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Fluorescence detection of a protein-bound 2Fe2S cluster

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A variety of mitochondrial diseases have been correlated with reduced activities of enzymes that require iron-sulfur metalloclusters to function [1]. While the chemistry and structure of Fe-S proteins have been intensively studied using absorbance, circular dichroism, electron paramagnetic resonance, Mössbauer, and Raman spectroscopy [2-7], these approaches have not been helpful in diagnosing mitochondrial diseases that arise from Fe-S cluster biosynthesis defects. These spectroscopic methods cannot measure Fe-S cluster synthesis kinetics for individual proteins in biopsies, since multiple metalloproteins are present that give rise to the observed spectra. Furthermore, the lack of signals for some Fe-S clusters (diamagnetic centers), a requirement for high metallocluster concentrations, and the need for cryogenic temperatures present challenges when using these spectroscopic tools to study the kinetics of metallocluster synthesis (and degradation) reactions [2-7]. Here, we describe a novel fluorescent assay for monitoring the Fe-S cluster content of a protein that lacks many of these limitations (Figure 1A). By using green fluorescent protein (GFP) variants to report on the 2Fe2S content of human glutaredoxin 2 (Grx2), our approach should be useful for studying Fe-S cluster degradation and synthesis reactions in complex samples.

Grx2 is a glutathione-dependent oxidoreductase that is monomeric when it lacks a metallocluster and dimeric upon coordinating a 2Fe2S cluster [8, 9]. Since GFP variants have been reported that exhibit altered fluorescence upon dimerization [10] and metal binding [11, 12], we hypothesized that Grx2 dimerization could be used to alter the fluorescence of GFP-Grx2 protein fusions. To first test whether GFP-Grx2 oligomerizes, protein overexpressed in *Escherichia coli* was purified and subjected to gel filtration analysis. Like Grx2 [9], GFP-Grx2 elutes as two peaks (Figure 1B). A comparison of the elution volumes for these peaks with standards indicates that GFP-Grx2 (M_r = 46.5 kDa) exhibits apparent molecular weights (56 and 130 kDa) consistent with the presence of an elongated monomer and dimer.

To examine whether the two GFP-Grx2 species differ in cofactor content, their absorbance and circular dichroism (CD) spectra were compared. The absorbance spectrum of monomeric GFP-Grx2 contains two peaks (280 and 470 nm) that are interpreted as arising from the extinction of aromatic amino acids and the GFP chromophore, respectively (Figure

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1C). Dimeric GFP-Grx2 additionally absorbs across the visible spectrum with a distinct peak at 320 nm, similar to Fe-S cluster bound Grx2 [9]. Monomeric and dimeric GFP-Grx2 also exhibit distinct CD spectra (Figures 1D and 1E). The spectrum for monomeric GFP-Grx2 has one ellipticity maximum (280 nm) and one minimum (470 nm). In contrast, dimeric GFP-Grx2 has a spectrum with four ellipticity maxima (260, 305, 440, and 560 nm) and two minima (370 and 470 nm). The additional peaks observed in this spectrum occur at similar wavelengths and relative intensities as those attributed to the 2Fe2S cluster in dimeric Grx2 [9], implicating this species as a metallocluster-bridged dimer $(GFP\text{-}Grx2)_2$-2Fe2S.

To determine if 2Fe2S clusters affect GFP-Grx2 fluorescence, we acquired spectra for identical concentrations of monomeric and dimeric protein. Although both spectra exhibit maximal excitation and emission at similar wavelengths (Figure 2A), the fluorescence intensity of $(GFP\text{-}Grx2)_2$-2Fe2S is 48% lower than GFP-Grx2. To test if other fluorescent proteins are more sensitive as 2Fe2S biosensors, protein fusions having Grx2 fused to blue (BFP) and cyan (CFP) fluorescent proteins were characterized. Both BFP-Grx2 and CFP-Grx2 are produced as mixtures of monomeric and dimeric proteins, and they exhibit absorbance and CD spectra consistent with the dimer having a 2Fe2S cluster (*data not shown*). In addition, the dimeric forms of these proteins exhibit reduced fluorescence compared with the monomers (Figure 2B and 2C), similar in magnitude to that observed with GFP-Grx2 (BFP-Grx2 = 36% and CFP-Grx2 = 51%).

The ability of GFP-Grx2 fluorescence to report on changes in the cofactor content of Grx2 was investigated by characterizing spectral changes that arise from removing glutathione (GSH) and adding ascorbate, conditions that destabilize the 2Fe2S cluster coordinated by Grx2 [9]. Under these conditions, the fluorescence of $(GFP\text{-}Grx2)_2$-2Fe2S increased 71% after a 10 hour incubation. In contrast, the fluorescence of monomeric GFP-Grx2 only increased 4% over the same time course (Figure 3A). A fit of the fluorescence increase for $(GFP\text{-}Grx2)_2$-2Fe2S to a single exponential yields a rate that is identical to the rate at which the ellipticity for this protein diminishes (Figure 3B). This indicates that this fluorescence increase occurs concomitantly with 2Fe2S cluster loss.

To determine the conditions where CD and fluorescence are capable of accurately measuring changes in the 2Fe2S content of a protein, we examined the effect of GFP-Grx2 dimer concentration on the coefficient of variation (CV) of its fluorescence and ellipticity. Steady-state measurements over a range of GFP-Grx2 dimer concentrations revealed that the CV for fluorescence is less than 1% when protein concentrations are ≥200 nM and only 2.6% for 100 nM $(GFP\text{-}Grx2)_2$-2Fe2S (Figure 4). In contrast, the CV for ellipticity is >27-fold higher than that of fluorescence for protein concentrations ranging from 100 nM to 2 μM and 16-fold higher at the highest concentration analyzed (5 μM). This indicates that fluorescence analysis will provide a more accurate estimate of the changes in the 2Fe2S cluster content of GFP-Grx2 when submicromolar protein concentrations are used for analysis.

We have shown that the fluorescence of our engineered biosensor composed of GFP and Grx2 is sensitive to changes in the 2Fe2S content of Grx2. Our approach has several advantages over existing methods for monitoring the kinetics of changes in the Fe-S cluster content of a protein, including the ability to: i) measure changes in the cofactor content of a protein present at submicromolar concentrations in a reaction, ii) detect changes in the cofactor content in small volumes that are compatible with high-throughput methods, and iii) assess the cofactor content of a single protein in complex reactions such as cell extracts. Further improvements in the sensitivity of our biosensor using directed evolution strategies [13] will aid in developing assays that can analyze Fe-S cluster synthesis rates in

biopsy samples and diagnose patients with mitochondrial myopathies that arise from defects in Fe-S cluster assembly [14].

Experimental Section

Materials

Escherichia coli, enzymes for DNA manipulation, oligonucleotides, and bacterial growth media were from EMD Biosciences, New England Biolabs, Fischer Scientific, and BD Biosciences, respectively. All other reagents were from Sigma-Aldrich.

Plasmid construction

Gene fusions encoding full-length fluorescent proteins fused to the N-terminus of the processed mitochondrial isoform of Grx2 (residues 42-164)[15] were cloned into pET28b (EMD Biosciences) using SacI and NotI sites to produce vectors that express $(His)_6$ tagged fusion proteins.

Protein expression and purification

Rosetta2 *Escherichia coli* harboring vectors for expressing fusion proteins were grown in LB at 37°C, induced with IPTG (0.1 mM) at $A_{600} \approx 1$, and grown for 6 to 18 h at 23°C. Harvested cells were resuspended in PBSIG (50 mM phophate pH 7.0, 300 mM NaCl, 10 mM imidazole, 2 mM GSH) containing $MgCl_2$ (1 mM) lysozyme (300 μg/mL), and DNase I (2 U/mL). Resuspended cells were frozen at −80°C, thawed, and centrifuged at 40k × g. Cleared lysate was applied to a nickel talon affinity column (Qiagen, Inc) equilibrated with PBSIG. Protein was eluted using imidazole (250 mM), dialyzed against HEG buffer (50 mM Hepes 7.0, 0.5 mM EDTA, and 2 mM GSH), and stored at −80°C.

Gel filtration chromatography

Protein molecular masses were estimated by comparing their elution to standards of known molecular weight on a Superdex 200 column equilibrated in HEG buffer using an AKTA FPLC system (GE, Inc.). The standard curve was generated using amylase (200 kDa), alcohol dehydrogenase (158 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Spectroscopy

Absorbance, CD, and fluorescence spectra were acquired using a Varian Cary 50 UV/Vis spectrophotometer, a JASCO 815 spectropolarimeter, and a SLM AMINCO Series 2 spectropolarimeter. For kinetic measurements, GSH was removed from GFP-Grx2 samples by loading 1 mL of dimer (24 μM) or monomer (32 μM) on a HiTrap desalting column preequilibrated in HE buffer (50 mM HEPES 7.5 and 0.5 mM EDTA), protein eluted from this column was diluted to 3 mL in HE buffer, ascorbate was added to a final concentration of 5 mM, and ellipticity and fluorescence changes were monitored at 25°C using a JASCO 815 spectropolarimeter having a FMO-427 Scanning Emission Monochromator.

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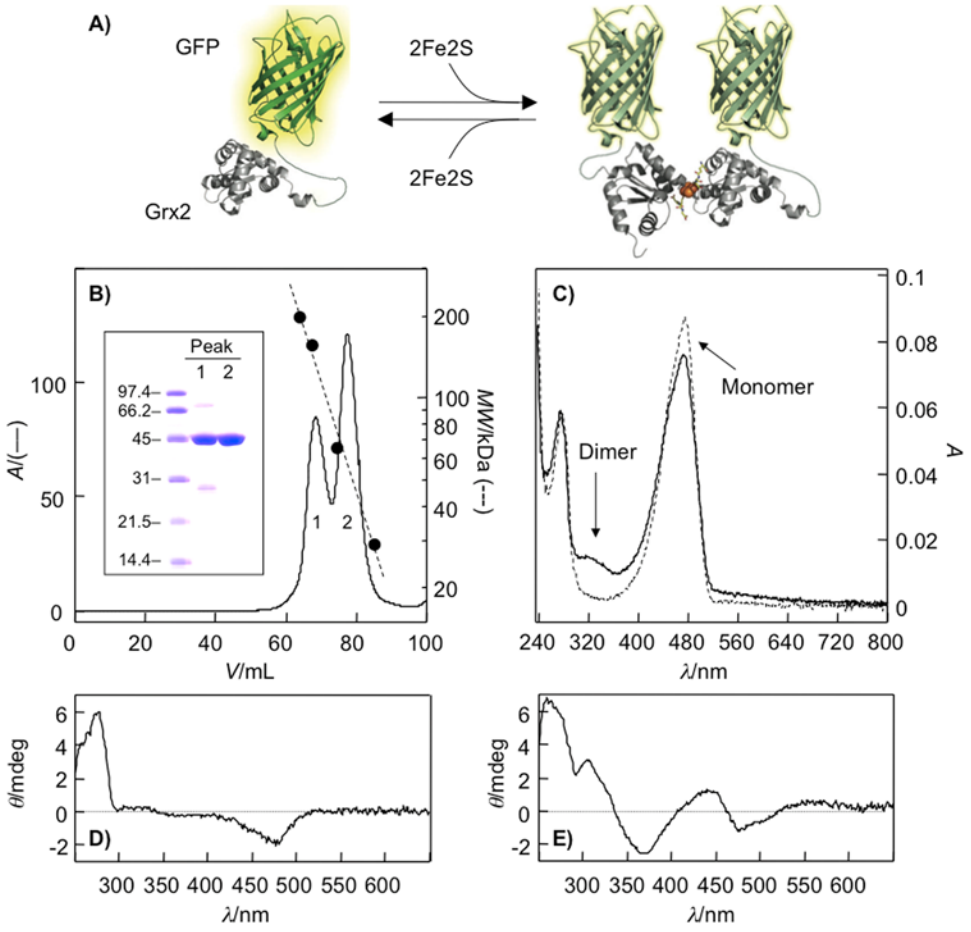

Figure 1.

A) GFP-Grx2 fusion proteins exhibit quenched fluorescence upon iron-sulfur cluster binding and dimerization. B) The elution profile of purified GFP-Grx2 (16 mg) chromatographed using a Superdex 200 column in HEG buffer is compared with protein standards of known molecular weight (*MW*). *Inset*, SDS-PAGE analysis of GFP-Grx2 in each elution peak (5 μg each). C) Absorption spectra for monomeric and dimeric GFP-Grx2 (2 μM each). Circular dichroism spectra for D) monomeric and E) dimeric GFP-Grx2 (8.4 μM each). Spectra are shown for samples in HEG buffer that have been baseline corrected for buffer absorbance and ellipticity.

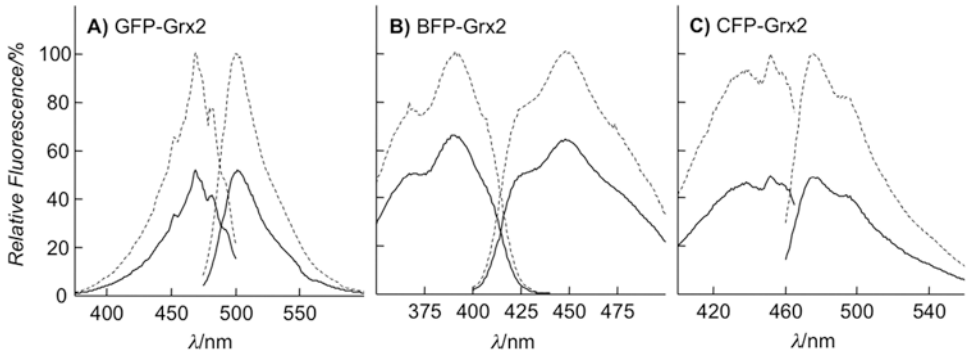

Figure 2.

Effect of dimerization on the fluorescence of A) GFP-Grx2, B) BFP-Grx2, and C) CFP-Grx2. Spectra were acquired in HEG buffer using emission and excitation wavelengths that yielded the highest fluorescence intensity. The fluorescence of each dimeric fusion protein (solid lines) is reported relative to that of the corresponding monomeric protein (dashed lines).

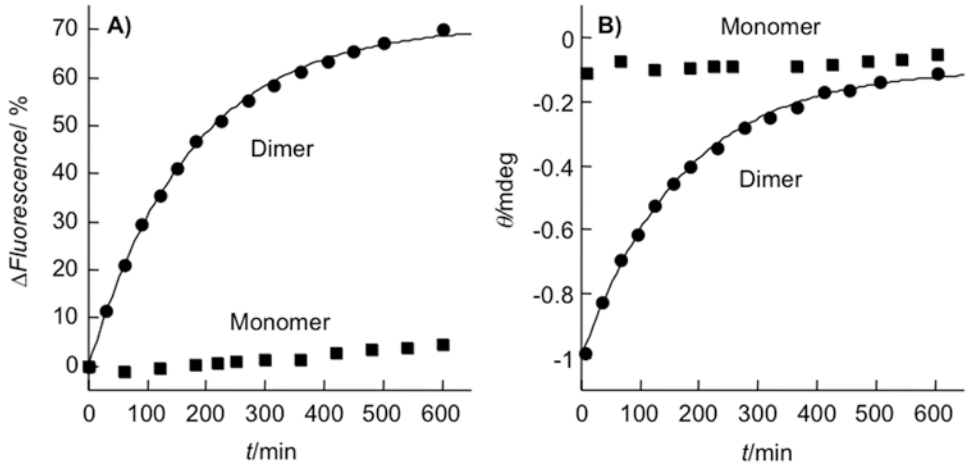

Figure 3.

Kinetics of A) fluorescence and B) ellipticity changes in dimeric (●) and monomeric (■) GFP-Grx2 arising from treatment with ascorbate (5 mM). Fluorescence (λ_{ex} = 470 nm and λ_{em} = 506 nm) is reported relative to that measured for each sample one minute after addition of ascorbate. Ellipticity was monitored from 360 to 370 nm, baseline corrected for HE buffer, and measurements from 360 to 370 nm were averaged to improve the signal to noise. The solid lines represent single exponentials having a k_{obs} = 0.006 min^{-1}.

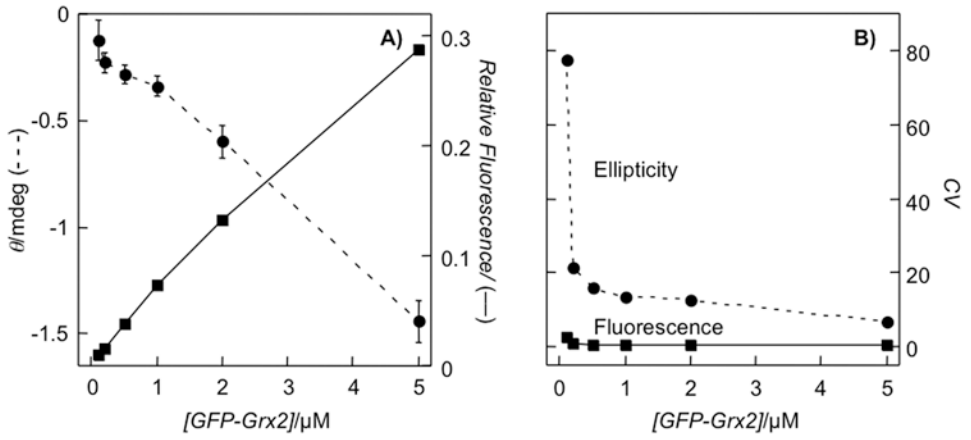

Figure 4.

The effect of protein concentration on the A) average magnitude and B) coefficient of variance (*CV*) of GFP-Grx2 dimer fluorescence (■) and ellipticity (●). Fluorescence (λ_{ex} = 470 nm and λ_{em} = 506 nm) and ellipticity (370 nm) were measured using identical signal averaging times, and average values were calculated using the results from ten measurements at each protein concentration (0.1, 0.2, 0.5, 1, 2, and 5 μM). Error bars representing ±1 standard deviation are only shown where they fall outside of the symbols used.