

# Artificial Chaperone-assisted Refolding of Carbonic Anhydrase B\*

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**We recently reported a new approach to protein refolding that utilizes a pair of low molecular weight folding assistants, a detergent and a cyclodextrin (Rozema, D., and Gellman, S. H. (1995) *J. Am. Chem. Soc.* 117, 2373–2374). Here, we provide a detailed study of carbonic anhydrase B (CAB) refolding assisted by these “artificial chaperones.” When CAB is heated in the presence of a competent detergent, or when guanidinium-denatured CAB is diluted to nondenaturing guanidinium concentration in the presence of such a detergent, the detergent forms a complex with the non-native protein, thereby preventing aggregation. CAB is unable to refold from the detergent-complexed state, but folding can be induced by introduction of a cyclodextrin, which strips the detergent away from the protein. Use of artificial chaperones provides excellent yields of reactivated CAB under conditions that lead to little or no reactivation in the absence of the refolding assistants. Our studies show that the detergent can capture the unfolded protein even at submicellar concentrations, but that not all CAB-detergent complexes lead efficiently to refolded enzyme upon introduction of the stripping agent. Effective refolding appears to require that detergent stripping occur as rapidly as possible; intrinsically slow methods of detergent removal (dialysis or use of macroscopic adsorbents) are less effective than cyclodextrin at inducing renaturation upon detergent removal. The detailed characterization of artificial chaperone-assisted CAB refolding reported here should guide the application of this strategy to other proteins.**

The process by which protein molecules achieve their native conformations is a subject of fundamental and practical importance. Fundamental interest in the “protein folding problem” arises because we do not yet understand how a complex network of noncovalent interactions can specify one particular compact conformation for an intrinsically flexible polypeptide (1), how the polypeptide rapidly finds that compact conformation (2, 3), or why such a purely noncovalent process involves relatively large kinetic barriers (4). Practical interest in the “protein refolding problem” stems from the fact that proteins overproduced by genetically engineered cells are often obtained in non-native forms (*e.g.* inclusion bodies), and the use of such proteins for basic research or biotechnological applications requires that the native conformation be achieved (5, 6).

Revolutionary advances in genetic manipulation techniques have made protein refolding an increasingly pressing problem,

but there have been relatively few efforts to devise general renaturation strategies. In a common scenario, an overproduced protein is purified in inclusion body form, the inclusion bodies are solubilized with a chemical denaturant (typically guanidinium or urea), and refolding is attempted by removing the denaturant, via dilution or dialysis. For many proteins, however, denaturant removal leads predominantly or completely to protein aggregation rather than refolding (5, 6). One strategy for overcoming aggregation is to immobilize the non-native protein on a solid support before denaturant removal; immobilization prevents the protein molecules from finding one another during the folding process (7). Covalent side chain modifications intended to enhance the solubility of non-native protein forms have also been employed with some success (8).

In 1986, Tandon and Horowitz (9) introduced a new strategy for refolding chemically denatured proteins, in which a detergent is included in the solution used to dilute guanidinium chloride to nondenaturing concentration. This strategy can improve refolding yields of several proteins (10); detailed evaluation has focused on rhodanese. Mechanistic studies indicate a transient association between non-native rhodanese and detergent micelles, and the process has been called “micelle-assisted refolding” (11).

We will use the term “dilution additive technique” to describe the general approach pioneered by Horowitz and co-workers (9–11). Several laboratories have subsequently examined dilution additives other than detergents. Cleland *et al.* (12) have shown that polyethylene glycol can promote refolding of several proteins upon denaturant removal. Detailed mechanistic studies of polyethylene glycol refolding assistance with carbonic anhydrase B (CAB) indicate that the polyethylene glycol interacts transiently with a compact folding intermediate (13, 14). This intermediate has a strong tendency to self-associate, which is the origin of the protein aggregation that competes with unimolecular refolding during denaturant removal.

In recent years it has become clear that protein folding *in vivo* is often controlled by other proteins. Such folding assistance comes in several forms, including catalysis of proline *cis-trans* isomerization and disulfide interconversion (15). One of the most interesting types of folding assistance involves the “chaperones,” a diverse group of proteins that promotes proper folding by inhibiting competing aggregation of the substrate protein (15, 16). The detailed mechanisms of these assisted refolding processes are not yet clear. The prokaryotic GroEL/GroES system, and its mitochondrial analogues Hsp60/Hsp10, have been among the most extensively studied of the natural chaperone systems. These chaperones appear to operate via a two-step mechanism. In the first step, GroEL, as a 14-mer, captures non-native states of the substrate protein, thus preventing substrate aggregation; the substrate protein will not spontaneously refold from this GroEL-bound state. In the second step, release and concomitant refolding of the substrate protein are triggered by the binding to GroEL of additional agents, MgATP and K<sup>+</sup>, and, in at least some cases, the co-chaperone GroES (as a 7-mer).

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Chaperone activity *in vitro* has been demonstrated in two experimental modes. Many proteins are irreversibly denatured by heating, but the presence of GroEL prevents this irreversible process (17, 18). After cooling, the GroEL-substrate complex yields native substrate protein when the appropriate additives are introduced. This thermal experiment is presumably related to the "heat shock" activity of chaperones, prevention of irreversible protein inactivation at elevated growth temperatures. The second type of *in vitro* chaperone assay involves Gdm<sup>1</sup>- or urea-denatured substrate proteins that aggregate rather than refolding upon dilution to nondenaturing Gdm or urea concentrations. In such cases, dilution in the presence of GroEL leads to formation of a GroEL-substrate complex, from which native protein can be elicited by the triggering agents (19, 20).

Inspired by the two-step mechanism of the GroE system, we have devised a new approach to refolding that makes use of low molecular weight "artificial chaperones" (21). In the first step, the non-native target protein is captured by a detergent under conditions that would normally lead to irreversible protein aggregation (heating or denaturant removal). The substrate protein cannot spontaneously refold from the detergent-complexed state. In the second step, removal of detergent from the protein is triggered by addition of a cyclodextrin, allowing the protein to refold. Preliminary data have shown that two structurally diverse proteins can be successfully refolded via the artificial chaperone method: carbonic anhydrase B (CAB), from the thermally denatured state, and citrate synthase, from the Gdm-denatured state (21). Here, we present a detailed mechanistic study of artificial chaperone-assisted CAB refolding, from both the thermally denatured and Gdm-denatured states. Because we seek a refolding method that is applicable to recalcitrant proteins, we have focused on conditions that provide little or no refolded CAB in the absence of artificial chaperone assistance.

#### MATERIALS AND METHODS

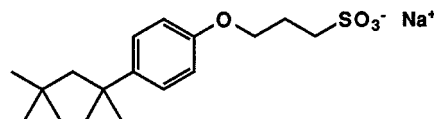
CAB, CTAB (99%), pNPAC, and SDS (99%) were purchased from Sigma. GdmCl was purchased as Sequanal grade from Pierce. Orange OT (75%), propane sultone (98%), NaH (60% dispersion in mineral oil), CTAHS (99%), and 4-(*tert*-octyl)phenol (97%) were purchased from Aldrich. STS (99%) was purchased from Lancaster Synthesis (Windham, NH).  $\alpha$ -Cyclodextrin,  $\beta$ -cyclodextrin, and methyl- $\beta$ -cyclodextrin were gifts from American Maize Products, Inc. (Hammond, IN). Orange OT was recrystallized from ethanol. Coomassie Blue G-250 and 0.22- $\mu$ m acetate filters were purchased from Fisher. Centricon concentrators were purchased from Amicon (Beverly, MA).  $\alpha$ - and  $\beta$ -Cyclodextrins were recrystallized from water. All other materials were used as obtained from suppliers.

Protein concentration for native CAB was determined by absorbance at 280 nm with an extinction coefficient of  $1.83 \text{ (mg/ml protein)}^{-1} \text{ cm}^{-1}$  (22). The concentration of GdmCl stock solutions was determined by measuring the refractive index (23).

In a typical thermal denaturation study, 0.043 mg/ml CAB, 0.57 mM detergent, and 23 mM Tris sulfate, pH 7.75, were heated to 70 °C for 6 min. After cooling for 10 min, the CAB solutions were diluted with addition of water or stock solutions of cyclodextrin to make solutions of 0.03 mg/ml CAB, 0.4 mM detergent, 16 mM Tris sulfate, pH 7.75, and 4.8 mM cyclodextrin. After sitting overnight, the solutions were assayed for enzymatic activity.

For many studies, the concentration of CAB,  $\beta$ -cyclodextrin, or detergent was varied. When the CAB concentration was raised, the samples were diluted prior to assay by addition of 16 mM Tris sulfate, pH 7.75, to bring the CAB concentration to 0.03 mg/ml. In experiments aimed at determining the effect of ionic strength on refolding yields, Na<sub>2</sub>SO<sub>4</sub> was added in various concentrations before heating.

For some heat denaturation studies,  $\beta$ -CD was added in two parts:



STRUCTURE 1.

the first addition was 10 min after heating, and the second addition was 10 min after the first. The total volume of  $\beta$ -cyclodextrin added was equal in all cases and resulted in solutions of 0.03 mg/ml CAB, 0.4 mM CTAB, 16 mM Tris sulfate, pH 7.75, and 4.8 mM  $\beta$ -CD.

For GdmCl refolding studies, 30 mg/ml CAB was denatured for 16 h in 5 M GdmCl. The CAB solution was then diluted by a factor of 700 with Tris sulfate buffer containing detergent, to give concentrations of 0.043 mg/ml CAB, 23 mM Tris sulfate, pH 7.75, and 0.57 mM detergent. After 10 min,  $\beta$ -cyclodextrin stock solution was added to bring the final concentrations to 0.030 mg/ml CAB, 16 mM Tris sulfate, pH 7.75, 0.4 mM detergent, and 4.8 mM  $\beta$ -cyclodextrin. After sitting overnight, the solutions were assayed for enzymatic activity.

Activity of the CAB solutions was determined using a pNPAC ester-ase assay (24), in which 45  $\mu$ l of 52 mM pNPAC in dry acetonitrile was added to 0.45 ml of CAB solution to make a solution of 0.026 mg/ml CAB, 14 mM Tris sulfate, pH 7.75, 4.4 mM  $\beta$ -CD, and 4.7 mM pNPAC. After 10 s of mixing, the increase in the hydrolysis product *p*-nitrophenolate was monitored by measuring the increase in absorbance at 400 nm as a function of time. The absorbance of the solution was measured every second for 30–60 s using a Model 8452 diode array spectrophotometer (Hewlett-Packard, Mountain View, CA). The yield of refolded CAB was determined by comparison to the rate of pNPAC hydrolysis by the native enzyme under identical conditions.

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 (25). For far-UV, 197–255 nm, 1-mm path length cells were used, and a reference sample containing buffer, detergent, and cyclodextrin was subtracted from the signal. For near-UV, 255–320 nm, 10-mm path length cells were used.

Intrinsic protein fluorescence and light scattering were measured using an SLM 8000 spectrophotometer. The sample cuvettes were 3  $\times$  3 mm. For intrinsic fluorescence measurements, the excitation wavelength was at 280 nm. For light scattering measurements, all samples were filtered through a 0.22- $\mu$ m acetate filter prior to heating or dilution from GdmCl. The irradiation ("excitation") and detection ("emission") wavelengths were 350 nm. Excitation and emission slits were 4 mm.

To determine the CMC of a detergent in enzyme buffer, 0.3-ml samples of the detergent at various concentrations in 23 mM Tris sulfate, pH 7.75, were gently rocked in the presence of solid orange OT (26). After 2 days, the remaining undissolved orange OT was filtered off through a cotton plug. 0.2-ml aliquots of the filtrate were diluted with 0.8 ml of absolute ethanol, and absorbance at 500 nm was measured in a 10-mm path length cuvette. The absorbance at 500 nm was plotted as a function of detergent concentration, which gave a graph consisting of two linear regions. The intersection of the lines defined by these linear regions was taken to define the CMC of the detergent.

To isolate refolded CAB, 2.0 ml of refolded CAB solution was filtered through a 0.22- $\mu$ m acetate filter and then concentrated with a  $M_r = 10,000$  cutoff Centricon filter to a volume of 0.25–0.5 ml. Comparisons between stock solutions of native CAB and refolded CAB solutions were made using the Coomassie Blue dye binding method (27), absorbance at 280 nm, and specific activity.

Detergent 1 (Structure 1) was synthesized by reaction of 4-(*tert*-octyl)phenol with propane sultone. *tert*-Octylphenol (2.78 g, 13.5 mmol) was dissolved in 50 ml of distilled, dry tetrahydrofuran. To this solution was added a slurry of sodium hydride (originally 0.75 g of 60 weight % dispersion in mineral oil, 20 mmol of NaH; mineral oil removed by three 10-ml washes with tetrahydrofuran) in 15 ml of tetrahydrofuran. The slurry was allowed to stir for 15 min. Propane sultone (1.2 ml, 13.5 mmol) was then added, and the solution was allowed to stir overnight. The excess sodium hydride was quenched with careful addition of 5 ml of methanol, and the mixture was allowed to stir for 30 min. The solvents were then removed by rotary evaporation. The white solid residue was dissolved in water, and the product was precipitated by addition of solid sodium chloride. Detergent 1 was then recrystallized from chloroform/ethanol, to yield 2.0 g, 42% yield. <sup>1</sup>H NMR (D<sub>2</sub>O/CH<sub>3</sub>OH, 300 MHz):  $\delta$ 0.66 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 1.24 (s, 6H, C(CH<sub>3</sub>)<sub>2</sub>), 1.60 (s, 2H, CH<sub>2</sub>), 2.12 (m, 2H, SCH<sub>2</sub>), 2.93 (m, 2H, CH<sub>2</sub>), 3.87 (t,  $J = 6.3$  Hz, 2H, CH<sub>2</sub>), 6.78 (m, 2H, ArH), 7.20 (m, 2H, ArH). <sup>13</sup>C NMR (D<sub>2</sub>O/CH<sub>3</sub>OH, 300

<sup>1</sup> The abbreviations used are: Gdm, guanidinium; CAB, carbonic anhydrase B; STS, sodium tetradecyl sulfate; CTAB, cetyltrimethylammonium bromide; CTAHS, cetyltrimethylammonium hydrogen sulfate; POE(10)L, CH<sub>3</sub>(CH<sub>2</sub>)<sub>11</sub>(OCH<sub>2</sub>CH<sub>2</sub>)<sub>10</sub>OH; pNPAC, *para*-nitrophenyl acetate; CMC, critical micelle concentration.

MHz) 842.9 ( $\text{CH}_2$ ), 50.0 ( $\text{CH}_3$ ), 50.4 (C), 56.0 (C), 66.6 ( $\text{CH}_2$ ), 67.3 ( $\text{CH}_3$ ), 75.1 ( $\text{CH}_2$ ), 84.9 ( $\text{CH}_2$ ), 132.2 (CH), 145.5 (CH), 160.7 (C), 174.3 (C). IR (KBr pellet) 2956 (m), 2894 (m), 1513 (m), 1474 (m), 1256 (m), 1188 (s), 1052 (s)  $\text{cm}^{-1}$ .

## RESULTS

**Unfolded CAB Is Captured by Some, but Not All, Detergents under Conditions That Would Otherwise Lead to Protein Aggregation**—Heating 0.043 mg/ml CAB to 70 °C for 6 min, or denaturing 30 mg/ml CAB in 5 M GdmCl followed by 1000-fold dilution with buffer, causes most of the protein to aggregate, as indicated by an increase in light scattering intensity (monitored with a fluorimeter). Four detergents were evaluated for their ability to prevent this aggregation: CTAB, STS, SDS, and POE(10)L. All three of the ionic detergents can, at sufficient concentration, inhibit CAB aggregation upon heating; CTAB and SDS can inhibit aggregation also upon Gdm dilution (STS could not be evaluated via light scattering for the Gdm dilution mode, because mixing STS and GdmCl causes precipitation). In contrast, the nonionic detergent POE(10)L does not inhibit protein aggregation upon heating or Gdm dilution.

The failure of POE(10)L to capture the unfolded protein can be rationalized in terms of a competition between detergent self-association and detergent-protein interaction. Detergents with nonionic polar segments, like POE(10)L, tend to self-associate more avidly than do detergents with charged polar segments, like CTAB, SDS, and STS (28). This difference is manifested in critical micelle concentrations: nonionic detergents often have lower CMC values than charged detergents with comparable hydrophobic segments. (This trend results from the fact that self-association is opposed by polar group repulsion for charged detergents, but not for nonionic detergents.) The more strongly a detergent aggregates to form micelles, the less likely that detergent is to bind to the unfolded protein (29). It has been noted that nonionic detergents are typically less potent denaturing agents than ionic detergents (29), and this trend matches the distinction we observe for inhibition of non-native CAB aggregation.

Light scattering data suggest that soluble CAB-detergent complexes are formed upon heating the protein in the presence of CTAB, STS, or SDS or diluting the Gdm-denatured protein in the presence of CTAB or STS. Assays for enzymatic activity show that the protein in these complexes is completely inactivated (see below). The circular dichroism (CD) of these detergent complexes was examined in order to determine whether there was any residual conformational order in the detergent-complexed protein. CD measurements are difficult with CTAB-containing solutions, because of absorption by bromide ion; therefore, we used cetyltrimethylammonium monohydrogen sulfate (CTAHS) for these studies. (Control studies indicated very similar behavior for CTAB and CTAHS as artificial chaperones.) Fig. 1 shows far-UV CD data for native CAB and for the complexes formed by heating CAB with CTAHS, SDS, or STS. All three complexes show greater negative ellipticity than the native protein. Very similar far-UV CD data have been reported for CAB-SDS complexes formed by mixing the two components at room temperature (30). The large negative ellipticity of the CAB-detergent complexes has been attributed to enhanced  $\alpha$ -helix formation in the detergent-complexed state relative to the native state (30). The CAB-CTAHS complexes formed by heating and by diluting Gdm-denatured protein in the presence of the detergent are indistinguishable by far-UV CD (not shown), which indicates that the two methods of generation yield similar complexes; this conclusion is supported by reactivation studies discussed below.

Near-UV CD data for native CAB and the complexes with CTAHS and STS, formed by heating, are shown in Fig. 2. The

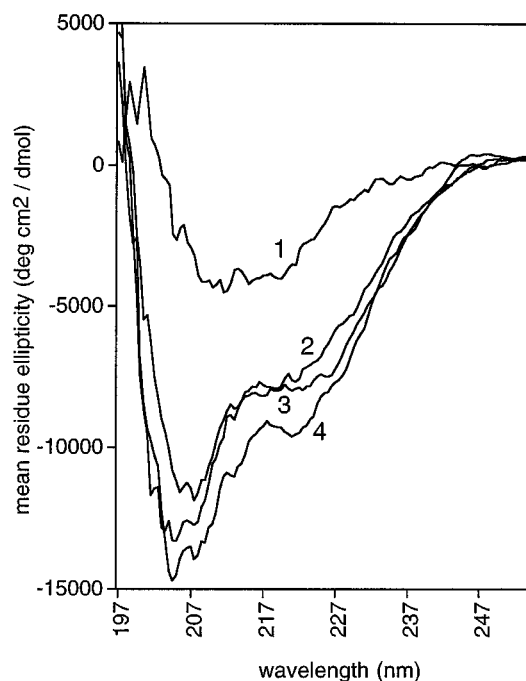


FIG. 1. Far-UV circular dichroism of native CAB (1) and the thermally generated CAB-SDS (2), CAB-CTAHS (3), and CAB-STs (4) complexes. Solutions contained 0.15 mg/ml CAB, 0.4 mM detergent (when present) and 16 mM Tris sulfate, pH 7.75. Each spectrum was obtained with a 1-mm path length cell and is the average of eight scans.

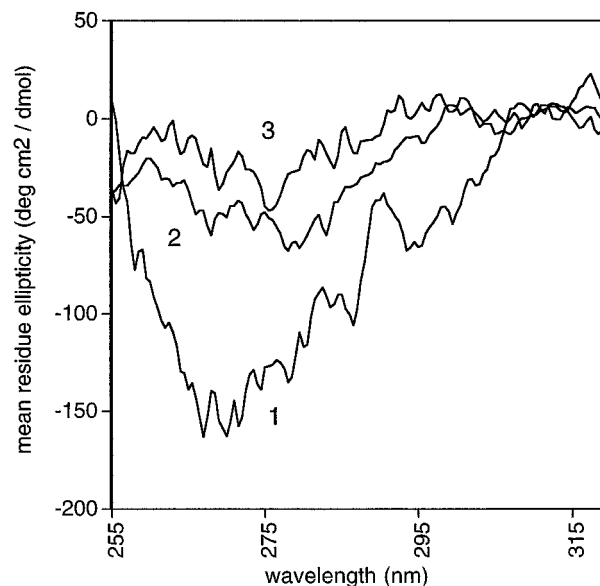


FIG. 2. Near-UV circular dichroism of native CAB (1) and the thermally generated CAB-CTAHS (2) and CAB-STs (3) complexes. Solutions contained 0.15 mg/ml CAB, 0.4 mM detergent (when present), and 16 mM Tris sulfate, pH 7.75. Each spectrum was obtained with a 10-mm path length cell and is the average of eight scans.

detergent complexes show very little ellipticity in this region, which is consistent with previously reported data for CAB-SDS complexes (30). The CTAHS complex formed by dilution of Gdm-denatured CAB was similar to the CTAHS complex formed by heating (data not shown). Near-UV ellipticity is generally taken as an indicator of tertiary structure, while far-UV ellipticity is taken as an indicator of secondary structure. It is interesting that the CAB complexes with CTAHS, SDS, and STS, which appear to contain considerable secondary structure but little tertiary structure, are at least superficially

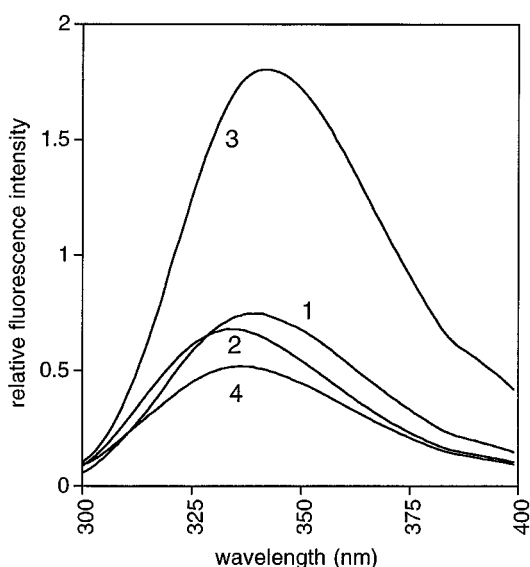


FIG. 3. Intrinsic fluorescence of native CAB (1) and the thermally generated CAB-SDS (2), CAB-CTAB (3), and CAB-STs (4) complexes. Samples were irradiated at 280 nm, with a 4-mm slit width. Solutions contained 0.029 mg/ml CAB, 0.4 mM detergent (when present), and 16 mM Tris sulfate, pH 7.75. Each spectrum was obtained with a  $3 \times 3$  mm cell.

similar to "molten globule" states of CAB (31) and other proteins (32).

Intrinsic fluorescence data for native CAB and for the CTAB, STS, and SDS complexes are shown in Fig. 3 (excitation at 280 nm). Each of the detergent complexes differs substantially from the native protein, which is consistent with the near-UV data in indicating that native tertiary structure is not retained in the detergent complexed states. The detergent complexes also differ from one another: the CTAB complex (which is identical with the CTAHS complex (not shown)) displays considerably enhanced fluorescence intensity, relative to the native enzyme, while the SDS and STS complexes display diminished intensity relative to the native state. Variations in emission maximum are small but significant among the spectra in Fig. 3, and, again, the CTAB complex (red-shifted relative to native CAB) differs from the SDS and STS complexes (blue-shifted relative to native CAB). Intrinsic protein fluorescence arises largely from tryptophan residues, and control studies with *N*-acetyl-L-tryptophanamide show that this small molecule's fluorescence maximum and intensity are identical in the presence of CTAB and STS. Therefore, the fluorescence differences among the detergent complexes presumably reflect differences in the structure of the complexed protein.

*Cyclodextrins Induce Efficient Renaturation from Some, but Not All, CAB-Detergent Complexes*—Table I shows the effects of various manipulations on the enzymatic activity of CAB. Activity was monitored via pNPAC hydrolysis (24), and all measurements are normalized to native CAB activity. When 0.043 mg/ml CAB is heated to 70 °C for 6 min in pH 7.75 buffer, only approximately 2% of the native activity is observed (after the solution has cooled). We conclude that the enzyme has been completely inactivated by this heat treatment, because a similar level of hydrolysis is observed in buffer alone (21). Denaturing 30 mg/ml CAB in 5 M GdmCl, followed by 1000-fold dilution with pH 7.75 buffer, leads to approximately 8% of native hydrolysis activity; thus, this protocol inactivates most but not all of the enzyme.

No enzymatic activity is observed after CAB has been heated in the presence of 0.57 mM CTAB, STS, SDS, or POE(10)L, but addition of 12 eq of  $\beta$ -cyclodextrin (relative to detergent), after

TABLE I  
CAB refolding

Protocol: activity was judged in terms of relative initial rate of pNPAC hydrolysis; all values were normalized to the initial rate of the native protein. For thermal denaturation, 0.35-ml aliquots of 0.043 mg/ml CAB, 23 mM Tris sulfate, pH 7.75, and, when indicated, 0.57 mM detergent, were heated to 70 °C for 6 min. The samples were then allowed to cool for 15 min. Then, 0.15 ml of 16 mM  $\beta$ -cyclodextrin, or 0.15 ml of water, was added, to give final concentrations of 0.03 mg/ml CAB, 16 mM Tris sulfate, 0.4 mM detergent (when present), and 4.8 mM  $\beta$ -cyclodextrin (when present). After  $\beta$ -cyclodextrin addition, solutions were allowed to stand overnight before assay. For Gdm denaturation, 30 mg/ml CAB in 5 M GdmCl was allowed to stand for  $\geq 16$  h, and then 0.5- $\mu$ l aliquots were diluted with Tris sulfate buffer, containing detergent when indicated, to give concentrations of 0.043 mg/ml CAB, 7 mM Gdm, 23 mM Tris sulfate, pH 7.75, and 0.57 mM detergent, when indicated. Then, 150  $\mu$ l of 16 mM aqueous  $\beta$ -cyclodextrin, or 150  $\mu$ l of water, was added, to give final concentrations of 0.03 mg/ml CAB, 5 mM Gdm, 16 mM Tris sulfate, 0.40 mM detergent, when indicated, and 4.8 mM  $\beta$ -cyclodextrin, when indicated. Samples were allowed to stand overnight before assay. For the control experiments, the reaction solutions were assembled in the same way, but no heating or Gdm denaturation was performed.

Controls	
Sample	Relative rate
Native CAB	1.00
+CTAB <sup>a</sup>	0.89 $\pm$ 0.05
+STS	0.01 $\pm$ 0.01
+POE(10)L <sup>a</sup>	1.06 $\pm$ 0.04
Thermal denaturation	
CAB alone <sup>a</sup>	0.02 $\pm$ 0.01
+CTAB <sup>a</sup>	0.03 $\pm$ 0.02
+CTAB; then $\beta$ -CD <sup>a</sup>	0.81 $\pm$ 0.02
+CTAB; then $\alpha$ -CD	0.80 $\pm$ 0.04
+SDS	0.06 $\pm$ 0.01
+SDS; then $\beta$ -CD	0.15 $\pm$ 0.02
+STS	0.01 $\pm$ 0.01
+STS; then $\beta$ -CD	0.78 $\pm$ 0.06
+POE(10)L <sup>a</sup>	0.02 $\pm$ 0.01
+POE(10)L; then $\beta$ -CD <sup>a</sup>	0.03 $\pm$ 0.01
Gdm denaturation	
CAB alone	0.08 $\pm$ 0.01
+CTAB	0.02 $\pm$ 0.01
+CTAB; then $\beta$ -CD	0.74 $\pm$ 0.07
+SDS	0.05 $\pm$ 0.01
+SDS; then $\beta$ -CD	0.28 $\pm$ 0.07
+STS	0.01 $\pm$ 0.01
+STS; then $\beta$ -CD	0.61 $\pm$ 0.05
+POE(10)L	0.19 $\pm$ 0.03
+POE(10)L; then $\beta$ -CD	0.21 $\pm$ 0.01

<sup>a</sup> Result originally published in Ref. 21.

cooling, causes a regain of approximately 80% native activity for the CTAB and STS samples. Achievement of these maximum renaturation levels required that the solutions stand overnight after  $\beta$ -cyclodextrin addition, before assay. When a CAB-CTAB solution was allowed to stand only 15 min after  $\beta$ -cyclodextrin addition, approximately 45% of the original enzymatic activity was detected, and approximately 72% activity was detected when the solution had stood 2 h after  $\beta$ -cyclodextrin addition.

The reactivation of CAB after heating in the presence of 0.57 mM SDS (approximately 15%) is much lower than the reactivation obtained with STS or CTAB, and there is no significant regain in activity for the POE(10)L sample. The lack of reactivation with POE(10)L is not surprising, since light scattering data showed that this detergent was unable to prevent heat-induced CAB aggregation. The relatively low extent of CAB reactivation from the SDS complex is more surprising, because light scattering data suggest that 0.57 mM SDS and 0.57 mM

STS are similarly effective in preventing heat-induced aggregation, and because the CAB complexes of SDS and STS show similar features in the far- and near-UV CD and intrinsic fluorescence spectra (Figs. 1–3).

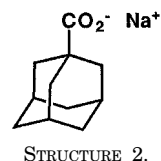
Use of artificial chaperones to aid refolding of CAB from the Gdm-denatured state gives similar but not identical results to those obtained with the thermal denaturation mode. Dilution of Gdm-denatured CAB in the presence of 0.57 mM CTAB does not regenerate enzymatic activity, but addition of 12 eq of  $\beta$ -cyclodextrin to the resulting CAB-CTAB complex leads to approximately 80% reactivation. Significantly lower reactivation (approximately 60%) is observed upon addition of  $\beta$ -cyclodextrin to the CAB-STs complex generated from Gdm-denatured protein. This distinction between CTAB and STS contrasts with the very similar reactivation yields from these two detergents in the thermal mode. We attribute the lower yield obtained when STS is used with Gdm-denatured CAB to the fact that addition of STS to a GdmCl-containing solution causes precipitation of the detergent (see above).

The identical reactivation yields from CAB-CTAB complexes generated by heating or by dilution of Gdm-denatured enzyme supports our conclusion that these two routes produce similar protein-detergent complexes. Most of our subsequent experiments were focused on protein-detergent complexes generated by heating; we assume that the conclusions from these experiments apply also to complexes generated from Gdm-denatured CAB, in the absence of interaction between Gdm and the detergent.

Light scattering studies were performed to determine the fate of the approximately 20% of the enzymatic activity not recovered after addition of  $\beta$ -cyclodextrin to the CAB-CTAB and CAB-STs complexes. A modest increase in light scattering intensity was detected upon  $\beta$ -cyclodextrin addition, relative to the starting CAB-detergent complex, which suggests that the unrecovered activity reflects protein that has aggregated rather than refolded. Thus, addition of  $\beta$ -cyclodextrin to the CAB-detergent complexes seems to set off competing intermolecular (aggregation) and intramolecular (folding) processes, both presumably involving association of hydrophobic peptide surfaces.

Control studies involving native CAB show that anionic detergents SDS and STS completely inactivate the enzyme upon mixing at room temperature (Table I), which is consistent with earlier findings for CAB-SDS interactions (30) and with the generally recognized denaturing powers of alkyl sulfate detergents (29). In contrast, CAB is almost fully active in the presence of cationic detergent CTAB at room temperature. This observation, along with the stability and inactivity of the CAB-CTAB complex formed by heating or dilution from the Gdm-denatured state, indicates that there is a kinetic barrier at room temperature between native CAB in the presence of CTAB and the CAB-CTAB complex. An analogous kinetic barrier exists between the chaperone GroEL and native substrate proteins: the chaperone will not denature properly folded substrates (16). The stability of native CAB in the presence of CTAB at room temperature is interesting because we are unaware of other reports, for any protein, of detectable kinetic barriers between native states and unfolded states induced by low molecular weight denaturants.

**Cyclodextrin Removes Detergent from the CAB-Detergent Complexes by Competitive Binding**— $\alpha$ -Cyclodextrin was found to be as effective as  $\beta$ -cyclodextrin in the second step of the refolding protocol (Table I). We used this similarity to test our hypothesis that the function of the cyclodextrin is to strip detergent away from the protein-detergent complex. Both  $\alpha$ - and  $\beta$ -cyclodextrin bind strongly to detergents containing lin-



ear alkyl chains (33, 34); however, these cyclodextrins differ dramatically in their affinities for bulkier hydrocarbon moieties (35). The adamantyl group, for example, is very well matched to and strongly bound by the central cavity of  $\beta$ -cyclodextrin, but this group is too large to be enclosed by  $\alpha$ -cyclodextrin and, therefore, not strongly bound in this case (36). When  $\beta$ -cyclodextrin and adamantane carboxylate (Structure 2) were added simultaneously to the CAB-CTAB complex, no regain of enzymatic activity was observed; however, adding adamantane carboxylate along with  $\alpha$ -cyclodextrin to the CAB-CTAB complex did not diminish the refolding yield (approximately 80%). Presumably, adamantane carboxylate inhibits the refolding activity of  $\beta$ -cyclodextrin because the adamantyl group is so strongly bound by  $\beta$ -cyclodextrin that detergent binding is precluded, while there is no inhibitory effect on the refolding activity of  $\alpha$ -cyclodextrin because the adamantyl group is not bound in this case. The selective inhibition of  $\beta$ -cyclodextrin's artificial chaperone activity by adamantane carboxylate supports our hypothesis that cyclodextrins function as detergent-stripping agents.

A second test of our explanation for cyclodextrin function involved use of a detergent that can be bound strongly by  $\beta$ -cyclodextrin but not by  $\alpha$ -cyclodextrin. Triton X-100 fulfills this requirement, because the bulky *p*-*t*-octylphenyl hydrophobic moiety is expected to fit well into the  $\beta$ -cyclodextrin cavity, but this hydrophobic group should be too large for strong complexation by  $\alpha$ -cyclodextrin. (These expectations are based on the difference in cyclodextrin affinities for *p*-*t*-butylphenol;  $\beta$ -cyclodextrin binds this molecule strongly, but  $\alpha$ -cyclodextrin does not (35).) Triton X-100, however, is not able to capture heat-denatured CAB; this failure is presumably related to the failure of the nonionic detergent POE(10)L. We synthesized anionic detergent **1**, which contains the *p*-*t*-octylphenyl hydrophobic group, because it seemed possible that **1**, like other ionic detergents, would be an effective artificial chaperone for CAB. We determined the CMC of **1** in refolding buffer to be approximately 4.5 mM, by monitoring solubilization of the dye orange OT (26). (Orange OT is insoluble in aqueous solution, and the CMC is assigned as the concentration at which dye solubilization begins. For typical detergents, CMC values determined in this way agree with values determined by other methods (28).) When 0.043 mg/ml CAB was heated to 70 °C for 6 min in the presence of 4.7 mM **1**, protein aggregation was prevented. Addition of 2.3 eq of  $\beta$ -cyclodextrin (relative to **1**) to the soluble CAB-**1** complex resulted in approximately 70% reactivation, while addition of  $\alpha$ -cyclodextrin to the CAB-**1** complex resulted in only approximately 15% reactivation. The poor performance of  $\alpha$ -cyclodextrin relative to  $\beta$ -cyclodextrin with detergent **1** is consistent with our proposal that the cyclodextrin promotes refolding by competitively binding the detergent away from the CAB-detergent complex.

Sequential addition of the two artificial chaperones, detergent and cyclodextrin, is required to promote CAB refolding, which provides a third line of evidence in support of our stripping agent hypothesis. When CAB was heated in the presence of both CTAB and  $\beta$ -cyclodextrin, no activity was regained after cooling. This failure presumably results from the fact that the detergent is strongly bound by the cyclodextrin and therefore unavailable to capture the heat-unfolded protein prior to aggregation. (Model studies suggest that the affinity of  $\beta$ -cyclo-

dextrin for typical hydrophobic amino acid side chains is approximately  $10^2 \text{ M}^{-1}$  (37, 38) while the affinity of  $\beta$ -cyclodextrin for detergents with long alkyl tails is  $\geq 10^3 \text{ M}^{-1}$  (33, 39). Interestingly, the timing of the detergent addition is not crucial. When CAB was heated in the absence of any additive (to generate an inactive aggregated state), and CTAB was added after the solution had cooled, followed by  $\beta$ -cyclodextrin, approximately 80% of the original enzymatic activity was regained. This finding indicates that CTAB can disrupt the aggregated state of CAB, which contrasts with our previous observation that CTAB does *not* substantially disrupt that native state of CAB. Thus, the aggregated state is kinetically (and perhaps thermodynamically) less stable than the native state toward CTAB.

CAB refolded with the assistance of CTAB and  $\beta$ -cyclodextrin was purified via a two-step ultrafiltration protocol. After refolding was complete, the solution was passed through a  $0.22\text{-}\mu\text{m}$  filter, to remove aggregated protein (verified by light scattering measurements), and then through a  $M_r = 10,000$  cutoff filter. This second filtration was intended to allow the low molecular weight artificial chaperones to pass, but to retain the refolded CAB. The purified protein showed identical specific activity to the starting native protein, as judged by pNPac hydrolysis (CAB quantified by UV absorbance at 280 nm):  $9.7 \pm 0.7 \times 10^3$  units/mg for starting CAB, and  $9.5 \pm 0.4 \times 10^3$  units/mg for refolded CAB. Nondenatured CAB and purified, refolded CAB were indistinguishable by far-UV CD, near-UV CD, and intrinsic fluorescence (21), which suggests that the refolded protein has native-like secondary and tertiary structure. The yield of refolded CAB was approximately 70% after the two ultrafiltration steps (based on protein quantification by absorbance at 280 nm and by Coomassie Blue staining), which is only slightly lower than the "crude" yield estimated before purification (Table I).

**Comparison of the Artificial Chaperone Method with Other Refolding Techniques Employing Detergent or Cyclodextrin**—CAB refolding from the Gdm-denatured state can be enhanced to a modest degree by using nonionic detergents in the diluting buffer. Table I shows that dilution of CAB from the Gdm-denatured state in the presence of 0.57 mM POE(10)L leads to recovery of approximately 20% enzymatic activity (addition of  $\beta$ -cyclodextrin after dilution causes no further reactivation). This behavior stands in contrast to the result of diluting CAB from the Gdm-denatured state in the presence of 0.57 mM CTAB, SDS, or STS; with these ionic detergents and no  $\beta$ -cyclodextrin addition, there is less residual enzymatic activity than is observed after dilution of Gdm-denatured CAB in the absence of detergent. The atypical behavior of POE(10)L appears to be an example of the micelle-assisted refolding process discovered by Horowitz and co-workers (9–11). The concentration of detergent in the dilution buffer is crucial in this refolding strategy (40); therefore, we examined CAB refolding yield as a function of POE(10)L concentration over the range 0.01 to 5 mM. A maximum of approximately 25% reactivation was observed for dilution of Gdm-denatured CAB in the presence of 1 mM POE(10)L. Horowitz *et al.* (11) found lauryl maltoside to be particularly effective in micelle-assisted refolding. We found that lauryl maltoside also allowed micelle-assisted CAB refolding, with a maximum of approximately 25% reactivation at 20 mM detergent. Thus, with neither of these nonionic detergents was the efficiency of micelle-assisted CAB reactivation as high as could be obtained via the artificial chaperone technique (with ionic detergents). It is interesting to note that POE(10)L was unable to promote CAB refolding to any extent in thermal denaturation experiments (Table I).

After our original description of the artificial chaperone tech-

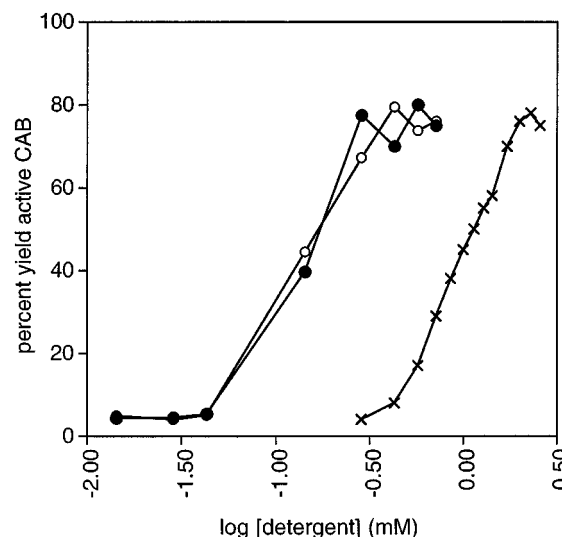


FIG. 4. Yield of refolded CAB, as determined by pNPac hydrolysis activity after  $\beta$ -cyclodextrin addition, as a function of the logarithm of the concentration of CTAB (●), STS (○), or SDS (x) present at the time of heating. Solutions contained 0.026 mg/ml CAB, 14 mM Tris sulfate, pH 7.75, 4.4 mM  $\beta$ -cyclodextrin, and 4.7 mM pNPac. After  $\beta$ -cyclodextrin addition, solutions were allowed to stand overnight before assay. The lines are arbitrary.

nique appeared (21), Karupiah and Sharma (41) reported that cyclodextrin alone, used as a dilution additive, could promote CAB refolding from the Gdm-denatured state. Most of the refolding conditions employed by these workers, however, allowed substantial reactivation yields in the *absence* of cyclodextrin. In contrast, only approximately 8% CAB activity is recovered upon dilution from the Gdm-denatured state under the conditions employed for the experiments in Table I. Under our stringent conditions, inclusion of 4.8 mM  $\alpha$ - or  $\beta$ -cyclodextrin in the dilution buffer did not increase the reactivation yield; inclusion of 100 mM  $\alpha$ -cyclodextrin provided a very modest improvement, to approximately 18% reactivation.

**Assisted Refolding Requires Cooperative Interaction among Detergent Molecules at the Capture Step, but Capture Can Be Achieved below the Detergent's CMC**—The efficiency of CAB refolding shows a critical dependence on the concentration of detergent at the "capture step." The importance of detergent concentration on assisted refolding was examined by monitoring reactivation yields after  $\beta$ -cyclodextrin addition, because it is difficult to quantify the relative extents of CAB-detergent complex formation and CAB aggregation, upon heating.

The effect of CTAB, STS, or SDS concentration on the extent of CAB reactivation after thermal denaturation (0.043 mg/ml protein at the heating step) is shown in Fig. 4. For STS and CTAB, refolding assistance becomes apparent above 0.05 mM detergent. As detergent concentration is raised further, this assistance rapidly reaches a maximum, at approximately 0.3 mM. For SDS, refolding assistance is observed above 0.5 mM detergent, and the maximum yield is achieved at approximately 2 mM. The curves in Fig. 4 look qualitatively like the parameter *versus* concentration curves commonly used to determine the critical micelle concentrations (CMC) of detergents (28): the appearance and maximization of refolding assistance over a small concentration range suggests that this assistance depends upon cooperative detergent aggregation. The CMC values for CTAB and STS implied by the curves in Fig. 4, approximately 0.1 mM, are lower than the CMC values of these detergents in the absence of protein. We determined the "protein-free" CMC values in the buffer employed for CAB refolding via a standard method, solubilization of the hydrophobic dye



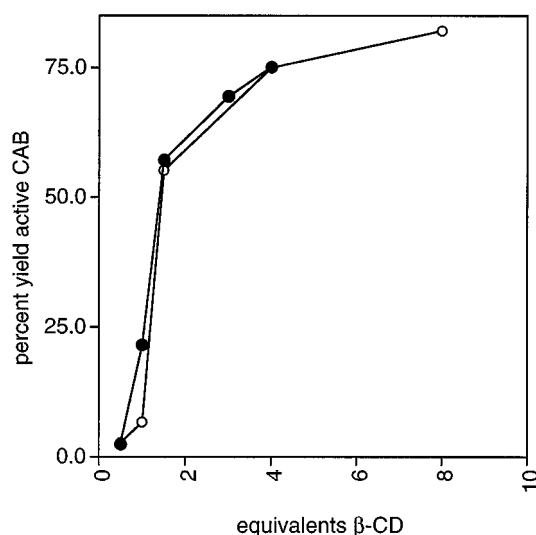


FIG. 5. Yield of refolded CAB, as determined by pNPac hydrolysis activity after  $\beta$ -cyclodextrin addition, as a function of the number of equivalents of  $\beta$ -cyclodextrin relative to the detergent CTAB. Solutions contained 0.026 mg/ml CAB, 14 mM Tris sulfate, pH 7.75, 0.18 mM (●) or 0.36 mM (○) CTAB, and 4.7 mM pNPac. After  $\beta$ -cyclodextrin addition, solutions were allowed to stand overnight before assay. The lines are arbitrary.

orange OT (26). In the refolding buffer, CTAB has a CMC of approximately 0.6 mM, and STS has a CMC of approximately 2.0 mM, in the absence of protein. Thus, the CMC of each detergent is significantly higher than the lowest concentration at which refolding assistance is observed. These results suggest that the soluble protein-detergent complexes, intermediates in the refolding process, are mixed protein-detergent micelles.

Fig. 4 shows that SDS can be as effective an artificial chaperone as STS or CTAB, but that optimal CAB refolding yield requires at least 2.0 mM detergent at the heating step. Thus, the relatively low yield of CAB reactivation shown for SDS in Table I results from suboptimal SDS concentration (0.57 mM at the heating step). Light scattering studies show that protein aggregation is efficiently suppressed when CAB is heated in the presence of 0.57 mM SDS; therefore, the low yield of refolded CAB obtained from this suboptimal SDS concentration arises from an increase in aggregation relative to refolding at the stripping step, rather than from a failure to capture the unfolded protein at the heating step. A similar profile of refolding failure can be observed for CTAB and STS at some suboptimal concentrations. These findings suggest that the nature of the protein-detergent complex is dependent upon detergent concentration.

It is possible to convert a suboptimal CAB-detergent complex into a more efficiently stripped complex by introduction of additional detergent after the capture step. Thus, if the CAB-SDS complex is formed with 0.57 mM SDS at the time of heating, and if additional SDS is introduced after the solution has cooled (to 2.3 mM total SDS), addition of cyclodextrin leads to the full 80% reactivation. Conversion of the suboptimal to the optimal CAB-SDS complex is a relatively slow process: at least 1 day is required for reorganization of the CAB-SDS complex to a state from which efficient refolding can occur. Intrinsic fluorescence measurements revealed only modest differences between the CAB complexes formed with 0.57 or 2.0 mM SDS; the latter had a slightly greater fluorescence intensity.

**Refolding Yield Shows a Critical Dependence on Cyclodextrin-Detergent Ratio**—Fig. 5 shows the variation in recovery of CAB activity from thermally generated CTAB complexes as a

function of the number of equivalents of  $\beta$ -cyclodextrin (relative to detergent) employed in the stripping step. Results are shown for two different initial detergent concentrations; the similarity of the curves indicates that  $\beta$ -cyclodextrin functions by interacting with the detergent, rather than with the protein. Addition of 0.5 or 1.0 eq of  $\beta$ -cyclodextrin causes no reactivation, but addition of 2.0 eq provides approximately 55% reactivation, and  $>2$  eq of  $\beta$ -CD leads to a further increase in yield. (In our preliminary communication on artificial chaperones (21), we suggested that 2 eq of  $\beta$ -cyclodextrin per detergent were sufficient for optimum reactivation of CAB; the data in Fig. 5 show that larger quantities of  $\beta$ -cyclodextrin are actually required.) This critical behavior presumably reflects the fact that co-aggregation of the detergent and the protein is a cooperative phenomenon.

**Rapid Introduction of Cyclodextrin Is Required for Optimal Refolding Yield**—Fig. 6 provides data from a series of experiments in which refolding from the CAB-CTAB complex was induced by addition of a total of 12 eq of  $\beta$ -cyclodextrin (relative to the amount of detergent). In all but one of the experiments, the  $\beta$ -cyclodextrin was added in two portions, with a 10-min interval between additions. The horizontal axes in Fig. 6, A and B, indicate the number of  $\beta$ -cyclodextrin equivalents in the first addition (the rightmost data point in each case represents addition of all 12 eq of  $\beta$ -cyclodextrin at once). Fig. 6A shows that the maximum CAB refolding yield is obtained when all of the  $\beta$ -cyclodextrin is added at once. A substantial diminution of yield is observed if the initial addition is only 1–3 eq of  $\beta$ -cyclodextrin. Fig. 6B shows the same series of experiments, monitored by light scattering. The minimum in recovered CAB activity in Fig. 6A matches the maximum in  $\beta$ -cyclodextrin-induced light scattering in Fig. 6B. Similar behavior was observed for CAB-STS complexes (not shown). We interpret the data in Fig. 6 to indicate that addition of too little  $\beta$ -cyclodextrin causes only partial stripping of the detergent, which in turn leads to significant protein aggregation. The aggregation induced by addition of too little  $\beta$ -cyclodextrin occurs very rapidly: maximal light scattering intensity is observed within 10 s of the  $\beta$ -cyclodextrin addition.

**Detergent Removal by Dialysis or with a Macroscopic Adsorbent Does Not Lead to Efficient Refolding from the CAB-CTAB Complex**—Dialysis is a common technique for removing detergent from proteins, and it was therefore important to compare this conventional method to detergent stripping by cyclodextrin. When the thermally generated CAB-CTAB complex was dialyzed three times against 300 volumes of buffer (24 h each), the dialysis bag contained visible protein precipitate, and recovered CAB activity was only 25% (versus 81% reactivation upon rapid addition of 12 eq of  $\beta$ -cyclodextrin). The yield was not improved when the dialysis buffer contained 0.5 mM  $\text{ZnCl}_2$ , which indicates the low efficiency of CAB renaturation did not result from removal of the enzyme's essential  $\text{Zn}^{2+}$  ion during dialysis. Dialysis of detergent from the CAB-STS complex also led to lower refolding yield (49%) than rapid addition of cyclodextrin. The observation that proportionally more aggregation occurs during dialysis than during rapid addition of cyclodextrin is consistent with the results of experiments in which the rate of  $\beta$ -cyclodextrin addition was varied (see above); in both cases, slow removal of detergent from the CAB-CTAB complex promotes protein aggregation.

The artificial chaperone method might be streamlined if the detergent-stripping agent were a macroscopic solid, which could be separated from the refolded protein by simple filtration. We explored three adsorbents as stripping agents, Bio-Beads<sup>®</sup> SM-2 (from Bio-Rad), Calbisorb<sup>™</sup> beads (from Calbiochem), and a  $\beta$ -cyclodextrin-epichlorhydrin copolymer (from

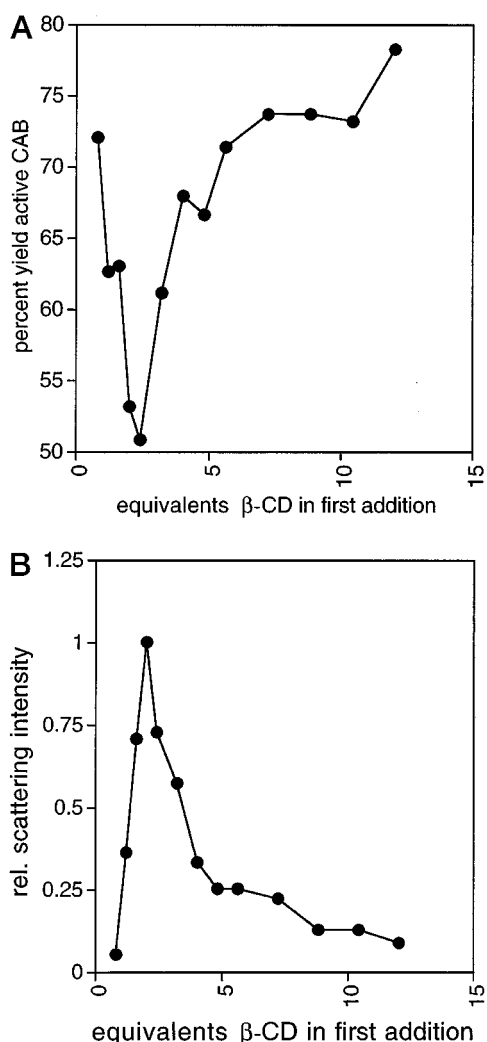


FIG. 6. A, yield of refolded CAB, as determined by pNPac hydrolysis activity after complete  $\beta$ -cyclodextrin addition, as a function of the number of equivalents of  $\beta$ -cyclodextrin, relative to the detergent CTAB, introduced during the first of two additions. The two cyclodextrin additions were separated in time by 10 min, except for the rightmost data point, which represents addition of all of the cyclodextrin at once. Solutions contained 0.026 mg/ml CAB, 14 mM Tris sulfate, pH 7.75, 0.36 mM SDS, 4.7 mM pNPac, and a total of 4.4 mM  $\beta$ -cyclodextrin (12 eq relative to detergent). After  $\beta$ -cyclodextrin addition, solutions were allowed to stand overnight before assay. B, relative light scattering intensity after complete  $\beta$ -cyclodextrin addition, as a function of the number of equivalents of  $\beta$ -cyclodextrin, relative to the detergent CTAB, introduced during the first of two additions. The two cyclodextrin additions were separated in time by 10 min, except for the rightmost data point, which represents addition of all of the cyclodextrin at once. Solutions contained 0.029 mg/ml CAB, 16 mM Tris sulfate, pH 7.75, 0.40 mM CTAB, and a total of 4.8 mM  $\beta$ -cyclodextrin (12 eq relative to detergent). Samples were analyzed in  $3 \times 3$  mm cells, irradiating and monitoring at 350 nm (4 mm slit width). In both cases, the lines are arbitrary.

Aldrich). The Calbiosorb<sup>TM</sup> beads provided almost no reactivation from the CAB-CTAB complex, and the other two adsorbents provided 20–25% reactivation yields. Starting from the CAB-STS complex, Bio-Beads<sup>®</sup> SM-2 provided 23% reactivation, Calbiosorb<sup>TM</sup> provided 13% reactivation, and the  $\beta$ -cyclodextrin-epichlorohydrin copolymer provided 36% reactivation. These relatively poor performances may stem from the fact that the rate of detergent stripping by the macroscopic adsorbents is necessarily slower than stripping by a soluble agent.

**Nonchaotropic Salts Can Profoundly Influence Refolding Yield**—Fig. 7 shows the effect of  $\text{Na}_2\text{SO}_4$  concentration (in the initial solution) on recovery of CAB activity after thermal de-

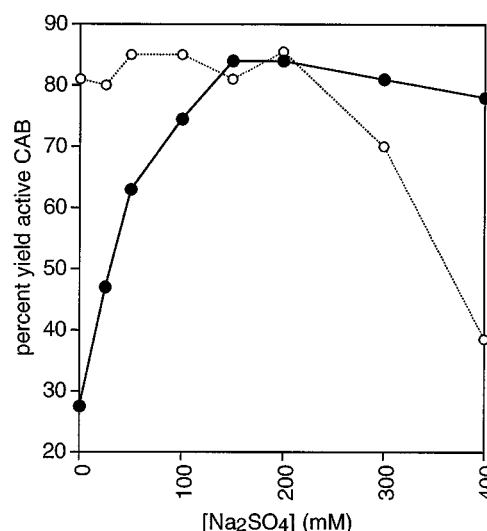


FIG. 7. Yield of refolded CAB, as determined by pNPac hydrolysis activity after  $\beta$ -cyclodextrin addition, as a function of the concentration of  $\text{Na}_2\text{SO}_4$  present at the time of heating. Solutions contained 0.026 mg/ml CAB, 14 mM Tris sulfate, pH 7.75, 0.36 mM SDS (●) or STS (○), 4.7 mM pNPac, and 4.4 mM  $\beta$ -cyclodextrin. After  $\beta$ -cyclodextrin addition, solutions were allowed to stand overnight before assay. The lines are arbitrary.

naturation in the presence of 0.57 mM SDS or STS. As indicated in Table I, approximately 15% activity is recovered with 0.57 mM SDS as artificial chaperone in the absence of  $\text{Na}_2\text{SO}_4$ . Fig. 7 shows that addition of 150 mM  $\text{Na}_2\text{SO}_4$  to the initial protein-SDS solution, before heating, causes SDS at this low concentration to be as effective an artificial chaperone as STS or CTAB in the absence of added salt. A further increase in  $\text{Na}_2\text{SO}_4$  concentration, up to 400 mM, has relatively little effect on CAB refolding yield with SDS. The  $\text{Na}_2\text{SO}_4$  need not be present at the time of heating: 0.57 mM SDS prevents thermally induced CAB aggregation, and addition of  $\text{Na}_2\text{SO}_4$  to the resulting CAB-SDS complex after cooling leads to the same refolding yield, after cyclodextrin addition, as having the  $\text{Na}_2\text{SO}_4$  present at the time of heating. (The effect of  $\text{Na}_2\text{SO}_4$  added after heating is fully exerted within a few minutes.) This result implies that  $\text{Na}_2\text{SO}_4$  causes some type of restructuring of the CAB-SDS complex.

The effects of added  $\text{Na}_2\text{SO}_4$  on assisted refolding with STS are quite different from the results with SDS. Up to 200 mM, added  $\text{Na}_2\text{SO}_4$  has relatively little effect on refolding yield, with STS, but at higher  $\text{Na}_2\text{SO}_4$  concentrations, the refolding yield diminishes. Similar effects were observed when salt was added to CTAB-CAB solutions before heating, but very high  $\text{Na}_2\text{SO}_4$  concentrations (approximately 1 M) were required before a deleterious effect was observed.

**Strategies for Maximizing Protein Concentration in the Refolding Process**—For many applications, it is advantageous to conduct refolding at protein concentrations that are as high as possible. In contrast, our only concern in choosing conditions for the experiments described above was that the protein be sufficiently concentrated that refolding fails in the absence of artificial chaperones. Not surprisingly, if the CAB concentration is raised while all the artificial chaperone concentrations are held constant, the yield of reactivated enzyme decreases. With the CTAB/ $\beta$ -cyclodextrin combination, for example, raising the CAB concentration at the time of heating from 0.043 mg/ml to 0.15 mg/ml causes the refolding yield to drop from 80% to 30%; with the STS/ $\beta$ -cyclodextrin combination, the yield drops from 80% to 60%.

CAB refolding at higher protein concentrations can be made more efficient by increasing the concentrations of the artificial



chaperones and by adding  $\text{Na}_2\text{SO}_4$ . The naturally occurring cyclodextrins have limited water solubility (approximately 130 and 16 mM, respectively, for  $\alpha$ - and  $\beta$ -cyclodextrin), so high concentrations of the stripping agent require the use of commercially available cyclodextrin derivatives that are more soluble. We have found "methyl- $\beta$ -cyclodextrin" (mostly tetradecamethyl- $\beta$ -cyclodextrin; methyl groups at the 2- and 6-hydroxyls of the glucose rings) to be particularly useful. When 1.5 mg/ml CAB was heated to 70 °C for 6 min in the presence of 28 mM CTAB and 100 mM  $\text{Na}_2\text{SO}_4$  and 7 eq (relative to CTAB) of methyl- $\beta$ -cyclodextrin (final concentration = 200 mM) was added after the solution had cooled, 67% of the CAB activity was recovered. A nearly identical yield was obtained when 30 mg/ml CAB denatured in 5 M GdmCl was diluted 14-fold in the presence of 40 mM CTAB, followed by addition of 7 eq of methyl- $\beta$ -cyclodextrin. In this latter case, the yield of active enzyme without artificial chaperones was 10%, and there was no recovery of active enzyme in the former case (thermal denaturation) without artificial chaperones.

#### DISCUSSION

The utility of the artificial chaperone technique will depend upon the number of other proteins that can be refolded in this way. (Preliminary observations show that this technique is effective with citrate synthase (21), a dimeric enzyme with no structural homology to CAB; thus, the method is not limited to the protein we have focused upon here.) The data reported above allow us to define features of artificial chaperone-assisted refolding that are likely to be general, and, therefore, to suggest strategies for application to other proteins.

The choice of stripping agent appears to be constrained. The observation that the detergent must be removed from the protein-detergent complex as rapidly as possible seems to require a water-soluble stripping agent that can be added in excess. This observation suggests also that stripping processes limited by rate of diffusion to or across a two-dimensional interface will be intrinsically ineffective, which is consistent with our finding that detergent removal via dialysis or a macroscopic stripping agent gives poor refolding yields. The naturally occurring cyclodextrins and various synthetic derivatives are the only water-soluble molecules that have been shown to bind detergents tightly, to our knowledge. It is possible that appropriately functionalized calixarenes and/or other water-soluble molecules containing nonpolar cavities might also serve as stripping agents. Alternative stripping agents, however, will have to display selectivity for the detergent relative to nonpolar amino acid side chains, as is the case with  $\alpha$ - and  $\beta$ -cyclodextrin.

The choice of detergent is crucial for successful refolding. Our data reveal that there are two distinct ways in which a detergent can fail as an artificial chaperone: (i) the detergent does not prevent protein aggregation at elevated temperature or upon removal of denaturant; (ii) the detergent forms a soluble complex with the unfolded protein, but stripping of detergent from the complex leads to aggregation rather than refolding. The first type of failure is rationalized in terms of a competition between detergent-detergent association (micelle formation) and detergent-protein association (capture of the unfolded state). This failure mode appears to be insurmountable, at least for the CAB-POE(10)L combination (unfolded CAB is not "captured" when as much as 5 mM POE(10)L is present). The second type of failure is displayed with CAB by SDS at suboptimal concentrations. This failure mode can be overcome by altering the refolding conditions (e.g. raising detergent concentration and/or adding a nonchaotropic salt). The physical basis of this second failure mode is unclear. The "competent" and "incompetent" CAB-SDS complexes seem to be similar when examined by CD or intrinsic fluorescence, but we

assume that some structural difference must underlie the functional difference. We suspect that the structural variation involves the number of protein molecules per protein-detergent complex, and that complexes that lead to greater refolding will prove to have fewer protein molecules. This hypothesis should be testable via dynamic light scattering and/or analytical ultracentrifugation studies of protein-detergent complexes formed under various conditions.

Our findings suggest a general protocol for applying the artificial chaperone technique to new proteins. Such applications will most commonly involve proteins that have been obtained in a urea- or Gdm-denatured state, and that aggregate rather than refolding when the denaturant is removed via dilution or dialysis. For refolding new proteins, it will be crucial to investigate a set of detergents with a range of CMC values. POE(10)L, Triton X-100, and other nonionic detergents have low CMCs, while ionic detergents with a net charge have relatively high CMCs, especially if the hydrophobic "tail" is short, as with SDS. Zwitterionic detergents, like members of the zwittergent series, have intermediate CMCs. Preliminary studies may be most fruitful if the detergents are examined for their ability to prevent protein aggregation, which can be monitored as an increase in light scattering intensity. Since there is no simple relationship between the extent of light scattering and the extent of protein aggregation, one must examine a sample of fully aggregated protein in order to define the appropriate light scattering intensity "end point." If possible, examination of the native protein, to define the "no aggregation" limit in light scattering intensity, will also be helpful.

Once detergents have been identified that prevent protein aggregation upon urea or guanidinium removal, refolding can be attempted by introducing cyclodextrin. The success of these attempts can be monitored by appearance of native protein activity, if this activity is easily assayed. Alternatively, refolding attempts can be followed, at least initially, by monitoring light scattering intensity; little or no increase in light scattering upon addition of cyclodextrin is a good sign, suggesting that detergent stripping has not caused the protein to precipitate. The presence of soluble protein in the resulting solution can be probed via fluorescence.

Efforts to refold a new protein should begin with a relatively low protein concentration. (The dilution limit will often be defined by the minimum concentration at which native activity can be conveniently and sensitively assayed.) If successful refolding is achieved in this first phase of testing, it is relatively straightforward to search for conditions that allow refolding at higher protein concentrations, e.g. by raising detergent concentration and/or by introducing nonchaotropic salts. Our results suggest that nonchaotropic salt additives should *not* be used in the initial search for refolding conditions. For CAB at relatively low concentration, refolding yields can be either improved (SDS) or diminished (STS) by addition of  $\text{Na}_2\text{SO}_4$ ; both of these detergents were initially identified as potential artificial chaperones by their ability to prevent CAB aggregation upon heating, in the absence of  $\text{Na}_2\text{SO}_4$ .

*The Artificial Chaperone Method versus Use of Detergent or Cyclodextrin as a Dilution Additive*—Horowitz *et al.* (9–11, 40) have shown that protein refolding from Gdm- or urea-denatured states can be promoted by inclusion of a detergent in the diluting buffer. This process has been termed "micelle-assisted refolding," because the detergent must be in a micellar state and because successful refolding seems to involve transient interaction between the unfolded or partially folded protein and detergent micelles. The micelles appear to act as a hydrophobic buffer, blocking exposed hydrophobic surfaces on the non-native protein sufficiently to prevent aggregation, but not

so avidly that the intramolecular association of hydrophobic surfaces necessary for folding is prevented. This delicate balance among alternative hydrophobic interactions may make it difficult to identify conditions for efficient micelle-assisted refolding of some proteins. Indeed, we were able to obtain only 25% reactivation from Gdm-denatured CAB with this approach, while 80% CAB reactivation was achieved with the artificial chaperone method.

Despite a superficial resemblance between the micelle-assisted and artificial chaperone refolding methods, the two approaches are quite different. In particular, the roles of the detergent in the two techniques are distinct. When the detergent is used as an artificial chaperone, it must capture the non-native protein under conditions that would otherwise lead to protein aggregation. In the micelle-assisted approach, the detergent must perform the more difficult task of disrupting intermolecular but not intramolecular association of hydrophobic protein surfaces. Starting from the chemically denatured protein, refolding via the artificial chaperone strategy is a two-step process, with the protein-detergent complex as a stable intermediate. Micelle-assisted refolding, on the other hand, is a single step process, in which stable association between detergent and protein would be counterproductive. (Zardeneta and Horowitz (42) have reported a two-step variation on the micelle-assisted method, in which unfolded rhodanese is captured by binding to phospholipid vesicles, and refolding is induced by addition of a detergent, which disrupts the vesicle and frees the protein.)

Karuppiiah and Sharma (41) have reported high reactivation yields from Gdm-denatured CAB with cyclodextrins as dilution additives, but their experiments were conducted under conditions that allowed substantial reactivation in the absence of cyclodextrins. For example, when 10 mg/ml CAB denatured in 6.8 M GdmCl was diluted 20-fold in the presence of 100 mM  $\alpha$ -cyclodextrin, approximately 95% of the enzymatic activity was recovered, and dilution in the presence of 1 mM  $\alpha$ -cyclodextrin provided approximately 60% reactivation, but approximately 40% activity was recovered in the *absence* of cyclodextrin. These results are consistent with earlier findings of Cleland and Wang (43), who showed that CAB could be refolded efficiently from the Gdm-denatured state if the final Gdm concentration was kept fairly high (0.3 to 1.0 M). Under the more stringent refolding conditions employed in our studies (0.007 M Gdm after dilution), only 8% CAB reactivation is observed without a dilution additive, and refolding assistance from cyclodextrin as a dilution additive is severely limited.

After the present study was submitted for publication, a report appeared from Wetlaufer and Xie (44) on the use of surfactants to assist CAB refolding from the Gdm-denatured state. (More recently, these workers have demonstrated that careful manipulation of temperature can enhance CAB refolding yields (45).) Again, virtually all experiments were carried out under conditions that allow substantial refolding yield without any dilution additive (final GdmCl concentrations 0.25 to 1.0 M). In contrast, our experiments were designed on the assumption that refolding of CAB (or other commercially available enzymes) will be most useful as a model for the renaturation of recalcitrant proteins if one works under conditions that provide little or no reactivation in the absence of the prospective refolding assistant(s).

**Conclusion**—We have shown that a pair of small molecules, a detergent and a stripping agent, can assist the refolding of

the enzyme CAB. We designate the low molecular weight additives artificial chaperones, because our search for such refolding assistants was inspired by the two-step mechanism of the GroEL/S chaperone proteins (16). Artificial chaperone-assisted refolding can provide very efficient refolding of CAB under conditions that otherwise lead predominantly to CAB aggregation. Efficient refolding is a prerequisite for structural and functional characterization of any protein isolated initially in a non-native form; therefore, if the artificial chaperone method proves to be useful with recalcitrant proteins, this method could become an important tool in basic protein science.

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