Protein refolding assisted by self-assembled nanogels as novel artificial molecular chaperone

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Received 18 July 2003; revised 26 August 2003; accepted 9 September 2003

First published online 19 September 2003

Edited by Felix Wieland

Abstract Molecular chaperone-like activity for protein refolding was investigated using nanogels of self-assembly of cholesterol-bearing pullulan. Nanogels effectively prevented protein aggregation (i.e. carbonic anhydrase and citrate synthase) during protein refolding from GdmCl denaturation. Enzyme activity recovered in high yields upon dissociation of the gel structure in which the proteins were trapped, by the addition of cyclodextrins. The nanogels assisted protein refolding in a manner similar to the mechanism of molecular chaperones, namely by catching and releasing proteins. The nanogels acted as a host for the trapping of refolded intermediate proteins. Cyclodextrin is an effector molecule that controls the binding ability of these host nanogels to proteins. The present nanogel system was also effective at the renaturation of inclusion body of a recombinant protein of the serine protease family.

Key words: Artificial chaperone; Protein refolding; Inclusion body; Hydrophobized polysaccharide; Cyclodextrin; Nanogel

1. Introduction

In the field of biotechnology, there is currently much interest in producing large amounts of biologically active recombinant proteins in heterologous systems. However, in many cases, problems such as the formation of insoluble inclusion bodies are encountered. Such aggregates are usually separated from the host cell and solubilized with a denaturant (urea or GdmCl) in vitro, and refolding is attempted by removing the denaturant. One strategy for successful refolding is to prevent such non-specific aggregation by using reagents that either inhibit aggregation or aid in refolding. Sugars [1], proline [2], cyclodextrins [3], micelles [4], reversible micelles [5], and liposomes [6] have been reported to prevent the irreversible aggregation of partially refolded proteins, and they are also known to assist in protein refolding. Two-step systems employing surfactants and cyclodextrins [7,8] or surfactants and cycloamylose [9] are referred to as 'artificial chaperone' techniques, and are more effective than typical small molecular systems. However, with such systems, it can be difficult to completely remove the surfactants after treatment. Water-soluble polymers such as polyethylene oxide [10], functional nanosphere [11], and size exclusion chromatography matrices [12] also increase the recovery yield of native proteins during refolding. These polymers appear to act as a hydrophobic buffer by blocking the exposed hydrophobic surface on the denatured protein just enough to prevent aggregation. Excessively strong binding to the intermediate would prevent folding to the native conformation. Thus, the delicate balance required between the various folding processes leads to the low efficiency and low generality of these systems. In the refolding process in living systems, molecular chaperones selectively trap heat-denatured proteins or their intermediates in order to prevent irreversible aggregation due to macromolecular host (molecular chaperone)–guest (protein) interactions [13,14]. Then, with the aid of ATP and another co-chaperone, the host chaperone releases the protein in its refolded form.

To simulate the function of molecular chaperones, we designed amphiphilic self-assembled nanogels that behave as a host for a guest protein. Hydrophobized polysaccharides such as cholesterol group-bearing pullulan (CHP) spontaneously form hydrogel nanoparticles (nanogels) in water by intramacromolecular self-association [15–17]. These nanogels form complexes with various soluble proteins or enzymes in water [18,19]. We developed a novel artificial chaperone system using CHP nanogels (nanogel method) [20]. The nanogels were able to form stronger complexes with heat-denatured proteins than were native proteins such as molecular chaperones. The complexed proteins were effectively released in their refolded native form upon dissociation of the nanogels and in the presence of cyclodextrin [15]. Therefore, the nanogels were revealed to possess heat shock protein-like activity that was similar to the mechanism of molecular chaperones, i.e. they were able to catch and release proteins. In this paper, we report the refolding of GdmCl-denatured model proteins such as carbonic anhydrase and citrate synthase by using the nanogel system. In addition, the refolding of inclusion

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01028-7

bodies overproduced in *Escherichia coli* was also investigated using this artificial molecular chaperone system.

2. Materials and methods

2.1. Materials

Bovine carbonic anhydrase (CAB) and *p*-nitrophenyl acetate (*p*NPA) were purchased from Sigma. Porcine heart citrate synthase (CS) was purchased from Roche. Dithiothreitol (DTT), ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt (EDTA·2Na), acetyl-CoA trilithium salt, oxaloacetic acid, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrin (γ-CD) were purchased from Wako Pure Chemicals Co. 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) was purchased from Mitsubishi Chemical. Methyl-β-cyclodextrin (methyl-β-CD) was purchased from Tokyo Kasei Kogyo. CHP, which was substituted by 1.2 cholesteryl moieties per 100 glucose units of pullulan (MW 108 000), was synthesized as previously reported [15].

2.2. Denaturation and refolding of CAB

Native CAB was dissolved in 6 M GdmCl, 50 mM Tris-sulfate buffer (pH 7.5) at 30 mg/ml (final concentration). After denaturation for 16 h at 25°C, the mixture was diluted rapidly 1000-fold by the 50 mM Tris-sulfate solution, which contained 5.0 mg/ml of CHP nanogels; the mixture was then left undisturbed for at least 2 h at 37°C. The final concentration of CAB was 0.03 mg/ml in 6 mM GdmCl, 50 mM Tris-sulfate (pH 7.5). The protein concentration of native CAB was determined by absorbance at 280 nm with a coefficient of 1.83 mg protein/ml/cm. CHP nanogel solutions were prepared by sonication methods reported elsewhere [15,17]. Finally, cyclodextrin was added as a powder to 1.0 ml of CHP nanogels and CAB complexed solution, and the resulting solution was then mixed gently. This solution was maintained for at least 24 h at 37°C. The enzyme activity of CAB was determined by the *p*NPA hydrolysis assay [21]. Ten μl of 100 mM *p*NPA solution was added to 1.0 ml of the final concentration of the CAB solution (0.03 mg/ml). The increase in absorbance at 400 nm was measured as a function of time using a Hitachi U-2010 (Japan). The refolding yield of CAB was determined by comparing the initial rate of enzyme activity with the initial rate of native enzyme activity.

2.3. Denaturation and refolding of CS

Native CS was dissolved in 6 M GdmCl, 150 mM Tris–HCl buffer (pH 7.6), 0.75 mM EDTA, and 40 mM DTT at 2.4 mg/ml (final concentration). After denaturation for 1 h at 25°C, the mixture was diluted rapidly 70-fold with 150 mM Tris–HCl solution containing CHP nanogels (2.4 mg/ml); the mixture was then allowed to stand for 1 h at 25°C. The final concentration of CS was 0.034 mg/ml in 150 mM Tris–HCl buffer (pH 7.6) containing 86 mM GdmCl, 0.57 mM DTT, and 0.75 mM EDTA. Finally, the mixture was diluted 1.4-fold by various concentrations of cyclodextrin solutions; the resulting mixtures were then allowed to stand for 6 h at 25°C. The final concentration of CS was 0.024 mg/ml in 150 mM Tris–HCl buffer (pH 7.6) containing 60 mM GdmCl, 0.40 mM DTT and 0.75 mM EDTA. The enzyme assay of the CS solution was performed using an acetyl-CoA assay [22]. The protein concentration of CS was determined by absorbance at 280 nm with a coefficient of 1.75 mg protein/ml/cm. The refolded CS solution (15 μl) was added to 0.76 ml of substrate solution containing 0.023 mM acetyl-CoA, 0.5 mM oxaloacetic acid, and 0.12 mM DTNB in 150 mM Tris–HCl (pH 7.6) buffer. The increase in absorbance at 412 nm was measured as a function of time. The refolding yield of CS was determined by comparing the initial rate of enzyme activity with the initial rate of native enzyme activity.

2.4. HPSEC analysis of nanogels and nanogel–CAB complexes

Denatured CAB was diluted 1000-fold by 5.0 mg/ml CHP nanogels and was left to stand for 2 h at 37°C. 100 μl of this solution was applied to a HPSEC system at 30°C. The elution buffer was the same as the injection buffer. The flow rate was 0.75 ml/min. The HPSEC system used for this study was composed of a CCPS dual pump (Tosoh, Tokyo, Japan), a RI-8020 refractive index detector (Tosoh), a UV-8020 UV detector (Tosoh), a CO-8020 column oven (Tosoh), and a SD-8022 degasser (Tosoh). HPSEC was performed using a TSK-GEL G4000SWXL column (Tosoh).

2.5. Resolution and renaturation of inclusion body proteins

Approximately 0.5 mg of washed inclusion bodies (prosemin) was dissolved in 20 ml of 8 M urea, 0.2 M NaCl, and 20 mM Tris-sulfate (pH 8.0) solution and the mixture was left to stand for 1 h at room temperature to a final concentration of approximately 0.025 mg/ml prosemin, 8.0 M urea, 0.2 M NaCl, and 20 mM Tris-sulfate (pH 8.0). This solution was then sonicated for 5 min in a water bath at room temperature and was left to stand for several minutes. All insoluble materials in the denaturation solution were removed by centrifugation for 20 min at 3500×*g* and were collected as the supernatant solution. The supernatant was diluted 8.0-fold by 8.0 mg/ml CHP nanogel solution and the solution was left to stand for at least 1 h at room temperature (final concentration, 1 M urea, 7.0 mg/ml nanogel). All insoluble materials in the nanogel–protein complexed solution were removed by centrifugation for 20 min at 3500×*g*. The nanogel–protein complex supernatant solution was diluted 1.3-fold by 71.3 mM HP-β-CD solution to give a final concentration of 5.6 mg/ml CHP nanogels, 14.3 mM HP-β-CD, 0.2M NaCl, and 20 mM Tris-sulfate (pH 8.0). Two hours later, this solution was centrifuged at 3500×*g* for 20 min to remove the precipitate. After purification by a His tag purification system (Talon Purification Kit, Clontech), the refolding solution was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Western blotting) and by the enzymatic activity [23].

The refolded prosemin solution was applied to a 12.5% SDS–PAGE gel and electrophoresed. The separated proteins were transferred onto polyvinylidene difluoride membrane and incubated under microwave shaking at 25°C with anti-human prosemin rabbit antibody diluted 1000-fold with PBS-Tween 20. After a wash with the same buffer, the membrane was treated with alkaline phosphatase-labeled goat anti-rabbit IgG under the same condition. After washing, immunoreactivity was visualized with a NBT/BCIP mixture. Prosemin protease activities were measured by MCA-labeled synthetic peptide substrates, i.e. Boc-Q-A-R-MCA, Boc-F-S-R-MCA, Bz-R-MCA, Boc-V-L-K-MCA, Pyr-G-R-MCA, P-F-R-MCA, Boc-V-P-R-MCA, Z-R-R-MCA, R-MCA, or Z-F-R-MCA, in 20 mM Tris–HCl, pH 8.0, containing 0.2 M NaCl at 37°C after the treatment with enterokinase (150 kDa, Roche) and gel-filtrated for removal of enterokinase. The fluorescence was measured at 460 nm emission, excited at 380 nm using a Cytofluor plate reader.

3. Results and discussion

3.1. Complexation of refolding intermediates of unfolded CAB and CS with nanogels

The preparation and characterization of CHP nanogels have been reported elsewhere [17]. The size of the nanogels was approximately 20–30 nm, as determined by a static light scattering method, as previously reported. CAB (molecular

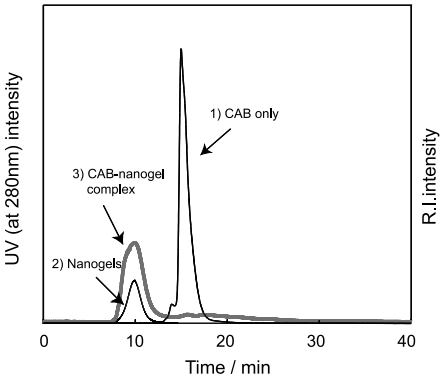

Fig. 1. Size exclusion chromatogram of CAB and the nanogel–CAB complexes. The sample conditions were (1) 0.03 mg/ml native CAB detected by UV, (2) 4.8 mg/ml CHP nanogels detected by RI, (3) 4.8 mg/ml CHP nanogels complexed with the chemically denatured CAB detected by UV. Elution rate was 0.75 ml/min at 30°C. The elution buffer was 50 mM Tris-sulfate buffer (pH 7.5).

weight 30 000) as a model protein is a monomeric metalloenzyme, and its secondary structure consists primarily of β-sheets [24]. CAB was unfolded and inactivated completely by 5 M GdmCl dissolved in 50 mM Tris-sulfate, pH 7.5. The refolding of CAB (30 mg/ml) was performed by rapid dilution in renaturation buffer consisting of 50 mM Tris-sulfate, pH 7.5 in the absence and presence of the nanogels. In the absence of the nanogels, CAB irreversibly aggregated and only 18% of the original enzymatic activity was recovered. The exposure of the hydrophobic surface of the refolding intermediate resulted in irreversible aggregation. On the other hand, in the presence of the nanogels, such aggregation was significantly prevented, although the enzymatic activity did not recover. That result was due to the complexation between the nanogels and the refolding intermediates of unfolded CAB. Complexation between CAB and the nanogels was confirmed by HPSEC. Fig. 1 indicates that the nanogels trapped CAB, and that CHP nanogel–CAB complexes were formed.

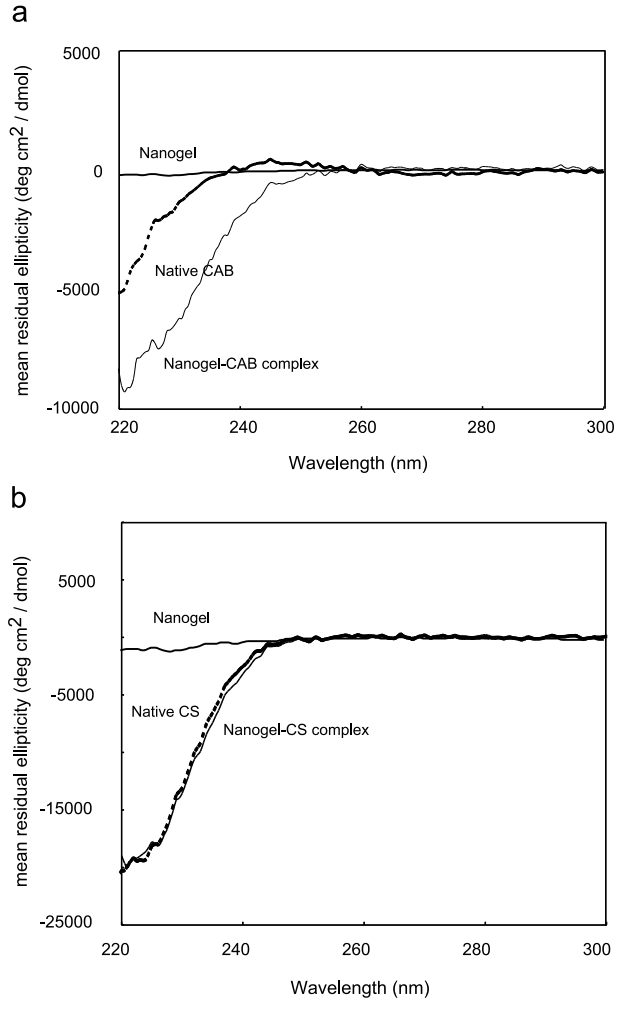

Fig. 2. UV c.d. spectra of CAB (a) and CS (b) complexed with the nanogels. a: Native CAB solution contained 0.03 mg/ml of CAB in 50 mM Tris-sulfate buffer (pH 7.5), and 6 mM GdmCl. The nanogel–CAB complex solution contained 4.8 mg/ml CHP nanogels, 0.03 mg/ml CAB in 50 mM Tris-sulfate buffer (pH 7.5) and 5 mM GdmCl. b: Native CS solution contained 0.02 mg/ml of CS in 150 mM Tris–HCl (pH 7.6), 0.75 mM EDTA, and 60 mM GdmCl. The nanogel–CS complex solution contained 4.8 mg/ml CHP nanogels and 0.02 mg/ml CS in 150 mM Tris-sulfate buffer (pH 7.6), 60 mM GdmCl, and 0.75 mM EDTA.

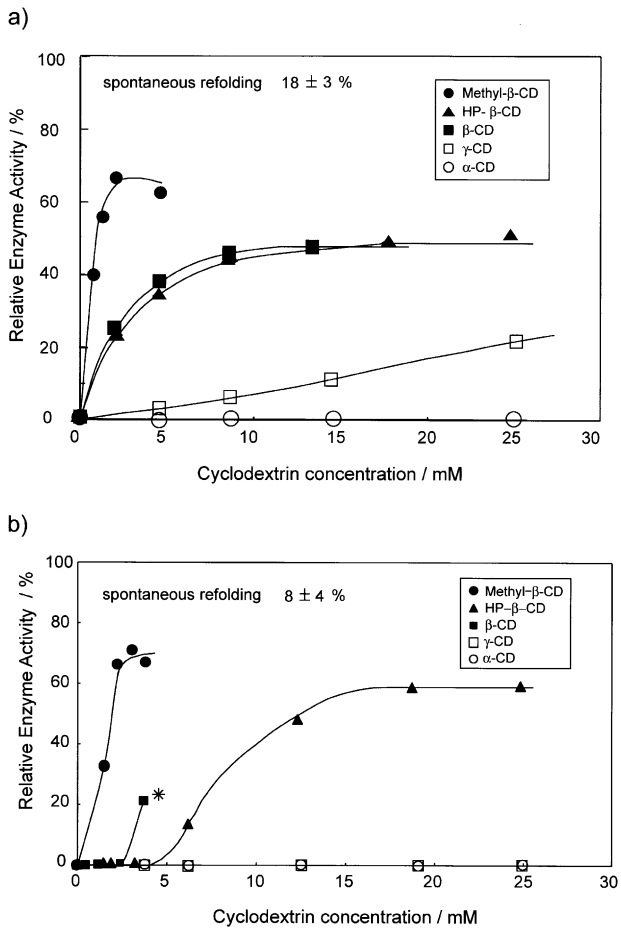

Fig. 3. Refolding of GdmCl-denatured CAB (a) and CS (b) with nanogel assistance as a function of cyclodextrin concentrations at the releasing step. Experimental conditions were as described in Section 2. *The experiment could not be carried out above the concentration in this condition due to the problem of the solubility of β-CD.

In the simple buffer dilution method, the only small peak of the folded CAB was observed by HPSEC (data not shown) because the precipitates and the aggregates of denatured CAB were removed by filtration (0.22 μm) before injection of the column. No spontaneous dissociation of CAB was observed, even after storage for a week at room temperature. The secondary structure of complexed CAB was investigated by circular dichroism (c.d.) spectroscopy. Native CAB is known to consist of 6.6% α-helices and 30.9% β-sheets [25]. After complexation, the α-helical structure appear to increase (Fig. 2a). Goto and coworkers previously reported that the α-helical intermediate of denatured proteins is a non-hierarchical intermediate of β-sheet proteins such as CAB [26]. The nanogels (4 μM) did not bind to native CAB (2 μM), even after incubation for 24 h at 25°C. Therefore, the nanogels selectively interacted with the CAB refolding intermediate.

CS (molecular weight 100 000 as a dimer) is a homodimeric enzyme and its secondary structure is primarily helical. There is no structural homology comparable to that of CAB. CS requires quaternary structure formation (homodimerization) for enzyme activity to occur [27]. In the absence of the nanogels, CS irreversibly formed aggregates, and only 8% of the original enzymatic activity was recovered by the GdmCl de-

naturation and dilution method. Such aggregation was also prevented in the presence of the nanogels, although the enzymatic activity was not recovered. The near UV c.d. spectrum of the CHP nanogel–CS complex did not differ greatly from that of the native form (Fig. 2b). Nanogels captured denatured CS possessing a secondary structure similar to that of native CS.

The CHP nanogels trapped refolding intermediates of both CAB and CS. As a result, protein aggregation was inhibited in both cases. This effect was similar to that observed with a molecular chaperone system (e.g. the GroEL system) in nature.

3.2. *Refolding of enzymes due to the addition of cyclodextrin*

Nanogels formed by the self-assembly of CHP macromolecules have been shown to dissociate upon the addition of cyclodextrins [28]. The cross-linking domains provided by the hydrophobic interactions of cholesteryl groups in the nanogels become disrupted by the encapsulation of a cholesteryl moiety into the cyclodextrin cavity. CHP nanogels have been shown to dissociate within 5 min [28]. The dissociation of nanogels might subsequently induce the release of proteins as well as protein refolding [20].

Fig. 3a shows the recovery of the enzymatic activity of CAB after the addition of various types and concentrations of cyclodextrins. The dissociation of nanogels depended on the concentration and type of cyclodextrin. It is known that the affinity of β-CD for cholesterol is higher than that of both α-CD and γ-CD [29]. Therefore, β-CD was the most effective cyclodextrin for achieving the dissociation of CHP nanogels. We also investigated β-CD derivatives such as HP-β-CD and methyl-β-CD. Methyl-β-CD was the most effective refolding reagent. The higher refolding ability of methyl-β-CD was due

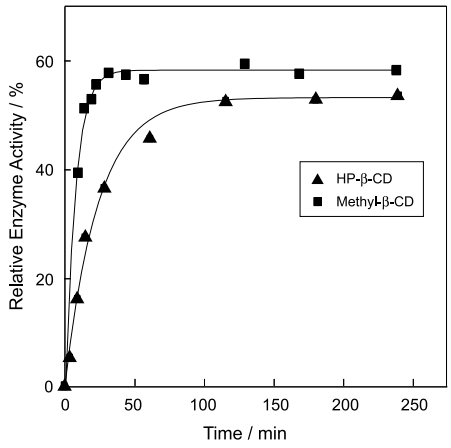

Fig. 4. The time course of reactivation of CS after the addition of cyclodextrins. Refolding kinetics were monitored for each cyclodextrin by activity. Denatured CS was diluted rapidly 70-fold in buffer solution containing CHP nanogels (2.4 mg/ml) and the mixture was then allowed to stand for 1 h at 25°C. The mixed solution was diluted 1.4-fold by each cyclodextrin (HP-β-CD or methyl-β-CD). After addition of cyclodextrins, enzyme activities were assayed for each time with the acetyl-CoA enzyme assay (detailed in Section 2). For all samples, $t = 0$ corresponds to the time of addition of cyclodextrin to the nanogel–CS complex solution.

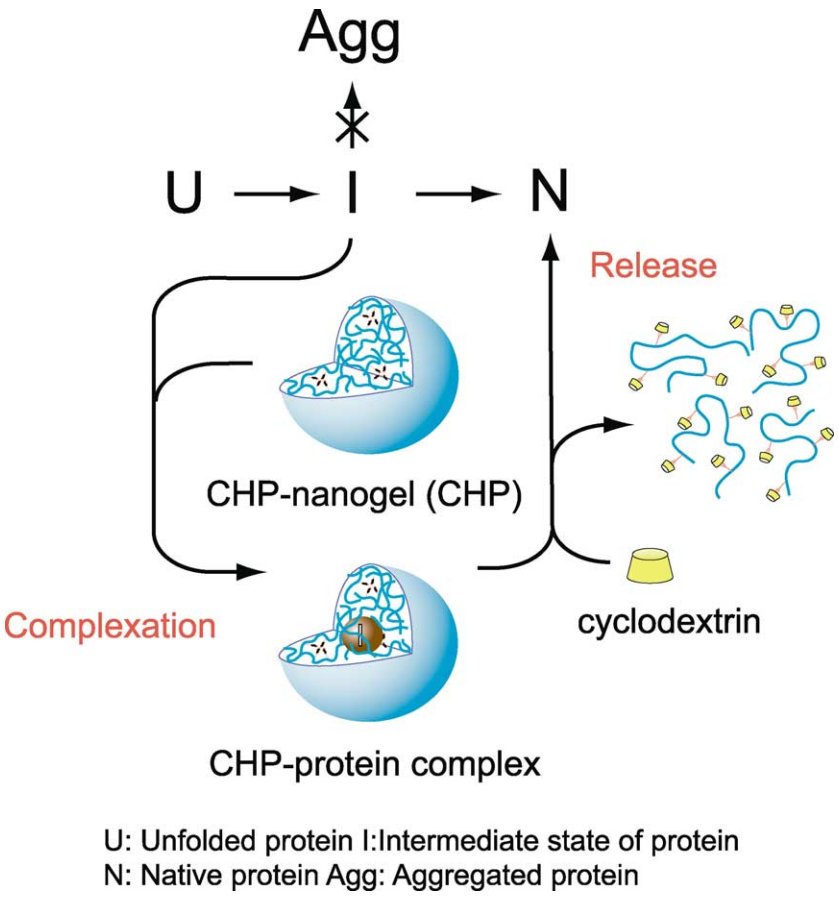

Fig. 5. Schematic representations of artificial molecular chaperones.

to the more effective dissociation of the nanogel–protein complex. Under these conditions, almost all of the nanogels dissociated at concentrations below 2.0 mM of methyl-β-CD. However, in the case of both β-CD and HP-β-CD, concentrations exceeding 10 mM led to the dissociation of all the nanogels.

About the nanogel–CS complex solution, refolding experiments were also carried out in the presence of various cyclodextrins (Fig. 3b). The maximum refolding activity (up to 76%) was observed in the presence of methyl-β-CD. Fig. 4 shows the time course of recovery of enzymatic activity (refolding) after the addition of HP-β-CD and methyl-β-CD. Under these conditions, all nanogels were dissociated by CD. The refolding curves shown in the figure were well fit by a single exponential function. The refolding half-life values $t_{1/2}$ deduced from these data were 5.1 min for methyl-β-CD and 17.0 min for HP-β-CD. These data indicate that the rate of dissociation of nanogels was controlled by cyclodextrin, and also the CHP nanogel–CD complex dissociating products of nanogels affects the rate of refolding. Usually, the refolding process lasts a few minutes when it takes place without nanogels. The releasing rate of trapped proteins is likely to be important for preventing the aggregation and reaggregation of refolding intermediates. Slow folding kinetics indicate that the aggregation of released protein is prevented in the cage of nanogel–CD complexes. We were able to control the rate of release of the proteins from the nanogels by selecting the hydrophobic groups on the nanogels and also by choosing the type of cyclodextrin used. Fig. 5 provides a representation of the catching and releasing mechanism of artificial molecular chaperones.

3.3. Refolding of inclusion body proteins

Using the current nanogel system, we also performed the refolding of inclusion body proteins that are overproduced in *E. coli*. We selected prosemin (BSSP4) with a His tag fragment; prosemin is a recombinant protein of the serine protease family, and is found in the mouse brain [23]. During synthesis of the proteins in *E. coli*, inclusion bodies were formed and refolding of the protein was not successful by the standard dilution refolding methods. First, the inclusion bodies were dissolved in 8 M urea (Fig. 6, lane 1). After dilution of the solution in the buffer and in the absence of nanogels, most proteins reaggregated, and no protein was observed upon SDS–PAGE (Fig. 6, lane 3). The aggregated protein was not observed in the stacking gel because all insoluble aggregated prosemin was removed as precipitates by centrifugation. However, in the presence of the nanogels, reaggregation was inhibited, and almost all of the prosemin was left in the supernatant (Fig. 6, lane 2) due to the formation of nanogel–protein complexes. After the addition of cyclodextrin to the supernatant, the solution was applied to the His tag resin. The proteins adsorbed to the His tag resin and the nanogels were eluted. The nanogels were separated from the proteins by this process. No trapping of proteins to the column was observed without the addition of cyclodextrin to the supernatant after the dilution. This finding provides evidence of the complexation of nanogels with prosemin during the dilution–refolding process. Then, the proteins were eluted with a histidine buffer (Fig. 6, lane 4). From this result, about 20% of the complexed protein was re-solved in the solution. Protease enzyme activity was also recovered. The present

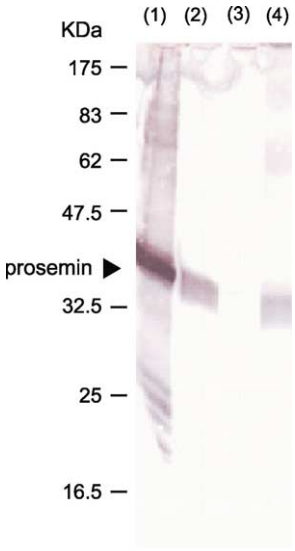

Fig. 6. Western blotting analysis of the refolding of prosemin inclusion bodies using the present CHP nanogel system. Lane 1, inclusion bodies in 8 M urea solution; lane 2, dilution of denatured inclusion bodies by the buffer in the presence of CHP nanogels; lane 3, simple dilution of denatured inclusion bodies by the buffer in the absence of CHP nanogels; lane 4, after the addition of cyclodextrin to the CHP nanogel–protein complexed solution, purification on a His tag column was carried out.

nanogel system was effective at renaturation of inclusion body proteins with biological activity.

3.4. Conclusions

Nanogel systems have been used in order to assist with protein refolding as a novel molecular chaperone-inspired system. The amphiphilic nanogels selectively trapped non-native proteins and cyclodextrin acted as an effector molecule to control the binding ability of the host to proteins. It is possible to design various functional nanogels such as surface-modified (cationic as well as ionic) and stimulus-sensitive (heat, light) nanogels. We are able to immobilize nanogels (artificial chaperone) by various surfaces such as beads for chromatography. The immobilized artificial chaperone could be useful in refolding chromatography and batchwise renaturation. The nanogel system is considered to be promising as an efficient and versatile technique for protein refolding in the post-genome era.

Acknowledgements: The authors are grateful to Shigemichi Nishikawa, Invitrotech Co., Ltd and Wakenyaku Co., Ltd for his helpful discussion. This work was supported by a Grant-in-Aid for Scientific Research in Priority Areas from the Ministry of Education, Science, Culture and Sports, Japan, and also by PRESTO of Japan Science and Technology.

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