Disulfide-mediated modifications of roGFP and their impact on its use as a redox sensor

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Abstract

Variants of the original green fluorescent protein (GFP) with engineered cysteine residues have been widely used to assess cellular redox state. Named roGFP to reflect its redox sensitivity, this protein exhibits a ratiometric shift in its excitation fluorescence spectrum depending on the reversible formation of an internal disulfide bond. A new generation of roGFP with an additional insertion near one of the redox-sensitive cysteine residues (roGFP-iX) was recently developed to examine the redox states of subcellular compartments with higher reduction potential. Studies have used a particular version of roGFP-iX with leucine as the inserted residue, roGFP-iL, to measure the endoplasmic reticulum (ER) redox state in living cells. We show here that roGFP-iL undergoes unexpected disulfide-mediated modifications, some of which affect its excitation spectrum. These disulfide-based modifications represent additional redox states of roGFP-iL other than the fully oxidized, disulfide-bonded monomer and the fully reduced monomer with dithiols. With the tendency to undergo disulfide-based modifications that are potentially redox-sensitive, roGFP-iL no longer exists as a two-state redox switch. The additional redox states exhibiting intermediate excitation fluorescence spectra make it highly unlikely for roGFP-iL to function as a ratiometric redox sensor.

Introduction

Oxidative folding involves the formation of disulfide bonds in proteins as they adopt native conformations. It often occurs in the endoplasmic reticulum (ER) where the redox environment is conducive to disulfide bond formation and enzymes are present to facilitate the oxidative folding process. Thiol oxidoreductases such as Ero1 (Endoplasmic Reticulum Oxidoreductin) and PDI (Protein Disulfide Isomerase) are known to play a key role in oxidative folding in eukaryotes. (Tu, Ho-Schleyer, Travers, & Weissman, 2000) Other ER oxidoreductases such as the QSOX (quiescin sulfhydryl oxidase) superfamily, PRDX4 (Peroxiredoxin), VKOR (vitamin K epoxide reductase), cytochrome P450s, flavin-containing monooxygenases, prolyl and lysyl hydroxylases, NOX4 (NADPH oxidase), L-gulonolactone oxidase, Erv2 (Essential for Respiration and Viability), and PDI homologues also influence ER redox state and oxidative folding. (Kodali & Thorpe, 2010) (Wajih, Hutson, & Wallin, 2007) (Margittai & Bánhegyi, 2010; Sevier & Kaiser, 2002) (Csala, Margittai, & Bánhegyi, 2010) Electron acceptors including dehydroascorbic acid, hydrogen peroxide, metalloproteins, FAD, vitamin E, reactive oxygen species, vitamin K, cystamine help drive oxidative folding. (Margittai & Bánhegyi, 2010; Sevier & Kaiser, 2002) (Csala et al., 2010) Glutathione may act as an electron acceptor, but is also required to maintain ER oxidoreductases in a reduced state and to reduce non-native disulfide bonds. (Jessop & Bulleid, 2004) (Chakravarthi & Bulleid, 2004) (Molteni et al., 2004)

The redox state of the ER influences the formation of disulfide bonds; a more oxidized state favors disulfide bond formation while a more reduced state disfavors disulfide bond formation. (Braakman, Helenius, & Helenius, 1992; Marquardt, Hebert, & Helenius, 1993) Additionally, ER redox state may affect oxidative protein folding by modifying the activity of Ero1. (Sevier et al., 2007) Thus, alteration of the ER redox state can influence oxidative folding.

In many proteins, disulfide bonds are integral to a protein's stability and structure and, consequently, its function. (Betz, 1993) (Wedemeyer, Welker, Narayan, & Scheraga, 2000) Failure to form the necessary disulfide bonds may affect a protein's function and lead to a diseased state. Several diseases are associated with altered disulfide bond formation. For instance, adiponectin, a hormone secreted by adipocytes, exists in three forms: trimer, hexamer, and 18-mer. The hexamer and 18-mer consist of, respectively, two and six trimer units held together by disulfide bonds. (Tsao et al., 2003) In patients with type 2 diabetes, the ratio of the

18-mer to total adiponectin is decreased relative to non-diabetic individuals. (Hotta et al., 2000) This relative decrease in the 18-mer could be due to an altered ER redox environment. ER stress characterized by induction of the unfolded protein response (UPR) is present in adipocytes and hepatocytes from obese rodents and humans who are susceptible to develop type 2 diabetes. (Ozcan et al., 2004) Altered ER redox environment was previously shown to accompany induction of ER stress. (Merksamer, Trusina, & Papa, 2008)

Additionally, alteration of the ER redox state and its subsequent effect on oxidative folding leads to activation of a compensatory UPR in the ER. The ER chaperone BiP binds unfolded or misfolded proteins that accumulate during redox imbalance, and activate the UPR. (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000) Reduction of disulfide bridges in ATF6, a main component of the UPR, is necessary for its activation. (Nadanaka, Okada, Yoshida, & Mori, 2007) UPR activation can counteract changes in ER redox state through activation of UPR components such as Nrf2 that contribute to redox homeostasis. (Cullinan & Diehl, 2004) Failure to compensate ER stress by activation of UPR can result in programmed cell death and is present in many diseases. (Kaufman, 2002) (Marciniak & Ron, 2006)

Despite its importance in protein folding and disease, however, the precise redox state of the ER remains largely elusive. While it is known that the environment in the ER is more oxidizing than that in the cytoplasm, measuring ER redox potential remains technically challenging.

A redox sensitive GFP, roGFP, was recently generated (Hanson et al., 2004) (Lohman & Remington, 2008) and has been used to study ER redox state. (Schwarzer et al., 2007) (Merksamer et al., 2008) (Delic, Mattanovich, & Gasser, 2010) In these redox sensitive GFPs (roGFPs), two cysteine residues were engineered into adjacent beta-strands on the surface of the protein barrel of wild type GFP. Hanson 2004 One of the original roGFPs, roGFP1, was later modified by single amino acid insertions to have an increased midpoint reduction potential, generating the roGFP-iX family. (Lohman & Remington, 2008) GFP has two excitation peaks that correspond to the neutral and anionic forms of the GFP chromophore. roGFP exhibits a ratiometric shift in the excitation fluorescence spectrum depending on the formation of an internal disulfide bond between the two engineered cysteine residues. Crystal structures of members of the roGFP-iX family suggest that the oxidized form favors the neutral chromophore

(excitation maximum at ~ 390 nm) while the reduced form favors the anionic chromophore (excitation maximum at ~ 475 nm).

The roGFP-iX family of redox sensors have higher reduction potentials than roGFP1 due to the conformational strain placed on disulfide bond formation by the extra amino acid insertion. (Lohman & Remington, 2008) The most oxidizing of the roGFP-iX family, roGFP-iL, has a reduction potential that may allow it to detect oxidizing changes in ER. When roGFP-iL was targeted to ER in cultured 3T3-L1 fibroblasts or adipocytes, it was unexpectedly found to have multiple disulfide-based covalent modifications (Mauser *et al*., unpublished observations). The present study was initiated to determine if roGFP-iL undergoes disulfide-mediated modifications *in vitro* and whether such modifications affect the fluorescent properties of roGFP-iL. Here we show that roGFP-iL can indeed exist in alternate oxidized forms *in vitro*, which can exhibit an excitation spectrum different from that of oxidized roGFP-iL monomer. Under such modifications, roGFP-iL is no longer a ratiometric redox sensor.

Methods

Expression and purification of roGFP-iL

His-tagged roGFP-iL in pQE30 vector was a gift from Dr. James Remington. (Lohman & Remington, 2008) The plasmid was expressed in BL-21 competent cells (Stratagene). Protein expression was induced by IPTG. The cells were harvested by centrifugation and lysed by freeze/thaw and sonication. Cell debris was removed by centrifugation. The supernatant was loaded onto a nickel-charged HiTrap Chelating column (GE Healthcare) and eluted with a step gradient of imidazole. RoGFP-iL was purified to homogeneity with an additional anion exchange chromatography step using quarternary ammonium resin and elution with a continuous sodium chloride gradient.

Preparation of in vitro roGFP-iL samples

Reduced and oxidized lipoic acid or reduced and oxidized glutathione were combined in 20 mM HEPES, 150 mM NaCl, and 1 mM EDTA, pH 7.0 to generate buffers with the indicated redox potentials using the Nernst equation. RoGFP-iL was added to a final concentration of 25 μM. Nitrogen was flowed into the reaction tube. The reaction was incubated at room temperature in the dark for the indicated time to prevent photobleaching.

Excitation Spectra

Excitation spectra were determined using a SLM Aminco T-format fluorescence spectrometer model 8000 with 8100 software. RoGFP-iL was diluted to a final concentration of 1.5 uM in 20 mM HEPES, 150 mM NaCl, and 1 mM EDTA at pH 7.0. Excitation scans from 350 nm to 500 nm were taken while emission was kept constant at 505±5 nm. Excitation spectra were normalized to the fluorimeter reference and to the isosbestic point of roGFP-iL (427 nm).

Non-reducing SDS PAGE

Samples were heated to 70°C for 15 min in non-reducing loading buffer. Equal amounts of protein were loaded and fractionated in either 12% or 4-12% polyacrylamide gels in MES-based SDS running buffer. Gradient 4-12% Bis-Tris gels were purchased from Invitrognen (Carlsbad, CA). Proteins on the gel were either stained with Coomassie Brilliant Blue and visualized using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) or transferred to nitrocellulose membrane for western analysis.

Western analysis

Western blots were performed following standard protocols. (Burnette, 1981) Membranes were stained with Ponceau S. to visualize proteins. Primary antibodies were 1:1000 dilutions of anti-glutathione (Arbor Assays), 1:1000 anti-myc epitope 9B11 (Cell Signaling Technology, Danvers MA), and 1:1000 anti-BiP (Cell Signaling Technology, Danvers MA). Secondary antibodies were IRDye 800CW Goat anti-Mouse IgG, IRDye 800CW Donkey anti-Rabbit IgG (Licor), and HRP Goat anti-Mouse (Research Products International, IL) in 1:2000 dilutions. Blots were visualized using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) or enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL).

Gel filtration chromatography

RoGFP-iL dimers and monomers were separated on a Superdex 75 column (GE Healthcare Biosciences, Piscataway, NJ). The column was equilibrated and eluted with 20 mM HEPES buffer, 150 mM NaCl, 1 mM EDTA, pH 7.0. The column was run at 1 mL/min at room temperature.

Calculated dimmer spectrum

Densitometry was performed using the Odyssey software to quantify the intensity of the bands corresponding to roGFP dimers, reduced monomers, and oxidized monomers. The percent dimer, reduced monomer, and oxidized monomer were determined to be 53.8%, 21.5%, and 24.8%, respectively, of the sample analyzed. The dimer spectrum was calculated by subtracting the contribution of the oxidized and reduced monomers from the total spectrum (*i.e.,* the spectrum corresponding to the sample on which densitometry analysis was performed) through the following equation:

$$\text{dimer spectrum} = \frac{\text{total spectrum} - (\text{percent reduced})(\text{reduced spectrum}) - (\text{percent oxidized})(\text{oxidized spectrum})}{\text{percent dimer}}$$

This calculation assumes equal fluorescence emission efficiency for the three species.

Results

RoGFP-iL has different excitation spectra in glutathione redox buffer compared to lipoic acid redox buffer (Figure 1). The differences in excitation spectra were more pronounced in the more reducing redox environments of -250 and -280 mV reduction potentials (Figure 1). Because glutathione is abundant in cells and because we aimed to use roGFP-iL to measure redox state in living cells, we looked for the cause of this difference.

In order to eliminate the possibility that the samples were becoming oxidized by air, it was confirmed that samples prepared in a glove box with degassed buffers or treated with N-ethylmaleimide (NEM) to preserve free thiols from further disulfide bond formation showed identical excitation spectra to that of samples prepared on the open bench and incubated under nitrogen. (Data not shown.) Additionally, it was confirmed that possible intrinsic fluorescence of reduced or oxidized lipoic acid or glutathione was not contributing to differences between the spectra of roGFP-iL in glutathione versus lipoic acid redox buffer. (Data not shown.)

In both glutathione redox buffer and lipoic acid redox buffer, roGFP-iL formed dimers (Figures 2 and 4, bottom panel). Additionally, in glutathione redox buffer, roGFP-iL became glutathionylated (Figure 2). Glutathionylation was strongest at -220 mV and was not observed at -280 mV or -160 mV.

Addition of lipoic acid redox buffer to roGFP-iL in glutathione redox buffer caused roGFP-iL to exhibit an excitation spectrum characteristic of roGFP-iL in lipoic acid redox buffer (Figure 3), eliminated glutathionylation of roGFP-iL (Figure 4, top) and decreased dimer formation (Figure 4, bottom).

In order to examine covalent roGFP-iL dimers' fluorescent property, we used size exclusion column chromatography to separate roGFP-iL dimers from monomers. RoGFP-iL dimers have an excitation spectrum that is intermediate between the fully reduced and fully oxidized excitation spectra (Figure 5).

Discussion

These results show that roGFP-iL undergoes disulfide-mediated modifications, some of which affect its excitation spectrum.

Ratiometric GFP-based reporters of redox state have previously been generated. In these redox sensitive GFPs (roGFPs), two cysteine residues were engineered into adjacent beta-strands on the surface of the protein barrel of wild type GFP. Hanson 2004 One of the original roGFPs, roGFP1, was later modified by single amino acid insertions to have an increased midpoint potential, generating the roGFP-iX family. (Lohman & Remington, 2008) RoGFP has two excitation peaks that correspond to the neutral and anionic forms of the GFP chromophore. RoGFP exhibits a ratiometric shift in the excitation spectrum depending on the formation of an internal disulfide bond between the two engineered cystine residues. It has been assumed that the oxidized form favors the neutral chromophore (excitation maximum at ~ 390 nm) while the reduced form favors the anionic chromophore (excitation maximum at ~ 475 nm).

RoGFP has been used to determine ambient redox potential in subcellular compartments. (Cheng et al., 2010) (Brach et al., 2009; Jiang et al., 2006; Meyer, 2008; Meyer & Brach, 2009; Meyer et al., 2007; Michaelson, Shi, Ward, & Rodney, 2010; Wolf, Asoh, Ohsawa, & Ohta, 2008; Yu et al., 2009) (Schwarzländer, Fricker, & Sweetlove, 2009) (Chi, Waypa, Mungai, & Schumacker, 2010; Haga, Remington, Morita, Terui, & Ozaki, 2009) (Desireddi, Farrow, Marks, Waypa, & Schumacker, 2010) (Gutscher et al., 2009) (Waypa et al., 2010) (Rhee, Chang, Jeong, & Kang, 2010; Rosenwasser et al., 2010; Wedgwood et al., 2010) (Loor et al., 2010b) (Loor et al., 2010a) A few groups have used roGFP to measure ER redox state (Schwarzer et al., 2007)

(Merksamer et al., 2008) (Delic et al., 2010). Schwarzer *et al.* demonstrated that the ratio of oxidized to reduced roGFP1 as a function of reduction potential is similar between roGFP1 *in vitro* and cytosolic roGFP1 *in vivo* in cystic fibrosis nasal epithelial cells and CFTR-corrected cells in terms of the shape of the curve, suggesting similar redox responsiveness. However, the ratio was smaller *in vivo* compared to *in vitro*. (Schwarzer et al., 2007) They could observe reducing changes in the ER after adding DTT, but not oxidizing changes after adding H_2O_2. They determined ER-targeted roGFP1 to be approximately 94% oxidized *in vivo* in cultured epithelial cells. Merksamer *et al.* used roGFP2 to measure reducing changes caused by DTT, tunicamycin, inositol starvation, and overexpression of the secretory protein CPY variants in yeast. They reported that eroGFP2 was 96.9% oxidized (± 0.3% SD) in vivo. Thus, the reporter could not indicate oxidizing changes. Delic *et al.* tested roGFP-iE and roGFP-iL in the ER of the yeast Pichia pastoris. They report that roGFP-iL and roGFP-iE were not fully oxidized in the ER of unstressed cells and were able to measure both oxidizing and reducing changes.

This current study with roGFP-iL shows that, in addition to the oxidized and reduced monomeric forms, roGFP-iL exists in alternative oxidized forms. RoGFP-iL dimer has an excitation spectrum that is intermediate to the oxidized and reduced monomeric spectra. RoGFP-iL also formed a mixed disulfide conjugate with glutathione. It is currently unclear whether glutathionylation of roGFP-iL affects its excitation spectrum. However, because glutathionylation of roGFP-iL occurs preferentially at reduction potentials around -250 mV, the reduction potential dependence of roGFP-iL glutathionylation may affect the ability of the GFP to accurately report redox potential.

Presence of these modified versions of roGFP has not been documented in previous studies with either roGFP-iL or the parent roGFPs, roGFP1 and roGFP2. Even though wild type GFP is highly susceptible to formation of dimers, dimer formation has only been observed in roGFP1 and roGFP2 during crystallization of the proteins at concentration greater than 1 mM and was considered to be an artifact of crystal packing. (Cannon & Remington, 2006; Hanson et al., 2004) The fluorescence properties of these dimers have also not been reported by the respective studies. Crystal structures of oxidized and reduced roGFP-iE and roGFP-iR show that the redox-active cysteine residues are located on a surface with a dense cluster of hydrophobic side chains. Disulfide bonds between two roGFP-iL monomers are likely buried within the hydrophobic interface contributed by both roGFP-iL monomers and as a result stabilize the

dimer. We recently generated a mutant roGFP-iL with A206K substitution to reduce the hydrophobicity of the region near the redox-active cysteine residues. Preliminary studies of A206K roGFP-iL showed a dramatically decreased tendency to form dimers compared with parent roGFP-iL.

While these modifications of roGFP-iL indicate that roGFP-iL is not likely to function as a ratiometric reporter of redox state, it may still be useful in indicating relative changes in reduction potential. Additionally, *ex vivo* data (not shown) indicate that these modifications may make roGFP-iL useful in detecting changes in the ER redox state in living cells. The reported midpoint potential of roGFP-iL is -229±5 mV (Lohman & Remington, 2008), and the expected midpoint reduction potential of the ER is about -180 mV. (Hwang, Sinskey, & Lodish, 1992) Thus, we expected ER localized roGFP-iL to be fully oxidized and poorly efficient in detecting oxidizing changes in the ER. However, in cultured differentiated 3T3-1L cells expressing ER localized roGFP-iL, we could monitor transient development of oxidizing conditions upon treatment with the strong oxidizing agent diamide. (Sarkar *et al*., unpublished data) We speculate that the intermediate fluorescence spectra of roGFP-iL dimers and conjugates may contribute to the ability to measure oxidizing changes in the ER at the expense of losing their ratiometric fluorescence properties.

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Figure Legends

Figure 1
Excitation scan monitoring emission at 505 nm of roGFP-iL incubated for 3 hours at a reduction potential of -160 mV (a), -190 mV (b), -220 mV (c), -250 mV (d), or -280 mV (e) generated by lipoic acid (dotted lines) or glutathione (solid lines). Data were normalized to isosbestic point (427 nm) and smoothened.

Figure 2
roGFP-iL incubated for 3 hr at the indicated reduction potential generated by glutathione. Top: Western blot using an anti-glutathione antibody Bottom: Coomassie stain of a duplicate gel

Figure 3
Excitation scan monitoring emission at 505 nm. roGFP-iL incubated for 2 hr with 2.5 mM reduced glutathione (thin dashes), 2 hr with 2.5 mM reduced lipoic acid (bold dashes), or 2 hr with 2.5 mM reduced glutathione followed by 2 hr with 2.5 mM reduced lipoic acid (solid). Data normalized to isobestic point (427 nm) and smoothened.

Figure 4
Excitation scan monitoring emission at 505 nm. roGFP-iL incubated for 2 hr with 2.5 mM reduced glutathione (thin dashes), 2 hr with 2.5 mM reduced lipoic acid (bold dashes), or 2 hr with 2.5 mM reduced glutathione followed by 2 hr with 2.5 mM reduced lipoic acid (solid). Data normalized to isobestic point (427 nm) and smoothened. Top: Western blot using an anti-glutathione antibody Bottom: Ponceau S. stain

Figure 5
Excitation scan monitoring emission at 505 nm of roGFP-iL dimer (solid) or monomer (dotted) fractions from a Supradex75 column.

Figures

Figure 1

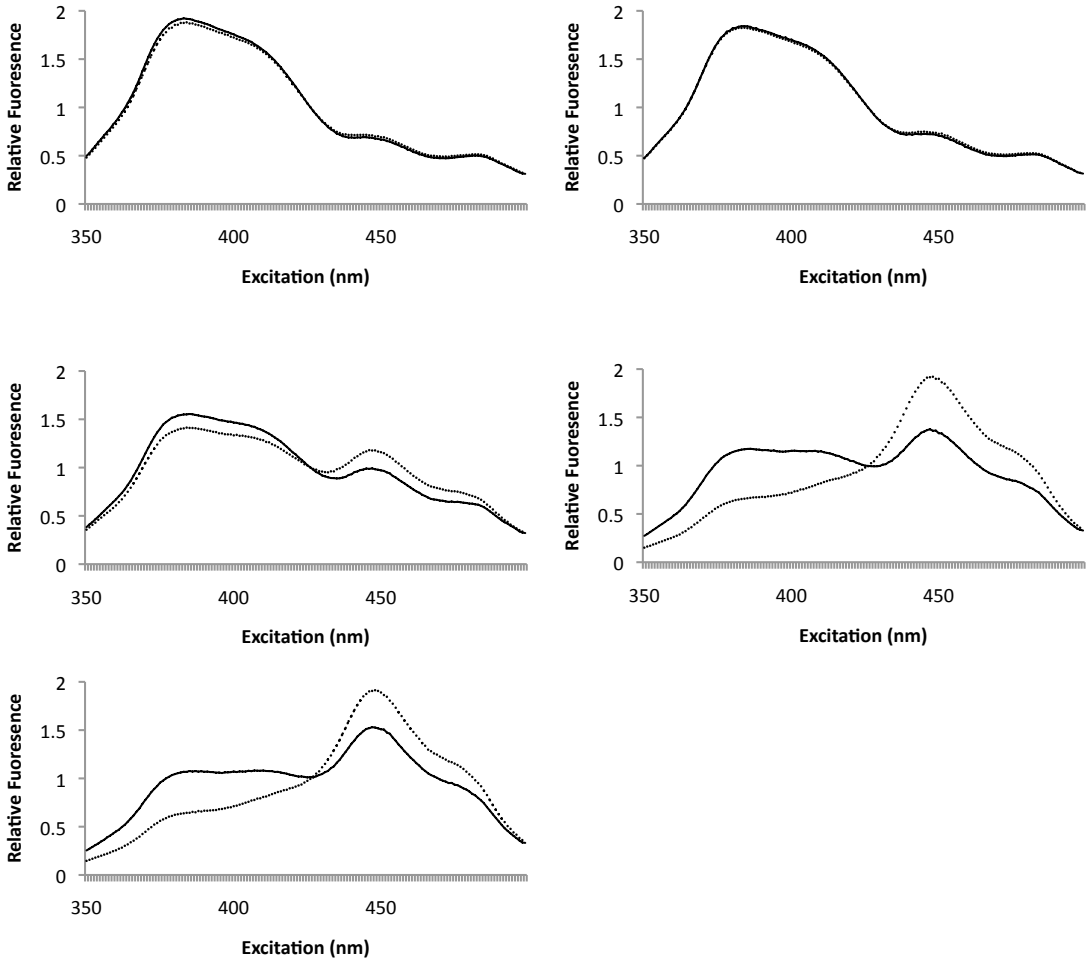


Figure 2

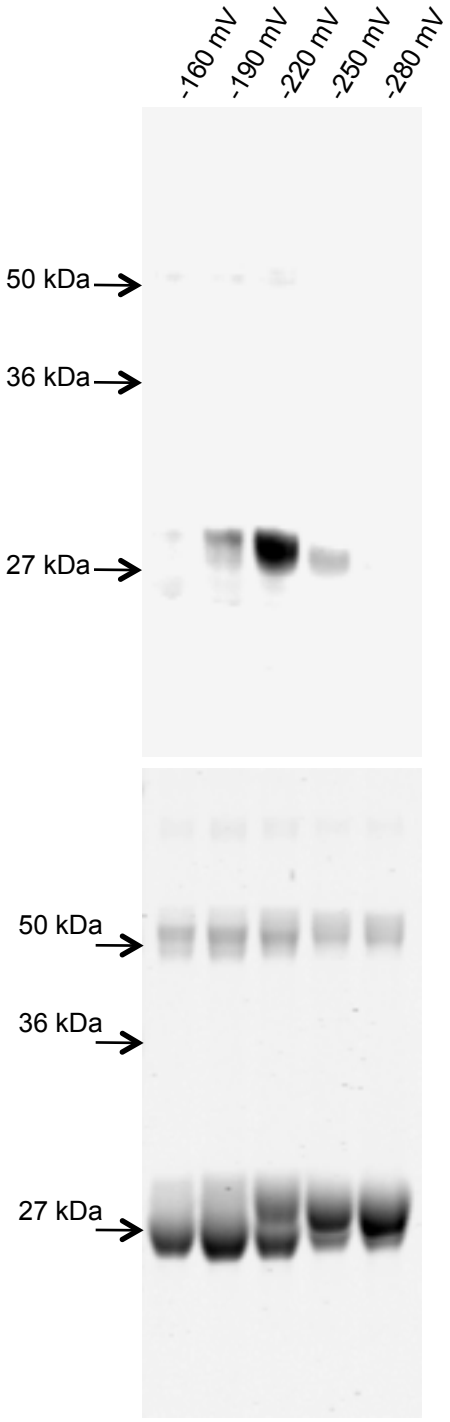

-160 mV
-190 mV
-220 mV
-250 mV
-280 mV
50 kDa
36 kDa
27 kDa
50 kDa
36 kDa
27 kDa

Figure 3

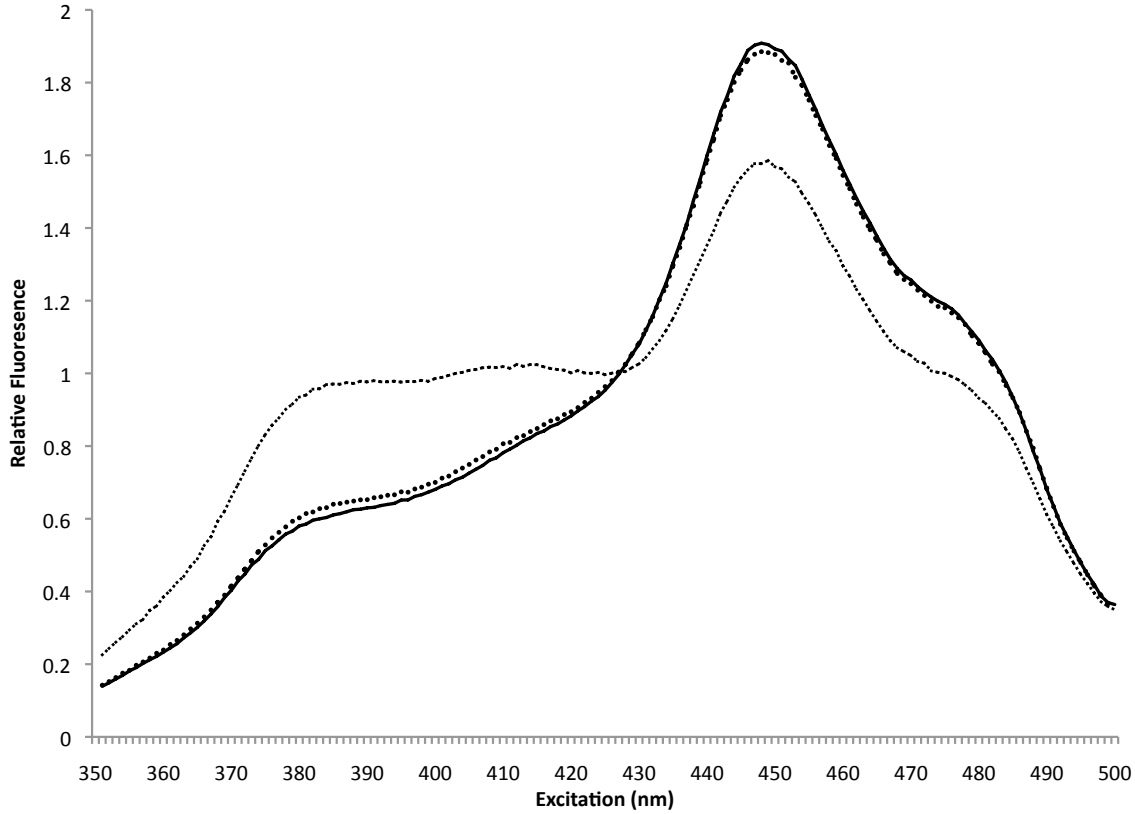

Relative Fluoresence
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
350
360
370
380
390
400
410
420
430
440
450
460
470
480
490
500
Excitation (nm)

Figure 4

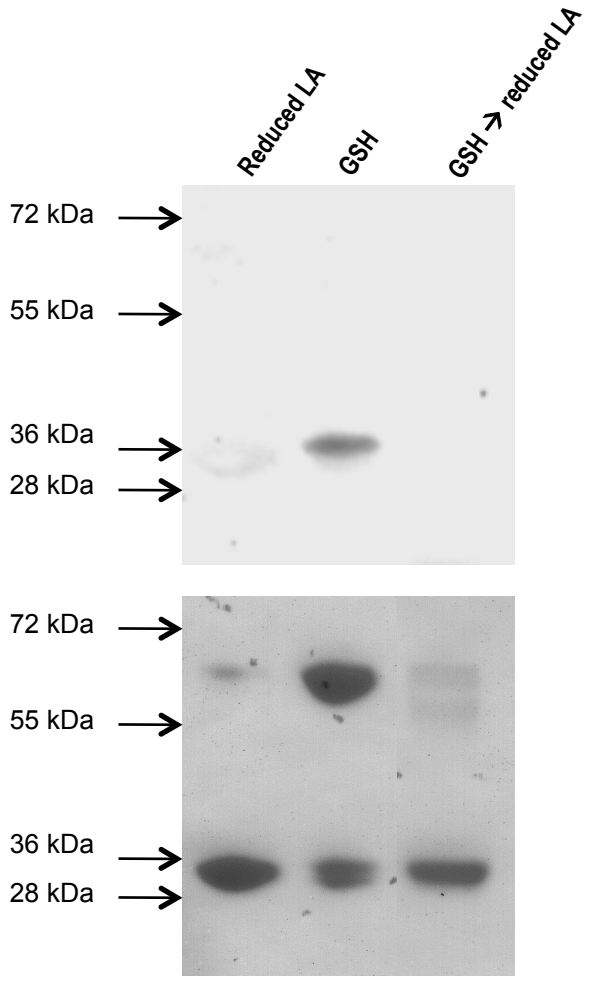
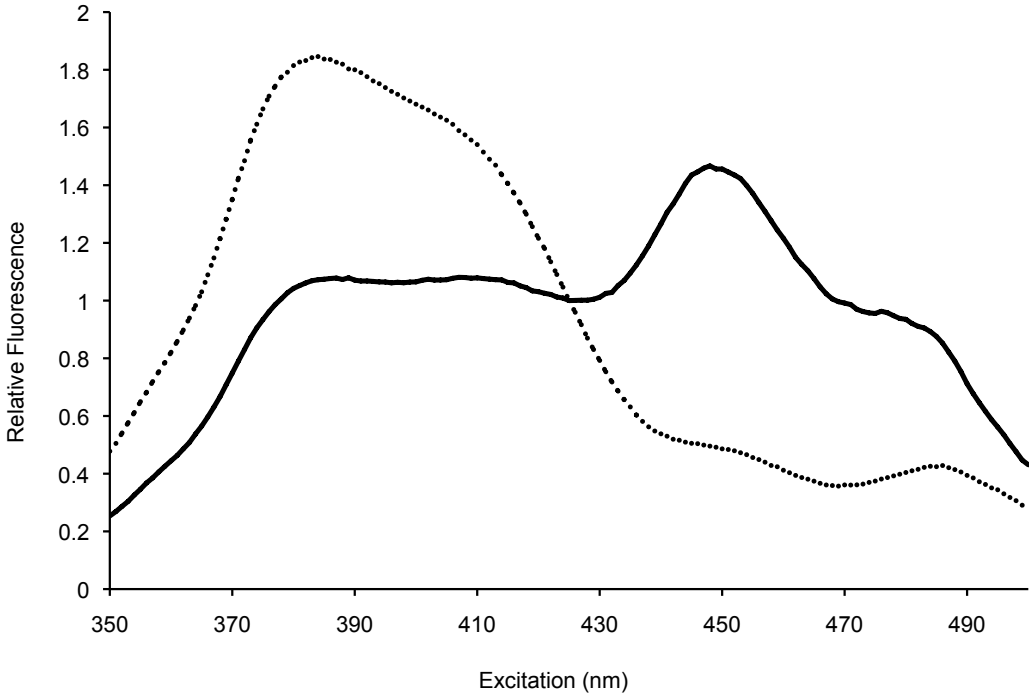

Reduced LA
GSH
GSH → reduced LA
72 kDa
55 kDa
36 kDa
28 kDa
72 kDa
55 kDa
36 kDa
28 kDa

Figure 5


Relative Fluorescence
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
350
370
390
410
430
450
470
490
Excitation (nm)