Polyvinylpyrrolidone 40 Assists the Refolding of Bovine Carbonic Anhydrase B by Accelerating the Refolding of the First Molten Globule Intermediate

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Protecting proteins from aggregation is one of the most important issues in both protein science and protein engineering. In this research, the mechanism of enhancing the refolding of guanidine hydrochloride-denatured carbonic anhydrase B by polyvinylpyrrolidone 40 (PVP40) was studied by both kinetic and equilibrium refolding experiments. The reactivation and refolding kinetics indicated that the rate constant of refolding the first refolding intermediate (I₁) to the second one (I₂) is promoted by the addition of PVP. Fluorescence quenching studies further indicated that PVP could bind to the aggregation-prone species I₁, resulting in the protection of the exposed hydrophobic surface, a minimization of the protein surface, and more importantly, an increase of the refolding rate of I₁. These properties were quite different from those of poly(ethylene glycol) (PEG), which has been shown to have a strong and stoichiometric binding to I₁ and does not interfere with the refolding pathway. Unlike PEG, the binding of PVP to I₁ does not block the aggregation pathway directly but decreases the energy barrier for I₁ to refold to I₂ and thus reduces the accumulation of I₁. These results suggested that PVP works by a quite different mechanism from those well established ones in chaperones and chemical promoters. PVP is more like a folding catalyst rather than a chemical chaperone. The distinct mechanism of enhancing protein aggregation by PVP is expected to facilitate the attempt to develop new chemical compounds as well as new strategies to protect proteins from aggregation.

Non-native aggregation, an off-pathway product during unfolding/refolding that has been associated with more than 20 serious degenerative diseases (1), is also a challenge in protein industry engineering (2). Numerous studies have been carried out to identify the determinants and driving force of protein aggregation. In general, protein molecules are prone to stick together if considerable hydrophobic residues are exposed (3), and it has been widely accepted that the intermolecular cross-β motif is the core structure of aggregates (1, 4). Protein folding studies have suggested that the partially denatured states, particularly the “molten globule”-like intermediates, play a crucial role in the formation of aggregation (1, 5–8). The occurrence of aggregation, which facilitates non-native intermolecular interactions, competes with the refolding pathway, which facilitates the correct assembly of intramolecular interactions to the native state (9). In this case, the yield of correctly folded proteins is gradually affected by the nonproductive off-pathway aggregation.

The tendency of a protein to aggregate in aqueous solution is affected by its physicochemical properties (10), the existence of chaperones (11), co-solutes (12), and environmental conditions (13). Various strategies have been developed to stabilize the native protein (13) or to enhance protein refolding from inclusion bodies in vitro (14). Among these, the utilization of chemical additives, including osmolytes, surfactants, and water-soluble polymers, is of particular interest because of their good biocompatibility and wide applicability (15–23). The mechanism by which these chemical additives prevent protein aggregation has been well established (24–27), and most of these additives are thought to act as chemical chaperones to prevent aggregation-prone species from sticking together during protein refolding. For example, poly(ethylene glycol) (PEG), one of the most widely used water-soluble polymers in assisting protein refolding in vitro (28), was found to specifically bind to the first refolding intermediate of bovine carbonic anhydrase B (CAB) and to perturb the self-association of the aggregation-prone intermediate (29). The stoichiometric interaction between PEG and the first molten globule (MG) intermediate of CAB results in a PEG-protein complex, which reduces the non-native dimers but does not affect the refolding rate of the first MG intermediate (30). These properties are thought to coincide with the mechanisms in chaperonin-mediated protein folding.

Although blocking the off-pathway process can effectively enhance the refolding yield, the efficiency of various chemical additives is usually different from case to case and is protein- and solution condition-dependent (18, 22, 23, 31). Thus the development of new strategies is still a challenge in the protein aggregation problem. In this research, the mechanism of enhancing refolding of guanidine hydrochloride (GdnHCl)-denatured CAB by polyvinylpyrrolidone (PVP) was studied by both kinetic and equilibrium experiments. PVP, a water-soluble polymer, has been widely used as in pharmaceutics because of its low toxicity (32, 33). PVP is a linear polymer with a structure similar to the structure of PEG but with more hydrophobic side chains. Considering that protein aggregation comes from intermolecular hydrophobic interactions, PVP should have the ability to bind the exposed hydrophobic parts of protein refolding intermediate by nonspecific interactions, which might protect the protein from aggregation and facilitate protein refolding. Consistent with this analysis, several studies have shown that the exist-

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3. The abbreviations used are: PEG, poly(ethylene glycol); PVP, polyvinylpyrrolidone; CAB, carbonic anhydrase B; GdnHCl, guanidine hydrochloride; SEC, size exclusion chromatography; MG, molten globule.

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ence of PVP can protect proteins from thermal aggregation (34–37). However, it was also found that PVP could promote heat-induced aggregation in some cases (38). These properties led us to the hypothesis that PVP might interact with the protein intermediate(s) in a different way than PEG. In this research, the effect of PVP on protein refolding was evaluated using the well studied CAB as a model protein. In both kinetic and equilibrium studies, the refolding of CAB has been found to involve two intermediates (6, 39). The first MG refolding intermediate (I1) is prone to aggregate and can self-associate to yield off-pathway dimer, but the second refolding intermediate (I2) is not aggregation-prone (40). Similar to the study concerning the PEG-CAB complex (30), it was found that PVP could also bind to the first MG refolding intermediate. The binding of PVP to the first MG refolding intermediate resulted in protection of the exposed hydrophobic surface, a decrease of the protein volume, and more importantly, an increase of the refolding rate of the first MG refolding intermediate. The mechanism by which PVP enhances protein refolding is quite different from those well established ones in osmolyte-, surfactant-, or chaperonin-mediated protein refolding. The present research sheds new light on further development of new strategies in assisting protein refolding by organic compounds.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine CAB (EC 4.2.1.1), GdnHCl, polyvinylpyrrolidone with a molecular mass of 40 kDa (PVP40), Tris base, sulfuric acid, p-nitrophenol acetate, N-acetyltryptophan, and acrylamide were purchased from Sigma-Aldrich. All buffers and protein solutions were prepared by using ultrapure water from a MilliQ water purification system (Millipore Corp., Bedford, MA).

**Preparation of the Denatured CAB**—Denatured CAB samples for rapid dilution refolding were prepared by incubating the native CAB in denaturation buffer (containing 100 mM Tris sulfate, pH 7.5, and 5 M GdnHCl) at 6 h at 25 °C.

**Aggregation Experiments**—The aggregation experiments were carried out at 25 °C through rapid dilution of GdnHCl-denatured CAB into Tris sulfate buffer with various concentrations of PVP40. The final concentration of CAB was 0.3 mg/ml. It has been proposed that the light scattering intensity is proportional to the amount of the protein in the aggregated form (41, 42), and thus the aggregation of CAB was monitored by measuring the light absorption at 400 nm with an Ultraspex 4300 pro UV-visible spectrophotometer from Amersham Biosciences.

**Esterase Activity and Reactivation Kinetics**—The CAB activity was determined by using the esterase activity assay described by Pocker and Stone (43). The refolding samples for activity measurements were prepared by rapid dilution of the denatured CAB into dilution buffer containing different concentrations of PVP40 at a mixing ratio of 1:10. The activities of refolding samples were measured after 1 h of equilibration. The details regarding the kinetic experiments have been described elsewhere (44). In brief, standard buffer was added to the GdnHCl-denatured CAB to initiate the refolding of the protein. Samples were removed at given times, and the activity of the samples was recorded immediately using a cell with a 1-cm light path length on an Ultraspex 4300 pro UV-visible spectrophotometer as a function of time. The rate of the reaction was obtained by recording the intensity increase in the absorption at 348 nm for 1 min using the maximum linear rate. The dead time before measurement was 5 s. As a control, no significant difference of native CAB activity was found between samples with or without the addition of PVP40.

**Stopped-flow Measurements**—The samples for stopped-flow measurements were prepared in 100 mM Tris sulfate buffer, pH 7.5, with or without 1% PVP40. The changes in fluorescence upon refolding were monitored using a stopped-flow apparatus (Applied Photophysics Ltd., Surrey, United Kingdom) at 25 °C. The mixing ratio of the GdnHCl-denatured CAB and the standard buffer was 1:10 (v/v), and the final protein concentration was 0.1 mg/ml. The fluorescence emission intensity was monitored at wavelengths above 320 nm using a 320-nm cut-off filter by excitation at 296 nm with a slit width of 1 nm. The dead time of the instrument was determined to be about 10 ms.

**Equilibrium Intrinsic Fluorescence**—The denatured CAB was diluted into 100 mM Tris sulfate buffer containing certain concentrations of GdnHCl by a mixing ratio of 1:100 with or without 1% PVP40. The final protein concentration was 0.1 mg/ml. The samples were equilibrated for 15 h before measurement. Then the intrinsic fluorescence of each solution was measured on an F-2500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with a 5-nm slit width for both excitation and emission. The excitation and emission wavelengths were 296 and 340 nm, respectively (45, 46). The ratio of the fluorescence intensity at 340 nm of the samples with given concentrations of GdnHCl (F) to that of the native protein (F0) was defined as the relative fluorescence (F/F0) (29). The fluorescence of N-acetyltryptophan with different concentrations of GdnHCl and PVP40 in Tris sulfate buffer was also measured as control experiments.

The effect of PVP40 on the fluorescence of the first MG refolding intermediate (I1) of CAB was carried out by diluting the denatured CAB into a buffer containing 2.0 M GdnHCl with different concentrations of PVP40 (0.05–4%) in the refolding solution, and the fluorescence of each solution was measured after equilibration for 6 h. The final protein concentration was 0.05 mg/ml. The relative fluorescence was defined as the variation in the fluorescence between the samples containing PVP40 (F) and the sample without PVP40 (F0). F0 was taken as 100% to normalize all data to show the percentage of change caused by different concentrations of PVP40. The effect of PVP on CAB fluorescence and the number of binding sites between PVP40 and the first MG intermediate of CAB were analyzed using the method described by Pesce et al. (47) (see also “Results”).

**CAB Fluorescence Quenched by Acrylamide**—The samples were prepared by diluting the denatured CAB into 100 mM Tris sulfate buffer containing different concentrations of GdnHCl and 1% PVP40. After 15 h of equilibration, each refolding sample was divided equally into two samples. The two samples were mixed with stock solutions with or without acrylamide, respectively. The final concentration of acrylamide was 0.5 M. The mixed solutions were equilibrated for an additional 2 h. In consideration of the possible volume change caused by the addition of acrylamide, the protein concentration was corrected using the samples without acrylamide. The ratio of the fluorescence without acrylamide to that in the presence of acrylamide (F/F0) was used to monitor the quenching effect of acrylamide. The concentration-dependent effect of acrylamide was evaluated by samples with or without PVP at three GdnHCl concentrations: 1.0, 1.4, and 2.0 M. The concentration range of acrylamide was 0.05–0.5 M.

**Size Exclusion Chromatography**—Size exclusion chromatography (SEC) was performed on a Tricorn high performance column (10/300 GL) attached to an AKTA purifier (Amersham Biosciences) at 4 °C with the fractionation range for globular proteins between 10,000 and 200,000. The column was first equilibrated with 2 column volumes of elution buffer (2.0 M GdnHCl, 0.1 M Tris sulfate with or without 1% PVP40) to characterize the impact of PVP40 on the elution volume of the first intermediate present in 2 M GdnHCl. When analyzing the transient forming dimer during the refolding of denatured CAB in 5 M GdnHCl by rapid dilution to 1 M GdnHCl, the Tricorn high performance column was pretreated with 2 column volumes of elution buffer.
The optimal concentration of PVP40 for the reactivation of CAB was most beneficial in assisting the reactivation of GdnHCl-denatured CAB. Capability to inhibit aggregation, 1% PVP40 was also found to be the concentration of PVP to prevent aggregation is 1%. Consistent with the previous studies (18), serious aggregation was observed during the refolding of CAB even at a final concentration of 0.3 mg/ml (Fig. 1A). These distinct effects of PVP on protein refolding had not been observed in other osmolytes, polymers, or surfactants (18, 22, 26, 27, 48), which suggested that PVP might assist protein refolding by a mechanism different from the well established one of PEG. To verify this hypothesis, the effect of PVP on CAB refolding was studied by both kinetic and equilibrium studies using a final CAB concentration in the refolding buffer of 0.1 mg/ml. This concentration was chosen because it was dependent on the final concentration of the protein in the refolding buffer. When the CAB concentration was 0.1 mg/ml in the refolding buffer, the best concentration of PVP was about 1.8%, whereas an optimal concentration was about 0.2% when the CAB concentration was 3 mg/ml (Fig. 1B). These results showed that no aggregation will occur at this concentration (40).

Effect of PVP40 on CAB Reactivation Kinetics—The kinetics of reactivation was investigated by diluting the denatured CAB into the refolding buffer, and the recovery of activity was measured as a function of refolding time. The results of the control experiment showed that the presence of PVP40 would not affect the esterase activity of native CAB. As presented in Fig. 2, the refolding of denatured CAB shows a typical biphasic process, which is quite consistent with previous studies (6). The final recovery of the enzyme activity was about 90% of the native enzyme for samples both with and without the addition of PVP. However, the activity recovery of the sample with PVP40 was much faster than that of the sample without PVP40. To further characterize whether the fast phase or the slow phase was affected by the addition of PVP in the dilution buffer, the kinetic parameters of the reactivation were obtained by fitting the data using a biphasic model. The rate constants of the fast and slow phases were (1.4 ± 0.1) × 10^{-3} s^{-1} and (4.2 ± 0.3) × 10^{-4} s^{-1} for the samples without PVP40 and (3.0 ± 0.1) × 10^{-3} s^{-1} and (2.8 ± 0.2) × 10^{-4} s^{-1} for the samples in the presence of PVP40, respectively.

Effect of PVP40 on CAB Refolding Kinetics by Stopped-flow Experiments—To further investigate whether the formation of the first refolding intermediate (I1) might be affected by the presence of PVP40, but the second refolding intermediate (I2) was not. To further characterize this deduction, the refolding kinetics was studied by stopped-flow experiments.

RESULTS

Effect of PVP40 on CAB Reactivation and Aggregation—To characterize the effect of PVP on CAB refolding, the aggregation and reactivation of CAB in the presence of various concentrations of PVP40 was investigated first. Consistent with previous studies (18), serious aggregation was observed during the refolding of CAB even at a final concentration of 0.3 mg/ml (Fig. 1A). We were surprised to find that aggregation of CAB during refolding was inhibited by the presence of PVP40 in the refolding buffer with concentrations lower than 2%, but aggregation was promoted with PVP40 concentrations higher than 2%. The optimal concentration of PVP to prevent aggregation is 1%. Consistent with the turbidity measurements, which showed that 1% PVP40 had the best capability to inhibit aggregation, 1% PVP40 was also found to be the most beneficial in assisting the reactivation of GdnHCl-denatured CAB. The optimal concentration of PVP40 for the reactivation of CAB was obtained by fitting the data using a biphasic model. The rate constants of the fast and slow phases were (1.4 ± 0.1) × 10^{-3} s^{-1} and (4.2 ± 0.3) × 10^{-4} s^{-1} for the samples without PVP40 and (3.0 ± 0.1) × 10^{-3} s^{-1} and (2.8 ± 0.2) × 10^{-4} s^{-1} for the samples in the presence of PVP40, respectively.

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the equilibrium refolding of CAB had a plateau between 1.5 and 2.0 M GdnHCl. Consistent with a previous study (39), the relative fluorescence curve of CAB at 340 nm was observed. The GdnHCl concentration was 0.5 M and 0.1 mg/ml, respectively. The time course of refolding was 500 or 5 s (shown as inset).

samples without PVP40, which suggested that PVP40 might have the effect of stabilizing CAB. For the control samples, no significant impact of PVP40 was found on the fluorescence of the native protein or that of tryptophan (data not shown).

Binding of PVP40 to I1 by Fluorescence and SEC Analysis—As shown in Fig. 5, when the PVP40 concentration was lower than 0.6%, an increase of the relative fluorescence of I1 was observed with the increase of PVP40 concentrations. Because PVP40 has little fluorescence-enhancing effect on free tryptophan, the enhancing of CAB fluorescence by PVP was mainly due to the binding of PVP to the protein. However, as the PVP concentration exceeded 0.6%, it was found that the intensity of the fluorescence of I1 gradually decreased, accompanied by a red shift. This unusual behavior of PVP40 on the intrinsic fluorescence of I1 suggested that PVP40 could act as a ligand of I1 at low concentrations but as a denaturant at high concentrations. In this case, the curve in Fig. 5B could not reach the saturation point, and the binding constant could not be calculated.

To further verify the assumption that PVP40 can specifically bind to I1, but not to I2, the quenching effect of acrylamide was studied using protein solutions with or without PVP40. As shown in Fig. 6A, the great difference of the quenching ability of acrylamide between the samples with or without the addition of PVP40 was found at GdnHCl concentrations above 1.5 M. The data on acrylamide quenching also suggested that PVP40 did not interact with I2, which was formed at GdnHCl concentrations below 1.43 M in equilibrium refolding studies of CAB (49). The concentration-dependent quenching effect of acrylamide shown in Fig. 6B indicated that acrylamide had the same quenching abilities in the samples with and without PVP when refolded to buffers with 1.0 or 1.4 M GdnHCl. In contrast, a significant variance could be observed between samples with and without PVP when

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refolded into buffers with 2 M GdnHCl. This result was quite consistent with that shown in Fig. 6A.

To determine what effect PVP40 has on the volume change in different species occurring during CAB refolding, SEC analysis was performed for the native refolding intermediate I1 and the off-pathway dimer formed by the self-association of I1 (39, 40). The elution profiles in Fig. 7 show the effect of PVP40 on the native protein, I1, formed in 2 M GdnHCl, and the dimer formed in the refolding buffer containing 1 M GdnHCl. The results shown in Fig. 7, A and B, clearly indicate that the volume of I1 was reduced by the addition of PVP, whereas the native protein was not. Previous studies have shown that the unfolded species could exist for 200 ms after the initiation of refolding in buffers containing 1 M GdnHCl (45, 50); thus the peaks observed in Fig. 7C should come from species with a compact structure but not the unfolded state. A calibration of the multiple species in Fig. 7C suggested that the peak contains a monomer and a dimer, which is similar to the elution profile reported by Cleland and Wang (51). Therefore, the strong dimer peak observed for samples both with and without the addition of PVP40 (Fig. 7C) suggested that PVP40 has poor ability to inhibit the formation of aggregation-prone dimers. It was interesting to find that the transient dimer formed during CAB refolding was also affected by PVP40, which suggests that PVP might have the property of binding all aggregation-prone species.

**DISCUSSION**

In general, unproductive aggregation is generated from the intermolecular interactions of the exposed hydrophobic parts of polypeptide chains. The formation of non-native oligomers has been recognized as the initial step of aggregation (1, 13, 39). Thus the protection of the aggregation-prone species by additives can effectively weaken the off-pathway aggregates and enhance the yield of protein refolding. In the case of CAB, it has been found that the first refolding intermediate, I1, is an aggregation-prone species and it can form into a dimer, a multimer, or large aggregates (39, 40). It has been found that many organic compounds can act as chemical chaperones to assist protein refolding and protect proteins from unproductive aggregation (15–23). These chemical folding aids are thought to act through a mechanism similar to the one in protein chaperones, which captures the denatured protein under conditions that would otherwise lead to aggregation but does not interfere with renaturation. In particular, PEG can specifically bind to I1 and increase the refolding yield by forming a new species (I1/PEG complex) that has a low aggregation probability.

Similar to PEG, PVP also has an amphiphilic property but with more hydrophobicity. The highly polar amide group of PVP monomer confers its hydrophilic and polar-attracting properties, whereas the apolar methylene and methyne group in the backbone and the ring contribute to its hydrophobic properties. In this research, it was found that PVP40 could enhance the refolding of GdnHCl-denatured CAB in a concentration-dependent manner (see Fig. 1). The optimal concentration of

**FIGURE 5.** A, intrinsic fluorescence emission spectra of PVP effect of CAB fluorescence in 2 M GdnHCl. The fluorescence was measured at the same experimental settings as in Fig. 4. The CAB concentration was 0.05 mg/ml, equilibrated at 2 M GdnHCl with various concentrations of PVP. PVP concentrations are labeled beside the spectral lines.  B, effect of different concentrations of PVP on the relative fluorescence of CAB in 2 M GdnHCl. The relative fluorescence was defined as the variation in the fluorescence between the samples containing PVP40 (F) and the sample without PVP40 (F0). F0 was taken as 100% to normalize all data to show the percentage changes caused by different concentrations of PVP40.

**FIGURE 6.** Effect of PVP40 on the fluorescence quenching of CAB equilibrium refolding solutions by acrylamide. A, the quenching effect of 0.5 M acrylamide on refolded CAB with (squares) or without (circles) 1% PVP40 monitored as a function of GdnHCl concentration. B, the concentration-dependent quenching effect of acrylamide on samples with (open) or without (filled) 1% PVP40 when refolded in buffers with a GdnHCl concentration of 1.0 M (squares), 1.4 M (triangles), and 2.0 M (circles). The fluorescence of the refolding samples with or without acrylamide was measured as described in the legend for Fig. 4, and the ratio of the fluorescence without acrylamide to that with acrylamide (F0/F) was used to monitor the quenching effect of acrylamide.
PVP40 was dependent on the protein concentration in the refolding buffer, and no stoichiometric relationship was found between the concentration of PVP40 and CAB. This result was quite consistent with the previous observations that PVP40 could inhibit protein aggregation at a relatively low concentration (34–37) but promote aggregation at a high concentration (38). The observation that the increased reactivation was accompanied with the decrease of aggregation during refolding led to the hypothesis that a relatively low PVP40 concentration might have the property of blocking unproductive aggregation. Similar to the mechanism of PEG-enhanced protein refolding, the ability of PVP40 to protect proteins from aggregation was also found to be associated with the interactions between PVP40 and the aggregation-prone intermediate I1. However, further investigation through both kinetic and equilibrium refolding studies indicated that PVP40 enhances the refolding of CAB by a quite different mechanism from that of osmolytes such as PEG (30) and other surfactants (18).

**PVP Enhances Protein Refolding by Accelerating the Refolding of Aggregation-prone Species**—Because CAB has one exposed Trp residue in its native state (52) and six exposed Trp residues in the first refolding intermediate state (53), the changes in the intrinsic fluorescence were used to monitor the effect of PVP40 on CAB. In agreement with previous studies (39), the equilibrium refolding without PVP was found to be a typical three-state transition. Similar to the effect of PEG (30), an increase in the intrinsic fluorescence was found for the samples with 1% PVP40 added (see Fig. 4). In general, chaperone-assisted protein refolding is usually characterized by strong interactions between the chaperone and the protein substrate, and no interference of the refolding pathway has been observed (18, 30, 54). Interestingly, the kinetic analysis (see Figs. 2 and 3) clearly indicated that the existence of 1% PVP affected neither the rate of formation of the first intermediate nor the refolding of I2 to the native state, but it accelerated the formation of I1 from I0 in the folding pathway. The gradually increased rate constant $k_{12}$ might reduce the accumulation of aggregation-prone intermediate I1 and result in the apparent effect of a decrease of aggregation and an enhancement of refolding, as shown in Fig. 1. These results suggested that PVP could not be taken as a chaperone but is more like an “artificial enzyme.”

A comparison of the rate constants of CAB reactivation and refolding indicated that the rate constants of the fast phase of CAB reactivation was similar to $k_{12}$ of refolding, which suggested that the refolding of I2 was the rate-limiting step of CAB reactivation. The existence of the slow phase of CAB reactivation and the time scale of the rate constant further revealed that the reactivation was slower than the refolding of CAB. This result suggested that a slow adjustment step was necessary for CAB to recover its activity even though the structure had refolded to its native-like state (tentatively called N*) as monitored by fluorescence. The fact that only the fast phase of CAB reactivation was slightly accelerated by the addition of PVP also confirmed that PVP accelerated the formation of I2 from I1, resulting in a relatively fast recovery of the CAB activity.

**Associations of PVP Molecules to Aggregation-prone Species**—The conclusion that only I1 was affected by the addition of PVP was also verified by equilibrium refolding (see Fig. 4), the enhancing effect of PVP on the intrinsic fluorescence of I1 (see Fig. 5), the alteration of the quenching ability of acrylamide by PVP (see Fig. 6), and SEC analysis (see Fig. 7). All of these results suggested that PVP could specifically capture the aggregation-prone species I1. Unfortunately, it is impossible to obtain the dissociation constant and binding sites of PVP to I1 because PVP acts as a bifunctional reagent to I1. Low concentrations of PVP increased the fluorescence of I1 as an enhancer, whereas I1 was denatured when PVP concentrations exceeded 0.6%, resulting in a fluorescence red shift (see Fig. 5). Nevertheless, it is clear the binding of PVP to I1 was not stoichiometric. The stoichiometric binding of PEG to I1 significantly reduced the formation of aggregation-prone dimers and aggregates. However, the binding of PVP to I1 might have little effect on the formation of aggregation-prone products. As revealed by Fig. 7C, the SEC analysis showed that the presence of PVP could not inhibit the formation of transient dimer and thus PVP did not affect the aggregation-prone property of I1.

PVP might interact with the amino acid residue side chains or the polypeptide backbone of aggregation-prone species. The well-established mechanisms of protein protection by osmolytes might provide clues to explain the effect of PVP.

**Two major mechanisms have been characterized to explain the effects of osmolytes: “preferential hydration”**, (25), which indicates that the exclusion of the cosolvent molecules from the protein surface leads to a minimization of the protein surface and thus increases the protein stability; and “solvophobic thermodynamic force” (24, 27), which indicates that the unfavorable interaction between the osmolytes and the peptide backbone raises the free energy of the denatured state and thus stabilizes the native state. In this research, it was found that the binding of PVP molecules to I1 resulted in a reduction of its surface, as revealed by SEC (Fig. 7B) and fluorescence analysis (Figs. 4–6). Moreover, the increase of $k_{2}$ determined by kinetic analysis (Fig. 3) suggested that a decrease of the energy barrier between I1 and I2 might be induced by the binding of PVP. These results suggested that the binding of PVP to aggregation-prone species might have a similar mechanism to that of osmolytes. This conclusion could also explain previous findings of increased thermal stability by PVP (34–37). However, the property of specific binding to aggregation-prone species by PVP is similar to that of PEG but is not found in osmolytes.

The failure of high concentration PVP to protect proteins from aggregation (Fig. 1) might also be due to its property of binding to aggregation-prone species. As presented in Fig. 7, the volumes of both the intermediate I1 and the aggregation-prone dimer were reduced by the addition of PVP. This result was quite consistent with the previous study.
A Working Model of Enhancing Protein Refolding by PVP—The mechanisms of prevention of aggregation by the two kinds of polymers, PEG and PVP, are quite different although they have similar properties; both are amphiphilic and water-soluble, and they have hydrophobic moieties capable of binding to proteins. Both PEG and PVP can specifically bind to the first intermediate. For PEG, this interaction facilitates the refolding of I₁ to I₂, whereas the binding to the dimer might facilitate the aggregation pathway. This hypothesis was verified by the observation of the protein concentration dependence of the optimal concentration of PVP to protect proteins from aggregation. The higher protein concentration had a relatively high accumulation of I₁ and the aggregation pathway. This hypothesis was verified by the observation of the protein concentration dependence of the optimal concentration of PVP to protect proteins from aggregation. 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