Monothiol Glutaredoxins Function in Storing and Transporting
[Fe₂S₂] Clusters Assembled on IscU Scaffold Proteins

Priyanka Shakamuri,† Bo Zhang,‡ and Michael K. Johnson*‡

Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, United States

Supporting Information

**ABSTRACT:** In the bacterial ISC system for iron–sulfur cluster assembly, IscU acts as a primary scaffold protein, and the molecular co-chaperones HscA and HscB specifically interact with IscU to facilitate ATP-driven cluster transfer. In this work, cluster transfer from *Azotobacter vinelandii* [Fe₂S₂]₂⁺ cluster-bound IscU to apo-Grx5, a general purpose monothiol glutaredoxin in *A. vinelandii*, was monitored by circular dichroism spectroscopy, in the absence and in the presence of HscA/HscB/Mg-ATP. The results indicate a 700-fold enhancement in the rate of [Fe₂S₂]₂⁺ cluster transfer in the presence of the co-chaperones and Mg-ATP, yielding a second-order rate constant of 20,000 M⁻¹ min⁻¹ at 23 °C. Thus, HscA and HscB are required for efficient ATP-dependent [Fe₂S₂]₂⁺ cluster transfer from IscU to Grx5. The results support a role for monothiol Grxs in storing and transporting [Fe₂S₂]₄⁺ clusters assembled on IscU and illustrate the limitations of interpreting *in vitro* cluster transfer studies involving [Fe₂S₂]-IscU in the absence of the dedicated HscA/HscB co-chaperone system.

The ubiquitous and essential IscU protein serves as the primary scaffold for cysteine desulfurase-mediated iron–sulfur cluster assembly in the ISC machinery for cluster biogenesis that is used by many bacteria and in eukaryotic mitochondria.¹⁻³ Under reconstitution conditions, the initial product is a stable form containing one [Fe₂S₂]₂⁺ cluster per homodimer, [Fe₂S₂]-IscU, which is slowly converted under aerobic and anaerobic conditions.⁶ Studies have shown that IscU-type proteins have critical roles in *Saccharomyces cerevisiae* have demonstrated an important role for Grx5 in Fe–S cluster biogenesis.¹³ Yeast Grx5 is a member of a ubiquitous and well-defined class of monothiol Grx5s with CGFS active sites that exhibit low glutathione-dependent thiol–disulfide oxidoreductase activity.¹⁴ Rather, ⁵²Fe-radiolabeled immunoprecipitation studies have indicated a role in facilitating transfer of Fe–S clusters assembled on Isu1, a yeast homologue of IscU.¹⁶ Moreover, spectroscopic and structural studies have shown that monothiol Grx5s can bind subunit-bridging [Fe₂S₂]₂⁺ clusters, ligated by the active-site cysteines of each monomer and two glutathiones, that can be rapidly transferred to physiologically relevant acceptor proteins.¹³,¹⁷,¹⁸ However, there is currently no direct evidence for [Fe₂S₂]₂⁺ cluster transfer from IscU to apo monothiol Grx5. In this work, we present direct spectroscopic evidence for rapid, ATP-driven, [Fe₂S₂]₂⁺ cluster transfer from [Fe₂S₂]-IscU to apo-Grx5 in the presence of HscA and HscB using recombinant proteins from *Azotobacter vinelandii*. The results demonstrate the critical role that HscA and HscB play in facilitating cluster transfer from [Fe₂S₂]-IscU to monothiol Grx5s and suggest an important role for monothiol Grxs in the trafficking of [Fe₂S₂]₂⁺ clusters assembled on IscU. The experimental methods for expressing, purifying, and assaying the proteins used in this work and the protocols used for cluster transfer reactions are described in the Supporting Information.

The marked differences in the CD spectra of the [Fe₂S₂]₂⁺ centers in *A. vinelandii* IscU and Grx5s red and blue spectra, respectively, in Figure 1, make this the method of choice for monitoring cluster transfer between these two proteins. No cluster transfer was observed from [Fe₂S₂]-Grxs to apo-IscU over a period of 3 h using a 2-fold excess of [Fe₂S₂]₄⁺ clusters per apo-IscU dimer, in the presence of physiologically relevant levels of glutathione (3 mM). However, the reverse reaction involving cluster transfer from [Fe₂S₂]-IscU to apo-Grx5 does occur, albeit very slowly, in the presence of 3 mM glutathione. This is shown in Figure 1, which indicates ∼30% cluster transfer over a period of 3 h, using IscU containing 0.9 [Fe₂S₂]₄⁺ cluster per homodimer and a 1.7-fold excess of dimeric Grxs per IscU [Fe₂S₂]₂⁺ cluster. In contrast, in the presence of HscA, HscB, Mg-ATP, and KCl (required for optimal ATPase activity of HscA), the CD spectrum of [Fe₂S₂]-IscU is perturbed by binding to HscA and HscB, as previously observed.

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observed,7 and the rate of cluster transfer from [Fe2S2]-IscU to apo-Grx5 is dramatically enhanced, going to completion within 6 min of initiating the reaction by the addition of Mg-ATP, see Figure 2.

Rate constants for [Fe2S2]-IscU to apo-Grx5 cluster transfer in the absence and in the presence of the co-chaperones, Mg-ATP and KCl, were quantitatively assessed by fitting CD intensities as a function of time to second-order kinetics based on the initial concentrations of donor and acceptor, see Figure 3. The rate constant increases ∼700-fold, from 30 to 20 000 M−1 min−1, on addition of the co-chaperones, Mg-ATP and KCl. This is much greater than the ∼20-fold increase (from 36 to 800 M−1 min−1) in the rate of cluster transfer from [Fe2S2]-IscU to apo-IscFdx that occurs on addition of the same co-chaperones.7 This suggests that [Fe2S2]-IscU is unlikely to be the immediate [Fe2S2] cluster donor for maturation of IscFdx, which functions as an essential electron donor for ISC-mediated cluster assembly in A. vinelandii.19 Rather, as discussed below, IscFdx may receive [Fe2S2] clusters directly from Grx5.

The ability of Grx5 to rapidly and quantitatively accept [Fe2S2]2+ clusters from [Fe2S2]-IscU in the presence of the co-chaperones, in an ATP-dependent reaction, supports a physiological role for monothiol Grx’s in the trafficking of [Fe2S2]2+ clusters that are assembled on IscU. Monothiol Grx’s therefore have the capacity to store and/or deliver [Fe2S2]2+ clusters assembled on U-type scaffold proteins. Interestingly, slow and reversible [Fe2S2] cluster exchange via direct protein interaction between human Isu and Grx2, in the absence of the human Fe−S cluster biogenesis co-chaperone system (HSPA9 and HSC20)20,21 and Mg-ATP, has recently been reported by monitoring loss or gain in disulfide oxidoreductase activity and isothermal titration calorimetry.22 Although human Grx2 (CSYC active site) is a dithiol Grx and has not been implicated in Fe−S cluster biogenesis, it has been shown to exist in a mononuclear apo form with high disulfide oxidoreductase activity.

Figure 1. Time course of cluster transfer from A. vinelandii [Fe2S2]-IscU (45 μM in [Fe2S2]2+ clusters) to apo-Grx5 (150 μM in monomer) monitored by UV−visible CD spectroscopy at 23 °C. (A) CD spectra recorded at 0, 7, 20, 40, 60, 80, 120, and 180 min after adding [Fe2S2]-IscU to apo-Grx5 in 100 mM Tris-HCl buffer, pH 7.8, with 3 mM glutathione. (B) Simulated CD spectra corresponding to quantitative [Fe2S2]2+ cluster transfer from [Fe2S2]-IscU to apo-Grx5 in 10% increments. Δε values are based on the [Fe2S2]2+ cluster concentration, and the path length was 1 cm.

Figure 2. Time course of cluster transfer from A. vinelandii [Fe2S2]-IscU to apo-Grx in the presence of 0.10 mM A. vinelandii HscA and HscB, 40 mM MgCl2, 2 mM ATP, and 150 mM KCl monitored by UV−visible CD spectroscopy at room temperature. CD spectra were recorded at 3, 6, 10, 14, 18, 22, 26, 30, 40, 50, and 60 min after the addition of Mg-ATP to the reaction mixture. All other conditions are the same as described in Figure 1.

Figure 3. Comparison of the kinetics of cluster transfer from A. vinelandii [Fe2S2]-IscU to apo-Grx5 in the presence and in the absence of HscA/HscB/ATP. All conditions are the same as described in Figures 1 and 2. The data in the presence of HscA/HscB/ATP (■) were obtained by continuously monitoring the CD intensity at 460 nm after initiation of the reaction with Mg-ATP, and the solid line is a best-fit simulation to second-order kinetics with a rate constant of 20 000 M−1 min−1. The data in the absence of HscA/HscB/ATP (●) were obtained by monitoring the difference in the CD intensity at 457 and 408 nm, and the solid line is a best-fit simulation to second-order kinetics with a rate constant of 30 M−1 min−1.
activity and a [Fe₃S₄]²⁺ cluster-bridged dimer, ligated by the first active-site cysteine of each Grx2 monomer and two glutathiones, which lacks disulfide oxidoreductase activity. In light of the stability of the cluster-bound form with respect to cluster transfer and sensitivity to oxidative stress, the [Fe₃S₄]²⁺ cluster on human Grx2 has been proposed to function as a sensor that responds to oxidative stress by activating the disulfide oxidoreductase activity via cluster degradation.

A [Fe₃S₄]²⁺ cluster storage function for monothiol Grx’s may be required under Fe-replete conditions, and the extent of cluster loading may be an important sensor of the cellular Fe–S cluster status. This latter hypothesis is supported by the accumulating evidence that the Fe regulon in yeast is controlled by the extent of [Fe₂S₂] cluster-loading of the cytosolic Grx3 and Grx4 monothiol glutaredoxins. In S. cerevisiae, the sensing mechanism involves interaction of the [Fe₃S₄]²⁺ cluster-bound form of the Grx3 or Grx4 homodimer with a BolA-type protein, termed Fra2, to form a less labile [Fe₃S₄]²⁺ cluster-bound Grx3/4-Fra2 heterodimer that prevents accumulation of the Aft transcription factor in the nucleus, where it functions in activating Fe uptake systems. A related Fe or Fe–S cluster regulatory function may also occur in bacteria since a stable [Fe₃S₄]²⁺ cluster-bound Grx4/BolA heterodimer has been reported in Escherichia coli, where Grx4 is the sole monothiol Grx. An homologous BolA protein is also present in A. vinelandii. Alternatively, either the [Fe₃S₄]²⁺ cluster-bound monothiol Grx homodimer or the Grx-BolA heterodimer may serve to regulate Fe–S cluster biogenesis in bacteria by acting as the [Fe₃S₄]²⁺ cluster donor for IscR, which acts as a transcriptional repressor of the entire isc operon in its [Fe₃S₄]²⁺ cluster-bound form.

In addition to a potential [Fe₃S₄]²⁺ cluster storage or sensing role for monothiol Grxs, the available evidence for rapid cluster transfer to physiologically relevant acceptor proteins suggests a role as a delivery system for clusters assembled on primary scaffold proteins. This was first demonstrated in plant chloroplasts, in which [Fe₃S₄]²⁺ cluster-bound monothiol Grx514 was found to rapidly and quantitatively transfer its [Fe₃S₄]²⁺ cluster to apo plant Fdx with a second-order rate constant of 20 000 M⁻¹ min⁻¹ at 23 °C. Subsequently, cluster transfer from [Fe₃S₄]²⁺-Grx4 to apo-IscFdx in E. coli was demonstrated, although the rate constant was not determined. Based on the CD studies shown in Figure 4, intact and quantitative cluster transfer from [Fe₃S₄]²⁺-Grx5 to apo-IscFdx also occurs in A. vinelandii, with a second-order rate constant of 2100 M⁻¹ min⁻¹ at 23 °C, indicating that Grx5 is a viable intermediate carrier protein for delivering [Fe₃S₄]²⁺ clusters assembled on Grx5 to apo-IscFdx.

Much work needs to be done to identify specific cluster acceptor proteins for [Fe₃S₄]²⁺ cluster-bound forms of monothiol Grx’s. These could be other proposed carrier proteins such as A-type and Nfu-type proteins and/or specific apo Fe–S proteins and enzymes. Identifying the specificity of [Fe₃S₄]²⁺ cluster-bound forms of monothiol Grx’s with respect to acceptor proteins is under active investigation in our laboratory. In addition, since IscU functions as a catalyst for Fe–S cluster assembly, it is clearly important to develop a robust in vitro catalytic system that includes the co-chaperones for investigating cluster assembly on target proteins using Fe(II) and cysteine as the primary substrates.

![Figure 4. Time course of cluster transfer from A. vinelandii [Fe₃S₄]²⁺-Grx5 (32 μM in [Fe₃S₄]²⁺ clusters) to apo-IscFdx (48 μM) monitored by UV–visible CD spectroscopy at 23 °C.](http://dx.doi.org/10.1021/ja306061x)
Notes
The authors declare no competing financial interest.

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■ REFERENCES