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Characterization of glutaredoxin Fe-S cluster binding interactions using circular dichroism spectroscopy

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Abstract

Monothiol glutaredoxins (Grxs) with a conserved Cys-Gly-Phe-Ser (CGFS) active site are iron-sulfur (Fe-S) cluster binding proteins that interact with a variety of partner proteins and perform crucial roles in iron metabolism including Fe-S cluster transfer, Fe-S cluster repair, and iron signaling. Various analytical and spectroscopic methods are currently being used to monitor and characterize glutaredoxin Fe-S cluster-dependent interactions at the molecular level. The electronic, magnetic, and vibrational properties of the protein-bound Fe-S cluster provide a convenient handle to probe the structure, function, and coordination chemistry of Grx complexes. However, some limitations arise from sample preparation requirements, complexity of individual techniques, or the necessity for combining multiple methods in order to achieve a complete investigation. In this chapter, we focus on the use of UV-visible circular dichroism spectroscopy as a fast and simple initial approach for investigating glutaredoxin Fe-S cluster-dependent interactions.

Keywords

Circular dichroism (CD); iron-sulfur (Fe-S) cluster; monothiol glutaredoxin (Grx); BolA-like protein; Fe-S cluster transfer; pH titration

1. Introduction

The ubiquitous monothiol CGFS glutaredoxin (Grx) proteins play important roles in iron metabolism by assisting in cellular iron sensing, and Fe-S cluster biosynthesis, transfer, repair and storage (Couturier, Przybyla-Toscano, Roret, Didierjean, & Rouhier, 2015). A wide variety of CGFS glutaredoxin isoforms have been identified in both prokaryotes and multiple compartments of eukaryotes, all with the ability to either assemble or acquire an Fe-S cluster, which is subsequently stored or transferred to target proteins. CGFS Grxs with a single GRX domain are found in prokaryotes and the mitochondria of eukaryotes, while multidomain Grxs with one to three GRX domains linked to an N-terminal thioredoxin (TRX)-like domain are found exclusively in eukaryotes (Couturier, Jacquot, & Rouhier, 2009). In the absence of a binding partner, CGFS Grxs primarily form homodimers with a bridging [2Fe-2S] cluster ligated by the conserved cysteine in the CGFS motif from each monomer and two glutathione (GSH) molecules stabilized by the GSH binding pocket of

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each Grx monomer (Iwema et al., 2009). Cellular processes involving CGFS Grxs often proceed as Fe-S cluster-dependent protein-protein interactions via a mechanism that employs structural rearrangements and ligand exchange. Two primary types of Grx Fe-S cluster-dependent interactions have been identified thus far in which Grxs interact with one or two binding partners to form (1) a heterocomplex with the Fe-S cluster ligated at the interface of the Grx domain(s) and the interacting partner protein as illustrated by the Grx-BolA complexes (Banci, Camponeschi, Ciofi-Baffoni, & Muzzioli, 2015; Dlouhy et al., 2016; Li et al., 2009; Li, Mapolelo, Randeniya, Johnson, & Outten, 2012; Nasta, Giachetti, Ciofi-Baffoni, & Banci, 2017; Roret et al., 2014; Uzarska et al., 2016; Yeung et al., 2011), or (2) the formation of an entirely new Fe-S cluster coordinated protein complex obtained via complete and intact transfer of the Fe-S cluster from Grx to the interacting partner (Banci et al., 2014; Banci, Camponeschi, et al., 2015; Banci, Ciofi-Baffoni, et al., 2015; Bandyopadhyay et al., 2008; Fidai, Wachnowsky, & Cowan, 2016; Mapolelo et al., 2013; Poor et al., 2014; Qi & Cowan, 2011; Shakamuri, Zhang, & Johnson, 2012; Vranish, Das, & Barondeau, 2016; Vranish et al., 2015; Xia et al., 2015; Yeung et al., 2011; Zhang et al., 2013).

Biological Fe-S clusters are commonly coordinated by cysteines via the sulfur atom of the protein thiol moiety. However, sometimes non-cysteinyll ligands such as histidine, serine or water are used as ligands. Owing to the fact that the structural, electronic and vibrational properties of Fe-S clusters in metalloenzymes depend on the type of ligands and protein conformation in the vicinity of the Fe-S cofactor, spectroscopic methods such as UV-visible absorption, circular dichroism (CD), electron paramagnetic resonance (EPR), resonance Raman, Mossbauer, and NMR are commonly employed when characterizing Fe-S containing proteins and/or Fe-S cluster-dependent interactions in vitro. Taken together and in association with analytical methods and site directed mutagenesis, these techniques provide information on the number, oxidation state and structure type of the cluster involved, as well as the amino acid residues coordinating the cluster in both the initial and final complexes. However, no one spectroscopic probe available can accomplish all these criteria at the present moment and complementary techniques often need to be employed.

Most Grxs identified thus far have been shown to ligate a $[2\text{Fe-2S}]^{2+}$ cluster that can be observed easily using UV-visible absorption and CD spectroscopies. Although $[3\text{Fe-4S}]^{+}$ and $[4\text{Fe-4S}]^{2+}$ cluster-coordinating Grxs have also been reported (Zhang et al., 2013), for simplicity these will not be discussed here. Solutions of oxidized $[2\text{Fe-2S}]^{2+}$ cluster-containing Grxs absorb light strongly, exhibiting a reddish-brown color and broad UV-visible absorption spectra consisting of multiple overlapping absorption bands through the visible and near UV regions. These bands reflect many unresolved S to Fe^{3+} charge transfer electronic transitions, with greater intensity closer to the near UV region (Fig. 1A). Due to the high similarities and lack of resolved features in the absorption spectra of $[2\text{Fe-2S}]^{2+}$ cluster-containing proteins, UV-visible absorption spectroscopy is generally not the method of choice when monitoring and/or characterizing Grx Fe-S cluster-dependent interactions, as it cannot distinguish between Fe-S clusters within subtle changes in the protein surroundings.

On the other hand, the interaction of the achiral Fe-S cluster with the chiral protein moiety generates a suitable handle for characterization via CD absorption spectroscopy (Stephens et al., 1978). This method measures the difference in absorption of left- and right-polarized light as a function of wavelength for a given chiral chromophore. CD absorption is observed continuously for [2Fe-2S] cluster proteins, and the spectral range spans the near IR, visible, and near UV. In the UV range, CD reports on protein secondary structure, whereas in the visible and near UV range, CD reports on the electronics of the Fe-S cluster. In contrast to their corresponding absorption spectra, the CD spectra of [2Fe-2S] cluster-containing proteins are significantly more structured due to both positive and negative bands with well-defined peaks accompanied by shoulders and inflections. Thus, overlapping bands in the absorption spectrum can be clearly resolved by CD if their signs are different. Due to its ability to report on subtle changes in cluster coordination environment, CD is an elegant diagnostic tool for investigating Fe-S cluster structural rearrangements orchestrated by protein-protein interactions and/or ligand exchanges. In addition, unlike other spectroscopic methods which require concentrated protein solutions, denaturing solvents, and/or low temperature conditions, CD absorption spectroscopy provides the advantage of measurements on dilute protein solutions under physiological conditions.

Given these advantages, UV-visible CD spectroscopy has been routinely and extensively employed as a method to assess Fe-S cluster-dependent interactions in biological systems. Due to its reliability, simplicity, and unique sensitivity to Fe-S cluster coordination changes, this technique has been our preferred approach to investigate the interaction between CGFS-type glutaredoxins and BOLA-like proteins from both prokaryotes (Dlouhy et al., 2016; Mapolelo et al., 2013) and eukaryotes (Li et al., 2011; Li et al., 2009; Li et al., 2012; Poor et al., 2014). In this chapter, we will outline several CD-focused approaches for characterizing and probing different Fe-S dependent interactions in which CGFS Grxs participate.

2. General equipment

- Anaerobic chamber (glove box)
- 1-cm / 0.5-mL quartz spectrophotometer cell (semi-micro cuvette) sealed with a rubber septum to maintain anaerobicity when needed (Starna Cells, Inc.)
- Pipettes and pipette tips
- CD spectropolarimeter (Jasco) equipped with a temperature controlling (Peltier) device to maintain the temperature below 10 °C when needed
- Spectra Manager™ (Jasco) for instrument control, data acquisition, and data processing
- Microsoft Excel, GraphPad Prism, or similar graphing software for graphing, spectral analysis, and curve fitting via non-linear regression

3. General notes

1. CD spectra are measured over the visible and near-UV range (290 – 700 nm).

2. Typical CD parameters include: sensitivity, 100 mdeg; data pitch, 1 nm; scanning mode, continuous; scanning speed, 200 nm/min; response, 2 sec; band width, 10 nm.
3. Semi-micro quartz cuvettes are used to minimize the volume of protein solution required.
4. In the case of samples requiring anaerobic conditions, solutions should be manipulated and cells should be filled inside an anaerobic chamber. Cells should be sealed with a septum as shown in Fig. 2B.
5. The concentration of the [2Fe-2S] cluster in each sample is obtained by dividing the concentration of total iron in the sample by two. The iron concentration is typically determined using the ferrozine assay (Riemer, Hoepken, Czerwinska, Robinson, & Dringen, 2004). In addition, the [2Fe-2S] concentration can be estimated with UV-visible absorption spectroscopy from the dominant band in the 390–430 nm range. The extinction coefficient for this peak is typically in the range of 8–11 mM⁻¹ cm⁻¹ per [2Fe-2S] cluster for Grx homodimers and 5–9 mM⁻¹ cm⁻¹ per [2Fe-2S] cluster for Grx heterocomplexes (Bandyopadhyay et al., 2008; Dlouhy et al., 2016; Li et al., 2011; Li et al., 2009; Li et al., 2012; Nasta et al., 2017; Roret et al., 2014; Zhang et al., 2013), similar to other [2Fe-2S]²⁺ cluster proteins (Dailey, Finnegan, & Johnson, 1994). Fe-S cluster loading and protein purity can thus be assessed from the A₃₉₀:A₂₈₀ ratio calculated from the UV-visible absorption spectrum.
6. When using a semi-micro cuvette (1-cm pathlength), Fe-S cluster concentrations should be adjusted to the optimum range (± 100 mdeg) in the UV and visible region (typically 30 – 100 μM Fe-S cluster).
7. If protein stability is affected by temperature, the samples should be kept on ice at all times, and the temperature should be maintained below 10 °C while scanning the CD spectrum using the Peltier device.
8. The same cuvette should be used for recording all of the spectra whenever possible to avoid reading errors arising from intrinsic cuvette absorption when subtracting the blank.
9. Handle cuvettes with gloves and wipe with a disposable wiper (e.g. Kimwipes®) before recording each spectrum in order to avoid introducing errors from fingerprint marks.
10. 50 mM Tris-MES (pH 8.0) or 50–100 mM Tris-HCl (pH 7.4–8.0) buffer solutions are generally used in our group to purify and characterize CGFS Grxs and their complexes. GSH (1 – 5 mM) is sometimes added to the buffer in order to improve Fe-S cluster stability for Grx homodimers (Bandyopadhyay et al., 2008; Dlouhy et al., 2016; Poor et al., 2014). However, the effect of GSH on Grx heterocomplexes varies depending on the specific proteins. For example, GSH does not appear to affect the stability of the [2Fe-2S]²⁺ cluster in *S. cerevisiae* Grx3-Fra2 or Grx4-Fra2 heterodimers (Li et al., 2011; Li et al., 2009), but

destabilizes the $[2\text{Fe-2S}]^{2+}$ cluster in *E. coli* Grx4-IbaG heterodimers (Dlouhy et al., 2016).

4. Fe-S cluster-dependent complex formation

The results for our studies on the *S. cerevisiae* Grx3/4-Fra2 interaction are used in this section to illustrate the use of UV-visible CD spectroscopy to rapidly and efficiently probe Fe-S cluster-dependent complex formation. By comparing the CD spectra of the $[2\text{Fe-2S}]$ centers of the as-purified $[2\text{Fe-2S}]$ Grx3/4 homodimers and the complex obtained upon co-expressing Grx3/4-Fra2 or titrating $[2\text{Fe-2S}]$ Grx3/4 homodimer with apo Fra2, we were able to distinguish and characterize the two Fe-S-containing complexes, and ultimately conclude that Grx3/4 and Fra2 interact by forming a $[2\text{Fe-2S}]$ cluster-bridged heterodimer (Li et al., 2009). Furthermore, when coupled with site directed mutagenesis, these CD-monitored interactions can be used to explore the role of specific amino acid residues in Fe-S cluster-dependent interactions (Li et al., 2011).

4.1 Characterization of Fe-S cluster binding in a copurified protein complex

Genetic and biochemical studies indicated that *S. cerevisiae* Grx3/4 and Fra2 interact *in vivo* and are required for regulation of iron homeostasis (Kumanovics et al., 2008; Lesuisse et al., 2005; Ojeda et al., 2006; Pujol-Carrion, Belli, Herrero, Nogues, & de la Torre-Ruiz, 2006). To characterize the iron-dependent interactions between Grx3/4 and Fra2 at the molecular level, Grx3 or Grx4 were co-expressed with Fra2 in *E. coli* for overexpression and purification, and the purified complexes were subjected to a detailed biochemical characterization. *E. coli* cells co-expressing recombinant Grx3 and Fra2 or Grx4 and Fra2 exhibited a reddish-brown color (Fig. 2A) that was maintained throughout the purification procedure (Fig. 2B). This color is a strong indication of the presence of a Fe-S cluster-binding protein. For comparison, *E. coli* cells expressing Fra2 alone, which does not bind an Fe-S cluster, is shown in Fig. 2A (left panel), resulting in a colorless purified protein solution (Fig. 2B, left panel). The SDS-PAGE gel of the colored fractions indicated that Grx3/4 and Fra2 co-eluted as a complex (Li et al., 2009). The shift in the intensity and position of the CD absorption bands of the as-purified heterocomplexes versus the as-purified $[2\text{Fe-2S}]$ -Grx3/4 homodimers demonstrated that the Grx3/4-Fra2 interaction included an $[2\text{Fe-2S}]$ center with a distinctly different coordination environment (Fig. 1A). However, comprehensive analytical and spectroscopic characterization of the purified Grx3-Fra2 and Grx4-Fra2 complexes was necessary to further characterize the protein-protein and metal-protein interactions (Li et al., 2009). These results confirmed the presence of $[2\text{Fe-2S}]$ cluster-bridged Grx3-Fra2 and Grx4-Fra2 heterodimers, in which the Fe-S centers were ligated by one active site cysteine contributed by Grx3 or Grx4, one glutathione (GSH) molecule, and two ligands (one histidine and one cysteine) contributed by the Fra2 partner (Fig. 1B) (Li et al., 2011; Li et al., 2009).

4.1.1 Buffers and reagents

- Purified proteins with known concentrations (both protein and Fe-S cluster concentration)

- Sample buffers: same as the buffers used for concentrating the protein complexes after the final purification step

4.1.2 Procedure

1. Scan and record the UV-visible CD absorption spectrum of the sample buffer for the baseline normalization of all spectra in the 1-cm cuvette. This spectrum is called the blank hereafter.
2. Clean the quartz cuvette after each sample by rinsing with water to avoid contamination. Flush cuvette with ethanol and blow nitrogen to completely dry the cuvette between different samples.
3. For each Fe-S-containing protein sample prepare ~ 350 μL of 30 – 100 μM final Fe-S cluster concentration in the cuvette and record the UV-visible CD absorption spectrum.
4. Subtract the corresponding blank from the spectrum of each sample.

4.1.3 Data analysis

1. Export files and convert the observed CD ellipticity units (θ_{obs} in millidegrees) to the differential molar extinction coefficient ($\Delta\epsilon$ in $\text{M}^{-1} \text{cm}^{-1}$) using Equation 4.1.1. $\Delta\epsilon$, also known as the molar circular dichroism, is the difference in molar extinction coefficients for left vs. right polarized light ($\Delta\epsilon = \epsilon_{\text{L}} - \epsilon_{\text{R}}$). In Equation 4.1.1, 32980 is the ellipticity to absorbance conversion factor, l is the cuvette path length in cm, and $[\text{Fe-S}]$ is the molar concentration of the $[\text{2Fe-2S}]$ cluster in the sample:

$$\Delta\epsilon = \frac{\theta_{\text{obs}} \times 10^6}{32980 \times l \times [\text{Fe-S}]} \quad \text{Equation 4.1.1}$$

2. Use GraphPad Prism or similar graphing software to plot the UV-visible CD spectra for each sample.
3. Overlap all spectra together for comparison and analysis (Fig. 1A). Differences in the intensity and/or position of CD absorption bands are indicative of changes in the Fe-S cluster coordination environments.

4.2 Characterization of Fe-S cluster complex formation via titration

As another approach, Fe-S cluster-dependent binding interactions can be investigated by separately expressing and purifying the potential partner proteins, followed by CD-monitored titration of the holo protein with the apo partner. For instance, CD-monitored titration of purified $[\text{2Fe-2S}]$ Grx3 with apo Fra2 has been performed to probe the stoichiometry, binding affinity, and cluster coordination changes that occur upon interaction of these partner proteins (Li et al., 2011). A typical CD-monitored titration reaction is performed by incubating a fixed amount of holo protein with varying amounts of its apo partner and scanning the UV-visible CD absorption spectra (Fig. 3).

4.2.1 Buffers and reagents

- Refer to section 4.1.1

4.2.2 Procedure

1. Follow steps 1–2 in section 4.1.2 to record the blank.
2. Record the UV-visible CD absorption spectrum of the Fe-S cluster-containing holo protein.
3. Prepare ~ 350- μ L reactions with 30 – 100 μ M fixed final cluster concentration of holo protein and different concentrations (0.125 – 5-fold excess) of apo-protein binding partner to be titrated in 1.5-mL microcentrifuge tubes.
4. Allow reactions to reach equilibrium by incubating for 5–15 minutes at room temperature, with occasional stirring.
5. Record the UV-visible CD absorption spectra for each reaction.

4.2.3 Data analysis

1. Export files and convert the observed CD units (θ_{obs} in millidegrees) to molar CD units (ϵ in $\text{M}^{-1} \text{cm}^{-1}$) using Equation 4.1.1 (see section 4.1.3 above).
2. Use GraphPad Prism or similar graphing software to plot the UV-visible CD spectra for each sample.
3. Overlap all spectra together for comparison and analysis (Fig. 3A).
4. In order to determine the stoichiometry of the binding interaction between partner proteins, plot the difference in the ϵ_{obs} values at two different wavelengths that exhibit the maximum changes as a function of [2Fe-2S]:apo protein ratio (Fig. 3A inset). The shape of this binding curve provides information about the nature of the binding interaction. In the case of the Grx3-Fra2 interaction, the initial binding curve is a straight line, indicating that Fra2 binding to [2Fe-2S] Grx3 is stoichiometric under these conditions. In other words, the K_d for this interaction is below the concentration of [2Fe-2S] Grx3 homodimer used in the assay (50 μ M) and therefore too low to be directly measured under these conditions. However, the intercept of the initial binding curve and the saturation binding plateau provides a measure of the binding stoichiometry.

4.2.4 Notes

1. For a comprehensive and accurate analysis of the ligand exchange reaction, the products of the final titration should be subsequently isolated using appropriate chromatography methods, and subjected to analytical, biochemical and spectroscopic analyses in order to assess the oligomeric state and Fe:S:protein ratio of each product. These additional steps were used to confirm formation of the [2Fe-2S]-bridged Grx3-Fra2 heterodimer and release of the apo-Grx3 monomer (Fig. 3B) (Li et al., 2011).

5. Thermodynamic and kinetic characterization of Fe-S cluster transfer reactions

CGFS glutaredoxins, either alone or in concert with BolA proteins, have been shown to accept, store, and deliver Fe-S clusters to a wide variety of acceptor proteins (Banci et al., 2014; Banci, Camponeschi, et al., 2015; Banci, Ciofi-Baffoni, et al., 2015; Bandyopadhyay et al., 2008; Boutigny et al., 2013; Frey, Palenchar, Wildemann, & Philpott, 2016; Hoffmann et al., 2011; Iñigo et al., 2016; Kim, Chung, Kim, Lee, & Roe, 2010; Mapolelo et al., 2013; Melber et al., 2016; Mühlenhoff et al., 2010; Poor et al., 2014; Shakamuri et al., 2012; Ströher et al., 2016; Uzarska et al., 2016; Vranish et al., 2016; Xia et al., 2015; Zhang et al., 2013). In *S. cerevisiae*, transfer of a [2Fe-2S] cluster from Grx3/4-Fra2 to the transcription factor Aft1/2 is proposed to be the key mechanism for regulating Aft1/2 activity in response to iron (Poor et al., 2014). We monitored the thermodynamics of Fe-S cluster transfer from Grx3-Fra2 to Aft2 by incubating [2Fe-2S] Grx3-Fra2 with Aft2 at different ratios and measuring the UV-visible CD spectra, following a procedure similar to the one described in section 4.2.

In order to determine whether the rate of Fe-S cluster transfer is physiologically relevant, CD-monitored Fe-S cluster transfer reactions can be also followed kinetically either by scanning the full UV-visible CD absorption spectrum of the two interacting proteins with time (full wavelength scan), or by monitoring the changes in CD at a single fixed wavelength with time (fixed wavelength scan). Although both methods can be used for the kinetic evaluation of the reaction, in the case of a very fast Fe-S cluster transfer, the latter would be better for capturing the initial changes in the CD spectrum due to the shorter time between scans, therefore ensuring a more accurate determination of the kinetic rate constant. We are describing here the procedure for each of these methods.

5.1 CD-monitored thermodynamic analysis of Fe-S cluster transfer reactions

Similar to the Fe-S cluster complex formation titrations described in section 4.2, by varying the amount of acceptor protein added to the [2Fe-2S] cluster donor, thermodynamic analysis of transfer of an intact Fe-S cluster from an acceptor to a donor can be assessed. Similar equipment, procedures, data analysis and notes described for the Grx-Fra2 Fe-S complex titrations (see section 4.2) are employed when determining the stoichiometry and binding ratio of the intact cluster transfer from a donor to acceptor (Fig. 4). Additional equipment and procedures are described below.

5.1.1 Additional equipment

- Concentrator (e.g. Amicon® stirred cell or centrifugal concentrator)

5.1.2 Buffers and reagents

- Purified proteins with known Fe-S cluster concentrations for the donor and protein concentration for the acceptor
- Interaction buffer: same as the buffer used for concentrating the protein samples after the final purification step. If different buffers are used when purifying the

two proteins to be used in the study, choose the buffer suitable to the least stable protein. For the [2Fe-2S]-Grx3-Fra2 to Aft2 cluster transfer reaction, 50 mM Tris-HCl pH 8.0, 500 mM NaCl buffer solution was used considering that Aft2 stability decreases with decreasing salt concentration.

- Reductant (i.e. dithiothreitol (DTT) or tris-(2-carboxyethyl)-phosphine (TCEP))

5.1.3 Procedure

1. Follow steps 1–2 in section 4.1.2 to obtain the CD spectrum of the blank solution.
2. Pre-reduce the acceptor protein by incubating on ice with reductant (5–10 mM DTT or 40 mM TCEP) for approximately 30 min. Stir occasionally. This step is necessary in order to break any disulfide bonds and ensure that all thiol moieties involved in ligating the Fe-S cluster are reduced and available.
3. Buffer exchange to remove excess reductant by repeated dilution-concentration cycles, using either a stirred cell or centrifugal concentrator inside the anaerobic chamber.
4. Determine the protein concentration using the Bradford assay (BioRad).
5. Record the UV-visible CD spectrum of the Fe-S cluster-containing holo protein.
6. Prepare ~ 350-μL reactions with 30 – 100 μM fixed final cluster concentration of holo protein and different concentrations (0.125 – 5-fold excess) of pre-reduced apo-protein to be titrated in 1.5-mL microcentrifuge tubes.
7. Allow reactions to reach equilibrium by incubating 5–15 min at room temperature, with occasional stirring.
8. Record the UV-visible CD absorption spectra for each reaction.

5.1.4 Data analysis

1. See section 4.2.3.

5.1.5 Notes

1. Control experiments lacking the acceptor protein should be carried out in order to demonstrate that the changes in CD occur as a result of the interaction rather than Fe-S cluster instability of the holo donor protein.
2. As a control for cluster disassembly/reassembly, the Fe-S donor can be replaced with equivalent amounts of Fe²⁺ and S²⁻ and the cluster transfer reaction repeated. Lack of a distinct [2Fe-2S] cluster signal for this control reaction indicates that the Fe-S cluster is specifically transferred as an intact unit from the donor to acceptor protein. Intact cluster transfer can also be confirmed by including EDTA (~ 0.1–1 mM) in the transfer mixture to chelate free iron.
3. Excess reductant should be removed prior to the Fe-S cluster transfer reaction to avoid any interference in the transfer reaction. In particular, the strong reductant

DTT has been shown to accelerate Fe-S cluster transfer reactions in a non-physiological manner (Vranish et al., 2016). If required, GSH (1–10 mM) can be included in the Fe-S transfer reaction instead to better mimic intracellular conditions.

4. The Fe-S cluster transfer can be theoretically simulated by adding the CD spectra of the holo-donor protein and the CD spectrum of acceptor protein in 10% increments (Bandyopadhyay et al., 2008). A close correlation between the experimental and simulated data it indicates that an intact and direct cluster transfer reaction occurred.

5.2 CD-monitored kinetic analysis of Fe-S cluster transfer reactions based on full wavelength scans

Changes in the intensity and position of peaks that occur with time in the UV-visible region of the CD spectra upon mixing a Fe-S cluster donor and its potential acceptor partner are readily observed in vitro and are a definitive indication of changes in the Fe-S cluster coordination. Similar to the Fe-S cluster-dependent interactions described in section 4, when coupled with site-directed mutagenesis, CD-monitored Fe-S cluster transfer experiments offer information not only on the presence of a Fe-S cluster transfer, but also on the molecular details of the mechanism by which the reaction occurs.

5.2.1 Additional equipment

- Air tight Hamilton syringes and rubber stopper
- Concentrator (e.g. Amicon® stirred cell or centrifugal concentrator)
- Timer

5.2.2 Buffers and reagents

- See section 5.1.2.

5.2.3 Procedure

1. All samples are to be prepared in an anaerobic chamber.
2. Select “spectrum measurement” mode in the CD software and select the range of wavelength to be measured.
3. Follow steps 1–2 in section 4.1.2 to obtain the CD spectrum of the blank solution.
4. Transfer donor protein into the cleaned and dried cuvette, seal with a rubber septum to maintain anaerobicity (Fig. 2B), and take outside of the anaerobic chamber.
5. Scan and record the CD spectrum of the donor protein.
6. Bring cuvette back into the anaerobic chamber and transfer donor protein to a microcentrifuge tube.

7. Rinse cuvette thoroughly with distilled water and dry as described in section 4.1.2, step 2.
8. Pre-reduce the acceptor protein and buffer exchange to remove excess reductant as described in section 5.1.3, steps 2–4.
9. Calculate the volume of donor and acceptor proteins needed to prepare ~ 350 μL reaction having a 1:2 [2Fe-2S]:acceptor protein ratio, and a final [2Fe-2S] cluster concentration of 30 – 100 μM .
10. Prepare the acceptor protein by diluting into the interaction buffer to the concentration and volume calculated in step #9, and place into the 1-cm semi-micro cuvette sealed with a rubber septum.
11. Record the UV-visible CD absorption spectrum of the acceptor protein.
12. Using a Hamilton airtight syringe with a needle, obtain the calculated amount of Fe-S cluster donor protein in the glove box, cap the needle with a rubber stopper (Fig. 5, left), then take it outside to the CD spectrophotometer.
13. Remove stopper from the syringe needle and quickly insert the needle in the cuvette containing the acceptor protein through the rubber stopper used to seal the cuvette (Fig. 5, right).
14. Inject donor protein into the acceptor protein.
15. Start timer simultaneously with injecting the donor protein.
16. Mix quickly by inverting the cuvette 3–4 times.
17. Place cuvette inside the cuvette compartment, close the instrument lid and start scanning the CD spectrum.
18. Record the time that passes from the moment the reaction is initiated (donor protein is injected) until the scan is started. This will be called the “lag time” hereafter.
19. When scan is done, start scanning again, and record the time each scan is started.
20. Continue scanning spectra and recording the time until no changes in CD are observed.

5.2.4 Data analysis

1. Export files and convert the observed CD units (θ_{obs}) to ϵ ($\text{M}^{-1} \text{cm}^{-1}$) using Equation 4.1.1. For the $[Fe-S]$ term in this equation, use the protein concentration for the acceptor protein, and the Fe-S cluster concentration for the donor protein and the cluster transfer reaction data points.
2. Use graphing software (Microsoft Excel, GraphPad Prism, etc.) to plot the CD spectra collected for the donor protein, and for each of the time points recorded during the Fe-S cluster transfer reaction.
3. Overlap all spectra together for comparison and analysis (Fig. 6A).

4. Plot the change in CD values (ϵ_{obs}) at a wavelength position exhibiting the largest spectral changes, as a function of time. Add the “lag time” to each of the time values recorded during data collecting.
5. Use kinetics simulation software (e.g. Chemical Kinetics Simulator, COPASI, etc.) to fit the data to a kinetic model.

5.2.5 Notes

1. In order to minimize the amount of time that passes from the moment the donor protein is injected into the cuvette containing the acceptor protein, until the CD spectrum is scanned, have the software and computer set up and ready for scanning prior to injection.
2. The Jasco CD spectrophotometer also has an “interval scan measurement” scanning mode that can be used for scanning the spectrum at designated time intervals.

5.3 CD-monitored kinetic analysis of Fe-S cluster transfer reactions at a single wavelength

A more direct kinetic measurement for the rate of Fe-S cluster transfer reactions is obtained when the changes in CD are monitored in real time. The same equipment, buffers, reagents and notes described for the “full wavelength scan” method (see section 5.2) are also needed when employing the single wavelength time course measurement for monitoring Fe-S cluster transfers. The procedure is similar, except changes in CD at only one wavelength are monitored. However, a wavelength scan of the interaction is necessary prior to employing the single wavelength method in order to be able to select the wavelength where the most dramatic changes in the CD values occur during the Fe-S cluster transfer reaction.

5.3.1 Additional equipment

- Refer to section 5.2.1

5.3.2 Procedure

1. Use the results obtained from the “wavelength scan” method described above to select the wavelength at which the changes in CD values with time are the greatest.
2. Select “Time course measurement” in the CD software and use the wavelength selected in step 1 to set up the instrument.
3. Prepare samples in the anaerobic chamber as described in section 5.2.3., steps 3–9.
4. Transfer the calculated amount of acceptor protein to the cuvette, seal cuvette using a rubber septum (Fig. 2B), and bring it outside of the anaerobic chamber.
5. Place cuvette into the cuvette compartment and close lid. Record CD value reading.

6. Using a Hamilton airtight syringe with a needle, obtain the calculated amount of Fe-S cluster donor protein in the glove box, cap the needle with a rubber stopper (Fig. 5, left), then take it outside to the CD spectrophotometer.
7. Remove stopper from the syringe needle and quickly insert the needle in the cuvette containing the acceptor protein through the rubber stopper used to seal the cuvette (Fig. 5, right).
8. Inject donor protein into the acceptor protein.
9. Simultaneously start the timer.
10. Mix quickly by inverting the cuvette 3–4 times.
11. Place cuvette inside the cuvette compartment, close the instrument lid and start the program.
12. Record the time that passes from the moment the donor protein is injected into the cuvette until the scan is started.
13. Continue recording until no changes in CD value occur and a plateau is reached.

5.3.3 Data analysis

1. Export the file and use software suitable for curve fitting to plot the change in CD values (ϵ_{obs}) as a function of time. Subtract the CD value recorded in Section 5.3.1, step #5, from each of the CD values on the Y-axis.
2. Using a kinetics simulation software, estimate the rate constant and fit the data to a kinetic curve.

5.3.4 Notes

1. Some cluster transfer reactions occur extremely fast, making it difficult to determine the rate constant accurately. In such cases, a CD spectrometer equipped with a rapid mixer such as the stopped-flow accessories available from Jasco or Applied Photophysics (e.g. RX2000 stopped-flow accessory) is needed. These systems are capable of observing reactions on the 2.1 ms and 10 ms or higher scale, respectively.

6. CD-monitored pH titrations of Fe-S cluster complexes

CD spectroscopy is also an effective tool for characterizing the effects of pH on the coordination environment of a [2Fe-2S] cluster. If an amino acid residue that either directly coordinates or is located sufficiently close to the [2Fe-2S] center has a dissociable proton that is solvent accessible, the spectroscopic features of the Fe-S center may reflect measurable changes as a function of pH. Such behavior has been reported using CD, UV-visible absorption, NMR, and resonance Raman spectroscopy for Rieske-type [2Fe-2S] cluster proteins (Cys₂His₂ coordination) (Iwasaki, Imai, Urushiyama, & Oshima, 1996; Konkle et al., 2009; Kuila et al., 1992; Lin et al., 2006), and using resonance Raman spectroscopy and UV-visible absorption for the mitochondrial membrane [2Fe-2S] mitoNEET protein family (Cys₃His₁ coordination) (Tirrell et al., 2009; Zuris et al., 2010).

Subtle changes in the CD spectra of *Azotobacter vinelandii* [3Fe-4S] cluster ferredoxin (Cys₄ coordination) as a function of pH have also been reported due to protonation of a histidine introduced in place of a phenylalanine in the vicinity of the cluster (Chen et al., 2002). We have used this approach to study the interaction between CGFS Grxs and BolA proteins in *E. coli*. Specifically, we have compared the pH dependence of the CD spectra of the *E. coli* [2Fe-2S]-Grx4 homodimer to the [2Fe-2S]-Grx4-IbaG heterodimer (IbaG is a BolA homologue). This approach confirmed histidine ligation to the Fe-S cluster in the Grx4-IbaG complex and allowed calculation of the p*K*_a of the dissociable proton (Dlouhy et al., 2016). Protonation/deprotonation of a nitrogen atom in the histidine imidazole ring is proposed to induce subtle changes in the protein environment surrounding the cluster in the [2Fe-2S]-Grx4-IbaG complex, leading to marked differences in the spectroscopic properties of the isolated complex as a function of pH (Fig. 7). In comparison, the *E. coli* [2Fe-2S]-Grx4 homodimer with all-Cys ligation does not exhibit these pH-dependent changes (Dlouhy et al., 2016).

6.1. Additional Equipment

- Micro pH meter to measure pH of low volume samples (< 500 µl)

6.2 Buffers and Reagents

- 50 mM Tris-MES, 150 mM NaCl with pH ranging from 5.5 to 9.0 in 0.1 pH unit increments

6.3 Procedure

1. Mix 40–100 µM Fe-S cluster-containing protein with sample buffer at different pH values in separate chilled microcentrifuge tubes. The final dilution of concentrated protein to buffer should be at least 1:10 to obtain a final pH close to the respective buffer pH.
2. Allow the protein to reach conformational equilibrium by incubating for at least 30 min, with occasional stirring, either at room temperature or on ice, depending on thermodynamic stability.
3. Using a micro pH meter, read and record pH of each sample before scanning.
4. Transfer to 1-cm quartz semi-micro cuvette and scan CD absorption spectrum.
5. Rinse cuvette with distilled water and dry after scanning the spectrum of each buffer and/or protein sample as described in section 4.1.2, step 2 to avoid contaminating the samples or diluting the protein concentration.
6. Acquire and subtract the CD spectrum of the sample buffer from the CD spectrum of each sample.

6.4 Data Processing and p*K*_a Determination

1. Export files and use GraphPad Prism or similar curve fitting software to plot, overlap and compare the CD spectra obtained at different pH values. Additionally, plot the change in CD values (θ_{obs} or ϵ_{obs}) at a wavelength

exhibiting the greatest spectral shift as a function of pH. Often the changes in the CD spectral features may be subtle due to rising peaks overlapping disappearing peaks and/or slight conformational changes around the Fe-S center. In such cases, the difference in the CD values at two different wavelengths should be plotted as a function of pH (Fig. 7B).

2. Determine the pK_a and the number of protons involved in the conversion from the conformation observed at low pH to the one observed at high pH, by fitting the data to an ideal titration curve obtained using Hill's equation defined by Equation 6.1. This is a traditional model used to measure cooperativity in protein-ligand interactions in which the observed CD intensity (CD_{obs}) is fit to a sigmoidal curve simulated by the Hill's coefficient (n), the pK_a , and the CD intensity of the protonated (CD_P) and deprotonated (CD_D) forms of the protein complex.

$$CD_{obs} = CD_D + (CD_P - CD_D) \left[\frac{(10^{pH})^n}{(10^{pH})^n + (10^{pK_a})^n} \right] \quad \text{Equation 6.1}$$

3. or:

$$CD_{obs} = CD_D + \frac{(CD_P - CD_D)}{1 + 10^{n(pK_a - pH)}}$$

4. In this equation, the Hill coefficient (n) represents the cooperativity of the proton dissociation. When $n = 1$, the equation is a standard Henderson-Hasselbalch protonation function.

6.5 Notes

- For recording the blank, scanning one buffer solution at any pH value should suffice because the pH of the buffer has insignificant effects on the CD spectrum.
- Due to pH variations as a function of temperature, all samples need to be handled at the same or very close temperature during preparation and scanning.
- In some cases, protein degradation can occur at pH values below 6 or above 8.5. If the degradation is not severe, centrifuge the sample for 1 min at 7000–8000 rpm, then transfer the supernatant to a new microcentrifuge tube before reading the pH and scanning the CD spectrum.
- The existence of two separate protonation events as observed for Rieske [2Fe-2S] proteins with two His ligands to the Fe-S cluster requires fitting the titration curve to the sum of two Henderson-Hasselbalch equations (Konkle et al., 2009).

7. Summary and Conclusions

CD spectroscopy has emerged as a useful and preferred tool for studying Fe-S cluster-dependent interactions due to its unique ability to capture slight changes in the conformational properties of and around the Fe-S centers in a protein environment. Unlike other spectroscopic techniques currently available, CD spectroscopy requires small protein sample volumes at low concentrations, with measurements performed under physiological conditions. These advantages are particularly important since some Fe-S clusters – containing proteins are difficult to purify in large quantities with an intact Fe-S cluster due to Fe-S cluster instability and sensitivity to oxygen. Various types of Fe-S cluster-dependent interactions can be investigated, and a wide range of information can be gleaned using CD spectroscopy ranging from primary protein-protein interaction data to detailed information on Fe-S cluster transfer mechanisms at the molecular level. However, in spite of the valuable information acquired using CD spectroscopy, further studies are often necessary to complement this approach and answer questions regarding the fine details of Fe-S cluster-dependent interactions.

The versatility of CGFS Grxs in carrying out different types of Fe-S cluster-dependent interactions and the compilation of data available to date, renders them an ideal model for illustrating the use of CD spectroscopy in studying similar cellular processes.

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Figure 1.

(A) Comparison of the UV-visible absorption (top) and CD spectra (bottom) of as-purified [2Fe-2S] Grx3 (gray line) and [2Fe-2S] Grx3-Fra2 (black line). Adapted with permission from (Li et al., 2009). Copyright (2009) American Chemical Society. (B) Models for Grx3 homodimer and Grx3-Fra2 heterodimer.

Figure 2.

Overexpression and purified samples of Fra2 and Grx3-Fra2 heterodimer. **(A)** Cell pellets obtained upon expression of recombinant *S. cerevisiae* Fra2 (*left*) and coexpression of Grx3 with Fra2 (*right*) in *E. coli*. **(B)** UV-visible absorption/CD samples of purified Fra2 (*left*) and Grx3-Fra2 (*right*) in a semi micro cuvette.

Figure 3.

(A) Titration of apo Fra2 into the [2Fe-2S] Grx3 homodimer. As purified [2Fe-2S] Grx3 is shown as a thick black line. Arrows indicate the direction of changes in CD intensity with increasing apo Fra2 concentration. The inset shows the maximum difference in CD intensity (at 463 and 403 nm) as a function of the Fra2:Grx3 [2Fe-2S]²⁺ cluster ratio. This research was originally published in (Li et al., 2011) © the American Society for Biochemistry and Molecular Biology. (B) Model for conversion of [2Fe-2S] Grx3 homodimer to [2Fe-2S] Grx3-Fra2 heterodimer.

Figure 4.

Thermodynamic analysis of the Fe-S cluster transfer from *S. cerevisiae* [2Fe-2S] Grx3-Fra2 to Aft2 monitored by UV-visible CD spectroscopy. **(A)** Different ratios of acceptor:donor proteins are incubated and allowed to reach equilibrium. The CD spectrum of the reaction at each ratio is recorded, then the difference in CD intensity between two different wavelengths exhibiting the largest variations (400 and 472 nm) is plotted as a function of the Aft2:[2Fe-2S] ratio (*inset*). *Blue line* is [2Fe-2S]-Fra2-Grx3 without addition of Aft2, *black lines* indicate titration with 0.25–3.5 excess Aft2, and *red line* is 5:1 [Aft2]:[2Fe-2S] ratio. Arrows indicate the direction of intensity changes with increasing [Aft2]. Taken with permission from (Poor et al., 2014). Copyright © 2012, National Academy of Sciences. **(B)** Model of the Fe-S cluster transfer reaction from [2Fe-2S]-Fra2-Grx3 to Aft2.

Figure 5. Set up for Fe-S cluster transfer reactions. (*Left*) Hamilton syringe filled inside anaerobic chamber with [2Fe-2S] – Grx3-Fra2 and sealed with a rubber stopper to maintain anaerobicity. (*Right*) Hamilton syringe inserted via the rubber septum into the cuvette containing pre-reduced anaerobic Aft2.

Figure 6. CD-monitored wavelength scan and fixed wavelength kinetic analysis (*inset*) for the Fe-S cluster transfer reaction (*thin grey lines*) from *Arabidopsis thaliana* [2Fe-2S]-GrxS14 (*thick black line*) to *Arabidopsis thaliana* SufA1. Adapted with permission from (Mapolelo et al., 2013). Copyright © 2012, Royal Society of Chemistry.

Figure 7.

CD-monitored pH titration studies on *E. coli* [2Fe-2S]²⁺ Grx4-IbaG. **(A)** CD spectra of the protein complex equilibrated in buffer at the indicated pH values. **(B)** The difference in CD intensity at two wavelengths indicated by the arrows plotted as a function of pH was used to determine the pK_a of the dissociable proton near the Fe-S cluster. Taken with permission from (Dlouhy et al., 2016). Copyright (2016) © American Chemical Society.