

Supporting Information

Quantification of effect of DTT on exchange kinetics.

Table S1. **Relative rate constants compared with the rate constant of β LG with stoichiometrically added DTT.** We performed an exchange experiment with the PDG-PEG_{10K}-SS-Pyr at pH 6.0 using DTT alone

<i>Conditions of exchange</i>	<i>$k_{exch} : k_{exch} (\beta LG + 0.95 \text{ equ. DTT})$</i>
DTT Alone	3
β LG + 1.2 equiv DTT	2
β LG + 0.5 equiv DTT	1

(at the same molarity, 0.2mM diluted to 0.1mM during the exchange), β LG with 0.95 equivalents of DTT (just as in all the experiments), β LG with 1.2 equivalents of DTT, and β LG with 0.5 equivalents of DTT. The pyr-2-thione release for this experiment was followed for 30 minutes rather than 24 hours.

Comparing the rate constants of each group against the condition of β LG + 0.95 equivalents of DTT, DTT alone and β LG with excess DTT (1.2 equivalents) significantly increased the rate constant by 2-3 times. Adding only half the necessary DTT, such that only half of the β LG would have been separated, the rate constant does not change significantly.

These differences in rate constants indicate that DTT alone reacts much faster than β LG, and that excess DTT does react with the disulfide macromer, affecting the rate constant. However, if not enough DTT is added the rate constant is not very different than close to stoichiometric amounts. This, combined with information from literature (Cleland, J. Biochem.1963) that cyclized DTT, which forms after it reduces a disulfide bond, is sterically favorable and not likely to revert to its linear or active form, indicates that DTT is not contributing to the reaction with the PDG-PEGx-SS-Pyr when added stoichiometrically. β LG dimerizes again 24 hrs after being reconstituted in PBS and separated with stoichiometric amounts of DTT, and the reacted DTT is not removed (Table S2). Also, in all of the exchange experiments, β LG does not undergo complete exchange, even after 24 hrs (Figure 3) . All of these facts support the hypothesis that DTT can be stoichiometrically added to pre-react with dimerized β LG without affecting the kinetics of disulfide exchange.

Table S2. **Mole fraction of free thiols measured by Ellman's Reagent.** The three thiols were reconstituted in pH 7.4 buffer at 0.2mM. DTT was added to β LG at 0.95 equivalents immediately after reconstitution and allowed to react for 30 minutes. The number of free thiols in each solution was measured using Ellman's reagent after 30 min, 1 h 45 min, and 24 h (the length of the exchange experiment) to determine extent of free dimerization in solution.

	<i>Conditions prior to reaction</i>	<i>1 hr. 45 minutes after reconstitution</i>	<i>24 hrs. after reconstitution</i>
GSH	0.93	0.89	0.11
β LG (+ DTT)	1.03	1.09	0.12

BSA	0.36	0.34	0.32
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The initial concentrations of each thiol (0.2mM) based on mass, were compared to the number of free thiols measured by Ellman's Reagent at different times. When the solutions are left at 0.2 mM, initially all of the expected GSH and β LG have free thiols (mole fraction ~ 1) while BSA has roughly 40% free. BSA is known to be a mixture of monomer, dimer, and sometimes higher order tetramer and hexamer. The fraction of each depends on vendor, purification method, and lot. The solutions were allowed to incubate at room temperature at 0.2mM, while some of each solution was used for exchange experiments diluted to 0.1mM for 24 h. These unreacted solutions were measured again with Ellman's Reagent after 1h 45 min. and again after 24 h. While there is no significant loss of free thiols after 1hr 45 min. for any of the thiols, after 24 hrs. the GSH and β LG free thiols are decreased to roughly 10% and the BSA is relatively unchanged (decreasing only 4%). The rate constants are fairly high for GSH and β LG, such that all of the reactions are complete before 1h 45 min, thus the dimerization of those thiols should be insignificant in the calculation of rate constants of disulfide exchange. BSA, though being mostly oxidized/dimerized to begin with, is relatively stable, not continuing to dimerize over the course of the exchange experiment. The remaining free thiols in the exchange solutions were also measured by Ellman's after the 24 h reaction. During the reaction, free thiols attack the activated disulfides, releasing pyridine-2-thione, a thioketone that cannot react with other free thiols. Any free thiols remaining in the solution are unreacted thiols from the original source. Thus moles of pyridine-2-thione, free thiols, and oxidized/dimerized thiols should be conserved. Assuming moles that are unaccounted for by Ellman's or the pyridine-2-thione release are either oxidized or dimerized, the relative changes in oxidized/dimerized thiols after the reaction are as expected and likely unable to affect the rate constants for disulfide exchange.

Table S3. Mole percent tracked through the reaction by Ellman's Reagent and pyridine-2-thione (pH=7.4, thiols reacted with PDG-PEG_{4k}-SS-Pyr).

GSH Before rxn			After rxn	
			Pyr-2-thione released	73%
Free thiols	95%		Free thiols	17%
Oxidized/dimerized	5%		Oxidized/dimerized	10%
βLG Before rxn			After rxn	
			Pyr-2-thione released	30%
Free thiols	100%		Free thiols	50%
Oxidized/dimerized	0%		Oxidized/dimerized	20%
BSA Before rxn			After rxn	
			Pyr-2-thione released	3%
Free thiols	35%		Free thiols	29%
Oxidized/dimerized	65%		Oxidized/dimerized	68%

It is relevant to note that, similar to the protein solutions incubated for 24hrs, there is some increase in the oxidized/dimerized component of GSH and β LG, but this would not have occurred until the maximum exchange has completed and there is still a considerable amount of

free thiol remaining. The BSA also has a very slight, probably insignificant increase, and its extent of reaction is still very low.