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Protein refolding using stimuli-responsive polymer-modified aqueous two-phase systems

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Abstract

The function of a stimuli-responsive polymer was studied for the utilization of protein unfolding and refolding in protein separation using aqueous two-phase systems (ATPS). Poly(ethylene glycol) (PEG) bound to a thermo-reactive hydrophobic head (poly(propylene oxide)-phenyl group (PPO-Ph group)) was used as the functional ligand to modify the PEG phase of the aqueous two-phase systems. Firstly, refolding of carbonic anhydrase from bovine (CAB) was examined in the presence of PPO-Ph-PEG at various temperatures. The refolding yield of CAB was strongly enhanced and aggregate formation was suppressed by addition of PPO-Ph-PEG at a specific temperature (50–55°C). The change in the local hydrophobicity of CAB and PPO-Ph-PEG was characterized using the aqueous two-phase partitioning method and a hydrophobic fluorescent probe. The local hydrophobicity of CAB was maximized at 60°C. The local hydrophobicity of PPO-Ph-PEO was also found to be increased above 45°C. A simple model for CAB refolding, which includes (i) PPO-Ph-PEG complex formation and CAB in the intermediate state and (ii) refolding and release of native CAB from the PPO-Ph-PEG surface, is suggested based on the evaluated surface hydrophobicity.

Keywords: Aqueous two-phase systems; Stimuli-responsive separation; Protein folding

1. Introduction

Study of the protein folding problem is as old as Anfinsen's experiments [1]. Extensive studies on the intermediate state have been carried out in recent years because of its intriguing features and practical importance in the refolding process. The protein in this state is thought to have a secondary structure like the native state, but not the close packed tertiary structure [2,3]. Recently, a systematic approach to the protein folding process was reported in a series of works related to the evaluated values for proteins in various conformations [4–6]. The variation of the surface properties of proteins (e.g., surface net and local hydrophobicity) during the denaturation and refolding processes can be evaluated quantitatively using the aqueous two-phase partitioning method [4–6]. The local hydrophobicity of a protein was found to play an important role not only in the protein denaturation or aggregate-formation processes [4–6], but also in the interaction with liposomes [7–9], heat shock proteins (GroEL and GroES) [10], and hydrophobic ligands [11]. Based on these evaluated properties, the efficiency of the protein refolding process can be improved.

It is also possible to use molecular chaperones, which are defined as a family of unrelated cellular proteins that mediate the correct assembly of other polypeptides [12], in order to improve the efficiency

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of the protein refolding process. Some heat shock proteins (HSPs), which are produced by cells under various stresses, are classified in terms of molecular chaperones. DnaK, DnaJ, GroEL and GroES are typical representatives of HSPs, produced in *E. coli* cells under heat stress conditions. HSPs are known to play important roles in protein folding in vivo and in vitro. Recently, more attention has been paid to the use of various synthetic polymers in vitro [13,14], which is one of the most useful approaches for the modification and stabilization of the protein structure and for enhancement of the refolding of the unfolded protein. In a previous study, the separation process was successfully achieved as a novel separation process for reactivated proteins by combining the functions of HSPs with aqueous two-phase partitioning systems (Fig. 1a) [15]. The efficiency of protein recovery in the active state can be improved by using the functions of such a natural chaperone machinery.

It has been reported that many other ligands, such as amphiphilic polymers and polyols, could also have such chaperone-like functions [15–20]. The stimuli-responsive polymer has similar functions to those of the natural chaperone machinery, which can react and adapt itself to environmental stimuli. Recently, most research attention has been focused on the polymers that can spontaneously and reversibly change their structure and properties in response to external chemical and/or physical stimuli such as pH and temperature. These polymers, called smart polymers [21] or stimuli-responsive polymers, sense a stimulus as a signal, judge the magnitude of this signal and then alter their function in response to the corresponding stimuli. It is thought that stimuli-responsive ligands which assist and enhance protein refolding can be designed and used as an artificial chaperone instead of natural chaperones.

It is also interesting and reasonable to use a stimuli-responsive polymer (Fig. 1b) both as an artificial chaperone and as well as a modifier of the top phase of an aqueous two-phase system in order to improve the previous separation process using both GroEL functions and aqueous two-phase systems [15]. The final purpose of this study was to develop a protein refolding process using aqueous two-phase systems modified with a stimuli-responsive polymer, which has a chaperone-like function. The polymer, which has a PEG chain and a stimuli-responsive hydrophobic head (poly(propylene oxide)-phenyl group), was first synthesized for the design of an artificial chaperone. A carbonic anhydrase from bovine (CAB) was selected as a model

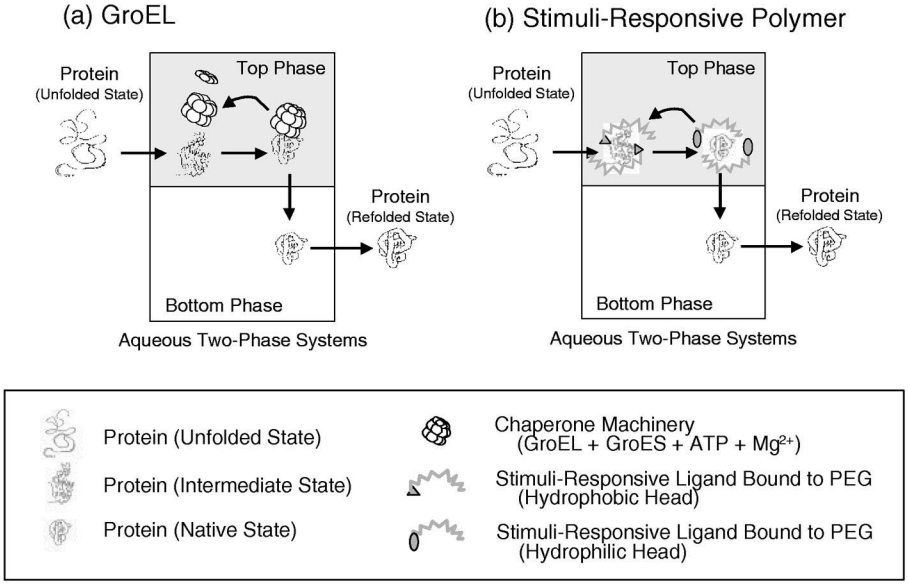

Fig. 1. Concept of protein unfolding and refolding in protein separation using aqueous two-phase systems modified with (a) a molecular chaperone (GroEL) and (b) stimuli-responsive polymers (concept from Ref. [15]).

protein. The effect of the addition of the polymer on CAB refolding was investigated at various temperatures. A possible mechanism for protein folding assisted by a stimuli-responsive polymer is elaborated. Based on these results, the possibility of developing a protein refolding process using aqueous two-phase systems modified with a stimuli-responsive polymer was investigated.

2. Experimental

2.1. Materials

Carbonic anhydrase from bovine (CAB, EC 4.2.1.1, 28,800 molecular mass) was purchased from Sigma (St. Louis, MO, USA). Guanidine hydrochloride (GuHCl) used as a denaturant of CAB was purchased from Wako Pure Chemical Industries (Osaka, Japan). The phase-forming polymers in aqueous two-phase systems such as dextran 100–200k (100,000–200,000 molecular mass) and poly(ethylene glycol) (PEG) 1540, 4k, 8k (1500, 3000, 8000 molecular mass) were purchased from Wako. Triton X-405 was purchased from Sigma. All other reagents used in this study were of analytical grade.

2.2. Synthetic polymers

The structural formula of the ligand used in this study is shown in Fig. 2. The ligands used here have a hydrophobic head (such as stimuli-responsive polymer; poly(propylene oxide), PPO) and a hydrophilic polymer chain (poly(ethylene glycol); PEG) chain with the same molecular weight (average molecular weight about 8k).

PPO was first brominated using PBr_3. The brominated PPO (number of propylene oxide units, 33) was added to dry *N*,*N*-dimethylformamide under nitrogen atmosphere. Phosphorus tribromide was added dropwise to this solution in an ice bath. The solution was stirred for 12 h at 40°C. Methanol and distilled water were added dropwise to this solution in an ice bath to deactivate any excess phosphorus tribromide. The desired compound was distilled in vacuo and a viscous liquid was obtained.

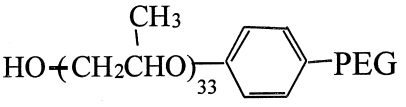

Fig. 2. Structure of PPO-Ph-PEG.

Phenyl-PEG (Ph-PEG) was obtained by transalkylation of Triton X-405 [22]. Under nitrogen atmosphere, toluene was added to a solution containing Triton X-405, phenol and anhydrous aluminum chloride. The solution was stirred for 6 h at room temperature. Methanol and distilled water were added dropwise to this solution in an ice bath to deactivate any excess aluminum chloride. The solvent was removed in vacuo, and then diethyl ether was added to reprecipitate the final product as a white powder after filtration.

Poly(propylene oxide) bromide was reacted with Ph-PEG. Under nitrogen atmosphere, dry *N*,*N*-dimethylformamide was added to a solution containing poly(propylene oxide) bromide, Ph-PEG and anhydrous aluminum chloride. The solution was stirred for 12 h at 40°C. Methanol and distilled water were added dropwise to this solution in an ice bath to deactivate any excess aluminum chloride. The solvent was removed in vacuo, and then diethyl ether was added to reprecipitate the product as a white powder. Formation of the final product was confirmed from IR spectra.

2.3. Refolding of CAB in the presence of functional ligands and in ligand-modified aqueous two-phase systems

For protein refolding, 60 μl of denatured protein solution (5 *M* GuHCl) was diluted with 0.1 *M* Tris–hydrochloride (Tris–HCl) buffer (pH 8.0) with and without PPO-Ph-PEG (0.1 m*M*) to a total volume of 3 ml [2,8]. The refolding experiments were performed at a final GuHCl concentration of 0.1 *M* at various temperatures (25–60°C). It has already been shown that CAB activity corresponds to the conformational change in the tertiary and secondary structures during the CAB unfolding process with 5 *M* GuHCl [4] and CAB refolding can be monitored by activity measurements [4,8]. The activity of CAB was measured together with aggregate formation at 25°C. CAB refolding was also performed in aqueous two-phase systems with and without PPO-Ph-PEG. The systems used for CAB

refolding consisted of PEG 4k (9%)/dextran 100–200k (9%) in the presence of 0.1 m*M* PPO-Ph-PEG, which is partitioned to the upper phase. CAB refolding was carried out at 25 and 52°C with completely mixed two-phase systems. The activity of CAB was determined from the hydrolysis rate of the substrates (*p*-nitrophenyl acetate; *p*-NPA, 1 m*M*) at a wavelength of 348 nm in 50 m*M* Tris–HCl buffer with 5 m*M* ethylenediaminetetraacetic acid (EDTA) [23]. The refolding of CAB could be stopped by the addition of EDTA [24]. CAB aggregate formation during the refolding process was monitored by measuring the optical density of the solution [15].

2.4. Analysis

The local hydrophobicity (*LH*) of proteins was determined by the aqueous two-phase partitioning method [25]. The local hydrophobicity of proteins and ligands was determined from the fluorescent intensity of 8-anilino-1-naphthalene-sulfonate (ANS; $\lambda_{ex} = 400$ nm, $\lambda_{em} = 470$ nm). The change in fluorescence intensity of the proteins and functional ligands was determined as a function of ANS concentration. The value at the plateau of the curve versus the ANS concentration was used as the local hydrophobicity of the polymers. Fluorescent spectra were measured by excitation at 400 nm on an FP-777 (Jasco, Japan) with a thermo-regulated cell compartment.

3. Results and discussion

3.1. Effect of the addition of functional ligands on CAB refolding

The effect of the addition of stimuli-responsive ligands (PPO-Ph-PEG) on the refolding of CAB denatured with GuHCl was investigated in order to check the possibility of the application of the unfolding, refolding, and separation processes in aqueous two-phase systems.

3.1.1. Typical refolding behavior of CAB under specific conditions

The effect of the addition of PPO-Ph-PEG on CAB refolding was investigated first. The refolding of CAB was initiated by dilution of denatured CAB solution. Fig. 3 shows the time course of CAB activity (Fig. 3a) and optical density (OD_{340}) of a solution (Fig. 3b) in the presence and absence of 0.1 m*M* PPO-Ph-PEG at 50°C. As shown in Fig. 3a, the CAB activity increased with increasing time in the absence of PPO-Ph-PEG and the values reached a plateau (45%) within 10 min. Similarly, in the presence of PPO-Ph-PEG, the refolding yield of CAB increased to 78% within 10 min. The value at the plateau was 1.7 times greater than that in the absence of PPO-Ph-PEG. A molecular chaperone has previously been reported to enhance protein refolding [3,10,15]. The refolding yield of CAB in the presence of a molecular chaperone, GroELp [15], is also shown in Fig. 3a. The refolding yield of CAB in the presence of PPO-Ph-PEG was found to be similar to that in the presence of GroELp, showing that PPO-Ph-PEG has a similar function as the molecular chaperone.

CAB aggregate formation was also studied by measuring the optical density of the solution, as

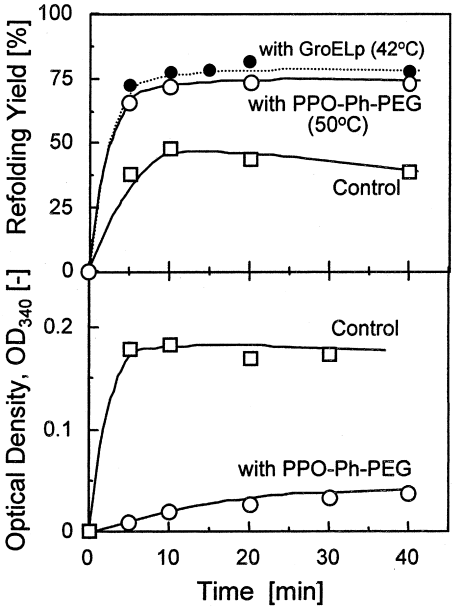

Fig. 3. Time course of (a) CAB activity and (b) optical density during the CAB refolding process at a temperature of 50°C. CAB refolding was initiated by dilution of denatured CAB (5 *M* GuHCl) with the refolding buffer (100 m*M* Tris–HCl, pH 8.0). The final concentration of CAB and GuHCl was 0.1 mg/ml and 0.1 *M*, respectively. The concentration of PPO-Ph-PEG was 0.1 m*M*.

shown in Fig. 3b. The increase in the turbidity of the solution can be interpreted as the formation of aggregation in the solution. After the initiation of CAB refolding, the optical density in the absence of PPO-Ph-PEG increased with increasing time and the OD_{340} values reached 0.5. On the other hand, the OD_{340} values at the plateau indicated lower values than those in the absence of PPO-Ph-PEG, showing that formation of the CAB aggregation was prevented in the presence of PPO-Ph-PEG.

It was thus found that the addition of PPO-Ph-PEG improved the refolding yield of CAB and suppressed aggregate formation during the refolding process.

3.1.2. Temperature dependence of CAB refolding in the presence of functional ligands

The poly(propylene oxide) group (PPO), as well as the poly(ethylene oxide) group (PEO), is known as a stimuli-responsive polymer, which reacts and adopt its structure on recognition of a temperature change. The effect of temperature on the CAB refolding process was investigated in the presence of PPO-Ph-PEG.

CAB refolding was performed at various temperatures and the CAB activity and OD_{340} were measured under the above conditions (30–55°C). Fig. 4 shows the effect of temperature on (a) the reactivation yield and (b) aggregate formation (OD_{340}) after 40 min on CAB refolding. In the absence of PPO-Ph-PEG, the refolding yield did not change at temperatures of 25–45°C and decreased at higher temperatures (Fig. 4a). On the other hand, the refolding yield of CAB increased with increasing temperature and the values were maximized in a specific temperature range (48–52°C) in the presence of a functional ligand (PPO-Ph-PEG) (Fig. 4a). The maximal value of CAB activity was 85%. No CAB activity was observed at high temperature. It has been reported that a molecular chaperone, GroEL [3,10,15], and liposomes [8] can assist protein refolding. The refolding yield of CAB in the presence of GroELp [15] and liposomes [8] is also shown in Fig. 4a. Although the refolding yield of CAB with PPO-Ph-PEG was smaller than that with GroELp and liposomes at lower temperature (25–45°C), the value increased at higher temperatures (50–57°C). As shown in Fig. 4b, the OD_{340} values as a measure of

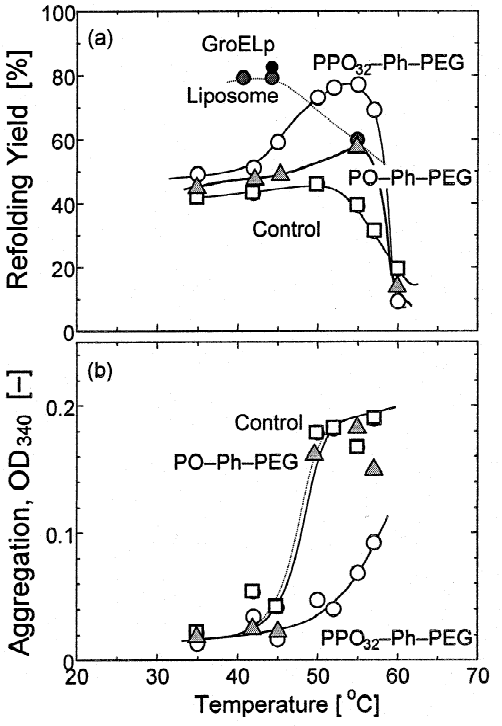

Fig. 4. Effect of temperature on (a) CAB activity and (b) optical density 40 min after CAB refolding. CAB refolding was initiated by dilution of denatured CAB (5 *M* GuHCl) with the refolding buffer (100 m*M* Tris–HCl, pH 8.0). The final concentration of CAB and GuHCl was 0.1 mg/ml and 0.1 *M*, respectively. The concentration of PPO-Ph-PEG was 0.1 m*M*.

CAB aggregate formation increased at temperatures above 48°C, where CAB aggregation was found to occur in the absence of PPO-Ph-PEG. On the other hand, aggregate formation was prevented in the temperature range from 48 to 55°C.

CAB is a monomeric protein and is often used for the study of protein refolding due to its slow refolding rate compared with other proteins. Cleland and Wang [26] investigated the conformational change and the formation of micro-sized inactive aggregates using a quasi-light-scattering method. It has also been demonstrated that the reactivation yield of a protein is enhanced by the addition of a molecular chaperon (such as GroELp), liposomes, and hydrophobic ligands (such as PEG and the Triton X series) [2,8,11,15,22]. Furthermore, it has also been reported that the local hydrophobicity of chaperones and other chaperone-like materials can

play an important role in the refolding and translocation of denatured proteins [7,9,15,22]. As shown in Fig. 2, PPO-Ph-PEG used in this study has a hydrophobic head group (PPO-Ph group) and a hydrophilic tail (PEG). The effect of the type of head group, which is covalently bound to the PEG molecule, has already been investigated [11]. The hydrophobicity of the head group was shown to be an important factor for interaction of CAB with the intermediate state. Similarly in this case, the local hydrophobicity of PPO-Ph-PEG at various temperatures was considered to be an effective factor governing the CAB refolding process.

3.2. Variation of the surface properties of proteins and functional ligands under heat stress

In order to study the mechanism of the stimuli-responsive ligand-assisted refolding process of CAB, the local hydrophobicity of CAB and PPO-Ph-PEG was evaluated using aqueous two-phase systems and, also, by the fluorescence of ANS. A minimal model for CAB folding in the presence of PPO-Ph-PEG under heating is presented based on the quantitative characteristics described above.

3.2.1. Temperature dependence of the local hydrophobicity of CAB

The local hydrophobicity (*LH*) of CAB was characterized using the aqueous two-phase partitioning method under heat stress conditions (50–70°C), as shown in Fig. 5. Although the *LH* values of native CAB were very low, the values increased at 60°C. At temperatures above 65°C, the *LH* values decreased. Under normal conditions (25°C), the hydrophobic amino acids of the protein are tightly packed inside the protein molecule, so the protein surface has a very hydrophilic nature. As the temperature increases, the structure of the protein is partly destroyed and some hydrophobic amino acids, which are buried inside, may be exposed on the protein surface. When the structure of the protein is only partially unfolded, the local hydrophobic site composed of hydrophobic amino acids appears on the limited protein surface area, so the local hydrophobicity of the protein increases significantly. Further destruction of the protein structure under heat stress above 65°C is, however, thought to induce the

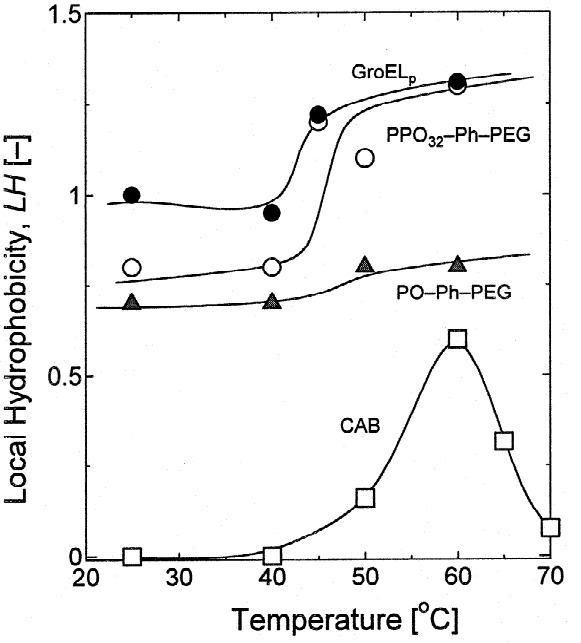

Fig. 5. Temperature dependence of the local hydrophobicity (*LH*) of PPO_{32}-Ph-PEG (○), PO-Ph-PEG (▲), GroELp (●), and CAB (□). The *LH* values of proteins (GroELp and CAB) were determined using the aqueous two-phase partitioning method [25]. Those of ligands (PPO-Ph-PEG and PO-Ph-PEG) were determined from the fluorescent intensity of ANS.

disappearance of the local hydrophobic site on the protein surface and make the fully unfolded protein surface more hydrophobic. It has already been reported that CAB in the intermediate state has strong local hydrophobic sites, while that of the fully unfolded state has a larger surface net hydrophobicity and that similar profiles of the surface properties of CAB were observed under heat stress conditions [6,9]. Among the possible conformations, it has been reported that CAB in the intermediate state has a strong local hydrophobic site, while that of the fully unfolded state has a larger surface net hydrophobicity [5,6,15]. The results for the local hydrophobicity of CAB under heat stress correspond well with previous findings.

3.2.2. Temperature dependence of the local hydrophobicity of PPO-Ph-PEG

The stimuli-responsive ligand PPO-Ph-PEG, as well as the protein CAB, can also adapt themselves to a given environmental change through a structural

change. The local hydrophobicity of PPO-Ph-PEG was also characterized using a hydrophobic fluorescent probe (ANS). Fig. 5 shows the value of the local hydrophobicity of the stimuli-responsive polymer (PPO-Ph-PEG) plotted versus temperature. The *LH* values increased gradually with increasing temperature (below 45°C) (Fig. 5). The values increased sharply at a temperature of 45°C and reached a plateau above 45°C. PPO-Ph-PEG was thus found to be stimuli responsive and to have, at least, two distinct states, indicating that the phase transition temperature was around 45°C. The *LH* values of GroELp are also plotted versus temperature in Fig. 5. Similarly, a phase transition temperature is observed in the case of GroELp, implying that PPO-Ph-PEG is expected to have a similar function to GroELp.

PPO-PEG block copolymers (called Pluronic), which can change structure in response to a temperature change, have been well characterized. Their conformation and structure change at the phase transition temperature [27]. Although the structure of block copolymers (PPO-PEG) is different from the above polymer (PPO-Ph-PEG), a similar tendency in stimuli-responsive behavior can be observed. Variation of the polymer conformation is mainly caused by the dehydrating effect of the PPO part of the molecule. The local hydrophobicity of Ph-PEG was measured and the values plotted against temperature (Fig. 5) in order to investigate the effect of the PPO group on the local hydrophobicity. The local hydrophobicity of PO-Ph-PEG did not change in this temperature region, although a slight increase in the hydrophobicity could be observed. It was found that the PPO group of the ligands could cause a phase transition of the local hydrophobicity. The phase transition in the local hydrophobicity value of PPO-Ph-PEG at 45°C may be caused by a specific part (PPO group) of the polymer.

3.3. Simple model for CAB refolding in the presence of PPO-Ph-PEG under heat stress

As shown in Fig. 4a, the refolding yield of CAB is maximized in the presence of PPO-Ph-PEG at temperatures between 48 and 52°C. CAB aggregate formation is also inhibited under similar conditions (Fig. 4b). The model for protein folding assisted by a chaperone has already been investigated, showing GroEL bound to the target protein in the intermediate state and refolded and released from its surface due to structural fluctuations [8,15]. In our series of works, we have shown that CAB refolding is dependent on the local hydrophobicity of CAB and additives (such as GroEL and liposomes) [2,8,11,15]. The surface properties of both CAB and PPO-Ph-PEG were characterized, especially in relation to their local hydrophobicity. The temperature regions where the local hydrophobicity of (i) CAB and (ii) PPO-Ph-PEG increased are (i) 60°C and (ii) above 45°C. Under specific conditions, the complex between CAB in the intermediate state and PPO-Ph-PEG is thought to be formed because of a hydrophobic interaction. CAB aggregation is considered to be suppressed because the intermolecular interaction of CAB is reduced by the formation of the complex between CAB and PPO-Ph-PEG (Fig. 3b, Fig. 4b). After complex formation between CAB and PPO-Ph-PEG, CAB in the intermediate state refolds to the native state and native CAB is released from the polymer surface (Fig. 3b, Fig. 4b) because of the sharp change in the surface properties of the polymer, which may be caused by the change in temperature. A minimal and simple model for the functional ligand-assisted refolding of the protein, involving the two steps (i) complex formation and (ii) release and refolding of CAB from the polymer surface, is thus suggested.

3.4. CAB refolding in PPO-Ph-PEG-modified aqueous two-phase systems under heat stress

Based on the above results, the possibility of achieving protein refolding using an aqueous two-phase system (ATPS) modified with stimuli-responsive polymer (Fig. 1b) was finally investigated. PEG derivatives are known to be partitioned to the PEG phase in ATPS. CAB refolding was performed in PEG 4k (9%)/dextran 100–200k (9%) aqueous two-phase systems with and without 0.1 m*M* PPO-Ph-PEG at the optimal temperature (52°C). The refolding yields of CAB in various two-phase systems are summarized in Table 1, together with those in the single-phase systems. Generally, the refolding yield of CAB was reduced to 23–25% in PEG 4k (9%)/dextran 100–200k (9%) two-phase systems as compared with that in the control system. However, the

Table 1
Refolding yield of CAB in aqueous two-phase systems in the presence and absence of functional ligands. CAB refolding was initiated by dilution of denatured CAB (5 *M* GuHCl) with the aqueous two-phase systems. Final concentration of CAB and GuHCl, 0.1 mg/ml and 0.1 m*M*, respectively

System[a]	Additives	Temp. (°C)	Refolding yield (%)
Tris–HCl buffer	None	25	43
Tris–HCl buffer	None	52	40
Tris–HCl buffer	0.1 m*M* PPO-Ph-PEG	25	48
Tris–HCl buffer	0.1 m*M* PPO-Ph-PEG	52	76
PEG 4k (9%)/dextran 100–200k (9%)	None	25	23
PEG 4k (9%)/dextran 100–200k (9%)	None	52	25
PEG 4k (9%)/dextran 100–200k (9%)	0.1 m*M* PPO-Ph-PEG	25	24
PEG 4k (9%)/dextran 100–200k (9%)	0.1 m*M* PPO-Ph-PEG	52	41
PEG 4k (9%)/dextran 100–200k (9%) +0.1 *M* KPi and 5 m*M* Triton X-405[b]	1 μ*M* GroEL	42	78

[a] The systems were buffered by 100 m*M* Tris–HCl (pH 7.5).
[b] Data from Yano [15].

value increased to 41% in PEG 4k (9%)/dextran 100–200k (9%) two-phase systems with 0.1 m*M* PPO-Ph-PEG at 52°C. The increase in the refolding yield may be caused by the chaperone function of PPO-Ph-PEG, as indicated in the previous section (Fig. 3a, Fig. 4a). It has been reported that CAB refolding is enhanced under optimal conditions in aqueous two-phase systems modified with a molecular chaperone, GroELp, by exploiting the functions of both GroEL and ATPS. Although the refolding yield in ATPS modified with PPO-Ph-PEG is lower than that in GroELp-modified ATPS and in the single-phase solution of PPO-Ph-PEG (Table 1), and further investigations are needed, the possibility of protein refolding in aqueous two-phase systems modified with PPO-Ph-PEG has thus been demonstrated.

4. Conclusion

The possibility of the stress-mediated refolding of CAB was investigated using a stimuli-responsive polymer (PPO-Ph-PEG) in order to achieve protein unfolding and refolding in protein separation using aqueous two-phase systems (Fig. 1). In the presence of PPO-Ph-PEG, the refolding yield of CAB increased 1.7 times and aggregate formation was suppressed when suitable heating to increase the local hydrophobicity of both PPO-Ph-PEG and CAB was selected. Based on the model for CAB refolding, CAB refolding in aqueous two-phase systems modified with stimuli-responsive PPO-Ph-PEG was finally performed; the refolding yield increased by selecting suitable systems and operating conditions.

5. Nomenclature

LH	Local hydrophobicity ($=\Delta \ln K_{pr}$) determined from the partitioning behavior of proteins in aqueous two-phase systems [25]
OD_{340}	Optical density of the solution at 340 nm as a measure of the aggregate formed in solution
R_y	CAB refolding yield

6. List of abbreviations

ANS	8-Anilino-1-naphthalene-sulfonate
ATPS	Aqueous two-phase systems
CAB	Carbonic anhydrase from bovine
GroELp	GroEL purified using a previous method [15]
GuHCl	Guanidine hydrochloride
p-NPA	*p*-Nitrophenylacetate

PEG	Poly(ethylene glycol)
Ph-PEG	Phenyl-poly(ethylene glycol)
PO-Ph-PEG	(Propylene oxide)-phenyl-poly(ethylene glycol)
PPO	Poly(propylene oxide)
PPO-Ph-PEG	Poly(propylene oxide)-phenyl-poly-(ethylene glycol)

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