Artificial Chaperone-Assisted Refolding of Denatured-Reduced Lysozyme: Modulation of the Competition between Renaturation and Aggregation†

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ABSTRACT: Conditions that promote renaturation of an unfolded protein also promote protein aggregation, in many cases, because these competing intramolecular and intermolecular processes are driven by similar networks of noncovalent interactions. The GroEL/GroES system and related biological chaperones facilitate the renaturation of substrate proteins by minimizing the aggregation pathway. We have devised a two-step method in which small molecules, "artificial chaperones," facilitate protein refolding from a chemically denatured state. In the first step, the protein is captured by a detergent as guanidinium chloride is diluted to a non-denaturing concentration; formation of a protein−detergent complex prevents both protein aggregation and proper refolding. In the second step, a cyclodextrin strips detergent from the protein, allowing the protein to refold. Here we describe the first application of this method to a protein that must form disulfides in the native state. Lysozyme (hen egg white) can be refolded from the Gdm-denatured, DTT-reduced state in good yields at final protein concentrations as high as 1 mg/mL with the artificial chaperone method. Several mechanistic aspects of artificial chaperone-assisted refolding have been probed, and a detailed mechanism for the kinetically controlled stripping step is proposed.

Many studies of protein folding focus on systems that are well-behaved in the test tube: the particular protein and conditions are chosen so that proper folding is the dominant process upon removal of denaturing conditions [e.g., dilution of a concentrated urea or guanidinium chloride (GdmCl)[1] solution]. While such studies provide insight on important aspects of the folding process, crucial questions involving the competition between intramolecular and intermolecular association of peptide surfaces are side-stepped by this experimental strategy (Jaenicke, 1993). Two developments have stimulated rising interest in the competition between folding and aggregation. First, it has become clear that biological systems have a sophisticated machinery, the chaperone proteins, for encouraging protein folding by discouraging aggregation (Hartl, 1996). Second, the refinement of genetic engineering techniques has made the heterologous expression of proteins routine, and such proteins must often be folded from a chemically denatured state (Cleland, 1993; Georgiou & DeBernardez-Clark, 1991).

We have recently described a new approach for controlling the competition between renaturation and aggregation in vitro (Rozema & Gellman, 1995, 1996). This method employs small molecules, a detergent and a cyclodextrin, to guide the folding process. We refer to these low molecular weight assistants as "artificial chaperones," because the development of this technique was inspired by the mechanism of the GroEL/GroES chaperone system. Although the details of the GroEL/GroES mechanism are still the subject of debate, the general outline of a two-step mechanism seems to be established (Hartl, 1996). In the first step, GroEL captures the non-native substrate protein by binding to exposed hydrophobic surfaces. This binding prevents both aggregation and proper folding of the substrate protein. In the second step, release of the substrate and concomitant folding are triggered by interaction between GroEL and ATP; GroEL−GroES interaction is also important for the release of some substrate proteins. Substrate folding may require multiple binding−release cycles. The artificial chaperone method, too, has two distinct steps (Scheme 1). In the first step, a detergent captures the non-native protein under conditions that would otherwise lead to aggregation, e.g., rapid dilution from a concentrated GdmCl solution. Formation of the protein−detergent complex prevents both protein aggregation and renaturation. In the second step, the detergent is stripped from the protein by addition of a cyclodextrin, allowing at least some of the protein to fold.

Small molecules have previously been employed as folding assistants in vitro, but the artificial chaperone method represents a fundamentally new strategy. Most previous uses of small molecules conform to what we call the "dilution additive method" (Scheme 1). In this one-step technique, the small molecule is introduced in the solution used to dilute urea or GdmCl to a non-denaturing concentration. The additive is believed to function by transient association with the non-native protein, which selectively diminishes the intermolecular attraction between peptide segments that leads to aggregation, relative to the intramolecular attraction that drives folding (Cleland et al., 1992a,b; Cleland & Randolph, 1992; Zardeneta & Horowitz, 1992). Successful dilution additives have included detergents (Tandon & Horowitz, 1986, 1988; Wetlaufer & Xie, 1995), other amphiphiles

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[1] Abbreviations: CTAB, cetyltrimethylammonium bromide; CTAHS, cetyltrimethylammonium hydrogen sulfate; TTAB, tetradecylammonium bromide; Z 3-14, Zwittergent 3-14 [$CH_3(CH_2)_{13}N(CH_3)_2CH_2CH_2CH_2SO_3$]; POE(10)L, $CH_3(CH_2)_{11}(OCH_2CH_2)_{10}OH$; GdmCl, guanidinium chloride; DTT, dithiothreitol; CD, circular dichroism; PEG, polyethylene glycol; CMC, critical micelle concentration.

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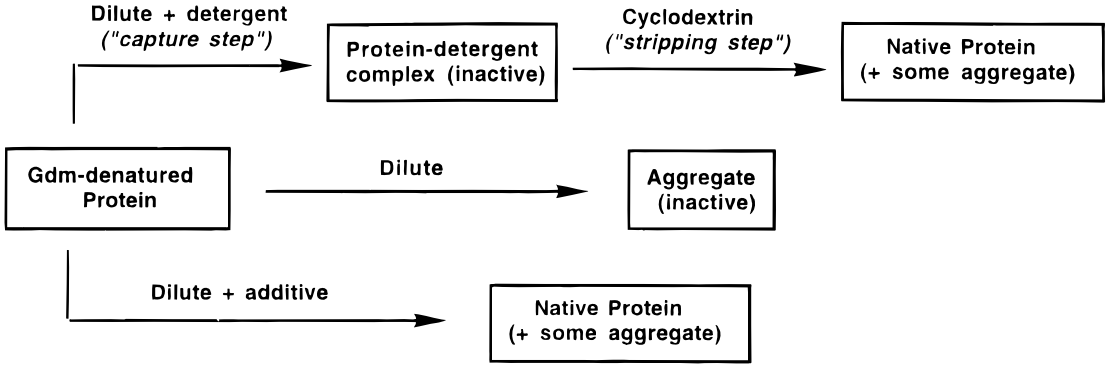
Scheme 1: Artificial Chaperone-Assisted Protein Refolding (Uppermost Path) vs Dilution Additive-Assisted Protein Refolding (Lowermost Path)



(Goldberg et al., 1996; Wetlaufer & Xie, 1995), cyclodextrins (Karuppiah & Sharma, 1995), and polyethylene glycol (PEG) (Cleland & Wang, 1990; Cleland et al., 1992a,b).

We have shown that the artificial chaperone method facilitates renaturation of two non-homologous proteins, carbonic anhydrase B (CAB) and citrate synthase (Rozema & Gellman, 1995, 1996). In this paper, we expand the method to lysozyme, which, unlike CAB and citrate synthase, contains disulfide links in the native state. Lysozyme from hen egg white has 129 residues; the crystal structure shows two domains, one composed largely of four α-helices, and the other composed largely of a triple-stranded antiparallel β-sheet (Blake et al., 1965). If lysozyme is denatured without reducing the four disulfides, refolding by rapid dilution is a very efficient process (Goldberg et al., 1994), and this efficiency has allowed extensive characterization of this renaturation process (Dobson et al., 1994). Renaturation of lysozyme by rapid dilution from the reduced state, however, is inefficient unless protein concentration is very low, because of competing aggregation (Goldberg et al., 1991; Fischer et al., 1993).

MATERIALS AND METHODS

Materials. Hen egg white lysozyme (three-times recrystallized and lyophilized), dried *Micrococcus lysodeikticus* cells, glutathione (GSH) (95%), oxidized glutathione (GSSG) (98%), dithiothreitol (DTT) (99%), polyoxyethylene(10 units) lauryl ether (POE(10)L), *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Z 3-14), tetradecyltrimethylammonium bromide (TTAB), and cetyltrimethylammonium bromide (CTAB) (99%) were obtained from Sigma (St. Louis, MO). Sequanal grade guanidinium chloride (GdmCl), HEPES buffer, and electrophoresis grade Tris base were obtained from Fisher Scientific (Pittsburgh, PA). 1,3-Dibromopropane, 4-(*tert*-octyl)phenol, trimethylamine, and Orange OT were obtained from Aldrich. Bio-Beads were obtained from Bio-Rad. Calbiosorb was obtained from Calbiochem. α-Cyclodextrin, β-cyclodextrin, epichlorohydrin β-cyclodextrin polymer, and methyl-β-cyclodextrin were gifts from American Maize Products Inc. (Hammond, IN). α- and β-Cyclodextrins were recrystallized from water. Orange OT was recrystallized from ethanol. All other materials were used as obtained from suppliers.

Methods

Protein concentration for native lysozyme was determined by absorbance at 280 nm with an extinction coefficient of 2.37 (mg/mL protein)$^{-1}$ cm^{-1} (Wetlaufer *et al*., 1974). The concentration of GdmCl stock solutions was determined by measuring the refractive index of the solution (Pace, 1986).

Denaturation−Reduction of Lysozyme. A solution of 50 mg/mL lysozyme in 6 M GdmCl, 100 mM Tris sulfate, pH 8.5, and 30 mM DTT was prepared by addition of 3 μL of 167 mg/mL lysozyme stock solution to 7 μL of 8.7 M GdmCl, 143 mM Tris sulfate, and 43 mM DTT solution. After vigorous mixing of the solution by vortexing, the solution was allowed to sit for at least 5 h.

Renaturation−Oxidation of Lysozyme. 1 μL of denatured−reduced lysozyme solution was placed on the side of a microcentrifuge tube that contained various volumes and concentrations of aqueous detergent and GSH/GSSG. Mixing of the two solutions was accomplished by rapid vortexing. 10 min after mixing, 75−300 μL of water or β-cyclodextrin solution was added, and then the mixture was vortexed to bring the sample to final conditions before assay. For the study of cyclodextrin as a dilution additive, 1 μL of denatured−reduced lysozyme was diluted directly into buffer containing cyclodextrin.

Copper sulfate-catalyzed oxidation of denatured−reduced lysozyme was performed by dilution of 50 mg/mL of denatured−reduced lysozyme in 6 M GdmCl and 30 mM DTT to 0.071 mg/mL lysozyme, 8.6 mM GdmCl, 0.04 mM DTT, 0.071 mM $CuSO_4$, 1.43 mM EDTA, and 0 or 5.7 mM CTAB. The solutions were allowed to sit for various periods of time, and then β-cyclodextrin was added to make a solution of 0.05 mg/mL lysozyme, 6.0 mM GdmCl, 0.03 mM DTT, 0.05 mM $CuSO_4$, 1.0 mM EDTA, 0 or 4.0 mM CTAB, and 4.8 mM β-cyclodextrin. After sitting 40 h, the solutions were assayed for enzymatic activity.

To investigate solid stripping agents, 0.175 mL of a solution of 0.071 mg/mL lysozyme, 143 mM Tris sulfate, pH 8.5, 8.6 mM GdmCl, 0.043 mM DTT, 5.7 mM GSH, 0.57 mM GSSG, 1.43 mM EDTA, and 1.1 mM CTAB, which was generated by dilution of denatured−reduced lysozyme, was added to 50 mg of Bio-Beads or epichlorohydrin β-cyclodextrin polymer or to a 0.2 mL slurry of Calbiosorb beads. The solutions were gently rocked overnight and then assayed for enzymatic activity.

Native Samples. Native samples were prepared from 50 mg/mL stock solutions that did not contain GdmCl, Tris sulfate, or DTT. The lysozyme was diluted to 0.286 or 0.072 mg/mL lysozyme in the presence of 143 mM Tris sulfate, pH 8.5, and 1.43 mM EDTA. The solutions were then diluted further by the addition of water or aqueous solutions

of β-cyclodextrin to make solutions of 0.2 or 0.05 mg/mL lysozyme, 100 mM Tris sulfate, pH 8.5, and 1.0 mM EDTA.

Assay of Enzymatic Activity. The assay of lysozyme was a modification of a published procedure (Jolles, 1962). Depending on the concentration of the stock solution, 500 μL of *M. lysodeikticus* cell suspension was added to 1−10 μL of lysozyme solution in a methacrylate 1 mL cuvette to bring the lysozyme concentration to 1−2 μg/mL. The mixture was vigorously shaken and allowed to sit for 25 s before assay. The decrease in light scattering intensity of the solution was then measured by following the decrease in apparent absorbance of the solution at 450 nm. The rate of the decrease in light scattering intensity was found to be directly proportional to the amount of active lysozyme in the sample.

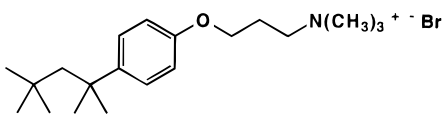
The *M. lysodeikticus* suspension was prepared by mixing 0.16 mg of dried *M. lysodeikticus* cells per mL of 50 mM sodium phosphate buffer, pH 6.2. The suspension was filtered through filter paper and stored frozen in 10 mL aliquots. Prior to assay, the solutions were warmed to room temperature and vigorously shaken to ensure a resuspension of all insoluble material.

Measurement of Refolding Kinetics. The measurement of refolding kinetics was a modification of a published procedure (Saxena & Wetlaufer, 1970). At various times in the course of refolding, 20 μL aliquots of the refolding lysozyme solutions were added to 20 μL of 100 mM acetic acid solutions. Acidification stops the refolding of lysozyme by stopping the exchange of disulfide bonds. The lysozyme samples were then assayed for enzymatic activity within 1 h after acidification of the refolding mixture. A plot of enzymatic activity as a function of the time of acidification was fit to an exponential decay function by Graph Pad Prism version 1.03 curve fitting program (Graph Pad Software, Inc.).

Fluorescence Measurements. Intrinsic protein fluorescence spectra were measured using a Hitachi F-4500 spectrophotometer. The quartz cuvettes were 3 × 3 mm. All samples were prepared according to procedures described above. Excitation and emission slits were 5 nm. The fluorescence was measured by excitation at 280 nm.

Circular Dichroism Spectra. An OLIS-modified Cary UV60 instrument was used for all CD experiments. The calibration factor of the instrument was adjusted using aqueous solutions D-10-camphorsulfonic acid (Cassim & Yang, 1969). The CD spectra were taken 1 h after dilution from denaturing−reducing solution. For all measurements a reference sample containing buffer, detergent, and cyclodextrin was subtracted from the CD signal.

Synthesis of Detergent TOPPA. 4-(*tert*-Octyl)phenol (4.6



TOPPA

gm, 22 mmol) was dissolved in 75 mL of THF that had been distilled from sodium metal under nitrogen. To this solution was added 1 equiv of solid KOH (1.4 gm, 22 mmol) and 1 equiv of 1,3-dibromopropane (2.3 mL, 22 mmol). The mixture was allowed to stir for 4 days. The solid was then filtered off, and the THF was removed by rotary evaporation. The resulting oil was then placed under high vacuum for 1 h to remove any unreacted 1,3-dibromopropane. The oil was then dissolved in 10 mL of 1,4-dioxane. The solution was partitioned equally into three screw-cap vials. Trimethylamine gas was bubbled through the solution at 0 °C until there was approximately a 2 mL increase in volume (6 mL, 66 mmol). After 3 days, the dioxane was removed by rotary evaporation. The residue was then washed with 200 mL of diethyl ether and dissolved in 200 mL of acetone. The acetone solution was filtered, and the acetone was removed by rotary evaporation. The product was recrystallized from ethyl ether/acetone solutions to produce 1.0 g of white crystals (13% yield): mp 173−175 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.70 (s, 9H, $C(CH_3)_3$), 1.33 (s, 6H, $C(CH_3)_2$), 1.69 (s, 2H, CH_2), 2.3 (m, 2H, NCH_2), 3.52 (s, 9H, $N(CH_3)_3$), 3.86 (m, 2H, CH_2), 4.09 (t, J = 3.0 Hz, 2H, CH_2), 6.78 (ABq, J = 8.5 Hz, 2H, ArH), 7.20 (ABq, J = 8.5 Hz, 2H, ArH). ^{13}C-NMR ($CDCl_3$, 300 MHz): δ 23.6 (CH_2), 31.6 (CH_3), 31.8 (CH_3), 32.3 (C), 38.0 (C), 53.7 (CH_3), 56.9 (CH_2), 63.8 (CH_2), 64.8 (CH_2), 113.7 (CH), 127.3 (CH), 143.2 (C), 155.5 (C). IR (KBr pellet): 3455 (s), 3391 (s), 2961 (m), 2864 (m), 1627 (m), 1514 (s), 1289 (s) cm^{-1}. HRLSIMS m/z 306.2799 (calcd for $C_{20}H_{36}NO^+$ 306.2797).

Orange OT Dye-Uptake Determination of CMC. To a 0.3 mL solution of 143 mM Tris sulfate, pH 8.5, 1.43 mM EDTA, and 0.71−35.7 mM detergent TOPPA was added solid orange OT. After gentle rocking of each solution for 48 h, the solid orange OT was filtered off through a cotton plug. A 0.2 mL aliquot of each filtrate was diluted with 0.3 mL of absolute ethanol. The absorbance of these solutions was measured at 500 nm using a 10 mm pathlength cell. A_{500} was plotted as a function of detergent concentration, which resulted in a graph consisting of two linear regions. The intersection of these two regions was taken to define the CMC of detergent TOPPA.

RESULTS

Reduced−Denatured Lysozyme Can Be Efficiently Folded via the Artificial Chaperone Protocol, if Ionic Detergents Are Used in the Capture Step. Table 1 shows the results of artificial chaperone-assisted refolding of lysozyme with several detergents, and appropriate control experiments. Refolding trials and control studies were assessed in terms of enzymatic activity, in a standard assay, and each result is normalized to the activity of native lysozyme.

Only ca. 21% lysozyme activity was recovered via unassisted refolding under the conditions employed in Table 1: denaturation of 50 mg/mL lysozyme in 6 M GdmCl, 30 mM dithiothreitol (DTT), 10 mM Tris sulfate, pH 8.5, followed by 250-fold dilution with 100 mM Tris sulfate buffer, pH 8.5, containing 4 mM glutathione (GSH) and 4 mM glutathione disulfide (GSSG). Aggregated protein was visible in the final solution. Our observation that aggregation predominates over folding at a final lysozyme concentration of 0.2 mg/mL is consistent with earlier findings (Goldberg et al., 1991). The artificial chaperone strategy, however, substantially improves the refolding yield: inclusion of 5.7 mM CTAB in the dilution buffer, and subsequent addition of 4 equiv of methyl-β-cyclodextrin (relative to detergent) to the resulting lysozyme−CTAB complex, provided ca. 85% of the original enzymatic activity. (We used the synthetic derivative methyl-β-cyclodextrin because of the low aqueous

Table 1: Lysozyme Refolding[a]

sample	relative rate
Controls	
native lysozyme	1.00
+ methyl-β-CD	0.90 ± 0.07
+ CTAB	1.00 ± 0.05
+ TTAB	1.12 ± 0.08
+ Z 3-14	0.96 ± 0.09
+ POE(10) L	0.97 ± 0.11
After Gdm Denaturation	
no additives	0.21 ± 0.03
no detergent; then methyl-β-CD	0.28 ± 0.04
+ CTAB	0.10 ± 0.07
+ CTAB; then methyl-β-CD	0.85 ± 0.05
+ TTAB	0.00 ± 0.01
+ TTAB; then methyl-β-CD	0.89 ± 0.03
+ Z 3-14	0.14 ± 0.04
+ Z 3-14; then methyl-β-CD	0.87 ± 0.07
+ POE(10)L	0.21 ± 0.05
+ POE(10)L; then methyl-β-CD	0.21 ± 0.04

[a] Protocol: Lysozyme, 50 mg/mL, was denatured for 5 h in 6 M GdmCl, 30 mM DTT, 100 mM Tris sulfate, pH 8.5. This solution was then diluted to 0.29 mg/mL lysozyme, 34 mM GdmCl, 0.17 mM DTT, 143 mM Tris sulfate, pH 8.5, 1.43 mM EDTA, 5.7 mM GSH, 5.7 mM GSSG, and, when indicated, 5.7 mM detergent (0.175 mL aliquots). After 10 min, these aliquots were diluted with 0.075 mL of aqueous methyl-β-cyclodextrin or water to give 0.2 mg/mL lysozyme, 24 mM GdmCl, 0.12 mM DTT, 100 mM Tris sulfate, pH 8.5, 1.0 mM EDTA, 4.0 mM GSH, 4.0 mM GSSG, and, when indicated, 4.0 mM detergent and 16.5 mM methyl-β-cyclodextrin. These solutions stood overnight before assay (Jolles, 1962). The "control" samples were never denatured.

solubility of β-cyclodextrin itself.) The cationic detergent TTAB (shorter homologue of CTAB) and the zwitterionic detergent Z 3-14 were similarly effective. The nonionic detergent POE(10)L, in contrast, did not enhance lysozyme reactivation, because this detergent does not prevent protein aggregation. Thus, as observed with CAB (Rozema & Gellman, 1996), ionic detergents can serve as artificial chaperones for lysozyme folding, but nonionic detergents are not successful in this role. (We did not examine anionic detergents because of their tendency to precipitate in the presence of GdmCl.)

The optimal GSH:GSSG ratio varies with lysozyme concentration. We were surprised by this observation because a 10:1 GSH:GSSG ratio has been recommended for general use in renaturation studies (Jaenicke & Rudolph, 1989). Saxena and Wetlaufer (1970) originally discovered that a combination of oxidized and reduced thiol facilitates lysozyme refolding from the reduced state, apparently by catalyzing thiol−disulfide interchange. In experiments involving final lysozyme concentrations of ≤0.015 mg/mL, these workers found that a 10:1 GSH:GSSG ratio was optimal (Saxena & Wetlaufer, 1970), and this ratio has been adopted by other workers trying to refold disulfide-containing proteins from the reduced state. For artificial chaperone-assisted folding at 0.05 mg/mL final lysozyme concentration, we find that the 10:1 ratio gives high yields, but experiments conducted at 0.2 mg/mL final protein concentration required a 1:1 ratio for the best yields.

It is often desirable to conduct protein refolding at as high a final concentration as possible. The final lysozyme concentration in the artificial chaperone protocol can be raised if the detergent and cyclodextrin concentrations are also raised. For example, when 50 mg/mL lysozyme denatured-reduced in 6 M GdmCl, 30 mM DTT, was diluted 35-fold with buffer containing 17 mM CTAB, and methyl-β-cyclodextrin was subsequently added, to give final concentrations of 12 mM CTAB, 150 mM methyl-β-cyclodextrin, and 1.0 mg/mL lysozyme, 57% of the enzymatic activity was recovered. When the same dilution protocol was followed, but the detergent was omitted in the first step, only 6% of the enzymatic activity was recovered.

The protein−detergent complexes formed during the capture step have native-like secondary structure, according to far-UV circular dichroism (CD) data, but the tertiary structure has been altered, according to near-UV CD and intrinsic fluorescence data. Figure 1a shows far-UV CD data for native lysozyme and for the complex formed between lysozyme and cetyltrimethylammonium hydrogen sulfate (CTAHS), upon 150-fold dilution of DTT-reduced, Gdm-denatured lysozyme with a solution containing the detergent. CTAHS was used for the CD studies because the strong absorption of the bromide ion of CTAB can lead to poor-quality data. CTAHS provides refolding yields similar to those reported in Table 1 for CTAB. The similarity of the curves in Figure 1a suggests that the protein component of the lysozyme−CTAHS complex has secondary structure similar to that of the native enzyme. This observation is particularly interesting given that there are presumably no disulfides in this lysozyme−CTAHS complex, since the GSH/GSSG mixture was not added. The apparently native-like secondary structure of the CTAHS-complexed lysozyme stands in contrast to the non-native secondary structure previously observed for CTAHS-complexed CAB (Rozema & Gellman, 1996).

Figure 1b shows near-UV CD data for native lysozyme and the lysozyme−CTAHS complex formed by dilution of DTT-reduced, Gdm-denatured protein (no GSH/GSSG added). The lysozyme−CTAHS complex displays significant circular dichroism in this region, but the variation from the native lysozyme data suggests that the tertiary packing in the detergent complex is different from that in the native enzyme. The intrinsic fluorescence data in Figure 1c support the conclusion that tertiary structure in the detergent-complex lysozyme is different from native tertiary structure. Detergent-complexed protein shows much higher fluorescence intensity than native lysozyme, suggesting that the fluorescing side chains (presumably tryptophan) are less subject to internal quenching in the detergent complex than in the native folded state. The fluorescence maximum for both samples is 341 nm, implying significant protection of tryptophan residues from aqueous solvation. In contrast, GdmCl-denatured, DTT-reduced lysozyme displays a fluorescence maximum at 350 nm (Raman et al., 1996), which is typical of water-exposed tryptophan.

Interactions between CTAB and native lysozyme have previously been examined by CD (Subramanian et al., 1984). Subramanian et al. report that addition of CTAB to lysozyme at pH 9 causes increased ellipticity in the far-UV region, which was attributed to increased α-helix formation, but that no such effect was observed at pH 5 or pH 7. The far-UV CD comparison between native lysozyme and the lysozyme−CTAHS complex shown in Figure 1a was carried out at pH 8.5, and we cannot detect an increase in ellipticity in the complex; mixing native lysozyme and CTAHS under these conditions also does not cause significant far-UV CD changes (not shown). The previous workers also detected CTAB-induced changes in the near-UV CD upon mixing detergent

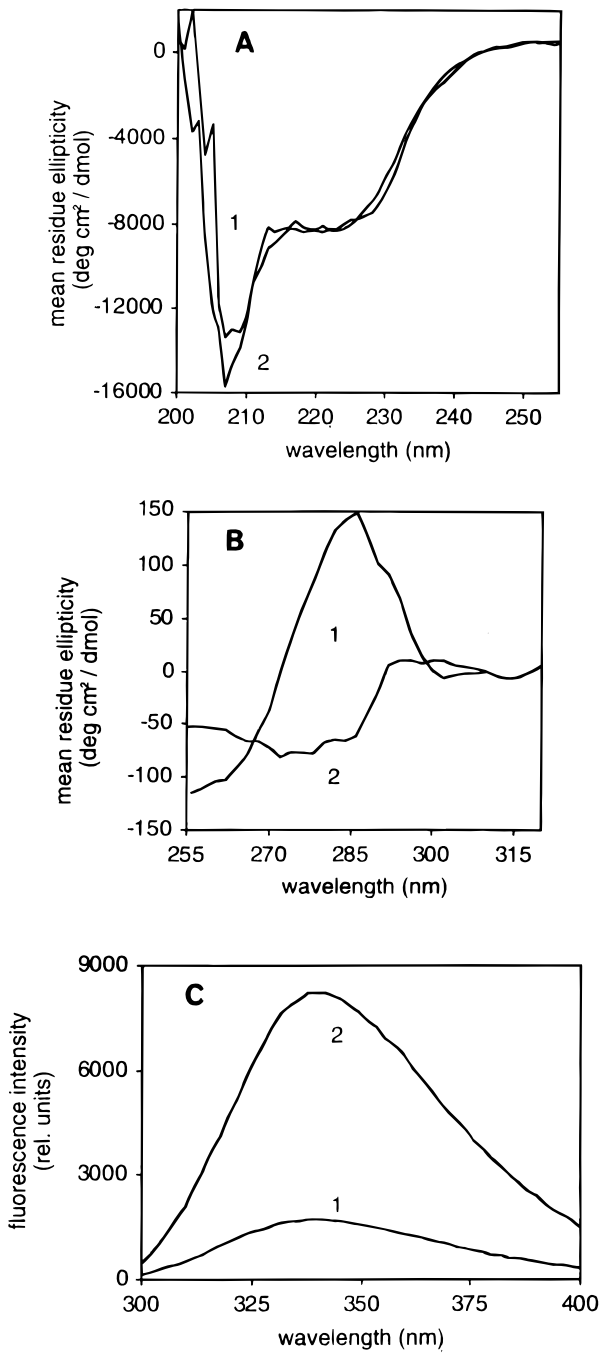


FIGURE 1: (A) Far-UV CD spectra of native lysozyme (curve 1) and lysozyme−CTAHS complex formed by capture from the denatured−reduced state (curve 2). The two curves are indistinguishable within the uncertainty of the data. The lysozyme−CTAHS complex was formed by diluting a sample of 50 mg/mL lysozyme, 6 M GdmCl, 100 mM Tris sulfate, pH 8.5, and 30 DTT by a factor of 150−0.333 mg/mL lysozyme, 83 mM Tris sulfate, pH 8.5, 40 mM GdmCl, 0.2 mM DTT, and 0.83 mM EDTA, and 6.67 mM CTAB. The native sample was prepared from a 50 mg/mL lysozyme stock solution that did not contain GdmCl, Tris sulfate, or DTT. The native stock solution was diluted to 0.333 mg/mL lysozyme, 50 mM Tris sulfate, and 0.5 mM EDTA. Path length of the cell was 1.0 mm. (B) Near-UV CD spectra of native lysozyme (curve 1) and lysozyme−CTAHS complex formed by capture from the denatured−reduced state (curve 2). The samples were prepared as described above. Pathlength of the cell was 10 mm. (C) Intrinsic fluorescence spectra of native lysozyme (curve 1) and lysozyme−CTAHS complex formed by capture from the denatured−reduced state (curve 2). The samples were prepared as described above. The fluorescence spectra were measured using a 3 × 3 mm quartz cell. The samples were irradiated at 280 nm.

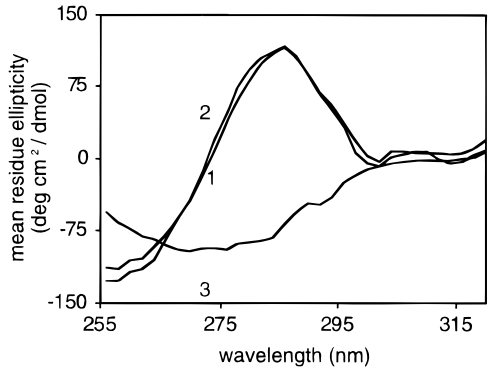


FIGURE 2: Near-UV CD spectra of native lysozyme at 0.333 mg/mL lysozyme in 83 mM Tris sulfate pH 8.5 and 0.83 mM EDTA (curve 1) and the lysozyme−CTAHS complex formed by the addition of CTAHS to native lysozyme (curve 3) at 0.333 mg/mL lysozyme, 83 mM Tris sulfate, pH 8.5, 0.83 mM EDTA, and 6.7 mM CTAHS. Curve 2 is the spectrum of lysozyme−CTAHS complex after addition of β-cyclodextrin, to generate a solution of 0.2 mg/mL lysozyme, 50 mM Tris sulfate, pH 8.5, 0.5 mM EDTA, 4.0 mM CTAHS, and 16.5 mM methyl-β-cyclodextrin. Path length of the cell was 10 mm.

and native lysozyme at pH 9 (Subramanian et al., 1984). As shown in Figure 2, we observe similar effects of CTAHS on the near-UV CD spectrum of native lysozyme, and this native lysozyme−CTAHS combination displays increased fluorescence intensity, but no shift in fluorescence maximum, relative to the native enzyme alone (data not shown). These spectroscopic effects of added CTAHS suggest an underlying conformational change, but the enzymatic activity of native lysozyme is not diminished in the presence of CTAHS (data not shown). Addition of cyclodextrin to the solution containing native lysozyme and CTAHS regenerates the near-UV CD and intrinsic fluorescence signatures of the native enzyme in the absence of detergent. It is interesting to note that the near-UV CD spectrum of native lysozyme in the presence CTAHS (curve 3 of Figure 2), a fully active species, is similar to that of the inactive lysozyme−CTAHS complex generated during the capture step (curve 2 of Figure 1b).

Cooperative Interaction among Detergent Molecules Is Required in the Capture Step. Figure 3 shows the effect of CTAB concentration at the capture step on the final regain of lysozyme activity after addition of the cyclodextrin stripping agent. Data are shown for two series of experiments, with differing protein concentrations. In one series, the lysozyme concentration was 0.071 mg/mL after capture and 0.05 mg/mL after stripping, and in the second series the lysozyme concentration was 0.29 mg/mL after capture and 0.20 mg/mL after stripping.

The sigmoidal shape of the curves in Figure 3 indicates that cooperative interaction among detergent molecules is required for formation of the protein−detergent complex. Cooperative detergent self-association to form micelles is a hallmark of detergent behavior; the "critical micelle concentration" (CMC) is the minimum concentration at which micelles occur (Myers, 1992). Artificial chaperone-assisted CAB refolding displays a similar sigmoidal dependence on detergent concentration, and the protein can be captured well below the detergent's CMC, which suggests that the protein−detergent complex is a mixed micelle (Rozema & Gellman, 1996). The data in Figure 3 support this conclusion, since the principal effect of raising the lysozyme concentration 4-fold is to shift the midpoint of the sigmoidal curve roughly

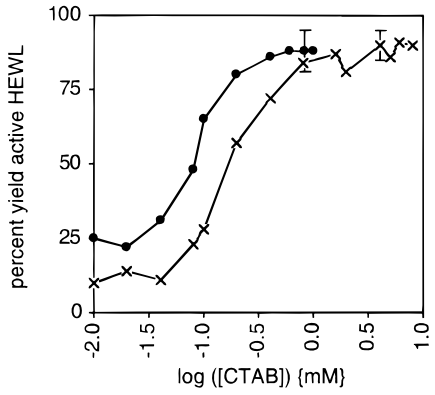


FIGURE 3: Yield of refolded lysozyme (recovered enzymatic activity) from the artificial chaperone method, after addition of cyclodextrin, as a function of detergent concentration at the time of dilution of lysozyme from denaturing solution. 50 mg/mL lysozyme in 6 M GdmCl, 30 mM DTT, 100 mM Tris sulfate, pH 8.5 was diluted by one of two procedures. Option one (●): diluted to 0.071 mg/mL lysozyme, 8.6 mM GdmCl, 0.043 mM DTT, 143 mM Tris sulfate, pH 8.5, 5.7 mM GSH, 0.57 mM GSSG, 1.43 mM EDTA, and 0.014−2.28 mM CTAB. 10 min after dilution from denaturing−reducing solution, β-cyclodextrin was added to generate solutions of 0.05 mg/mL lysozyme, 6 mM GdmCl, 0.03 mM DTT, 100 mM Tris sulfate, pH 8.5, 4.0 mM GSH, 0.4 mM GSSG, 1.0 mM EDTA, 0.01 to 1.6 mM CTAB, and 4.8 mM β-cyclodextrin. Option 2 (×): diluted to 0.28 mg/mL lysozyme, 34 mM GdmCl, 0.17 mM DTT, 143 mM Tris sulfate, pH 8.5, 5.7 mM GSH, 5.7 mM GSSG, 1.43 mM EDTA, and 0.014 to 11.4 mM CTAB. Ten min after dilution from denaturing−reducing solution, methyl-β-cyclodextrin was added to generate solutions of 0.05 mg/mL lysozyme, 6 mM GdmCl, 0.03 mM DTT, 100 mM Tris sulfate, pH 8.5, 4.0 mM GSH, 4.0 mM GSSG, 1.0 mM EDTA, 0.01−8.0 mM CTAB, and 16.5 mM methyl-β-cyclodextrin. In all samples, the lysozyme solutions were allowed to refold 16 h prior to assay. Representative error bars are shown. The lines connecting the data points are arbitrary.

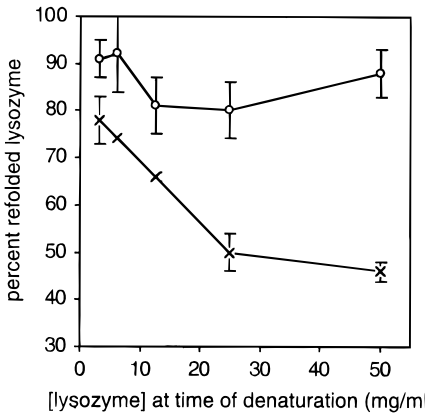


FIGURE 4: Yield of refolded lysozyme as a function of initial lysozyme concentration, *i.e.*, at time of denaturation, for artificial chaperone-assisted (○) and unassisted (×) refolding. A solution of 50 mg/mL solution of denatured−reduced lysozyme in 6 M GdmCl, 30 mM DTT, and 100 mM Tris sulfate, pH 8.5, was diluted with a solution of 6 M GdmCl and 100 mM Tris sulfate to generate four additional denatured lysozyme solutions: 25 mg/mL lysozyme and 15 mM DTT; 12.5 mg/mL lysozyme and 7.5 mM DTT; 6.25 mg/mL lysozyme and 3.75 mM DTT; and 3.12 mg/mL lysozyme and 1.87 mM DTT. These solutions were then diluted into a 0.7 mL buffer solution to generate 0.071 mg/mL lysozyme, 137 mM GdmCl, 0.043 mM DTT, 143 mM Tris sulfate, pH 8.5, 1.4 mM EDTA, 5.7 mM GSH, 0.57 mM GSSG, and 0 or 1.14 mM CTAB. GdmCl was added in order to generate a constant concentration of GdmCl in all samples. After 10 min, 0.3 mL of β-cyclodextrin was added to generate a sample of 0.05 mg/mL lysozyme, 96 mM GdmCl, 0.03 mM DTT, 100 mM Tris sulfate, pH 8.5, 1.0 mM EDTA, 4.0 mM GSH, 0.4 mM GSSG, 0 or 0.8 mM CTAB, and 0 or 4.8 mM β-cyclodextrin. The lines connecting the data points are arbitrary.

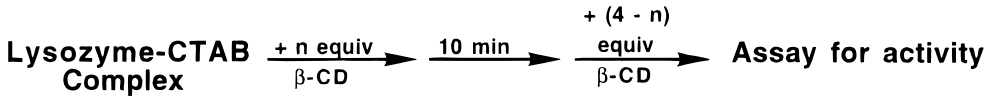
4-fold along the detergent concentration axis. These results indicate that the amount of detergent required for efficient refolding is determined by the protein concentration, and not by the detergent's CMC.

Detergent Can "Rescue" Lysozyme from the Aggregated State. If lysozyme is induced to aggregate by dilution from the DTT-reduced, Gdm-denatured state with a solution that contains GSH and GSSG but no detergent, then addition of CTAB will solubilize the aggregated protein, and subsequent addition of methyl-β-cyclodextrin provides the usual high refolding yield. Thus, there is not a significant kinetic barrier between aggregated protein plus detergent and the protein−detergent complex. In contrast, a substantial kinetic barrier protects native lysozyme from spontaneous denaturation by detergent, as indicated by the control studies in Table 1.

The data in Figure 4 illustrate an important practical consequence of detergent's ability to reverse lysozyme aggregation. In this experiment, denatured and reduced lysozyme was refolded by dilution to a final protein concentration of 0.05 mg/mL from *varying* initial protein concentrations. At this relatively low final protein concentration, unassisted lysozyme refolding can be quite efficient (up to 80%), if the initial protein concentration is also relatively low. The unassisted folding yield drops substantially, however, as the initial protein concentration rises. A similar effect has been reported by Goldberg et al. (1991), who attributed the inverse relationship between initial lysozyme concentration and unassisted folding yield to a very fast commitment to protein aggregation upon dilution of the denaturant. These workers speculated that manual mixing can introduce folding conditions (higher pH in their case) more rapidly than non-native protein molecules are separated from one another; therefore, at high initial lysozyme concentrations the irreversible pathway to aggregation is embarked upon too rapidly to allow efficient folding. Figure 4 shows that under artificial chaperone assistance, folding efficiency is *not* significantly influenced by initial protein concentration. Building upon the hypothesis of Goldberg et al. (1991), we propose that this difference in behavior reflects a switch from kinetic control of folding vs aggregation, for the dilution step of unassisted refolding, to thermodynamic control of protein−detergent complex formation vs aggregation, for the capture step of artificial chaperone-assisted refolding. (In the artificial chaperone method, the principal dilution occurs during the capture step, and only a small additional dilution occurs at the cyclodextrin addition step; for the artificial chaperone-assisted refolding results in Figure 4, the lysozyme concentrations were identical in all samples after the capture step.)

Rapid Stripping of the Detergent Is Required for Optimal Refolding. Figure 5 shows results from a series of experiments in which methyl-β-cyclodextrin was added in two portions to a lysozyme−CTAB complex formed in the presence of GSH and GSSG. In each case, a total of 4 equiv of methyl-β-cyclodextrin was used, relative to the detergent, and the two methyl-β-cyclodextrin additions were separated by 10 min, as summarized in Scheme 2. Figure 5 plots the ultimate yield of regained enzymatic activity vs the number of methyl-β-cyclodextrin equivalents (relative to CTAB) *in the first addition*. The rightmost point corresponds to addition of all methyl-β-cyclodextrin at once. The data in

Scheme 2: Protocol for Cyclodextrin Double Addition Experiments (See Figure 5)



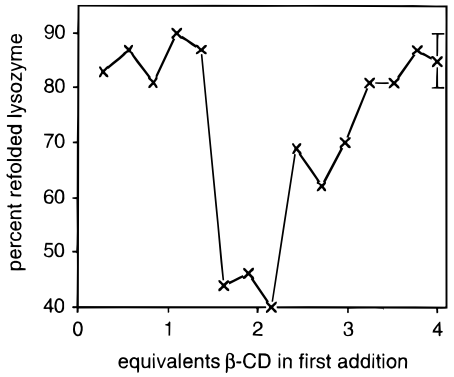


FIGURE 5: Yield of lysozyme from artificial chaperone-assisted refolding as a function of the number of equiv of methyl-β-cyclodextrin, relative to detergent, added in the first of two additions (see Scheme 3). 50 mg/mL lysozyme in 6 M GdmCl, 100 mM Tris sulfate, pH 8.5, 30 mM DTT was diluted to a 0.175 mL solution of 0.28 mg/mL lysozyme, 34 mM GdmCl, 0.17 mM DTT, 143 mM Tris sulfate, pH 8.5, 1.43 mM EDTA, 5.7 mM GSH, 0.57 mM GSSG, and 5.7 mM CTAB. 10 min after dilution from denaturing solution 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or 75 μL of 55 mM methyl-β-cyclodextrin was added to the lysozyme−CTAB solution. 10 min after the first addition of cyclodextrin, a second portion of methyl-β-cyclodextrin was added, to bring the volume to 0.25 mL and the final concentrations to 0.2 mg/mL lysozyme, 24 mM GdmCl, 0.12 mM DTT, 100 mM Tris sulfate, 1.0 mM EDTA, 4.0 mM GSH, 4.0 mM GSSG, 4.0 mM CTAB, and 16.5 mM methyl-β-cyclodextrin. A representative error bar is shown. The lines connecting the data points are arbitrary.

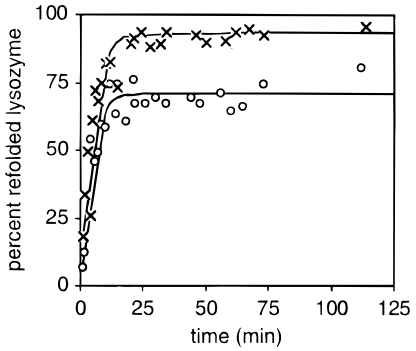


FIGURE 6: Kinetics of lysozyme refolding via unassisted (○) and artificial chaperone-assisted (×) pathways (see Scheme 4). Denatured−reduced lysozyme at 3.12 mg/mL in a solution containing 1.9 mM DTT, 6 M GdmCl, and 100 mM Tris sulfate, pH 8.5, was diluted into either a 1 mL or 0.7 mL buffer solution to generate two sets of samples. One set, 1.0 mL volume, contained 0.05 mg/mL lysozyme, 0.03 mM DTT, 100 mM Tris sulfate, pH 8.5, 4.0 mM GSH, 0.4 mM GSSG, 1.0 mM EDTA, and 4.8 mM β-cyclodextrin after mixing. The other set of samples, 0.7 mL, contained 0.071 mg/mL lysozyme, 0.043 mM DTT, 143 mM Tris sulfate, pH 8.5, 5.7 mM GSH, 0.57 mM GSSG, 1.43 mM EDTA, and 1.14 mM CTAB after mixing. After 10 min, to the sample with volume of 0.7 mL, was added β-cyclodextrin to generate a solution containing 0.05 mg/mL lysozyme, 0.03 mM DTT, 100 mM Tris sulfate, pH 8.5, 4.0 mM GSH, 0.4 mM GSSG, 1.0 mM EDTA, 0.8 mM CTAB, and 4.8 mM β-cyclodextrin after mixing. For the samples that did not contain detergent, $t = 0$ corresponds to the time of dilution from GdmCl solution. For the samples that contained CTAB, $t = 0$ corresponds to the time of the addition of the β-cyclodextrin. At various times after $t = 0$, aliquots of the refolding solutions were assayed for enzymatic activity. The data were fit to an exponential decay function. For curve (○) $y = -79.9e^{-0.254t} + 70.8$ and for curve (×) $y = -87.8e^{-0.174t} + 93.1$.

Figure 5 show that the final yield of reactivated lysozyme is substantially diminished if roughly half of the methyl-β-cyclodextrin is added in the first portion. Comparable behavior was previously observed for CAB refolding from the CTAB complex (Rozema & Gellman, 1996); apparently, slow removal of detergent from the protein−detergent complex is deleterious to proper refolding.

Macroscopic detergent adsorbents provide poor refolding yields from the lysozyme−CTAB complex, which is presumably another manifestation of the requirement for rapid stripping. When the lysozyme−CTAB complex containing 0.05 mg/mL protein (formed in the presence of GSH/GSSG) was gently rocked with solid adsorbents for 16 h, the following extents of enzymatic reactivation were observed: Calbiosorb, ca. 3%; Bio-Beads SM-2, ca. 23%; β-cyclodextrin−epichlorohydrin copolymer (from Aldrich), ca. 26%. In contrast, addition of soluble β-cyclodextrin to this complex provides ca. 87% reactivation. We assume that the key difference between the soluble stripping agent and the macroscopic adsorbents is that detergent removal by the latter is limited by the rate of detergent diffusion to a water−solid interface and therefore is intrinsically slow.

Kinetics of Lysozyme Reactivation: Artificial Chaperones Do Not Alter the Rate-Determining Step. The rate of lysozyme renaturation can be determined by monitoring enzymatic activity as a function of time after removal of the denaturing conditions. In order to determine whether assistance by artificial chaperones affects the rate-limiting step for refolding, we needed to identify conditions under which unassisted refolding is efficient, so that unassisted and assisted folding could be directly compared. Previous workers have shown that unassisted refolding is efficient if the initial and final protein concentrations are relatively low (Goldberg et al., 1991; Saxena & Wetlaufer, 1970). The experiments we employed for kinetic comparison of assisted and unassisted refolding started from 3.1 mg/mL lysozyme reduced and denatured with 2 mM DTT and 6 M GdmCl. Dilution of this solution 62-fold with buffer containing 5.7 mM GSH and 0.57 mM GSSG provides ca. 71% regain of enzymatic activity (unassisted refolding). Artificial chaperone-assisted refolding could also be conducted from this starting point: 46-fold dilution with a solution containing 1.1 mM CTAB, 5.7 mM GSH, and 0.57 mM GSSG generated a catalytically inactive lysozyme−CTAB complex (0.07 mg/mL protein), and further addition of methyl-β-cyclodextrin (6 equiv relative to detergent) provided ca. 93% regain of enzymatic activity.

Figure 6 shows enzymatic reactivation as a function of time for the two refolding protocols. For unassisted refolding, $t = 0$ corresponds to dilution of the DTT-reduced, Gdm-denatured lysozyme; for artificial chaperone-assisted refolding, $t = 0$ corresponds to addition of methyl-β-cyclodextrin to the lysozyme−CTAB complex. Each data set can be fit by a single exponential function. The deduced $t_{1/2}$ value for unassisted refolding is 163 ± 55 s, which is similar to the $t_{1/2}$ values previously reported for lysozyme refolding under

related conditions (Goldberg et al., 1991). For artificial chaperone-assisted refolding, $t_{1/2} = 214 \pm 60$ s, which is indistinguishable from the unassisted half time, within experimental uncertainty. Thus, artificial chaperones do not appear to change the rate-determining step for refolding relative to unassisted refolding.

Timing of Disulfide Formation in Artificial Chaperone-Assisted Refolding. The GSH/GSSG combination is required for lysozyme renaturation under the conditions described above (Saxena & Wetlaufer, 1970). If GSH/GSSG is not added, attempts to refold lysozyme from the DTT-reduced, Gdm-denatured state provide <5% regain of enzymatic activity, with or without artificial chaperones. For all of the artificial chaperone-assisted refolding results described above, the GSH/GSSG was introduced in the capture step. With this protocol, the native disulfides could form either in the lysozyme−detergent complex, or after the cyclodextrin strips detergent from the protein.

To probe the timing of disulfide formation, we examined a variant of the artificial chaperone conditions employed in Table 1: instead of introducing the GSH/GSSG along with the detergent, we added GSH/GSSG along with the methyl-β-cyclodextrin. This modified protocol gave ca. 61% reactivation vs ca. 85% when GSH/GSSG was introduced in the capture step. The fact that most of the enzymatic activity was regained in the modified protocol is consistent with previous reports that the oxidation and disulfide shuffling promoted by GSH/GSSG occurs very rapidly during lysozyme folding (Saxena & Wetlaufer, 1970). The decrease in folding yield that accompanies late GSH/GSSG addition, however, suggests that at least some of the native disulfide bond formation occurs in the lysozyme−CTAB complex. Far-UV CD data for a lysozyme−detergent complex (Figure 1) suggest native-like secondary structure, and it is therefore possible that the detergent-bound state of the protein is preorganized for proper disulfide formation.

A recent report indicates that single-chain F_V proteins (sF_V; engineered antibody derivatives) can be preorganized for proper disulfide formation in a detergent-complexed state (Kurucz et al., 1995). The recombinant sF_V described, initially obtained in inclusion body form with all four cysteine residues reduced, was solubilized with the detergent sodium lauroylsarcosine (SLS). Air oxidation of the cysteine side chains in the SLS-complexed state, catalyzed with $CuSO_4$, provided high yields of the correct intramolecular disulfide pairing (Kurucz et al., 1995).

We applied the metal-catalyzed cysteine oxidation strategy to the reduced lysozyme−CTAB complex. Treatment of a complex containing 0.071 mg/mL protein with 0.07 mM $CuSO_4$ for 1 h, followed by addition of methyl-β-cyclodextrin, provided 35% regain of enzymatic activity. Thus, irreversible oxidation of the lysozyme−detergent complex leads to some native disulfide pairing, but this process is inferior to the disulfide shuffling allowed by GSH/GSSG (Saxena & Wetlaufer, 1970) in promoting proper disulfide formation in lysozyme. Interestingly, when the lysozyme-CTAB complex was allowed to sit with the $CuSO_4$ for 16 h before cyclodextrin addition, only 5% enzymatic activity was regained. Kurucz et al. (1995) allowed their copper-catalyzed oxidations to run overnight. Acharya and Taniuchi (1976) have reported that Cu^{2+}-catalyzed oxidation of reduced lysozyme leads mostly to an inactive form with incorrect disulfides.

Table 2: Dependence of Refolding Yield on Relationship between Detergent and Cyclodextrin Sizes[a]

+ CTAB; then α-CD	0.83 ± 0.13
+ CTAB; then methyl-β-CD	0.91 ± 0.07
+ TOPPA; then α-CD	0.50 ± 0.06
+ TOPPA; then methyl-β-CD	0.85 ± 0.06

[a] Protocol for CTAB experiments: Lysozyme, 50 mg/mL, was denatured in 6 M GdmCl, 30 mM DTT, 100 mM Tris sulfate, pH 8.5. This solution was then diluted to 0.071 mg/mL lysozyme, 8.6 mM GdmCl, 0.04 mM DTT, 143 mM Tris sulfate, pH 8.5, 1.43 mM EDTA, 5.7 mM GSH, 0.57 mM GSSG, and 1.14 mM CTAB (0.700 mL). After 10 min, these aliquots were diluted with 0.300 mL of aqueous α- or methyl-β-cyclodextrin, to give final concentrations of 0.05 mg/mL lysozyme, 5.0 mM GdmCl, 0.03 mM DTT, 100 mM Tris sulfate, pH 8.5, 1.0 mM EDTA, 4.0 mM GSH, 0.4 mM GSSG, 0.8 mM CTAB, and 4.8 mM cyclodextrin. The protocol for the TOPPA experiments was similar, except that the TOPPA concentration was 11.4 mM after the first dilution and 8.0 mM after the second dilution. The final cyclodextrin concentration was 33.3 mM.

Mechanistic Role of the Cyclodextrin. Addition of cyclodextrin to CAB−detergent complexes promotes renaturation because the cyclodextrin selectively binds the detergent, thereby stripping it from the protein, which is then able to fold (Rozema & Gellman, 1996). Demonstrating this mechanistic role in CAB refolding depended upon differences in the binding properties of the α- and β-cyclodextrin cavities. Similar studies have been conducted with lysozyme, to determine whether the cyclodextrin function is general in the artificial chaperone method.

Table 2 shows that methyl-β-cyclodextrin and α-cyclodextrin are similarly effective as stripping agents in promoting refolding from the lysozyme−CTAB complex, as expected in light of reports that that underivatized β-cyclodextrin and α-cyclodextrin have similar affinities for detergents with linear alkyl tails (Palepu & Reinsborough, 1988; Turco Liveri et al., 1992). The difference in cyclodextrin cavity sizes becomes a significant factor, however, with more bulky binding partners, e.g., the branched alkylphenyl moiety of the detergent Triton X-100: bulky substrates are bound strongly by the β-CD cavity but not by the smaller α-CD cavity (Matsui et al., 1985). Since nonionic Triton X-100 itself was not expected to be an effective artificial chaperone for lysozyme, we prepared cationic analogue TOPPA as a probe for the mechanistic role of the cyclodextrin. [TOPPA has a CMC of ca. 10 mM in the refolding buffer (no protein), as determined by solubilization of the hydrophobic dye orange OT (Schott, 1964).]

Methyl-β-cyclodextrin is superior to α-cyclodextrin in inducing enzymatic reactivation from the lysozyme-TOPPA complex (Table 2). These experiments required methyl-β-cyclodextrin rather than β-cyclodextrin itself, because of the relatively high concentrations of TOPPA necessary for efficient capture of the protein upon dilution from the Gdm-denatured state, but we assume that the binding properties of β-cyclodextrin and methyl-β-cyclodextrin will be similar. The superiority of the cyclodextrin with the larger cavity is consistent with the mechanistic role previously proposed for the cyclodextrin, (Rozema & Gellman, 1995, 1996) involving selective binding to the detergent component of the protein−detergent complex.

Comparison of Folding Assistance by Artificial Chaperones with Assistance Provided by Cyclodextrin or Detergent in the Dilution Additive Mode. Detergents do not promote lysozyme folding in the dilution additive mode (Scheme 1),

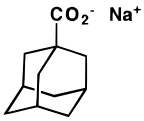
as far as we can tell. [In contrast, non-detergent zwitterionic amphiphiles have recently been shown to enhance refolding of reduced, denatured lysozyme in the dilution additive mode (Goldberg et al., 1996), as have the additives sarcosine, glycerol, ammonium sulfate, glucose, and *N*-acetylglucosamine (Maeda et al., 1996).] Horowitz and co-workers have shown that detergents, as dilution additives, promote rhodanese refolding from the Gdm-denatured state (Tandon & Horowitz, 1986, 1988), but there have been very few other reports of "micelle-assisted" refolding in the decade since the first rhodanese results were published. With CAB, only modest dilution additive refolding assistance by nonionic detergents has been observed (Rozema & Gellman, 1996; Wetlaufer & Xie, 1995). Our observations with lysozyme support our earlier speculation (Rozema & Gellman, 1996) that refolding assistance by detergents in the dilution additive mode may be limited in scope.

Methyl-β-cyclodextrin, at relatively high concentrations, is very effective at promoting lysozyme refolding in the dilution additive mode. For example, 250-fold dilution of 50 mg/mL DTT-reduced, Gdm-denatured lysozyme (to 0.2 mg/mL) with a buffer solution containing 4 mM GSH, 4 mM GSSG, and 16 mM methyl-β-cyclodextrin led to ca. 50% regain of enzymatic activity, while only ca. 21% activity was regained when the diluting buffer did not contain methyl-β-cyclodextrin (Table 1). When the concentration of methyl-β-cyclodextrin was raised to 80 mM, the recovery of active lysozyme was >90%. Our examination of cyclodextrin as a dilution additive was inspired by the recent discovery that CAB refolding is promoted by cyclodextrins in the dilution additive mode (Karuppiah & Sharma, 1995). In contrast to the effectiveness in lysozyme refolding, however, we showed that cyclodextrin folding assistance in the dilution additive mode for CAB is quite modest (Rozema & Gellman, 1996).

Dilution additive folding assistance by cyclodextrin cannot explain the folding assistance we observe with sequential introduction of detergent and cyclodextrin in the artificial chaperone mode. In the artificial chaperone protocol, the principal dilution of the chemically denatured protein occurs at the capture step (first step), and only a small additional dilution occurs when the cyclodextrin is added to the protein−detergent complex (Scheme 1). As indicated in Table 1, if detergent is omitted from the first dilution, and cyclodextrin is added subsequently, as usual in the artificial chaperone protocol, the poor regain in lysozyme activity is indistinguishable from the unassisted folding level. These results reveal an important difference between the folding assistance provided by cyclodextrin in the dilution additive mode and the folding assistance provided by sequential introduction of detergent and cyclodextrin in the artificial chaperone mode: detergent can reverse protein aggregation brought on by rapid dilution from the chemically denatured state, while cyclodextrin cannot reverse such aggregation.

Karuppiah and Sharma (1995) proposed that folding assistance from cyclodextrin as a dilution additive stems from transient complexation by the cyclodextrin of exposed by hydrophobic side chains on the polypeptide. These workers reported that aromatic amino acids could inhibit α-cyclodextrin's ability to prevent CAB aggregation upon dilution from the Gdm-denatured state, which was interpreted to support the transient complexation hypothesis. This interpretation seems unlikely, however, given that only ca. 5 mol % of the "inhibitor," relative to cyclodextrin, was reportedly present in these experiments. Even if every "inhibitor" molecule occupied a cyclodextrin cavity, there would still have been plenty of unoccupied cyclodextrin to interact with the folding protein.

In order to probe the mechanism by which lysozyme folding is assisted by methyl-β-cyclodextrin in the dilution additive mode, we examined the effect of adamantanecarboxylate on this process. Adamantanecarboxylate is ex-



Adamantanecarboxylate

tremely tightly bound by β-cyclodextrin itself (Eftink et al., 1989), and we expect similarly tight binding to methyl-β-cyclodextrin. When 50 mg/mL DTT-reduced, Gdm-denatured lysozyme was diluted to 0.8 mg/mL in the presence of 83 mM methyl-β-cyclodextrin, ca. 51% of the enzymatic activity was regained. When a similar dilution was carried out with 83 mM adamantanecarboxylate included (i.e., one full equivalent relative to the cyclodextrin), the yield was not significantly altered. Based on the reported binding constant of β-cyclodextrin itself for adamantanecarboxylate in aqueous solution (ca. 10^4 M^{-1}; Eftink et al., 1989), we estimate that 99% of the methyl-β-cyclodextrin cavities are occupied by adamantyl groups under these conditions. (We could not use excess adamantanecarboxylate in these experiments because this compound, alone, inhibits lysozyme refolding; the origin of this inhibition is unclear.) Since the added adamantanecarboxylate does not suppress methyl-β-cyclodextrin's ability to promote refolding in the dilution additive mode, we conclude, in contrast to Karuppiah and Sharma (1995), that the cyclodextrin *does not* function by transient complexation of exposed hydrophobic groups on the non-native protein. Instead, this folding assistance must involve transient interaction between the protein and the exterior surface of the cyclodextrin. We suggest that the cyclodextrin plays a role similar to that proposed for PEG as a dilution additive folding assistant (Cleland et al., 1992a,b; Cleland & Randolph, 1992).

DISCUSSION

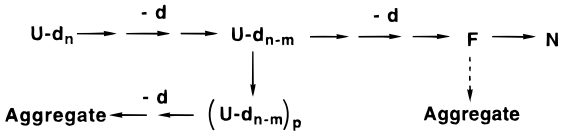
Comparison of Artificial Chaperone-Assisted Refolding of CAB and Lysozyme. There are several important operational parallels between assisted refolding of lysozyme and assisted refolding of CAB (Rozema & Gellman, 1996), the other well-characterized target for the artificial chaperone method. (i) Ionic detergents are required for effective capture of the non-native protein upon dilution from concentrated GdmCl solution. Nonionic detergents apparently do not interact strongly enough with the unfolded protein to prevent protein aggregation. (ii) Detergent molecules must interact cooperatively with one another in order to form the protein−detergent complex, but the amount of detergent required for capture is determined by the protein concentration, rather than by the detergent's CMC. The protein−detergent complexes appear to be mixed micelles. (iii) Detergents that are effective artificial chaperones, e.g., CTAB for both lysozyme and CAB, do not necessarily unfold the native enzyme at room temperature; however, these detergents do spontaneously dissolve aggregated protein. (iv) The role of

the cyclodextrin is to bind tightly to the nonpolar segment of the detergent, thereby stripping the detergent away from the protein−detergent complex. (v) Rapid detergent stripping is required for optimal refolding yield; slow addition of the stripping agent seems to promote protein aggregation relative to refolding. This feature of the artificial chaperone process accounts for the superiority of soluble stripping agents, since the removal of detergent by macroscopic adsorbents or dialysis is rate limited by diffusion to or across an interface.

Kinetic vs Thermodynamic Control of Competing Intermolecular and Intramolecular Processes in the Artificial Chaperone Method. Each of the two steps of the artificial chaperone protocol creates a competition among inter- and intramolecular noncovalent interactions, and the result of this competition can be determined either by kinetic or by thermodynamic factors. In the first step, rapid dilution of the GdmCl to non-denaturing concentration allows at least three noncovalent processes to occur: intramolecular assembly of peptide surfaces ("folding"), intermolecular assembly of peptide surfaces ("aggregation"), and formation of the protein−detergent complex ("capture"). A combination of thermodynamic and kinetic factors appears to determine the course of the lysozyme capture step. Partitioning between aggregated and captured forms of the protein is thermodynamically controlled, since detergent can spontaneously dissolve aggregated lysozyme. Thus, even if some protein initially aggregates when the GdmCl is diluted, the detergent will reverse this aggregation. Partitioning between folded and captured forms must be kinetically controlled, however, because native lysozyme is not spontaneously disrupted by the detergents employed here. Approximately 21% of lysozyme refolds via an unassisted pathway upon dilution from concentrated GdmCl solution, under the conditions employed to construct Table 1. Inclusion of detergent in the diluting buffer can diminish the extent of unassisted refolding (clearest for the detergent TTAB), which indicates that capture can occur before folding is complete. (Partitioning between folded and aggregated forms in unassisted refolding is kinetically controlled, as indicated by the effect of initial protein concentration on the unassisted folding data shown in Figure 4.) The behavior of CAB (Rozema & Gellman) parallels that of lysozyme: partitioning between aggregation and capture is thermodynamically controlled, but partitioning between folding and capture is kinetically controlled, at least with CTAB [anionic detergents like SDS spontaneously denature CAB at room temperature (McCoy & Wong, 1981)].

The second step of the artificial chaperone protocol, too, is controlled by both kinetic and thermodynamic factors. Upon addition to the protein−detergent complex, the cyclodextrin must choose between the detergent and the non-native protein as binding partner. Precedent indicates that both α- and β-cyclodextrin will thermodynamically prefer to bind to the hydrophobic segment of a detergent, relative to the hydrophobic side chains displayed by a protein (Inoue et al., 1983; Palepu & Reinsborough, 1988; Turco Liveri et al., 1992; Takahashi et al., 1986). As the thermodynamically controlled stripping of detergent occurs, the polypeptide is confronted by two alternative pathways, folding and aggregation. The partitioning between these two pathways must be kinetically controlled, since there is no evidence of spontaneous interconversion between the native and aggregated states of either CAB or lysozyme.

Scheme 3: Proposed Mechanism for Cyclodextrin-Induced Folding from a Protein−Detergent Complex (See Text)



Mechanistic Hypothesis for the Stripping Step (Scheme 3). If capture is achieved, the efficiency of artificial chaperone-assisted refolding is determined by the kinetically controlled fate of the protein during the stripping process. Scheme 3 presents a hypothetical mechanism for the events that can occur upon addition of cyclodextrin to a soluble protein−detergent complex. Several alternative pathways are shown, only some of which may be relevant in any particular case. All proposed mechanistic paths begin with U-d_n (upper left corner of Scheme 3), which represents non-native protein (U for "unfolded") bound to some number (n) of detergent molecules (d). We postulate that protein−detergent complexes that ultimately provide high folding yields contain only one protein, which is consistent with nearly all relevent literature on protein−detergent complexes (Anathapadmanabhan, 1993).

The uppermost left-to-right path in Scheme 3 illustrates the route to native protein, N. Upon cyclodextrin addition, detergent molecules are removed in stepwise fashion from the protein−detergent complex. According to this mechanistic hypothesis, removal of the final detergent molecule yields a non-native form of the protein, F, which then folds to the native state. Some folding is likely to occur during the stripping process, which would mean that the first detergent-free state, F, is at least partially folded. It is also conceivable that the protein folds completely before the final detergent molecule is removed, i.e., that native state N is the only detergent-free form of the protein to appear in solution. Since, however, the artificial chaperone protocol does not appear to affect the rate-determining step of lysozyme refolding, relative to unassisted refolding (Figure 6), we postulate that the assisted and unassisted folding pathways proceed through a common folding intermediate, which presumably occurs after the final detergent molecule has been removed in the assisted pathway.

Non-productive alternative routes branch downward from the folding pathway in Scheme 3. A crucial branch point in our hypothetical mechanism occurs after detergent has been partially stripped from the protein, to form U-d_{n-m} (this intermediate may correspond to a range of protein−detergent stoichiometries, rather than a single stoichiometry). At this point, we propose that the protein−detergent complex develops the capacity to self-associate, which generates complexes containing multiple protein molecules, (U-d_{n-m})$_p$. Further detergent stripping from these multiprotein complexes leads unavoidably to protein aggregation, because the protein molecules are preassembled.

A second non-productive branch point from the folding pathway could occur if a detergent-free non-native form of the protein, F or a later folding intermediate, had a propensity to aggregate. In light of the requirement for rapid detergent stripping with both lysozyme and CAB (Rozema & Gellman, 1996), and the relatively long $t_{1/2}$ for folding of these proteins, we speculate that this branch point is not significant in either

case. If this late branch point were a major diversion from the folding pathway of CAB or lysozyme under the conditions examined, it is not clear how the artificial chaperone protocol could overcome this problem.

Two puzzling features of the artificial chaperone-assisted folding of CAB and lysozyme are explained by the mechanism in Scheme 3, and more specifically by the first branch point, associated with partially stripped form U-d_{n-m}: (i) the requirement that cyclodextrin addition to the protein−detergent complex be rapid for optimum folding yield; (ii) the observation, most carefully explored with CAB, that suboptimal detergent concentrations prevent protein aggregation at the capture step, but generate protein−detergent complexes that lead largely to protein aggregation rather than folding upon introduction of cyclodextrin. According to the mechanism we have proposed, the requirement for rapid cyclodextrin addition arises because it is essential to minimize the amount of aggregation-prone complex(es) U-d_{n-m} present at any given time. A large excess of cyclodextrin enhances the rate of further stripping of U-d_{n-m} relative to the rate of self-association of U-d_{n-m}. The behavior of protein−detergent complexes generated by capture at suboptimal detergent concentrations is explained by proposing that these complexes exist largely in forms like $(U\text{-}d_{n-m})_p$, so that aggregation predominates upon cyclodextrin addition.

Previous studies of unassisted refolding vs aggregation for lysozyme (Goldberg et al., 1991), for CAB (Cleland & Wang, 1990), and for other proteins (Brems, 1988) have suggested that aggregation results from early and short-lived folding intermediates. On the basis of these conclusions, we speculate that the initial state of the protein released from the detergent, F, is different from the early folding intermediates generated upon dilution from the chemically denatured state. This proposal suggests a possible mechanistic parallel between the function of artificial and biological chaperones because different biological chaperones appear to release their substrate proteins in distinct conformational states (Tian et al., 1995).

Other Recent Efforts to Refold Denatured−Reduced Lysozyme. Although lysozyme refolding has been a subject of interest for many years (Saxena & Wetlaufer, 1970; Wetlaufer et al., 1974), this enzyme has recently become popular for evaluation of new refolding strategies. Maeda et al. (1994, 1995, 1996) have examined refolding from the urea-denatured lysozyme reduced with 2-mercaptoethanol. Among other things, these workers found that unassisted refolding yields upon simple dilution are improved if the residual urea concentration is left moderately high, e.g., 1 M (Maeda et al., 1994). These findings parallel the observation that moderately high residual GdmCl or urea promote unassisted CAB refolding (Cleland & Wang, 1990; Semisotnov et al., 1990). Maeda et al. also found that slow dialysis of urea provides high refolding yields at concentrations as high as 5 mg/mL (Maeda et al. 1995) and that a number of low molecular weight compounds assist lysozyme refolding, as dilution additives, in a rapid dilution protocol (Maeda et al., 1996). Goldberg et al. (1996) have reported that several non-detergent sulfobetaines promote lysozyme refolding in the dilution additive mode. Batas and Chaudhuri (1996) have described the use of size-exclusion chromatography for refolding of chemically denatured lysozyme. Raman et al. (1996) have reported that the cysteine/cystine redox couple is superior to the GSH/GSSG couple for refolding of GdmCl-denatured, DTT-reduced lysozyme by rapid dilution to moderate final protein concentrations (≤0.25 mg/mL). These workers do not appear to have varied the thiol:disulfide ratio from 10:1, which is interesting in light of our observation that 1:1 GSH:GSSG is superior at 0.2 mg/mL for artificial chaperone-assisted refolding.

Conclusion. The results reported here expand the scope of the artificial chaperone method by showing that protein refolding can be efficiently assisted when formation of multiple disulfide bonds is required. Operational similarities between assisted refolding of lysozyme and CAB, nonhomologous proteins, raise the possibility that the artificial chaperone technique will be useful with other proteins as well. Mechanistic elucidation of this mode of folding assistance should facilitate application of the technique to new proteins.

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