in both prokaryotes and eukaryotes using high throughput and directed biochemical approaches.^{1, 2} Spectroscopic and biochemical analyses of Grx3/4-BolA complexes from yeast, plants and humans demonstrate formation of [2Fe-2S]-bridged heterocomplexes with Grx3/4 providing two Cys

ligands from the CGFS active site and GSH, while the BolA orthologs provide at least one His ligand that is conserved in all BolA protein family members.^{19–22} A molecular function for this Grx-BolA heterocomplex has been established in *S. cerevisiae*, with genetic and biochemical evidence indicating that cytosolic [2Fe-2S]-bridged Grx3-Fra2 heterodimers deliver an Fe-S cluster to the transcriptional regulator Aft1/2 to promote its dissociation from DNA and export from the nucleus.^{2, 23–27} Similarly, the orthologous complex in humans, namely GLRX3-BOLA2, was shown to transfer [2Fe-2S] clusters to the cytosolic Fe-S cluster biogenesis factor anamorsin.²⁸

Less is known about the specific roles of CGFS Grxs and BolA proteins in prokaryotes, including the model gram-negative bacterium *E. coli*. Previous studies have shown that the single CGFS Grx in *E. coli*, namely GrxD/Grx4, is ubiquitously expressed, yet with increased expression under stress conditions including iron starvation and stationary phase.²⁹ Likewise, deletion of Grx4 leads to hypersensitivity to iron deficiency.³⁰ In addition, the *grxD* mutant exhibits synthetic lethality in combination with mutations in the *isc* (iron sulfur cluster) assembly pathway, suggesting that Grx4 functions with the alternate Fe-S cluster assembly pathway encoded by the *suf* (sulfur utilization factor) operon, which is expressed primarily under oxidative stress and iron starvation conditions.³¹ Indeed, *E. coli* Grx4 binds a [2Fe-2S] cluster as a homodimer³ and was shown to help repair the Fe-S cluster on MiaB, a radical SAM enzyme involved in methylthiolation of certain tRNAs, which appears to utilize a sacrificial [4Fe-4S] cluster.³² Grx4 also forms a [2Fe-2S] bridged heterodimer with *E. coli* BolA similar to Grx-BolA complexes characterized from other organisms; however, the specific function of this complex is unclear.³⁰ BolA proteins also possess a putative nucleic acid binding domain based on structural analysis.³³ As such, the *E. coli* BolA protein has been implicated as a global transcriptional regulator of biofilm development via modulation of flagellum and cell wall biosynthesis genes.³⁴

The *E. coli* genome encodes a second BolA-like protein, YrbA, that currently has no reported function. However, a recent study demonstrated that the *yrbA* gene is induced under acid stress conditions and deletion of *yrbA* caused acid stress sensitivity. Consequently, *yrbA* was renamed *ibaG* (influenced by acid gene).³⁵ Similar to BolA, IbaG may have a function in cell wall biosynthesis, as genes found in the vicinity of *ibaG* are involved in maintenance of the outer membrane and cell wall. In addition, similar to *grxD*, *ibaG* displays an aggravating interaction with *isc* pathway components in a genome-wide synthetic lethality/sickness screen, suggesting that IbaG may function with the Suf proteins in Fe-S cluster assembly or sulfur donation.³¹ However, the potential role of IbaG in Fe-S cluster metabolism and its interaction with Grx4 have not previously been addressed. Here we present biochemical and spectroscopic data characterizing the interaction between IbaG and Grx4. We have established that Grx4 and IbaG form a heterodimer in the apo form as well as in the holo form with a [2Fe-2S] cluster bridging the two proteins. Furthermore, mutagenesis data indicate that IbaG binds to the Fe-S cluster through a conserved His residue similar to other Grx-BolA complexes characterized in eukaryotes. Spectroscopic and mutagenesis results also implicate a second His located at or near the cluster coordination environment. Together, this evidence suggests that IbaG and Grx4 function together in pathways that involve Fe-S cluster metabolism similar to other Grx4-BolA binding partners.

EXPERIMENTAL PROCEDURES

Plasmid Construction

The ORF of *E. coli* Grx4 was amplified from *E. coli* genomic DNA by PCR using the primers shown in Table S1 and cloned into the NcoI and BamHI sites of pRSFDuet-1 (Novagen) to generate pRSFDuet-1-Grx4. The ORF of *E. coli* BolA or IbaG was amplified from *E. coli* genomic DNA by PCR using the primers shown in Table S1 and cloned into pRSFDuet-1 at the NdeI and XhoI sites to generate pRSFDuet-1-BolA, or the NdeI and KpnI sites to generate pRSFDuet-1-IbaG. A dual expression plasmid for Grx4-IbaG was made by cloning IbaG into the NdeI and KpnI sites of pRSFDuet-1-Grx4 to create pRSFDuet-1-Grx4/IbaG. IbaG amino acid substitutions were created by site-directed mutagenesis of pRSFDuet-1-IbaG or pRSFDuet-1-Grx4/IbaG (QuikChange Mutagenesis kit, Stratagene) using primers listed in Table S1.

Protein Expression and Purification

Overexpression of Grx4 was performed in the *E. coli* BL21(DE3) strain in 1L of LB media at 30 °C shaking until the $A_{600} = 0.6$ –0.8 followed by induction with 1 mM isopropyl β -D-thiogalactoside (IPTG). The cells were collected 18 hr after induction and resuspended in 50 mM Tris/MES, pH 8.0, 5 mM GSH, sonicated, and centrifuged to remove cell debris. The cell-free extract was loaded onto a Q Sepharose anion-exchange column (GE Healthcare) equilibrated with 50 mM Tris/MES, pH 8.0, 5 mM GSH. The protein was eluted with a 0–1.0 M NaCl gradient using 50 mM Tris/MES, pH 8.0, 5 mM GSH, 1 M NaCl. Fractions containing Grx4 were pooled and $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 1 M. The sample was loaded onto a Phenyl Sepharose column (GE Healthcare) equilibrated with 50 mM Tris/MES, pH 8.0, 5 mM GSH, 1 M $(\text{NH}_4)_2\text{SO}_4$, 100 mM NaCl. The protein was eluted with a 1.0–0 M $(\text{NH}_4)_2\text{SO}_4$ gradient, and fractions containing Grx4 were concentrated and loaded onto a HiLoad Superdex 75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris/MES, pH 8.0, 5 mM GSH, 150 mM NaCl. The purest fractions of [2Fe-2S] Grx4 homodimer and apo-Grx4 monomer, as judged by SDS-PAGE and UV-visible spectroscopy, were collected separately and concentrated with the addition of 5% (v/v) glycerol and stored at –80 °C. All purifications were carried out under anaerobic conditions ($\text{O}_2 < 5$ ppm) in a glovebox (Coy Laboratory Products).

Coexpression and copurification of Grx4 with IbaG was performed with the pRSFDuet-1-Grx4/IbaG expression plasmid transformed into *E. coli* BL21(DE3), using the procedure described above for Grx4, except no GSH was added to the purification buffers. Purification of apo-BolA and apo-IbaG was performed as described previously for yeast apo-Fra2.²⁰ All IbaG variants (either alone or coexpressed) were purified using the same procedure as the WT form. The identities of all proteins described above were verified by proteolytic digestion with trypsin and mass spectrometry analysis.

Biochemical Analyses

Protein concentrations were determined by the Bradford Assay (Bio-Rad) using bovine serum albumin as the standard. Iron concentrations were determined using the colorimetric ferrozine assay.³⁶ Acid-labile sulfur concentrations were determined using published

methods.^{37, 38} For GSH measurements, the purified Fe-S protein complexes were denatured and precipitated with 1% 5-sulfosalicylic acid, and GSH in the supernatant was measured by the 5, 5'-dithiobis(2-nitrobenzoic acid) -GSSG reductase cycling assay as described previously.³⁹

Analytical and Spectroscopic Methods

Analytical gel filtration analyses were performed on a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with 50 mM Tris/MES, pH 8.0, 150 mM NaCl, 5 mM GSH and calibrated with the low molecular weight gel filtration kit (GE Healthcare) as described previously.²⁰ Mass spectrometry analysis of purified proteins was determined using a Bruker UltraFlex MALDI-TOF/TOF mass spectrometer. A saturated solution of sinapinic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as the matrix.

UV-visible absorption spectra were recorded using a Beckman DU-800 spectrophotometer. CD spectra were recorded under anaerobic conditions on identical samples using a Jasco J-715 or J-800 spectropolarimeter (Jasco, Easton, MD). X-band EPR spectra were recorded using a ESP-300D spectrometer (~9.6 GHz, Bruker, Billerica, MA) equipped with an ESR900 continuous flow cryostat (Oxford Instruments, Concord, MA). Spectra were quantified under nonsaturating conditions by double integration against a 1.0 mM CuEDTA standard. Resonance Raman spectra were recorded at 20 K as previously described using an Instruments SA Ramanor U1000 spectrometer coupled with a Coherent Sabre argon ion laser, with 20 μ l frozen droplets of ~1.5–2.5 mM sample mounted on the cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator.⁴⁰ All spectroscopy data shown are representative of 2 or more independent experiments.

Fe-S Cluster Stability Assays

[2Fe-2S] cluster degradation for as-purified Grx4 and Grx4-IbaG complexes was quantified by monitoring the change in A_{410} over two hours for samples in the presence and absence of both GSH and air. Experiments were performed in 50 mM Tris/MES, pH 8.0, 150 mM NaCl buffer, and [2Fe-2S] was 35–40 μ M at time = 0 minutes. Aerobic samples were scanned at ten minute intervals for the first 60 minutes, followed by two additional scans after 90 and 120 minutes. Anaerobic samples were scanned at 30 minute intervals for the entire two-hour experiment. GSH was used at a concentration of 5 mM.

CD-Monitored pH Titration of [2Fe-2S] Complexes

pH-dependent [2Fe-2S]²⁺ cluster ligation changes were monitored by UV-visible CD spectroscopy with [2Fe-2S] cluster concentration was kept constant at 38.2 μ M. Holo complexes of Grx4 and Grx4-IbaG were anaerobically equilibrated in 50 mM Tris/MES buffer at a pH range of 5.5–8.1 for 4 hrs at 4 °C before recording CD spectra. The pH of each sample was determined using a semi-micro pH electrode (Thermo Scientific Orion) immediately before the experiments to account for any drift over time. The temperature was maintained at 5 °C using a water bath cooling system to avoid pH shifts arising from temperature variations during sample scanning. The titration data was fit by nonlinear least-squares analysis using GraphPad Prism 7.0 to the Hill equation: $M = \text{Min} + (\text{Max}-\text{Min})/$

$(1+10^{((\log K_a - \text{pH}) * n)))$, where M is the CD intensity, M_{min} is the minimum CD intensity, M_{max} is the maximum CD intensity and n is the Hill coefficient.

CD-Monitored Titration of [2Fe-2S] Cluster-bound Grx4 with IbaG

The titration of [2Fe-2S]²⁺ cluster-bound Grx4 with apo-IbaG was monitored under anaerobic conditions at room temperature using UV-visible CD spectroscopy. Reactions were carried out in 50 mM Tris/MES, pH 8.0, 5 mM GSH, with the [2Fe-2S] cluster concentration kept constant at 100 μM and IbaG:[2Fe-2S] ratios varying from 0 to 4. Samples were equilibrated for 5 min at room temperature after addition of IbaG prior to recording CD spectra, followed by analytical gel filtration analyses on a Superdex 75 10/300 GL column (GE Healthcare) as described above to determine the oligomerization state of Grx4-IbaG complexes.

Isothermal Titration Calorimetry

Binding characteristics of the apo Grx4-BolA and Grx4-IbaG complexes were determined by ITC, using a VP-ITC titration microcalorimeter (MicroCal, Inc.). Experiments were performed at 26 °C by titrating a 1.24 mM solution of either BolA or IbaG in the syringe into a 62.1 μM solution of apo-Grx4 in the adiabatic cell, with all proteins prepared in 50 mM Tris-HCl, pH 8.0. For the BolA titration into Grx4, the initial four injections were at 2 μL , followed by four injections at 4 μL , four injections at 6 μL , and finally 25 injections at 10 μL . For the IbaG titration into Grx4, the initial injection was at 1 μL , followed by 59 additional injections at 3 μL . Data analysis was performed using the Origin Software (MicroCal) to calculate the binding stoichiometry, dissociation constant, and change in enthalpy and entropy of the titrations.

RESULTS

Grx4 and IbaG copurify as a complex that coordinates a [2Fe-2S] cluster

Given the strong connection between CGFS Grxs and BolA proteins in Fe-S cluster biogenesis and cellular Fe-S trafficking, we assessed whether *E. coli* Grx4 formed a [2Fe-2S]-bridged complex with the BolA paralog IbaG. Coexpression and purification of IbaG with Grx4 yielded a reddish-brown protein complex with UV-visible absorption peaks that suggest binding of a [2Fe-2S] cluster (Figure 1A, Figure 2A). Expression and purification of Grx4 alone for comparison also yielded a [2Fe-2S] bound complex with spectral characteristics identical to a previous report (Figure 2A), but distinct from [2Fe-2S]-Grx4-IbaG.³⁰ Iron, acid-labile sulfur, and GSH analysis of the Grx4 complex confirms binding of two GSH molecules per cluster, with the complex containing ~0.75 [2Fe-2S] cluster per homodimer (Table 1). Analysis of the Grx4-IbaG complex shows only one GSH molecule binding per cluster and the complex containing ~0.5 [2Fe-2S] cluster per heterodimer. The UV-visible absorption spectra of Grx4-IbaG and the previously reported Grx4-BolA are fairly similar, with a shoulder ~320 nm and a broad peak ~410 nm.³⁰ Comparison of the Grx4 and Grx4-IbaG CD spectra shows distinct spectral features, particularly peak intensity differences in the 300–500 nm range, suggesting differences in the cluster binding environments (Figure 2A). Expression and purification of IbaG alone yields a colorless sample that lacks an Fe-S cluster as determined by iron and acid-labile

sulfur measurements. Reconstitution of IbaG with an Fe-S cluster in the presence or absence of GSH was attempted using our previously reported methods²⁰ with no success. In this respect, IbaG is similar to other BolA proteins as there is no reported BolA homologue that binds an Fe-S cluster in the absence of a Grx partner.

Based on observations during purification and the Fe-S cluster yields reported above, it appears that the Fe-S cluster in the Grx4-IbaG complex is relatively unstable despite exclusion of oxygen, suggesting that IbaG destabilizes the Fe-S cluster upon binding to Grx4. To confirm these observations and directly compare the relative stabilities of the oxidized Fe-S clusters in Grx4 and Grx4-IbaG, the purified $[2\text{Fe-2S}]^{2+}$ complexes were exposed to air in the presence or absence of GSH at physiological concentrations (5 mM).⁴¹ As shown in Figure S1, the Fe-S cluster in $[2\text{Fe-2S}]^{2+}$ Grx4-IbaG is less stable than $[2\text{Fe-2S}]^{2+}$ Grx4, especially in the presence of GSH, as shown by loss of the Fe-S cluster absorbance signal over time in the 300–600 nm region.

The stabilities of reduced $[2\text{Fe-2S}]^{1+}$ clusters in Grx4 and Grx4-IbaG were also investigated by UV-visible absorption and EPR (Figure 2B). Anaerobic reduction with a 5-fold excess of dithionite resulted in irreversible bleaching of the UV-visible absorption features associated with the $[2\text{Fe-2S}]^{2+}$ cluster in Grx4. None of the UV-visible absorption features characteristic of a valence-localized $[2\text{Fe-2S}]^{1+}$ cluster were observed and oxidation by air failed to restore the UV-visible absorption spectrum of the $[2\text{Fe-2S}]^{2+}$ cluster. Moreover, no EPR signals indicative of a $[2\text{Fe-2S}]^{1+}$ cluster were observed on anaerobic reduction with a 5-fold excess of dithionite and incubating for 5 min prior to freezing. However, when samples of $[2\text{Fe-2S}]$ Grx4 at 0 °C were treated with stoichiometric dithionite and frozen immediately after mixing, a weak axial EPR signal accounting for approximately 5% of cluster content was observed, with $g_{\parallel} = 2.03$ and $g_{\perp} = 1.94$ ($g_{\text{av}} = 1.97$) and relaxation properties indicative of a $[2\text{Fe-2S}]^{1+}$ cluster. However, the spectrum was not observed in samples frozen 1 min after dithionite addition, indicating that the reduced cluster is a transient intermediate on the pathway of reductive cluster degradation. Analogous transient EPR signals with similar g -values have been reported for other dithionite-reduced $[2\text{Fe-2S}]$ cluster-containing Grxs and have been interpreted in terms reductive cluster degradation due to the instability of the $[2\text{Fe-2S}]^{1+}$ cluster.²⁰

The reduced $[2\text{Fe-2S}]^{1+}$ cluster in the Grx4-IbaG heterodimer is semi-stable and gives rise to a near axial $S = 1/2$ resonance, $g_{1,2,3} = 2.01, 1.92, \text{ and } 1.87$ ($g_{\text{av}} \sim 1.93$) accounting for up to 20% of the cluster content in samples reduced anaerobically at room temperature with stoichiometric dithionite and frozen rapidly. Hence, the $[2\text{Fe-2S}]^{1+}$ cluster in the Grx4-IbaG is much less stable than in other Grx-BolA heterodimers, such as *Sc*Grx3-Fra2 and *At*GrxS14-BolA1, which have stable $[2\text{Fe-2S}]^{1+}$ clusters accounting for 100% of the cluster content on reduction with excess dithionite.^{20, 22} Some indication of the ligation of $[2\text{Fe-2S}]^{1+}$ clusters can be inferred by g_{av} values and g -value anisotropy which are very sensitive to changes in ligation at the valence-localized Fe(II) site.^{42–44} In particular, a decrease in the g_{av} value for the reduced cluster, coupled with the observation of low energy (250–275 cm^{-1}) bands in the resonance Raman spectrum of the oxidized $[2\text{Fe-2S}]^+$ cluster (see below), has proven to be diagnostic of cysteine-to-histidine ligand replacement at the reducible Fe site. For example, $[2\text{Fe-2S}]^{1+}$ clusters with all-cysteine ligation have $g_{\text{av}} \sim 1.97$,

whereas g_{av} decreases to ~ 1.93 when one cysteine is replaced by a histidine and to ~ 1.90 when two cysteines at the Fe(II) site are replaced by histidine. Therefore, it is likely that the $[2Fe-2S]^{1+}$ cluster in Grx4/IbaG has one histidine and three cysteine ligands. However, it is not possible to rule out loss of a histidine ligand on reduction or the presence of an oxygenic ligand in place of one of the cysteines ligating the localized-valence Fe(III) site, which would be expected to have only a minor effect on the g_{av} value.^{42, 43, 45}

Resonance Raman and pH-dependent CD spectroscopy suggest histidine ligation in $[2Fe-2S]$ Grx4-IbaG

Resonance Raman spectroscopy using 488 and 514 nm excitation was used to confirm the presence and assess the ligation of the $[2Fe-2S]^{2+}$ clusters in Grx4 and Grx4-IbaG (Figure 3). The spectra obtained for $[2Fe-2S]$ Grx4 are very similar to those reported for the monothiol Grx3 from *S. cerevisiae*.²⁰ The Fe-S stretching frequencies for this cluster are similar to other characterized $[2Fe-2S]$ clusters with complete cysteinyl ligation.^{20, 46, 47} In contrast, the resonance Raman spectra of $[2Fe-2S]$ Grx4-IbaG are more characteristic of $[2Fe-2S]^{2+}$ clusters with one or two His ligands based on $[2Fe-2S]^{2+}$ centers in Grx3-Fra2, mitoNEET, and wild-type or variant Rieske-type proteins with one or two His ligands.^{20, 48–51} Histidine ligation is evident by multiple bands in the low energy region ($250\text{--}300\text{ cm}^{-1}$) due to mixing of Fe-N(His) stretching with low energy Fe-S stretching modes and internal modes of coordinated cysteine residues. The spectra observed for the $[2Fe-2S]^{2+}$ cluster in Grx4-IbaG are broad and poorly resolved, most likely due to heterogeneity in the cluster environment. However, the frequencies are more similar to those observed for the $[2Fe-2S]^{2+}$ cluster in *AtGrxS14-BolA1*, which was interpreted as having two His ligands at the reducible Fe site based primarily on EPR data for the reduced sample,²² than those reported for *ScGrx3-Fra2* which has one His ligand. In particular, the number of resolved bands in the low energy region ($250\text{--}300\text{ cm}^{-1}$), which contains bands with significant Fe-N(His) stretching character, appears to be diagnostic of the number of histidine ligands at the unique Fe site: three for two histidines; two for one histidine; one for no histidines.²² For example, *AtGrxS14-BolA1* and *EcGrx4-IbaG* have three bands (250 , 282 , and 296 cm^{-1} and 262 , 286 , and 300 cm^{-1} , respectively), *ScGrx3-Fra2* has two bands (275 and 300 cm^{-1}) and *EcGrx4* has one band (288 cm^{-1}). In contrast, the EPR spectrum for $[2Fe-2S]^{1+}$ center in *ScGrx3-Fra2* is almost identical to that reported herein for the $[2Fe-2S]^{1+}$ center in *EcGrx4-IbaG*, suggesting that both have one His ligand in the reduced state. However, as discussed below, this may be a consequence of dissociation of one of the two His ligands upon cluster reduction.

Histidine residues ligating an Fe-S cluster have an available nitrogen that can change protonation state by changes in solvent pH. Since this change perturbs the cluster coordination environment, it can be monitored spectroscopically. In contrast, cysteine cluster ligands do not change protonation with changes around biological pH. Hence CD-monitored pH titrations of $[2Fe-2S]$ Grx4-IbaG and $[2Fe-2S]$ Grx4 were carried out in the pH range $5.5\text{--}8.1$ (Figure 4). The $[2Fe-2S]$ Grx4-IbaG complex shows an increase in the bands at 391 (peak 1) and 564 nm (peak 3) and a shift in the negative band at 515 nm to 481 nm (peak 2) with increasing pH (Figure 4A). The differences in CD intensity between peaks 1 and 2 and peaks 2 and 3 as a function of pH are fit by theoretical plots with a $pK_a \sim 6.4$ (Figure 4B).

This result again supports cluster coordination by one or two His residues. CD-monitored pH titration of [2Fe-2S] Grx4 did not give any changes in the CD spectra, other than a small decrease in intensity due to cluster instability at high pH (Figure S2).

Complex formation between Grx4 and IbaG is dimeric and independent of Fe-S cluster binding

In order to determine the stoichiometry between Grx4 and IbaG, the molecular masses of apo-IbaG, apo and holo Grx4, and apo and holo Grx4-IbaG complexes were measured by size-exclusion chromatography and MALDI-TOF mass spectrometry. The MALDI-TOF results for as-purified IbaG were consistent with the theoretical molecular mass of 9.5 kD; however, IbaG runs as a single peak corresponding to 16.7 kD on a size exclusion column (Figure 5A, Table 2). Although this latter measurement falls between the expected monomer and dimer masses for IbaG, the reported NMR solution structure of IbaG is a monomer (PDB 1NY8). Therefore, IbaG likely runs at a higher than expected apparent molecular mass by size exclusion chromatography, which is consistent with previous reports for other BolA family proteins.^{20, 21, 30} Likewise, apo-Grx4 has an apparent molecular mass (20.9 kD) that is higher than the theoretical mass (12.8 kD), similar to other single domain CGFS Grxs reported to be monomers in the apo form.^{21, 30} Comparison of calculated masses indicates that both apo and holo Grx4 form a heterodimeric complex with IbaG (Table 2). These data suggest that complex formation between Grx4 and IbaG occurs regardless of the presence of the Fe-S cluster, similar to the previously reported BolA-Grx4 interaction.³⁰

To further examine the interaction between IbaG and Grx4, apo-IbaG was incubated with apo and holo Grx4 and characterized by size-exclusion chromatography and CD spectroscopy. As shown in the chromatograms in Figure 5A, the individual apo-proteins elute as single peaks, while IbaG-Grx4 mixtures elute as multiple peaks. A 2:1 mixture of apo-IbaG and apo-Grx4 produces two peaks corresponding to apo-IbaG-Grx4 heterodimer (24.4 kD apparent mass) as well as excess apo-IbaG eluting as a monomer (16.7 kD). Incubation of apo-IbaG with holo-Grx4 generates three peaks corresponding to [2Fe-2S]-IbaG-Grx4 heterodimer (30.1 kD), apo-IbaG-Grx4 heterodimer (24.4 kD), and apo-IbaG monomer (16.7 kD). SDS-PAGE analysis of the eluted fractions confirms these peak assignments (data not shown). CD spectroscopy was also used to monitor changes in the Fe-S cluster coordination environment upon titration of [2Fe-2S]-Grx4 with IbaG (Figure 5B). The addition of increasing equivalents of IbaG resulted in loss of the [2Fe-2S] Grx4 homodimer spectrum and formation of a weak spectrum that matches the [2Fe-2S] Grx4-IbaG heterodimer (see Figure 2A). Taken together, these results demonstrate that IbaG titration with [2Fe-2S] Grx4 homodimer promotes formation of the [2Fe-2S] Grx4-IbaG heterodimer as well as the apo Grx4-IbaG heterodimer. However, given the size of the apo-heterodimer peak in the chromatogram (Figure 5A, dotted red line) and the weaker [2Fe-2S] CD signal after addition of IbaG (Figure 5B), it appears that the majority of the Grx4-IbaG heterodimer is isolated in the apo form. These results verify that the interaction between Grx4 and IbaG is not Fe-S cluster dependent, and that IbaG destabilizes the [2Fe-2S] cluster on Grx4.

To verify and quantify the thermodynamic characteristics of the interaction of IbaG with apo-Grx4, isothermal titration calorimetry was performed, with *E. coli* apo-Grx4 + BolA and yeast apo-Grx3 + Fra2 as controls. BolA and IbaG both displayed a strong exothermic interaction when binding to apo-Grx4 (Table 3 and Figure S3). Data were fit to a one binding site model, consistent with the 1:1 interactions found by gel filtration above. Corresponding K_d values were comparable, with IbaG at $3.4 \pm 0.1 \mu\text{M}$ binding to apo-Grx4, and BolA at $4.6 \pm 0.9 \mu\text{M}$. These affinities are 5–6 fold tighter than observed for the yeast Grx3-Fra2 interaction at a K_d of $20.8 \pm 6.6 \mu\text{M}$, and are similar to values obtained for *A. thaliana* BolA1-GrxS14 ($7 \pm 2 \mu\text{M}$).²² The protein-protein interaction for the yeast homologues also demonstrated a decrease in the amount of heat released during the interaction, indicating a weaker binding event. It was not possible to determine binding constants for [2Fe-2S] Grx4 + IbaG. Isothermal titration calorimetry only measures changes in heat, thus multiple binding events occurring simultaneously (Grx4 homodimer disassembly, apo and holo heterodimer assembly) cannot be separated to assign individual K_d values. In addition, Fe-S cluster degradation would make any results unreliable. Nevertheless, together these data confirm that the Grx4-IbaG (and Grx4-BolA) interaction is independent of Fe-S cluster binding, and that this complex behaves differently than the yeast homologues, possibly indicating a distinct function for the apo heterodimers in *E. coli*.

IbaG His63 is an Fe-S cluster ligand in the [2Fe-2S] Grx4-IbaG heterodimer

Recently, a phylogenetic classification of BolA proteins was generated based on homologues from various organisms.^{52, 53} BolA proteins are separated into four classes based on conserved key amino acids, in particular His and Cys residues thought to act in Fe-S cluster binding with Grxs. IbaG is in the divergent BolA4 class found only in prokaryotes and photosynthetic eukaryotes, while BolA is in the broader BolA1 class found in both prokaryotes and eukaryotes that utilize aerobic metabolism. A sequence alignment of these two families highlights these potential Fe-S binding ligands (Figure S4). While IbaG contains no cysteines, IbaG and the other BolA1/4 family members contain the conserved histidine known to be a cluster ligand in the yeast Grx3-Fra2 complex (His63 in IbaG),¹⁹ as well as the histidine conserved in the H/C loop of BolA1/4 proteins (His27).^{52, 53} To determine whether these residues were critical for Fe-S cluster binding with Grx4, both His27 and His63 was changed by site-directed mutagenesis to either an Ala (H27A, H63A) or a Cys (H27C, H63C) and the effects on Fe-S coordination by the Grx4-IbaG complex were investigated spectroscopically. The most dramatic effects on the UV-visible absorption and CD spectra were observed for the H63A/C substitutions (Figure 6A). Although these variants all copurified with Grx4 (Figure 1B) and bound a [2Fe-2S] cluster, the UV-visible absorption and CD spectra were markedly different than the WT IbaG-Grx4 heterodimer, and in fact closely resembled the [2Fe-2S] Grx4 homodimer. In the UV-visible absorption spectra, distinct peaks were present at 322 and 412 nm that matched the Grx4 homodimer spectrum (Figure 6A, top), while the CD spectra displayed a strong negative band at 400 nm similar to [2Fe-2S] Grx4 (Figure 6A, bottom). Furthermore, these H63 variant complexes exhibit greater Fe-S cluster stability in air in the presence of GSH than the WT Grx4-IbaG complex (Figure S1), suggesting a coordination environment more similar to Grx4 homodimers. These results suggest that while the IbaG H63A/C variants still interact with Grx4, IbaG may not be providing ligands to the Fe-S cluster in these variants. Resonance

Raman confirms this interpretation, as the spectra for [2Fe-2S] Grx4-IbaG H63A is very similar to that of the [2Fe-2S] Grx4 homodimer (Figure 6B). Moreover, the GSH content in these mutants is ~1 GSH per Fe, which is the same as the Grx4 homodimer (two GSH per [2Fe-2S]), but distinct from the cluster on the Grx4-IbaG heterodimer (one GSH per [2Fe-2S]) (see Table 1). Taken together, these results demonstrate that His63 is a key ligand that is essential for binding the [2Fe-2S] cluster across the Grx4-IbaG heterodimer interface.

Interestingly, the spectra of the H27A and H27C variants exhibited significant differences compared to WT IbaG-Grx4 (Figure 6A). The UV-visible absorption spectra of the H27A/C complexes show better resolved peaks at ~320 and ~410 nm, and a shoulder at ~530 nm rather than ~580 nm. While similar features are observed in the H63A/C complexes and the Grx4 homodimer, these also have a pronounced shoulder at 460 nm, which is not observed for the H27A/C variants. Moreover, the CD spectra for the Grx4-IbaG H27A/C complexes more closely match the WT Grx4-IbaG heterodimer than the Grx4 homodimer. Nevertheless, the [2Fe-2S]²⁺ centers in the WT, H27A, and H27C Grx4-IbaG complexes all have distinctive CD spectra in the 300–550 region. Overall, these results suggest that His27 is located at or near the [2Fe-2S]²⁺ cluster and influences the coordination environment of the cluster.

In addition to these conserved histidine residues, IbaG has a third histidine residue (His20) that was substituted to probe the effects on Fe-S cluster binding. The role of a serine (Ser26) located adjacent to His27 was also addressed since serine residues have also been shown to act as cluster ligands, although rarely.⁵⁴ The IbaG H20A and S26A variants all copurified with Grx4 and bound a [2Fe-2S] cluster (Figure 1B and data not shown). The UV-visible absorption and CD spectra for these IbaG variant complexes closely matched the WT complex (Figure S5), effectively ruling out these two residues as Fe-S cluster ligands.

DISCUSSION

In this study we provide evidence that Grx4, the sole CGFS monothiol Grx in *E. coli*, forms a complex with the BolA paralog IbaG. Grx4 and IbaG copurify as a [2Fe-2S] cluster bound heterodimer, with spectroscopic properties distinct from the published Grx4 homodimer but similar to the Grx4-BolA heterodimer.³⁰ In addition, several features of the Grx4-IbaG interaction are comparable to Grx-BolA interactions previously published for the *S. cerevisiae*, human, and plant homologues. In all cases, the strictly conserved His located in the loop between the α 3 helix and β 3 sheet of the BolA protein is a key cluster ligand.^{19, 21, 22} Furthermore, titration of [2Fe-2S] Grx homodimers with BolA proteins results in displacement of a Grx monomer and a GSH to form the His-ligated cluster.^{19, 21} Interestingly, previous studies have shown the GSH molecules in Fe-S-bridged Grx homodimers are in dynamic equilibrium with GSH in solution.⁵⁵ This labile GSH coordination may allow access to the Fe-S cluster by competing ligands from IbaG, facilitating formation of the heterodimer. Spectroscopic analysis of the *E. coli* Grx4-IbaG complex indicates the presence of one or two His ligated to the cluster, provided by IbaG. The key His ligand from IbaG was identified as His63, based on distinct changes in the CD and UV-visible spectra of IbaG His63A/C complexes. Substitution of this His for Ala or Cys led to a spectroscopic signature that matched the [2Fe-2S] Grx4 homodimer, as well as

increase in the GSH content of the complex. Thus, in the absence of His63, a cluster coordination site with all Cys ligands similar to the homodimer is favored over the heterodimer formation. Since IbaG lacks any Cys residues, the cluster must be ligated solely by Grx4 and GSH in these mutant complexes, even though IbaG His63A/C variants still coelute with the Fe-S-bound complex (Figure 1B).

The other ligand provided by BolA proteins in Grx-BolA complexes is proposed to be either a His or a Cys found in the H/C loop between $\beta 1$ and $\beta 2$.^{22, 53} In the *E. coli* Grx4-IbaG complex, the spectroscopic data are consistent with IbaG providing either one or two His ligands, with EPR of the reduced $[2\text{Fe-2S}]^{1+}$ center favoring the former and resonance Raman of the oxidized $[2\text{Fe-2S}]^{2+}$ cluster favoring the latter. We used site-directed mutagenesis in an attempt to distinguish between these two possibilities. In contrast to the His63A/C forms that promote formation of an IbaG-associated $[2\text{Fe-2S}]^{2+}$ cluster-bound Grx4 homodimer, substitution of the other conserved His located in the H/C loop (His27) maintained stable $[2\text{Fe-2S}]^{2+}$ cluster-bound Grx4-IbaG(H27A/C) heterodimeric complexes, albeit with UV-visible absorption and CD spectra showing significant differences compared to WT. While this indicates that His63 is more important than His27 for stabilizing the $[2\text{Fe-2S}]^{2+}$ cluster-bound heterodimer complex, it also shows that His27 influences the coordination environment of the cluster and may be a ligand to the $[2\text{Fe-2S}]^{2+}$ cluster. Such a conclusion appears to be in conflict with the EPR spectrum of the $[2\text{Fe-2S}]^{1+}$ cluster in the reduced WT Grx4-IbaG heterodimer, which is very similar to that observed for yeast Grx3-Fra2 and characteristic of one histidyl and three cysteinyl ligands,²⁰ rather than the anomalous “Rieske-type” spectrum ($g = 2.02, 1.96, 1.65, g_{\text{av}} = 1.88$) observed for the $[2\text{Fe-2S}]^{1+}$ cluster in *A*GrxS14-BolA1, which was interpreted as having two His ligands at the reducible Fe site.²² However, these observations can be reconciled by dissociation of His27 from the localized-valence Fe(II) site on reduction of the Grx4-IbaG heterodimer. Reductive dissociation of a weakly coordinated His ligand is probably not physiologically relevant, as it seems unlikely that the reduced form of any $[2\text{Fe-2S}]$ cluster-bound Grx-BolA heterodimers is present *in vivo*. Since we tested for other possible cluster ligands in this region but found no definitive hits, this leads us to propose that His27 is the other cluster ligand provided by IbaG. Our results mirror previously reported observations for the yeast $[2\text{Fe-2S}]$ Grx3-Fra2 complex.¹⁹ In that case, substitution of the conserved Cys in the H/C loop of Fra2 had only minor effects on the spectroscopic properties of the Fe-S heterocomplex, yet no other potential cluster ligands were identified. As yet, there is no published atomic-level structure of any holo Grx-BolA complex to help resolve the issue of the nature of the fourth cluster ligand.

Ligation of the Fe-S cluster in Grx4-IbaG by His residues is also supported by pH-dependent changes observed in the CD spectrum for the $[2\text{Fe-2S}]$ Grx4-IbaG heterodimer (Figure 4), presumably due to changes in the His protonation state. In contrast, the CD spectrum of the all Cys ligated $[2\text{Fe-2S}]$ Grx4 homodimer is unaffected by pH (Figure S2). The pK_a of the pH titration curve for $[2\text{Fe-2S}]$ Grx4-IbaG is ~ 6.4 , which is lower than the resting physiological cytosolic pH range reported for *E. coli* ($\sim 7.2\text{--}7.8$).^{56, 57} However, the cytosolic pH is reported to transiently fall as low as pH 5.5 under acid stress, as would be encountered during enteric colonization by pathogenic *E. coli* strains.⁵⁶ These acid conditions would presumably impact the Grx4-IbaG Fe-S coordination environment,

possibly altering the lability or binding affinity of the Fe-S cluster. Interestingly, previous genetic studies have implicated *ibaG* in resistance against acid stress,³⁵ which might involve the pH-sensitive [2Fe-2S]-bridged Grx4-IbaG heterodimer.

One key difference noted for the *E. coli* Grx4-IbaG interaction compared to the yeast and human homologues is the stronger protein-protein interaction of the apo proteins. Apo Grx4-IbaG (as well as apo Grx4-BolA) were found to have a higher binding affinity in the range of 3–5 μ M compared to the yeast apo-Fra2-Grx3 interaction (21 μ M) (Table 3) and the human apo-BOLA2-GLRX3 interaction (25 ± 15 μ M).²⁸ The strength of this interaction explains the predominance of apo heterodimers in addition to holo-heterodimers following titration of [2Fe-2S] Grx4 with apo-IbaG. In comparison, titration of yeast Grx3 or human GLRX3 with their BolA binding partners results in complete conversion to the holo-heterocomplexes with little or no apo heterocomplexes detected.^{19, 21} The relatively strong interaction between IbaG and Grx4 may indicate a possible physiological function for the apo heterodimer, as suggested for *A. thaliana* BolA1-GrxS14, which has a similar dissociation constant (7 ± 2 μ M).²²

The physiological relevance of the physical interaction between IbaG and Grx4 is supported by genetic evidence linking both proteins with the function of the SUF pathway in Fe-S cluster biogenesis: an *ibaG* mutant is synthetically sick and a *grx* mutant is synthetically lethal in combination with *isc* mutants.³¹ The SUF pathway is an alternative system for building Fe-S clusters during oxidative stress and iron starvation conditions that complements the housekeeping Fe-S biogenesis pathway encoded by the ISC operon.⁵⁸ These findings together with our interaction studies suggest that the Grx4-IbaG complex functionally interacts with the SUF pathway in Fe-S cluster biogenesis. Nevertheless, the specific roles of Grx4 and IbaG individually or as a complex in Fe-S metabolism are not well characterized. In general, *ibaG* mRNA levels are relatively low with *ibaG* expression highest at mid exponential phase, lowest in stationary phase, and increased with acid stress.³⁵ This expression pattern may reflect a possible regulatory role for IbaG that modifies Grx4 Fe-S delivery and/or redox activity under specific growth conditions. Both *E. coli* Grx4 homodimers and Grx4-BolA heterodimers were previously shown to transfer Fe-S clusters to apo-ferredoxin *in vitro*.³⁰ The Grx4-IbaG complex could likewise deliver Fe-S clusters assembled by the SUF pathway to specific target proteins. However, the physiological relevance of this activity for either BolA or IbaG is not established. Alternatively, a role for CGFS Grxs and BolA proteins in regulating sulfur trafficking has also been proposed previously based on physical and genetic interactions. For example, the sulfurtransferase SufE1 in *A. thaliana* contains a C-terminal BolA domain, shown to interact with monothiol Grxs. Based on these findings, the *A. thaliana* BolA-Grx interaction is suggested to regulate SufE1 sulfurtransferase activity via conformational changes or masking of the active site cysteine.⁵² Along these lines, *E. coli* Grx4 has been shown to form a disulfide with the sulfur trafficking proteins CsdE and SufE *in vivo*, linking Grx4 function with regulation of sulfur trafficking.^{59, 60} Thus, IbaG/BolA may potentially regulate the activity of Grx4 via binding and/or blocking of the redox-active cysteine.

Several reports have also suggested that *E. coli* BolA acts as transcription factor since it has a nucleic acid-binding helix-turn-helix motif and was shown to bind to and regulate genes

involved in cell wall and flagellum biosynthesis.^{34, 61, 62} However, in an attempt to establish the effect of Grx4 on any potential IbaG/BolA DNA binding activity, we performed electrophoretic mobility shift assays with IbaG, BolA, and their holo and apo complexes with Grx4 and found no DNA binding activity using the *mreB* promoter region and binding buffer reported previously (data not shown).⁶¹ Alternatively, the connection between BolA/IbaG proteins and cell wall biogenesis could be related to their function in Fe-S cluster assembly and/or sulfur trafficking. In fact, two [4Fe-4S]-dependent enzymes, namely IspH and IspG, are required for the biosynthesis of isoprenoids, which are used to synthesize the lipid carrier that functions in cell wall peptidoglycan synthesis. Previous studies demonstrated that these Fe-S enzymes require either the SUF or the ISC assembly pathways for Fe-S cluster insertion, depending on the growth conditions.⁶³ Thus BolA and/or IbaG may facilitate or otherwise regulate Fe-S cluster delivery to these target proteins. In any case, further study of Grx4/IbaG proteins and complexes is needed to determine their specific cellular functions, especially in Fe-S cluster assembly. Nevertheless, the results presented here link the function of IbaG with Grx4 via formation of both apo and Fe-S-bridged heterodimers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Grx	glutaredoxin
Trx	thioredoxin
GSH	reduced glutathione
Fe-S	iron-sulfur
SUF	sulfur utilization factor
ISC	iron sulfur cluster
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
CD	circular dichroism
EPR	electron paramagnetic resonance
ITC	isothermal titration calorimetry
IPTG	isopropyl β -D-thiogalactoside

References

1. Couturier J, Przybyla-Toscano J, Roret T, Didierjean C, Rouhier N. The roles of glutaredoxins ligating Fe-S clusters: Sensing, transfer or repair functions? *Biochim Biophys Acta*. 2015; 1853:1513–1527. [PubMed: 25264274]
2. Li H, Outten CE. Monothiol CGFS glutaredoxins and BolA-like proteins: [2Fe-2S] binding partners in iron homeostasis. *Biochemistry*. 2012; 51:4377–4389. [PubMed: 22583368]
3. Iwema T, Picciocchi A, Traore DA, Ferrer JL, Chauvat F, Jacquamet L. Structural basis for delivery of the intact [Fe2S2] cluster by monothiol glutaredoxin. *Biochemistry*. 2009; 48:6041–6043. [PubMed: 19505088]
4. Mapolelo DT, Zhang B, Randeniya S, Albetel AN, Li H, Couturier J, Outten CE, Rouhier N, Johnson MK. Monothiol glutaredoxins and A-type proteins: partners in Fe-S cluster trafficking. *Dalton Trans*. 2013; 42:3107–3115. [PubMed: 23292141]
5. Muhlenhoff U, Gerber J, Richhardt N, Lill R. Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *EMBO J*. 2003; 22:4815–4825. [PubMed: 12970193]
6. Uzarska MA, Dutkiewicz R, Freibert SA, Lill R, Muhlenhoff U. The mitochondrial Hsp70 chaperone Ssq1 facilitates Fe/S cluster transfer from Isu1 to Grx5 by complex formation. *Mol Biol Cell*. 2013; 24:1830–1841. [PubMed: 23615440]
7. Kim KD, Chung WH, Kim HJ, Lee KC, Roe JH. Monothiol glutaredoxin Grx5 interacts with Fe-S scaffold proteins Isa1 and Isa2 and supports Fe-S assembly and DNA integrity in mitochondria of fission yeast. *Biochem Biophys Res Commun*. 2010; 392:467–472. [PubMed: 20085751]
8. Banci L, Brancaccio D, Ciofi-Baffoni S, Del Conte R, Gadepalli R, Mikolajczyk M, Neri S, Piccioli M, Winkelman J. [2Fe-2S] cluster transfer in iron-sulfur protein biogenesis. *Proc Natl Acad Sci USA*. 2014; 111:6203–6208. [PubMed: 24733926]
9. Brault A, Mourer T, Labbe S. Molecular basis of the regulation of iron homeostasis in fission and filamentous yeasts. *IUBMB Life*. 2015; 67:801–815. [PubMed: 26472434]
10. Outten CE, Albetel AN. Iron sensing and regulation in *Saccharomyces cerevisiae*: Ironing out the mechanistic details. *Curr Opin Microbiol*. 2013; 16:662–668. [PubMed: 23962819]
11. Muhlenhoff U, Molik S, Godoy JR, Uzarska MA, Richter N, Seubert A, Zhang Y, Stubbe J, Pierrel F, Herrero E, Lillig CH, Lill R. Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster. *Cell Metab*. 2010; 12:373–385. [PubMed: 20889129]
12. Haunhorst P, Hanschmann EM, Brautigam L, Stehling O, Hoffmann B, Muhlenhoff U, Lill R, Berndt C, Lillig CH. Crucial function of vertebrate glutaredoxin 3 (PICOT) in iron homeostasis and hemoglobin maturation. *Mol Biol Cell*. 2013; 24:1895–1903. [PubMed: 23615448]
13. Xia H, Li B, Zhang Z, Wang Q, Qiao T, Li K. Human glutaredoxin 3 can bind and effectively transfer [4Fe-4S] cluster to apo-iron regulatory protein 1. *Biochem Biophys Res Commun*. 2015; 465:620–624. [PubMed: 26296460]
14. Huynen MA, Spronk CA, Gabaldon T, Snel B. Combining data from genomes, Y2H and 3D structure indicates that BolA is a reductase interacting with a glutaredoxin. *FEBS Lett*. 2005; 579:591–596. [PubMed: 15670813]
15. Couturier J, Jacquot JP, Rouhier N. Evolution and diversity of glutaredoxins in photosynthetic organisms. *Cell Mol Life Sci*. 2009; 66:2539–2557. [PubMed: 19506802]
16. Baker PR 2nd, Friederich MW, Swanson MA, Shaikh T, Bhattacharya K, Scharer GH, Aicher J, Creadon-Swindell G, Geiger E, MacLean KN, Lee WT, Deshpande C, Freckmann ML, Shih LY, Wasserstein M, Rasmussen MB, Lund AM, Procopis P, Cameron JM, Robinson BH, Brown GK, Brown RM, Compton AG, Dieckmann CL, Collard R, Coughlin CR 2nd, Spector E, Wempe MF, Van Hove JL. Variant non ketotic hyperglycinemia is caused by mutations in *LIAS*, *BOLA3* and the novel gene *GLRX5*. *Brain*. 2014; 137:366–379. [PubMed: 24334290]
17. Cameron JM, Janer A, Levandovskiy V, Mackay N, Rouault TA, Tong WH, Ogilvie I, Shoubridge EA, Robinson BH. Mutations in iron-sulfur cluster scaffold genes *NFU1* and *BOLA3* cause a fatal deficiency of multiple respiratory chain and 2-oxoacid dehydrogenase enzymes. *Am J Hum Genet*. 2011; 89:486–495. [PubMed: 21944046]

18. Haack TB, Rolinski B, Haberberger B, Zimmermann F, Schum J, Strecker V, Graf E, Athing U, Hoppen T, Wittig I, Sperl W, Freisinger P, Mayr JA, Strom TM, Meitinger T, Prokisch H. Homozygous missense mutation in *BOLA3* causes multiple mitochondrial dysfunctions syndrome in two siblings. *J Inher Metab Dis*. 2013; 36:55–62. [PubMed: 22562699]
19. Li H, Mapolelo DT, Dingra NN, Keller G, Riggs-Gelasco PJ, Winge DR, Johnson MK, Outten CE. Histidine 103 in Fra2 is an iron-sulfur cluster ligand in the [2Fe-2S] Fra2-Grx3 complex and is required for in vivo iron signaling in yeast. *J Biol Chem*. 2011; 286:867–876. [PubMed: 20978135]
20. Li H, Mapolelo DT, Dingra NN, Naik SG, Lees NS, Hoffman BM, Riggs-Gelasco PJ, Huynh BH, Johnson MK, Outten CE. The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation. *Biochemistry*. 2009; 48:9569–9581. [PubMed: 19715344]
21. Li H, Mapolelo DT, Randeniya S, Johnson MK, Outten CE. Human glutaredoxin 3 forms [2Fe-2S]-bridged complexes with human BOLA2. *Biochemistry*. 2012; 51:1687–1696. [PubMed: 22309771]
22. Roret T, Tsan P, Couturier J, Zhang B, Johnson MK, Rouhier N, Didierjean C. Structural and spectroscopic insights into BOLA-glutaredoxin complexes. *J Biol Chem*. 2014; 289:24588–24598. [PubMed: 25012657]
23. Kumanovics A, Chen OS, Li L, Bagley D, Adkins EM, Lin H, Dingra NN, Outten CE, Keller G, Winge D, Ward DM, Kaplan J. Identification of *FRA1* and *FRA2* as genes involved in regulating the yeast iron regulon in response to decreased mitochondrial iron-sulfur cluster synthesis. *J Biol Chem*. 2008; 283:10276–10286. [PubMed: 18281282]
24. Poor CB, Wegner SV, Li H, Dlouhy AC, Schuermann JP, Sanishvili R, Hinshaw JR, Riggs-Gelasco PJ, Outten CE, He C. Molecular mechanism and structure of the *Saccharomyces cerevisiae* iron regulator Aft2. *Proc Natl Acad Sci USA*. 2014; 111:4043–4048. [PubMed: 24591629]
25. Ueta R, Fujiwara N, Iwai K, Yamaguchi-Iwai Y. Iron-induced dissociation of the Aft1p transcriptional regulator from target gene promoters is an initial event in iron-dependent gene suppression. *Mol Cell Biol*. 2012; 32:4998–5008. [PubMed: 23045394]
26. Chen OS, Crisp RJ, Valachovic M, Bard M, Winge DR, Kaplan J. Transcription of the yeast iron regulon does not respond directly to iron but rather to iron-sulfur cluster biosynthesis. *J Biol Chem*. 2004; 279:29513–29518. [PubMed: 15123701]
27. Ueta R, Fujiwara N, Iwai K, Yamaguchi-Iwai Y. Mechanism underlying the iron-dependent nuclear export of the iron-responsive transcription factor Aft1p in *Saccharomyces cerevisiae*. *Mol Biol Cell*. 2007; 18:2980–2990. [PubMed: 17538022]
28. Banci L, Camponeschi F, Ciofi-Baffoni S, Muzzioli R. Elucidating the Molecular Function of Human BOLA2 in GRX3-Dependent Anamorsin Maturation Pathway. *J Am Chem Soc*. 2015; 137:16133–16143. [PubMed: 26613676]
29. Fernandes AP, Fladvad M, Berndt C, Andresen C, Lillig CH, Neubauer P, Sunnerhagen M, Holmgren A, Vlamis-Gardikas A. A novel monothiol glutaredoxin (Grx4) from *Escherichia coli* can serve as a substrate for thioredoxin reductase. *J Biol Chem*. 2005; 280:24544–24552. [PubMed: 15833738]
30. Yeung N, Gold B, Liu NL, Prathapam R, Sterling HJ, Willams ER, Butland G. The *E coli* monothiol glutaredoxin GrxD forms homodimeric and heterodimeric FeS cluster containing complexes. *Biochemistry*. 2011; 50:8957–8969. [PubMed: 21899261]
31. Butland G, Babu M, Diaz-Mejia JJ, Bohdana F, Phanse S, Gold B, Yang W, Li J, Gagarinova AG, Pogoutse O, Mori H, Wanner BL, Lo H, Wasniewski J, Christopoulos C, Ali M, Venn P, Safavi-Naini A, Sourour N, Caron S, Choi JY, Laigle L, Nazarians-Armavil A, Deshpande A, Joe S, Datsenko KA, Yamamoto N, Andrews BJ, Boone C, Ding H, Sheikh B, Moreno-Hagelseib G, Greenblatt JF, Emili A. eSGA: *E. coli* synthetic genetic array analysis. *Nat Methods*. 2008; 5:789–795. [PubMed: 18677321]
32. Boutigny S, Saini A, Baidoo EE, Yeung N, Keasling JD, Butland G. Physical and functional interactions of a monothiol glutaredoxin and an iron sulfur cluster carrier protein with the sulfur-donating radical S-adenosyl-L-methionine enzyme MiaB. *J Biol Chem*. 2013; 288:14200–14211. [PubMed: 23543739]

33. Kasai T, Inoue M, Koshiba S, Yabuki T, Aoki M, Nunokawa E, Seki E, Matsuda T, Matsuda N, Tomo Y, Shirouzu M, Terada T, Obayashi N, Hamana H, Shinya N, Tatsuguchi A, Yasuda S, Yoshida M, Hirota H, Matsuo Y, Tani K, Suzuki H, Arakawa T, Carninci P, Kawai J, Hayashizaki Y, Kigawa T, Yokoyama S. Solution structure of a BolA-like protein from *Mus musculus*. *Protein Sci.* 2004; 13:545–548. [PubMed: 14718656]
34. Dressaire C, Moreira RN, Barahona S, Alves de Matos AP, Arraiano CM. BolA is a transcriptional switch that turns off motility and turns on biofilm development. *MBio.* 2015; 6:e02352–02314. [PubMed: 25691594]
35. Guinote IB, Moreira RN, Freire P, Arraiano CM. Characterization of the BolA homolog IbaG: a new gene involved in acid resistance. *J Microbiol Biotechnol.* 2012; 22:484–493. [PubMed: 22534295]
36. Riemer J, Hoepken HH, Czerwinska H, Robinson SR, Dringen R. Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal Biochem.* 2004; 331:370–375. [PubMed: 15265744]
37. Beinert H. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. *Anal Biochem.* 1983; 131:373–378. [PubMed: 6614472]
38. Broderick JB, Henshaw TF, Cheek J, Wojtuszewski K, Smith SR, Trojan MR, McGhan RM, Kopf A, Kibbey M, Broderick WE. Pyruvate formate-lyase-activating enzyme: strictly anaerobic isolation yields active enzyme containing a [3Fe-4S](+) cluster. *Biochem Biophys Res Commun.* 2000; 269:451–456. [PubMed: 10708574]
39. Outten CE, Culotta VC. Alternative start sites in the *Saccharomyces cerevisiae* *GLR1* gene are responsible for mitochondrial and cytosolic isoforms of glutathione reductase. *J Biol Chem.* 2004; 279:7785–7791. [PubMed: 14672937]
40. Cospér MM, Jameson GN, Hernandez HL, Krebs C, Huynh BH, Johnson MK. Characterization of the cofactor composition of *Escherichia coli* biotin synthase. *Biochemistry.* 2004; 43:2007–2021. [PubMed: 14967041]
41. Ostergaard H, Henriksen A, Hansen FG, Winther JR. Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein. *EMBO J.* 2001; 20:5853–5862. [PubMed: 11689426]
42. Bertrand P, Guigliarelli B, Gayda JP, Beardwood P, Gibson JF. A ligand-field model to describe a new class of 2Fe-2S clusters in proteins and their synthetic analogs. *Biochim Biophys Acta.* 1985; 831:261–266.
43. Guigliarelli B, Bertrand P. Application of EPR spectroscopy to the structural and functional study of iron-sulfur proteins. *Adv Inorg Chem.* 1999; 47:421–497.
44. Link TA. The structures of Rieske and Rieske-type proteins. *Adv Inorg Chem.* 1999; 47:83–157.
45. Werth MT, Cecchini G, Manodori A, Ackrell BA, Schroder I, Gunsalus RP, Johnson MK. Site-directed mutagenesis of conserved cysteine residues in *Escherichia coli* fumarate reductase: modification of the spectroscopic and electrochemical properties of the [2Fe-2S] cluster. *Proc Natl Acad Sci USA.* 1990; 87:8965–8969. [PubMed: 2174169]
46. Fu W, Drozdowski PM, Davies MD, Sligar SG, Johnson MK. Resonance Raman and magnetic circular dichroism studies of reduced [2Fe-2S] proteins. *J Biol Chem.* 1992; 267:15502–15510. [PubMed: 1639790]
47. Han S, Czernuszewicz RS, Kimura T, Adams MWW, Spiro TG. Fe₂S₂ protein resonance Raman-spectra revisited - structural variations among adrenodoxin, ferredoxin, and red paramagnetic protein. *J Am Chem Soc.* 1989; 111:3505–3511.
48. Kounosu A, Li Z, Cospér NJ, Shokes JE, Scott RA, Imai T, Urushiyama A, Iwasaki T. Engineering a three-cysteine, one-histidine ligand environment into a new hyperthermophilic archaeal Rieske-type [2Fe-2S] ferredoxin from *Sulfolobus solfataricus*. *J Biol Chem.* 2004; 279:12519–12528. [PubMed: 14726526]
49. Kuila D, Schoonover JR, Dyer RB, Batie CJ, Ballou DP, Fee JA, Woodruff WH. Resonance Raman studies of Rieske-type proteins. *Biochim Biophys Acta.* 1992; 1140:175–183. [PubMed: 1280165]

50. Rotsaert FJ, Pikus JD, Fox BG, Markley JL, Sanders-Loehr J. N-isotope effects on the Raman spectra of Fe(2)S(2) ferredoxin and Rieske ferredoxin: evidence for structural rigidity of metal sites. *J Biol Inorg Chem*. 2003; 8:318–326. [PubMed: 12589567]
51. Tirrell TF, Paddock ML, Conlan AR, Smoll EJ Jr, Nechushtai R, Jennings PA, Kim JE. Resonance Raman studies of the (His)(Cys)3 2Fe-2S cluster of MitoNEET: comparison to the (Cys)4 mutant and implications of the effects of pH on the labile metal center. *Biochemistry*. 2009; 48:4747–4752. [PubMed: 19388667]
52. Couturier J, Wu HC, Dhalleine T, Pegeot H, Sudre D, Gualberto JM, Jacquot JP, Gaymard F, Vignols F, Rouhier N. Monothiol glutaredoxin-BolA interactions: redox control of *Arabidopsis thaliana* BolA2 and SufE1. *Mol Plant*. 2014; 7:187–205. [PubMed: 24203231]
53. Willems P, Wanschers BF, Esseling J, Szklarczyk R, Kudla U, Duarte I, Forkink M, Nootboom M, Swarts H, Gloerich J, Nijtmans L, Koopman W, Huynen MA. BOLA1 is an aerobic protein that prevents mitochondrial morphology changes induced by glutathione depletion. *Antioxid Redox Signal*. 2013; 18:129–138. [PubMed: 22746225]
54. Johnson DC, Dean DR, Smith AD, Johnson MK. Structure, function, and formation of biological iron-sulfur clusters. *Annu Rev Biochem*. 2005; 74:247–281. [PubMed: 15952888]
55. Berndt C, Hudemann C, Hanschmann EM, Axelsson R, Holmgren A, Lillig CH. How does iron-sulfur cluster coordination regulate the activity of human glutaredoxin 2? *Antioxid Redox Signal*. 2007; 9:151–157. [PubMed: 17115894]
56. Wilks JC, Slonczewski JL. pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by green fluorescent protein fluorimetry. *J Bacteriol*. 2007; 189:5601–5607. [PubMed: 17545292]
57. Slonczewski JL, Rosen BP, Alger JR, Macnab RM. pH homeostasis in *Escherichia coli*: measurement by 31P nuclear magnetic resonance of methylphosphonate and phosphate. *Proc Natl Acad Sci USA*. 1981; 78:6271–6275. [PubMed: 7031646]
58. Boyd ES, Thomas KM, Dai Y, Boyd JM, Outten FW. Interplay between oxygen and Fe-S cluster biogenesis: insights from the Suf pathway. *Biochemistry*. 2014; 53:5834–5847. [PubMed: 25153801]
59. Bolstad HM, Botelho DJ, Wood MJ. Proteomic analysis of protein-protein interactions within the Cysteine Sulfinate Desulfinate Fe-S cluster biogenesis system. *J Proteome Res*. 2010; 9:5358–5369. [PubMed: 20734996]
60. Bolstad HM, Wood MJ. An in vivo method for characterization of protein interactions within sulfur trafficking systems of *E. coli*. *J Proteome Res*. 2010; 9:6740–6751. [PubMed: 20936830]
61. Freire P, Moreira RN, Arraiano CM. BolA inhibits cell elongation and regulates MreB expression levels. *J Mol Biol*. 2009; 385:1345–1351. [PubMed: 19111750]
62. Guinote IB, Matos RG, Freire P, Arraiano CM. BolA affects cell growth, and binds to the promoters of penicillin-binding proteins 5 and 6 and regulates their expression. *J Microbiol Biotechnol*. 2011; 21:243–251. [PubMed: 21464593]
63. Vinella D, Brochier-Armanet C, Loiseau L, Talla E, Barras F. Iron-sulfur (Fe/S) protein biogenesis: phylogenomic and genetic studies of A-type carriers. *PLoS Genet*. 2009; 5:e1000497. [PubMed: 19478995]

Figure 1. Grx4 and IbaG overexpressed in *E. coli* copurify as a complex. SDS-PAGE analysis of purified (A) [2Fe-2S] Grx4-IbaG WT heterodimer and (B) and [2Fe-2S] Grx4-IbaG heterodimers with the indicated IbaG variants.

Figure 2.

(A) Comparison of UV-visible absorption (top) and CD (bottom) spectra of purified [2Fe-2S] Grx4-IbaG heterodimer (red line) with [2Fe-2S] Grx4 homodimer (black line). ϵ and $\Delta\epsilon$ values are based on [2Fe-2S] concentration. (B) EPR spectra of dithionite-reduced [2Fe-2S] Grx4 and [2Fe-2S] Grx4-IbaG recorded at 20 K and 2 mW microwave power with a microwave frequency of 9.572 GHz and modulation amplitude of 0.63 mT.

Figure 3.

Comparison of resonance Raman spectra of [2Fe-2S] Grx4 (left) and [2Fe-2S] Grx4-IbaG (right) with 514-nm and 488-nm excitation. Samples (~2 mM in the [2Fe-2S] cluster) were in 50 mM Tris/MES, pH 8.0, 150 mM NaCl, 5 mM GSH and were in the form of a frozen droplet at 22 K. Each spectrum is the sum of 100 scans, with each scan involving photon counting for 1 s at 0.5 cm⁻¹ increments with 7 cm⁻¹ spectral resolution. Bands due to lattice modes of ice have been subtracted from all spectra.

Figure 4.

CD-monitored pH titration of [2Fe-2S] Grx4-IbaG (A) in the range between pH 5.51 and 8.08. The arrows at selected wavelengths indicate the direction of the change in intensity with an increase in pH. [2Fe-2S] concentration was kept constant at 38 μ M. (B) % CD signal change is based on the maximal difference in ϵ between peaks 1 (391 nm) and 2 (481 nm) or peaks 2 and 3 (564 nm) in (A) as a function of pH. The points were fit to theoretical curves with a pK_a of 6.36 ± 0.08 (blue line) and 6.43 ± 0.09 (red line). Both pK_a values are similar within experimental error. The Hill slopes for these fits (1.6 and 1.4, respectively) suggest cooperativity in the pH-dependent coordination changes.

Figure 5.

Size-exclusion chromatography and CD spectroscopy analysis of Grx4-IbaG interactions. (A) Gel filtration chromatograms (0.5–1 mg loaded) of apo-IbaG (solid black line), apo-Grx4 (solid blue line), [2Fe-2S] Grx4 (solid red line), apo-Grx4 + apo-IbaG (dotted blue line), and [2Fe-2S]-Grx4 + apo-IbaG (dotted red line). IbaG and Grx4 were mixed in a ratio of 2:1. The elution positions of molecular mass standards are shown at the top of the chromatogram. (B) Titration study of [2Fe-2S] Grx4 homodimer with apo IbaG monitored by UV-visible CD spectroscopy. CD spectra for [2Fe-2S] cluster-bound Grx4 homodimer (red line) titrated with a 0.67–4-fold excess of IbaG (legend indicates IbaG:[2Fe-2S] ratio). The arrows at selected wavelengths indicate the direction of the change in intensity with an increase in IbaG concentration. ϵ values are based on initial [2Fe-2S] concentration, 100 μ M. *Inset*: Model of interaction between [2Fe-2S] Grx4 homodimer and IbaG to form apo and holo Grx4-IbaG heterodimers.

Figure 6.

(A) UV-visible absorption (top panel) and CD spectra (bottom panel) of [2Fe-2S] Grx4-IbaG H27A (solid blue), H27C (dotted blue), H63A (solid green) and H63C (dotted green) variants compared to WT (red) and [2Fe-2S] Grx4 (black). ϵ and $\Delta\epsilon$ values are based on [2Fe-2S] concentration. (B) Comparison of resonance Raman spectra of [2Fe-2S] Grx4 and Grx4-IbaG H63A using 457.9-nm excitation. Samples were ~2 mM [2Fe-2S] cluster and were in the form of a frozen droplet at 22 K. Each spectrum is the sum of 100 scans, with each scan involving photon counting for 1 s at 0.5 cm⁻¹ increments with 7 cm⁻¹ spectral resolution. Bands due to lattice modes of ice have been subtracted from both spectra.

Table 1

Fe, S²⁻, and GSH measurements in purified Fe-S complexes.

Sample	Fe ^I	S ^I	GSH ^I	Fe:S:GSH
[2Fe-2S] Grx4-IbaG	1.0 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	1 : 0.9 : 0.4
[2Fe-2S] Grx4	1.5 ± 0.3	1.5 ± 0.2	1.1 ± 0.2	1 : 1 : 0.7

^IValues are reported as mol Fe, S²⁻, or GSH per mol of homodimer or heterodimer. Data are the average of three independent samples.

Table 2Molecular mass determination of individual proteins and complexes.¹

Sample	Complex	Theoretical (Da)	Gel filtration (Da)	MALDI (Da)
apo Grx4	Monomer	12748 (–1 st Met) ²	20900 ± 320	12705 ± 49
apo IbaG	Monomer	9452	16700 ± 150	9462 ± 11
apo Grx4-IbaG	Heterodimer	22200	24400 ± 230	22171 ± 171
[2Fe-2S] Grx4	Homodimer	26286	41800 ± 110	ND ³
[2Fe-2S] Grx4-IbaG	Heterodimer	22683	30100 ± 170	ND ³

¹Data are the average of three independent samples.²The MALDI result for apo-Grx4 matches closest to the theoretical mass of Grx4 without the N-terminal Met residue (12,748), which is likely removed during *E. coli* expression.³ND: not determined

Table 3

Thermodynamic binding parameters of *E. coli* (Ec) BolA/IbaG to Grx4 and *S. cerevisiae* (Sc) Fra2 to Grx3.

Sample	N (sites)	K_d (μ M)	H (kcal mol ⁻¹)	S (cal mol ⁻¹ K ⁻¹)
EcGrx4 + EcIbaG	0.70 $\pm$ 0.01	3.4 $\pm$ 0.1	-5.9 $\pm$ 0.1	5.40
EcGrx4 + EcBolA	0.97 $\pm$ 0.03	4.6 $\pm$ 0.9	-3.1 $\pm$ 0.1	14.1
ScGrx3 + ScFra2	0.40 $\pm$ 0.19	20.8 $\pm$ 6.6	-10.1 $\pm$ 5.4	-12.4