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***Schizosaccharomyces pombe* Grx4 Regulates the Transcriptional Repressor Php4 via [2Fe-2S] Cluster Binding†**

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Abstract

The fission yeast *Schizosaccharomyces pombe* expresses the CCAAT-binding factor Php4 in response to iron deprivation. Php4 forms a transcription complex with Php2, Php3, and Php5 to repress the expression of iron proteins as a means to economize iron usage. Previous *in vivo* results demonstrate that the function and location of Php4 are regulated in an iron-dependent manner by the cytosolic CGFS type glutaredoxin Grx4. In this study, we aimed to biochemically define these protein-protein and protein-metal interactions. Grx4 was found to bind a [2Fe-2S] cluster with spectroscopic features similar to other CGFS glutaredoxins. Grx4 and Php4 also copurify as a complex with a [2Fe-2S] cluster that is spectroscopically distinct from the cluster on Grx4 alone. *In vitro* titration experiments suggest that these Fe-S complexes may not be interconvertible in the absence of additional factors. Furthermore, conserved cysteines in Grx4 (Cys172) and Php4 (Cys221 and Cys227) are necessary for Fe-S cluster binding and stable complex formation. Together, these results show that Grx4 controls Php4 function through binding of a bridging [2Fe-2S] cluster.

TOC image

Grx4 forms a cysteine-ligated [2Fe-2S] binding complex with the transcriptional repressor Php4 to regulate transcription of iron utilization genes.

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Introduction

Maintenance of iron homeostasis is an essential function for most organisms given the critical role for this metal in cell metabolism. Low intracellular iron levels hamper the activity of iron-dependent enzymes, while iron overaccumulation can disrupt other metal trafficking pathways and catalyze formation of reactive oxygen species. Consequently, cells must closely regulate iron uptake and usage to maintain a balance between sufficient and excess levels.

The fission yeast *Schizosaccharomyces pombe* employs two transcription factors to control cellular iron levels, Fep1 under iron replete conditions, and Php4 under iron deprivation.¹ The actions of Php4 and Fep1 are coordinated at the transcriptional level since Fep1 and Php4 reciprocally regulate expression of each other in an iron-dependent manner.² Fep1 is a GATA-type transcriptional repressor that mainly controls genes involved in iron acquisition.³ When cells are iron-replete, Fep1 binds to GATA sequences in these genes and represses their transcription, downregulating iron transport systems. When cells are iron deficient, Php4 acts as a transcriptional repressor with the CCAAT-binding complex.⁴ In *S. pombe*, Php2, Php3 and Php5 form a heterotrimer that binds CCAAT cis-acting elements to activate gene expression. Genes regulated by this complex encode for proteins involved in iron-dependent metabolic pathways such as the TCA cycle, mitochondrial respiration, amino acid biosynthesis, heme biosynthesis, and iron-sulfur cluster assembly.⁵ Under iron-deplete conditions, Php4 is recruited to the CCAAT-binding complex, causing it to switch from an activator to a repressor. The main function of Php4 is therefore iron conservation: when cellular iron is limiting, expression of genes encoding iron-dependent proteins is repressed.

While expression of Php4 at the transcriptional level is controlled by Fep1, Php4 is also regulated in an iron-dependent manner at the protein level. The cytosolic CGFS glutaredoxin Grx4 controls both Fep1 and Php4 function since Fep1- and Php4-regulated genes are constitutively repressed in a *grx4* strain, causing sensitivity to iron depletion.⁶⁻⁹ In the case of Php4, Grx4 regulates interaction with the Php2/3/5 complex as well as the exportin Crm1.⁸ Under iron-deplete conditions, Php4 accumulates in the nucleus and binds to the

CCAAT-binding complex (Php2/3/5) to repress iron utilization genes. When iron levels increase, Php4 dissociates from Php2/3/5 and is relocalized to the cytosol by Crm1. Yeast two-hybrid mapping experiments revealed that Grx4 physically interacts with the C-terminal region of Php4, through the N-terminal thioredoxin-like (TRX) and C-terminal glutaredoxin-like (GRX) domains of Grx4.¹⁰ Association with the TRX domain is relatively weak and constitutive, while association with the GRX domain is iron-dependent and requires Cys172 of the conserved CGFS active site. Transcriptional analysis of Php4-regulated genes in strains expressing C172S/A Grx4 mutants indicates that Cys172 is absolutely required for Grx4-dependent inhibition of Php4 activity.^{6,9,10} In Php4, Cys221 and Cys227 in the C-terminus are specifically required for interaction with the GRX domain of Grx4, and may be involved in iron-mediated inactivation of Php4.¹⁰

It has been well-established that both prokaryotic and eukaryotic CGFS Grxs form [2Fe-2S]-bridged homodimers with the CGFS active site Cys and two glutathione (GSH) molecules serving as the iron ligands.^{11–22} Furthermore, the *S. cerevisiae* orthologs of *S. pombe* Grx4, namely Grx3/4, form [2Fe-2S]-bridged heterodimers with the BolA protein Fra2, which function in delivering an inhibitory Fe-S cluster to the transcriptional activators Aft1 and Aft2.²³ Given this precedent for *S. cerevisiae* Grx3/4 forming [2Fe-2S]-bridged complexes with iron regulatory proteins, and the previous studies in *S. pombe* documenting the *in vivo* Cys-dependent physical and functional interactions between Grx4 and Php4, these two proteins are proposed to form a Fe-S binding complex that inhibits Php4 activity in iron-replete conditions.^{10,24} However, characterization of this complex at the molecular level has not yet been reported.

In order to understand how Grx4 physically interacts with and regulates Php4, we expressed and characterized both proteins using biochemical, analytical, and spectroscopic techniques. We determined that as-purified Grx4 binds a [2Fe-2S] cluster similar to other CGFS glutaredoxins. While Php4 expressed alone does not co-purify with iron or an Fe-S cluster, it does bind a [2Fe-2S] cluster in complex with Grx4. In addition, we found that the conserved Cys residues in Grx4 (C172) and Php4 (C221/C227) are required for Fe-S cluster binding and strengthen the protein-protein interaction between Php4 and Grx4. Taken together, these results suggest that Grx4 regulates Php4 function through complex formation with a bridging [2Fe-2S] cluster.

Experimental

Plasmid construction

The open reading frame (ORF) of *S. pombe* Php4 was amplified from *S. pombe* genomic DNA by PCR and cloned into the NcoI and SalI sites of pRSFDuet-1 (Novagen) to create pRSFDuet-1-Php4, subsequently generating 6xHis-tagged Php4. The ORF of *S. pombe* Grx4 was amplified from *S. pombe* genomic DNA by PCR and cloned into the NdeI and XhoI sites of pRSFDuet-1 to create pRSFDuet-1-Grx4. Dual expression plasmids for Php4-Grx4 were made by inserting the Php4 ORF into the pRSFDuet-1-Grx4 plasmid. Php4 (C221A, C227A) and Grx4 (C172A) mutants were created by site-directed mutagenesis of the above plasmids.

Protein Expression and Purification

Overexpression of Grx4 was performed in the *E. coli* BL21(DE3) strain in LB media at 37 °C. Cells were grown to $A_{600} = 0.6\text{--}0.8$, then were induced with 20 μM isopropyl β -D-thiogalactosidase (IPTG) and incubated at 25 °C. Cells were collected 18 h after induction and resuspended in 50 mM Tris-HCl, pH 8.0, followed by sonication and centrifugation to remove cell debris. The cell-free extract was subjected to ammonium sulfate precipitation with the protein salting out in the 40% cut. The protein pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 750 mM $(\text{NH}_4)_2\text{SO}_4$ and loaded onto a Phenyl Sepharose column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 750 mM $(\text{NH}_4)_2\text{SO}_4$, 100 mM NaCl. The protein was eluted with a 750-0 mM $(\text{NH}_4)_2\text{SO}_4$ gradient, and fractions containing Grx4 as judged by SDS-PAGE and UV-visible spectroscopy were collected and concentrated. Concentrated protein was loaded onto a HiLoad Superdex 75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl. The purest fractions of [2Fe-2S] Grx4 as judged by SDS-PAGE and UV-visible spectroscopy were collected, concentrated, and stored at -80°C . Coexpression of Php4 with Grx4 was performed with the pRSFDuet-1-Php4-Grx4 expression plasmid transformed into *E. coli* BL21(DE3) using the procedure described above for Grx4.

Overexpression of Php4 was performed in the *E. coli* BL21(DE3) strain in LB media at 37 °C. Mid-logarithmic-phase cells ($A_{600} = 0.6\text{--}0.8$) were induced with 1 mM IPTG and grown at 16 °C. Cells were collected 18 h after induction and resuspended in 50 mM MES, pH 6.0, followed by sonication and centrifugation to remove cell debris. The cell-free extract was loaded onto a SP FF cation-exchange column (GE Healthcare) equilibrated with 50 mM MES, pH 6.0. Most of the protein eluted in the wash step and was collected and exchanged into 50 mM Tris-HCl, pH 8.0 buffer. This sample was loaded to a DEAE anion-exchange column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0. The protein was eluted with a 0–1 M NaCl gradient using 50 mM Tris-HCl, pH 8.0, 1 M NaCl. Fractions containing Php4 as judged by SDS-PAGE were collected, concentrated, and loaded onto a HiLoad Superdex 75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl. The purest fractions of Php4 as judged by SDS-PAGE were collected, concentrated, and stored at -80°C . All Php4 and Grx4 mutants (either alone or coexpressed) were purified using the same procedure as the wild-type forms. All purifications were carried out under anaerobic conditions ($\text{O}_2 < 5\text{ ppm}$) in a glovebox (Coy Laboratory Products), with the exception of Php4 WT and mutants (expressed without Grx4). *S. cerevisiae* Fra2^{1–35} was overexpressed in *E. coli* and purified as previously described.²⁵

Biochemical Analyses

Protein concentrations were determined by the Bradford Assay (Bio-Rad) using bovine serum albumin as the standard. Iron and acid-labile sulfur concentrations were measured as previously described.¹³ 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.²⁶ We note that buffers used for Grx4 and Php4 purification did not contain any GSH, so any GSH bound to the protein complexes is derived from the *E. coli* cells.

Analytical and Spectroscopic Methods

Analytical gel filtration analyses were performed on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM GSH and calibrated with a high molecular weight calibration kit (GE Healthcare). The same set of fractions was collected for all proteins eluted from gel filtration, and were analyzed by SDS-PAGE in order to compare relative elution times.

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-815 spectropolarimeter (Jasco, Easton, MD). EPR spectra were recorded using a Bruker EMX plus spectrometer (Bruker, Billerica, MA), equipped with an Oxford ESR900 continuous flow cryostat (Oxford Instruments, Oxfordshire, UK), and quantified under nonsaturating conditions by double integration against a 1.0 mM CuEDTA standard. The samples were reduced under anaerobic conditions by addition of stoichiometric sodium dithionite and frozen within five minutes in liquid nitrogen. EPR conditions were as follows: microwave frequency, ~9.6 GHz, modulation frequency, 100 kHz, modulation amplitude, 10 G, microwave power, 10 mW, and temperature 10–20 K.

Iron-sulfur cluster reconstitutions

Purified Grx4 or Php4-Grx4 was incubated with 20-fold excess ferrous ammonium sulfate, 20-fold excess L-cysteine, a catalytic amount of IscS, and 5 mM GSH in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl under anaerobic conditions on ice for 2 h. Excess reagents were purified out using a Phenyl Sepharose column (GE Healthcare) with a decreasing linear gradient from 750 to 0 mM $(\text{NH}_4)_2\text{SO}_4$, and the red-colored fractions were pooled and concentrated.

Surface plasmon resonance

Binding kinetics of apo Php4-Grx4 complexes were determined by surface plasmon resonance (SPR), using a Biacore 3000 (Biacore, GE Healthcare). His-Php4 (WT) was immobilized on a Sensor Chip NTA activated with Ni^{2+} according to the manufacturer's instructions. Grx4 (WT or C172A) was injected over the flow cell for 9.3 min at 9–10 different concentrations between 25 $\mu\text{g}/\text{ml}$ (0.9 μM) and 20 mg/ml (740 μM) using a flow rate of 35 $\mu\text{l}/\text{min}$. The assays were run at 25° C in 50 mM Tris-HCl, pH 8.0, 0.005% Tween-20 with a blank injection (buffer only) between each run. Bound protein was removed with a 60-sec wash of 25 mM NaOH using a flow rate of 15 $\mu\text{l}/\text{min}$. The NTA sensor chip was regenerated with 300 mM EDTA. Global data analysis was performed using BIAEvaluation 4.1 (Biacore Software) applying a Langmuir 1:1 binding model with baseline drift to calculate k_{on} (association rate constant) and k_{off} (dissociation rate constant). A linear baseline drift was included to account for leaching of His-Php4 from the sensor surface during the association and dissociation phases.

CD-monitored titrations of Php4 and Grx4

Several titration methods were used in an attempt to convert between Grx4 homodimer and Php4-Grx4 heterocomplexes. All samples were prepared and scanned under anaerobic conditions with 1 mM GSH, and the $[\text{Fe-2S}]$ content was kept constant at 50 μM . Php4 was

pre-incubated with 5 mM DTT to reduce any disulfides formed during purification, and then desalted anaerobically before mixing with [2Fe-2S] Grx4. Method 1: Increasing amounts of Php4 were added to [2Fe-2S] Grx4 up to a 2.5 : 1 ratio of Php4 to [2Fe-2S]. Method 2: [2Fe-2S] Grx4 was incubated first with 2-fold molar excess *S. cerevisiae* Fra2 to generate a [2Fe-2S] Grx4-Fra2 heterodimer. Php4 was added to this mixture in 2-fold and 5-fold molar excesses relative to [2Fe-2S]. A 1:2 mixture of [2Fe-2S] Grx4:Fra2 was used as a control.

Results and discussion

Grx4 binds a [2Fe-2S]²⁺ cluster

In a recent report, recombinant *S. pombe* Grx4 was reconstituted with an Fe-S cluster, which requires GSH and is sensitive to the presence of oxygen.⁶ However, only the UV-visible spectrum of this Fe-S cluster complex was provided, which showed a single peak ~400 nm, as well as two shoulders between 500–700 nm, indicative of a mixture of [2Fe-2S] and linear [3Fe-4S] clusters.²⁷ Previously published spectra of [2Fe-2S] Grxs purified under strictly anaerobic conditions typically have two distinct peaks at ~320 and ~410 nm, with a characteristic shoulder at ~445 nm.^{11,13,28,29} To confirm that *S. pombe* Grx4 binds a bona fide [2Fe-2S]²⁺ cluster similar to previously characterized Grxs, we overexpressed and purified recombinant *S. pombe* Grx4 from *E. coli*. The purified protein had a reddish-brown color, and was investigated by spectroscopic and biochemical methods. The UV-visible absorption spectrum of as-purified Grx4 is nearly identical to other monothiol glutaredoxins from *S. cerevisiae* (*Sc* Grx3) and *E. coli* (*Ec* Grx4), with peaks at 323, 413, and 445 nm, indicative of an [2Fe-2S]²⁺ cluster (Fig. 1). As expected, the CD spectrum for *S. pombe* Grx4 was also comparable to *Sc* Grx3 and *Ec* Grx4. Positive peaks at 312, 460, and 550–590 nm and negative peaks at 348, 408, and 518 nm are characteristic of [2Fe-2S]²⁺-bridged Grx homodimers.

Grx4 and Php4 copurify as a complex

To characterize the interactions between Php4 and Grx4 *in vitro*, Php4 was initially expressed and purified separately. Soluble recombinant Php4 was extracted from *E. coli*, yielding apo, monomeric protein, with no apparent color in the protein extract. To determine if Grx4 forms a stable complex with Php4, the proteins were coexpressed in *E. coli*. The induced cells had a distinct reddish-brown color, similar to cells overexpressing Grx4 alone, which was not observed when Php4 was expressed alone. Moreover, Php4 and Grx4 were found to copurify as a stable complex with a reddish-brown color. The individual proteins and the Php4-Grx4 complex were subjected to size-exclusion chromatography to confirm complex formation. The same fractions were collected from the elution of each protein or complex, and analyzed by SDS-PAGE (Fig. 2). Php4 expressed alone runs as a monomer in fractions 5–7 (theoretical mass: 34.4 kD, apparent mass: 30–40 kD). [2Fe-2S] Grx4 elutes in fractions 4–5 (theoretical [2Fe-2S] homodimer mass: 55.0 kD, apparent mass: 60–90 kD) and higher order oligomers in fractions 1–3 (>90 kD). Coexpression of Php4 with Grx4 shows no sign of the Php4 monomer but instead results in co-elution of the two proteins in fractions 4–6 (theoretical heterodimer mass 63.1 kD; apparent mass, 60–90 kD) and higher order oligomers in fractions 2–3 (>90 kD). We note that the apparent molecular masses for Grx4 expressed alone and the Grx4-Php4 heterocomplex were difficult to determine since

samples with Grx4 tended to aggregate and/or adsorb to the column during the experiment, resulting in Grx4 elution across a broad range of fractions. Nevertheless, comparison of Php4 expressed alone versus with Grx4 shows a clear shift in the elution time, indicating formation of a complex between Php4 and Grx4 with a higher apparent molecular mass than Php4 alone.

Php4-Grx4 binds a [2Fe-2S] cluster with a coordination environment distinct from [2Fe-2S] Grx4

The identity of the chromophore on the purified Php4-Grx4 complex was investigated and compared to Grx4. Both the absorption and CD spectra of the Php4-Grx4 complex suggest the presence of a [2Fe-2S] cluster; however, the spectra of the Fe-S cluster on Php4-Grx4 are significantly different from those of the Fe-S center on Grx4 alone (Fig. 3A). In particular, the dominant visible absorption band for Php4-Grx4 is blue shifted to 408 nm compared to the equivalent band for Grx4 at 413 nm. In addition, Php4-Grx4 has a shoulder ~ 320 nm instead of a distinct peak as seen for Grx4 (Fig. 3A, top). These differences are reflected in the CD spectrum of Php4-Grx4, which exhibits changes in the intensity and wavelengths of the visible CD bands (Fig. 3A, bottom). Overall, comparison of the excited state electronic structures of the [2Fe-2S]²⁺ centers in Php4-Grx4 and Grx4 alone suggest differences in the coordination environment and/or chirality of the [2Fe-2S] centers.

To confirm binding of an Fe-S cluster in the Php4-Grx4 complex and investigate the role of GSH in cluster ligation, we measured iron and acid-labile sulfide in the Grx4 and Php4-Grx4 complexes. These analyses show an Fe:S ratio close to 1:1 for Grx4 and Php4-Grx4 with ~0.42 [2Fe-2S] clusters per Grx4 dimer and ~0.41 [2Fe-2S] clusters per Php4-Grx4 (Table 1). Interestingly, the GSH measurement of the Php4-Grx4 complex suggests only 1 GSH bound per [2Fe-2S] cluster (Fe:S:GSH ratio of 1:1:0.4) in comparison to the Grx4 alone, which is closer to 2 GSH per cluster (Fe:S:GSH ratio of 1:1:0.8). These clear differences in GSH coordination to the clusters in Grx4 and Php4-Grx4 may reflect the distinct coordination environments observed in the UV-visible absorption and CD spectra.

The EPR spectra of Grx4 and Php4-Grx4 provide information about ligand identity, redox properties and stability of the reduced [2Fe-2S]⁺ cluster in each complex (Fig. 3B). Both samples were unstable to reduction as evidenced by the absence of an EPR signal upon addition of excess sodium dithionite. Thus, samples were reduced with stoichiometric amounts of dithionite under anaerobic conditions, and frozen under liquid nitrogen within five minutes. The [2Fe-2S]⁺ Grx4 homodimer gave a rhombic $S = \frac{1}{2}$ EPR signal, $g_{1,2,3} = 2.00, 1.94, \text{ and } 1.90$ ($g_{av} \sim 1.95$), accounting for less than 10% of the cluster content (Fig. 3B, top). This signal is similar to other [2Fe-2S]-bridged CGFS Grxs, which are also known to be reductively labile, resulting in a low number of spins per cluster.^{13,28,29} [2Fe-2S]⁺ Php4-Grx4 also gives a rhombic $S = \frac{1}{2}$ EPR signal, $g_{1,2,3} = 2.00, 1.96, \text{ and } 1.92$ ($g_{av} = 1.96$), accounting for ~20% of the cluster content (Fig. 3B, bottom). Therefore, as with [2Fe-2S] Grx4, the [2Fe-2S] cluster on Php4-Grx4 is reductively labile. The g_{av} values and g -value anisotropy provide information about the ligation of [2Fe-2S]⁺ clusters, which are sensitive to ligation changes at the Fe(II) site.¹³ [2Fe-2S]⁺ clusters with all-cysteine ligation exhibit $g_{av} \sim 1.95\text{--}1.97$, whereas g_{av} decreases to $\sim 1.93\text{--}1.94$ when one cysteine is replaced by a

histidine.¹³ Given these trends, *S. pombe* Grx4 likely binds its [2Fe-2S] cluster through all-cysteine ligation from the active site CGFS cysteines and the cysteine moiety of two GSH molecules, as demonstrated for other CGFS Grxs. Similarly, the signal obtained for Php4-Grx4 is also characteristic of a [2Fe-2S]⁺ cluster with all-cysteine ligation. Taken together, these results confirm that Php4 binds a bridging [2Fe-2S]²⁺ cluster with Grx4.

[2Fe-2S] Php4-Grx4 and [2Fe-2S] Grx4 are not interconvertible *in vitro*

We previously demonstrated that *S. cerevisiae* [2Fe-2S] Grx3/Grx4 homodimers interact with the cytosolic protein Fra2 to form heterodimeric complexes.^{13,25,30} Fra2 is a member of the BolA family of proteins that serve as functional bindingpartners for CGFS Grxs in a variety of organisms.¹⁴ Grx3/4-Fra2 heterodimers, in turn, regulate activity of the *S. cerevisiae* Fe- responsive transcription factors Aft1/Aft2 via specific transfer of the [2Fe-2S] cluster from Grx3-Fra2 to Aft2.²³ The stoichiometric conversion of [2Fe-2S] Grx3 homodimer to [2Fe-2S] Grx3-Fra2 heterodimer upon titration with Fra2 as well as Fe-S cluster transfer from Grx3-Fra2 to Aft2 was monitored by CD spectroscopy, providing a convenient handle to observe changes in Fe-S cluster coordination.^{23,25} Since *S. pombe* [2Fe-2S] Grx4 and [2Fe-2S] Grx4-Php4 complexes have distinct CD spectra, we used a similar approach to determine if [2Fe-2S] Grx4 is converted to the [2Fe-2S] Php4-Grx4 complex upon titration with purified Php4. Addition of up to 2.5-fold molar excess of Php4 to [2Fe-2S] Grx4 did not yield any significant changes in the CD spectrum (Fig. 4A), suggesting that Php4 does not bind the cluster or alter the Fe-S cluster coordination environment under these conditions. The addition of 5 mM DTT to this mixture, which has been shown to accelerate Fe-S cluster transfer reactions,³¹ also did not facilitate formation of the [2Fe-2S] Php4-Grx4 heterocomplex (data not shown). These results demonstrate that [2Fe-2S] Grx4 cannot be converted to the [2Fe-2S] Php4-Grx4 complex by simple titration with Php4.

In *S. cerevisiae*, the iron-responsive transcription factor Aft2 preferentially interacts with and accepts an Fe-S cluster from [2Fe-2S] Grx3-Fra2 heterodimers rather than [2Fe-2S] Grx3 homodimers.²³ To determine whether Fra2 has a similar role in facilitating interaction between *S. pombe* Php4 and Grx4, we tested whether a Grx4-Fra2 heterodimer is capable of interacting with Php4. Although *in vivo* data argue against a role for *S. pombe* Fra2 in the Php4-Grx4 interaction,³² we tested the Grx4-Php4-Fra2 interaction by mixing [2Fe-2S] Grx4 with Fra2 prior to adding Php4 (Fig. 4B). The *S. cerevisiae* (*Sc*) and *S. pombe* (*Sp*) Fra2 proteins are orthologs (39% identical, 63% similar), and *Sc* Fra2 can complement *Sp* Fra2 function under iron depletion conditions *in vivo* (Fig. S1, ESI[†]). Therefore, *Sc* Fra2 was used in the titration with *Sp* Grx4 since this purified protein was available. Mixing [2Fe-2S] *Sp* Grx4 with a two-fold excess of *Sc* Fra2 resulted in distinct changes in the CD spectrum (Fig. 4B, gray line), demonstrating that interaction of Fra2 with [2Fe-2S] Grx4 alters the coordination environment of the Fe-S cluster, similar to the effect observed upon titration of [2Fe-2S] *Sc* Grx3 with *Sc* Fra2.²⁵ However, addition of Php4 to this mixture in both 2- and 5-fold excesses did not yield any further changes compared to the [2Fe-2S] Grx4 + Fra2 spectrum (Fig. 4B, compare gray and green lines). Thus, these results suggest that

[†]Electronic Supplementary Information (ESI) available. See DOI:

Fra2 does not facilitate interaction of Php4 with Grx4 under these conditions, consistent with the *in vivo* findings previously reported.³²

We next asked if Fe-S complex formation occurred in the reverse direction by testing whether the [2Fe-2S] Php4-Grx4 heterocomplex could be converted to the [2Fe-2S] Grx4 homodimer by titration with apo-Grx4 (Fig. S2A, ESI[†]). Addition of excess Grx4 did not convert [2Fe-2S] Php4-Grx4 to the [2Fe-2S] Grx4 homodimer, indicating that these forms are not interconvertible under these conditions. Furthermore, we tested whether pre-mixed apo Php4-Grx4 accepts a cluster from [2Fe-2S] Grx4 homodimer. *In vivo*, Php4 and Grx4 interact under iron deficiency conditions, thus the apo complex may need to be formed before cluster transfer.¹⁰ However, by titrating apo Php4-Grx4 into [2Fe-2S] Grx4, we saw no evidence for Fe-S cluster transfer (Fig. S2B, ESI[†]). These results suggest that the conversion of [2Fe-2S] Grx4 to [2Fe-2S] Php4-Grx4 may require some other protein or factor found in cells.

Finally, we tested whether formation of the Fe-S-bridged Grx4 homodimer or Php4-Grx4 complex is preferred upon anaerobic cysteine desulfurase-mediated Fe-S reconstitution of apo Php4-Grx4. As a control, a sample of apo-Grx4 was subjected to reconstitution in the absence of Php4, generating a CD signal that closely overlapped the spectrum for as-purified [2Fe-2S] Grx4 (Fig. 5, blue lines). However, Fe-S reconstitution of a 1:1 mixture of Php4 and apo-Grx4 produced a sample with CD peaks that more closely matched as-purified [2Fe-2S] Php4-Grx4 (with some intensity differences), with little indication of [2Fe-2S] Grx4 homodimer formation (Fig. 5, black lines). These results suggest that [2Fe-2S] Php4-Grx4 is the thermodynamically preferred cluster-bound complex under these conditions.

Grx4 C172 and Php4 C221/C227 are required for [2Fe-2S] cluster binding and stable complex formation

The conserved CGFS active site in *S. pombe* Grx4 includes the cysteine residue (Cys172) that serves as an Fe-S cluster ligand in previously characterized CGFS Grxs from other organisms.^{11–13} A recent study demonstrated that *S. pombe* Grx4 cannot be reconstituted with an Fe-S cluster when this cysteine is changed to a serine (C172S).⁶ To confirm this result, we tested whether a C172A substitution would affect Fe-S cluster-binding in the as-purified Grx4 homodimer alone or in complex with Php4. Grx4 (C172A) overexpressed alone or with Php4 was found to be predominantly insoluble. Insoluble protein pellets did not have any color to indicate Fe or Fe-S cluster binding. Protein was extracted from inclusion bodies with urea, followed by a stepwise dialysis to remove urea and allow for protein refolding. This sample purified without an Fe-S cluster and was found to be relatively unstable. Although Grx4 (C172A) still copurified with Php4 to some extent (Fig. S3), the ratio of Php4 to Grx4 is visibly reduced compared to the WT complex (compare relative Php4 and Grx4 band intensities in Php4-Grx4 complexes in Fig. 2 and Fig. S3, ESI[†]). Presumably, the Php4-Grx4 interaction is not completely abolished by mutating this Cys due to the presence of the TRX domain-dependent interaction detected *in vivo*.¹⁰

As mentioned previously, Php4 contains two conserved cysteines, Cys221 and Cys227, which are required for iron-dependent interaction with Grx4 *in vivo*.¹⁰ These cysteines are hypothesized to act as Fe-S cluster ligands in the Php4-Grx4 complex.¹ We tested this by

substituting both cysteines with alanine (C221/227A) and determining if Php4 still interacted with Grx4. Php4(C221/227A) overexpressed and purified alone is a relatively soluble protein. When coexpressed with Grx4, Php4(C221/227A) copurifies with Grx4 to a certain extent as shown by SDS-PAGE analysis (Fig. S3, ESI[†]), although the sample elutes from the analytical gel filtration column across several fractions, suggesting multiple aggregation states. Due to this heterogeneity, we were unable to reliably determine the stoichiometry of the complex by analytical gel filtration. The Fe-S cluster yield from this as-purified complex was low, so the sample was subjected to Fe-S reconstitution using the as-purified complex or a 1:1 ratio of Php4 (C221/227A) and Grx4 purified separately and mixed together. Both approaches yielded a CD spectrum with features almost identical to that of [2Fe-2S] Grx4 (Fig. 5, compare red and blue lines), although with lower peak intensities. These results suggest that while Php4 (C221/227A) can still interact with Grx4, it no longer participates in binding the [2Fe-2S] cluster. Taken together, these data provide evidence that Grx4 Cys172 and Php4 Cys221 and Cys227 are required for Fe-S cluster binding and complex stability.

In order to quantify the stability of the Php4-Grx4 complex and measure the effects of substituting key Cys residues in Grx4 or Php4, we measured the kinetic parameters for the binding interaction between Grx4 and Php4 using surface plasmon resonance (SPR) (Fig. 6, Table 2). The apo forms of the proteins were used for these experiments due to the sensitivity of the Fe-S clusters to oxygen-induced degradation. With His-Php4(WT) as the immobilized ligand and Grx4(WT) or Grx4(C172A) as the analyte, we measured the association (k_{on}) and dissociation (k_{off}) rate constants for the interaction in order to calculate the equilibrium dissociation constant ($K_D = k_{off}/k_{on}$). These experiments demonstrated that the Grx4(C172A) variant binds to Php4 with a 26-fold lower affinity than WT Grx4 (897 μ M vs. 34.1 μ M) (Table 2). This result is consistent with yeast two-hybrid data indicating that Cys172 in Grx4 plays a key role in stabilizing the Grx4-Php4 interaction *in vivo*.¹⁰ As stated earlier, this previous yeast two-hybrid study also demonstrated that Cys221 and Cys227 in Php4 are required for interaction with the GRX domain of Grx4. We attempted to measure the *in vitro* binding interaction between His-Php4(C221,227A) and Grx4 using SPR, but were unable to obtain consistent results with this Php4 variant as the immobilized ligand, possibly due to heterogeneity in the purified Php4(C221,227A) sample. In any case, the SPR results clearly show a significant role for Grx4 Cys172 in stabilizing the Php4-Grx4 interaction, even in the absence of Fe-S cluster binding.

Conclusions

Previous *in vivo* studies in *S. pombe* established that the CGFS glutaredoxin Grx4 binds to and regulates Php4 function in response to iron bioavailability.^{6,8,10} The strength of this interaction is iron-responsive and dependent on several conserved Cys residues, Cys172 in Grx4 and Cys221 and Cys227 in Php4. Based on these data, we sought to characterize the iron-dependent interaction between Grx4 and Php4 *in vitro*. In this study we report that recombinant Grx4 expressed alone binds a [2Fe-2S]²⁺ cluster with spectroscopic features almost identical to previously characterized CGFS Grxs. When coexpressed, Php4 and Grx4 copurify as a complex with a bound [2Fe-2S]²⁺ cluster. Analysis via UV-visible absorption, CD, and EPR spectroscopies indicate the presence of a [2Fe-2S]²⁺ cluster in the Php4-Grx4

complex that is distinct from that of $[2\text{Fe-2S}]^{2+}$ Grx4. However, g_{av} values from the EPR signals suggest complete cysteine ligation for both cluster types. Furthermore, both of these clusters are relatively labile based on overall cluster-loading on the proteins after purification (only ~40% for each), as well as spin quantitation of their EPR signals upon reduction.

It was shown previously that *E. coli*, *S. cerevisiae*, and human $[2\text{Fe-2S}]$ Grxs can be converted to $[2\text{Fe-2S}]$ Grx-BolA heterocomplexes upon addition of the BolA protein binding partner.^{15,25,28,29,33,34} Furthermore, $[2\text{Fe-2S}]$ -bridged Grx homodimers and $[2\text{Fe-2S}]$ -bridged Grx-BolA heterocomplexes can transfer Fe-S clusters to specific apo acceptor proteins.^{11,15,18,23,27,33,35,36} Of note, the $[2\text{Fe-2S}]$ Grx3-Fra2 complex in *S. cerevisiae* specifically transfers its cluster to the transcription factor Aft2 when these proteins are mixed.²³ Given these previous findings, we sought to determine if $[2\text{Fe-2S}]$ Grx4 would convert to $[2\text{Fe-2S}]$ Php4-Grx4, or if there was a cluster transfer in a similar fashion. However, contrary to previous studies demonstrating facile conversion of $[2\text{Fe-2S}]$ Grx homodimers to $[2\text{Fe-2S}]$ -bridged heterocomplexes, several attempts to produce a transition between complexes were not successful. Addition of Php4 to $[2\text{Fe-2S}]$ Grx4 did not allow formation of the $[2\text{Fe-2S}]$ Php4-Grx4 complex. Similarly, addition of apo-Grx4 to $[2\text{Fe-2S}]$ Php4-Grx4 did not drive formation of the $[2\text{Fe-2S}]$ Grx4 homodimer. Mixing $[2\text{Fe-2S}]$ Grx4 with apo-Php4-Grx4 complex did not facilitate cluster transfer, nor did mixing $[2\text{Fe-2S}]$ Grx4 with Php4 in the presence of Fra2 protein. Intriguingly, Fe-S cluster reconstitution of a mixture of Php4 and Grx4 primarily produces the $[2\text{Fe-2S}]$ Php4-Grx4 complex rather than $[2\text{Fe-2S}]$ Grx4. Although the $[2\text{Fe-2S}]$ Php4-Grx4 complex appears to be thermodynamically favored under the Fe-S reconstitution conditions we tested, it is possible that there is a kinetic barrier to interconversion from $[2\text{Fe-2S}]$ Grx4 to $[2\text{Fe-2S}]$ Grx4-Php4 that requires additional factors and/or specific conditions to overcome. We note that this phenomenon was observed for the delivery of an Fe-S cluster to Aft2 in *S. cerevisiae*. Although the Fe-S cluster in the *S. cerevisiae* $[2\text{Fe-2S}]$ Grx3 homodimer is relatively labile, this complex was inefficient at delivering an Fe-S cluster to Aft2, while the $[2\text{Fe-2S}]$ Grx3-Fra2 heterodimer performed this task effectively. In *S. pombe*, as Php4 and Grx4 constitutively interact regardless of iron binding, it seems likely that a transfer protein delivers the $[2\text{Fe-2S}]$ cluster to apo Php4-Grx4 in the nucleus.¹⁰ This possibility is supported by *in vivo* fluorescence imaging experiments demonstrating predominant localization of functional GFP-tagged Grx4 to the nucleus in both iron-starved and iron-replete cells.^{7,32} Presumably, Grx4 is in the apo form and associates with Php4 at the Php2/3/5 complex in iron-starved conditions.

The results presented herein provide further evidence for CGFS Grxs as dynamic sensors of cellular iron status and build on the current model for Fe-dependent inhibition of Php4 via Grx4 binding (Fig. 7).^{1,2,8,10,24} The *in vitro* studies reported here taken together with the following previously published *in vivo* observations support this proposed regulation mechanism: (1) deletion of *grx4* or depletion of GSH results in constitutive repression of Php4-regulated genes suggesting regulation of Php4 by both Grx4 and GSH,^{5,8} (2) export of GFP-Php4 from the nucleus to the cytosol visualized by fluorescence microscopy is iron-dependent and requires Grx4,⁸ and (3) the iron-dependent interaction between Grx4 and Php4 detected by yeast two-hybrid and bimolecular fluorescence complementation assays is dependent on Cys172 in Grx4 and Cys221/227 in Php4.¹⁰ Our *in vitro* protein-protein and

Fe-S binding analyses support this model and provide additional molecular details about the coordination chemistry of the binding interaction. In the proposed model shown in Fig. 7, Grx4 and Php4 constitutively interact through the TRX domain of Grx4 under iron-deplete conditions.^{8,10} Php4 is able to bind to and inactivate the Php2/3/5 transcriptional complex (Fig. 7, top). When iron levels are sufficient, the Php4-Grx4 complex binds a [2Fe-2S] cluster. Since Grx4 and Php4 interact constitutively *in vivo*, it seems likely that a [2Fe-2S] cluster is delivered to this complex to convert it from apo to holo, rather than Php4 binding to and converting [2Fe-2S] Grx4. Fe-S cluster binding and complex stability are dependent on conserved cysteines in these proteins: Cys172 in Grx4 and Cys221 and Cys227 in Php4. Binding of this Fe-S cluster facilitates inactivation and relocation of Php4 to the cytosol, allowing the Php2/3/5 complex to activate transcription of iron utilization genes (Fig. 7, bottom). This mode of interaction is somewhat different from *S. cerevisiae* Aft2 that receives an Fe-S cluster from the Fra2-Grx3 complex to form a [2Fe-2S]-bridged Aft2 homodimer.²³ To our knowledge, this study is the first report of a CGFS Grx regulating a transcription factor through direct binding without an accessory protein such as BOLA/Fra2. Thus, the CGFS Grxs in these two evolutionarily divergent organisms apparently have a common role in iron regulation, albeit via different mechanisms. Further studies are needed to determine the identity of the [2Fe-2S] cluster donor protein for the Php4-Grx4 complex and the mechanism of transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance to metallomics

In the fission yeast *Schizosaccharomyces pombe*, the transcription factor Php4 acts to limit iron utilization under conditions of iron deficiency by repressing expression of genes involved in iron-rich metabolic pathways. The glutaredoxin Grx4 binds to Php4 *in vivo* and regulates its repressor activity and subcellular localization in an iron-dependent manner. We report here the molecular details of this Php4-Grx4 interaction, providing insight into the mechanisms of iron regulation in this model eukaryote.

Fig. 1.

UV-visible absorption (top) and CD spectra (bottom) of [2Fe-2S] Grx4 from *S. pombe* (*Sp*, blue line), [2Fe-2S] Grx3 from *S. cerevisiae* (*Sc*, black line),¹³ and [2Fe-2S] Grx4 from *E. coli* (*Ec*, red line).²⁷ ϵ and $\Delta\epsilon$ values are based on [2Fe-2S] concentration. *Inset*, model for [2Fe-2S] Grx4 demonstrating ligation of the cluster by the GRX domain.

Fig. 2.

Php4 and Grx4 coelute as a complex. SDS-PAGE analysis of gel filtration fractions collected for purified Php4 expressed alone (*top*), Grx4 expressed alone (*middle*), and Php4 coexpressed with Grx4 (*bottom*). Identical fractions from each analytical gel filtration experiment were loaded for comparison of elution volumes. In this experiment, larger proteins/complexes elute earlier and smaller ones later. The theoretical masses of Php4 and Grx4 are 34.3 and 27.1 kD, respectively. Masses (in kD) of MW standards used in the SDS-PAGE gel are shown on the left. The elution position and apparent mass (in kD) of the molecular weight standards used to calibrate the gel filtration column are shown across the bottom.

Fig. 3. [2Fe-2S] Grx4 and [2Fe-2S] Php4-Grx4 complexes are distinct. (A) Comparison of UV-visible absorption (top) and CD spectra (bottom) of [2Fe-2S] Php4-Grx4 heterodimer (black line) with [2Fe-2S] Grx4 homodimer (blue line). ϵ and $\Delta\epsilon$ values are based on [2Fe-2S] concentration. (B) Comparison of the X-band EPR spectra of dithionite-reduced [2Fe-2S] Grx4 (top) with [2Fe-2S] Php4-Grx4 (bottom). Spectra recorded at 10–20 K and 10 mW microwave power with a microwave frequency of 9.60 GHz and modulation amplitude of 10 G.

Fig. 4.

CD-monitored titrations of [2Fe-2S] Grx4 with Php4 and *S. cerevisiae* Fra2. (A) CD spectra for [2Fe-2S] Grx4 (blue line) titrated with 0.1 – 2.0 molar excess Php4 (gray lines). The red line corresponds to 2.5 molar excess Php4 added to [2Fe-2S] Grx4. As-purified [2Fe-2S] Php4-Grx4 is shown as a black line. (B) CD spectra for as-purified [2Fe-2S] Grx4 (blue line) incubated with 2-fold molar excess of *Sc* Fra2 (gray line), or 2-fold excess of *Sc* Fra2 then 5-fold excess of Php4 (green line). ϵ values are based on the $[2Fe-2S]^{2+}$ cluster concentration [75 μM for (A) and 50 μM for (B)].

Fig. 5.

Comparison of CD spectra of as-purified [2Fe-2S] Grx4 (blue solid line) and [2Fe-2S] Php4-Grx4 (black solid line) with reconstituted [2Fe-2S] Grx4 (blue dotted line), [2Fe-2S] Php4-Grx4 (black dotted line), and [2Fe-2S] Php4(C221/227A)-Grx4 (red line).

Fig. 6.

Biacore response data for WT (**A**) and C172A (**B**) Grx4 binding to His-Php4 captured on a biosensor surface. The association phase, in which Grx4 samples were flowed across immobilized His-Php4, occurs from 50 to 610 s (9.3 min injection time), while the dissociation phase was recorded from 610 to 860 s. Experimental data (shown in black) represent the responses of varying concentrations of Grx4; 0.92 μM to 553 μM in (**A**), and 9.23 μM to 738 μM in (**B**). Global fit analyses of the sensorgrams to a 1:1 binding model with pseudo first order kinetics are shown in red (Table 2).

Fig. 7.

Proposed model for regulation of Php4 via Grx4. During iron starvation conditions, Grx4 interacts with Php4 through the TRX domain, independent of iron. Php4 is free to bind and modify the function of the Php2/3/5 transcriptional complex in the nucleus, repressing iron utilization genes. Under iron replete conditions, the Php4-Grx4 complex binds a [2Fe-2S] cluster. [2Fe-2S] Php4-Grx4 is released from the Php2/3/5 complex and exported from the nucleus, allowing transcriptional activation of iron utilization genes.

Table 1

Fe, S²⁻, and GSH measurements for purified Fe-S cluster proteins. Values are reported per dimer. Data are the average of three independent samples.

Protein	Fe	S ²⁻	GSH	Fe:S:GSH
Grx4	0.84 ± 0.18	0.83 ± 0.10	0.70 ± 0.17	1 : 1 : 0.8
Php4-Grx4	0.81 ± 0.05	0.78 ± 0.08	0.32 ± 0.06	1 : 1 : 0.4

Table 2

SPR kinetic measurements for the interaction between His-Php4 and WT or C172A Grx4. The rate constants k_{on} and k_{off} (and standard error of the fit) were determined by global fitting of sensorgrams in Fig. 6 using BIAevaluation software. $K_D = k_{\text{off}}/k_{\text{on}}$.

Ligand	Analyte	k_{on} ($\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_D (μM)
His-Php4	Grx4	64.3 (0.6)	$2.19 (0.04) \times 10^{-3}$	34.1 (0.08)
His-Php4	Grx4(C172A)	10.2 (0.1)	$9.16 (0.10) \times 10^{-3}$	897 (16)