Protein Particulates: Another Generic Form of Protein Aggregation?

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ABSTRACT Protein aggregation is a problem with a multitude of consequences, ranging from affecting protein expression to its implication in many diseases. Of recent interest is the specific form of aggregation leading to the formation of amyloid fibrils, structures associated with diseases such as Alzheimer's disease. The ability to form amyloid fibrils is now regarded as a property generic to all polypeptide chains. Here we show that around the isoelectric point a different generic form of aggregation can also occur by studying seven widely different, nonrelated proteins that are also all known to form amyloid fibrils. Under these conditions gels consisting of relatively monodisperse spherical particulates are formed. Although these gels have been described before for β-lactoglobulin, our results suggest that the formation of particulates in the regime where charge on the molecules is minimal is a common property of all proteins. Because the proteins used here also form amyloid fibrils, we further propose that protein misfolding into clearly defined aggregates is a generic process whose outcome depends solely on the general properties of the state the protein is in when aggregation occurs, rather than the specific amino acid sequence. Thus under conditions of high net charge, amyloid fibrils form, whereas under conditions of low net charge, particulates form. This observation furthermore suggests that the rules of soft matter physics apply to these systems.

INTRODUCTION

The aggregation of proteins into often insoluble aggregates is a major problem in the processing of proteins on both large and small scales. Aggregation can severely decrease the yields of protein expression (1), is a nuisance in the milk industry (2), and is involved in a variety of human diseases, including several such as Alzheimer's disease, likely to affect aging populations increasingly (3). When properly controlled, however, protein aggregation often results in novel materials with many potential uses. Examples are common in the food industry, where protein aggregates and their gels are used to structure and texture foods (4–6) and more recently in the field of biomaterials, where protein- and peptide-containing systems are put to use in increasingly varied applications (7). A fundamental understanding of the driving forces and mechanisms involved in protein aggregation, however, is still outstanding (4,8).

We have recently been studying the aggregation behavior of the milk protein β-lactoglobulin under different conditions. Away from the protein's isoelectric point, heated solutions of the protein form clear fibrillar gels, whereas near its isoelectric point the gels are opaque and of a particulate nature (9,10). Mixed gels can be formed at intermediate pH. The fibrillar gels have been characterized in some detail. The fibrillar material has been found to be indistinguishable from amyloid fibrils (9,11), a form of protein aggregate normally associated with diseases such as Alzheimer's disease or the transmissible spongiform encephalopathies. Amyloid fibrils are long (several micrometer), thin (usually 5–15 nm), and unbranched polymeric forms of protein, which contain large amounts of a cross-β structure in their core (12,13). Beyond the proteins involved in amyloid diseases, many other proteins, when incubated under conditions in which partially denatured conformations are populated, form fibrils with indistinguishable properties. There are no known similarities between these proteins other than their ability to form amyloid fibrils. This has led to the suggestion that the formation of amyloid fibrils is a generic property of the polypeptide chain and suggests that all partially folded protein molecules exhibit generic behavior, similar to synthetic polymers (3,14).

In contrast to the fibrillar gels formed away from the isoelectric point, the partial denaturation of β-lactoglobulin near its isoelectric point is known to result in the formation of gels, consisting of a network of protein aggregates surrounded by liquid (15). The protein aggregate network consists of relatively monodisperse spherical particles, thus the gels are often called 'particulate gels', consisting of 'particulates' (16). These particulates bear no resemblance to amyloid fibrils and have not been described as having medical relevance. As the gel strength is highly dependent on the particulate size and this size is easily changed, the gels are of tremendous importance to the food industry (5). The particulates typically have diameters of hundreds of nanometers (15). Their internal structure is thought to contain large amounts of nonnative β-sheet, although it is unclear if this is intra- or intermolecular (17–19). Various models have been proposed describing the formation of the β-lactoglobulin particulates. One model suggests that the basic network structure leading to gelation of the solution is formed early on in the aggregation process and is not significantly

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changed by continued incubation and aggregation (17). A different model suggests a mechanism similar to polymer radical chemistry, comprising initiation, propagation, and termination stages (20). Given the apparent importance of the formation of disulphide bonds (21–24), it is possible that cysteine radicals correspond to the radical species described by Roefs and de Kruif (20). We have recently proposed a nucleation and growth mechanism in which unfolded protein molecules form a nucleus which then grows into the particulates observed microscopically (25). The model correctly explains and predicts particle size trends by rationalizing the observed dependence of particle size on both the solution conditions (pH and ionic strength) and the heating regime (heating rate, final incubation temperature, and incubation time) (9,10,15). What is striking is that this mechanism, like the other proposed mechanisms, is not specific to β-lactoglobulin: all proteins have an isoelectric point and will be at least partially unfolded at elevated temperatures. It could therefore be argued that like amyloid fibril formation, the formation of these particulate gels should be a generic property of all polypeptide chains. Similar structures have indeed been observed in whey protein isolate, the mixture of proteins from the whey fraction of milk (26). It is not clear, however, whether the particulates are composed solely of β-lactoglobulin or whether other proteins present in whey also formed particulates. Hen lysozyme gels formed by a two-step heating process are also known to contain spherical structures (27).

Given the capability of β-lactoglobulin to form both fibrillar and particulate gels under partially denaturing solution conditions but at different pH, we set out to investigate whether this duality exists for other protein systems. We studied the particulate formation of seven proteins, all of which differ substantially from each other, but are all known to form amyloid fibrils. After incubation at a pH near each protein's isoelectric point, particulates could be seen to be formed by all of the proteins. This observation strongly suggests that the formation of particulates, like that of amyloid fibrils, is a generic property of all polypeptide chains. These results allow us to speculate on the general principles underlying all ordered forms of protein aggregation.

MATERIALS AND METHODS

Chemicals and solutions

Human transthyretin was a gift from Margaret McCammon, α-synuclein was a gift from Lisa Cabrita and Robert Clay Rivers (all at the Department of Chemistry, University of Cambridge). The proteins were expressed in *Escherichia coli* and purified using ion exchange chromatography (28). All other proteins and chemicals were obtained in the highest purity available (>90% pure) from Sigma-Aldrich (Poole, UK) and used without further purification. β-lactoglobulin was bought as a mixture of genetic types A and B and used as such. All protein solutions had the same mass concentration of 30 mg/mL or 3% (w/v) and were brought to the pH shown in Table 1 using 1 M solutions of NaOH or HCl. All pH values are $\pm$0.05 pH unit. The pH values were based on calculations of the isoelectric point of each protein using the amino acid sequence and their associated pK_a values (29), as indicated in Table 1. With the exception of insulin, the solutions before heating were all clear and showed no obvious signs of isoelectric precipitation, even though they are near the pI of each protein. Insulin did show some cloudiness in its solutions, suggestive of some precipitation occurring.

Particulate formation

Aliquots of 30 μL from the protein solutions were placed in 40 μL aluminum DSC pans and sealed. Samples were heated in a PerkinElmer DSC7 (PerkinElmer, Beaconsfield, UK) differential scanning calorimeter (DSC) to permit carefully controlled thermal histories to be applied. Given that the proteins would be unfolding and aggregating concurrently, analysis of the DSC traces would be complex and this was not done. The heating regime consisted of equilibrating at 20°C for 1 min, heating to 90°C at a rate of 100°C/min, holding at 90°C for 30 min, cooling to 20°C at a rate of 25°C/min, and finally holding at 20°C for 1 min.

Conversion efficiency

To measure the amount of protein that had formed particulates, samples that had been heated were diluted to 1 mL in pH 2.0 H_2O. Under these conditions, any protein not in aggregates would dissolve. Electron microscopy measurements of the particulates have shown that particulates suspended in pH 2.0 H_2O have not decreased in size after 6 h (not shown). Given that the conversion efficiency experiment was done in <1 h, any contribution from dissolved particulates to the soluble protein fraction is likely to be negligible. After centrifugation for 10 min at 12,100 $\times$ g in a ''minispin'' centrifuge (Eppendorf, Cambridge, UK), the supernatant was removed and its absorbance at 280 nm measured. This was converted into a concentration using extinction coefficients taken from the UniProt Knowledge Database (http://www.expasy.ch). The absorbance measurements were taken at pH 2.0 H_2O; control experiments have shown that absorbance values measured in pH 2.0 H_2O and in 6 M guanidine.HCl with 0.02 M Na_2HPO_4 at pH 6.7 are indistinguishable (not shown).

TABLE 1 Relevant characteristics of the proteins used

Protein	Molecular mass/kDa	Secondary structure	Oligomerization state	Cys residues	pI	Incubation pH
Bovine β-lactoglobulin	18.3	Largely β-sheet	Dimer	5	4.8	5.30
Bovine serum albumin	66.4	α-helix	Monomer	35	5.8	5.80
Bovine insulin	5.5	α-helix	Mono-, di-, or hexamer	6	5.5	5.50
Horse heart myoglobin	16.9	α-helix	Monomer	0	7.7	7.80
Hen egg white lysozyme	14.3	Mixed α-helix/β-sheet	Monomer	8	10.3	10.50
Human transthyretin	13.7	Largely β-sheet	Tetramer	1	5.3	5.30
Human α-synuclein	14.3	Natively unstructured	Monomer	0	4.6	4.65

Shown in Table 1 are the monomeric molecular weight, native secondary structure, native state oligomerization state, the number of Cys residues in each monomer, isoelectric point (pI), and the pH at which each protein was incubated.

Size measurement

After heating, the samples were transferred from the DSC pans onto aluminum sample stubs and placed inside the chamber of an XL30-FEG environmental scanning electron microscope (ESEM; FEI UK, Cambridge, UK). ESEM allows the imaging of materials without the need for extensive fixing or coating of the material (30). The microscope chamber was pumped down to a pressure of 0.5 Torr and flooded repeatedly with water vapor. Images were obtained at a chamber pressure of 1 Torr and an acceleration voltage of 10 kV. Four pictures were taken of each sample at different locations within the sample. On each picture, the diameters of at least 30 particles were measured using Scion Image (release alpha 4.0.3.2; Scion, Frederick, MD). It should be noted that, although diameters were measured, the average radius and the standard deviation in it are used in this work.

ATR-FTIR

Aliquots of 30 μL of the protein solutions in the presence of 0.1 M NaCl were placed in DSC pans and, after equilibration at 20°C for 1 min, heated to 80°C at a rate of 100°C/min, held at 80°C for 30 min, cooled to 20°C at a rate of 25°C/min, and finally held at 20°C for 1 min. These samples, along with identical unheated samples, were analyzed in a Bruker BioATRCell II using a Bruker Equinox 55 Fourier transform infrared spectroscopy (FTIR) spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector and a silicon internal reflection element (IRE). For each spectrum 256 interferograms were coadded at 2 cm^{-1} resolution, and the water background was independently measured and subtracted from each protein spectrum. Spectra were subjected to Fourier self-deconvolution using the Bruker Opus software to identify component band positions.

RESULTS

The proteins used, bovine β-lactoglobulin, bovine serum albumin, bovine insulin, horse heart myoglobin, chicken lysozyme, human transthyretin, and human α-synuclein are all known to form amyloid fibrils at a pH away from their isoelectric point and under conditions where they are partially folded (9,28,31–35). In their native state, the proteins are markedly different, as summarized in Table 1. Briefly, β-lactoglobulin (36) and myoglobin (31) have similar molecular weights but differing oligomerization states (dimer and monomer, respectively) and native structures (β-barrel and all helical, respectively). Lysozyme (37), transthyretin (28), and α-synuclein (38) are of similar molecular weight but again of different oligomerization states (monomer, tetramer, and monomer, respectively) and native structures (mixed α-helical and β-sheet, β-sheet and unstructured, respectively). Insulin is the smallest helical protein used at only 5.5 kDa and is found in a variety of oligomerization states, usually up to hexamers (39). Serum albumin is the biggest protein used and it is monomeric and largely helical (40).

It is well known that proteins tend to precipitate at a solution pH near the protein's isoelectric point. This process can easily be reversed by altering the pH; thus it is best described as a form of reversible association. Examining 3% (w/v) protein solutions by environmental scanning electron microscopy (ESEM) without heating showed that no regular structures had formed (not shown). To induce aggregation and structure formation, all proteins were incubated as 3% (w/v) solutions at a temperature of 90°C in the presence and absence of 0.1 M NaCl. Under these conditions molar concentrations vary and the proteins will be unfolded to different extents; however in the case of β-lactoglobulin it is known that this will only influence the aggregation rates and particle sizes, rather than the fundamental ability to form the particulates at all (15,26, 25). At 90°C all proteins used are at least partially unfolded: α-synuclein because it is a natively unfolded protein and the other proteins as demonstrated by fluorescence spectroscopy (2, 25, and Supplementary Information). After incubation, the solutions were examined by ESEM; all solutions were found to contain substantial numbers of particulates. Those formed in the presence of NaCl are shown in Fig. 1. There are no apparent qualitative differences between the particulates formed by one protein or another in the absence (not shown) or presence of 0.1 M NaCl (Fig. 1)—all are spherical objects with relatively monodisperse diameters. From Fig. 1 and other similar electron micrographs, it can be crudely appreciated that varying amounts of residual monomeric protein were left in the solutions. This deposited in between the particulates during the drying down of the solutions, resulting in the appearance of apparently connected particulates and often

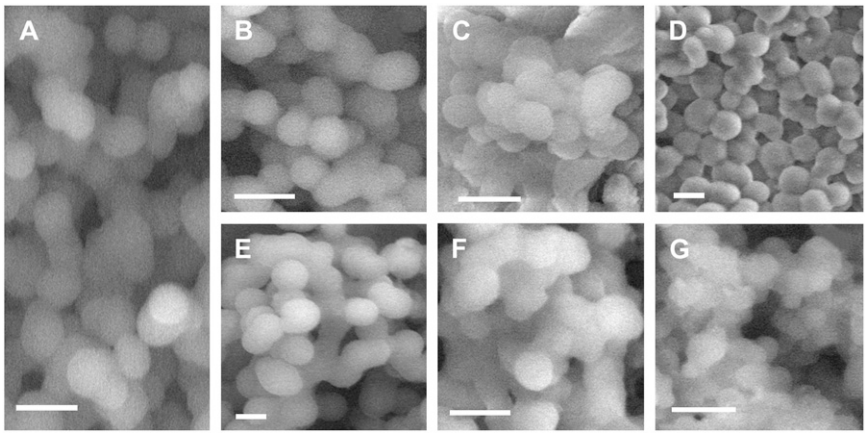

FIGURE 1 ESEM micrographs of the particulates formed by the various proteins used when heated to 90°C in the presence of NaCl. Scale bar on all images is 1 μm. Proteins shown are (*A*) bovine β-lactoglobulin; (*B*) bovine serum albumin; (*C*) bovine insulin; (*D*) horse heart myoglobin; (*E*) hen eggwhite lysozyme; (*F*) human transthyretin; and (*G*) human α-synuclein.

apparently amorphous films. This also suggests that monomeric protein concentration and kinetics are what limit particle size.

From electron micrographs similar to Fig. 1, the particulates' sizes were measured (Fig. 2). Different solutions of each protein did not give rise to significantly different measurements. There is a clear spread of average sizes, with insulin in the absence of NaCl forming the smallest particulates. It should be noted that insulin, from analysis of the electron micrographs, appeared to be the least efficient at forming particulates: more nonparticulate deposits, usually amorphous films, were found in preparations of insulin than any other proteins used. Most of the particulates appear to have a radius of ~250 nm. The average radius for β-lactoglobulin in the absence of NaCl was 215 $\pm$ 37 nm, which corresponds well to that reported previously (25). Incubation of the solutions in the presence of NaCl gave rise to larger particles than in its absence (Fig. 2). The size increase is within the error for bovine serum albumin and transthyretin but is significant in the case of insulin, β-lactoglobulin, α-synuclein, lysozyme, and myoglobin. It is not clear by what mechanism NaCl affects the size of the particulates, but this effect has been noted previously for β-lactoglobulin (26) and is under further investigation. It should be noted that at the ESEM chamber pressure used in this report, 1 Torr, the particulates were dry. To investigate if the particulates were significantly different in size when wet, particulates were imaged at higher chamber pressures of up to 5.5 Torr, where liquid water is present in the sample chamber (30). Water could be seen surrounding the particulates. Using careful control of the hydration state, a single site with 16 particulates was imaged at pressures between 5.5 Torr (wet particulates) and 1 Torr (dry particulates). The average size difference was <5% (not shown).

To investigate the internal structure of the particulates, attenuated total reflectance (ATR)-FTIR measurements were made of protein solutions before and after particulate formation. The level of conversion of protein into particulates for insulin was 42% converted; α-synuclein, 52%; transthyretin, 72%; bovine serum albumin, 85%; β-lactoglobulin, 98%; and lysozyme, 99%. The ultraviolet absorbance of free heme group, diffused out of partially unfolded myoglobin, precluded the determination of myoglobin conversion. Analysis of electron micrographs, however, reveals fewer nonparticulate protein deposits than observed for insulin, suggesting that conversion is significantly higher than that of insulin. The low conversion of insulin may be due in part to its lower solubility at the solution pH used. The normalized self-deconvoluted amide I spectra obtained are shown in Fig. 3. All spectra are well resolved, with clearly distinguishable secondary structure signatures. The native state spectra all correspond closely to what is expected based on the different proteins' native structures. For example, the spectrum of β-lactoglobulin is dominated by a strong peak at ~1630 cm^{-1}, corresponding to a predominance of β-sheet structure (36,41). The spectrum of myoglobin is characterized by a band at ~1655 cm^{-1}, corresponding to helical structures (31,41). The spectrum of α-synuclein features a mixture of structures, although a significant contribution comes from a band at 1645 cm^{-1}, which corresponds to random structure (38,41). For insulin, however, which was the only protein to undergo significant precipitation from solution under the conditions used, substantial disordered structure was indicated (as characterized by a band centered on 1643 cm^{-1}) that is not present in the crystal structure. Spectra of the heated samples revealed that the particulates contained similar levels of ordered secondary structure to the unheated proteins. In the cases of insulin, lysozyme, and α-synuclein, secondary structure bands present in the unheated samples persisted in the particulate spectra, with little change. For bovine serum albumin, myoglobin, and transthyretin there was an increase in the content of β-sheet as indicated by the increase in intensity of bands positioned between 1620 and 1630 cm^{-1}. The β-lactoglobulin particulates demonstrated a decrease in

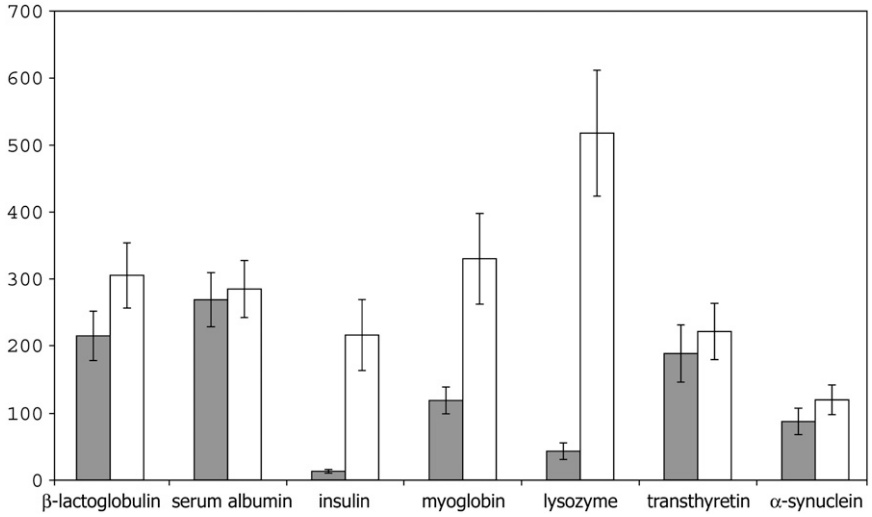

FIGURE 2 Particulate radii formed when protein solutions were heated to 90°C in the absence (■) and presence (□) of 0.1 M NaCl.

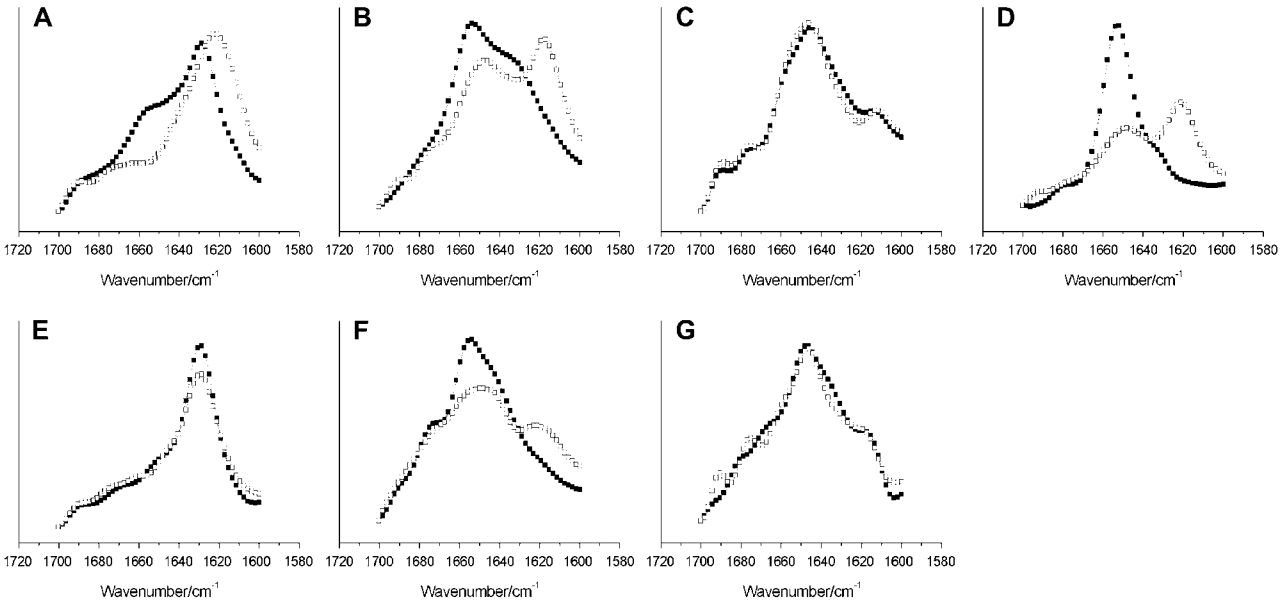

FIGURE 3 ATR-FTIR spectra of native (■) and particulate proteins (□). Proteins are (*A*) bovine β-lactoglobulin; (*B*)bovine serum albumin; (*C*) bovine insulin; (*D*) horse heart myoglobin; (*E*) hen eggwhite lysozyme; (*F*) human transthyretin; and (*G*) human α-synuclein.

helical structure (1653 cm^{-1}) and, as has previously been observed for this system, the β-sheet band in the aggregated form was shifted to lower frequencies relative to the soluble form (17,18,42). This shift may be indicative of the exchange of intramolecular β-sheets for intermolecular ones (41).

DISCUSSION

From Fig. 1 it can be seen that all the proteins tested form comparable particulates when the solutions are heated and subsequently investigated by electron microscopy. Included in these proteins were myoglobin, α-synuclein, and transthyretin. These proteins cannot polymerize through disulphide linkages, since they contain no (myoglobin and α-synuclein) or only one (transthyretin) Cys residue. The data presented here suggest, therefore, that disulphide bonds are not a necessary requirement for particulate formation. This may be different, however, under different conditions (23). Fig. 2 shows that the addition of 0.1 M NaCl to solutions of the proteins tested results in an increase in particulate size, in line with observations made for β-lactoglobulin (26). Given these observations and the spread of proteins studied in this work, ranging in size from 5.5 kDa to 66.4 kDa, with different native secondary structures and different native association states (Table 1), it seems likely that the ability to form particulate gels when incubated at elevated temperatures at or near the isoelectric point is common to potentially all polypeptide chains.

To investigate the mechanism by which particulates form further, their internal structure was studied by ATR-FTIR (Fig. 3). The particulate spectra show that there is no apparent common structure in the particulates; instead the particulates exhibit secondary structure rather similar to the protein's native state. This is in contrast to amyloid fibrils, where the secondary structure is always that of a cross-β-sheet, regardless of the protein contained within the fibrils (13). The lack of common secondary structure in the particulates suggests, therefore, that the protein is only partially unfolded before aggregation and that no or only minor reorganization occurs before, during, or after aggregation into the particulates. Indeed, at the temperatures we probed experimentally (up to 90°C), the proteins used in this report were at least partially unfolded (25, Supplementary Information). Given that the aggregation occurs near the protein's isoelectric point, there is little or no net charge and therefore a substantial barrier to aggregation, long-range charge-charge repulsion, has been removed. The protein molecules are, however, partially unfolded, exposing hydrophobic residues to the solvent, providing a potent driving force for aggregation. Together, these factors allow for a fast and nonspecific aggregation process. This, in turn, decreases the likelihood of substantial structural rearrangements during the aggregation process. Furthermore, because the aggregation is nonspecific, there is no directionality to the aggregates, resulting in the formation of three-dimensional spherical particles. At lengthscales larger than that of a protein molecule, therefore, the aggregates appear regular, whereas their internal structure is likely to be a fractal-like irregular structure.

These proteins were chosen, however, for their known abilities to form amyloid fibrils when incubated at elevated temperatures and at a pH away from their isoelectric point (9,28,31–35). As discussed in the Introduction section, it has

been suggested that the ability to form amyloid fibrils is a generic property of polypeptide chains (3,14). Rather similar to the particulates, amyloid fibrils form from partially unfolded protein (3,38,43). This partially unfolded state is, however, highly charged, resulting in long-range repulsion and slow aggregation. Aggregation only occurs when substantial structural reorganizations allow the formation of a favorable structure: the cross-β sheet. It is this structure that imposes essentially one-dimensional aggregation and is generic to all amyloid fibrils.

In some senses, this change in behavior, from amyloid fibril formation to particulate formation, can be likened to the aggregation of polymer colloids, whose state of aggregation is determined by the magnitude of electrostatic repulsion. Here in the presence of salt, i.e., when charges are screened, aggregation is rapid and results in the formation of irregular or fractal structures. In the absence of salt, charge-charge repulsion reduces the aggregation rate and more regular, crystal-like structures are formed (44).

We therefore propose that when a polypeptide chain is removed from its native conditions and some unfolding is allowed to occur, apparently generic behavior emerges. Thus, for example, under conditions of high charge on the protein chain, highly ordered amyloid fibrils are formed. Under conditions of low charge, such as those tested in this work, or high charge screening (8), particulates form, in a type of rapid and random aggregation. It is therefore the generic properties of the state the protein is in when aggregation occurs that give rise to certain aggregates forming, rather than any specific interactions.

The different properties of the partially unfolded state, of which charge is one, are imparted by the side chains. Under native conditions, the side chains of a protein determine the precise three-dimensional structure and stability of the native state (45). Conversely, they determine the conditions needed to destabilize the native state and give rise to more generic, less side-chain-specific behavior. Furthermore, under conditions where the native state is disrupted, the side chains will determine the rates and forms of aggregation through, for example, the charge, polarity, and hydrophobicity they carry (14). The side chains also influence the properties of the aggregates, giving rise to, for example, the observed specificity in the seeding of amyloid fibrils (46), the relative rates at which amyloid fibrils form (47), and morphology and other properties of amyloid fibrils formed under subtly different conditions (32,34,48–53).

The influence of the side chains, however, is now no longer exerted through specific interactions at specific locations in the protein backbone. Rather, the sum of all the side-chain attributes, including charge, hydrophobicity, and volume, result in an overall generic ''state'' of the partially folded protein. It is the generic, averaged physicochemical properties of this state of the polypeptide chain under specific solvent conditions that will determine the type of aggregate that will be formed. A similar approach has been used successfully in theoretical models and simulations limited to the amyloid fibril formation process (54). It is in this way that we can anticipate that the rules of soft matter physics will be more applicable to different forms of protein aggregation (spherical, fibrillar, or otherwise), without needing to take the molecular details so carefully into account.

Finally, the generality of the formation of particulates makes them an attractive target for nanoscience and nanotechnology. The proteins used self-assembly to form particulates of relatively uniform size. The size is easily controlled by altering heating or solution conditions. We are currently working on several proof-of-concept experiments to demonstrate potential uses for the particulates.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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