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Effects of Polymer Hydrophobicity on Protein Structure and Aggregation Kinetics in Crowded Milieu

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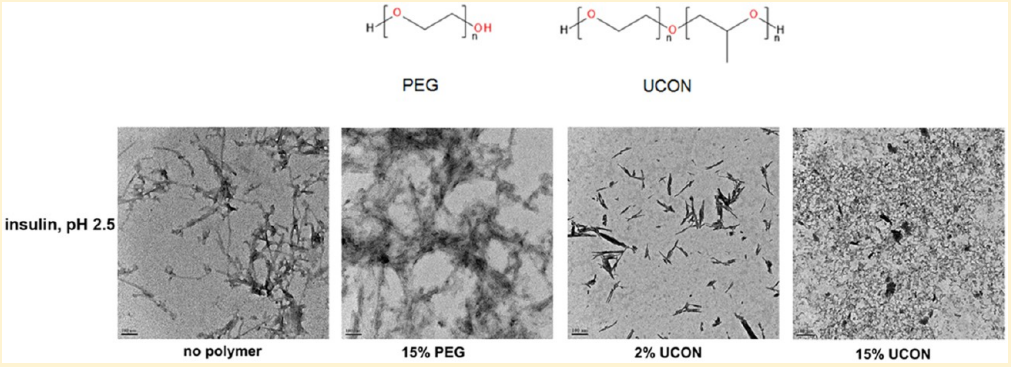
Supporting Information



ABSTRACT: We examined the effects of water-soluble polymers of various degrees of hydrophobicity on the folding and aggregation of proteins. The polymers we chose were polyethylene glycol (PEG) and UCON (1:1 copolymer of ethylene glycol and propylene glycol). The presence of additional methyl groups in UCON makes it more hydrophobic than PEG. Our earlier analysis revealed that similarly sized PEG and UCON produced different changes in the solvent properties of water in their solutions and induced morphologically different α-synuclein aggregates [Ferreira, L. A., et al. (2015) Role of solvent properties of aqueous media in macromolecular crowding effects. *J. Biomol. Struct. Dyn.*, in press]. To improve our understanding of molecular mechanisms defining behavior of proteins in a crowded environment, we tested the effects of these polymers on secondary and tertiary structure and aromatic residue solvent accessibility of 10 proteins [five folded proteins, two hybrid proteins; i.e., protein containing ordered and disordered domains, and three intrinsically disordered proteins (IDPs)] and on the aggregation kinetics of insulin and α-synuclein. We found that effects of both polymers on secondary and tertiary structures of folded and hybrid proteins were rather limited with slight unfolding observed in some cases. Solvent accessibility of aromatic residues was significantly increased for the majority of the studied proteins in the presence of UCON but not PEG. PEG also accelerated the aggregation of protein into amyloid fibrils, whereas UCON promoted aggregation to amyloid oligomers instead. These results indicate that even a relatively small change in polymer structure leads to a significant change in the effect of this polymer on protein folding and aggregation. This is an indication that protein folding and especially aggregation are highly sensitive to the presence of other macromolecules, and an excluded volume effect is insufficient to describe their effect.

Protein folding is a complex process that proceeds via a stochastic search for the most stable conformation corresponding to the natively folded state.[2] In many cases, a natively folded state does not exist or is never reached, and a protein remains fully or partially unfolded. Structures of both folding intermediates and a final folded state are known to be strongly dependent on the environment. In addition to other factors such as pH and the presence of salts, the presence of other biopolymers has a significant effect on protein folding *in vivo*. In fact, in the intracellular environment, macromolecules are

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present at overall concentrations of up to 300−400 mg/mL.[3] Earlier studies of the effects of biopolymers on the behavior of proteins were usually focused on either strong protein−polymer interactions (specific binding or charge−charge interactions) or macromolecular crowding. Macromolecular crowding is generally believed to be primarily driven by an excluded volume effect that works by decreasing the effective volume available for the proteins in the cell and thus increasing the effective protein concentration and promoting more compact protein conformations. However, the careful analyses of the macromolecular crowding effects in polymer solutions revealed that the excluded volume effect is not the only factor affecting the behavior of proteins in a crowded environment. The observed inconsistencies are commonly explained by the so-called "soft" interactions, such as electrostatic, hydrophobic, and van der Waals interactions, between the crowding agent and the protein, in addition to the hard nonspecific steric interactions.[4−8] It is believed that these interactions are similar to preferential solvation of proteins by organic solvents.[9]

Recently, we showed that changes in the solvent properties of aqueous media induced by the crowding agents might represent the molecular basis for these "soft" interactions.[1] This conclusion was based on the analysis of the solvatochromic properties of specific dyes sensitive to the solvent dipolarity/polarizability, hydrogen-bond donor acidity, and hydrogen-bond acceptor basicity of aqueous media in solutions of different polymers (dextran, PEG, Ficoll, UCON, and polyvinylpyrrolidone) with a polymer concentration of ≤40%, i.e., in the concentration range typically used to model crowding conditions *in vitro*.[1] This analysis revealed that solvent properties were affected by high polymer concentrations, indicating that the crowding agents do induce changes in solvent properties of aqueous media in a crowded environment.[1] Here we extended this research and examined the effects of two polymers with similar structures and different hydrophobicities on the structural properties and aggregation propensities of several proteins.

Most studies of the effects of crowding agents on protein folding have used hydrophilic polymers such as PEG, dextran, and Ficoll that primarily affect proteins via steric exclusion.[8,10] Here we used two polymers, PEG (MW 4400) and its derivative UCON (MW 5400). UCON is a 1:1 copolymer of ethylene glycol and 2-propylene glycol. Thus, the structures of these polymers are very similar except for the presence of an additional methyl group on every other monomer unit of UCON making it more hydrophobic (see Figure 1). This makes

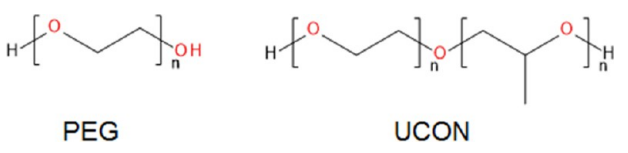

Figure 1. Structures of PEG and UCON polymers. The UCON polymer is composed of an equal proportion of ethylene glycol and propylene glycol monomer units distributed randomly.

it a good test case to see the effect of a modest change in polymer hydrophobicity on protein behavior in a crowded environment. We previously reported that the effects of UCON and PEG on the solvent properties of aqueous media are quite different.[1] We found that even this modest change is sufficient to significantly influence protein folding and especially aggregation.

MATERIALS AND METHODS

Materials. Recombinant α-synuclein was a gift from Dr. Munishkina (University of California, Santa Cruz, CA) and Dr. Rhoades (Yale University, New Haven, CT). Recombinant human insulin was from Akron Biotech (Boca Raton, FL). A commercially available mixture of core histones H2A, H2B, H3, and H4 from calf thymus (Calbiochem) was used without additional fractionation. UCON 50-HB-5100 (molecular mass of 4 kDa) was from Dow Corning. PEG (molecular mass of 4.5 kDa) was from Sigma. All other proteins and chemicals were from Sigma, Fisher Scientific, or VWR Scientific.

Methods. *Circular Dichroism (CD).* Far-UV CD (195−260 nm) spectra of proteins were measured using a JASCO J-815 spectropolarimeter at room temperature. A solution of protein (110 μL, 1 mg/mL) was placed into a 0.2 mm path length cell, and the CD spectra were recorded with a scan speed of 20 nm/min at a step size of 0.2 nm and a bandwidth of 1.0 nm under constant purging with nitrogen. Three spectra were accumulated and averaged for each sample. The same buffer was used for CD measurements and for protein aggregation experiments.

ANS Fluorescence. The protein solution (26.7 μL, 3 mg/mL, final concentration of 0.1 mg/mL) was mixed with buffer (final concentration of 10 mM Hepes, pH 7.5, final volume of 400 μL) containing either no polymer or 10% of the respective polymer by weight. The solution was incubated for 15 min at 24 °C, and then ANS fluorescence was measured. The excitation wavelength was 380 nm, and the emission spectrum was recorded in the range of 400−600 nm at a rate of 100 nm/min. Excitation and emission slits were 2.5 nm. Emission spectra were recorded in triplicate and averaged. Emission spectra were integrated, and their integrals are listed in Table 2.

Intrinsic Fluorescence Quenching. The protein solution (13.3 μL, 3 mg/mL, final concentration of 0.05 mg/mL) was mixed with buffer (final concentration of 10 mM Hepes, pH 7.5, 0.1 M NaCl, final volume of 400 μL) containing different concentrations of acrylamide (0−0.5 M). The solution was incubated for 1 h at 24 °C, and the intrinsic protein fluorescence was measured. The excitation wavelength was 280 nm, and the emission spectrum was recorded in the range of 295−350 nm at a rate of 50 nm/min. Excitation and emission slits were at 5 nm. Emission spectra were recorded in triplicate and averaged. The measurements were performed in duplicate for each sample. The data resulting from the quenching of the intrinsic fluorescence were analyzed using the Stern−Volmer equation (eq 1)

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (1)$$

where F_0 is the fluorescence emission in the absence of acrylamide, F is the emission at a specific concentration of acrylamide, K_{sv} is the dynamic quenching constant (Stern−Volmer constant), and [Q] is the concentration of acrylamide.

Protein Aggregation Assays. Conditions for protein aggregation were optimized for each protein. Aggregation of insulin (0.5 mg/mL) was conducted in glycine buffer (20 mM, pH 2.5) at 37 °C. The protein was dissolved directly in this buffer and incubated for 5 min prior to the start of the reaction. Aggregation of α-synuclein (0.25 mg/mL) was conducted either in 40 mM acetate buffer (pH 3.5) in the presence of 0.1 M NaCl at 37 °C or in 20 mM Hepes (pH 7.5) in the presence of 0.1 M NaCl and 0.025 mg/mL heparin sulfate at 40 °C. α-Synuclein was initially dissolved in 50 mM NaOH at 50 mg/mL, incubated

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in this solution for 1 min, and diluted to 4 mg/mL with water, and the resulting solution was added to the final reaction buffer.

Protein aggregation in the automated format was conducted in a reaction volume of 0.1 mL in black, flat-bottomed 96-well plates in the presence of 5 μM ThT. Two Teflon spheres (2.38 mm diameter, Engineering Laboratories, Oakland, NJ) were placed into each well of a 96-well plate. The reaction mixture containing protein and ThT (320 μL) was split into three wells (100 μL into each well), and the plates were covered with Mylar septum sheets (Thermo) and incubated while being continuously orbitally shaken at 280 rpm in an Infinite M200 Pro microplate reader (Tecan). The kinetics was monitored by top reading of fluorescence intensity every 3−8 min using 444 nm excitation and 485 nm emission filters. Data from replicate wells were averaged before the fluorescence was plotted versus time. The data were fit to a sigmoidal equation (eq 2) using Origin (OriginLab, Northampton, MA). The equation for this fit was as follows:[11,12]

$$F = A + B/\{1 + \exp[k(t - t_m)]\} \quad (2)$$

where A is the initial level of ThT fluorescence, B is the difference between the final level of ThT fluorescence and its initial level, k is the rate constant of amyloid accumulation (inverse hours), and t_m is the midpoint of the transition. The lag time (t_l) of amyloid formation was calculated as $t_l = t_m - 2/k$. The parameters derived from this equation are the yield of amyloid (B), the lag time (t_l), and the elongation rate (k) of amyloid. The initiation rate was defined as the inverse of the lag time. Although eq 2 gave good fits for the ThT kinetic profiles, the expression is strictly an empirical means of deriving kinetic parameters from the data and does not necessarily reflect the underlying complex kinetic scheme.

Electron Microscopy. Aliquots (10 μL) of protein solutions (0.1−0.3 mg/mL) were taken from the 96-well plates at the end of the incubation (40−80 h) and adsorbed onto 200 mesh Formvar/carbon-coated nickel grids for 3 min. The grids were washed with water (20 μL), stained with a fresh 2% uranyl acetate solution for 1 min, and washed with water again. The samples were analyzed with a JEM 1400 transmission electron microscope (JEOL) operated at 80 kV.

RESULTS

The polymers we chose were polyethylene glycol (PEG) and UCON (1:1 copolymer of ethylene glycol and propylene glycol with monomer units distributed randomly). Addition of propylene glycol monomer units increased the polymer hydrophobicity without significantly altering other polymer properties. We selected five folded proteins (insulin, β-lactoglobulin B, α-lactalbumin, lysozyme, and RNase B); two hybrid proteins, i.e., proteins contacting both ordered and intrinsically disordered regions (trypsinogen and α-chymotrypsinogen A), and three typical intrinsically disordered proteins, IDPs (α-casein, histones, and α-synuclein).

Folded proteins used in this study are commercially available proteins with a variety of secondary structures. Insulin is a small, primarily α-helical protein (molecular mass of 5.5 kDa). We tested it at both pH 2.5 where it is monomeric and pH 7.5 where it is hexameric.[13] β-Lactoglobulin B is a β-sheet rich protein (molecular mass of 18 kDa) present at high concentrations in milk.[14] α-Lactalbumin, a regulatory subunit of lactose synthase, is a primarily α-helical calcium-binding protein containing four disulfide bonds (molecular mass of 14 kDa).[15,16] Hen egg white lysozyme, a glycoside hydrolase, is a primarily α-helical globular protein (molecular mass of 14 kDa).[16] It shows a strong sequence and structural similarity to α-lactalbumin.[17] An important structural difference between these two proteins is the existence of easily accessible molten globule denaturation intermediates for α-lactalbumin but not for hen egg white lysozyme.[18] Ribonuclease B is an endonuclease with specificity for pyrimidine linkages. It is a glycoprotein (molecular mass of 14.7 kDa) composed of a mixture of α-helices and β-sheets.[19] Charges of these proteins at physiological pH vary significantly. Insulin, β-lactoglobulin B, and α-lactalbumin have a pI in the range of 4.5−5.5 and are negatively charged at neutral pH. Lysozyme has a pI of 11.3 and is highly positively charged at neutral pH, while ribonuclease B has a pI of 9.3 and is thus positively charged, as well.

We also used two hybrid proteins and three IDPs with different charge/hydropathy ratios.[20] Trypsinogen and chymotrypsinogen A are the precursor forms of the respective proteolytic enzymes. They contain both folded and disordered regions and can thus be considered hybrid proteins.[21,22] α-Caseins are highly phosphorylated, mostly disordered proteins (molecular mass of 22−25 kDa).[23] Core histones are lysine rich, highly charged IDPs that bind to DNA as octamers.[24] They exist in a variety of oligomeric states ranging from monomer (molecular mass of 14 kDa) to octamer depending on the assay conditions, with octamers being prevalent at neutral pH.[25−27] α-Synuclein is an intrinsically disordered, highly amyloidogenic protein. The conformational flexibility of this protein depends on pH, with more compact and flexible conformation at low pH and more extended conformation at neutral pH[6,28,29] because of the higher protein charge at neutral pH. We used α-synuclein at two pH values, pH 3.5 and 7.5. Far-UV CD spectra of IDPs (Figure 2) confirmed that they are predominantly unfolded under the assay conditions. Histones, trypsinogen, and chymotrypsinogen were positively charged at neutral pH (pI values of 9−11), while α-casein and α-synuclein were negatively charged (pI ∼4.6).

Secondary Structure Perturbation by Polymers Detected by Far-UV CD Spectroscopy. To assess the effect of polymers on the protein secondary structure, we used far-UV CD spectroscopy. All experiments were conducted at neutral pH [10 mM Hepes (pH 7.5)] with the exception of trypsinogen, insulin, and α-synuclein. Assays for trypsinogen were conducted at pH 3.0 (10 mM glycine) to prevent its cleavage by trace amounts of trypsin; insulin was assessed at both pH 2.5 (10 mM glycine) and pH 7.5, and α-synuclein was evaluated at pH 3.5 (10 mM citrate) and pH 7.5. A range of polymer concentrations (0−10%) was used. However, for the sake of presentation, the spectra of all proteins are shown here under three sets of conditions: without polymers, in the presence of 10% PEG, and in the presence of 10% UCON (Figure 2). More detailed dependences of CD spectra on polymer concentrations are shown for select proteins in the Supporting Information (Figure S1).

Effects of polymers on protein secondary structure were quite variable. Far-UV CD spectra of several folded proteins (insulin at pH 7.5, lysozyme, and ribonuclease B), and both hybrid proteins (α-chymotrypsinogen A and trypsinogen) were unchanged in the presence of both polymers (Figure 2 and Figure S1 of the Supporting Information). The spectral minimum of β-lactoglobulin B shifted from 214 to 217 nm in the presence of UCON (Figure 2C), indicating an alteration of the secondary structure, although we could not attribute this change to any specific structural transition. Spectra of insulin at pH 2.5 and of α-lactalbumin at pH 7.5 did change significantly upon the addition of UCON (Figure 2). CD spectra of these proteins are

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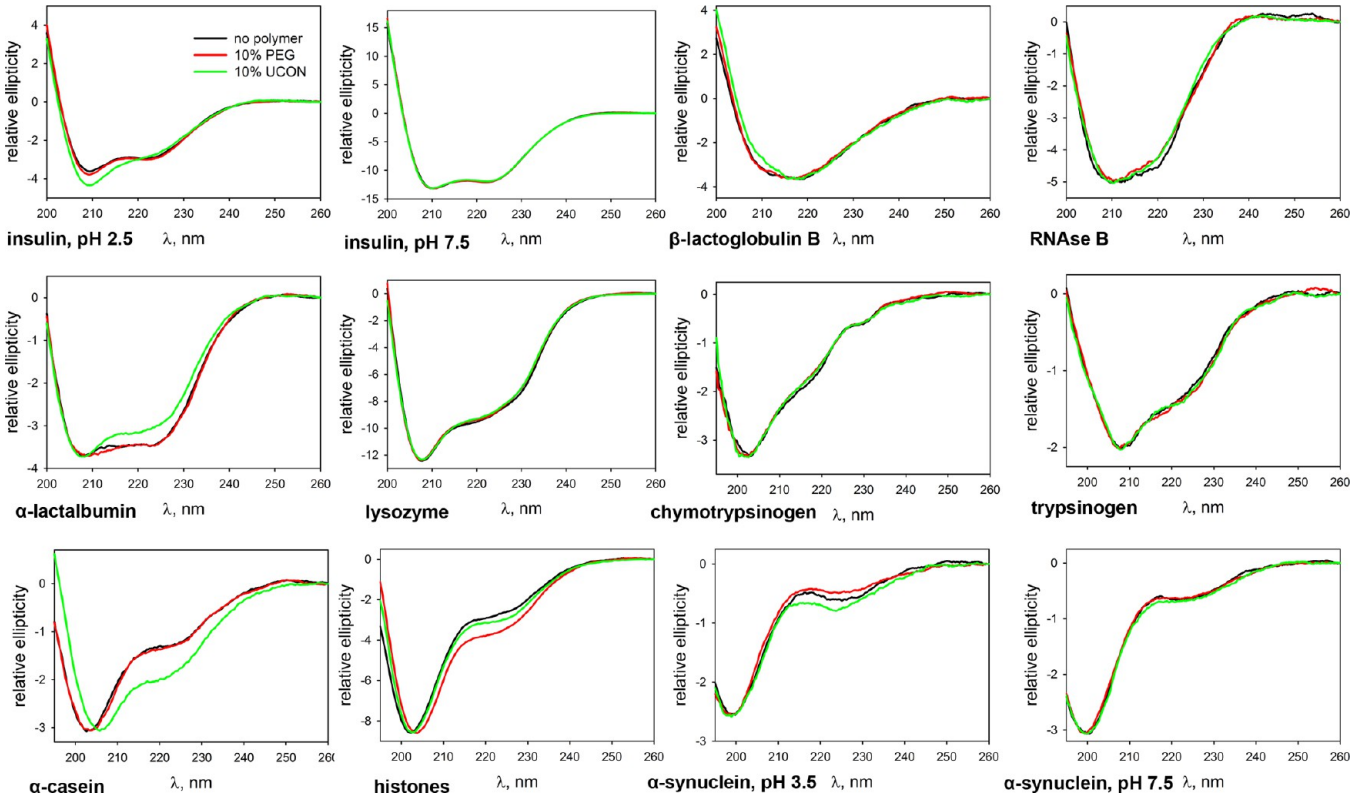

Figure 2. Far-UV circular dichroism spectra of proteins in the presence of PEG and UCON polymers: black, no polymer additives; red, 10% PEG; green, 10% UCON.

typical for primarily α-helical proteins with peaks at 208 and 222 nm. In the presence of UCON, the intensity of the peak at 222 nm progressively decreased in intensity with increasing UCON concentrations while the intensity of the peak at 205 nm increased. This shift is consistent with UCON-induced partial loss of the protein secondary structure. As for IDPs, spectra of α-synuclein at either pH 3.5 or 7.5 did not change significantly in the presence of either polymer. The CD spectrum of histones in the presence of PEG (but not UCON) showed the shift of the peak minimum from 202 to 204 nm and the increase in the intensity of the shoulder at 224 nm (Figure 2), indicating a possible increase in the level of ordered secondary structure. For α-casein, a similar but even larger spectral change was observed in the presence of UCON. These changes indicate an increased degree of protein folding in the presence of the respective polymers.

Overall, in the presence of PEG, spectral changes consistent with the excluded volume effect (protein folding) were observed for histones. CD spectra of other proteins remained essentially unchanged. We have also observed that UCON promoted partial loss of secondary structure in two folded proteins (insulin at pH 2.5 and α-lactalbumin) and gain of secondary structure in α-casein. We have also attempted to evaluate effects of polymers on the secondary structure of the proteins by deconvolution of the CD spectra using the K2D algorithm on the Dichroweb Web site.[30] On the basis of this analysis, we observed (Table 1) some structural changes in the presence of polymers for several proteins: insulin at pH 2.5 and β-lactoglobulin B, α-lactalbumin, α-casein, histones, and α-synuclein at pH 3.5. This result was similar to the result of the visual examination of the spectra.

Tertiary Structure Perturbation by Polymers Detected by Near-UV CD Spectroscopy. We confirmed our observations by examining the effect of polymers on the tertiary structure of these proteins via near-UV CD spectroscopy. Because IDPs do not have a stable tertiary structure, near-UV CD spectra of only folded and hybrid proteins were examined. Near-UV CD spectra arise from aromatic amino acid residues in a chiral environment, and decreased spectral intensity is an indication of the loss of tertiary structure. Spectra of most proteins remained essentially unchanged in the presence of both polymers (Figure 3 and Figure S2 of the Supporting Information). The intensities of the spectra of insulin at pH 7.5 and β-lactoglobulin B increased slightly in the presence of both polymers. Intensities of the spectra of α-lactalbumin, lysozyme, and chymotrypsinogen have decreased in the presence of both polymers, an indication of possible loss of some tertiary structure (Figure 3 and Figure S2 of the Supporting Information). The effect was especially pronounced for α-lactalbumin. Changes in the spectrum fine structure in the presence of polymers were observed for chymotrypsinogen, although these changes were relatively minor and could not be readily interpreted. No significant difference between the effects of PEG and UCON on near-UV CD spectra was observed for either of these proteins.

Structural Changes in the Presence of Polymers Assessed by ANS Binding and Quenching of Intrinsic Protein Fluorescence by Acrylamide. The fluorescence emission of 8-anilinonaphthalene-1-sulfonate (ANS) significantly increases and undergoes a blue shift upon binding to exposed hydrophobic surfaces of proteins. Partial denaturation or aggregation of proteins results in a significant increase in the level of ANS binding and fluorescence, and thus, ANS fluorescence can be used to monitor changes in the protein tertiary structure.[31,32] We examined ANS fluorescence (Table 2) in the presence of all the proteins in this study (at 0.1 mg/mL) in

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Table 1. Analysis of the Protein Secondary Structure Based on Deconvolution of Far-UV CD Spectra

		α-helix (%)	β-sheet (%)	random coil (%)
insulin (pH 2.5)	no polymer	62	6	32
	10% PEG	61	7	31
	10% UCON	69	4	27
insulin (pH 7.5)	no polymer	100	0	0
	10% PEG	100	0	0
	10% UCON	100	0	0
β-lactoglobulin B	no polymer	21	30	49
	10% PEG	19	32	49
	10% UCON	12	39	48
α-lactalbumin	no polymer	27	13	60
	10% PEG	25	15	60
	10% UCON	31	11	59
lysozyme	no polymer	26	19	56
	10% PEG	30	14	56
	10% UCON	30	13	57
RNase B	no polymer	23	40	36
	10% PEG	23	40	36
	10% UCON	23	40	36
α-chymotrypsinogen	no polymer	28	33	39
	10% PEG	28	33	40
	10% UCON	28	33	39
trypsinogen	no polymer	23	23	54
	10% PEG	24	22	55
	10% UCON	23	22	55
α-casein	no polymer	26	27	47
	10% PEG	22	27	50
	10% UCON	18	28	54
histones	no polymer	8	41	50
	10% PEG	9	35	56
	10% UCON	8	41	51
α-synuclein (pH 3.5)	no polymer	8	41	51
	10% PEG	8	44	48
	10% UCON	8	46	47
α-synuclein (pH 7.5)	no polymer	7	51	42
	10% PEG	7	51	42
	10% UCON	7	51	42

Table 2. ANS Fluorescence in the Presence of Proteins and Polymers[a]

	ANS fluorescence emission (%)		
	no polymer	10% PEG	10% UCON
insulin (pH 2.5)	273	115	106
insulin (pH 7.5)	746	90	102
β-lactoglobulin B	298	92	107
α-lactalbumin	185	79	148
lysozyme	101	84	103
RNase B	99	87	98
α-chymotrypsinogen	485	90	139
trypsinogen	901	135	132
α-casein	316	84	240
histones	106	83	102
α-synuclein p(H 3.5)	406	162	115
α-synuclein (pH 7.5)	108	83	104

[a]The fluorescence emission spectrum (excitationo at 380 nm, emission at 400−600 nm) was integrated, and the integral was normalized against the one obtained in the absence of protein.

the absence and presence of polymers (10% PEG or UCON). We found that addition of polymers (in the absence of proteins) resulted in a significant increase in ANS fluorescence intensity. The fluorescence of ANS also increased in the presence of several proteins (in the absence of polymers), although no blue shift of fluorescence was observed. Addition of proteins to ANS in the presence of polymers did not have significant additional effects on ANS fluorescence. Casein, trypsinogen, and chymotrypsinogen in the presence of UCON were the exceptions as addition of these proteins resulted in increased ANS fluorescence. These results indicate that while proteins bind ANS to a variable extent, the presence of much higher polymer concentrations results in ANS preferentially binding to polymers. However, a few proteins (especially α-casein) in the presence of UCON did present sufficient exposed hydrophobic surface to have an effect on ANS fluorescence. This observation confirms significant structural changes observed for α-casein in the presence of UCON by other methods (Figure 2).

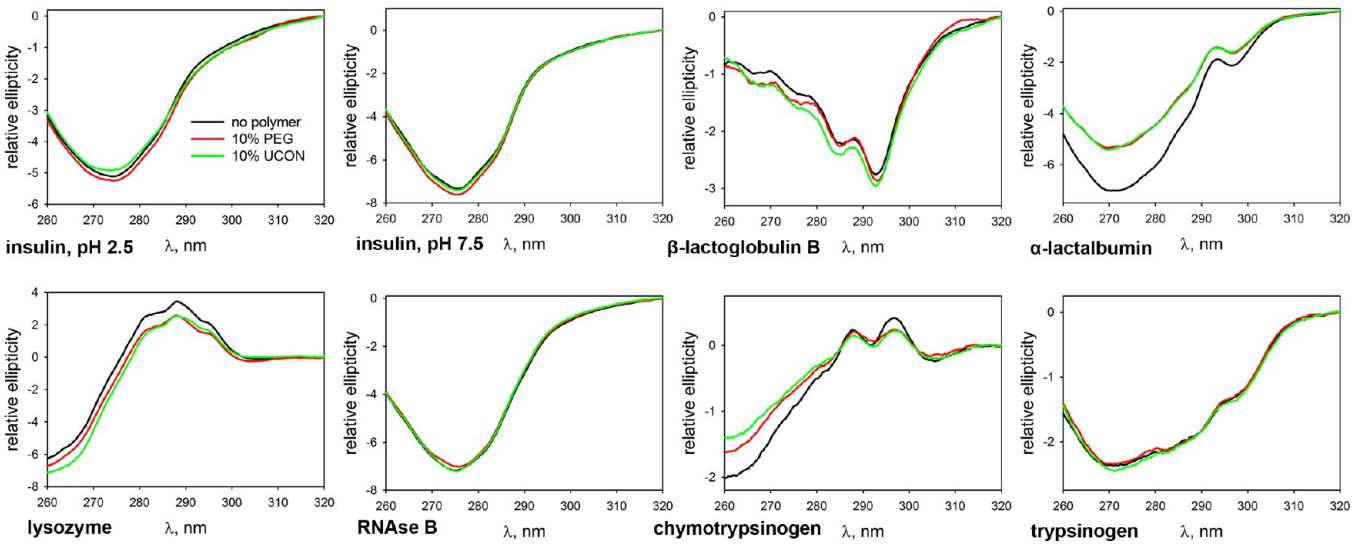

Figure 3. Near-UV circular dichroism spectra of natively folded and hybrid proteins in the presence of PEG and UCON polymers: black, no polymer additives; red, 10% PEG; green, 10% UCON.

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The intrinsic protein fluorescence is primarily due to their tyrosine and tryptophan residues. The solvent accessibility of these aromatic residues can be probed by analyzing the efficiency of quenching of their fluorescence by small molecules. Acrylamide is a common chemical quencher. Recent studies have shown that quenching by acrylamide can occur via through-space interactions (i.e., a type of orbital interaction resulting from direct spatial overlap of two orbitals), and acrylamide does not need to come into direct contact with the fluorophore.[33] Thus, the rate of quenching of intrinsic protein fluorescence by acrylamide is a measure of the proximity of the aromatic residues to the protein surface and hydrophobicity of the environment of these residues.

To assess the effect of PEG and UCON on the surface accessibility of aromatic residues and hydrophobicity of their environment, we have examined quenching of intrinsic protein fluorescence by acrylamide in the presence of these polymers. We found that for most proteins, both polymers increased acrylamide quenching rates, indicating an increased level of exposure of aromatic residues to solvent. Furthermore, in all cases studied, the effect of UCON was much stronger than that of PEG (Table 3 and Figure 4). For several proteins (β-lactoglobulin B, α-lactalbumin, and α-chymotrypsinogen A), PEG either had no effect on the efficiency of quenching or decreased it, whereas the presence of UCON consistently increased quenching rates, often by a significant margin. IDPs were especially sensitive to the presence of polymers, although small folded proteins (insulin and RNase B) were highly sensitive, as well. As expected, insulin was more accessible to acrylamide at pH 2.5 (where it is monomeric and partially unfolded) than at pH 7.5 (where it is oligomeric and primarily α-helical). Overall, these results confirmed the observation based on far-UV CD data that UCON promotes protein unfolding much more effectively than PEG and extended it across the full range of proteins we examined.

Table 3. Stern–Volmer Constants (K_{sv}, M^{-1}) for Acrylamide Quenching of Intrinsic Protein Fluorescence in the Presence and Absence of Polymers

	no polymer	10% PEG	10% UCON
insulin (pH 2.5)	13.5 ± 2.2	15.1 ± 3.9	23.3 ± 0.1
insulin (pH 7.5)	7.7 ± 2.4	12.1 ± 1.4	19.6 ± 3.5
β-lactoglobulin B	8.4 ± 0.1	5.3 ± 0.3	9.3 ± 0.9
α-lactalbumin	7.4 ± 0.9	7.1 ± 1.3	8.6 ± 1.5
lysozyme	7.1 ± 0.5	9.4 ± 1.7	9.2 ± 1.3
RNase B	7.1 ± 0.9	12.1 ± 0.8	22.1 ± 0.2
α-chymotrypsinogen	4.6 ± 1.0	4.5 ± 0.1	6.9 ± 0.5
trypsinogen	5.7 ± 0.7	9.9 ± 0.7	11.5 ± 2.7
α-casein	8.7 ± 0.6	17.3 ± 0.8	13.3 ± 3.6
histones	7.7 ± 0.4	9.2 ± 2.1	19.1 ± 1.1
α-synuclein (pH 3.5)	8.6 ± 2	9.8 ± 0.6	29.8 ± 1.0
α-synuclein (pH 7.5)	10.3 ± 2.2	10.6 ± 1.8	29 ± 0.6

Lack of Specific Interactions between Proteins and Polymers. The detection and analysis of protein interactions with other macromolecules generally depend on the choice of the appropriate technique to be used. Proving the lack of such interactions is much more difficult because it is always possible that the particular technique employed may be insufficiently sensitive. The only indirect approach allowing one to prove the lack of specific protein–polymer interactions seems to be analysis of protein partitioning in aqueous two-phase systems (ATPS) formed by pairs of different polymers.[34] It has been shown that the logarithms of partition coefficients of 12 different proteins, including α-chymotrypsinogen, lysozyme, trypsinogen, RNase B, and β-lactoglobulin B, in Ficoll-PEG and Dextran-UCON ATPS are linearly interrelated as[35]

$$\log K_i^{\text{Dex-UCON}} = b + a \times \log K_i^{\text{Ficoll-PEG}} \quad (3)$$

where $K_i^{\text{Dex-UCON}}$ and $K_i^{\text{Ficoll-PEG}}$ are partition coefficients for the ith protein in dextran-UCON and Ficoll-PEG ATPS, respectively, and a and b are constants. It should be mentioned that the partition coefficient of a protein in any given ATPS is commonly defined as the ratio of the protein concentration in the upper phase to that in the lower phase.

One should keep in mind that coefficients a and b in eq 3 depend upon the solvent features of the ATPS under comparison and therefore cannot be predicted. Equation 3 is an empirical relationship first established for partitioning of compounds of the same chemical nature in different organic solvent–water biphasic systems. In ATPSs, this equation holds for different biopolymers, such as proteins and nucleic acids, and small organic compounds.[1,34] The fulfillment of the relationship described by eq 3 for different proteins in ATPSs formed by different pairs of polymers is generally accepted as an indication that the proteins fitting the relationship do not participate in direct interactions with the phase-forming polymers, and that their distribution between the coexisting aqueous phases in each of two ATPSs under comparison is driven solely by differences between protein–water interactions in the two phases.[1,34] Therefore,

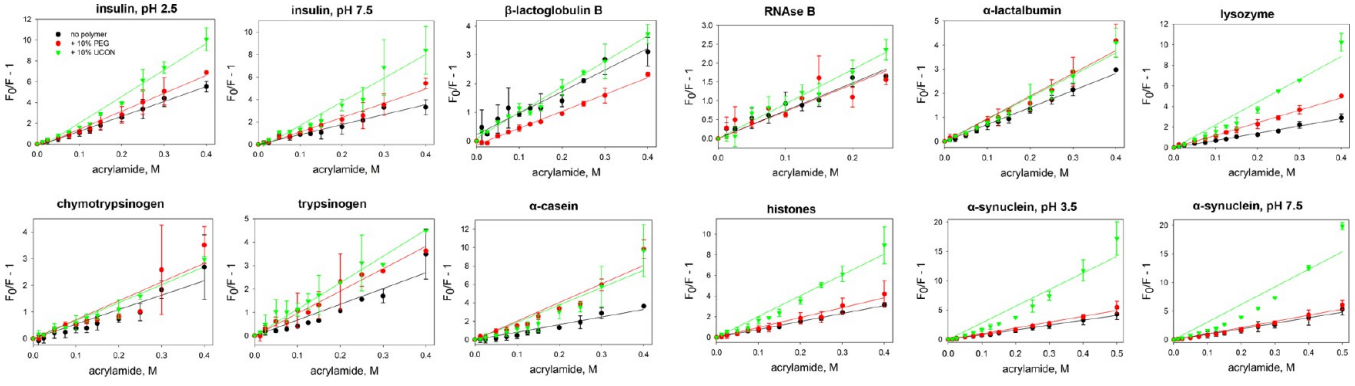

Figure 4. Intrinsic protein fluorescence quenching by acrylamide in the presence of PEG and UCON polymers: black, no polymer additives; red, 10% PEG; green, 10% UCON.

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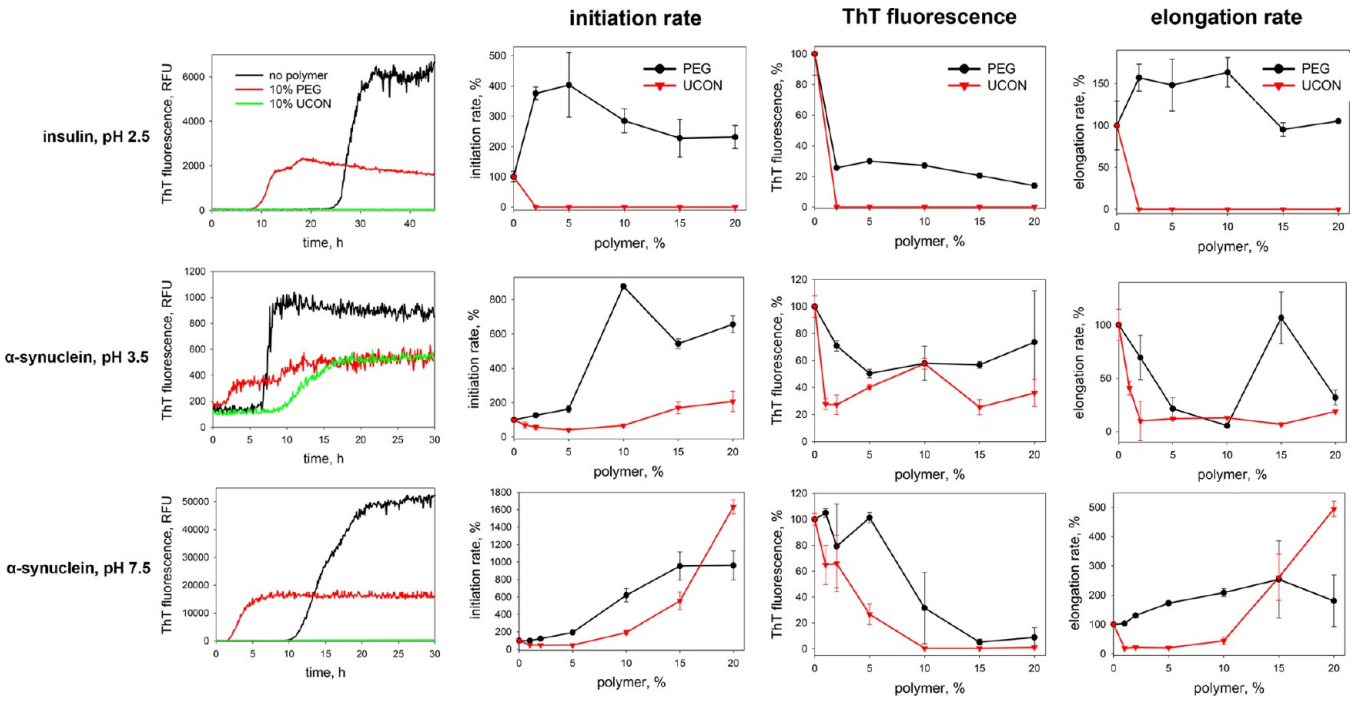

Figure 5. Effect of polymers on protein aggregation kinetics. Aggregation of insulin (pH 2.5) and α-synuclein (pH 3.5 and 7.5) was monitored by following ThT fluorescence. All experiments were conducted in triplicate, and the averages are shown. The dependence of kinetic parameters derived from these experiments (initiation and elongation rates of protein aggregation and ThT fluorescence intensity at the conclusion of the aggregation) on polymer concentration is also shown.

the data obtained for the proteins examined in our study were used for the purpose of checking if there are direct interactions between the protein of interest and UCON or PEG. The solvent properties of the aqueous media in the phases of ATPS cannot be classified in terms of "bad" and "good" solvent but may be quantified in terms of solvent ability to have dipole−dipole interactions, solvent ability to serve as a hydrogen bond donor and as a hydrogen bond acceptor. This issue, however, is beyond the scope of this study.

A similar relationship was reported[1] for 19 small organic compounds and seven different proteins, including α-synuclein, RNase B, and β-lactoglobulin B, in dextran-PEG and Ficoll-UCON ATPS. It follows from the constant values of coefficients a and b in eq 3 that either all different proteins and organic compounds examined interact with the polymers used for forming a given ATPS in an identical manner or there are no specific interactions between proteins and small compounds and phase-forming polymers at all. It was concluded[34] that there is no direct protein−polymer interactions for the proteins fitting the relationships described by eq 3, and that the unequal distribution of proteins in ATPS occurs due to different interactions of proteins with aqueous media with different solvent properties in the coexisting phases. This conclusion was confirmed[36−38] by the demonstration of the fact that the partition coefficient of a solute (including proteins) may be described quantitatively in terms of quantified solvent properties of the coexisting phases in ATPS, and even predicted with 90−95% accuracy.

Therefore, for six of ten proteins studied here, there is no doubt that these proteins do not participate in any direct interactions with PEG or UCON.

Effects of Polymers on the Protein Aggregation Kinetics. Effects of crowding agents on protein aggregation have been extensively investigated in the past. It has been shown that aggregation of many amyloidogenic proteins and peptides is accelerated by the presence of crowding agents.[28,39−42] This acceleration has been attributed to the excluded volume effect,[43,44] although the existence of weak interactions between proteins and polymers has been proposed, as well.[3,4,45,46] On the other hand, aggregation of stable folded proteins, such as lysozyme, has been inhibited by crowding agents presumably because of monomer stabilization.[41]

We have examined the effect of polymers on the kinetics of fibril formation by insulin and α-synuclein, under the conditions previously determined to be favorable for the conversion of these proteins to amyloid fibrils. Aggregation of insulin was examined at pH 2.5, where this protein is primarily monomeric. For α-synuclein, two sets of conditions were used: pH 3.5 and 7.5. Aggregation of α-synuclein at pH 7.5 was conducted in the presence of 25 μg/mL heparin sulfate, a negatively charged polysaccharide used to accelerate protein aggregation.[11,47−49] Under these conditions, proteins were efficiently converted to amyloid fibrils with the lag phase of several hours (Figure 5). Thioflavin T fluorescence was used to assess the protein aggregation kinetics. In control experiments (Figure S4 of the Supporting Information), we determined that polymers do not have a significant effect on the fluorescence of this dye in the absence of proteins.

From kinetic data, we found that aggregation of both insulin and α-synuclein was significantly accelerated in the presence of PEG with the initiation rate increasing 5−10-fold (Figure 5). ThT fluorescence decreased ~3-fold for fibrils grown in the presence of PEG (Figure 5 and Figure S3 of the Supporting Information), but we also found (Figure S4 of the Supporting Information) that addition of either PEG or UCON to preformed fibrils significantly decreases the fluorescence of ThT bound to them. Thus, the lower ThT fluorescence of fibrils in the presence of polymers is likely due to competition between polymers and fibrils for ThT binding and thus not related to the

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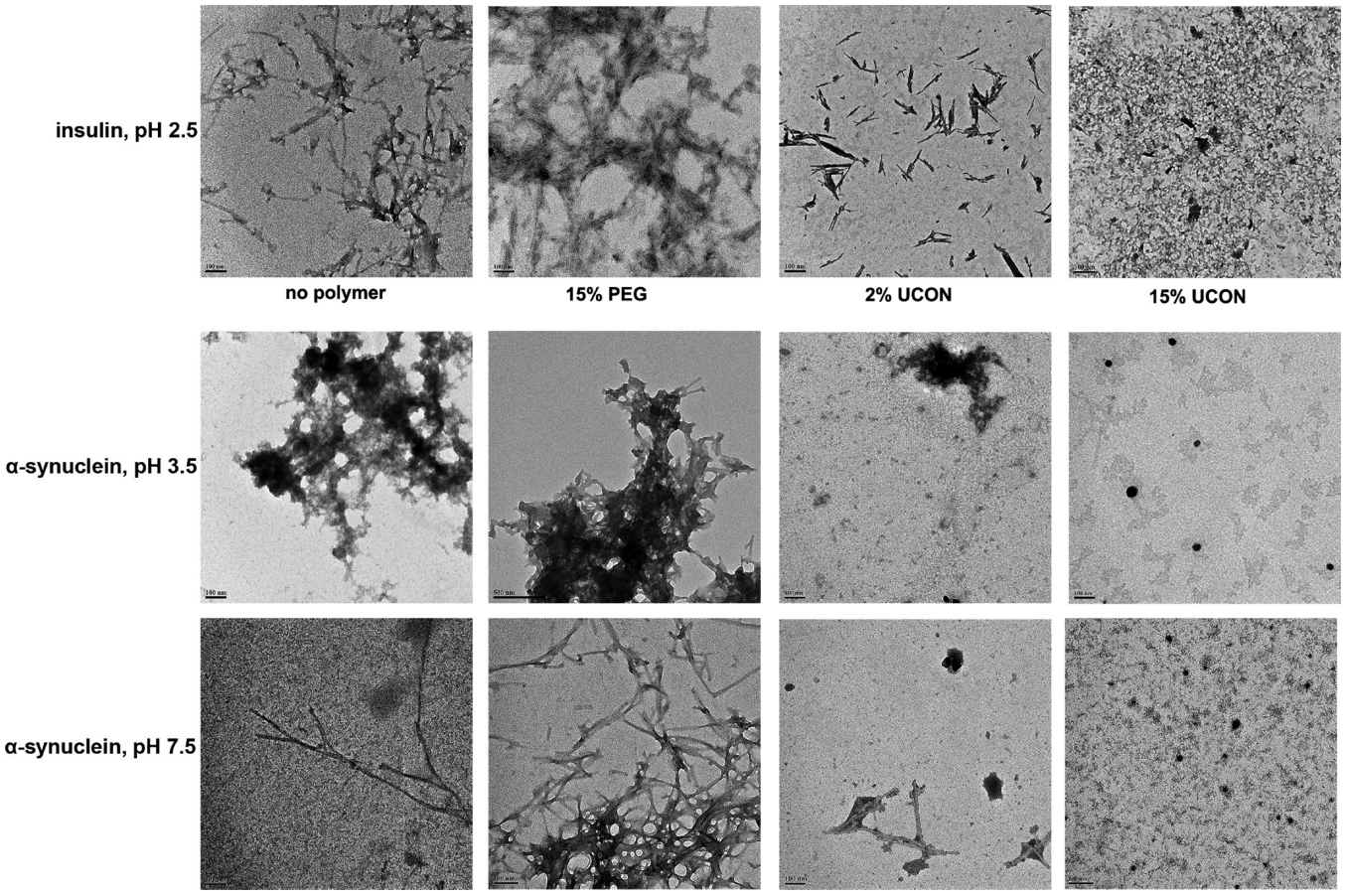

Figure 6. Morphology of amyloid fibrils of insulin and α-synuclein grown in the presence of either PEG or UCON polymers. Fibrils of insulin at pH 2.5, α-synuclein at pH 3.5, and α-synuclein at pH 7.5 were grown in the presence of polymers. The scale bars correspond to 100 nm.

mechanism of aggregation. Electron microscopy showed somewhat shorter fibrils in the presence of PEG for both proteins, but the overall fibrillar morphology was preserved (Figure 6).

Using a combination of kinetic data and electron microscopy, we found that UCON directed aggregation of both insulin and α-synuclein toward oligomeric structures. UCON completely inhibited aggregation of insulin to amyloid fibrils even at the lowest polymer concentration tested (Figure 5). Electron microscopy has shown that oligomeric aggregates were formed instead (Figure 6). The effect of UCON on the aggregation of α-synuclein was weaker (Figure 5 and Figure S3 of the Supporting Information), and some amyloid fibrils were still observed at low polymer concentrations. However, at higher UCON concentrations (10−15%), only α-synuclein oligomers were observed (Figure 6).

DISCUSSION

Here we have examined the effects of two polymers with different hydrophobicities (Figure 1) used as crowding agents on protein structure and aggregation. We found that with a few exceptions effects of both less hydrophobic (PEG) and more hydrophobic (UCON) polymers on the secondary and tertiary structures of proteins were relatively minor. These exceptions included α-lactalbumin and chymotrypsinogen that appeared to convert to a molten globule-like conformation in the presence of both polymers as seen from the significant decrease in intensity of near-UV CD along with smaller changes in the far-UV CD (Figures 2 and 3 and Figures S1 and S2 of the Supporting Information). Both of these proteins can be converted to molten globule-like states at neutral pH by thermal or guanidine-dependent denaturation.[18,50] Polymers also appeared to induce some ordered secondary structure in IDPs (PEG for histones and α-synuclein, UCON for α-casein), although these effects were not reflected in changes in the accessibility of aromatic residues in these proteins to acrylamide. On the other hand, for most proteins, both polymers had a significant effect on the solvent accessibility of the hydrophobic core measured by the quenching of the intrinsic fluorescence. For most proteins, Stern−Volmer constants increased in the presence of PEG and increased even further in the presence of UCON. The effects of UCON on IDPs were especially large. This could be an indication that some proteins (especially IDPs) in the presence of UCON (and to some extent PEG) undergo a structural transition that does not alter their secondary or tertiary structure but increases the level of exposure of buried hydrophobic residues.

Effects of polymers on protein aggregation were even more dramatic. While the presence of PEG decreased the duration of the lag phase of fibril formation and somewhat altered the fibril morphology, in the presence of UCON, fibril formation was almost completely inhibited, and various oligomers were formed instead. PEG may be acting on aggregating proteins via an excluded volume effect by increasing the effective protein concentration and stabilizing compact conformations.[8] The effect of UCON could be due to other factors such as changes in the sovent properties of aqueous media in solution.[1] It appears that aggregation intermediates are more sensitive to both crowding and other effects of polymers. This is not unexpected because of their oligomeric nature and the higher abundance of exposed hydrophobic surfaces.

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Because our analysis of the partition of target proteins in various ATPSs containing polymers of interest revealed the lack of specific protein−polymer interactions, the most probable explanation for the different effects of similarly sized PEG and UCON on the structural properties and aggregation behavior of proteins is the presence of the polymer-induced changes in solvent properties of aqueous media in a crowded environment. Overall, we found that increasing the hydrophobicity of PEG by adding a methyl group to every other monomer unit allowed the resulting polymer to affect proteins more effectively and inhibit protein aggregation to amyloid fibrils. This is an indication that protein folding and especially aggregation are highly sensitive to the presence of other macromolecules, and the excluded volume effect is insufficient to describe the effects of these biopolymers.

■ ASSOCIATED CONTENT

Supporting Information

Far-UV CD spectra of insulin, RNase B, α-casein, and α-synuclein in the presence of variable concentrations of PEG and UCON (Figure S1), near-UV CD spectra of folded and hybrid proteins in the presence of variable concentrations of PEG and UCON (Figure S2), kinetic curves for the aggregation of insulin and α-synuclein in the presence of variable concentrations of PEG and UCON (Figure S3), with aggregation monitored by following the ThT fluorescence and all experiments conducted in triplicate, and the effect of polymers on ThT fluorescence alone (A) or in the presence of 5 μM α-synuclein fibrils (B) (Figure S4). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00116.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CD, circular dichroism; IDP, intrinsically disordered protein; PEG, polyethylene glycol; UV, ultraviolet; ThT, thioflavin T.

■ REFERENCES

(1) Ferreira, L. A., Madeira, P. P., Breydo, L., Reichardt, C., Uversky, V. N., and Zaslavsky, B. Y. (2015) Role of solvent properties of aqueous media in macromolecular crowding effects. *J. Biomol. Struct. Dyn.*, 1−12.

(2) Dobson, C. M. (2004) Principles of protein folding, misfolding and aggregation. *Semin. Cell Dev. Biol. 15*, 3−16.

(3) Zhou, H. X., Rivas, G., and Minton, A. P. (2008) Macromolecular crowding and confinement: Biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys. 37*, 375−397.

(4) Wang, Y., Sarkar, M., Smith, A. E., Krois, A. S., and Pielak, G. J. (2012) Macromolecular crowding and protein stability. *J. Am. Chem. Soc. 134*, 16614−16618.

(5) Benton, L. A., Smith, A. E., Young, G. B., and Pielak, G. J. (2012) Unexpected effects of macromolecular crowding on protein stability. *Biochemistry 51*, 9773−9775.

(6) Breydo, L., Reddy, K. D., Piai, A., Felli, I. C., Pierattelli, R., and Uversky, V. N. (2014) The crowd you're in with: Effects of different types of crowding agents on protein aggregation. *Biochim. Biophys. Acta 1844*, 346−357.

(7) Sarkar, M., Li, C., and Pielak, G. J. (2013) Soft interactions and crowding. *Biophys. Rev. 5*, 187−194.

(8) Bhat, R., and Timasheff, S. N. (1992) Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Sci. 1*, 1133−1143.

(9) Timasheff, S. N. (2002) Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry 41*, 13473−13482.

(10) Arakawa, T., and Timasheff, S. N. (1985) Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry 24*, 6756−6762.

(11) Cohlberg, J. A., Li, J., Uversky, V. N., and Fink, A. L. (2002) Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from α-synuclein in vitro. *Biochemistry 41*, 1502−1511.

(12) Breydo, L., Bocharova, O. V., and Baskakov, I. V. (2005) Semiautomated cell-free conversion of prion protein: Applications for high-throughput screening of potential antiprion drugs. *Anal. Biochem. 339*, 165−173.

(13) Uversky, V. N., Garriques, L. N., Millett, I. S., Frokjaer, S., Brange, J., Doniach, S., and Fink, A. L. (2003) Prediction of the association state of insulin using spectral parameters. *J. Pharm. Sci. 92*, 847−858.

(14) Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C., Findlay, J. B., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., and Kraulis, P. J. (1986) The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature 324*, 383−385.

(15) Permyakov, E. A., and Berliner, L. J. (2000) α-Lactalbumin: Structure and function. *FEBS Lett. 473*, 269−274.

(16) McKenzie, H. A., and White, F. H., Jr. (1991) Lysozyme and α-lactalbumin: Structure, function, and interrelationships. *Adv. Protein Chem. 41*, 173−315.

(17) Acharya, K. R., Stuart, D. I., Walker, N. P., Lewis, M., and Phillips, D. C. (1989) Refined structure of baboon α-lactalbumin at 1.7 Å resolution. Comparison with C-type lysozyme. *J. Mol. Biol. 208*, 99−127.

(18) Kuwajima, K., Nitta, K., Yoneyama, M., and Sugai, S. (1976) Three-state denaturation of α-lactalbumin by guanidine hydrochloride. *J. Mol. Biol. 106*, 359−373.

(19) Williams, R. L., Greene, S. M., and McPherson, A. (1987) The crystal structure of ribonuclease B at 2.5-Å resolution. *J. Biol. Chem. 262*, 16020−16031.

(20) Uversky, V. N., Gillespie, J. R., and Fink, A. L. (2000) Why are "natively unfolded" proteins unstructured under physiologic conditions? *Proteins 41*, 415−427.

(21) Uversky, V. N., and Dunker, A. K. (2010) Understanding protein non-folding. *Biochim. Biophys. Acta 1804*, 1231−1264.

(22) Uversky, V. N. (2013) Under-folded proteins: Conformational ensembles and their roles in protein folding, function, and pathogenesis. *Biopolymers 99*, 870−887.

(23) Holt, C., and Sawyer, L. (1988) Primary and predicted secondary structures of the caseins in relation to their biological functions. *Protein Eng. 2*, 251−259.

(24) Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature 389*, 251−260.

(25) Munishkina, L. A., Fink, A. L., and Uversky, V. N. (2004) Conformational prerequisites for formation of amyloid fibrils from histones. *J. Mol. Biol. 342*, 1305−1324.

(26) Butler, A. P., Harrington, R. E., and Olins, D. E. (1979) Salt-dependent interconversion of inner histone oligomers. *Nucleic Acids Res. 6*, 1509−1520.

(27) Kawashima, S., and Imahori, K. (1980) Studies on histone oligomers. II. Reconstitution of heterotype histone oligomers and pH dependency of oligomer formation. *J. Biochem. 88*, 783−788.

(28) Munishkina, L. A., Cooper, E. M., Uversky, V. N., and Fink, A. L. (2004) The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *J. Mol. Recognit. 17*, 456−464.

(29) Uversky, V. N., Li, J., and Fink, A. L. (2001) Evidence for a partially folded intermediate in α-synuclein fibril formation. *J. Biol. Chem. 276*, 10737−10744.

DOI: 10.1021/acs.biochem.5b00116
Biochemistry 2015, 54, 2957−2966

(30) Whitmore, L., and Wallace, B. A. (2008) Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers 89*, 392−400.
(31) Hawe, A., Sutter, M., and Jiskoot, W. (2008) Extrinsic fluorescent dyes as tools for protein characterization. *Pharm. Res. 25*, 1487−1499.
(32) Bertoncini, C. W., and Celej, M. S. (2011) Small molecule fluorescent probes for the detection of amyloid self-assembly in vitro and in vivo. *Curr. Protein Pept. Sci. 12*, 205−220.
(33) Strambini, G. B., and Gonnelli, M. (2010) Fluorescence Quenching of Buried Trp Residues by Acrylamide Does Not Require Penetration of the Protein Fold. *J. Phys. Chem. B 114*, 1089−1093.
(34) Zaslavsky, B. (1994) *Aqueous Two-Phase Partitioning: Physical Chemistry and Bioanalytical Applications*, Marcel Dekker, New York.
(35) Madeira, P. P., Teixeira, J. A., Macedo, E. A., Mikheeva, L. M., and Zaslavsky, B. Y. (2008) "On the Collander equation": Protein partitioning in polymer/polymer aqueous two-phase systems. *J. Chromatogr. A 1190*, 39−43.
(36) Madeira, P. P., Reis, C. A., Rodrigues, A. E., Mikheeva, L. M., and Zaslavsky, B. Y. (2010) Solvent properties governing solute partitioning in polymer/polymer aqueous two-phase systems: Nonionic compounds. *J. Phys. Chem. B 114*, 457−462.
(37) Madeira, P. P., Reis, C. A., Rodrigues, A. E., Mikheeva, L. M., Chait, A., and Zaslavsky, B. Y. (2011) Solvent properties governing protein partitioning in polymer/polymer aqueous two-phase systems. *J. Chromatogr. A 1218*, 1379−1384.
(38) Madeira, P. P., Bessa, A., de Barros, D. P., Teixeira, M. A., Alvares-Ribeiro, L., Aires-Barros, M. R., Rodrigues, A. E., Chait, A., and Zaslavsky, B. Y. (2013) Solvatochromic relationship: Prediction of distribution of ionic solutes in aqueous two-phase systems. *J. Chromatogr. A 1271*, 10−16.
(39) Munishkina, L. A., Ahmad, A., Fink, A. L., and Uversky, V. N. (2008) Guiding protein aggregation with macromolecular crowding. *Biochemistry 47*, 8993−9006.
(40) Lee, C. F., Bird, S., Shaw, M., Jean, L., and Vaux, D. J. (2012) Combined effects of agitation, macromolecular crowding, and interfaces on amyloidogenesis. *J. Biol. Chem. 287*, 38006−38019.
(41) Ma, Q., Fan, J. B., Zhou, Z., Zhou, B. R., Meng, S. R., Hu, J. Y., Chen, J., and Liang, Y. (2012) The contrasting effect of macromolecular crowding on amyloid fibril formation. *PLoS One 7*, e36288.
(42) Yeung, P. S., and Axelsen, P. H. (2012) The crowded environment of a reverse micelle induces the formation of β-strand seed structures for nucleating amyloid fibril formation. *J. Am. Chem. Soc. 134*, 6061−6063.
(43) Sukenik, S., Politi, R., Ziserman, L., Danino, D., Friedler, A., and Harries, D. (2011) Crowding alone cannot account for cosolute effect on amyloid aggregation. *PLoS One 6*, e15608.
(44) Ellis, R. J., and Minton, A. P. (2006) Protein aggregation in crowded environments. *Biol. Chem. 387*, 485−497.
(45) Seeliger, J., Werkmuller, A., and Winter, R. (2013) Macromolecular Crowding as a Suppressor of Human IAPP Fibril Formation and Cytotoxicity. *PLoS One 8*, e69652.
(46) Zhang, D. L., Wu, L. J., Chen, J., and Liang, Y. (2012) Effects of macromolecular crowding on the structural stability of human α-lactalbumin. *Acta Biochim. Biophys. Sin. 44*, 703−711.
(47) Trexler, A. J., and Rhoades, E. (2010) Single molecule characterization of α-synuclein in aggregation-prone states. *Biophys. J. 99*, 3048−3055.
(48) Martin, D. J., and Ramirez-Alvarado, M. (2011) Glycosaminoglycans promote fibril formation by amyloidogenic immunoglobulin light chains through a transient interaction. *Biophys. Chem. 158*, 81−89.
(49) Motamedi-Shad, N., Monsellier, E., and Chiti, F. (2009) Amyloid formation by the model protein muscle acylphosphatase is accelerated by heparin and heparan sulphate through a scaffolding-based mechanism. *J. Biochem. 146*, 805−814.
(50) Chalikian, T. V., Volker, J., Anafi, D., and Breslauer, K. J. (1997) The native and the heat-induced denatured states of α-chymotrypsinogen A: Thermodynamic and spectroscopic studies. *J. Mol. Biol. 274*, 237−252.

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Biochemistry 2015, 54, 2957−2966