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Multi-block poloxamer surfactants suppress aggregation of denatured proteins

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Abstract

On the basis of elastic light scattering, we have compared the capacity of the multi-block, surfactant copolymers Poloxamer 108 (P108), Poloxamer 188 (P188), and Tetronic 1107 (T1107), of average molecular weight 4700, 8400, and 15,000, respectively, with that of polyethylene glycol (PEG, molecular weight 8000) to suppress aggregation of heat-denatured hen egg white lysozyme (HEWL) and bovine serum albumin (BSA). We also compared the capacity of P188 to that of PEG to suppress aggregation of carboxypeptidase A denatured in the presence of trifluoroethanol and to facilitate recovery of catalytic activity. In contrast to the multi-block copolymers, PEG had no effect in inhibiting aggregation of HEWL or of carboxypeptidase A with the recovery of catalytic activity. At very high polymer:protein ratios ($\geq$ 10:1), PEG increased aggregation of heat-denatured HEWL and BSA, consistent with its known properties to promote macromolecular crowding and crystallization of proteins. At a polymer:protein ratio of 2:1, the tetra-block copolymer T1107 was the most effective of the three surfactant copolymers, completely suppressing aggregation of heat-denatured HEWL. At a T1107:BSA ratio of 10:1, the poloxamer suppressed aggregation of heat-denatured BSA by 50% compared to that observed in the absence of the polymer. We showed that the extent of suppression of aggregation of heat-denatured proteins by multi-block surfactant copolymers is dependent on the size of the protein and the copolymer:protein molar ratio. We also concluded that at least one of the tertiary nitrogens in the ethylene-1,2-diamine structural core of the T1107 copolymer is protonated, and that this electrostatic factor underlies its capacity to suppress aggregation of denatured proteins more effectively than nonionic, multi-block poloxamers. These results indicate that amphiphilic, surfactant, multi-block copolymers are efficient as additives to suppress aggregation and to facilitate refolding of denatured proteins in solution. Because of these properties, multi-block, surfactant copolymers are suitable for application to a variety of biotechnological and biomedical problems in which refolding of denatured or misfolded proteins and suppression of aggregation are important objectives.

Keywords: Carboxypeptidase A; Light scattering; Lysozyme; Multi-block surfactant copolymer; Poloxamer; Protein denaturation; Serum albumin

1. Introduction

Refolding of proteins in non-native states and suppression of aggregation are important processes that occur under physiological conditions *in vivo* or that can be achieved through intervention *in vitro* with synthetic additives. A unifying principle common to both situations facilitating the recovery of biological function of proteins is mutual interaction of hydrophobic surfaces: *In vivo* the solvent exposed, hydrophobic residues of misfolded or denatured proteins interact with the surface of the inner cavity of chaperones [1,2]. In experimental systems *in vitro*, we have shown in preliminary studies that the exposed hydrophobic residues of denatured proteins can interact with the surface of hydrophobic parts of synthetic additives introduced to facilitate refolding [3]. While the presence of multi-component chaperones in the cytosol is sufficient to prevent accumulation of aggregates of misfolded proteins under ordinary conditions, abrupt accumulation of aggregates of proteins can

Abbreviations: BSA, bovine serum albumin; CPA, carboxypeptidase A; ClCPL, *O*-[*trans-para*-chloro-cinnamoyl]-L-β-phenyllactate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfo-nate; HEWL, hen egg white lysozyme; PEG, polyethylene glycol; TFE, trifluoroethanol

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occur through pathophysiological processes underlying disease [4,5] or, for instance, through thermal, electrical, or physical trauma requiring medical attention [6–8]. An important objective in our laboratory has been to develop use of multi-block, surfactant copolymers as synthetic agents administered through intravenous transfusion that can facilitate recovery from massive cell injury. A critical requirement of such polymers is the capacity to facilitate restoration of membrane function and to facilitate recovery of biological function and of the native structure of denatured proteins.

The chemical bonding structures of multi-block copolymers are schematically illustrated in Fig. 1. The surfactant copolymers known as Poloxamer 108 and Poloxamer 188 possess a tri-block structure comprised of two hydrophilic, polyoxyethylene (EO) segments and one central, hydrophobic, polyoxypropylene (PO) segment joined through ether oxygen linkages in an *a*:*b*:*a* construct. They are of average molecular weight 4700 and 8400, respectively. In contrast, the tetra-block copolymer Tetronic 1107, of average molecular weight 15,000, possesses a central ethylene-1,2-diamine skeleton to which the polyoxypropylene segments are joined to form a hydrophobic core with terminal, hydrophilic, polyoxyethylene segments flanking the hydrophobic core [9]. The T1107 copolymer is, thus, seen to contain two tertiary, organic nitrogens buried within the central hydrophobic core unlike the non-ionic P108 and P188 copolymers.

Of the tri-block surfactants, P188 has been used more extensively than P108 in pharmacological and physiological studies [10–14]. We have previously demonstrated that P188 significantly improves survival of cells following injury in which loss of membrane integrity occurs [6,7,10]. The results of these investigations, thus, demonstrate that the P188 copolymer is associated with a significant capacity to facilitate repair of damaged membranes. In addition, administration of P188 has been shown to mitigate the duration and pain associated with sickle-cell crises [15]. In contrast to multi-block poloxamer surfactants, PEG is comprised only of a hydrophilic polyoxyethylene chain and is non-ionic. Nonetheless, PEG, available as a commercial product over a broad range of molecular weight (200–500,000), has been employed to facilitate refolding of denatured proteins and to suppress their aggregation [16–18] and to restore nerve cell function by facilitating repair of damaged cell membranes [19–21]. The strikingly different character of poloxamer surfactants comprised of hydrophobic and hydrophilic segments from that of PEG suggests that they promote refolding of proteins and repair of cellular membranes through different mechanisms.

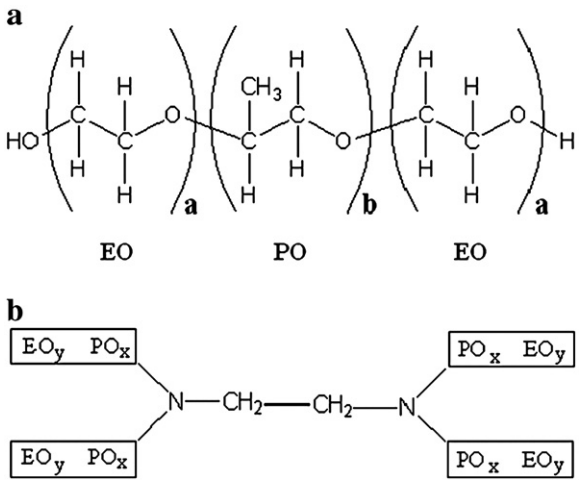

Fig. 1. Illustration of the chemical bonding structures of amphiphilic, surfactant copolymers employed in this study: (a) the tri-block copolymers P188 (*a*:*b*:*a*=75:31:75) and P108 (*a*:*b*:*a*=43:16:43); and (b) the tetra-block copolymer T1107 (*x*=78, *y*=243). PEG consists of only oxyethylene (EO) units and is classified, therefore, as a uni-block polymer. With reference to the structural formula a above for PEG-8000, *a*=181, *b*=0, as used in this study.

In a preliminary investigation, we compared the capacity of P188 and PEG (average molecular weight 8000) to facilitate recovery of the catalytic activity of heat-denatured HEWL [3]. We observed that the surfactant poloxamer restored catalytic action of the enzyme to a significant extent while PEG had no effect. Because our results with respect to PEG appeared to contradict the observations of others with respect to aggregation and refolding of denatured proteins [16–18,22–24], we have investigated these differences in greater detail. On the basis of elastic light scattering measurements, we have compared the capacity of P108, P188, and T1107 to that of PEG to suppress aggregation of thermally denatured HEWL and BSA. We have also compared the capacity of P188 to that of PEG to promote recovery of catalytic activity of chemically denatured carboxypeptidase A (CPA).

Because the polymers employed in this study differ in molecular weight, they differ from each other not only with respect to hydrodynamic volume and dimensions but also with respect to their size relative to each protein. Accordingly, we have paid close attention to the stoichiometry of added polymer to each protein in solution. PEG showed essentially no capacity to retard aggregation of the denatured proteins and did not restore catalytic function of denatured CPA. As the polymer:protein ratio was increased, light scattering results showed that PEG promoted only an increase in the molecular weight and concentration of protein aggregates, consistent with its characteristic properties of macromolecular crowding [25–27]. On the other hand, the capacity of the surfactant poloxamers to reduce accumulation of protein aggregates was significant and followed the order T1107>P188>P108.

2. Materials and methods

2.1. Materials

Lyophilized HEWL, fatty-acid free BSA, and bovine pancreatic CPA (Cox preparation) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The surfactant copolymers P108, P188, and T1107 were obtained from BASF Corporation (Mt. Olive, NJ, USA) and used as received. The mean molecular weights were 4700, 8400, and 15,000 for P108, P188, and T1107, respectively. PEG (average molecular weight 8000) and TFE were obtained from Sigma-Aldrich (Milwaukee, WI, USA). HEWL was dissolved in 150 mM KCl buffered to pH 7 with 55 mM potassium phosphate; BSA was dissolved in 150 mM KCl

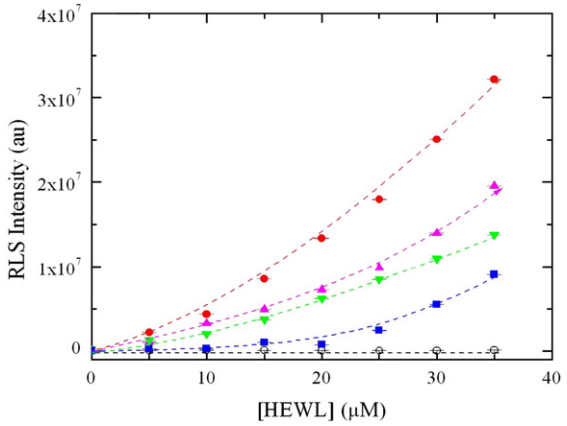

Fig. 2. Intensity of Rayleigh light scattering of HEWL solutions in the absence and presence of surfactant copolymers as a function of protein concentration. Each copolymer was added to a final 2:1 molar ratio with respect to protein. Conditions are: ○, scattering intensity prior to heating of HEWL-polymer solution; ●, HEWL solution after heating in the absence of added poloxamer; ▲, HEWL+P108; ▼, HEWL+P188; ■, HEWL+T1107. All protein solutions were optically clear after addition of the surfactant copolymer. In the absence of protein, the surfactant copolymers did not give rise to measurable changes in light scattering either prior to heating or after re-equilibration to 25 °C. HEWL solutions of concentration higher than 50 μM turned cloudy upon heating and consequently could not be investigated. The ordinate scale indicates the Rayleigh light scattering (RLS) intensity in absorption units (au).

buffered to pH 7 with 20 mM HEPES, and CPA was dissolved in 500 mM KCl buffered to pH 7.5 with 50 mM HEPES. De-ionized distilled water was used throughout. The specific esterolytic substrate of CPA *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (ClCPL) was synthesized in association with earlier studies [28]. Concentrated solutions of each polymer were prepared gravimetrically just before use in the same buffer as that of the protein to which they were added. No change in pH was detected after addition of the polymer to the protein solution.

2.2. Methods

Protein aggregation was monitored on the basis of Rayleigh light scattering at 500 nm with use of a Jobin-Yvon Horiba® FluoroMax-3 fluorometer (Edison, NJ, USA). To optimize monitoring of the intensity of scattered light, a band pass of 1 nm was used for incident and scattered light, with data collection programmed for a maximum of 5 trials, 10-s integration time, and a standard error of 3–5%. Prior to monitoring the scattering intensity of protein solutions, the scattering intensity for toluene was measured as a standard for a one-component solvent system.

Denaturation of HEWL and BSA was induced thermally while denaturation of CPA was induced with aid of TFE, for reasons explained below. 3.0-ml Solutions of HEWL or BSA diluted to the required concentration were placed in 15-ml Falcon tubes, as previously described [3]. The concentration of each protein was determined from the optical density at 280 nm using extinction coefficients of 3.65×10^4 and 4.36×10^4 M^{-1} cm^{-1} for HEWL and BSA, respectively, and 6.42×10^4 M^{-1} cm^{-1} for CPA [29]. In our preliminary study in which only HEWL was employed, we added the polymer to the enzyme solution after denaturation and temperature equilibration [3]. In the present study the polymer:protein ratio, at times exceeding 50:1, would have required considerably larger volumes of the surfactant copolymer to be added to the heat-denatured protein. In order to treat each copolymer–protein mixture identically and to avoid differential dilution of the protein solutions by addition of each polymer solution to achieve the required polymer:protein ratio, HEWL or BSA solutions were heated to 90 °C in a water-bath for 30 min, either with or without added polymer. The polymer:protein mixture was then placed in a 25 °C bath for 30 min for temperature re-equilibration. In each case, the intensity of light scattering was measured before heating and after re-equilibration to 25 °C.

Initial velocity data for the hydrolysis of ClCPL catalyzed by CPA were collected spectrophotometrically at 310 nm as described earlier [28]. TFE was added to 10% or 20% (v:v) final concentration, and the mixture of enzyme and TFE was incubated at 24 °C, as indicated in figure legends. To determine the capacity of P188 to facilitate recovery of catalytic activity after denaturation, aliquots of P188, dissolved in the 500 mM KCl buffered to pH 7.5 with 50 mM HEPES buffer, were added to the enzyme:TFE mixture.

3. Results

3.1. Lysozyme

Fig. 2 compares the Rayleigh light scattering intensity of solutions of HEWL prior to and after heating to 90 °C for 30 min followed by temperature re-equilibration to 25 °C. While the light scattering intensity for HEWL solutions did not vary significantly with protein concentration prior to heating, it is seen that the intensity increased significantly after heating and re-equilibration to 25 °C. Compared to the increase in light scattering intensity observed for HEWL solutions with no added polymer (labeled with closed circles), addition of all three surfactants resulted in significantly decreased light scattering intensity.

Fig. 3 correspondingly compares the capacity of surfactant copolymers to suppress aggregation of heat-denatured HEWL to that of PEG. Here we have employed HEWL at a concentration of 15 μM with polymer added in each case to a final 2:1 polymer:HEWL molar ratio. As evident also in Fig. 2 for 15 μM HEWL, the increase in light scattering intensity associated with heating to 90 °C and re-equilibration to 25 °C in Fig. 3 is essentially completely annihilated by the T1107 tetra-block copolymer while the P188 tri-block copolymer was slightly more effective than P108. PEG, on the other hand, was

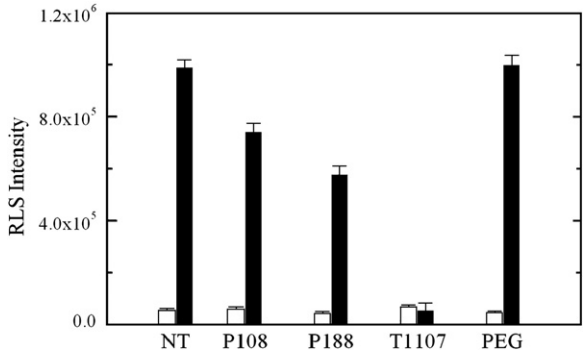

Fig. 3. Histogram comparison of the influence of surfactant polymers on the intensity of Rayleigh light scattering of HEWL after heating to 90 °C. The HEWL concentration was 15 μM in 150 mM KCl buffered to pH 7 with 50 mM potassium phosphate. In each case, the indicated polymer was added to a final 2:1 (polymer:protein) molar ratio, NT designating no treatment or addition of polymer. Each polymer:protein mixture, including that with PEG, was optically clear. Open bars represent the polymer:protein mixture prior to heating; solid bars represent the mixture after re-equilibration to 25 °C. The error bars at the top of each vertical rectangle of the histogram indicate the standard deviation of the average value observed for three separately prepared solutions.

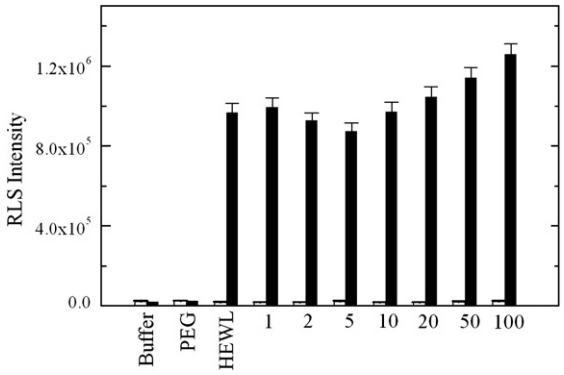

Fig. 4. Histogram comparison of the influence of PEG on the intensity of Rayleigh light scattering of HEWL after heating to 90 °C. Solutions of HEWL, as in Fig. 3, were prepared to a final protein concentration of 15 μM, and aliquots of PEG dissolved in an identical buffer were added. The resultant polymer:protein molar ratios 1 (1:1), 2 (2:1), … 100 (100:1) are indicated along the abscissa. Open rectangles represent light scattering prior to heating and solid, filled rectangles represent light scattering after heating and equilibration to 25 °C. PEG indicates the light scattering of a solution of PEG at the highest concentration used in the absence of protein, and HEWL indicates 15 μM protein in the absence of PEG. Other conditions were as described in Fig. 3.

associated with no reduction of light scattering of the protein solution after heating.

While the results in Fig. 3 pertain only to 2:1 polymer:protein mixtures, we have also investigated whether PEG has greater capacity to reduce the increase in light scattering at higher polymer:protein ratios. The results are shown in Fig. 4. As seen in this diagram, the light scattering intensity for PEG:HEWL mixtures after re-equilibration to 25 °C remains essentially constant up to a 10:1 molar ratio and does not differ significantly from that for heat-treated HEWL in the absence of PEG. In contrast, there is a monotonic increase in the intensity of light scattering of PEG:HEWL mixtures for mixtures with a PEG:HEWL ratio > 10:1.

3.2. Albumin

We have assumed that, in addition to the concentration of polymer relative to protein, i.e., the polymer:protein ratio, the molecular dimensions of the polymer relative to those of the hydrated protein in solution are also likely to influence its capacity to decrease light scattering intensity upon thermal denaturation of the protein. For this reason, we have compared the behavior of BSA to that of HEWL upon thermal denaturation in the presence and absence of each of the four polymers employed. With a molecular weight more than five-fold greater than that of HEWL, the dimensions of BSA as a hydrated, globular protein in solution are necessarily correspondingly greater.

Fig. 5 compares the influence of the tri-block P188 copolymer, the tetra-block T1107 copolymer, and PEG on the light scattering intensity of heat-denatured BSA upon re-equilibration of the solution to 25 °C. Similarly to the results illustrated for the P188 copolymer in Fig. 5, the P108 copolymer was also without effect in reducing light scattering intensity (data not shown). Of the multi-block surfactant copolymers, T1107 alone exhibited capacity to reduce the light scattering intensity of heat-denatured BSA. As evident in Fig. 5, the light scattering intensity is reduced with increasing T1107:BSA ratio, and the light scattering intensity of the mixture with a 10:1 copolymer:protein ratio was approximately 50% of that in the absence of the surfactant. However, a further increase in the T1107:BSA ratio up to 20:1 was without additional effect.

In contrast to the results associated with the multi-block T1107 copolymer, the light scattering intensity of PEG:BSA mixtures increased with increasing PEG:BSA ratio, as illustrated in Fig. 5, in sharp contrast to the influence of the tetra-block surfactant copolymer T1107. A similar influence of PEG on heat-denatured HEWL was noted in Fig. 4. The light scattering intensity of heat-denatured BSA in the presence of a 10^5-fold excess of PEG (equivalent to ca. 100 mM PEG) is nearly 3-fold greater than that of the protein in the absence of PEG. These observations demonstrate that the interaction of PEG with both proteins must differ substantively from that associated with the multi-block surfactant copolymers.

3.3. Carboxypeptidase A

As a Zn^{2+}-containing metalloenzyme, CPA presents a complex problem to identify conditions under which residual catalytic activity can be measured as an index of recovery of protein structure after denaturation. Heating to 90 °C causes release of the metal ion with irreversible loss of catalytic activity. Addition of excess Zn^{2+} to the protein solution to retard release of the active site metal ion results in protein precipitation upon heating. For these reasons, it was necessary to find a suitable

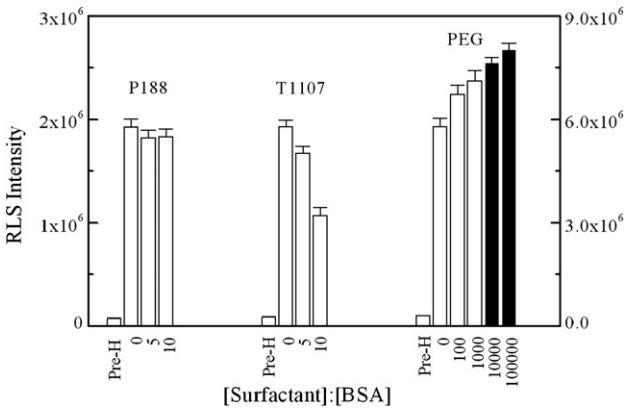

Fig. 5. Histogram comparison of the influence of surfactant polymers on the intensity of Rayleigh light scattering of BSA after heating to 90 °C. To BSA solutions, prepared in 150 mM KCl buffered to pH 7 with 20 mM HEPES, aliquots of each surfactant polymer were added to achieve the polymer:protein molar ratios indicated along the abscissa. The final protein concentration was 10 μM in each case. Each polymer–protein mixture was optically clear prior to and after heating to 90 °C. In contrast to HEWL, however, BSA solutions, up to concentrations approaching ~100 μM, showed no visible precipitation upon heating to 90 °C. The ordinate scale on the left applies to open vertical rectangles while the ordinate scale on the right-hand side applies to the two filled vertical rectangles for PEG:BSA ratios of 10,000:1 and 100,000:1. Pre-H indicates the light scattering of the solution prior to heating to 90 °C. The integral numbers under each rectangle indicate the polymer:BSA molar ratio of the solution.

chemical method for reversible denaturation of CPA. In the presence of 50% (v:v) TFE, the enzyme becomes completely inactive within 15 min at the concentrations approaching those required for the steady-state approximation. On the other hand, incubation of the enzyme in the presence of 20% (v:v) TFE allowed measurement of catalytic activity for several hours.

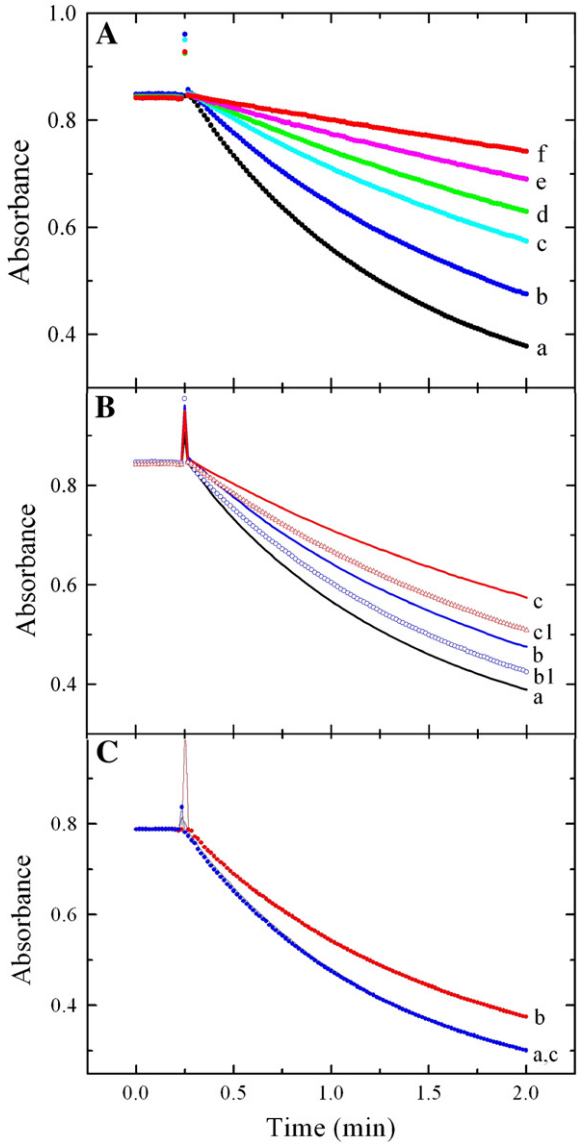

Fig. 6. Influence of P188 surfactant polymer and TFE on the catalytic activity of CPA. (A) Progress curves of the hydrolysis of ClCPL catalyzed by CPA at 24 °C in the presence of 20% (v:v) TFE. CPA and ClCPL concentrations were 1.7×10^{-7} M and 5.6×10^{-5} M, respectively. Traces correspond to the following conditions: *a*, absence of TFE; *b–f*, incubation for 30, 60, 90, 120, and 170 min, respectively, in 20% TFE prior to addition of substrate. (B) Catalytic activity of CPA in the absence of TFE (trace *a*) and after 30- and 60-min incubation in the presence of 20% TFE (traces *b* and *c*, respectively); correspondingly, traces *b*1 and *c*1 represent catalytic activity after incubation of the enzyme in the presence of 20% TFE with P188 added to a 1:1 polymer:CPA molar ratio. (C) Progress curves of the hydrolysis of ClCPL catalyzed by CPA, as in panel A, under the following conditions: *a*, CPA in absence of TFE; *b*, after 48-h incubation in the presence of 10% TFE at 24 °C; and *c*, 48-h incubation in the presence of 10% TFE and P188 added to a 1:1 polymer:CPA molar ratio.

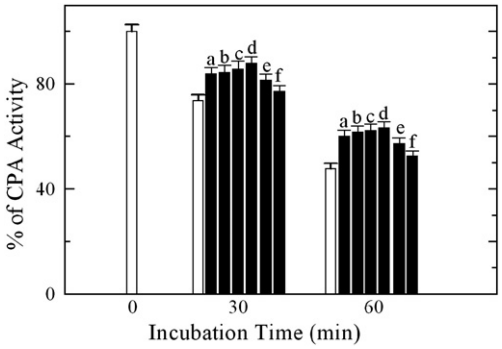

Fig. 7. Histogram comparison of residual catalytic activity of CPA remaining after denaturation in 20% TFE for 30 or 60 min, respectively, in the presence of P188 at the following P188:CPA ratios: *a*, 10:1; *b*, 5:1; *c*, 2:1; *d*, 1:1; *e*, 0.5:1 and *f*, 0.3:1. Open vertical rectangles provide comparison to conditions with no added TFE (0-min incubation time) and no added P188 at 30- and 60-min incubation in the presence of 20% TFE.

Therefore, we collected initial velocity data for the hydrolysis of ClCPL catalyzed by CPA as a function of P188:CPA or PEG:CPA molar ratio and as a function of incubation time of the enzyme in the presence of 10 or 20% (v/v) TFE.

Fig. 6 illustrates results quantifying the influence of TFE and the P188 surfactant copolymer on the catalytic activity of CPA. While incubation of the enzyme in 20% (v:v) TFE, as illustrated in Fig. 6A, resulted in a gradual decrease in residual catalytic activity for up to ~3 h, Fig. 6B shows that the P188 copolymer significantly retarded loss of catalytic activity associated with addition of TFE over the same time period. Furthermore, as seen in Fig. 6C, the presence of the copolymer at a 1:1 P188:CPA ratio completely protected the enzyme against denaturation in the presence of 10% (v:v) TFE. Fig. 7 compares the capacity of P188 to retard loss of catalytic activity of CPA in the presence of 20% (v:v) TFE. As seen in Fig. 7, as long as the P188:CPA molar ratio was ≥ 1.0, the protective influence of the copolymer, within experimental uncertainty, was maximal. PEG at equivalent polymer:CPA ratios exhibited no capacity to retard loss of catalytic activity in the presence of TFE.

4. Discussion

The importance of classical elastic light scattering, known as Rayleigh scattering, from particles in solution of dimension no greater than $\lambda/20$ (where λ is the wavelength of incident light) is that the intensity of the scattered radiation is proportional to the molecular weight and concentration of the solute particle [30]. Upon denaturation, the molecular dimensions of the globular proteins employed in this investigation do not change significantly with respect to the wavelength of incident light (in this case 5000 Å). Therefore, changes in the intensity of scattered light reflect directly variations in the concentration and molecular weight of aggregates of protein molecules. As the number of protein molecules increases in an aggregate or the concentration of aggregates increases, the more intensely is light scattered. With respect to the increase in light scattering upon denaturation, the behavior of all three multi-block

surfactant copolymers employed in this study was sharply distinguished from that of PEG. The surfactant copolymers suppressed aggregation of lysozyme and BSA upon thermal denaturation, as illustrated in (Figs. 2, 3 and 5), or loss of catalytic activity of CPA upon chemical denaturation, as illustrated in Figs. 6 and 7. On the other hand PEG either had no effect, as illustrated in Fig. 3, or caused an increase in light scattering, as illustrated in Figs. 4 and 5. The increase in light scattering of denatured proteins in the presence of PEG indicates a concomitant increase in the concentration or size of aggregates of the denatured protein. This latter observation is reflective of the well-established phenomenon of "macromolecular crowding" associated with PEG [25–27].

In preliminary studies we demonstrated that addition of the tri-block P188 copolymer to HEWL solutions in 2:1 copolymer: protein stoichiometry after heat denaturation restores enzyme activity up to 85% of that associated with the native protein [3]. The results in Fig. 3 show that the P188 polymer, when present in 2:1 copolymer:protein ratio during heat denaturation, suppresses HEWL aggregation by approximately 34% of that observed in the absence of poloxamer. The combined results, thus, demonstrate that the surfactant copolymer both facilitates refolding of the thermally denatured protein with recovery of catalytic activity and suppresses aggregation. While others have concluded that PEG facilitates refolding of proteins [16–18,22–24], our light scattering results unambiguously demonstrated that the protein remains highly aggregated in the presence of PEG. Comparable observations have been reported for heat-denatured HEWL in the presence of PEG (molecular weight 2000) [31]. Similarly, van den Berg and coworkers [32,33] have pointed out that macromolecular crowding agents increase aggregation of urea denatured HEWL. While we have not compared the influence of the P108 and T1107 copolymers on restoration of enzymatic activity, as in our earlier study [3], the results in Figs. 3 and 5 imply that their interaction with the heat-denatured protein similarly facilitates recovery of catalytic activity and suppression of aggregation. Our observations incisively demonstrate, therefore, a fundamental difference in the interaction of multi-block surfactant copolymers with denatured proteins from that associated with PEG.

In contrast to the polyoxyethylene, uni-block structure of PEG, the surfactant copolymers employed in this investigation are constructed of both hydrophilic and hydrophobic blocks (cf., Fig. 1). The different interaction of each surfactant copolymer compared to that of PEG must derive from the presence of the hydrophobic portion of the copolymer. A common characteristic of denatured or unfolded proteins that has emerged from a variety of chemical and physical studies is their tendency to have surfaces of hydrophobic amino acid side chains exposed to solvent. While ordinarily these residues are located in the interior regions of proteins and are shielded from bulk solvent, the unfolded form of a protein is likely best characterized as having a collapsed structure of multiple substrates in which different hydrophobic regions from the interior are in dynamic equilibrium with respect to the extent of solvent exposure. We suggest that the hydrophobic surfaces of exposed amino acid residues of the denatured proteins are attracted to the hydrophobic block regions of surfactant copolymers. The interaction between the hydrophobic regions of the surfactant copolymer with the hydrophobic side chains from the interior of the protein will be entropically favored because the more tightly hydrogen bonded water molecules on the surface of exposed hydrophobic side chains become displaced into bulk solvent. The interaction of the two hydrophobic surfaces with each other can then lead to hydrophobic collapse, nucleation, and secondary structure formation through a series of structural rearrangements. In this respect, the refolding of denatured proteins facilitated by multi-block surfactant copolymers is similar to the interaction between non-native forms of proteins in the cavity of bacterial chaperones. The action of bacterial chaperones also shifts the overall folding equilibrium away from aggregation.

While the behavior of the multi-block surfactant copolymers in general differed sharply from that of PEG, the tetra-block copolymer T1107 was more effective than P108 or P188 in suppressing aggregation of heat-denatured protein, as illustrated in (Figs. 2, 3 and 5). In fact, Fig. 3 shows that T1107 at a polymer:HEWL ratio of 2:1 completely suppressed aggregation of the heat-denatured enzyme in contrast to the lesser influence of the P108 and P188 copolymers. The chemical bonding structure of T1107 in Fig. 1 shows two tertiary nitrogens within the ethylene-1,2-diamine structural core of the central portion of the copolymer. Each nitrogen must possess, therefore, basicity comparable to that of the nitrogen in triethylamine. To our knowledge, no one has drawn attention to this feature of the central ethylene-1,2-diamine core structure of the T1107 surfactant copolymer. Nonetheless, we consider that at least one of the nitrogens must be protonated in the neutral pH range resulting in an overall charged character associated with the polymer. While it has been pointed out that amino acids, including spermine, and spermidine, hinder heat-induced inactivation and aggregation of HEWL [31], our attention has been brought to the greater capacity of alkyl esters of arginine to suppress aggregation of heat-denatured proteins and to enhance the solubility of aggregation-prone proteins [34]. In particular, the capacity of the ethyl ester of arginine to suppress aggregation was shown to depend on its positive charge. Through studies examining the capacity of arginine ethyl ester to suppress aggregation, it is thought that the hydrophobic portion of the side chain adjacent to the ethyl carboxylate group likely binds to solvent exposed hydrophobic residues of heat-denatured proteins. Since aggregation appears to occur through binding of solvent exposed hydrophobic residues between protein molecules, the protein-bound ethyl ester of arginine prevents heat-induced aggregation through an electrostatic effect. We suggest that the greater capacity of T1107 to suppress aggregation of heat-denatured proteins over that associated with the non-ionic, tri-block copolymers has its origin in a similar electrostatic effect. It is of interest to point out that there is a similar electrostatic influence in the binding of denatured or misfolded proteins that are recognized as "substrates" by the bacterial chaperone GroEL [35].

The results of our investigations reported here and earlier [3] on the influence of surfactant copolymers to suppress aggregation of denatured proteins and to facilitate recovery of the catalytic

activity of enzymes underline our interpretation that surfactant poloxamers do not promote refolding and recovery of function through macromolecular crowding, known as the excluded volume effect. The principle behind macromolecular crowding to facilitate refolding of proteins from non-native states depends sensitively on the available volume and the total fractional volume occupancy of "background," i.e., inert macromolecules [24–27]. While excluded volume theory predicts that the compact or native state of a protein molecule is stabilized under crowded conditions in comparison to the less compact, partially unfolded state, association rate constants may become orders of magnitude larger than in dilute solution. The consequence of these competing processes is aggregation of proteins in non-native states decreasing the yield of correctly refolded molecules. For these reasons, refolding experiments necessarily are carried out under very dilute conditions, e.g., ≤ 5 μM. In fact, correct refolding of HEWL is generally abolished at higher concentrations [32,33]. In this respect, our earlier results, demonstrating recovery of catalytic activity of heat-denatured 50 μM HEWL upon addition of P188 at a copolymer:protein ratio of 2:1 in comparison to no effect with PEG [3], is all the more significant.

The processes leading to aggregation of proteins in non-native states can be described through the equilibria in Eq. (1):

$$N \rightleftarrows U \rightleftarrows (U)_n \quad (1)$$

where N represents the protein in its lowest free energy, native conformation, U represents the unfolded protein, and $(U)_n$ represents aggregated forms of the unfolded protein, the formation of which is favored through association of surfaces of exposed hydrophobic amino acid side chains with each other. Through this simplified reaction scheme the differences between refolding of U to the native state N promoted through the excluded volume effect of inert macromolecules and refolding facilitated by poloxamer surfactants are readily understood. Macromolecular crowding influences U to refold to the more compact N state; however, macromolecular crowding under conditions of high concentrations of the unfolded protein readily leads to the association of U to form aggregates decreasing the yield of correctly refolded protein molecules. The extended volume effect is seen, thus, as resulting from a change in thermodynamic activity due to the presence of the "background" macromolecule in the solution and requires no physical contact between the unfolded protein and the inert macromolecule. On the other hand, poloxamer surfactant molecules interact physically with protein molecules in the U state through contact of solvent exposed hydrophobic residues with the hydrophobic units of the poloxamer, displacing the equilibrium between U and $(U)_n$ so as to suppress accumulation of aggregates. The associative interaction of the hydrophobic parts of poloxamer surfactants with the solvent exposed hydrophobic side chains of the unfolded protein leads to release of the protein into its native, fully hydrated conformation. In this manner, the poloxamer competitively binds to exposed hydrophobic side chains of a protein, preventing their association with hydrophobic, solvent exposed residues of other unfolded protein molecules. The extent of the displacement of the equilibrium between U and $(U)_n$ promoted by the poloxamer, therefore, depends on the thermodynamic favoredness of the hydrophobic poloxamer: protein interactions over the interactions of solvent exposed hydrophobic residues with other protein molecules.

Since the association of unfolded protein molecules leading to formation of aggregates occurs through the interaction of patches of solvent exposed hydrophobic residues with each other, the importance of the influence of electrostatic interactions, as discussed in the case of the alkyl esters of arginine [34], is readily explained through Eq. (1). When an unfolded protein molecule binds to the hydrophobic region of a charged poloxamer (P^+), an equilibrium of the type $U+P^+ \rightleftarrows U{:}P^+$ becomes established, removing the protein from participating in the $U \rightleftarrows (U)_n$ equilibrium. The resultant effect displaces the $U \rightleftarrows (U)_n$ equilibrium further away from formation of protein aggregates. The interactions of hydrophobic regions of the charged molecule with the solvent exposed residues of the protein in a non-native state must similarly lead to release of the protein in its fully hydrated, native state. Comparable to the suppression of aggregation of heat-denatured proteins promoted by α(s)-casein [36,37] and alkyl esters of arginine [34], we suggest that the greater capacity of the tetra-block surfactant copolymer T1107 to facilitate refolding compared to the non-ionic, tri-block copolymers P108 and P188, as illustrated in (Figs. 2, 3, and 5), is due to its hydrophobic segments containing at least one buried, protonated, organic nitrogen from its ethylene-1,2-diamine structural core. While totally non-ionic surfactant copolymers have been generally exploited for biological applications, the results of our investigations indicate that multi-block surfactant copolymers such as T1107 are superior in capacity to suppress aggregation of denatured proteins. In fact, it has been demonstrated that the T1107 surfactant also promotes repair of damaged cellular membranes [38–40]. The T1107 surfactant may consequently be the optimal, multi-block surfactant to achieve repair of cellular membranes and suppress aggregation of unfolded proteins in instances of massive cell injury.

5. Conclusions

Macromolecules, such as PEG, facilitate refolding of denatured proteins through an excluded volume effect generally called macromolecular crowding. However, under conditions of high protein concentrations, there is little accumulation of refolded proteins because the presence of such "inert background macromolecules" leads to greatly increased rates of association and aggregation of denatured proteins without recovery of function. Treatment of massive cell injury, as can occur in instances of burn, electrical shock, and physical trauma, requires counter-measures against widespread membrane damage, loss of cytosolic contents, and denaturation of proteins in the serum and interstitial fluid. Under such conditions, intravenous transfusion for fluid replacement can be used to introduce synthetic agents designed to facilitate membrane repair and recovery of function of denatured proteins. Although solutions of inert macromolecules operating according to the excluded volume effect have been shown to

facilitate membrane damage, they cannot be effective in refolding of proteins with recovery of function *in vivo* because of the high concentration of proteins in the interstitial fluid and blood. The results of our light scattering studies demonstrate that multi-block surfactant copolymers suppress aggregation under conditions of high protein concentrations because they facilitate refolding through a different mechanism. They have also been shown to facilitate membrane repair. For these reasons, we anticipate that the use of multi-block, surfactant copolymers administered through intravenous transfusion as synthetic agents to facilitate recovery from massive cell injury will become increasingly favored for clinical applications.

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