Supplemental Information

Challenges in the evaluation of thiol-reactive inhibitors of human protein disulfide isomerase

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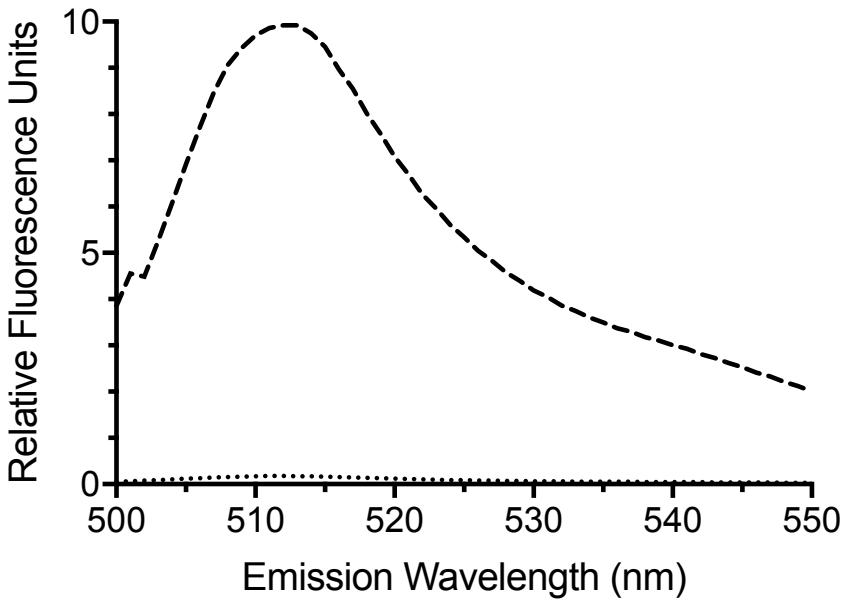

Fig. S1. Fluorescence of BD-SS and BD-SH exciting at 490 nm. Excitation and emission slit widths were 4 and 8 nm, respectively. BD-SS (150 nM in phosphate buffer) shows very low fluorescence (•••) until reduced by 10 mM DTT to BD-SH (15 min; – – –).

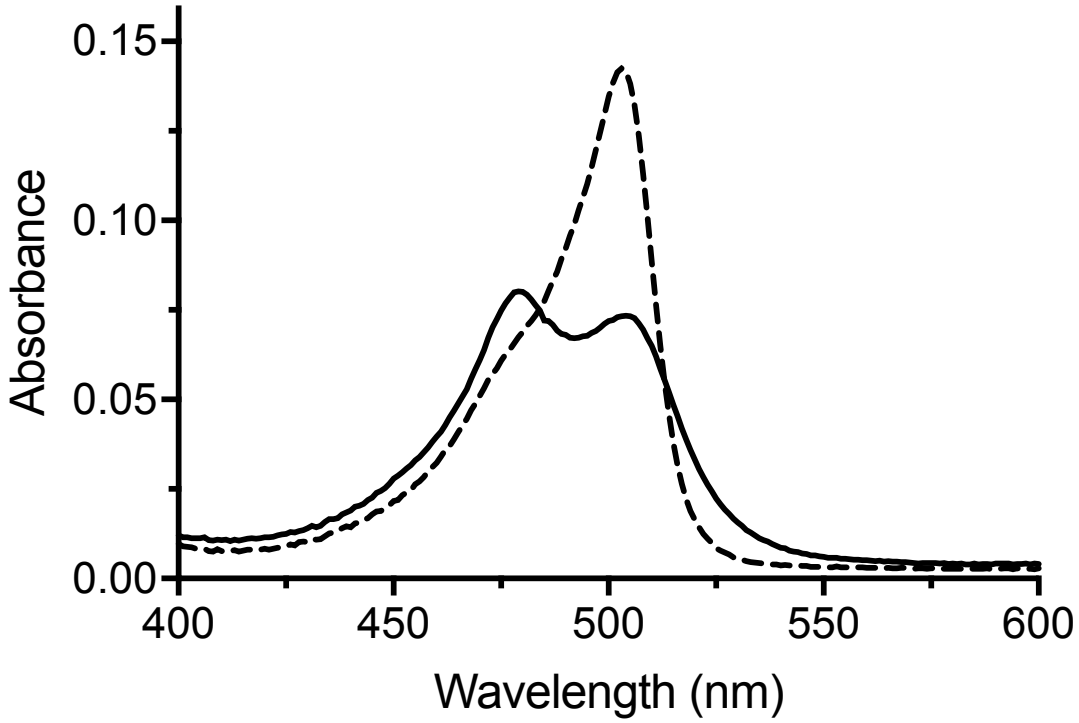

Fig. S2. Comparison of absorbance spectra of BD-SS and BD-SH. A solution of BD-SS (715 nM, phosphate buffer; ——) was incubated for 15 mins with excess DTT to produce BD-SH (– – –).

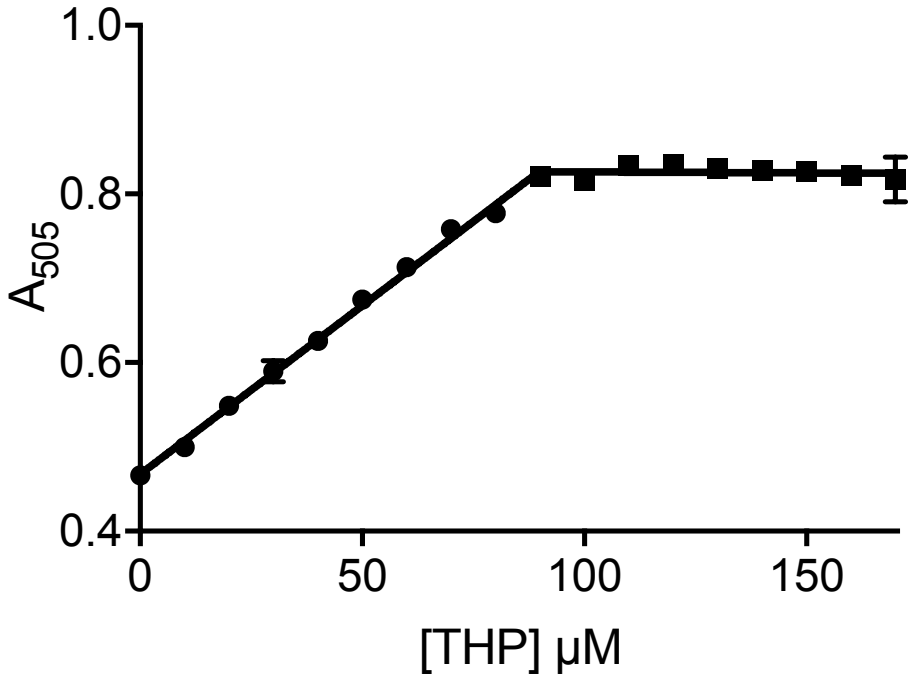

Fig. S3. Extinction coefficient determination for BD-SS. Aliquots of a stock solution of BD-SS were incubated in phosphate buffer for 3 h with 0 – 170 μM THP (previously calibrated with DTNB). The mixtures were then diluted 10-fold and absorbances at 505 nm were plotted as a function of the THP concentration. The results of 3 independent experiments are shown. The extinction coefficient for BD-SS was calculated from the inflexion point.

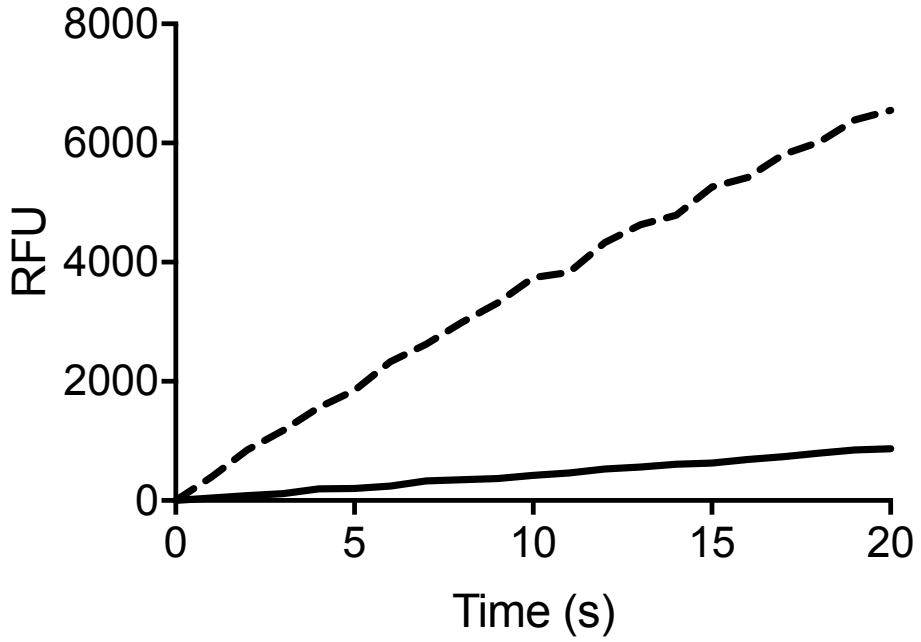

Fig. S4. Background fluorescence increase for Di-E-GSSG and BD-SS in the presence of 5 mM GSH. Di-E-GSSG was synthesized as described previously by Raturi and Mutus (*Free Radicals in Biology and Medicine* **43**, 2017, 62-70). A solution of 700 nM fluorophore was completely reduced by incubating with 5 mM GSH and 1 μM PDI for 45 min in order to set the gain of the plate reader (set to 90% by exciting at 490 nm, emitting at 513 nm for BD-SS; and exciting at 520 nm, emitting at 550 nm for Di-E-GSSG). Initial increase in fluorescence was measured by injecting 700 nM BD-SS (——) or 700 nM Di-E-GSSG (– – –) into a solution containing 5 mM GSH. Background fluorescence was subtracted and data represent an average of triplicate experiments. Rates were calculated to be 43.5 ± 0.3 RFU/s for BD-SS and 345.4 ± 3.4 RFU/s for Di-E-GSSG using a linear regression calculation over the first 20 s post-mixing.

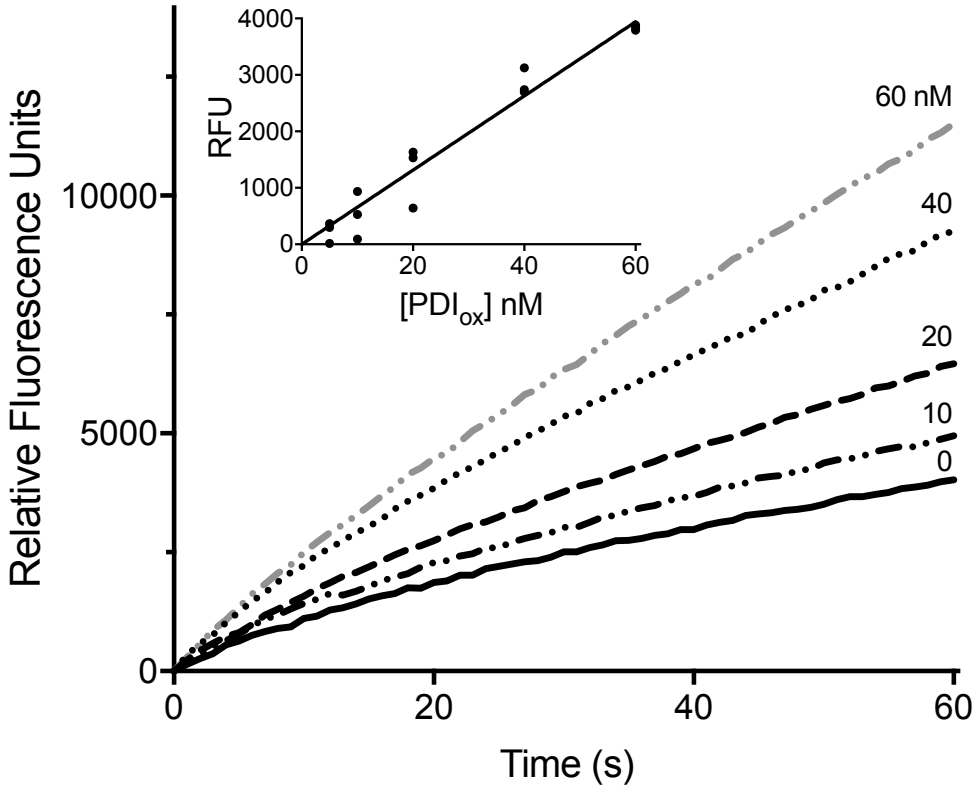

Fig. S5. Multiple turnover assay of PDI using TCEP as reductant, performed as described in the main text. BD-SS reduction is followed using 250 μM TCEP in buffer with the indicated concentrations of PDI_{ox}. The inset shows the dependence of the fluorescence at 30 s recorded in triplicate as a function of PDI_{ox} concentration after correction for the non-enzymatic reduction of BD-SS by TCEP.

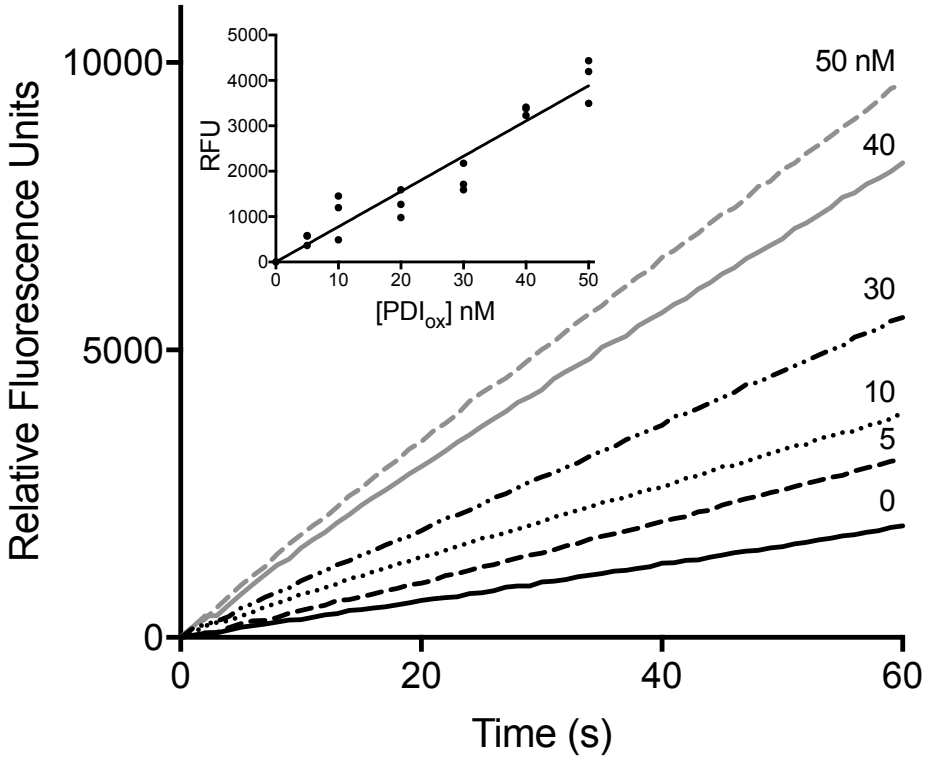

Fig. S6. Reduced RNase as a reductant in PDI multiple turnover assay. Reduced RNase was prepared as described in the main text. Assays followed BD-SS reduction following a 714 nM injection of BD-SS into a solution containing 10 μM rRNase thiols in buffer with the indicated concentrations of PDI_{ox}. The inset depicts the dependence of the fluorescence at 30 s recorded in triplicate as a function of PDI_{ox} concentration after correction for the non-enzymatic reduction of BD-SS by rRNase (see main panel).

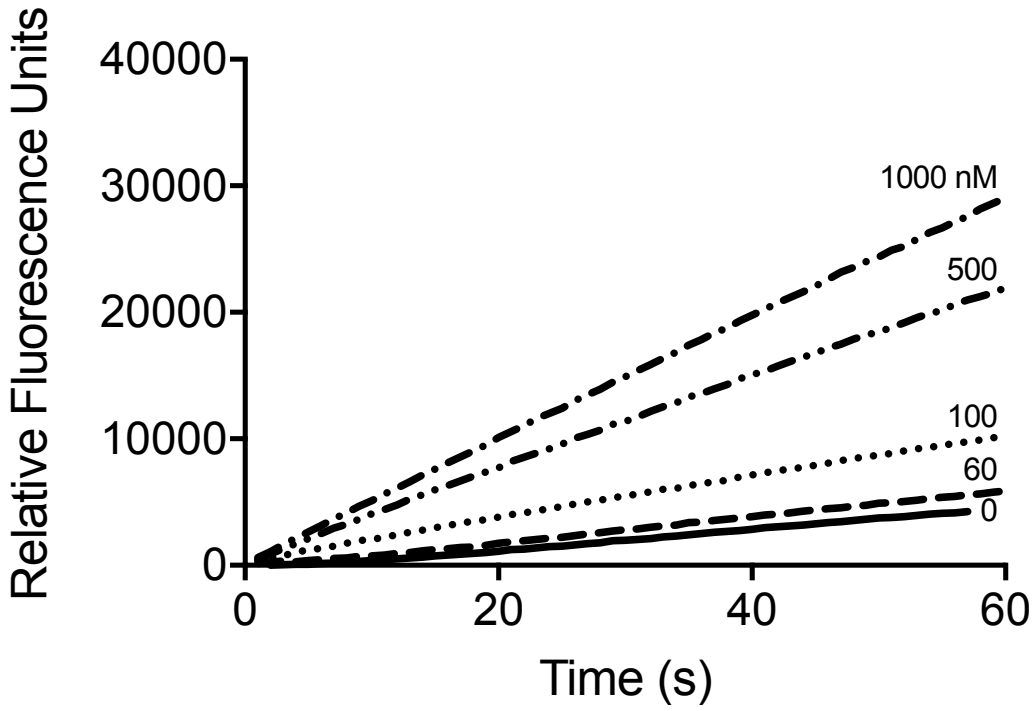

Fig. S7. The BD-SS assay can be extended to human thioredoxin. Multiple turnover assay in the presence of 5 mM GSH and 0-1000 nM human thioredoxin. Relative fluorescence was measured following the addition of 714 nM BD-SS in triplicate, corrected for background BD-SS fluorescence. Activity of the protein can be measured in the mid micromolar range.

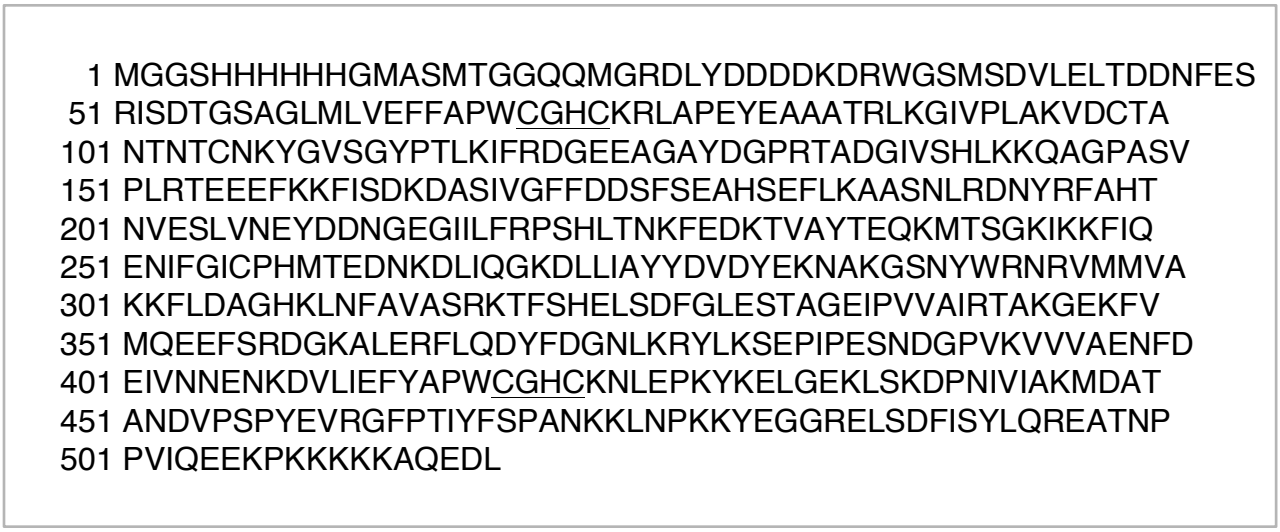

Fig. S8. ERp57 amino acid sequence and protein preparation. An *E. coli* codon optimized gene encoding the above amino acid sequence for 6X His-tagged human ERp57 was synthesized (Genewiz) and cloned into the expression vector pTRCHisA (Invitrogen) using NheI and HindIII sites. The sequence was verified before transfecting into the *E. coli* BL21(DE3) strain. Starter cultures were added to LB media supplemented with ampicillin (100 μg/mL) and grown to an OD_{600} of 0.6 at 37 °C before induction with 0.5 mM IPTG. Cells were harvested after 6 h and the pellet was resuspended in 50 mM potassium phosphate, pH 8.0, containing 300 mM NaCl. Following lysis by French press (2 passes at 10,000 psi) and sonication, the sample was centrifuged at 15,000 rpm for 30 min. The supernatant was removed immediately and incubated overnight with nickel agarose resin (GoldBio). After application to a column, protein was washed with 30 mL 50 mM potassium phosphate, pH 8.0 containing 300 mM NaCl, and 30 mL of the same buffer including 25 mM imidazole. Elutions of 50, 100, 200 and 300 mM imidazole in buffer were collected and fractions containing the purest protein, analyzed by SDS-Page, were dialyzed against 2 X 4 L of 50 mM potassium phosphate, 1 mM EDTA, pH 7.5. Once concentrated, the protein was stored at 4 °C. When needed, reduced protein was prepared as described for PDI in the main text.

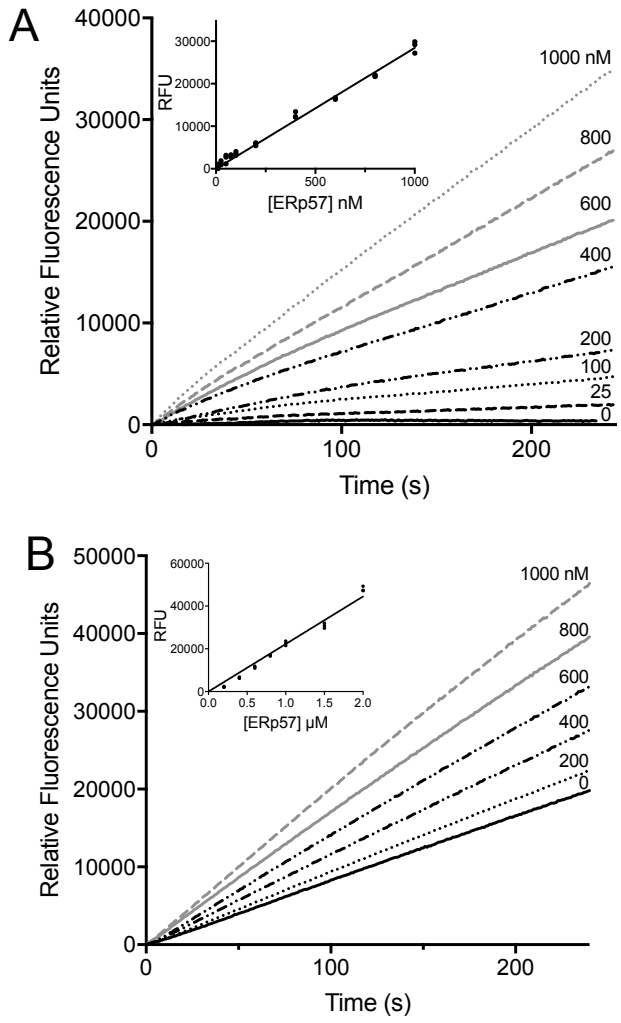

Fig. S9. Multiple turnover assay with ERp57. ERp57 was added to 5 μM DTT (A) or 5 mM GSH (B) in buffer and the relative fluorescence was monitored over 240 s following injection of BD-SS (to 714 nM) into the wells. All curves represent the average of triplicate experiments, corrected for background BD-SS fluorescence increase. The inset shows the relative fluorescence after 200 s as a function of ERp57 concentration.

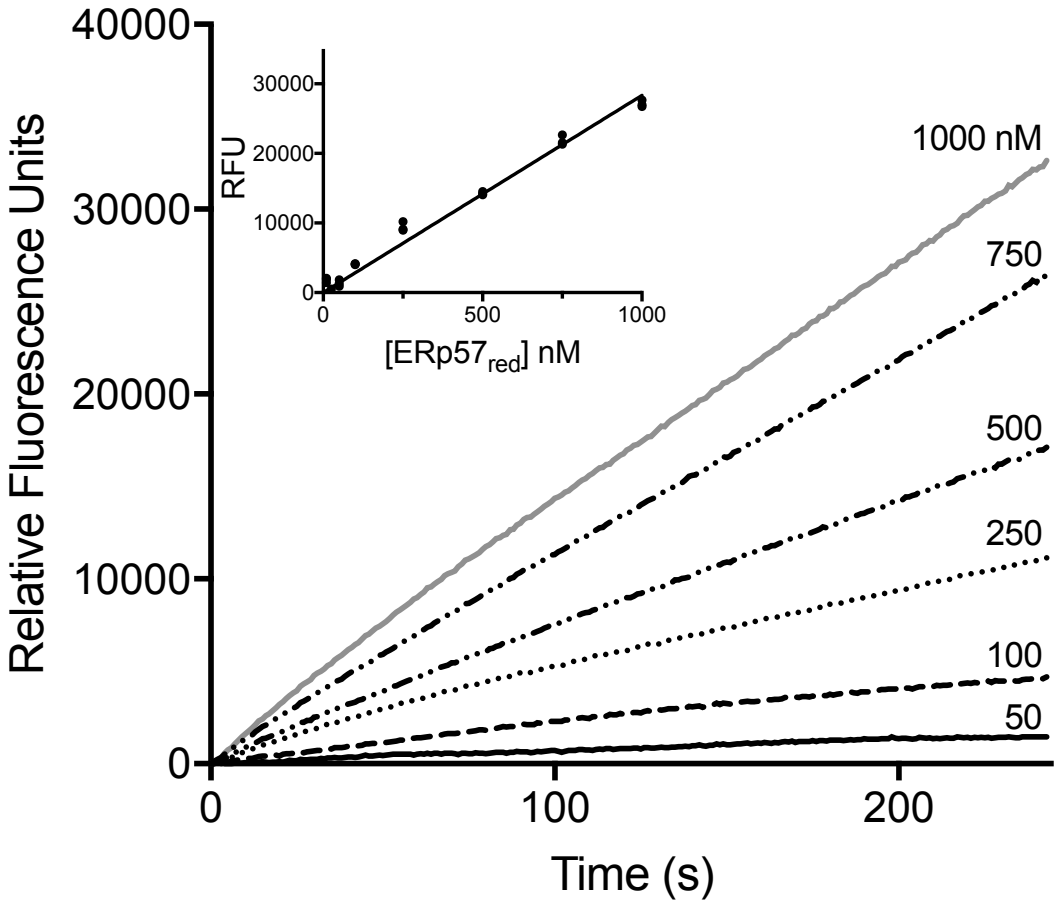

Fig. S10. **Reoxidation assay of ERp57.** Injections of 714 nM BD-SS were made into solutions containing a range $ERp57_{red}$ concentrations in buffer. The relative fluorescence was measured in triplicate over 240 s, corrected for BD-SS background. Inset depicts the relative fluorescence at 200 s as a function of $ERp57_{red}$ concentration.

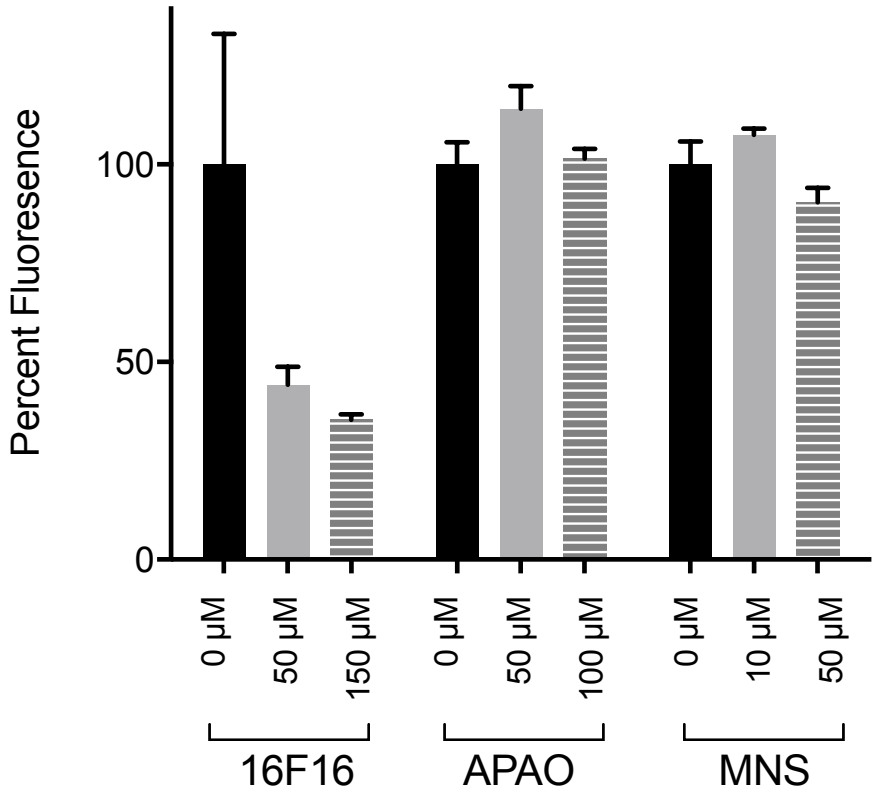

Fig. S11. PDI_{red} activity in the presence of 5 mM GSH and inhibitor. A solution of 75 nM PDI_{red} was incubated with 5 mM GSH and inhibitor (16F16 using 0, 50 and 100 μM; APAO using 0, 50 and 100 μM; and MNS using 0, 10, and 50 μM) for 15 min before the addition of 714 nM BD-SS. The percent fluorescence after 30 s, normalized to PDI_{red} activity in the absence of inhibitor was plotted for triplicate experiments.

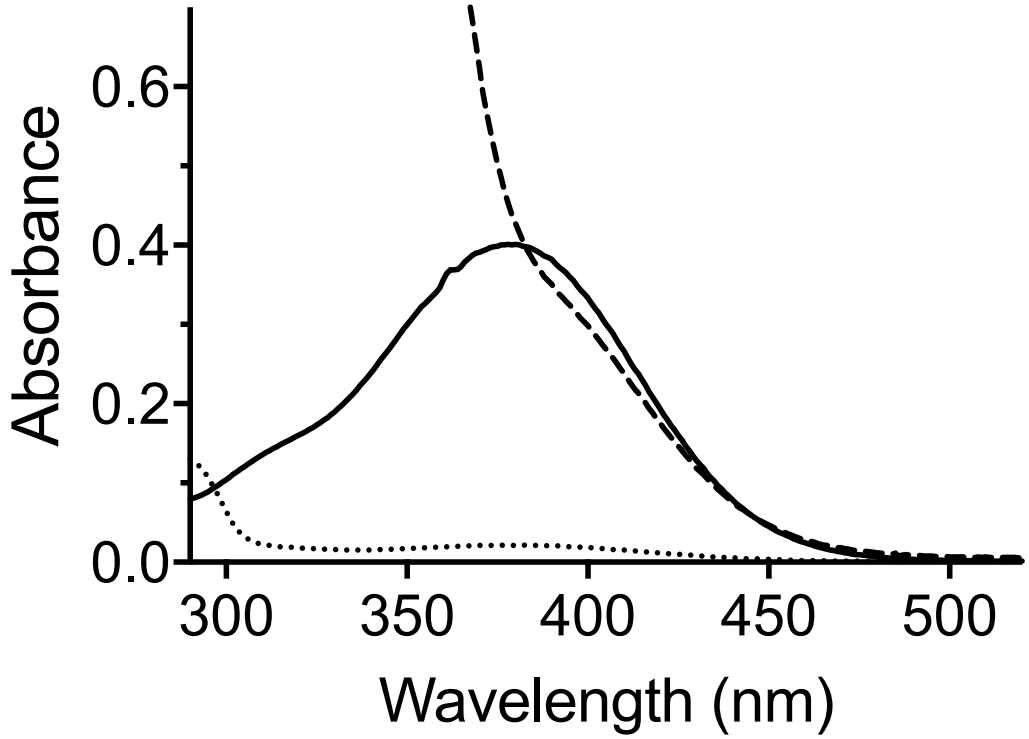

Fig. S12. Absorbance changes upon Michael adduct formation between GSH and MNS are reversed by NEM. MNS (—— ; 40 μM in buffer) is almost completely bleached upon addition of 400 μM GSH (•••). The kinetics of this reaction are followed at 378 nm (Figure 6; main text). After 1 h the addition of 5 mM NEM (50 μL to a final volume of 1.05 mL) reversed the bleaching (– – –).

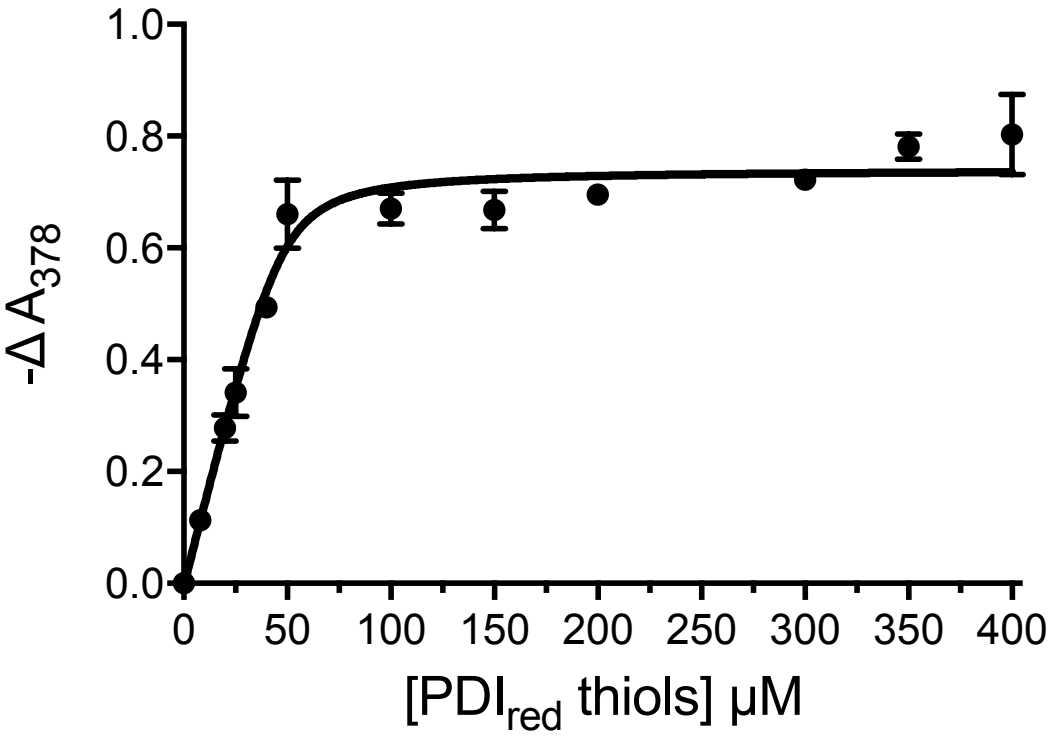

Fig. S13. **PDI_{red} addition to MNS.** PDI_{red} thiol concentration was measured before each use using DTNB to confirm full reduction (4 thiols). A 100 μM solution of MNS was titrated with 0 – 100 μM PDI_{red}, and absorbance at 378 monitored over 1 hour. Total change in absorbance, calculated as described in the main text, was plotted for triplicate data as a function of PDI_{red} thiol concentration.

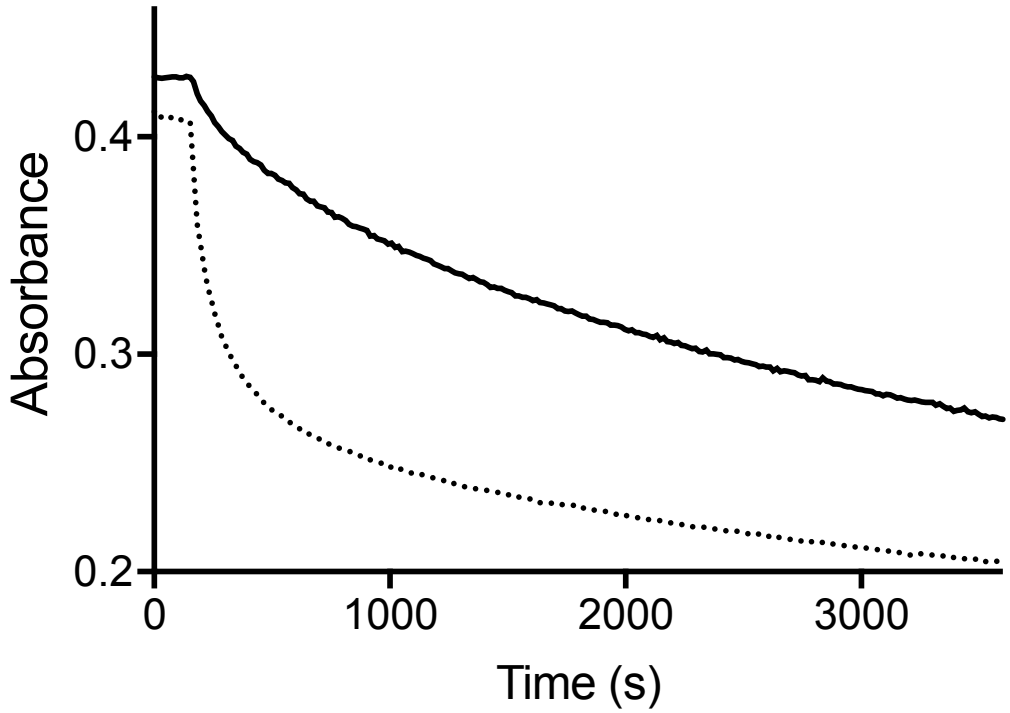

Fig. S14. Structured proteins interact with MNS. Solutions of aldolase and pyruvate kinase were prepared in buffer and water, respectively. A 20 μM aldolase solution was determined to have 40 μM surface accessible thiols via DTNB in phosphate buffer. Upon addition of 40 μM MNS, a decrease in absorbance at 378 nm is observed (•••). A 10 μM pyruvate kinase solution containing 10 μM thiols via DTNB thiol titer, was mixed with 40 μM MNS. A slow decrease in absorbance at 378 nm depicts conjugation of MNS to the protein (——). Curves for aldolase and pyruvate kinase represent duplicate and triplicate experiments, respectively.

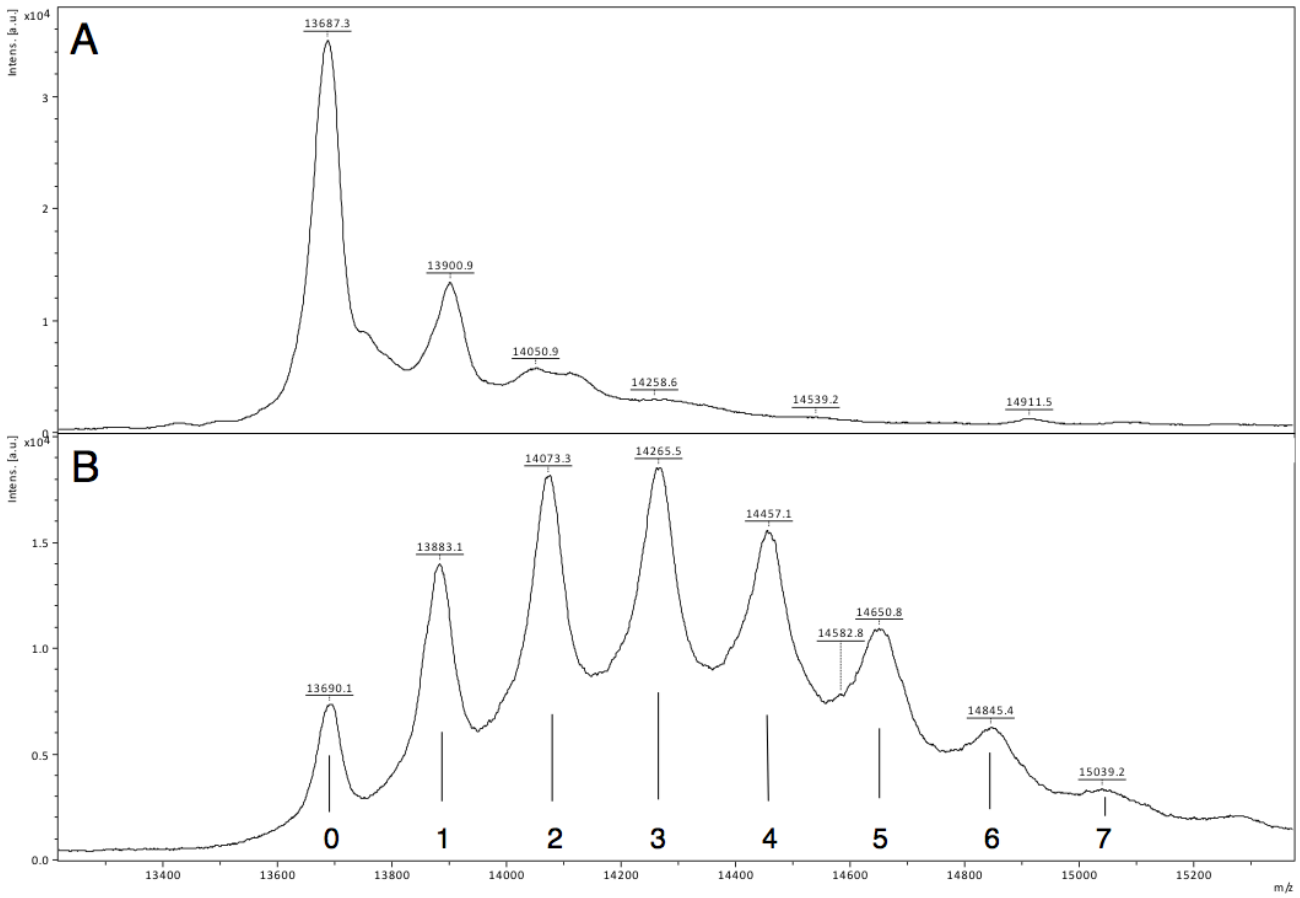

Fig. S15. MALDI-TOF spectra. Mass spectrometry was performed on a Bruker MicroFlex MALDI-TOF. A 10 μM solution of rRNase alone (A) or mixed with 70 μM MNS (B) in phosphate buffer, 0.1% TFA was incubated for 20 min at RT. To prepare the sample, a C18 Zip-Tip (Millipore) was first washed with acetonitrile, 0.1% trifluoroacetic acid (TFA), followed by a wash with 1:1 acetonitrile:water, 0.1% TFA and next equilibrated twice with water, 0.1% TFA. The protein solution was then drawn into the tip, and washed three times with water, 5% methanol, 0.1% TFA. A freshly-prepared matrix solution of saturated α-cyano-4-hydroxycinnamic acid (Sigma) in water was used to elute the sample onto a Bruker MSP 96 target ground steel BC plate (Part no. 8280799). Numerals shown on the spectrum (B) indicate the number of MNS additions to rRNase. (MNS = 193.16 g/mol). The ladder of masses reflects a partial loss of the labile Michael adducts during ionization of the sample.