Artificial Chaperones: Protein Refolding via Sequential Use of Detergent and Cyclodextrin

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The ability to overproduce natural or modified proteins in genetically engineered cells has revolutionized molecular biology and helped to create an expanding biotechnology industry. Expression techniques have been continually refined, but renaturation of the overproduced protein remains problematic in many cases.[1,2] We describe a new method in which sequential introduction of two low molecular weight agents promotes the adoption of a protein's native conformation under conditions that, in the absence of the additives, lead to aggregated ("misfolded") protein. In the first step, a detergent forms a complex with the non-native protein, preventing aggregation.[3] In the second step, a cyclodextrin strips the detergent away from the protein, allowing proper refolding.[4] Development of this two-step process was inspired by the mechanism of the GroE chaperone system.[5] The GroEL 14-mer binds to non-native states of substrate proteins, thus preventing aggregation, and substrate refolding is triggered by the binding of additional agents to the GroEL−substrate complex: MgATP, K^+, and, at least in some cases, the cochaperone GroES (as a 7-mer).

Table 1 summarizes results with thermally denatured carbonic anhydrase B (CAB). Heating 1.4 μM CAB in 40 mM aqueous Tris-sulfate, pH 7.75, to 70 °C for 6 min led to complete and irreversible denaturation of the enzyme,[6] as monitored by the initial rate of *p*-nitrophenyl acetate (pNPAc) hydrolysis[7] after cooling. An increase in light scattering[8] indicated that the protein had aggregated as a result of the heating. When heating was carried out in the presence of the cationic detergent cetyltrimethylammonium bromide [CTAB; $CH_3(CH_2)_{15}N(CH_3)_3$-Br],[9] no increase in light scattering was detected in the cooled solution, but the enzyme was inactive. Addition of β-cyclodextrin (β-CD), however, to the denatured protein−detergent complex caused reactivation of the enzyme, with a maximum yield of 81% recovered activity.[10] The reactivated enzyme could be purified by a two-step procedure: passage through a 0.22 μm filter, to remove large protein aggregates, and then concentration with a 10 000 MW cutoff filter, to remove detergent and CD. The resulting protein solution was identical to a solution containing non-denatured CAB at a similar concentration, as judged by circular dichroism, intrinsic fluorescence, and specific activity.[11] In contrast to CTAB, the nonionic detergent POE(10)L [$CH_3(CH_2)_{11}(OCH_2CH_2)_{10}CH$][9] was ineffective for CAB refolding.[12]

Table 1. Results for Thermally Denatured Carbonic Anhydrase B[a]

additives	rel init rate	additives	rel init rate
	Native Protein		
none	1.00	CTAB; β-CD	0.98 ± 0.04
β-CD	0.96 ± 0.04	POE(10)L	1.06 ± 0.04
CTAB	0.89 ± 0.05	POE(10)L; β-CD	1.00 ± 0.05
	Background (No Enzyme)		
none (buffer only)	0.01 ± 0.01	CTAB	0.01 ± 0.01
β-CD	0.02 ± 0.01		
	After Heating		
none	0.02 ± 0.01	CTAB; β-CD	0.81 ± 0.02
β-CD	0.03 ± 0.01	POE(10)L	0.02 ± 0.01
CTAB	0.03 ± 0.01	POE(10)L; β-CD	0.03 ± 0.01

[a] Protocol: for thermal denaturation, 350 μL aliquots containing 1.4 μM CAB (Sigma) in 23 mM Tris, pH 7.75, and, when indicated, 0.57 mM detergent, were heated to 70 °C for 6 min and then allowed to cool for 10 min. Next, 150 μL of 16 mM aqueous β-CD or 150 μL of water was added, to give final concentrations of 1.0 μM CAB, 0.4 mM detergent (when present), and 4.8 mM β-CD (when present). The resulting solutions were allowed to stand overnight before assay. For the native protein control experiments, the reaction solutions were assembled in the same way, but the heating step was omitted. For the background control experiments, the CAB was omitted. The assay, involving pNPAc hydrolysis, was performed as reported in ref 7 and monitored via production of *p*-nitrophenolate (absorbance at 400 nm).

pNPAc hydrolysis in the presence of β-CD alone or CTAB alone was well below the activity observed with native CAB. β-CD[13] and micelles[14] are known to promote pNPAc hydrolysis, but these control experiments show that "background" hydrolysis cannot account for the levels of activity we attribute to CAB renaturation. Control experiments indicate also that, at the concentrations used for refolding, CTAB and β-CD exert little or no deleterious effect on the activity of non-denatured CAB at room temperature, which suggests that neither additive disrupts the native conformation. The lack of a CD effect on native CAB is consistent with a report that low concentrations of β-CD exert only small effects on the thermal stabilities of globular proteins.[4c]

Table 2 shows results obtained with Gdm-denatured citrate synthase (CS). Dilution of Gdm-denatured GS by 100-fold, to 0.34 μM protein, led to a recovery of only 2% enzymatic activity, which is consistent with earlier reports that CS aggregates upon attempted refolding in this manner.[15] Dilution in the presence of nonionic detergent POE(10)L, followed by

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(2) Poly(ethylene glycol) enhances refolding yields: Cleland, J. L.; Builder, S. E.; Swartz, J. R.; Winkler, M.; Chang, J. Y.; Wang, D. I. C. *Bio/Technology* **1992**, *10*, 1013 and references therein.

(3) Detergents have occasionally been used for refolding; recent examples: (a) Zardeneta, G.; Horowitz, P. M. *J. Biol. Chem.* **1992**, *267*, 5811 and references therein. (b) Puri, N. K.; Crivelli, E.; Cardamone, M.; Fiddes, R.; Bertolini, J.; Ninham, G.; Brandon, M. R. *Biochem. J.* **1992**, *285*, 871. (c) Nguyen, L. H.; Jensen, D. B.; Burgess, R. R. *Protein Expression Purif.* **1993**, *4*, 425.

(4) There have been relatively few reports on protein−cyclodextrin interactions. (a) Lee, M.; Fennema, O. R. *J. Agric. Food Chem.* **1991**, *39*, 17. (b) Brewster, M. E.; Hora, M. S.; Simpkins, J. W.; Bodor, N. *Pharm. Res.* **1991**, *8*, 792. (c) Cooper, A. *J. Am. Chem. Soc.* **1992**, *114*, 9208. (d) Inhibition of lipase activity by detergents can be reversed by the addition of α-CD: Schomaecker, R.; Robinson, B. H.; Fletcher, P. D. I. *J. Chem. Soc., Faraday Trans. 1* **1988**, *84*, 4203.

(5) Reviews of chaperone function: (a) Hendrick, M. P.; Hartl, F. U. *Annu. Rev. Biochem.* **1993**, *62*, 349. (b) Stuart, R. A.; Cyr, D. M.; Craig, E.; Neupert, W. *Trends Biochem. Sci.* **1994**, *19*, 87.

(6) Previous studies of thermal CAB inactivation: McCoy, L. F.; Wong, K. P. *Biochemistry* **1981**, *20*, 3062 and references therein.

(7) Pocker, Y.; Stone, J. T. *Biochemistry* **1967**, *6*, 668.

(8) Light scattering studies were carried out with a fluorescence spectrophotometer, irradiating and observing at 350 nm. This technique has been widely used for examining protein aggregation; see, for example: Wiech, H.; Buchner, J.; Zimmerman, R.; Jakob, U. *Nature* **1992**, *358*, 169.

(9) All detergents were obtained from Sigma. CTAB and Triton X-100 were nominally 99% pure; no nominal purity was indicated for the POE(10)L.

(10) Very little CAB activity was recovered when both CTAB and β-CD were added before heating; ca. 14% CS activity was recovered when both POE(10)L and β-CD were added before Gdm dilution.

(11) Relevant data may be found in the supplementary material.

(12) For both CAB and CS, the identity and concentration of the detergent have been optimized, as has the concentration of CD. For optimum refolding of CAB, 2 equiv of β-CD per CTAB are required, and 4 equiv of β-CD per POE(10)L are required for CS. For both proteins, a minimum concentration of detergent is required, but this minimum is below the cmc of the detergent in the absence of protein.

(13) VanEtten, R. L.; Sebastian, J. F.; Clowes, G. A.; Bender, M. L. *J. Am. Chem. Soc.* **1967**, *89*, 3242.

(14) Fendler, J. H. *Membrane-Mimetic Chemistry*; John Wiley & Sons, Inc.: New York, 1982.

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Table 2. Results for Gdm-Denatured Citrate Synthase[a]

additives	rel init rate	additives	rel init rate
	Native Protein		
none	1.00	CTAB; β-CD	0.01 ± 0.01
β-CD	0.92 ± 0.09	POE(10)L	1.08 ± 0.15
CTAB	0.00 ± 0.01	POE(10)L; β-CD	1.06 ± 0.09
	After Unfolding and Dilution		
none	0.02 ± 0.01	CTAB; β-CD	0.13 ± 0.03
β-CD	0.01 ± 0.01	POE(10)L	0.01 ± 0.01
CTAB	0.01 ± 0.01	POE(10)L; β-CD	0.50 ± 0.06

[a] Protocol: CS (Boehringer), 24 μM, was denaturated for 1 h in 6 M guanidinium chloride containing 35 mM dithiothreitol. This solution was then diluted to give a solution containing 0.48 μM CS, 119 mM GdmCl, 0.7 mM DTT, 143 mM Tris-HCl, pH 8.0, 0.71 mM EDTA, and, when indicated, 0.57 mM detergent (70 μL aliquots). After 1 h, these aliquots were further diluted with 30 μL of 5.3 mM aqueous β-CD or 30 μL of water, to give final concentrations of 0.34 μM CS, 84 mM GdmCl, 0.49 mM DTT, 100 mM Tris-HCl, 0.5 mM EDTA, and, when indicated, 0.4 mM detergent and 1.6 mM β-CD. These solutions were allowed to stand overnight before being assayed as dscribed in ref 17.

addition of β-CD, led to 50% recovery of activity;[10] α-CD led to 29% recovery[16] (not shown). The reactivated protein could be purified by the two-step protocol described for CAB above, and the resulting material was identical to non-denatured CS as judged by circular dichroism, intrinsic fluorescence, and specific activity.[11] CTAB, which was optimal for CAB refolding, was less effective for CS refolding. CTAB inactivated native CS. In contrast, POE(10)L exerted no deleterious effect on native CS.[12]

The nonionic detergent Triton X-100 was effective in CS refolding (not shown), and the difference between β- and α-CD as second additive (19% reactivation vs background activity level, ca. 1%) provides evidence for the role of the CD. The nonpolar portion of Triton X-100 is a bulky *p-tert*-octylphenyl group. Related groups (e.g., *p-tert*-butylphenol) are bound much more strongly by β-CD than by α-CD, because the central cavity of α-CD is too small for the bulky guest.[18] Therefore, the observation that only β-CD enhances reactivation from the CS–Triton X-100 complex supports the hypothesis that the CD promotes refolding by stripping the detergent away from the polypeptide.

(15) (a) Buchner, J.; Schmidt, J.; Fuchs, M.; Jaenicke, R.; Rudolph, R.; Schmid, F. X.; Kiefhaber, T. *Biochemistry* **1991**, *30*, 1586. (b) Zhi, W.; Landry, S. J.; Gierasch, L. M.; Srere, P. A. *Protein Sci.* **1992**, *1*, 522.

(16) The similarity of α- and β-CD for promoting refolding from the CS-POE(10)L complex is consistent with reports that these two CDs have similar affinities for detergents with linear alkyl chains: (a) Liveri, V. T.; Cavallaro, G.; Giammona, G.; Pitarresi, G.; Puglisi, T.; Ventura, C. *Thermochim. Acta* **1992**, *199*, 125. (b) Palepu, R.; Reinsborough, V. C. *Can. J. Chem.* **1988**, *66*, 325.

(17) Srere, P. A.; Brazil, H.; Gonen, L. *Acta Chem. Scand.* **1963**, *17*, S129.

(18) (a) Matsui, Y.; Nishioka, T.; Fujita, T. *Top. Cur. Chem.* **1985**, *128*, 61. (b) Eftink, M. R.; Andy, M. L.; Bystrom, K.; Perlmutter, H. D.; Kriston, D. S. *J. Am. Chem. Soc.* **1989**, *111*, 6765.

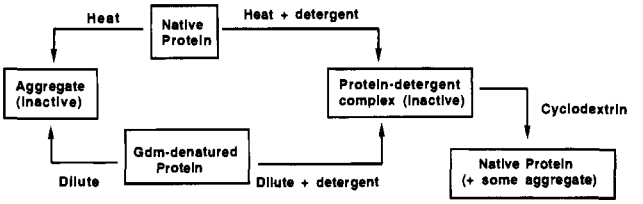

Figure 1. Schematic view of artificial chaperone-assisted protein refolding.

The analogy we have drawn between the GroE-assisted refolding and detergent/CD-assisted refolding is strengthened by the observation that the optimal detergents do not adversely affect native CAB or CS. Thus, there appears to be a substantial kinetic barrier at room temperature between native CAB in the presence of CTAB and the inactive CAB–CTAB complex, and between native CS in the presence of POE(10)L and the inactive CS–POE(10)L complex. An analogous kinetic barrier exists between native substrate proteins and their GroEL complexes.[5] The situation with CAB and CS and the relevant detergents is noteworthy because we are unaware of prior examples in which a native protein is kinetically protected from spontaneous inactivation by a low molecular weight denaturant.[19]

We have demonstrated a new strategy for protein renaturation that utilizes a pair of low molecular weight folding assistants, an "amphiphile" and a "stripping agent." Results with two structurally diverse proteins (CAB is a monomer with considerable β-sheet,[20] while CS is a dimer with almost exclusively α-helical secondary structure[21]) suggest that this strategy may be broadly applicable, with the optimal amphiphile and/or stripping agent varying from case to case.

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Supplementary Material Available: Far-UV and near-UV circular dichroism and intrinsic fluorescence comparisons between native CS and purified refolded CS, and between native CAB and purified, refolded CAB (3 pages). This material is contained in many libraries on microfiche, immediately follows this articles in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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