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Monothiol glutaredoxins and A-type proteins: Partners in Fe-S cluster trafficking

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Abstract

Monothiol glutaredoxins (Grxs) are proposed to function in Fe-S cluster storage and delivery, based on their ability to exist as apo monomeric forms and dimeric forms containing a subunit-bridging $[\text{Fe}_2\text{S}_2]^{2+}$ cluster, and to accept $[\text{Fe}_2\text{S}_2]^{2+}$ clusters from primary scaffold proteins. In addition yeast cytosolic monothiol Grxs interact with Fra2 (Fe repressor of activation-2), to form a heterodimeric complex with a bound $[\text{Fe}_2\text{S}_2]^{2+}$ cluster that plays a key role in iron sensing and regulation of iron homeostasis. In this work, we report on *in vitro* UV-visible CD studies of cluster transfer between homodimeric monothiol Grxs and members of the ubiquitous A-type class of Fe-S cluster carrier proteins (*Nif*IscA and SufA). The results reveal rapid, unidirectional, intact and quantitative cluster transfer from the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound forms of *A. thaliana* GrxS14, *S. cerevisiae* Grx3, and *A. vinelandii* Grx-nif homodimers to *A. vinelandii* *Nif*IscA and from *A. thaliana* GrxS14 to *A. thaliana* SufA1. Coupled with *in vivo* evidence for interaction between monothiol Grxs and A-type Fe-S cluster carrier proteins, the results indicate that these two classes of proteins work together in cellular Fe-S cluster trafficking. However, cluster transfer is reversed in the presence of Fra2, since the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound heterodimeric Grx3/Fra2 complex can be formed by intact $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from *Nif*IscA. The significance of these results for Fe-S cluster biogenesis or repair and the cellular regulation of the Fe-S cluster status are discussed.

Introduction

Monothiol Grxs with active-site cysteine-glycine-phenylalanine-serine (CGFS) amino acid sequences, are widely distributed in both prokaryotes and eukaryotes and constitute class II of the six classes of Grxs.^{1,2} In general, this class can further be divided into two subclasses: those with a single Grx domain (e.g. *Saccharomyces cerevisiae* (Sc) Grx5, *Homo sapiens* (Hs) Grx5, *Escherichia coli* (Ec) Grx4, *Azotobacter vinelandii* (Av) Grx5 and Grx-nif, and *Arabidopsis thaliana* (At) GrxS14 and GrxS15) and the multi-domain proteins comprising an N-terminal thioredoxin-like domain as well as one (Sc Grx3 and Grx4), two (Hs Grx3) or three (At GrxS17) Grx domains. All bacterial monothiol CGFS Grxs are of the single Grx domain type, while in eukaryotes, both the single Grx domain and the multi-domain Grxs

are present.^{1,3} Although monothiol CGFS Grxs are ubiquitous proteins, their precise role(s) remain to be elucidated. Traditionally, Grxs function in the reduction of protein disulfides or glutathionylated proteins.^{1,4} However, monothiol CGFS Grxs generally exhibit low levels of disulfide reductase activity and the available *in vivo* data suggest roles in Fe-S cluster biogenesis and/or Fe homeostasis.⁵⁻⁷

The initial evidence for a role for monothiol CGFS Grxs in Fe-S cluster biogenesis came from yeast (*Saccharomyces cerevisiae*) gene knockout studies. Deletion of the *grx5* gene which encodes for the mitochondrial monothiol CGFS Grx5, resulted in increased sensitivity to oxidative stress as a result of iron accumulation in the cell and deficient cluster assembly in at least two Fe-S cluster-containing proteins (aconitase and succinate dehydrogenase) leading to impaired respiratory growth.^{8,9} Moreover, radiolabelled ⁵⁵Fe immunoprecipitation studies of *grx5* knockout mutants revealed that Grx5 facilitates the transfer of Fe-S clusters preassembled on the Isu1 U-type Fe-S cluster scaffold protein to acceptor proteins.¹⁰ Other monothiol CGFS Grxs from prokaryotic or eukaryotic sources, when targeted to the yeast mitochondria, were able to rescue the defects of *grx5* mutants in *S. cerevisiae*, suggesting that this function is conserved in this class of proteins throughout evolution.^{11,12} Additional support for a role in Fe-S cluster biogenesis comes from the observation of specific interactions between yeast Grx5 with the Isa1, a mitochondrial A-type Fe-S cluster assembly protein, via yeast two-hybrid experiments.³ Interaction between Grx5 and both Isa1 and Isa2 was also recently demonstrated *in vivo* in *Schizosaccharomyces pombe* through bimolecular fluorescence complementation studies.¹³ Deleting the *grx5* gene in *S. pombe* resulted in a mutant that showed low activity for mitochondrial and cytoplasmic Fe-S cluster enzymes, and notably both Isa1 and Isa2 were able to complement the phenotype of the Δ *grx5* mutant. The function of the interaction between monothiol CGFS Grxs and A-type Fe-S cluster assembly proteins is explored in this work.

In addition to *in vivo* evidence for a role in Fe-S cluster biogenesis, a wide variety of recombinant monothiol CGFS Grxs have recently been shown to contain bound $[\text{Fe}_2\text{S}_2]^{2+}$ clusters as purified and the apo forms readily incorporate labile $[\text{Fe}_2\text{S}_2]^{2+}$ clusters during cysteine desulfurase-mediated cluster assembly under anaerobic conditions in the presence of glutathione (GSH).^{12,14,15} Analytical, spectroscopic, crystallographic and mutagenesis results have revealed that the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster is ligated by the active site cysteines of two Grx monomers and two GSH molecules.^{12,16} Moreover, ⁵⁵Fe radiolabeled immunoprecipitation studies have provided *in vivo* evidence for the presence of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters that are coordinated by the cysteines of GSH and the Grx CGFS motif in *Sc* Grx3 and *Sc* Grx4, two cytoplasmic yeast monothiol Grxs.¹⁷

The ability of $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-loaded Grxs to effect maturation of physiologically relevant apo ferredoxins (Fdx) via intact rapid cluster transfer,^{12,18} suggests a potential roles as scaffold proteins for the assembly and delivery of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters or as cluster carriers for the delivery of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters assembled on primary scaffold proteins. More recently, a cluster carrier or storage role for monothiol Grxs has been convincingly demonstrated by *in vitro* studies using proteins from *A. vinelandii* which demonstrated rapid, unidirectional and quantitative $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from the primary scaffold protein IscU to the general purpose monothiol CGFS Grx5 only in the presence of the dedicated HscA/HscB co-chaperone system and MgATP.¹⁸ Additionally, $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-loaded Av Grx5 was shown to be competent for maturation of apo Av Isc Fdx via intact cluster transfer at a much faster rate than $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-loaded IscU in the presence of HscA/HscB co-chaperone system and MgATP.¹⁸

In addition to a role in Fe-S cluster biogenesis, multi-domain monothiol CGFS Grxs have been shown to participate in regulation of Fe homeostasis in eukaryotes. The two yeast

cytosolic monothiol CGFS Grxs, *Sc* Grx3 and *Sc* Grx4, are involved in the regulation of a wide range of iron-responsive genes in yeast, termed the iron regulon, via an iron-dependent interaction with the iron-responsive transcriptional activator of ferrous transport, Aft.^{19–22} The Aft1 and Aft2 paralogs are located in the cytosol under iron-replete conditions and move to the nucleus under iron-depleted conditions, where they activate the expression of the Fe-responsive genes.^{23–25} The transcriptional activity of Aft is regulated by mitochondrial Fe–S cluster biosynthesis *via* a signaling pathway not only involving the cytosolic monothiol Grxs but also the aminopeptidase P-like protein Fra1 (Fe-repressor of activation-1) and Fra2 (Fe-repressor of activation-2), a member of the ubiquitous Bola family of proteins.^{19–22} Moreover, recent *in vitro* studies indicate that the Fe sensing mechanism involves a novel $[\text{Fe}_2\text{S}_2]^{2+}$ cluster at the subunit interface of a Grx/Fra2 heterodimer, that is coordinated by the active sites cysteines of GSH and the monothiol CGFS Grx and the conserved histidine of Fra2.^{7,15,26} The Fe and/or Fe–S cluster sensing role of the *S. cerevisiae* cytosolic monothiol Grxs, appears to be conserved in humans,²⁷ and other fungi that utilize Fe-responsive transcription factors, even though their iron-responsive transcription factors are structurally unrelated to the *S. cerevisiae* Aft proteins.^{7,28,29} Furthermore, *in silico* genomic analysis has shown that genes encoding monothiol CGFS Grxs and Bola-type proteins are frequently found in adjacent positions in many prokaryotic organisms.^{2,30} This suggests that the monothiol Grx-Bola interaction might constitute a general requirement for Fe and/or Fe–S cluster sensing in organisms in which the two genes are present, and is supported by the recent characterization of a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound *Ec* Grx4-Bola complex.³¹

In this work, we present additional *in vitro* evidence in support of a role for monothiol CGFS Grxs in Fe–S cluster biogenesis by demonstrating facile $[\text{Fe}_2\text{S}_2]^{2+}$ cluster exchange between monothiol Grxs and the ubiquitous A-type Fe–S cluster assembly proteins (termed IscA, NifIscA, and SufA in bacteria) which have been proposed to play an important role in both Fe and Fe–S cluster trafficking.^{32–35} $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound forms of homodimeric monothiol Grxs are shown to be efficient and rapid cluster donors for A-type proteins. Coupled with *in vivo* evidence for monothiol CGFS Grx/A-type protein interaction,^{3,13} these results indicate that monothiol Grxs and A-type Fe–S assembly proteins work together in cellular Fe–S cluster trafficking and repair. Interestingly the direction of cluster transfer was found to be reversed with heterodimeric yeast Grx3/Fra2 complex. *In vitro* experiments show that the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound form of the heterodimeric Grx3/Fra2 complex, can be formed by intact $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from an A-type protein, in addition to displacement or one Grx3 monomer by Fra2, as previously demonstrated.²⁶ This result raises the possibility that A-type proteins are involved with the mechanism of Fe and Fe–S cluster sensing in some organisms.

Experimental section

Materials

Materials used in this work were of reagent grade and were purchased from Fischer Scientific, Sigma-Aldrich Chemical Co, Invitrogen, or VWR International, unless stated otherwise.

Protein expression and purification

The *A. vinelandii* NifIscA gene, encoding the NifIscA protein was amplified by PCR method and inserted into the expression plasmid PT₇₋₇ as previously described.³⁶ The resulting plasmid, pDB570 was transformed into the *E. coli* host BL21(DE3) and induced for high level expression of *A. vinelandii* NifIscA and protein purification was carried out according to the published procedure.³⁶

The *A. thaliana* SufA1 (At1g10500) coding sequence was amplified by PCR from rosette cDNAs using AtSufA1for (5'CCCCCATGGCTGTTTCGATCCGCTTCGGTT3') and AtSufA1rev (5'CCCCGGATCCTCACATCTCGGCAGCAA3') primers and cloned into the pET3d vector. The amplified sequence encodes a protein, devoid of the first 53 amino acids, corresponding to the putative plastidial targeting sequence. Owing to the use of the NcoI restriction site, a codon for an alanine has been added in the primer to keep the sequence in frame. The N-terminal protein sequence of the recombinant proteins starts thus with MAVRSASV. The recombinant plasmid, containing *At* SufA1 was transformed into the *E. coli* host BL21(DE3) and induced for high level expression of *At* SufA1 according to the following procedure. Cells were grown in LB media containing 100 mg/mL ampicillin until OD600 reached 0.6 – 0.8 and expression was induced by addition of IPTG to a final concentration of 0.8 mM. The cells were grown for an additional 4 hours before harvesting by centrifugation and stored at –80 °C. The overexpressed protein was purified under both aerobic and anaerobic conditions. During the aerobic purification, cells (~15 g) were resuspended in 100 mM Tris-HCl, pH 7.8 (buffer A) with addition of 10 µg/mL phenylmethylsulphonyl fluoride (PMSF), 15 µg/ml DNase, and 5 ng/ml RNase. The cells were then disrupted by sonication on ice followed by centrifugation at 17,000 rpm for 1 hour at 4 °C. Soluble proteins were then precipitated with 40% of ammonium sulfate saturation and centrifuged as above. The resulting pellet was resuspended in buffer A and loaded onto a 25-mL Q-Sepharose column, equilibrated with buffer A. Elution of *At* SufA1 was achieved with a 0–100% NaCl gradient using 100 mM Tris-HCl with 1 M NaCl at pH 7.8. Fractions containing *At* SufA1 protein were collected and concentrated down to 3 mL using YM 10 Amicon ultrafiltration and loaded onto a Superdex-75 column, equilibrated with 100 mM Tris-HCl, 150 mM NaCl, pH 7.8. Anaerobic purification was carried out in a glove box under Ar (< 2 ppm O₂). Except for the Superdex-75 column, the same procedure described above for aerobic purification was used. UV-visible spectra indicated that the product was apo *At* SufA1 irrespective of aerobic or anaerobic purification. In order to reduce disulfides or polysulfides, aerobically purified apo *At* SufA1 and Av^{Nif}IscA were treated with 40mM tris(2-carboxyethyl)phosphine (TCEP) in the glove box under Ar atmosphere. Excess TCEP was removed using buffer A (10 mL × 4 times) in an YM10 Amicon ultrafiltration device prior to use for cluster reconstitution or cluster transfer studies.

Anaerobic purification of the reddish-brown cell-free extract containing *At* GrxS14 was carried out under Ar in a Vacuum Atmospheres glove box at O₂ levels < 2 ppm as previously described.¹² For protein expression and purification of *S. cerevisiae* Grx3 and Fra2, BL21(DE3) *E. coli* cells were transformed with pET21a-Grx3 and pET21a-Fra2 respectively and the resulting proteins produced in this strain were purified as previously described.¹⁵ Co-expression of Grx3 with Fra2 by transforming pET21a-Grx3 and pRSFDuet-1-Fra2 into the *E. coli* strain BL21(DE3) resulted in purification of the Fra2-Grx3 heterodimer complex. The reddish-brown cell-free extract containing Fra2-Grx3 was purified aerobically as previously described.¹⁵ For *A. vinelandii* Grx-nif, His-tagged Grx-nif was overexpressed in *E. coli* strain BL21(DE3) according to published procedure.¹² The reddish-brown cells were harvested by centrifugation at 5000 g for 15 min at 4 °C and stored at –80 °C until further use. Anaerobic purification of Grx-nif was carried out under Ar in a Vacuum Atmosphere glove box at O₂ levels < 2 ppm. 8 g of reddish-brown cells were thawed and resuspended in 50 mL of buffer A (50 mM Tris-HCl, pH 7.8, containing 1 mM GSH). 10 µg/mL PMSF, 15 µg/mL DNase (Roche) and 5 ng/mL RNase (Roche) were added to the mixture. The cells were lysed by sonication, and cell debris was removed by centrifugation at 39700 g for 1 hr at 4 °C. The reddish-brown cell-free extract containing Grx-nif was subjected to 40% ammonium sulfate cut followed by centrifugation. The resulting reddish-brown pellet was resolubilized in binding buffer (50 mM Tris-HCl, pH 7.8, containing 1 mM GSH, 0.5 M NaCl, 20 mM imidazole, and 10% glycerol) and loaded onto a 3×5 mL His-Trap HP column (GE Healthcare) previously equilibrated with binding buffer.

The column was washed with 10 column volumes of binding buffer before the protein of interest was eluted with a 20–500 mM imidazole gradient. The purest fractions, as judged by SDS-PAGE analysis, were eluted with 300 mM imidazole and were pooled together and concentrated by ultrafiltration using a YM-10 membrane.

Preparation of apo Grx-nif and Fra2-Grx3 heterodimer

Apo *A. vinelandii* Grx-nif was prepared by incubating the as-purified cluster-bound Grx-nif anaerobically with 50-fold excess EDTA and 20-fold excess potassium ferricyanide for 60 min on ice. The protein was then buffer exchanged into buffer A using a two sequential 5 mL desalting column (GE Healthcare) and the resulting apo-protein was concentrated via Amicon ultrafiltration using a YM-10 membrane. Apo Fra2-Grx3 heterodimer was prepared as previously described.¹⁵

Fe–S cluster reconstitution on Av NifIscA, At SufA1, At GrxS14, Sc Grx3, and Av Grx-nif

[Fe₂S₂]²⁺ cluster-bound homodimeric *A. vinelandii* NifIscA was prepared under anaerobic conditions starting with TCEP-pretreated apo protein via NifS-mediated cluster assembly in the presence of ferrous ammonium sulfate and L-cysteine and was purified as previously described.³⁶ A similar procedure was used for preparing the [Fe₂S₂]²⁺ cluster-bound homodimeric *At* SufA1. Reconstitution involved incubating apo *At* SufA1 (0.5 mM) with 8 mM L-cysteine and ferrous ammonium sulfate in the presence of catalytic Av NifS (10 μM) at room temperature in buffer A for 60 min and repurifying using a Q-Sepharose column. The resulting samples of Av NifIscA and *At* SufA1 contained 0.80 ± 0.10 and 0.62 ± 0.05 [Fe₂S₂] clusters per homodimer, respectively, based on protein and Fe determinations. Homogeneous samples of *Sc* Grx3 and *At* GrxS14 containing one [Fe₂S₂]²⁺ per homodimer were prepared as previously described.^{12,15} For Av Grx-nif, NifS-mediated reconstitution was carried out by incubating 1 mM apo Grx with 3 mM GSH, catalytic amounts of Av NifS (10 μM), 6-fold excess of both Fe(II) (ferrous ammonium sulfate) and L-cysteine. The reconstitution mixture was incubated under strictly anaerobic conditions for 2 hr on ice with occasional stirring, before being loaded on two sequential 5 mL Hi-Trap Q-Sepharose columns (GE Healthcare) previously equilibrated with buffer A and eluted with a 0.0–1.0 M NaCl gradient. The reddish-brown fractions were pooled and concentrated *via* Amicon ultrafiltration using a YM-10 membrane. The resulting samples contained 1.0 ± 0.1 [Fe₂S₂] cluster per homodimer based on protein and Fe determinations.

Analytical and spectroscopic methods

Protein concentrations were determined using bovine serum albumin as a standard (Roche) with BioRad Dc protein assay in conjunction with the microscale modified procedure of Brown et al.³⁷ Iron concentrations were determined after KMnO₄/HCl protein digestion as described by Fish,³⁸ using a 1000 ppm atomic absorption iron standard to prepare standard solutions of known Fe concentration (Fluka). Samples for all spectroscopic investigations were prepared under an argon atmosphere in a glove box (Vacuum Atmosphere, Hawthorne, CA) at O₂ levels < 2 ppm. UV–visible CD spectra were recorded under anaerobic conditions in septum-sealed 1 mm quartz cuvettes or 1 cm semi-micro cuvettes at room temperature using a JASCO J-715 spectropolarimeter (Jasco, Easton, MD). Kinetic data for cluster transfer experiments were analyzed using the Chemical Kinetics Simulator software package (IBM).

Fe–S cluster transfer experiments from monothiol CGFS Grxs to apo Av NifIscA or At SufA1

The time course of cluster transfer from [Fe₂S₂]²⁺ cluster-loaded forms of *At* GrxS14, *Sc* Grx3, and Av Grx-nif to either apo Av NifIscA or *At* SufA1 were monitored under anaerobic

conditions in 1 cm semi-micro cuvettes at room temperature using UV–visible CD spectroscopy. $\Delta\epsilon$ values are based on $[\text{Fe}_2\text{S}_2]^{2+}$ cluster concentrations of the reconstituted and repurified Grx samples used in this work, as determined by protein and Fe determinations. Mössbauer studies of $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-loaded *At* GrxS14 and *Sc* Grx3 have previously demonstrated that all the Fe in these samples is in the form of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters.^{12,15} The cluster transfer percentage in the kinetic plots were assessed based on the difference in the initial and resultant $[\text{Fe}_2\text{S}_2]$ cluster $\Delta\epsilon$ values at a fixed wavelength, with 100% corresponding to difference in the $\Delta\epsilon$ values for the $[\text{Fe}_2\text{S}_2]$ clusters exclusively on the donor and acceptor species. Reactions were carried out in 100 mM Tris-HCl buffer at pH 7.8 (2 mM DTT was also present in the *At* GrxS14-to-*At* SufA1 cluster transfer reaction mixture) and the final reaction mixture was 50 μM in apo *Av* ^{Nif}IscA or apo *At* SufA1 dimer and 50 μM in $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on homodimeric *At* GrxS14, *Sc* Grx3, or *Av* Grx-nif, unless otherwise indicated.

Fe–S cluster transfer experiments from *Av* ^{Nif}IscA to apo *Sc* Fra2-Grx3 heterodimer

The time course of cluster transfer from *Av* ^{Nif}IscA to apo *Sc* Fra2-Grx3 heterodimer was monitored under anaerobic conditions in 1 cm semi-micro cuvettes at room temperature using UV–visible CD spectroscopy. $\Delta\epsilon$ values are based on $[\text{Fe}_2\text{S}_2]^{2+}$ cluster concentrations on reconstituted and repurified *Av* ^{Nif}IscA, as determined by protein and Fe determinations.³⁵ Mössbauer studies of $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-loaded *Av* ^{Nif}IscA and *Sc* Fra2-Grx3 have previously demonstrated that all the Fe is in the form of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters.^{15,35} Reactions were carried out in 100 mM Tris-HCl buffer at pH 7.8 containing 2 mM GSH and the final reaction mixture was 50 μM in apo Fra2-Grx3 heterodimer and 50 μM in $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on homodimeric ^{Nif}IscA.

Results

In vitro cluster transfer from $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound monothiol CGFS Grxs to apo ^{Nif}IscA

The intensity and exquisite sensitivity of the UV-visible CD spectra of biological $[\text{Fe}_2\text{S}_2]^{2+}$ clusters to the asymmetry of the cluster environment, has made CD spectroscopy the method of choice for monitoring the kinetics of interprotein $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer.^{12,18,39} The $[\text{Fe}_2\text{S}_2]^{2+}$ centers on all three monothiol CGFS Grxs investigated in this work, *At* GrxS14, *Sc* Grx3, and *Av* Grx-nif, have distinct and intense UV-visible CD spectra compared to the $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on *At* SufA1 and *Av* ^{Nif}IscA, see Figure 1. UV-visible CD spectra with $\Delta\epsilon$ values quantified based on the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster concentrations, therefore provides a convenient method for quantitatively monitoring cluster transfer reactions involving these proteins based on changes in the $\Delta\epsilon$ values at discrete wavelengths.

UV-visible CD spectroscopy was used to investigate the potential role of monothiol Grxs in Fe–S cluster trafficking by monitoring cluster transfer experiments from three recombinant monothiol Grxs, plant chloroplast *At* GrxS14, yeast cytosolic *Sc* Grx3 and bacterial *Av* Grx-nif, to bacterial *Av* ^{Nif}IscA and from plant chloroplast *At* GrxS14 to *At* SufA1. The time course of CD-monitored, room-temperature cluster transfer reactions from the subunit-bridging $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound forms of the dimeric monothiol Grxs to the apo form of dimeric A-type proteins using a 1:1 donor:acceptor $[\text{Fe}_2\text{S}_2]^{2+}$ cluster stoichiometry, except for the *Av* Grx-nif to *Av* ^{Nif}IscA cluster transfer reaction which has a 1:1.67 donor:acceptor stoichiometry, are shown in Figure 2. In each case, rapid and quantitative cluster transfer occurs as judged by the CD $\Delta\epsilon$ values for the $[\text{Fe}_2\text{S}_2]^{2+}$ centers assembled on the A-type acceptor proteins, see Figure 1. The stoichiometric cluster transfers from $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound *At* GrxS14 and *Sc* Grx3 to apo *Av* ^{Nif}IscA and from *At* GrxS14 to *At* SufA1 are more than 95% complete before the first CD spectrum is recorded (after 3 min), suggesting a second order rate constant for cluster transfer of $50,000 \text{ M}^{-1}\text{min}^{-1}$. This lower limit

estimate of the rate constant is based on a more detailed kinetic analysis for the *At* GrxS14 to *At* SufA1 cluster transfer in which the time course was monitored continuously at 348 nm (see inset in Figure 2D). Based on the initial concentration of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on *At* GrxS14 (50 μM) and the concentration of the dimeric *At* SufA1 acceptor protein (50 μM), the data was well fit by simulated second order kinetics with a rate constant of 50,000 $\text{M}^{-1}\text{min}^{-1}$. The rate constant for the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from *Av* Grx-nif to apo *Av* NifIscA was assessed by continuously monitoring the time course at 343 nm, see inset in Figure 2C. Based on the initial concentration of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on *At* GrxS14 (42 μM) and the concentration of the dimeric *At* SufA1 acceptor protein (70 μM), the data was well fit by simulated second order kinetics with a rate constant of 22,000 $\text{M}^{-1}\text{min}^{-1}$. These rates of cluster transfer are clearly in the physiologically relevant range and are similar or significantly faster than those previously reported for $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound GrxS14 to apo plant-type ferredoxin (20,000 $\text{M}^{-1}\text{min}^{-1}$)¹² or HscA/HscB/ATP-mediated $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound IscU to apo Isc Fdx (800 $\text{M}^{-1}\text{min}^{-1}$).³⁹

The cluster transfer is unidirectional, as the reverse reaction involving reaction mixtures containing the same concentrations of $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound NifIscA and apo monomeric monothiol Grxs, in the presence of 3 mM GSH, showed no change in the CD spectrum after 60 min of reaction (data not shown). Control experiments monitored by UV-visible CD spectroscopy also revealed no change in the CD spectra of the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound GrxS14, Grx3 and Grx-nif in the absence of apo A-type proteins on incubating at room temperature for 30 min under anaerobic conditions respectively (data not shown). Additional CD evidence for intact cluster transfer was provided by the observation that the addition of 1 mM EDTA did not significantly affect the rate or extent of these cluster transfer reactions and that < 10% $[\text{Fe}_2\text{S}_2]$ cluster assembly on *Av* NifIscA or *At* SufA1 occurred after 30 min when cluster-loaded Grxs were replaced in the cluster transfer reaction mixture with equivalent amounts of S^{2-} and Fe^{2+} (data not shown). These control experiments demonstrate that the $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on the A-type proteins investigated in this study are derived from the $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on the Grxs via intact cluster transfer rather than via cluster degradation and reassembly on apo A-type proteins.

In vitro cluster transfer from $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound *A. vinelandii* NifIscA to apo *S. cerevisiae* Fra2-Grx3 heterodimer

Previous studies have shown that coexpression of recombinant *Sc* Fra2 with *Sc* Grx3 in *E. coli* results in purification of a stable $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-containing Fra2-Grx3 heterodimeric complex.¹⁵ The cluster can be removed in the presence of large excesses of metal chelators and ferricyanide to yield a stable apo form of the Fra2-Grx3 heterodimer.¹⁵ Moreover, recent *in vivo* mutagenesis studies of the cluster-ligating His and Cys residues on Fra2 and Grx3, respectively, indicate that $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound form of the Fra2-Grx3 heterodimeric complex plays a crucial role in controlling the Fe regulon in yeast.^{15,26} In an attempt to establish if the cluster-bound form of Fra2-Grx3 heterodimer can be generated by intact cluster transfer from a hitherto unidentified donor protein as well as via Fra2 displacement of one Grx3 monomer in the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound Grx3 homodimer, as previously demonstrated,²⁶ we have investigated the ability of $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound NifIscA to transfer a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster to the apo Fra2-Grx3 heterodimer.

The $[\text{Fe}_2\text{S}_2]^{2+}$ center on NifIscA has a distinct UV-visible CD spectrum compared to that of the $[\text{Fe}_2\text{S}_2]^{2+}$ center on Fra2-Grx3, making CD a very effective method for monitoring cluster transfer, see Figure 3. The time course of CD-monitored room-temperature cluster transfer from $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound NifIscA to apo Fra2-Grx3 heterodimer using a 1:1 donor:acceptor cluster stoichiometry in a buffer containing 2 mM GSH under anaerobic conditions is shown in Figure 4. These results indicate quantitative cluster transfer that is

complete after approximately 120 min as evidenced by 100% conversion of the donor CD spectrum to that of the acceptor CD spectrum on the basis of the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster $\Delta\epsilon$ values. More detailed kinetic analysis was carried out by monitoring the change in CD intensity as a function of time at 320 nm, see inset in Figure 4. Kinetic analysis based on initial $[\text{Fe}_2\text{S}_2]$ cluster concentration on the Nif^IscA donor (50 μM) and initial apo Fra2-Grx complex concentration (50 μM) indicates second order kinetics with a rate constant of 15,000 $\text{M}^{-1}\text{min}^{-1}$. The reverse reaction did not occur to an appreciable extent after 120 min and control experiments showed that the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster on Nif^IscA is stable over the time period of the reaction in the absence of the Fra2-Grx3 heterodimer and that equivalent amounts of Fe^{2+} and S^{2-} in place of cluster-loaded Nif^IscA result in < 5% $[\text{Fe}_2\text{S}_2]^{2+}$ cluster formation on the Fra2-Grx3 heterodimer dimer after 120 min. Hence, although not as fast as $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from monothiol CGFS Grxs to Nif^IscA , the results indicate that the Fra2-Grx3 heterodimer can receive clusters via intact cluster transfer from an A-type cluster donor and that Fra2 binding in place of one of the Grx3 monomers reverses the direction of cluster transfer with respect to Grx3/ Nif^IscA $[\text{Fe}_2\text{S}_2]^{2+}$ cluster exchange. We conclude that Fra2 or BolA-type protein binding converts monothiol Grxs from cluster donors to net cluster acceptors with respect to A-type proteins.

Discussion

Monothiol Grxs as cluster donors to A-type proteins in cellular Fe-S cluster trafficking

Recent *in vitro* and *in vivo* studies of the ubiquitous A-type Fe-S cluster biogenesis proteins indicate a role as cluster carriers for the delivery of clusters assembled on primary scaffold proteins (NifU in the NIF system; IscU in the iron sulfur cluster (ISC) system; SufB in the sulfur utilization factor (SUF) system) to acceptor proteins.^{33,35,40,41} Moreover, the ability of A-type proteins to bind mononuclear Fe^{2+} ,^{3,32,34} accept $[\text{Fe}_2\text{S}_2]^{2+}$ clusters formed on primary scaffold proteins,^{35,40–42} and reversibly convert between $[\text{Fe}_2\text{S}_2]^{2+}$ and $[\text{Fe}_4\text{S}_4]^{2+}$ cluster-bound forms in response to cellular redox status and/or oxygen levels,³⁵ are likely to be important for their key role in the maturation or repair of $[\text{Fe}_4\text{S}_4]$ clusters in mitochondrial proteins^{43–45} and in bacterial proteins under aerobic growth or oxidative stress conditions.^{46,47}

The work reported herein provides additional support for the proposal that A-type proteins function as Fe-S cluster carrier proteins by demonstrating that $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound monothiol CGFS Grxs are very efficient $[\text{Fe}_2\text{S}_2]^{2+}$ cluster donors for A-type proteins. Moreover, the recent demonstration of rapid, ATP-dependent cluster transfer from Av $[\text{Fe}_2\text{S}_2]^{2+}$ -IscU to Av Grx5 only in the presence of the dedicated HscA/HscB molecular co-chaperone system (Ssq1/Jac1 and HSPA9/HSC20 in yeast and human mitochondria, respectively),^{6,48} suggests that monothiol Grxs are likely to play a key role in storing and transporting $[\text{Fe}_2\text{S}_2]^{2+}$ clusters assembled on IscU primary scaffold proteins in the bacterial ISC system and in eukaryotic mitochondria.¹⁸ In contrast, we have failed to demonstrate ATP-dependent cluster transfer from Av $[\text{Fe}_2\text{S}_2]^{2+}$ -IscU to apo Av IscA in the presence of the dedicated HscA/HscB co-chaperone system (S. Randeniya, P. Shakamuri, and M. K. Johnson, unpublished work). Consequently it seems likely that monothiol Grxs mediate $[\text{Fe}_2\text{S}_2]^{2+}$ clusters transfer from IscU to IscA proteins in the bacterial ISC system and eukaryotic mitochondria. The means by which monothiol Grxs associated with the NIF and SUF systems obtain their $[\text{Fe}_2\text{S}_2]^{2+}$ clusters has yet to be determined, but is under active investigation in our laboratories.

The only partner proteins of potential physiologically relevance that have been shown to exhibit rapid $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer in this work are Av Grx-nif to Nif^IscA and chloroplast At GrxS14 to SufA1. However, A-type proteins are highly conserved in the bacterial NIF, ISC, and SUF Fe-S cluster assembly systems as well as in mitochondria and

chloroplasts,^{45,49–51} and monothiol Grxs are present in most bacteria and as well as the mitochondria, chloroplasts and cytosol of eukaryotes.⁵ Coupled with the evidence that monothiol Grxs exist in cluster-bound forms *in vivo* in the cytosol of yeast,¹⁷ and that monothiol Grxs and A-type proteins interact *in vivo* in yeast mitochondria,^{3,13} it seems likely that rapid $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from monothiol Grxs to A-type proteins is a physiologically relevant cluster transfer reaction. Moreover, the rapid $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer observed for the non-physiological chloroplast *At* GrxS14-to-*Av*^{Nif}IscA and *Sc* Grx3-to-*Av*^{Nif}IscA cluster transfer reactions indicates that this process is not specific to physiologically relevant cluster transfer partners. Indeed $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from cytosolic *Sc* Grx3 to an A-type protein is very unlikely to be physiologically relevant, as there is no evidence for an A-type Fe-S cluster protein in the yeast cytosol.^{50,52} Nevertheless, the observation of fast, efficient and quantitative cluster transfer from monothiol Grxs to A-type FeS cluster assembly proteins clearly support the proposal that monothiol Grxs and A-type proteins are partners in cellular Fe-S cluster trafficking.

A schematic proposal for the roles of monothiol Grxs and A-type proteins in cluster trafficking to facilitate the maturation or repair of $[\text{Fe}_4\text{S}_4]$ clusters via the ISC system for Fe-S cluster biogenesis is presented in Figure 5. The initial step involves ATP-dependent cluster transfer from $[\text{Fe}_2\text{S}_2]$ -IscU to apo-Grx in the presence of the dedicated HscA/HscB co-chaperone system.¹⁸ This is followed by cluster transfer from $[\text{Fe}_2\text{S}_2]$ -Grx to apo-IscA to yield $[\text{Fe}_2\text{S}_2]$ -IscA. Based on the available crystallographic data,⁵³ the $[\text{Fe}_2\text{S}_2]$ cluster in the IscA dimer is asymmetrically ligated by three cysteines from one monomer and one cysteine from the other monomer. This asymmetric ligation is likely to be crucial for the ability to reversibly convert between forms containing one $[\text{Fe}_2\text{S}_2]$ or one $[\text{Fe}_4\text{S}_4]$ per homodimer in response to cellular conditions as recently demonstrated with *Nif*IscA.³⁵ Anaerobic conditions, coupled with the presence a two-electron donor such as DTT, result in the conversion from $[\text{Fe}_2\text{S}_2]$ -*Nif*IscA to $[\text{Fe}_4\text{S}_4]$ -*Nif*IscA, which is reversed on exposure to air in the presence of apo-IscA.³⁵ Hence $[\text{Fe}_4\text{S}_4]$ -IscA is likely to be responsible for the maturation or repair of apo $[\text{Fe}_4\text{S}_4]$ cluster-containing proteins only under anaerobic conditions.

An alternative mechanism for the assembly or repair of oxygen-damaged $[\text{Fe}_4\text{S}_4]$ clusters that uses $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound A-type proteins is also proposed in Figure 5. This proposal originates from the recent *in vivo* evidence that an Fe-bound form of the Isa1-Isa2 dimer, along with $[\text{Fe}_2\text{S}_2]$ clusters ultimately supplied by Isu1 or Isu2, are essential for the maturation of $[\text{Fe}_4\text{S}_4]$ cluster-containing mitochondrial proteins in *S. cerevisiae* under both aerobic and anaerobic conditions.⁴⁵ Taken together with recent studies of the *E. coli* fumarate nitrate reduction regulatory protein, which demonstrate $[\text{Fe}_4\text{S}_4]^{2+}$ formation by addition of Fe^{2+} to a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster ligated by two cysteines and two cysteine persulfides,⁵⁴ this provides the basis for a mechanism for the maturation or repair of O_2 -damaged $[\text{Fe}_4\text{S}_4]$ cluster-containing proteins using A-type proteins, that may be operative under aerobic or anaerobic conditions.^{34,35} The starting point would be an apo protein containing two cysteine persulfides, generated by a cysteine desulfurase or via O_2 -induced degradation of a protein-bound $[\text{Fe}_4\text{S}_4]$ cluster, as originally demonstrated in aconitase by Kennedy and Beinert.⁵⁵ Incorporation of a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster, assembled initially on IscU and transferred to the acceptor protein by IscA via a monothiol Grx, would result in a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster ligated by two cysteines and two cysteine persulfides. The final step would involve *in situ* $[\text{Fe}_4\text{S}_4]^{2+}$ cluster assembly, by addition of Fe^{2+} supplied by Fe-bound IscA. This proposal is presented in the spirit of a working hypothesis that provides a basis for future experiments.

Monothiol Grxs as sensors of cellular iron and/or Fe–S cluster status

The proposal that monothiol Grxs provide a dynamic capacity for storage and transfer of $[\text{Fe}_2\text{S}_2]^{2+}$ clusters assembled on primary scaffold proteins also provides a rationalization for the involvement of the yeast cytosolic monothiol Grxs, *Sc* Grx3 and Grx4, in sensing the cellular Fe and Fe–S cluster status. *In vivo* studies have demonstrated that the yeast iron regulon responds to mitochondrial Fe–S cluster biosynthesis rather than cytosolic Fe levels,⁵⁶ and is regulated by the formation of an iron-dependent complex in the cytosol involving Grx3, Grx4, Fra1 and the BolA homolog, Fra2, that interacts with cytosolic Aft in the Fe replete form in order to prevent Aft migration into the nucleus where it activates the Fe regulon.^{20,22} The combination of *in vitro* and *in vivo* studies of wild-type and mutated proteins indicate that the Fe sensing mechanism involves the formation of a stable histidyl- and cysteinyl-ligated $[\text{Fe}_2\text{S}_2]^{2+}$ cluster at the subunit interface of the Fra2-Grx3/4 heterodimer.^{7,15,26} Consequently the concentration of stored Fe–S clusters on Grx3 and Grx4 can be assessed without significantly perturbing the apo/holo Grx equilibrium by interaction with Fra2, provided that Fra2 can replace one of the Grx molecules in the homodimer with high binding affinity and the cellular concentration of Fra2 is much less than that of Grx3 or 4. Hence the observation of high affinity binding of Fra2 to holo-Grx3/4 to form the Fra2-Grx3/4 heterodimeric complex provides a plausible hypothesis for Fe or Fe–S cluster sensing mechanism that controls the yeast Fe regulon.^{7,26}

An alternative sensing mechanism is suggested by the observation that the apo Fra2-Grx3 heterodimer is able to accept a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster from an A-type Fe–S cluster assembly protein. This mechanism is not relevant to the regulation of the yeast Fe regulon, because of the lack of A-type proteins in the yeast cytosol.^{50,52} However, the presence of BolA-type proteins in bacteria and in eukaryotic mitochondria and chloroplasts,^{26,30} which contain single domain monothiol Grxs as well as A-type proteins leaves open the possibility that $[\text{Fe}_2\text{S}_2]^{2+}$ cluster transfer from A-type proteins to apo Grx-BolA complexes may function as part of hitherto uncharacterized Fe or Fe–S cluster sensing mechanisms that are operative in bacteria, mitochondria and chloroplasts. This tentative proposal is supported by the report of a $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound Grx4-BolA complex in *E. coli*,³¹ and recent characterization of a *S. cerevisiae* $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound heterodimer involving mitochondrial Grx5 and the putative mitochondrial BolA-like protein, Aim1 (B. Zhang, A. Dlouhy, C. E. Outten, and M. K. Johnson, unpublished results). *In vitro* studies involving bacterial and yeast mitochondria Grx-BolA complexes and their corresponding A-type proteins are planned to test this hypothesis.

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Fig. 1.

Upper panel Comparison of the UV-visible CD spectra of the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound forms of *A. vinelandii* Nif^{IscA} (black line) and *A. thaliana* SufA1 (purple line). *Lower panel*: Comparison of the UV-visible CD spectra of the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound forms of *A. thaliana* GrxS14 (green line), *A. vinelandii* Grx-nif (red line), and *S. cerevisiae* Grx3 (blue line). Spectra were recorded under anaerobic conditions in sealed 0.1-cm cuvettes in 100 mM Tris-HCl buffer with 250 mM NaCl at pH 7.8. $\Delta\epsilon$ values are based on the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster concentrations.

Fig. 2.

Time course of cluster transfer from $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound forms of *At* GrxS14 (A), *Sc* Grx3 (B), and *Av* Grx-nif (C) to apo *Av* Nif^{IscA} and of *At* GrxS14 to *At* SufA1 (D) monitored by UV-visible CD spectroscopy under anaerobic conditions in semi-micro 1-cm cuvettes at room temperature. UV-visible CD spectra were recorded prior to addition of apo *Av* Nif^{IscA} or apo *At* SufA1 (thick line) and 3, 8, 12, 15, and 20 min after adding the apo A-type protein to the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound Grx (thin black lines). The *At* GrxS14-to-*Av* Nif^{IscA} (A), *Sc* Grx3-to-*Av* Nif^{IscA} (B), and *At* GrxS14-to-*At* SufA1 (D) cluster transfer reactions were carried out with a 1:1 donor:acceptor $[\text{Fe}_2\text{S}_2]$ cluster stoichiometry (50 μM apo A-type protein dimer/50 μM $[\text{Fe}_2\text{S}_2]^{2+}$ clusters on Grx). The *Av* Grx-nif-to-cluster transfer reaction was carried with a 1:1.67 donor:acceptor cluster stoichiometry (70 μM apo

$Av^{Nif}IscA/42 \mu M [Fe_2S_2]^{2+}$ clusters on $Av Grx-nif$). $\Delta\epsilon$ values are based on the initial $[Fe_2S_2]^{2+}$ cluster concentration.

Fig. 3.

Comparison of the UV-visible CD spectra of $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound *A. vinelandii* *NifIscA* (blue line) and $[\text{Fe}_2\text{S}_2]^{2+}$ -cluster bound *S. cerevisiae* Grx3-Fra2 (red line). Spectra were recorded under anaerobic conditions in sealed 0.1-cm cuvettes for $[\text{Fe}_2\text{S}_2]^{2+}$ -*NifIscA* in 100 mM Tris-HCl buffer with 250 mM NaCl at pH 7.8 and for the $[\text{Fe}_2\text{S}_2]^{2+}$ -Grx3-Fra2 complex in 50 mM Tris-MES buffer at pH 8.0. $\Delta\epsilon$ values are based on the $[\text{Fe}_2\text{S}_2]^{2+}$ cluster concentrations.

Fig. 4.

Time course of cluster transfer from *A. vinelandii* *NifIscA* to apo *S. cerevisiae* Grx3-Fra2 complex monitored by UV-visible CD spectroscopy under anaerobic conditions in semi-micro 1-cm cuvettes at room temperature. UV-visible CD spectra were recorded prior to addition of the apo Fra2-Grx3 complex (black line) and 5, 7, 10, 14, 17, 20, 25, 40, 60, 90, 120 min (thin gray lines) after adding the apo Fra2-Grx3 complex to $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound *NifIscA* in 1:1 $[\text{Fe}_2\text{S}_2]$ cluster donor:acceptor stoichiometry (50 μM apo Fra2-Grx complex/50 μM $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound *NifIscA*). $\Delta\epsilon$ values are based on the initial $[\text{Fe}_2\text{S}_2]^{2+}$ cluster concentration. The arrows indicate the direction of intensity change with time at selected wavelengths. The inset shows the kinetics of cluster transfer at 350 nm and the solid line is the best fit for second kinetics with a rate constant of $15000 \text{ M}^{-1}\text{min}^{-1}$ based on 50 μM concentrations for cluster donor and acceptor.

Fig. 5. Schematic proposal for the role of monothiol Grxs and A-type proteins in cluster trafficking leading to the maturation or repair of [Fe₄S₄] cluster-containing proteins in the ISC Fe-S cluster biogenesis system. See text for details.