Supplementary Information

Coupling effects of thiol and urea-type groups for promotion of oxidative protein folding

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Table of Contents

1. Materials

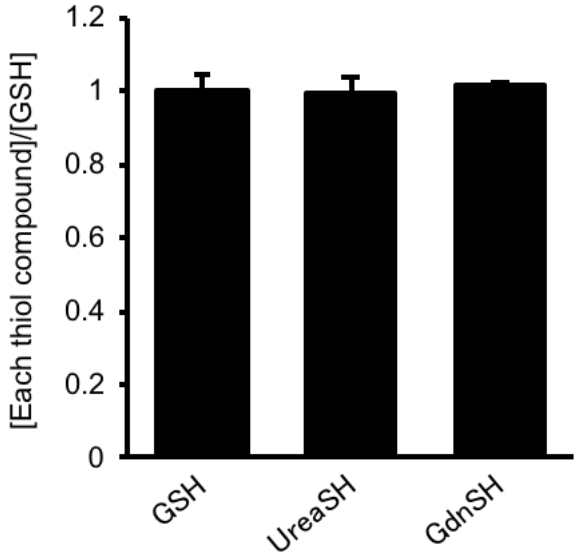
Deuterated solvents were purchased from Kanto Chemicals (Tokyo, Japan). 4-Acetamido-4-maleimidylstilbene-2,2 disulfonic acid (AMS) was purchased from Invitrogen (Carlsbad, CA, USA). Acetonitrile, 28% ammonia solution, 35% hydrochloric acid and 2-propanol were purchased from Kishida Chemical (Tokyo, Japan). Coomassie brilliant blue, 5,5'-dithiobis(2-nitrobenzoic Acid) (DTNB), 1,4-dithiothreitol (DTT), L-glutathione oxidized (GSSG), L-glutathione reduced (GSH) and guanidine hydrochloride (GdnHCl) were purchased from Nacalai Tesque (Kyoto, Japan). α-Cyano-4-hydroxycinnamic acid, cytidine 2':3'-cyclic monophosphate monosodium salt (cCMP) and ribonuclease A (RNaseA) from bovine pancreas, triethylamine (Et_3N) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*,*N'*-Bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, 1,1'-carbonyldiimidazole, cystamine dihydrochloride and gentisic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan). Bovine pancreatic trypsin inhibitor (BPTI) was purchased from Takara Bio (Shiga, Japan). Dry *N*,*N*-dimethylformamide (DMF), dry CH_2Cl_2 and dry tetrahydrofuran (THF) were purchased from Kanto Chemicals. Column chromatography was carried out with Silica Gel 60 (spherical, neutral, particle size: 63–210 μm) purchased from Kanto Chemicals. Deionized water (filtered through a 0.22 μm membrane filter, >18.2 MΩ cm) was purified in Purelab DV35 of ELGA (Buckinghamshire, UK) and a Milli-Q system of Merck Millipore (Burlington, MA, USA).

2. Instrumentation

Nuclear magnetic resonance (NMR) spectra were recorded on a JNM-ECX 400 spectrometer (^{1}H/400 MHz, ^{13}C/100 MHz) of JEOL (Tokyo, Japan), where the chemical shifts were determined with respect to a ^{1}H signal corresponding to the non-deuterated solvent and a ^{13}C signal corresponding to the solvent as an internal standard (^{1}H NMR: 7.24 ppm for $CDCl_3$, 4.67 ppm for D_2O, 3.29 ppm for CD_3OD; ^{13}C NMR: 77.16 ppm for $CDCl_3$, 47.68 ppm for CD_3OD). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) was performed on autoflex speed spectrometer of Bruker (Bremen, Germany). High-resolution electrospray ionization (HR ESI) TOF MS spectra were recorded on micrOTOF-Q II-S1 of Bruker with MeOH as a solvent. Analytical thin layer chromatography (TLC) was performed on precoated, glass-backed silica gel Merck 60 F254. Visualization of the developed chromatogram was performed by UV absorbance, Hanessian's stain or iodine. UV absorption spectra and temperature-dependent transmittance changes were recorded on V-650 UV-Vis spectrophotometer of JASCO (Tokyo, Japan) or U-3310 spectrophotometer of Hitachi High-Technologies (Tokyo, Japan). Reversed-phase high-performance liquid chromatography (RP-HPLC) was conducted with GL7400 HPLC system of GL Sciences (Tokyo, Japan) using TSKgel Protein C4-300 column of Tosoh Bioscience (φ4.6 × 150 mm, Tokyo, Japan).

3. Methods

1) Determination of thiol concentration for oxidative protein folding assay: To a solution of thiol (40 mM) in 10 mM HCl aq., Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid), 0.50 mM)[1] was added. Concentration of the thiol group was determined by measuring the absorbance at 412 nm at 30 °C with U-3310 spectrophotometer prior to its use for the oxidative protein folding assay.



Amounts of free thiol groups were determined by Ellman's reagent. Each amount includes both the free thiols of 1.0 mM reducing agent (GSH, GdnSH, or UreaSH) and 8.0 μM reduced substrate, RNase A. Bars represent means ± SEM from three independent measurements.

2) Preparation of reduced and denatured RNase A: RNase A was dissolved in a buffer (200 mM Tris-HCl, pH 8.7) containing 6.0 M GdnHCl and 100 mM DTT and incubated for 2 h at 25 °C. The resulting sample was dialyzed by 10 mM HCl aq. to remove the denaturing and reducing reagents.

3) RNase A refolding assay: Fully reduced and denatured RNase A (8.0 μM) was incubated for 3 h at 30 °C in the presence of 200 μM GSSG and 1.0 mM reducing agent in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5).[2] At selected time points during this incubation, aliquots (50 μL eadh) were taken from the reaction solution, which were immediately added to a buffer (150 μL, 50 mM Tris-HCl, 300 mM NaCl, pH 7.5) containing cCMP (final

concentration of cCMP = 0.60 mM) followed by the measurement of the linear increase in absorbance at 284 nm at 30 °C with U-3310 spectrophotometer. Values represent means ± SEM from three independent experiments.

4) Gel shift assay of RNase A folding: Oxidative folding of RNase A (8.0 μM) was carried out in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) containing 200 μM GSSG with/without 1.0 mM reducing agent. At selected time points, free thiols were blocked by the addition of Laemmli's 4×SDS-loading buffer[3] containing 10 mM AMS. Redox states of RNase A were separated by non-reducing 14% SDS-PAGE using WIDE RANGE gel (Nacalai Tesque). Proteins were detected by coomassie brilliant blue staining. The band intensities were analyzed by a ChemiDoc Touch imaging system and Image Lab (Bio-Rad).

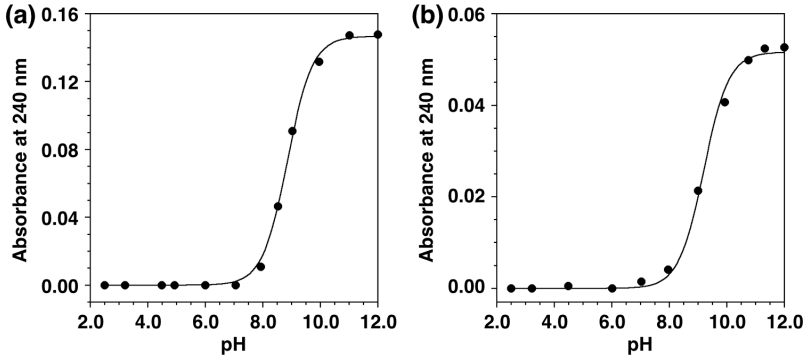
5) BPTI folding assay: Reduction and denaturation of BPTI was carried out as described previously.[4] Fully reduced and denatured BPTI (30 μM) was incubated at 30 °C in the presence of 200 μM GSSG and 1.0 mM reducing agent in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5), where the buffer was degassed by flushing N_2 prior to use. At selected time points, the reaction was quenched by adding an equal volume of 1 M HCl aq., which was then analyzed by RP-HPLC at a flow rate of 1.0 mL min^{-1} monitoring at 229 nm with a linear gradient elution (solvent A: 0.05% trifluoroacetic acid in water, solvent B: 0.05% trifluoroacetic acid in acetonitrile; percentages of solvent A: 95% at 0 min, 80% at 15 min, 30% at 115 min). The molecular mass values of the folding intermediates were determined by MALDI-TOF MS in a linear positive-ion mode using α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) as the matrix. The molecular mass was calculated using Protein-Prospector web server.[5]

6) Determination of thiol pK_a values: Stock solutions of citric buffer (sodium citrate and HCl for pH 2.0–4.0), phosphate buffer (Na_2HPO_4 and KH_2PO_4 for pH 5.0–8.0), borate buffers ($Na_2B_4O_7$ and HCl for pH 8.5–9.0, $Na_2B_4O_7$ and NaOH for pH 10.0–12.0) were prepared, and these buffers were degassed with N_2 for 1 h immediately prior to use. A stock solution of a thiol compound in degassed water (5.0 mM) were then prepared. Immediately after the aqueous solution of the thiol compound (20 μL) and a buffer (1.98 mL) were combined in a 1-cm thick quartz cuvette, the UV absorption spectrum of the sample was measured. The

pH value of the sample was measured by a HORIBA pH meter (9618S-10D), which had been calibrated prior to use with pH 4.01, 6.86 and 9.18 standard solutions (HORIBA 101-S). Absorbance at 240 nm was plotted in the function of the pH values, and the pK_a value of the thiol compound was calculated with Kaleidagraph software (version 4.5.2) by a curve fitting analysis using the following equation:

$$y = a + (m1)/(m2*10\text{^}(-x) + 1);\ m1 = b;\ m2 = 1000$$

where a is the absorbance below the pH 3 and b is the difference of absorbances above the pH 11 and below the pH 3, and curve fitting calculation provides m2 = pK_a.[6] For all analyses, r^2 values were higher than 0.99.



Absorbance changes at 240 nm of (a) GdnSH and (b) UreaSH depending on pH at 25 °C and curve fitting analyses.

7) Redox potential *E*°' measurements: *E*°' value of a thiol compound was determined by following the protocol described in a previous paper.[6] Buffer (100 mM Tris-HCl, 1.0 mM EDTA, pH 7.0) was treated by bubbling high purity Ar for longer than 1 h prior to use. DTT^{red} (60 μM, 2.5 mL) in the buffer was added to a disulfide (GdnSS or UreaSS, 60 μM, 2.5 mL) in the buffer under Ar, which was stirred at 25 ± 0.1 °C for 24 h. To quench the reaction, an aliquot of the reaction mixture (1 mL) was added to 1 M HCl aq. (200 μL), and the obtained sample solution was immediately analyzed by RP-HPLC (Tosoh TSKgel ODS-100V column, φ4.6 × 250 mm). The column was equilibrated with water containing 0.1% TFA at a flow rate of 1.2 mL min^{-1}. The RP-HPLC analysis was conducted with water containing 0.1% TFA (eluent A) and CH_3CN containing 0.1% TFA (eluent B) with a linear gradient (percentage of eluent B: 0–4% in 0–10 min, 4–7% in 10–20 min). The concentrations of the species at equilibrium were calculated from the observed peak areas and corresponding calibration curves.

The equilibrium constant K_{eq} for the reaction (eq. 1), described as eq. 2, was determined by averaging seven times of individual experiments following the above procedure.

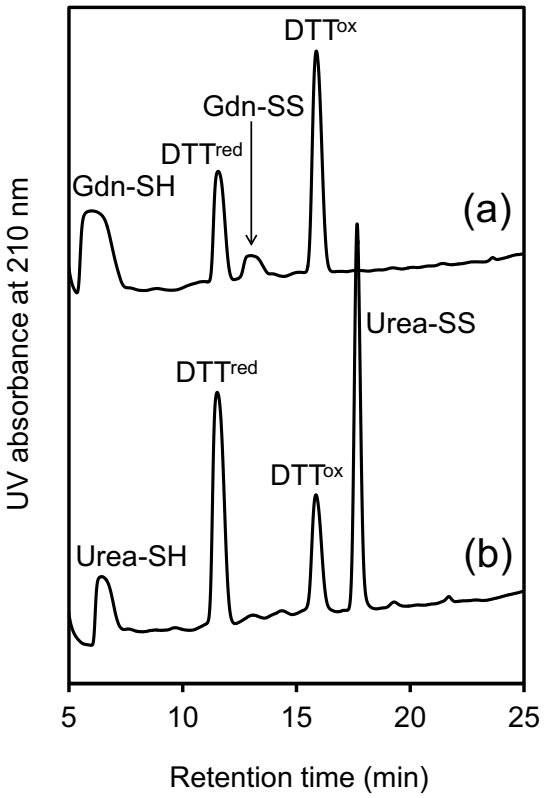
$$\text{Disulfide} + \text{DTT}^{red} \rightleftharpoons 2\text{Thiol} + \text{DTT}^{ox} \quad (1)$$

$$K_{eq} = \frac{[\text{Thiol}]^2[\text{DTT}^{ox}]}{[\text{Disulfide}][\text{DTT}^{red}]} \quad (2)$$

The redox potential $E^{\circ\prime}$ was calculated by the Nernst's equation (eq. 3)

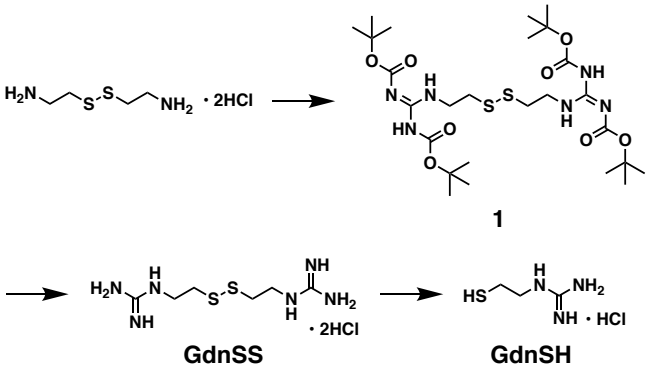
$$E^{\circ\prime} = E^{\circ\prime}_{DTT} + \frac{RT}{nF} \ln K_{eq} \quad (3)$$

where, n is the number of transferred electrons (n = 2), F is Faraday's constant (96500 C mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature (298 K), and $E^{\circ\prime}_{DTT}$ is the redox potential of DTT (–327 mV).



Representative RP-HPLC chromatograms of equilibrated mixtures of (a) GdnSS with DTT^{red} and (b) UreaSS with DTT^{red}. Reaction conditions were $[\text{disulfide}]^0 = [DTT^{red}]^0 = 30$ μM at 25 °C and pH 7.0 for 24 h.

4. Syntheses



1) Synthesis of bis(1,1-dimethylethyl) 3,12-bis{[(1,1-dimethylethoxy)carbonyl]amino}-7,8-dithia-2,4,11,13-tetraazatetradeca-3, 11-diene-1,14-dioate (1): To a dry DMF (15 mL) solution of cystamine dihydrochloride (340 mg, 1.51 mmol), *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine (993 mg, 3.20 mmol) and Et_3N (0.50 mL) were added under N_2, and the resulting mixture was stirred for 2 h at 25 °C. Then, CH_2Cl_2 (100 mL) was added to the mixture and the resulting mixture was washed with water (100 mL, three times) and brine (100 mL, once). The collected organic extract was dried over Na_2SO_4 and filtered off from insoluble substances through a filter paper. The filtrate was evaporated to dryness under reduced pressure, and the residual white solid was chromatographed on silica gel with EtOAc/hexane (1/4 to 1/1) as an eluent to allow isolation of **1** (397 mg, 0.623 mmol) as white solid in 41% yield.

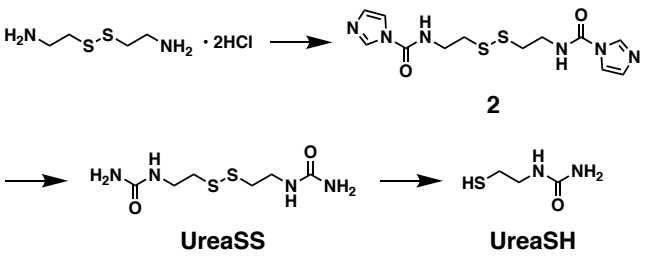
TLC (EtOAc/hexane = 1/4) R_f = 0.27; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 11.47 (brs, 2H), 8.63 (brs, 2H), 3.77 (q, J = 6.0, 6.4 Hz, 4H), 2.88 (t, J = 6.4 Hz, 4H), 1.46 (s, 36H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 163.57, 156.27, 153.20, 83.31, 79.43, 39.27, 37.16, 28.37, 28.16 ppm; HR ESI-TOF MS (MeOH, positive mode): m/z = 637.3070 (calculated m/z on the basis of the monoisotopic mass of $C_{26}H_{49}N_6O_8S_2$ $[M + H]^+$ = 637.3053).

2) Synthesis of 1,1'-[disulfanediylbis(ethane-2,1-diyl)]diguanidine dihydrochloride (GdnSS): To a MeOH (6 mL) solution of **1** (264 mg, 0.415 mmol), 1 M HCl aq. (20 mL) was added at 25 °C, and the resulting mixture was stirred for 3 h at 30 °C. Then, the mixture was evaporated to dryness under reduced pressure at 25 °C. Water (100 mL) was added to the residue, and the resulting mixture was washed with CH_2Cl_2 (100 mL, three times). The collected aqueous extract was evaporated to dryness under reduced pressure at 40 °C to allow isolation of GdnSS (108 mg, 0.349 mmol) as yellow oil in 84% yield.

^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 3.44 (t, J = 6.0, 6.4 Hz, 4H), 2.81 (t, J = 6.0, 6.4, 4H) ppm; ^{13}C NMR (125 MHz, D_2O, 25 °C): δ = 156.90, 39.78, 36.03 ppm; HR ESI-TOF MS (MeOH, positive mode): m/z = 237.0953 (calculated m/z on the basis of the monoisotopic mass of $C_6H_{17}N_6S_2$ $[M + H]^+$ = 237.0956).

3) Synthesis of 1-(2-mercaptoethyl)guanidine hydrochloride (GdnSH): To a degassed water (10 mL) solution of GdnSS (105 mg, 0.340 mmol), DTT (118 mg, 0.764 mmol) was added at 25 °C under N_2, and the resulting mixture was stirred for 18 h at 30 °C. Then, water (100 mL) was added to the residue, and the resulting mixture was washed with $CHCl_3$ (100 mL, four times) and a mixture of 2-propanol and $CHCl_3$ (10/70, 100 mL, four times). The collected aqueous extract was evaporated to dryness under reduced pressure at 40 °C to allow isolation of GdnSH (88 mg, 0.57 mmol) as colorless oil in 84% yield. DTNB assay revealed [reduced GdnSH]/[oxidized GdnSH] = 98/2.

^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 3.27 (t, J = 6.4 Hz, 4H), 2.60 (t, J = 6.4 Hz, 4H) ppm; ^{13}C NMR (125 MHz, D_2O, 25 °C): δ =156.90, 43.98, 22.94 ppm; HR ESI-TOF MS (MeOH, positive mode): m/z = 120.0594 (calculated m/z on the basis of the monoisotopic mass of $C_3H_{10}N_3S$ $[M + H]^+$ = 120.0595).



4) Synthesis of *N*,*N*′-[disulfanediylbis(ethane-2,1-diyl)]bis(1*H*-imidazole-1-carboxamide) (2): To a dry THF (20 mL) solution of cystamine dihydrochloride (608 mg, 2.69 mmol), 1,1'-carbonyldiimidazole (990 mg, 6.10 mmol) and Et_3N (1.69 mL) were added under N_2, and the resulting mixture was stirred for 18 h at 25 °C. Then, the mixture was evaporated to dryness under reduced pressure at 25 °C. The residual white solid was chromatographed on silica gel with CH_2Cl_2/MeOH (20/1 to 9/1) as an eluent to allow isolation of **2** (558 mg, 1.64 mmol) as white solid in 61% yield.

TLC (CH_2Cl_2/MeOH = 20/1) R_f = 0.063; ^{1}H NMR (400 MHz, CD_3OD, 25 °C): δ = 8.23 (s, 2H), 7.59 (t, J = 1.4 Hz, 2H), 7.04 (s, 1H), 3.66 (t, J = 6.9 Hz, 4H), 2.96 (t, J = 6.9 Hz, 4H)

ppm; ^{13}C NMR (125 MHz, CD_3OD, 25 °C): δ = 149.58, 136.12, 128.83, 116.51, 39.72, 36.74 ppm; MALDI-TOF MS (gentisic acid as the matrix, reflector positive mode): *m*/*z* = 341.102 (calculated *m*/*z* on the basis of the monoisotopic mass of $C_{12}H_{17}N_6O_2S_2$ $[M + H]^+$ = 341.085).

5) Synthesis of 1,1'-[disulfanediylbis(ethane-2,1-diyl)]diurea (UreaSS): To a MeCN (8 mL) solution of **2** (558 mg, 1.64 mmol), 28% NH_3 aq (15 mL) was added, and the resulting mixture was stirred for 18 h at 60 °C. Then, the mixture was evaporated to dryness under reduced pressure at 40 °C. Water (100 mL) was added to the mixture and the resulting mixture was washed with $CHCl_3$ (100 mL, four times). The collected aqueous extract was evaporated to dryness under reduced pressure at 25 °C to allow isolation of UreaSS (304 mg, 1.28 mmol) as white solid in 78% yield.
^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 3.31 (t, *J* = 6.4 Hz, 4H), 2.73 (t, *J* = 6.4 Hz, 4H) ppm; ^{13}C NMR (125 MHz, D_2O, 25 °C): δ = 161.46, 38.63, 37.76 ppm; HR ESI-TOF MS (MeOH, positive mode): *m*/*z* = 239.0634 (calculated *m*/*z* on the basis of the monoisotopic mass of $C_6H_{15}N_4O_2S_2$ $[M + H]^+$ = 239.0636).

6) Synthesis of 1-(2-mercaptoethyl)urea (UreaSH): To a degassed water (15 mL) solution of UreaSS (197 mg, 0.827 mmol), DTT (202 mg, 1.31 mmol) was added at 25 °C under N_2, and the resulting mixture was stirred for 30 min at 25 °C. Then, the resulting mixture was washed with a mixture of 2-propanol and $CHCl_3$ (10/70, 20 mL, five times). The collected aqueous extract was evaporated to dryness under reduced pressure at 40 °C followed by washing with *tert*-butyl methyl ether (10 mL, five times) and evaporation to allow isolation of UreaSH (64 mg, 0.53 mmol) as white solid in 32% yield. DTNB assay revealed [reduced UreaSH]/[oxidized UreaSH] = 98/2.
^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 3.16 (t, *J* = 6.4, 6.9 Hz, 2H), 2.51(t, *J* = 6.4 Hz, 2H) ppm; ^{13}C NMR (125 MHz, D_2O, 25 °C): δ = 161.53, 42.75, 24.04 ppm; HR ESI-TOF MS (MeOH, positive mode): *m*/*z* = 121.0431 (calculated *m*/*z* on the basis of the monoisotopic mass of $C_3H_9N_2OS$ $[M + H]^+$ = 121.0436).

5. Oxidative folding of BPTI

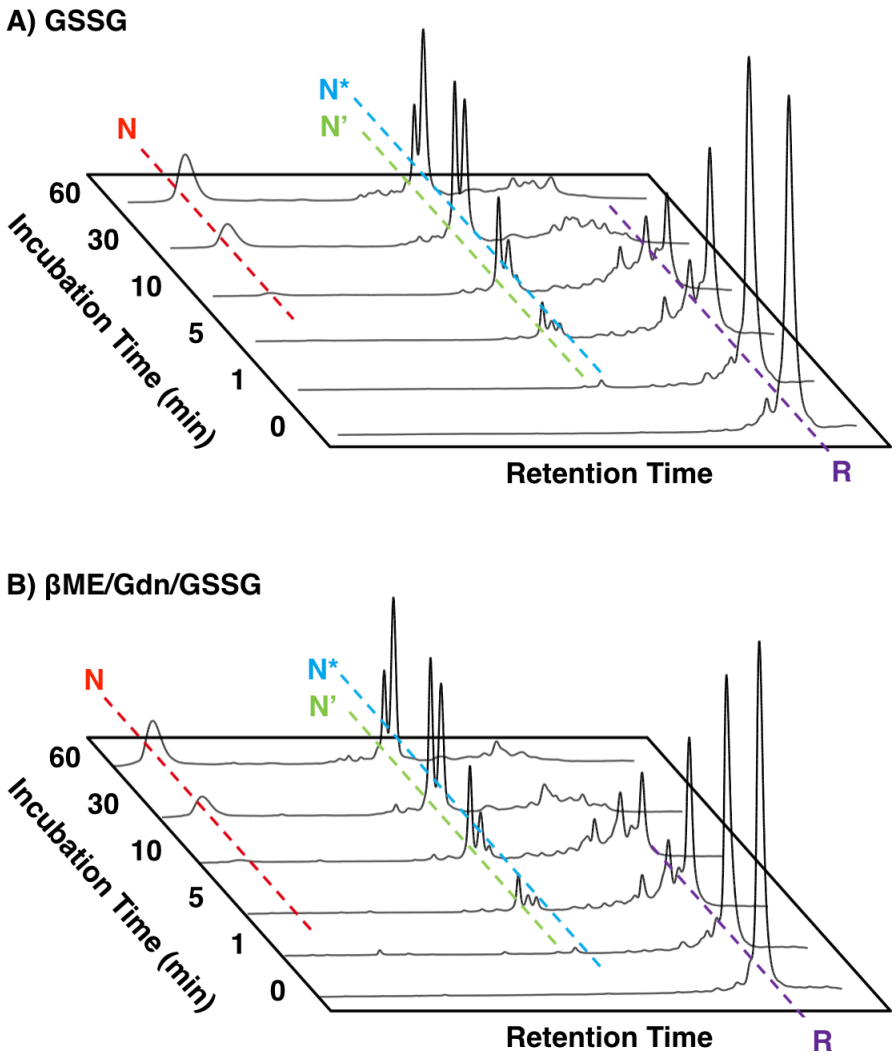


Fig. S1 Time-course reverse-phase HPLC analyses of oxidative folding of BPTI (30 μM) in the presence of (A) GSSG (0.20 mM) and (B) βME (1.0 mM), GdnHCl (1.0 mM) and GSSG (0.20 mM). N and R depict native and reduced forms of BPTI, respectively. Eluent buffers: water (containing 0.05% TFA) and CH_3CN (containing 0.05% TFA) with a linear gradient; flow rate: 1.0 mL min^{-1}; detection wavelength: 229 nm; temperature: 25 °C.

6. Aggregation suppression of lysozyme

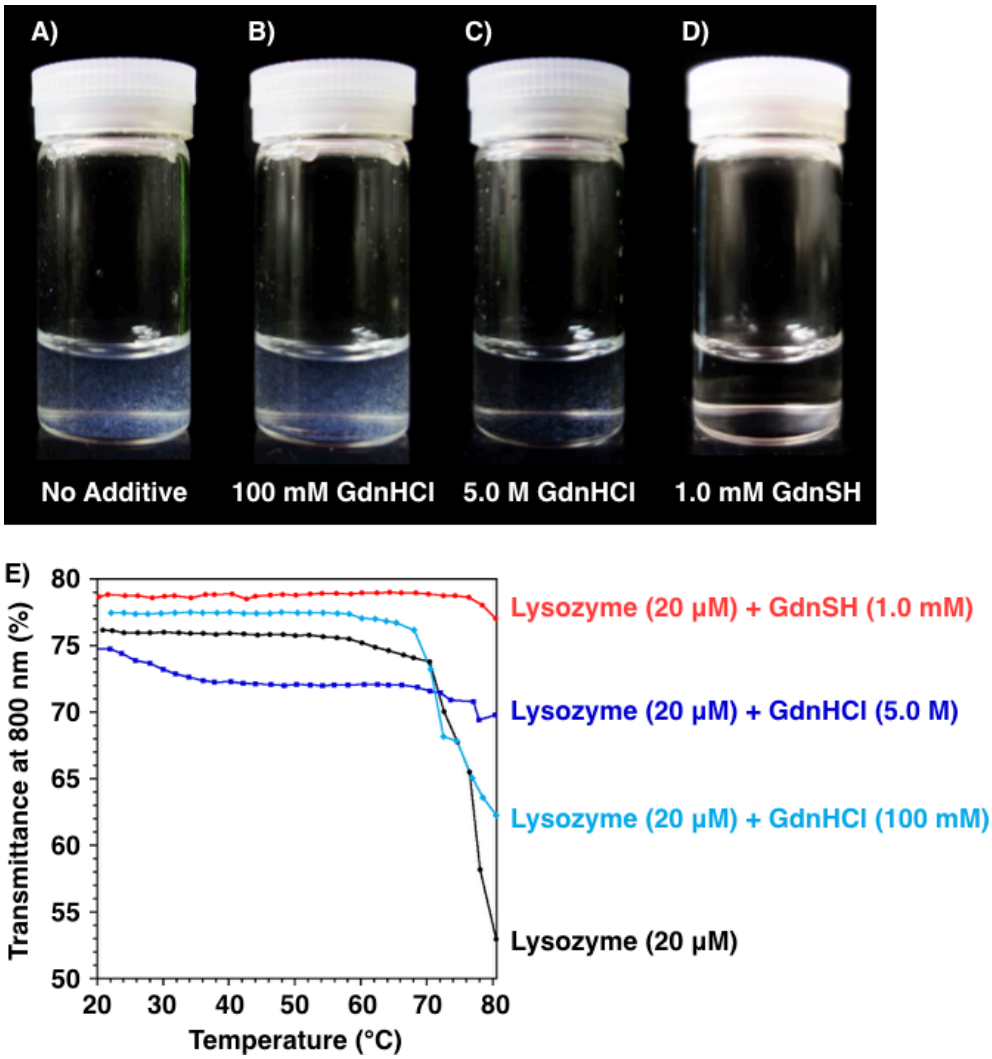


Fig. S2 Photographs of glass bottles containing lysozyme (20 μM) in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) after cooling from 95 °C to 25 °C with A) no additive, B) 100 mM guanidine-HCl (GdnHCl), C) 5.0 M GdnHCl, and D) 1.0 mM GdnSH. E) 800-nm Transmittance measurements of lysozyme (20 μM) in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) upon heating from 20 °C to 80 °C at the rate of 10 °C min^{-1} with (black line) no additive, (light blue line) 100 mM GdnHCl, (blue line) 5.0 M GdnHCl, and (red line) 1.0 mM GdnSH.

7. MALDI-TOF MS analysis of a mixture of GdnSH and GSSG

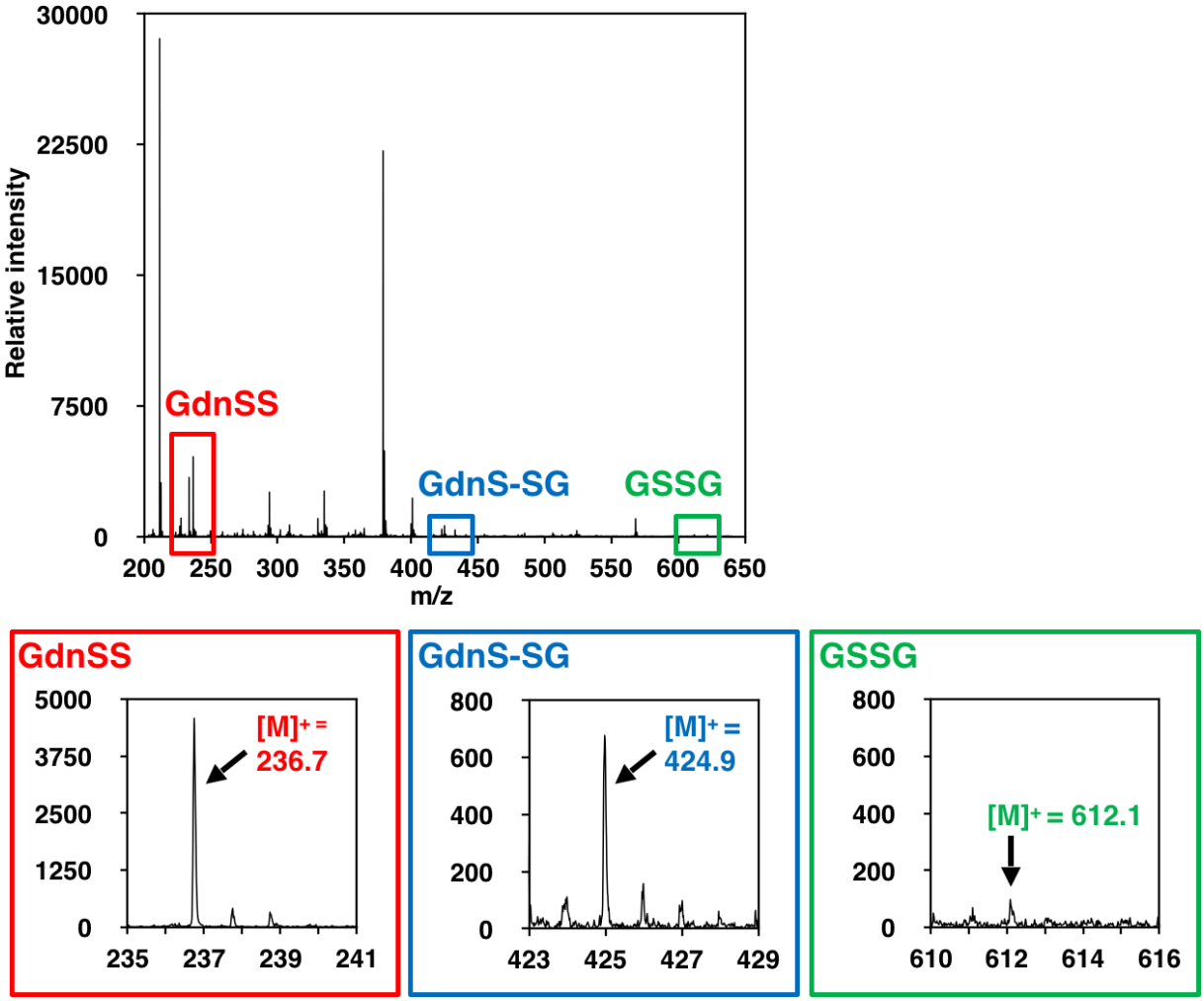


Fig. S3 MALDI-TOF MS spectrum of a mixture of GdnSH and GSSG in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5), with magnified views in the lower part. Calculated monoisotopic mass values of GdnSS: 236.1, GdnS-SG: 424.1, and GSSG: 612.2.

GdnSH and GSSG were incubated for 10 min in a buffer for equilibration, and the sample (1 μL) was dissolved in a matrix solution (α-cyano-4-hydroxycinnamic acid, 10 mg mL^{-1}, 1 μL) in a mixture of water/CH_3CN/trifluoroacetic acid (50/50/0.05) followed by air-drying on a target plate just before the measurement. Measurement modes: positive ion, reflector.

8. Oxidation of RNase A in the presence of GdnSH and GdnSS

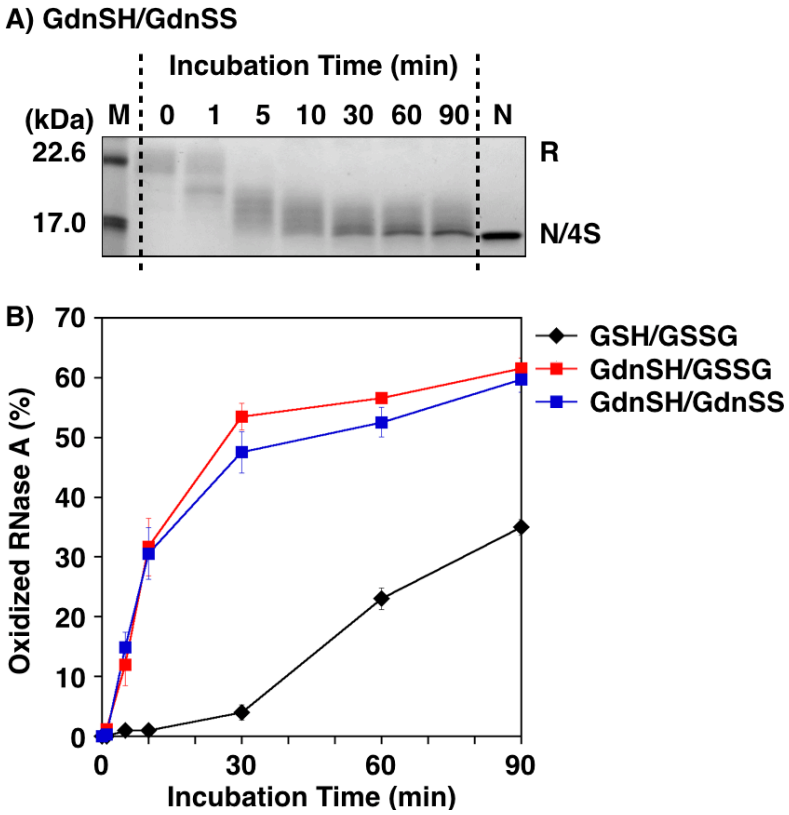


Fig. S4 A) A SDS-PAGE gel image monitoring the oxidation of RNase A (8.0 μM) in the presence of GdnSH (1.0 mM) and GdnSS (0.20 mM) in a buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5). B) A time-course change of the percentages of fully oxidized RNase A (8.0 μM) in the presence of GSH/GSSG (black diamonds), GdnSH/GSSG (red squares), and GdnSH/GdnSS (blue squares) quantified by the SDS-PAGE analyses (thiols: 1.0 mM, disulfides: 0.20 mM). Error bars indicate the means ± SEM of three independent experiments.

9. References

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