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Cycloamylose as an efficient artificial chaperone for protein refolding

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Abstract High molecular weight cyclic α-1,4-glucan (referred to as cycloamylose) exhibited an artificial chaperone property toward three enzymes in different categories. The inclusion properties of cycloamylose effectively accommodated detergents, which keep the chemically denatured enzymes from aggregation, and promoted proper protein folding. Chemically denatured citrate synthase was refolded and completely recovered it's enzymatic activity after dilution with polyoxyethylenesorbitan buffer followed by cycloamylose treatment. The refolding was completed within 2 h, and the activity of the refolded citrate synthase was quite stable. Cycloamylose also promoted the refolding of denatured carbonic anhydrase B and denatured lysozyme of a reduced form.

Key words: Cycloamylose; Refolding; Artificial chaperone; Citrate synthase; Carbonic anhydrase B; Lysozyme

1. Introduction

The process by which molecules achieve their native conformations, i.e. protein folding, is a subject of fundamental and practical importance, yet it remains one of the key unresolved issues in biochemistry [1]. Practical interest in the protein folding problem stems from the fact that proteins overproduced by genetically engineered cells are often obtained in non-native forms (e.g. inclusion bodies) [2] and the use of such proteins for basic research or biotechnological applications requires that the native conformation be achieved.

To improve the recovery of active proteins, the inclusion bodies are unfolded in denaturants such as urea or guanidine hydrochloride (GdmCl). The denatured protein is then refolded by removal of the denaturant, which is commonly performed by dialysis or dilution. This procedure is time consuming and, during refolding, many proteins tend to aggregate, which causes a significant reduction in the yield of active protein [3]. Though much effort has been expended to overcome this problem, no universal method has been established. One common approach, known as the 'dilution additive strategy', has been to include low molecular weight folding assistants in the buffer used to dilute the chemically denatured protein. A number of in vitro aggregation inhibitors or folding aids such as polyethylene glycol [4], polyamino acids [5], cyclodextrins [6] and detergents [7] have been reported to prevent aggregation and enhance protein folding.

The artificial chaperone technique [8] is mechanically distinct from other methods that employ small molecules to promote protein folding. This procedure inspired the two-step mechanism of GroEL/ES chaperone proteins [9,10]. GroE is the most prominent member of the molecular chaperone family. GroEL forms two heptamers and interacts with one heptamer of GroES. GroEL binds to the non-native state protein, thus preventing aggregation. The protein refolding is triggered by the addition of GroES, ATP, Mg^{2+} and K^{+}. The artificial chaperone technique introduced the sequential use of two low molecular weight agents to reconstitute the mimetic GroE system in vitro. In the first step, the aggregation of proteins is prevented by the addition of detergent molecules, which presumably shields the hydrophobic regions of the non-native protein. In the second step, cyclodextrin strips the detergent from the protein–detergent complex, allowing proper folding [11–13].

Cyclodextrins are cyclic α-1,4-glucans with degrees of polymerization of 6, 7 and 8; they are generally called α, β and γ-cyclodextrin, respectively. They have a hydrophobic central cavity and can accommodate various guest molecules to form an inclusion complex [14]. Besides these conventional cyclodextrins, larger homologs with a degree of polymerization from 17 to several hundred have recently become available [15]. Such high molecular weight cyclodextrins, referred to as cycloamylose in this paper, seemed to assume a single helical V-amylose conformation, with an anhydrophilic channel-like cavity [16]. As expected from their structure, cycloamyloses can form inclusion complexes with inorganic [17] and organic molecules [18]. Based on the available information, we expect cycloamylose to work as an effective artificial chaperone.

We describe here the chaperone-like activity of cycloamylose on protein refolding and present the best combination of detergent and cycloamylose for refolding three types of proteins (citrate synthase (CitSyn), carbonic anhydrase B (CAB) and lysozyme), which have no structural homology among them.

2. Materials and methods

2.1. Materials

Porcine heart CitSyn was purchased from Roche Molecular Biochemicals (Switzerland); CAB and lysozyme were from Sigma (St. Louis, MO, USA). The detergents, acetyl-CoA, 5,5′-dithiobis (nitrobenzoic acid), oxaloacetate, ρ-nitrophenyl acetate, DL-cystine and *Micrococcus lysodeikticus* were also purchased from Sigma. GroEL and

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GroES were purchased from Takara (Japan). All other reagents were of biochemical grade. The cycloamylose mixtures, CA(S) and CA(L), were provided by Ezaki Glico Co. (Osaka, Japan). CA(L) and CA(S) consist of cycloamylose with degrees of polymerization from 22 to 45, and over 50, respectively, and were produced as described previously [19].

2.2. Assay of CitSyn activity

Native or refolded CitSyn was added to a reaction mixture containing 150 mM Tris–HCl (pH 7.6), 0.022 μM acetyl-CoA, 0.48 mM oxalacetate and 0.11 mM 5,5′-dithiobis nitrobenzoic acid. The final concentration of CitSyn in the reaction mixture was 0.024 mg/ml. The increase in absorbance at 412 nm was measured every 0.5 s for 60 s using a spectrophotometer (Hitachi, Japan) at 25°C. The initial rate of change in absorbance was always linear. The yield of refolded CitSyn was determined by comparing the resulting initial rate with that of the native enzyme.

2.3. Assay of CAB activity

Forty-five μl of ρNPAC/23 mM Tris–sulfate (pH 7.8) was added to 450 μl of native CAB or refolded CAB solution. The increase in absorbance at 400 nm was measured. The refolding yield was determined as for CitSyn.

2.4. Assay of lysozyme activity

Twenty μl of native or refolded lysozyme solution was added to 480 μl of suspension of 0.16 mg/ml *M. lysodeikticus*/50 mM phosphate buffer (pH 6.2). The decrease in absorbance at 450 nm was measured. The refolding yield was determined as for CitSyn.

2.5. Denaturation and refolding

Unfolded CitSyn was prepared according to the procedure of Dougherty et al. [13]. The native enzyme was dissolved in 6 M GdmCl/145 mM Tris–HCl (pH 7.6) and 40 mM DTT at 2.4 mg/ml (final concentration). After 1 h denaturation of CitSyn at room temperature, freshly prepared detergent buffer (containing 0.1% detergent, 145 mM Tris–HCl, pH 7.6) was added to the denatured solution in order to dilute the denaturant (CitSyn concentration became 0.034 mg/ml) and the resulting mixture stood at room temperature for another 1 h. Next, 0.2 ml of stock solution of cycloamylose was added to the 0.8 ml of CitSyn/detergent complex solution. Small portions were withdrawn at different time intervals, the solution was centrifuged and the resultant supernatant was used as refolded CitSyn and assayed for enzymatic activity.

To check the refolding ability of natural chaperone GroE in this in vitro system, denatured CitSyn was diluted 100-fold in 0.1 M Tris (pH 8.0), 2 mM ATP, 10 mM $MgCl_2$ and 10 mM KCl buffer, containing 2 μM (final concentration) of GroEL, GroES or GroE complex.

CAB was denatured in 6 M GdmCl/23 mM Tris–sulfate (pH 7.8) for 16 h at 30 mg/ml (final concentration). After incubation at room temperature, 700 vol. of freshly prepared detergent buffer (containing 0.1% detergent, 23 mM Tris–sulfate, pH 7.8) was added, and the mixture was then incubated for another 1 h. The remaining steps were the same as those followed in the case of CitSyn.

Lysozyme was reduced and denatured in 6 M GdmCl and 5 mM DTT/23 mM Tris–acetate (pH 8.1) at 15 mg/ml (final concentration). After 16 h incubation at room temperature, 100 vol. of freshly prepared detergent buffer (0.1% detergent, 2 mM DL-cystine/23 mM Tris–acetate, pH 8.1) was added, and the mixture was then incubated for another 1 h. The remaining steps were the same as those followed in the case of CitSyn.

2.6. Protein concentration and light scattering

Protein concentrations were determined spectrophotometrically at 280 nm, with the extinction coefficients of 1.75 (mg of protein) ml/cm for CitSyn [20], 1.83 for CAB [21] and 2.37 for lysozyme [22]. Light scattering was measured using a fluorescence spectrophotometer (Jasco, Japan). Irradiation and detection wavelengths were 350 nm.

3. Results and discussion

3.1. Model proteins

Three proteins (CitSyn, CAB, lysozyme), that possess no structural homology to each other were selected as model proteins in this study. CitSyn is a dimeric protein composed of two identical subunits each with a molecular mass of 49 kDa and consists of a predominantly α-helical secondary structure [23]. CAB (30 kDa) is a metalloenzyme containing one zinc in its active center and constructed mostly with β-sheets [24], and lysozyme (15 kDa) has two β-sheets and requires four disulfide linkages to achieve its native state [25]. GdmCl-denatured enzymes completely lost their activity, and negligible refolding was observed following dilution with detergent buffer or cycloamylose. Control experiments also indicated that, at the concentration used for folding, most detergents and cycloamylose exert little or no effect on the activity of native proteins.

Table 1
Refolding of chemically denatured CitSyn

	CA(S) (%)	CA(L) (%)	α-Cyclodextrin (%)	β-Cyclodextrin (%)	γ-Cyclodextrin (%)
Non-ionic detergent:					
CnEχ					
C12E18	0	23.3	11.6	18.7	0
C12E8	0	18.7	8.8	8.9	0
C12E10	0	26.4	27.8	46.7	0
Brij 35	0	38.7	0	47.6	26.6
Lubro; PX	13.7	0	38.5	54.8	0
CnφEχ					
Triton X-100	5.9	3.8	0	56.7	37.6
Cn sorbitan Eχ					
Tween 20	76.5	54.8	28.7	57.6	26.4
Tween 40	100	100	47.6	100	7.5
Tween 60	100	100	64.3	100	6.5
Tween 80	92.6	93.7	54.3	87.6	0
Tween 81	19.2	12.8	13.2	16.5	13.2
Ionic detergent:					
CTAB	10.2	9.8	28.7	20.7	5.1
SB 3-14	0	8.9	19.8	37.6	0

GdmCl-denatured CitSyn was diluted with buffer containing detergent as described in the left column and incubated for 1 h at room temperature. In the next step, the molecules listed at the top of each column were added. The resulting solutions were allowed to stand overnight before assay. The yield of refolded CitSyn was determined by comparing the initial rate with that of native CitSyn (100%). Over 30 kinds of detergent were examined; those detergents that showed over 10% refolding activity with the combination of cycloamylose or cyclodextrin are shown.

3.2. Effect of detergent on denatured CitSyn

The dilution of denatured CitSyn by buffer without any additives resulted in increased light scattering, as observed with a fluorescence spectrophotometer, irradiating and observing at 350 nm, which indicated that the protein had aggregated [26]. After centrifugation of the diluted mixture, the decreased protein concentration was detected. It is necessary to prevent CitSyn from aggregation in order to allow the proper folding. The dilution additive method was the common method used to prevent this aggregation [4–7], but it took much time and the yield of the recovered activity was quite low. When the artificial chaperone method is used, aggregation is prevented by the formation of a protein–detergent complex.

In this study we examined the ability of several kinds of detergents in combination with the second agent, which removes the detergent from the complex, to keep denatured CitSyn from aggregation. Light scattering studies indicated that some of the protein aggregate and a loss of protein occurred even when the denatured CitSyn was diluted with detergent buffer. The extent of this loss depends greatly on the detergent used. Non-ionic detergents are optimal for CitSyn refolding (Table 1). A series of polyoxyethylenesorbitans (under the brand name: Tween) was most effective for the refolding of CitSyn. Tween 40 and 60 allowed almost 100% recovery of denatured CitSyn followed by stripping by cycloamylose, as described in Section 3.3. The addition of Triton X-100 and Tween to native CitSyn results in a 10–15% enhancement of activity.

3.3. Effect of large cyclic α-1,4-glucan on CitSyn–detergent complex

We examined the ability of cycloamylose as a stripping agent to mediate the extraction of detergent from the protein–detergent complex. Three types of cyclodextrin were also examined as a comparative study. The characteristics of these three cyclodextrins in making an inclusion complex were well studied. The degree of polymerization of CA(S) ranged from 22 to 45 and that of CA(L) was over 50. Both sizes of cycloamylose effectively worked (Table 1). CitSyn is known to be difficult to refold, and the attempt to achieve refolding of CitSyn by the dilution additive method was unsuccessful [27]. Nearly 65% of enzymatic recovery of CitSyn was obtained by the artificial chaperone method using Triton X-100 and β-cyclodextrin [13]. It was noteworthy that CitSyn refolded and recovered nearly 100% of its activity when diluted with Tween 40 or 60 followed by the addition of cycloamylose (Table 1). These results indicated that the combination of Tween 40 or 60 and cycloamylose makes the perfect artificial chaperone for refolding denatured CitSyn. The effective concentration of detergent was found to be between 0.1 and 0.01%, and the optimum concentration of cycloamylose was 0.4–0.8% (w/v, final concentration; data not shown). Cycloamylose stock solution was prepared as five times the concentrated one, and cycloamylose was easily dissolved in water at this concentration. Moreover, the cycloamylose solution was quite stable and much more resistant against aging than is cyclodextrin [28]. These excellent characteristics of cycloamylose give it the advantage as an artificial chaperone.

The effects of the natural molecular chaperone GroE on refolding of denatured CitSyn in vitro were also examined. No refolding was detected only on addition of GroEL and GroES. Although the addition of Mg^{2+} and ATP strongly increased the refolding yield, the yield remained at a low level (26%) compared with the combination of Tween 40 (or 60)-cycloamylose. Moreover the addition of GroE caused another problem. It was necessary to purify the target protein from the mixture of GroE, and this step is time consuming.

A remarkable feature of cycloamylose as an artificial chaperone was that it allowed prompt refolding. Fig. 1 shows the marked difference in the time course of refolding between cycloamylose and cyclodextrin. Denatured CitSyn was refolded within 2 h, and in the case of CA(L), over 50% of activity was recovered within 30 min. No such quick recovery has been achieved with other artificial chaperones. Although β-cyclodextrin was also effective in refolding CitSyn in combination with Tween 40 (or 60), complete refolding required overnight incubation.

Soon after the addition of cycloamylose to the detergent–CitSyn complex solution, cycloamylose may start to selectively extract detergent from the complex and accelerate the proper refolding of CitSyn. The addition of cycloamylose stock solution to the CitSyn–detergent complex solution produced a white sediment, and the sediment increased with time. This was not observed when adding cyclodextrins. CA(L) resulted in more sediment than did CA(S). This observation agreed with the observation that the cycloamylose formed an insoluble inclusion complex [18], and the solubility of the inclusion complex of CA(S) was much higher than that of CA(L).

The interaction of cycloamylose with detergents strongly depends on their hydrophobic moiety. Apparently, the size and charge of the detergent head groups contribute less to the detergent–cycloamylose interaction, since a large variety of head-groups is well tolerated. The structure of Tween, which contains sorbitan, is identical to that of other non-ionic detergents. The non-polar chain of the detergent has an important effect on its role. Tween 40 and 60 work very well, while Tween 20 does not work as well (Table 1), and these differences may come from the structure of the non-polar

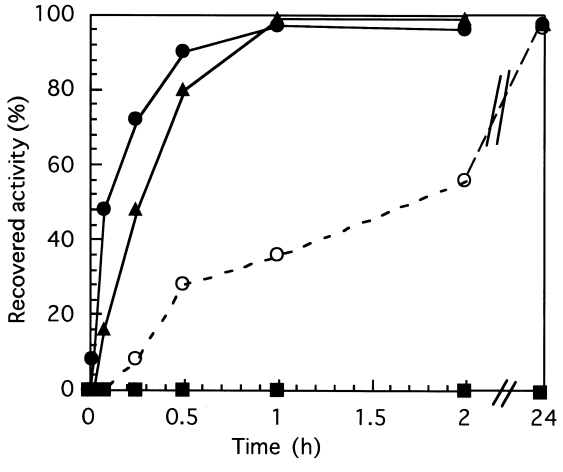

Fig. 1. Time-dependent recovery of CitSyn activity in cycloamylose-assisted refolding. Chemically denatured CitSyn was diluted with Tween 60 buffer and incubated for 1 h at room temperature. In the next step 0.6% (final concentration) of ▲: CA(S); ●: CA(L); ○: β-cyclodextrin; ■: no additive was added, and a small portion was withdrawn at the time indicated and assayed for enzymatic activity. All values are a percentage compared to the initial catalytic rate of native CitSyn.

Table 2
Refolding of chemically denatured CAB

	CA(S) (%)	CA(L) (%)	α-Cyclodextrin (%)	β-Cyclodextrin (%)	γ-Cyclodextrin (%)
Ionic detergent:					
CTAB	50.2	92.7	8.7	54.3	60.1
SB 3-14	42.1	82.1	23.4	56.1	41.2
SB 12	31.3	10.3	8.2	28.3	10.5
Myristyltrimethyl ammonium bromide	36.3	6.3	5.6	47.8	5.1
Hexadecyldimethylethyl ammonium bromide	42.5	11.3	0	50.1	0
Hexadecylpyridinium chloride monohydrate	33.8	7.5	0	32.5	0
Tetradecyl trimethyl ammonium bromide	43.2	11.2	0	53.8	0
Deoxycholate	16.3	12.5	21.3	18.8	25.0
Tauro cholate	23.4	18.8	0	25.0	0
CHAPS	30.0	25.3	20.0	33.8	25.0
CHAPSO	25.0	26.3	16.3	27.5	22.5
Deoxy BigCHAPS	27.5	27.5	22.5	28.8	31.3
Non-ionic detergent:					
CnEχ					
Brij 30	25.0	25.0	0	30.0	0
Brij 58	27.5	26.3	20.0	48.2	26.3
CnφEχ					
Triton X-100	23.8	25.0	21.3	31.3	32.5
NP 40	26.3	22.5	8.3	33.8	22.5
Cn sorbitan Eχ					
Tween 40	37.0	30.0	10.9	41.1	7.3
Tween 60	6.2	12.7	0	6.2	8.7

The refolding yield was determined as described in Table 1.

chain. An analysis of fatty acid composition reveals that 90% of the non-polar chain of Tween 40 is palmitic acid, and that of Tween 60 is a mixture of stearic acid and palmitic acid. The non-polar chain of Tween 20 is composed of monolaurate. The non-polar chain length had an important effect on interaction with cycloamylose. Cycloamylose easily formed a complex with long chain fatty acid [18], and the efficiency of an agent's stripping ability has a critical effect on the refolding yield. The structure of Tween may be most suitable for making a weak CitSyn–detergent complex that keeps CitSyn from aggregation, and the effective interaction with cycloamylose accelerates the proper folding.

3.4. Refolding of CAB and lysozyme

In contrast to the results obtained for CitSyn, no evidence of a significant recovery of denatured CAB was found by using non-ionic detergents. CAB preferred ionic detergent as the first agent (Table 2). Cetyltrimethylammonium bromide (CTAB) and *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB 3-14) were the most effective detergents. Fortunately, cycloamylose also worked well in stripping these ionic detergents and promoting refolding of CAB at a high yield.

Similar results were obtained with lysozyme. Reduced lysozyme, whose disulfide bond was completely disrupted, was impossible to refold without the recovery of the four disulfide bond in the correct form [29]. The dilution with SB 3-14 buffer containing 2 mM DL-cystine followed by the extraction of detergent by cycloamylose allowed a complete recovery of lysozyme activity (Table 3). These data demonstrate that reduced lysozyme could make a disulfide bond under this refolding condition. The combination of glutathione (GSH) and oxidized glutathione (GSSH) instead of DL-cystine was also examined. When GSH and GSSH were used at the ratio of 5:1, 80% activity compared to that of DL-cystine was achieved (date not shown). In both the case of CAB and lysozyme, the denatured protein refolding was also completed within 2 h. The dramatic effects of cycloamylose on CAB and lysozyme refolding, together with the remarkable effect on refolding of CitSyn, suggested that cycloamylose can be used as a general purpose artificial chaperone.

Triton X-100 is one of the most commonly used non-polar detergents. The non-polar portion of Triton X-100 is a bulky ρ-*tert*-octylphenyl group. A related group (e.g. ρ-*tert*-butylphenol) is bound much more strongly by β-cyclodextrin than by α-cyclodextrin, because the central cavity of α-cyclodextrin is too small for the bulky guest [30]. Rozema et al. suggested that this indicated that only β-cyclodextrin effec-

Table 3
Refolding of chemically denatured reduced lysozyme

	CA(S) (%)	CA(L) (%)	β-Cyclodextrin (%)
Ionic detergent:			
CTAB	68.9	91.8	68.9
SB 3-14	80.3	85.2	2.3
SB 12	34.8	42.4	40.1
Myristyltrimethyl ammonium bromide	68.7	66.7	68.7
Hexadecyldimethylethyl ammonium bromide	58.0	77.2	47.7
Hexadecylpyridinium chloride monohydrate	49.9	39.2	48.1
Dodesyltrimethyl ammonium bromide	35.4	14.6	10.4

Reduced and GdmCl-denatured lysozyme was diluted with buffer containing DL-cystine and the detergent listed in the left column, and the resulting mixture was incubated for 1 h at room temperature. The refolding yield was determined as described in Table 1.

tively promotes refolding by stripping Triton X-100 away from the protein–Triton X-100 complex [8], and that β-cyclodextrin was much more effective than α-cyclodextrin as the second additive. The limitation of the guest size on cycloamylose may not be so strict [16,28]. The interaction of detergent with cycloamylose in aqueous solution is not fully understood, but this can be speculated from the crystal structure of a cycloamylose containing 26 glucose residues (cyclomaltohexaicosaose, CA26) [16]. CA26 is comprised of two single helices of 12 glucoses each, in anti-parallel arrangement. The helices in CA26 have six glucoses per turn, thus contain central cavities with a similar diameter to that of α-cyclodextrin. However, the size of the helical channel might be different in cycloamylose having different glucose units, and expected to be flexible under aqueous condition. So the helices in cycloamylose might fit well to accommodate many kinds of detergent as guest molecules. The higher solubility of cycloamylose in water than that of conventional cyclodextrins is also favorable. These observations indicate that cycloamylose is expected to be a better detergent stripping agent in artificial chaperone technology.

Recently Sundari et al. suggested the chaperone-like property of dextrin-10, α-1,4-D-glucopyranoside chains, which possess amphiphilic surfaces [31]. The ability of dextrin-10 to accelerate the folding was not so high as that of cycloamylose, but it is also a useful molecule to expand the use of artificial chaperones.

3.5. Conclusion

Cycloamylose has the ability to extract many kinds of detergents from a detergent–protein complex. The interaction of a detergent with an enzyme is highly dependent on each protein. Once a weak interaction between a protein and detergent was achieved, cycloamylose worked well to strip the detergent from the protein–detergent complex and then promote the protein refolding. The application of cycloamylose as an artificial chaperone will be an effective tool in overcoming the folding problem of over-expression of target proteins. The ability of this procedure to recover CitSyn activity after denaturation suggests that this technique may be widely used to refold oligomeric as well as monomeric multi-domain proteins.

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