Catalysis of Protein Folding by an Immobilized Small-Molecule Dithiol

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The isomerization of non-native disulfide bonds often limits the rate of protein folding. Small-molecule dithiols can catalyze this process. Here, a symmetric trithiol, tris(2-mercaptoacetamidoethyl)amine, is designed on the basis of criteria known to be important for efficient catalysis of oxidative protein folding. The trithiol is synthesized and attached to two distinct solid supports via one of its three sulfhydryl groups. The resulting immobilized dithiol has an apparent disulfide $E^{\circ\prime} = -208$ mV, which is close to that of protein disulfide isomerase ($E^{\circ\prime} = -180$ mV). Incubation of the dithiol immobilized on a TentaGel resin with a protein containing non-native disulfide bonds produced only a 2-fold increase in native protein. This dithiol appeared to be inaccessible to protein. In contrast, incubation of the dithiol immobilized on styrene-glycidyl methacrylate microspheres with the non-native protein produced a 17-fold increase in native protein. This increase was 1.5-fold greater than that of a monothiol immobilized on the microspheres. Thus, the choice of both the solid support and thiol can affect catalysis of protein folding. The use of dithiol-decorated microspheres is an effective new strategy for preparative protein folding in vitro.

Introduction

Many proteins contain disulfide bonds between cysteine residues (*1*). In such proteins, the attainment of the proper disulfide bond pattern during folding is critical for function. Accordingly, much effort has been expended to improve the efficiency of in vitro folding for disulfide-bonded proteins (*2*).

The mechanism of oxidative folding includes both the oxidation of thiols and the isomerization of disulfide bonds. Often, protein disulfide bonds accumulate rapidly and the isomerization of oxidized intermediates limits the overall rate of protein folding (*3*, *4*). The active site of protein disulfide isomerase (PDI), which is the most efficient known catalyst of oxidative protein folding (*5*), employs a CXXC motif with a high disulfide $E^{\circ\prime}$ (−180 mV) and a low thiol pK_a (6.7) to catalyze this step (*6*−*8*).

In vitro, oxidative protein folding is usually initiated by the addition of reduced and unfolded protein to a redox buffer. The most commonly used redox buffer contains reduced and oxidized glutathione (GSH and GSSG, respectively). The glutathione system uses the mixed disulfide GSSG to provide oxidizing equivalents and the monothiol GSH to catalyze disulfide bond isomerization (*9*). The folding efficiency of this system shows a narrow optimum at concentrations around 1.0 mM GSH and 0.2 mM GSSG (*10*). These concentrations produce a solution potential similar to that of the endoplasmic reticulum ($E_{solution} = -180$ mV (*11*)). Increasing the total concentration of glutathione decreases the yield of correctly folded protein because the stability of mixed disulfides between glutathione and protein increases at higher glutathione concentrations (*12*, *13*). Monothiols with lower thiol pK_a such as *N*-methylmercaptoacetamide (NMA) or 4-mercaptobenzoic acid form less stable mixed disulfides and can be used at higher concentrations to give faster folding rates than glutathione (*14*, *15*).

In contrast to monothiols, dithiols can form cyclic disulfides and thus form less stable mixed disulfides. At a constant solution potential, the folding efficiencies of dithiol-based redox buffers should show optima at much higher concentrations of thiol and disulfide than is seen with monothiol-based systems. Indeed, reduced ribonuclease A (RNase A) can fold in a redox buffer consisting of 0.06 mM reduced dithiothreitol (DTT) and 100 mM oxidized DTT ($E_{solution} = -230$ mV) with minimal accumulation of mixed disulfides (*13*). The addition of reduced (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC, or Vectrase-P (*16*)) to a glutathione redox buffer increases the rate and yield of folding for an RNase A substrate containing non-native disulfide bonds (*14*).

Presentation of an appropriate dithiol by a solid support could be advantageous, facilitating both the separation of the catalyst from the folded protein and its recycling. Herein, a microsphere presenting a symmetric dithiol is shown to fold RNase A efficiently, and more efficiently than one presenting a monothiol. This immobilized dithiol is a new and useful tool for the production of proteins for pharmaceutical and biotechnical applications.

Materials and Methods

General. NovaSyn TG bromo resin (bromo-Tentagel, or Br-TG) was from NovaBiochem (San Diego, CA). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride, gly-

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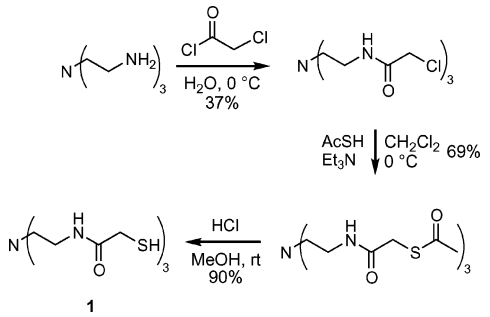

Figure 1. Synthetic route to tris(2-meraptoacetamidoethyl)-amine, trithiol **1**.

cidyl methacrylate, lithium hydride, lithium *tert*-butoxide, styrene, triethylamine, and tris(2-aminoethyl)amine were from Aldrich (Milwaukee, WI). Chloroacetyl chloride and thioacetic acid were from Acros Organics (Pittsburgh, PA). GSH and GSSG were from Sigma Chemical (St. Louis, MO). DTT was from Fisher (Pittsburgh, PA). NMA was from Fluka (Buchs, Switzerland). Dimethyl formamide (DMF) was from J. T. Baker (Phillipsburg, NJ). All other reagents were of commercial grade or better and were used without further purification.

Instrumentation. Ultraviolet and visible spectra were obtained with a Cary model 3 spectrophotometer equipped with a Cary temperature controller. NMR spectra were obtained with a Bruker AC-300 spectrometer. Mass spectra were obtained with a Micromass Q-TOF2 mass spectrometer.

Synthesis of Trithiol 1. Trithiol **1** was synthesized by a route similar to that used in previous syntheses of analogous dithiols (*17*, *14*), as described below (Figure 1).

Tris(2-chloroacetamidoethyl)amine. Chloroacetyl chloride (37.2 g, 0.33 mol) was added dropwise to water (0.50 L) containing tris(2-aminoethyl)amine (14.6 g, 0.10 mol) and K_2CO_3 (45.6 g, 0.33 mol). The solution was stirred on ice for 90 min. A white solid was collected by filtration and washed with 2 M K_2CO_3 (aqueous). Solid K_2CO_3 was added to the filtrate until the pH reached 11. A second crop of white solid was collected by filtration. The two crops were combined, and the tris(chloroacetyl) product (10.6 g, 37%) was judged to be pure by spectroscopic analysis: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.09 (br, 3H), 4.08 (s, 6H), 3.37 (q, J = 5.7 Hz, 6H), 2.65 (t, J = 5.6 Hz, 6H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.4, 53.5, 42.6, 37.6 ppm; MS (ESI) calculated for $C_{12}H_{22}Cl_3N_4O_3$ m/z 375.1, found 375.2.

Tris(2-acetylmercaptoacetamidoethyl)amine. Tris-(2-chloroacetamidoethyl)amine product (5.0 g, 13 mmol) was dissolved in dichloromethane (0.15 L), and the

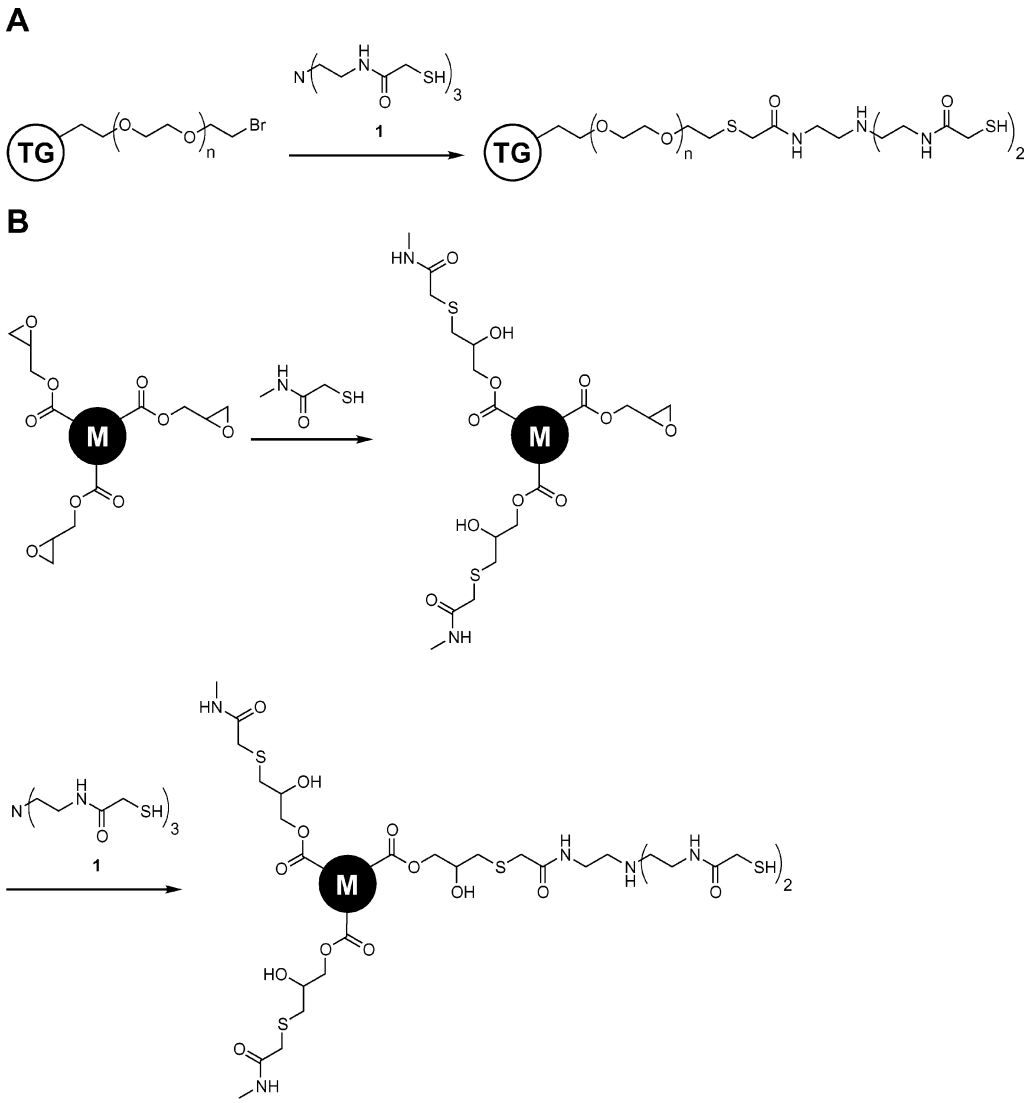

Figure 2. Scheme for coupling trithiol **1** to resins. (A) Bromo-TentaGel resin (TG). (B) Epoxy microspheres (M).

resulting solution was cooled to 0 °C. Thioacetic acid (5.7 mL, 80 mmol) was added dropwise and with stirring to the cooled solution. Then, triethylamine (11 mL, 80 mmol) was added dropwise and with stirring. The resulting yellow solution was allowed to warm slowly to room temperature and stirred under Ar (gas) for 40 h. The reaction was quenched by the addition of 2 M K_2CO_3 (aqueous). The layers were separated, and the organic layer was washed a second time with 2 M K_2CO_3 (aqueous). The organic layer was dried, filtered, and concentrated to yield a white solid. This solid was recrystallized twice from ethyl acetate. The tris(thioacetate) product (4.5 g, 69%) was judged to be pure by spectroscopic analysis: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.05 (br, 3H), 3.64 (s, 6H), 3.28 (q, J = 5.5 Hz, 6H), 2.56 (t, J = 5.6 Hz, 6H), 2.40 (s, 9H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 195.9, 168.2, 53.8, 37.9, 33.1, 30.1 ppm; MS (ESI) calcd for $C_{18}H_{31}N_4O_6S_3$ m/z 495.1, found 495.4.

Tris(2-mercaptoacetamidoethyl)amine. Tris(2-acetylmercaptoacetamidoethyl)amine (2.0 g, 4.0 mmol) was dissolved in 0.10 L of 1.2 M HCl in methanol. The resulting clear solution was incubated at room temperature under Ar (gas) for 20 h. The acetic acid byproduct was removed by bubbling Ar (gas) through the solution, and the solvent was removed under reduced pressure to yield a yellow oil. This oil was dissolved in a minimal volume of methanol, and the resulting solution was cooled to −78 °C. A white precipitate formed. During filtration at room temperature, the white precipitate redissolved, leaving a small amount of brown oil on the filter. The filtrate was dried under reduced pressure to yield a white solid. The trithiol **1** product (1.5 g, 90%) was judged to be pure by spectroscopic analysis: ^{1}H NMR (D_2O, 300 MHz) δ 3.25 ppm (t, J = 7.0 Hz, 6H), 3.08 (s, 6H), 2.67 (t, J = 6.5 Hz, 6H) ppm; ^{13}C NMR (D_2O, 75 MHz) 84.3, 65.4, 57.8 ppm; MS (ESI) calcd for $C_{12}H_{24}N_4O_3S_3$ m/z 368.1, found 366.8.

Coupling of Trithiol 1 to Bromo-TentaGel (Figure 2A). Br-TG (5 mg) was allowed to swell in water (1.0 mL) at room temperature for 1 h. The resin was then washed with 1.0 mL of 1.0 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.6. Trithiol **1** (0.9 mL of a 40 mM aqueous solution) and Tris-HCl buffer, pH 7.6 (0.10 mL of a 1.0 M solution), were added to the resin. The suspension was agitated gently on a platform shaker at room temperature for 2 h. The resin was separated by centrifugation. To block unreacted sites, the resin was incubated overnight at room temperature with NMA (1.0 mL of a 0.3 M aqueous solution in 0.3 M Tris-HCl buffer, pH 7.6). The resin was then washed extensively with water, and the coupling yield was determined by the reaction of resin-bound thiols with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (vide infra).

Synthesis of Epoxy Microspheres. A copolymer of styrene and glycidyl methacrylate forms microspheres in water that present surface epoxy groups (*18*). Styrene-glycidyl methacrylate microspheres were synthesized via a seeded emulsion polymerization. Both the styrene and the glycidyl methacrylate were distilled under reduced pressure in the dark before use. Styrene (11.4 mmol, 1.32 mL) and glycidyl methacrylate (12.6 mmol, 1.73 mL) were added to a flask containing degassed water (110 mL). Polymerization was initiated by the addition of aqueous 2,2′-azobis(2-methylpropionamidine) dihydrochloride (10 mL of a 6 mg/mL solution). The reaction was stirred under Ar (gas) at 70 °C for 2 h, during which the initially clear, colorless solution turned cloudy and white. Glycidyl methacrylate (21.1 mmol, 2.88 mL) was added to coat the styrene-glycidyl methacrylate seeds with surface epoxy groups. The polymerization was stirred under Ar (gas) at 70 °C for an additional 13 h. The epoxy microspheres were isolated by centrifugation at 20 000*g* for 10 min at 4 °C. The pellet was washed by three cycles of resuspension in water (120 mL) followed by centrifugation. The epoxy microspheres were dried under reduced pressure to give a fine, white solid (5.85 g, 98%).

Coupling of Dithiothreitol to Epoxy Microspheres. DTT was immobilized to microspheres via reaction with epoxy groups on the surface of the styrene-glycidyl methacrylate microspheres. DTT (1.0 g, 6.5 mmol) was added to LiH (0.11 g, 13 mmol) in dry DMF (3.5 mL). The resulting slurry was stirred on ice under Ar (gas) for 10 min and then transferred to a flask containing microspheres (0.10 g, 0.56 mmol total epoxide) dispersed in DMF (3.5 mL). The reaction was stirred on ice and under Ar (gas) for 4 h and then quenched by the addition of saturated NH_4Cl (aqueous). The microspheres were collected by centrifugation (20 000*g*, 20 min), and the pellet was washed three times with water (30 mL per wash). The pellet was then transferred to a 2 mL tube and washed by three more cycles of resuspension in water (1 mL per wash) followed by centrifugation for 2 min in a benchtop centrifuge (maximum speed). A final wash with 0.10 M potassium phosphate buffer, pH 7.6 (1.0 mL), was performed in a similar manner.

Coupling of Trithiol 1 to Epoxy Microspheres (Figure 2B). To minimize cross-linking of the trithiol to the epoxy microspheres (Figure 3B,C), a fraction of the epoxy groups were blocked. To accomplish this blocking, the monothiol NMA (10 μL, 0.11 mmol) was dissolved in dry DMF (1.0 mL) containing lithium *tert*-butoxide (10 mg, 0.12 mmol). The mixture was stirred on ice for 10 min. Microspheres (50 mg, 0.28 mmol total epoxide) were then added, and the blocking reaction was allowed to proceed on ice under Ar (gas) for 2 h. Trithiol **1** (46 mg, 0.13 mmol) in dry DMF (5 mL) and lithium *tert*-butoxide (20 mg, 0.24 mmol) were added sequentially to the NMA-capped microspheres. The coupling reaction was stirred on ice under Ar (gas) for 3 h and then quenched with saturated solution of NH_4Cl (aqueous). The microspheres were collected by centrifugation (20 000*g* for 20 min) and then washed extensively with water and 0.10 M potassium phosphate buffer, pH 7.6.

Determination of Coupling Yields. A solid support must be free of significant solution-phase thiol to assess coupling yields accurately. Accordingly, the solid support (TG resin or microsphere) was washed with 0.10 M potassium phosphate buffer, pH 7.6, and separated by centrifugation. The supernatant was collected, and its thiol content was assayed by the addition of DTNB (10 μL of a 10 mg/mL solution). This washing procedure was continued until the solution-phase thiol concentration was negligible.

The coupling yield was determined by the addition of DTNB (1.0 mL of a 10 mg/mL solution in 0.10 M potassium phosphate buffer, pH 7.6) to the washed solid support. The particles were dispersed in the DTNB solution by repeated inversion and then agitated on a platform shaker for 30 min at room temperature. The solid support was collected by centrifugation. The moles of immobilized thiol were calculated from the A_{412} of the supernatant by assuming that $\epsilon = 13\,600\ M^{-1}\,cm^{-1}$ (*19*).

Mixed Disulfide Formation by Immobilized Thiols. The tendency of immobilized thiols to form mixed disulfides was assessed by monitoring the amount of 2-nitro-5-thiobenzoic acid (NTB) released upon treatment of DTNB-oxidized resin with DTT. Briefly, a fully reduced, thoroughly washed resin was incubated with a

Table 1. Properties of Thiol-Coupled Resins

resin	coupling yield (mmol of thiol/g of resin)	mixed disulfides with NTB (%)	[active RNase A] (nM) from uncoupled resin	[active RNase A] (nM) from coupled resin	fold reactivation of sRNaseA
trithiol **1**−TentaGel	0.28	14	27 ± 2	54 ± 5	2.0 ± 0.3
DTT−microspheres	0.30	nd[b]	13 ± 2	143 ± 13	11 ± 3
trithiol **1**−microspheres[a]	0.056	17	13 ± 2	226 ± 40	17 ± 3

[a] Microspheres were partially blocked with NMA before coupling to trithiol **1**. [b] Not determined.

stoichiometric amount of DTNB relative to thiol. The DTNB solution was collected, and its NTB concentration ($[NTB]_1$) was measured by UV spectroscopy. The DTNB-oxidized resin was washed thoroughly and then incubated with an excess of DTT. The DTT solution was collected, and its NTB concentration ($[NTB]_2$) was measured likewise. The ratio of NTB released upon reduction to NTB released during oxidation gives the fraction of thiols that formed mixed disulfides with NTB. This ratio, $[NTB]_2/[NTB]_1$, is unity if all of the resin-bound thiols form stable mixed disulfides upon oxidation by DTNB. This ratio is zero if all of the resin-bound thiols form transient mixed disulfides and then cyclic disulfides upon oxidation by DTNB.

Disulfide Bond Isomerization Activity of Immobilized Thiols. Scrambled RNase A (sRNase A) results from the oxidation of the fully reduced protein in a denaturing solution such as 6 M guanidine-HCl. To determine the ability of immobilized thiols to catalyze disulfide bond isomerization in a protein, sRNase A (1.0 mL of a 0.5 mg/mL solution in 0.10 M Tris-HCl buffer, pH 7.6) was prepared as described previously (*56*) and added to fully reduced resin presenting 1.0 μmol of thiol. The resin was agitated gently at room temperature for 20 h, and the RNase A solution was then separated from the resin by centrifugation. The concentration of native RNase A was determined by spectrophotometric measurement of ribonucleolytic activity using poly(cytidylic acid) as the substrate, as described previously (*14*). The fold reactivation of RNase A was calculated relative to that from unmodified resin (i.e., resin presenting no thiol) and is the mean (± SD) of three experiments.

Determination of E_{resin}. The disulfide reduction potential of a dithiol immobilized on a solid support (E_{resin}) can be estimated by measuring the fraction of thiol groups remaining after incubation of the fully reduced resin in a redox buffer of known solution potential ($E_{solution}$). Trithiol-coupled TG resin was incubated in 0.10 M Tris-HCl buffer, pH 7.6 (0.90 mL), containing 6.5 mM GSH and 16 mM GSSG ($E_{solution} = -180$ mV) at room temperature for 1 h. The buffer was removed by centrifugation, and the resin was washed thoroughly with 50 mM Tris-HCl buffer, pH 7.6. The amount of thiol remaining on the resin was measured by using DTNB (vide supra). The value of E_{resin} was calculated from the amount of dithiol remaining after incubation in the redox buffer using eq 1, where $(\text{moles of dithiol})_{final}$ is equal the moles of dithiol remaining on the resin after incubation with the redox buffer and $(\text{moles of dithiol})_{initial}$ is equal to the moles of dithiol present on the fully reduced resin. The value of E_{resin} is reported as the mean ± SD of two experiments.

$$E_{resin} = E_{solution} - \frac{RT}{nF} \ln \frac{(\text{moles dithiol})_{final}}{(\text{moles dithiol})_{initial} - (\text{moles dithiol})_{final}} \quad (1)$$

Results

Design of Trithiol 1. Trithiol **1** (Figure 1) was designed as a means for presenting a dithiol of a defined structure on a solid support. Covalent attachment of trithiol **1** can occur via attack of one of its sulfur atoms on an electrophile presented by a solid support. Because trithiol **1** has C_3 symmetry, the attack of any one of its three sulfur atoms produces an immobilized dithiol of identical structure.

To maximize disulfide bond isomerization activity, a dithiol catalyst should have a low thiol pK_a (*20*), as only the thiolate form of a sulfhydryl group can participate in thiol−disulfide interchange reactions. Trithiol **1** has three mercaptoacetamido groups. Through-bond inductive effects within a mercaptoacetamido group decrease its thiol pK_a to 8.3 (*14*). At neutral pH, the tertiary amino group of trithiol **1** is cationic, which is likely to decrease the thiol pK_a still further. Thus, trithiol **1**-coupled microspheres likely have a thiol pK_a < 8.3. Protonation of its tertiary amino group has the additional benefit of increasing the hydrophilicity, and thus the solvent accessibility, of immobilized trithiol **1**, which is likely to enhance its reactivity with solution-phase molecules (*21*).

To maximize disulfide bond isomerization activity, a dithiol catalyst should also have a high disulfide $E^{o\prime}$ (*20*). If the disulfide $E^{o\prime}$ is too low, then the reduction of a protein disulfide bond will be favored over its isomerization. Oxidation of BMC forms a 10-membered cyclic disulfide containing two amide bonds. BMC has $E^{o\prime} = -240$ mV (*14*). Oxidation of trithiol **1** produces a 13-membered cyclic disulfide containing two amide bonds. Because oxidation of trithiol **1** forms a larger ring, its disulfide $E^{o\prime}$ should be greater than that of BMC. In addition, the lower pK_a of trithiol **1** should also increase its disulfide $E^{o\prime}$ (*20*).

Synthesis of Trithiol 1. The synthesis of trithiol **1** was accomplished in three steps with an overall yield of 23% (Figure 1).

Choice of Solid Support. Three criteria were considered in the choice of a solid support for the display of trithiol **1**. First, the support should have a high density of electrophilic groups. Second, the support should be amenable to trithiol coupling. Third, the support should be compatible with the aqueous buffer conditions required for protein folding. Br-TG resin (which is available commercially) and styrene-glycidyl methacrylate microspheres meet these criteria.

Trithiol-Coupled TentaGel. Br-TG resin is a composite of polystyrene and poly(ethylene glycol) (PEG) in which each PEG chain contains a terminal bromine atom (*22*). Br-TG was investigated as a solid support because of its high capacity (0.28 mmol Br/g resin) and water compatibility. Immobilization of trithiol **1** can occur through displacement of bromide, creating a stable thioether bond (Figure 2A). Coupling of a 30-fold excess of trithiol **1** to Bromo-TG resin gave a yield of 0.28 mmol of thiol/g of resin (Table 1), as measured by the reactivity of the coupled resin with DTNB.

One molecule of trithiol **1** can form up to three covalent bonds with the resin (Figure 3). A singly attached molecule presents a dithiol, a doubly attached molecule presents a monothiol; a triply attached molecule has no free thiols. To gauge the valency of the thiol groups

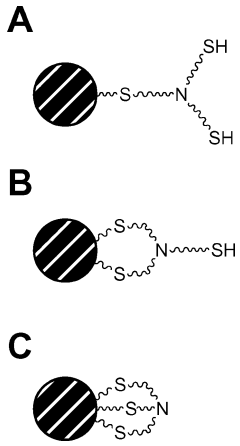

Figure 3. Different modes of trithiol immobilization. (A) Trithiol is singly attached to a resin and presents a dithiol. (B) Trithiol is doubly attached to a resin and presents a monothiol. (C) Trithiol is triply attached to a resin, and this fully cross-linked species presents no thiols.

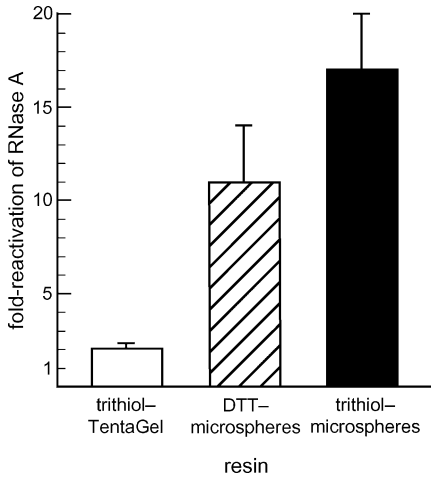

Figure 4. Reactivation of scrambled RNase A by reduced resins. Resin displaying 1.0 μmol of total thiol and unmodified resin were dispersed in parallel solutions of 0.10 M Tris-HCl, pH 7.6, containing sRNase A (0.5 mg/mL). The suspensions were gently agitated at room temperature for 20 h. The resin was removed by centrifugation, and the ribonucleolytic activity of each solution was measured with poly(cytidylic) acid as a substrate. The fold reactivation of RNase A is calculated from the difference in RNase A activity between resin displaying 1.0 μmol of total thiol and unmodified resin and is the mean (±SD) of three experiments.

presented by the coupled resin, the propensity of the coupled resin to form stable mixed disulfides was measured. Reduction of DTNB-oxidized resin by DTT indicated that only 14% of the thiols formed mixed disulfides with NTB (Table 1). Thus, the thiols on the surface of trithiol-coupled TG resin can readily form cyclic disulfides, which is indicative of dithiol presentation (Figure 3A).

The trithiol-coupled TG resin readily engages in thiol−disulfide interchange with small molecules such as glutathione, DTT, and DTNB. Incubation of the resin with sRNase A produced only a small increase in enzymatic activity (Figure 4 and Table 1). Incubation of sRNase A and uncoupled Br-TG resin in a solution of 1.0 mM GSH and 0.2 mM GSSG produced a 30-fold increase in enzymatic activity, indicating that the TG resin itself did not prevent RNase A from folding. Thus, trithiol-coupled TG resin is not an efficient catalyst of the thiol−disulfide interchange reactions necessary for the isomerization of non-native disulfide bonds.

Determination of E_{resin}. After incubation in a glutathione redox buffer with $E_{solution} = -180$ mV, the trithiol-coupled TG resin contained about 10% of its original thiol content. If the trithiol-coupled resin exclusively forms cyclic disulfides upon oxidation in this redox buffer, then the apparent $E_{resin} = -208 \pm 8$ mV.

Trithiol-Coupled Microspheres. Microspheres composed of styrene-glycidyl methacrylate copolymers are water-compatible and have a high capacity of electrophilic epoxy groups (5.6 mmol of epoxide/g of microsphere). The structural integrity of these microspheres stems from the burial of hydrophobic styrene units and the exposure of hydrophilic glycidyl methacrylate units. The surface enrichment of glycidyl methacrylate units provides a high density of exposed epoxy groups. Immobilization of a thiol can occur via its reaction with an epoxy group.

A 12-fold excess of DTT relative to total epoxide was coupled to these microspheres and gave a yield of 0.30 mmol of thiol/g of microsphere. Using identical coupling conditions, reaction of trithiol **1** with epoxy microspheres yielded 0.004 mmol of thiol/g of microsphere. Further, reduction of DTNB-oxidized resin with DTT showed that 65% of the thiol groups had formed mixed disulfides, indicating a low propensity for cyclic disulfide formation by the immobilized thiol groups.

The low yield of immobilized thiol and high propensity for the formation of stable mixed disulfides suggests that, during coupling, trithiol **1** often reacted with more than one microsphere epoxy group (Figures 3B,C). To decrease the likelihood of such cross-linking, the microspheres were first treated with the monothiol NMA (0.4 equiv relative to total epoxide) to block some of the reactive epoxy groups. Then, trithiol **1** (0.5 equiv relative to total epoxide) was coupled as before (Figure 2B). This procedure gave a yield of 0.056 mmol of thiol/g of microsphere, with 17% mixed disulfide formation upon oxidation with DTNB (Table 1).

The ability of thiol-presenting microspheres to fold sRNase A was assessed in the same manner as for the trithiol-coupled TG resin. In contrast to the results with TG resin, incubation of sRNase A with either the DTT-coupled microspheres or the trithiol-coupled microspheres resulted in a marked increase in enzymatic activity (Figure 4 and Table 1). The trithiol-coupled microspheres, however, gave a 1.5-fold greater increase in activity than did the DTT-coupled microspheres.

Discussion

Heterologous gene expression systems can provide ready access to large quantities of protein (*23*). Unfortunately, these large quantities can overwhelm the cellular folding machinery and result in a prevalence of misfolded, inactive protein (*24*−*26*). For those proteins that contain disulfide bonds (*1*), folding in the reducing environment of the bacterial cytosol is especially challenging. In *E. coli*, misfolded proteins tend to form insoluble aggregates (that is, inclusion bodies). To improve the yield of soluble protein from heterologous expression systems, Beckwith, Georgiou, and co-workers engineered *E. coli* strains to have improved oxidative folding capabilities (*27*). Such strains hold much promise for efficient production of soluble proteins containing disulfide bonds. Still, inclusion body formation does have advantages (*28*, *29*). Those advantages include the high yield and relative purity of the constituent protein and the ability to produce large quantities of proteins that are otherwise toxic to the cell.

The in vitro folding of proteins from insoluble aggregates commonly involves the dilution of denaturant-solubilized protein into solutions that promote protein folding (*29*). Oxidative folding is plagued by two deleterious side reactions: aggregation and non-native disulfide bond formation. To overcome these side reactions, cells use a full complement of chaperones (*30*, *31*) and foldases (*32*−*36*). Mimicking this strategy in vitro is problematic because chaperones and foldases are expensive and because their removal requires an additional separation step.

Several methods have been developed to circumvent these limitations. For example, the GroEL/GroES chaperone system was successfully incorporated into an automated, solution-phase protein-folding protocol (*37*). Although the chaperone could be recycled, its low stability prevented multiple uses. PDI has been immobilized on an agarose support that then demonstrated protein-folding activity in both column- (*38*) and batch-modes (*39*). The covalent attachment of PDI was achieved through the nonspecific reaction of lysine residues with activated agarose, a process that can render the active site inaccessible to substrate and thereby decrease the efficiency of catalysis. Finally, agarose gel beads decorated with a GroEL minichaperone and the foldases DsbA and peptidyl-prolyl isomerase were shown to facilitate the folding of proteins that are particularly intractable toward in vitro folding (*40*, *41*). These proteins were likewise immobilized through the random attachment of amino groups to activated agarose.

Small-molecule additives have also proven to be useful for increasing the efficiency of protein folding in vitro. For example, aggregation can be minimized by the addition of L-arginine (*42*). An artificial molecular chaperone system has been developed on the basis of the solubilization of unfolded proteins by detergent followed by the stripping of detergent from the protein by β-cyclodextrin (*43*). This method has also been adapted for use with insoluble, polymeric β-cyclodextrin (*44*).

Small-molecule thiol−disulfide couples are useful for in vitro oxidative protein folding (*9*, *45*). The disulfide can oxidize the reduced protein; the thiol can isomerize non-native intermediates. The isomerization of these intermediates often limits the rate of oxidative protein folding (*4*, *46*, *47*). Dithiols are more efficient at disulfide bond isomerization than monothiols (*8*, *13*, *14*). Thus, the attachment of trithiol **1** to a solid support could provide an efficient and convenient means to effect oxidative protein folding in vitro.

Trithiol **1** can be coupled to Br-TG resin in a variety of ways. If the trithiol displaces every bromine on the TG resin and every immobilized trithiol molecule is attached to the resin through only one of its thiols, then the coupling yield would be 2 mol of thiol/mol of bromine (or, 0.56 mmol of thiol/g of resin). Likewise, if the trithiol displaces every bromine on the resin and every immobilized trithiol is attached to the resin through two of its thiol groups, then the coupling yield should be 0.5 mol of thiol/mol of bromine (0.14 mmol of thiol/g of resin). Coupling of trithiol **1** to Br-TG resin produced a yield of 1 thiol group per bromine on the TG resin (0.28 mmol of thiol/g of resin). In extrema, this yield of immobilized thiol implies either that one-half of the coupled trithiol was singly attached while the other half was doubly coupled to the TG resin or that the trithiol was predominantly singly coupled to the TG resin but only coupled to half of the total sites. The observation that only 14% of the TG-bound thiols formed mixed disulfides upon oxidation with DTNB demonstrates that trithiol-coupled

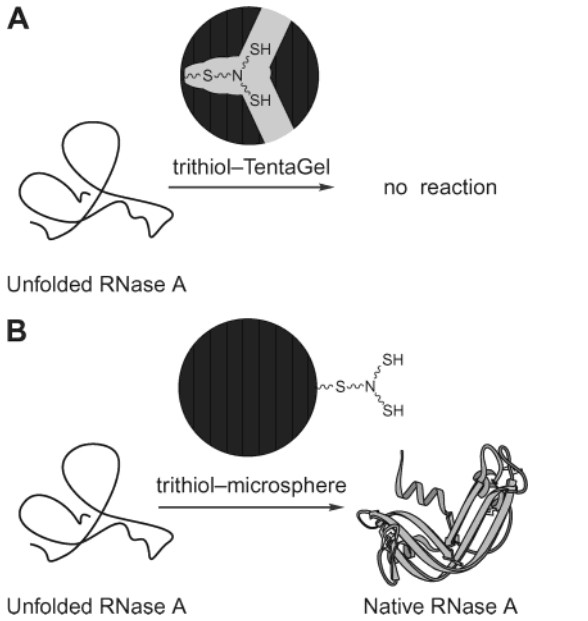

Figure 5. Model for the higher activity of trithiol **1** coupled to microspheres relative to trithiol **1** coupled to TentaGel resin. (A) Dithiols displayed on TG resin are located predominantly within pores of the resin. A protein such as RNase A cannot access these dithiols and thus does not fold efficiently. (B) Dithiols displayed on microspheres have a high surface density. RNase A can interact readily with these dithiols and thus folds efficiently.

TG resin effectively forms cyclic disulfides. Further, coupling reactions performed at lower ratios of trithiol **1**:Br-TG gave lower yields of coupled thiol per mg of resin, and these resin-bound thiols had a greater tendency to form mixed disulfides upon DTNB oxidation. The lower yields can be attributed to greater cross-linking of the trithiol (Figure 3B,C). The greater tendency of these coupling products to form mixed disulfides with NTB implies that cyclic disulfides are more likely to form within a molecule coupled at one site (Figure 3A) than between two molecules coupled at different sites.

To optimize its disulfide bond isomerization activity, the dithiol should have a high disulfide $E^{\circ\prime}$. Incubation of the fully reduced trithiol-coupled TG resin in a glutathione redox buffer with $E_{\text{solution}} = -180$ mV causes approximately 90% oxidation of the resin-bound thiols. This observation indicates that the trithiol-coupled TG resin has an apparent disulfide reduction potential of $E_{\text{resin}} = -208$ mV, which is close to that of the PDI active site ($E^{\circ\prime} = -180$ mV (*6*)).

Surprisingly, the trithiol-coupled TG resin has little protein disulfide bond isomerization activity. This resin can readily undergo thiol−disulfide interchange reactions with a variety of small molecules (e.g., DTT or DTNB), and the TG resin itself does not hinder RNase A folding significantly. These results indicate that thiols attached to TG are within pores and thus inaccessible to proteins (Figure 5A). Small molecules ($M_r < 1000$) can diffuse readily through these pores, but RNase A ($M_r = 13\,682$) cannot. Similar results were obtained with poly(ethylene glycol) polyacrylamide (PEGA) resin displaying a dithiol (data not shown). Previously, size exclusion was shown to be problematic in the detection of the interaction of a protein with small molecules synthesized directly on this solid support (*48*).

A copolymer of styrene and glycidyl methacrylate will form a microsphere in water. The styrene forms a hydrophobic core, and the hydrophilic glycidyl methacry-

late coats the solvent-exposed surface. Such microspheres provide a high surface density of electrophilic epoxy groups. The presentation of functional groups on the exterior of a microsphere circumvents the size exclusion limit presented by the porous TG resin. Indeed, microspheres coupled to DTT present monothiols on their surface and have been shown to have RNase A folding activity (*49*, *50*). Further, these DTT-coupled microspheres have a higher affinity for unfolded RNase A than for native RNase A (*50*), which serves to bring the protein in proximity to the thiol groups. DTT-coupled microspheres can be recycled readily and used for multiple folding reactions with only a slight loss of activity (*49*).

The previously reported coupling of DTT to epoxy microspheres was carried out in an unbuffered solution at pH 10 and 60 °C to produce a low yield of immobilized thiol (0.0074 mmol/g of microsphere) (*49*). These conditions are harsher than those used to couple trithiol **1** to Br-TG resin and necessitated by the difficulty of opening an epoxide relative to displacing a bromide. Using a strong base (H^-) and activation of the epoxy oxygen with a Lewis acid (Li^+) resulted in a 41-fold increase in DTT-coupling yield (0.30 mmol/g of microsphere) relative to that reported previously (*49*).

Yet, using these same conditions to couple trithiol **1** to the microspheres produced a resin that displayed few thiols (0.004 mmol/g of microsphere). Further, these trithiol-coupled microspheres had a high propensity for forming stable mixed disulfides upon oxidation with DTNB. These results indicate that the trithiol readily forms cross-links to the microsphere (Figure 3B,C). The proximity of thiol groups to the microsphere surface after the first coupling could increase the rate of attack by a second (and third) thiol group on neighboring epoxy groups. The larger number of atoms between thiols in trithiol **1** than in DTT (11 versus 4) could allow the immobilized thiols greater access to neighboring epoxy groups and thus increase the susceptibility of the trithiol to form cross-links (Figure 3B,C). Indeed, a similar increase in local concentration has been harnessed for the synthesis of homodimeric molecules from reactive monomers bound to the same polymer bead (*51*).

One advantage of microspheres relative to TG resin is the high surface density of their functional groups. This high surface density, however, also proved to be detrimental to efficient coupling and dithiol presentation. To improve the coupling efficiency, some of the surface density was sacrificed by blocking a fraction of the reactive epoxy groups with the monothiol NMA before coupling to trithiol **1** (Figure 2B). This strategy provided a 14-fold increase in the yield of immobilized thiol. Further, the propensity of this trithiol-coupled microsphere to form stable mixed disulfides decreased greatly (Table 1).

Whether coupled to DTT or trithiol **1**, the microspheres (in contrast to the trithiol-coupled TG resin) showed substantial disulfide bond isomerization activity (Figure 4). The higher activity of the trithiol-coupled microspheres relative to trithiol-coupled TG resin is likely to stem from the greater accessibility of dithiol on the microspheres (Figure 5B). The trithiol-coupled microspheres produced a 1.5-fold higher yield of active RNase A than did the DTT-coupled microspheres. Such a difference in yield between a dithiol and a monothiol has also been observed in the solution-phase folding of RNase A (*14*).

Catalysis of protein folding by trithiol **1**-coupled microspheres could be enhanced still further. Herein, the solid-phase reagents were used in a fully reduced form. Solution-phase reagents are typically a mixture of reduced and oxidized forms (such as GSH and GSSG). The use of trithiol **1**-coupled microspheres that display an appropriate mixture of reduced and oxidized forms could be especially advantageous.

Conclusions

Solid-phase reagents can be used to effect high-yielding transformations on solution-phase reactants without the need for purification. Indeed, the use of solid-supported reagents is an emerging strategy in synthetic organic chemistry (*52*−*54*). This strategy is also applicable to the facilitation of protein folding in vitro. Herein, we have shown that microspheres coupled to trithiol **1** catalyze native disulfide bond formation in a protein. Such microspheres are able to withstand exposure to organic solvents or high temperatures (*55*). This robust solid-phase reagent could be useful in numerous protein-folding protocols.

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