Supporting Information:

Reagentless Oxidative Folding of Disulfide-Rich Selenopeptides is Catalyzed by an Intramolecular Diselenide

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Abbreviations

TFA, trifluoroacetic acid; DTNP, 2,2'-dithiobis(5-nitropyridine); MTBE, methyl *tert*-butyl ether; HPLC, high performance liquid chromatography.

Results and Discussion

Inter- vs. Intra-molecularity

Several routes were considered to determine whether the catalysis of disulfide formation by an intramolecular diselenide was an inter- or intra-molecular effect. Interestingly, oxidation reactions appear to show both intermolecular and intramolecular character.

k_{ox} measures the rate of formation of the first disulfide bond, as this was derived from the depletion of the reduced peptide (bearing either six thiols *or* four thiols and a diselenide), and therefore represents the first oxidation step. k_{ox} is used exclusively to determine the inter- or intra-molecularity, as k_{native} also represents isomerization events as well as oxidation (see Data Analysis, below).

The invariance of k_{ox} over a 10-fold range in concentration suggests an intramolecular mechanism (Figure S1). However, if the reaction is exclusively intramolecular catalysis, then a 1:1 mixture of unmodified peptide with its diselenide-containing analog would give two distinct rates of depletion of the reduced peptide, both

being unchanged from their individual rates when folded separately. However, this was not the case, as is shown in Figure 2b and e (and insets), for GVIA and SIIIA, respectively.

Thus, the catalysis of disulfide bond formation by an intramolecular diselenide demonstrates both inter- and intra-molecular character. However, the co-folding reactions (combining an unmodified peptide with its diselenide-containing analog) may contain an artificial bias towards intermolecular catalysis, as the uncatalyzed folding of the unmodified peptides is extremely slow. Also, intermolecular diselenide catalysis differs in rate from catalysis with an intramolecular diselenide, despite the total diselenide and peptide concentrations being constant across conditions (Figure 3a and Table S1).

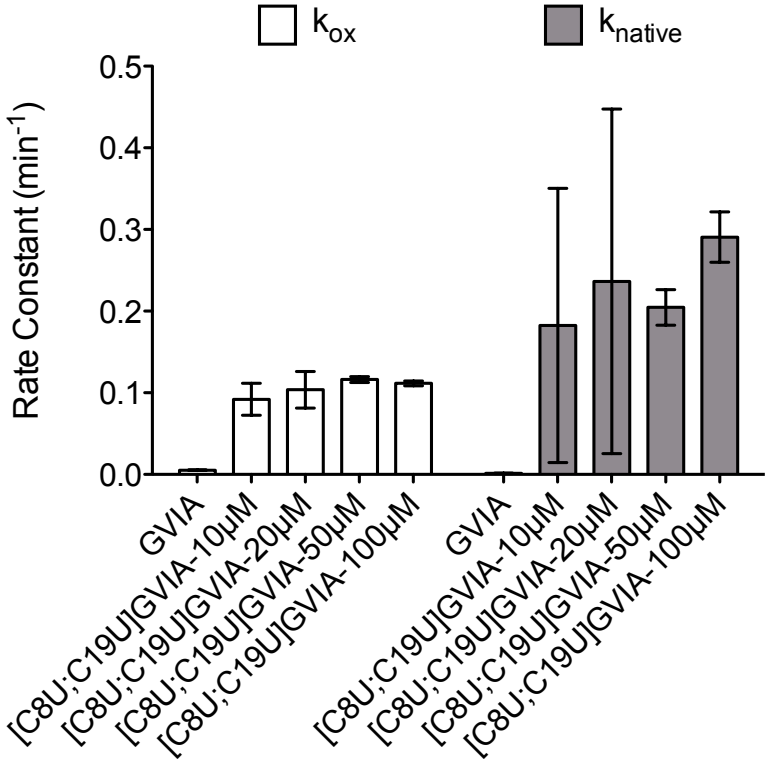


Figure S1: The effect of peptide concentration on folding rate and disappearance of the "linear" form for [C8U;C19U] GVIA. Equivalent rates for GVIA (at 20 μM) are also shown for comparison.

Numerical Values for Rate Constants

In the table below, we present the numerical values for the parameters involved in the analysis of the oxidative folding of GVIA and SIIIA and their selenopeptide analogs.

The results in the table below fit the data to the following equation, charting the extent of folding with respect to time, in minutes (given in Equation (1)).

$$[F](t) = \gamma \cdot (1 - \frac{k_{native}}{k_{native} - k_{ox}} e^{-k_{ox} \cdot t} + \frac{k_{ox}}{k_{native} - k_{ox}} e^{-k_{native} \cdot t})$$

γ is the final yield, accounting for the fact that not all of the terminally oxidized peptide achieves the native disulfide connectivity. k_{ox} provides the rate constant for the initial oxidation step, and k_{native} provides the rate constant for all subsequent oxidation and isomerization steps leading towards native disulfide formation.

Table S1: Kinetic Parameters

Folding Substrate	γ	k_{ox} (min^{-1})	k_{native} (min^{-1})
SIIIA	0.850	0.00201 ± 0.0000595	0.000355 ± 0.0000416
[C3U;C13U] SIIIA	0.801	0.0121 ± 0.000654	0.00521 ± 0.001863
[C4U;C19U] SIIIA	0.823	0.0257 ± 0.000960	0.0121 ± 0.00130
[C8U;C20U] SIIIA	0.625	0.0393 ± 0.00263	0.009942 ± 0.001793
GVIA	0.319	.00526 ± .000210	0.00132 ± 0.000279
10 μM [C8U;C19U] GVIA	0.673	0.0921 ± 0.0196	0.183 ± 0.1679
20 μM [C8U;C19U] GVIA	0.736	0.1038 ± 0.02238	0.236 ± 0.211
50 μM [C8U;C19U] GVIA	0.771	0.116 ± 0.00373	0.205 ± 0.0218
100 μM [C8U;C19U] GVIA	0.805	0.112 ± 0.00298	0.291 ± 0.0310
GVIA + 100 nM $CuCl_2$	0.169	0.0351 ± 0.00268	0.109 ± 0.590
[C8U;C19U] GVIA + 100 nM $CuCl_2$	0.723	0.110 ± 0.00395	0.147 ± 0.0165
SIIIA + 20 μM Selenocystine	0.799	0.0831 ± 0.00539	0.00516 ± 0.000479
GVIA + 20 μM Selenocystine	0.615	0.0573 ± 0.00598	0.00542 ± 0.00181
[C8U;C19U] GVIA + 20 μM Selenocystine	0.630	0.0948 ± 0.00590	0.185 ± 0.0536

Table S1: Numerical values for thermodynamic and kinetic parameters of the oxidation of SIIIA, GVIA and their selenopeptide analogs. *Note: The kinetic model used for these analyses is not valid for the copper(II)- and selenocystine-catalyzed oxidation reactions, but the model was used for these reaction systems for effective comparison with catalysis by an intramolecular diselenide.*

Unless otherwise indicated, the peptide concentration was 20 μM. γ is 'unitless,' and represents the fraction of total peptide that achieves the native disulfide connectivity. The units for k_{ox} and k_{native} are both min^{-1}.

Provided error ranges are standard errors.

Kinetic Traces of Folding with Linear Timescales

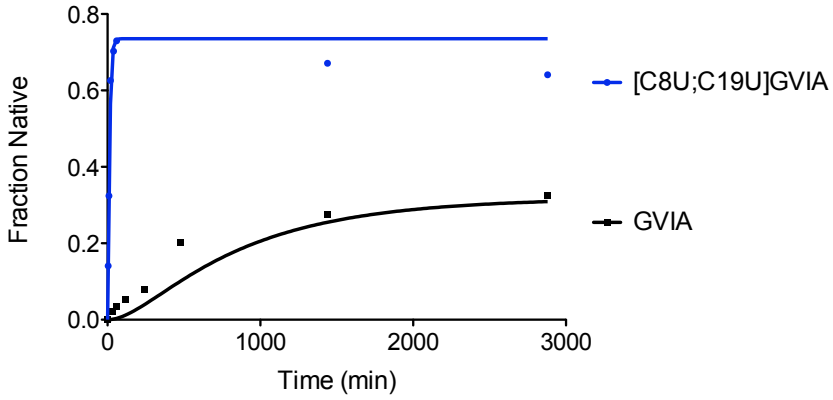


Figure S2: Kinetic traces of folding of GVIA and [C8U;C19U] GVIA, shown with a linear timescale.

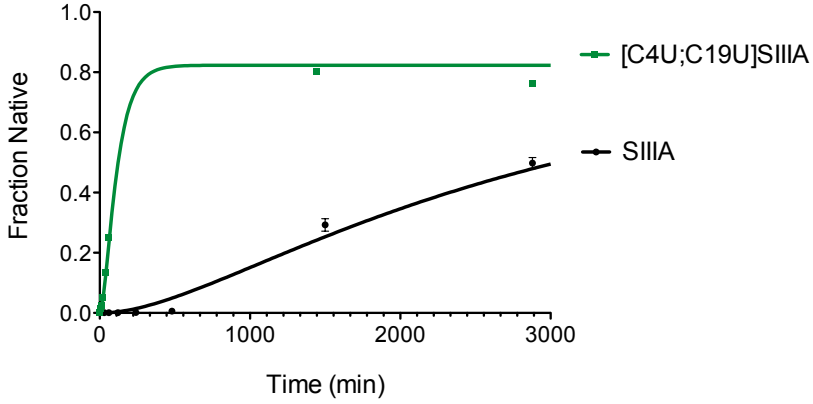
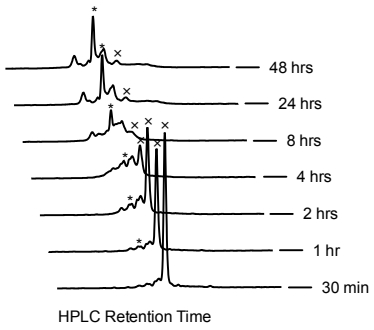


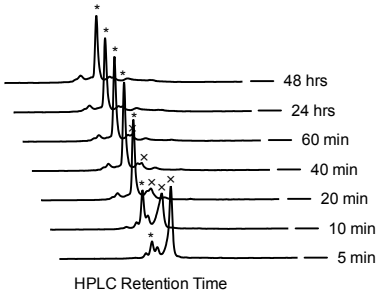
Figure S3: Kinetic traces of folding of SIIIA and [C4U;C19U] SIIIA, shown with a linear timescale.

Folding Chromatograms

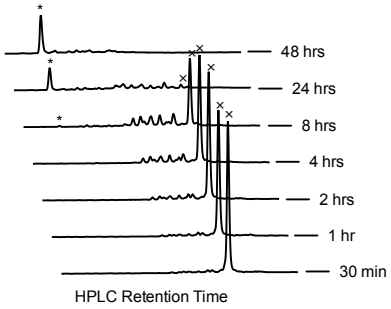
The figure below shows the HPLC chromatograms of reagentless folding reactions with respect to time for GVIA, [C8U;C19U] GVIA, SIIIA, [C3U;C13U] SIIIA, [C4U;C19U] SIIIA and [C8U;C20U] SIIIA. In all chromatograms, * indicates the native peak, and x indicates the reduced peptide.



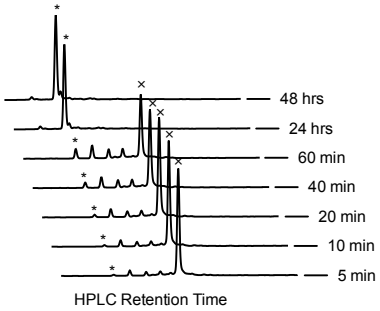
GVIA



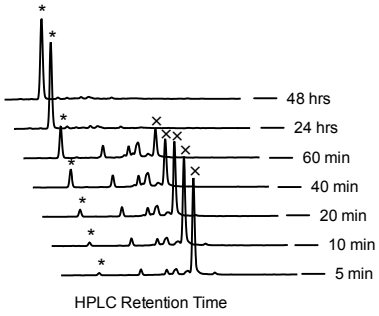
[C8U;C19U] GVIA



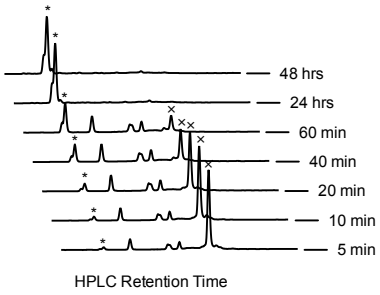
SIIIA



[C3U;C13U] SIIIA



[C4U;C19U] SIIIA



[C8U;C20U] SIIIA

Figure S4: Representative HPLC of folding chromatograms for each timepoint. * indicates the natively folded form, and x indicates the linear/diselenide-only form. Peaks were integrated and analyzed as described in the Data Analysis section.

Methods

Reduced Peptides

Peptides were synthesized on a single-channel automated peptide synthesizer using Rink Amide resins and standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. All cysteine residues were trityl protected, and all selenocysteine residues were 4-methoxybenzyl protected. Peptides were cleaved from the resin with either Reagent K (for non-selenocysteine-containing peptides) or Reagent K‡ (for selenocysteine containing peptides). Reagent K contained a 33:2:2:2:1 mixture of TFA : Phenol : water : thioanisole : 1,2-ethanedithiol; Reagent K‡ contained a 178:14:5:1.6:1 mixture of TFA : Phenol : water : thioanisole : DTNP. All peptides were cleaved for three hours. Cleaved peptides were filtered and precipitated with MTBE. Selenocysteine-containing peptides were then subjected to 2 hours of reduction (incubation in 50mM DTT, 0.1M Tris, pH 7.5 with 0.1mM EDTA), which was quenched by addition of formic acid to 8% (final). Peptides were then purified by HPLC on either a Waters 600 chromatograph or a Waters 2535 Quaternary Gradient Module, using a semi-preparative Vydac C18 column (218TP510). All peptides (reduced and oxidized) were purified using a gradient that ran from 5% to 35% B90 (89.9% acetonitrile, 10% water, 0.1% TFA), with the remainder being 0.1% TFA in water. The identify of the reduced peptides was then validated by electrospray ionization mass spectrometry.

Oxidative Folding

All oxidations were air-mediated, using molecular oxygen as the only oxidant, except as indicated.

Dried, reduced peptides were redissolved in 5% acetonitrile, 0.01% TFA (94.99% water) to 200 or 400 μM. In most cases, the peptide was dissolved to 200 μM; however, for folding reactions with a final peptide concentration of 100 μM, a more concentrated solution was used in order to limit the effect of the TFA on the final buffered pH.

Folding mixtures were then prepared, containing everything except the reduced peptides. Concentrations were chosen so that upon addition of the reduced peptides, the folding reactions would contain 0.1 M Tris, pH 7.5, 0.1 mM EDTA, as well as any other additives (the amount of EDTA was adjusted down to 1 nM for folding reactions containing 100 nM $CuCl_2$ as an oxidative catalyst). This mixture was allowed to thermally equilibrate for at least 5 minutes in a block set to 20^o C.

Peptide was then added to each folding reaction, and timepoints were taken as necessary. For selenocysteine-

containing peptides, timepoints were at 5, 10, 20, 40 and 60 minutes, and 24 and 48 hours. For peptides that did not contain selenocysteine (or an oxidative catalyst), timepoints were taken at 0.5, 1, 2, 4, 8, 24 and 48 hours. For folding reactions with copper(II) chloride as an oxidative catalyst, timepoints were taken at 10, 20, 40 and 60 minutes, and 2, 24 and 48 hours. For reactions with selenocystine as an oxidative catalyst, timepoints were taken at 5, 10, 20, 40, 60 minutes, 24 and 48 hours. Each timepoint contained 2 nmol of peptide, and was added to a tube containing 15% of the volume of the timepoint of 1 M HCl. This was then transferred to a pre-chilled HPLC vial, and 1.67 nmol was injected onto an analytical Vydac C18 column (218TP54) with a Waters e2695 Separations Module. For more concentrated folding reactions, 0.1% TFA (in water) was added to a final volume of 120 μl for increased precision in folding analysis. Samples are kept at 4^o C until they are run on the analytical HPLC. All timepoints were analyzed by analytical HPLC within 24 hours of the timepoint being taken.

All folding reactions were performed in 0.1 M Tris, pH 7.5. Upon quenching with HCl, the pH is lowered to approximately 1.45. Any further dilution that occurred with 0.1% TFA in water (only in more concentrated folding reactions) maintained the pH below 2.0.

Unless otherwise indicated, all folding reactions were performed with 20 μM peptide.

Data Analysis

The first step in data analysis was to use Empower 3 to perform HPLC peak integration. This provided the percent of the total peptide that was present as either reduced or native peptide at each timepoint. The elution times of native peptide are known from our previous work (**?**), and the elution times of reduced peptides were shown using reduced peptides that had been kept under acidic conditions, prohibiting disulfide formation.

Data were fit to a slightly modified kinetic model of the following chemical reaction, where R denotes the reduced peptide (bearing either six thiols *or* four thiols and a diselenide), I denotes any folding intermediates and F denotes folded peptide with the native disulfide connectivity.

$$\mathrm{R} \xrightarrow{k_{ox}} \mathrm{I} \xrightarrow{k_{native}} \mathrm{F}$$

This assumes that every oxidation is irreversible. The classical model for product formation in this reaction sequence is provided below, and is given in (**?**).

$$[F](t) = (1 - \frac{k_{native}}{k_{native} - k_{ox}} e^{-k_{ox} \cdot t} + \frac{k_{ox}}{k_{native} - k_{ox}} e^{-k_{native} \cdot t})$$

However, this had to be modified, since one of the assumptions is that all terminally oxidized forms are equivalent, which is not the case for our material (there are numerous folding isomers). Consequently, we also incorporated a multiplicative factor of γ, representing the fraction of the material that actually proceeds to the native disulfide connectivity (final yield). Thus, our analyses were done with Equation (1), below.

$$[F](t) = \gamma \cdot (1 - \frac{k_{native}}{k_{native} - k_{ox}} e^{-k_{ox} \cdot t} + \frac{k_{ox}}{k_{native} - k_{ox}} e^{-k_{native} \cdot t}) \quad (1)$$

DynaFit was used to solve Equation (1) for γ, k_{ox} and k_{native} simultaneously, using both the depletion of the reduced peptide ($\frac{d[R]}{dt}$) and the native formation ($\frac{d[F]}{dt}$). The maximum value was set to 0.95, as this was the approximate purity of the reduced peptides. The yield of the reaction (γ) was solved for by adding another component, wherein I goes to N (non-native folding products) at a rate of k_{non}. Because both reactions from intermediates to native product or intermediates to non-native folding products are assumed irreversible,

$$\gamma = \frac{k_{native}}{k_{native} + k_{non}}$$

Error values provided are formal standard errors, as given by DynaFit. Because the fitting software only used information on the depletion of the linear form (R) and the appearance of the native fold (F), the value of k_{non} is determined relative to the value of k_{native} in order to maintain a reasonable value for γ; consequently, errors were not calculated on the derivation of γ.

All computations were performed with DynaFit. Graphical preparations were done with PyMOL, ChemBioDraw and Prism 5.