Amyloid Fibril-Like Structure Underlies the Aggregate Structure across the pH Range for β-Lactoglobulin

Mark R. H. Krebs, Glyn L. Devlin, and Athene M. Donald*
Cavendish Laboratory, University of Cambridge, Cambridge, United Kingdom

ABSTRACT The protein β-lactoglobulin aggregates into two apparently distinct forms under different conditions: amyloid fibrils at pH values away from the isoelectric point, and spherical aggregates near it. To understand this apparent dichotomy in behavior, we studied the internal structure of the spherical aggregates by employing a range of biophysical approaches. Fourier transform infrared studies show the aggregates have a high β-sheet content that is distinct from the native β-lactoglobulin structure. The structures also bind the amyloidophilic dye thioflavin-T, and wide-angle x-ray diffraction showed reflections corresponding to spacings typically observed for amyloid fibrils composed of β-lactoglobulin. Combined with small-angle x-ray scattering data indicating the presence of one-dimensional linear aggregates at the molecular level, these findings indicate strongly that the aggregates contain amyloid-like substructure. Incubation of β-lactoglobulin at pH values increasingly removed from the isoelectric point resulted in the increasing appearance of fibrillar species, rather than spherical species shown by electron microscopy. Taken together, these results suggest that amyloid-like β-sheet structures underlie protein aggregation over a much broader range of conditions than previously believed. Furthermore, the results suggest that there is a continuum of β-sheet structure of varying regularity underlying the aggregate morphology, from very regular amyloid fibrils at high charge to short stretches of amyloid-like fibrils that associate together randomly to form spherical particles at low net charge.

INTRODUCTION

In the study of protein aggregation, the protein β-lactoglobulin presents an interesting conundrum because it has been documented that under different solution conditions the protein forms two apparently distinct and unrelated aggregate types. At pH values away from the isoelectric point, amyloid fibrils are formed that are indistinguishable from the structures observed in many diseases (1–3). On the basis of fiber diffraction evidence (4) and, more recently, x-ray crystallographic evidence (5), the underlying structure of amyloid fibrils has been revealed to comprise the cross β-sheet motif. At or near the isoelectric point pI, however, spherical aggregates are formed (1,2). In this work we study the hitherto unresolved internal structure of the spherical aggregates formed by β-lactoglobulin.

The spherical particles formed by β-lactoglobulin have radii ranging from ~100 to ~1500 nm; the size is controlled by the incubation conditions. Heating rate and holding temperatures in particular are known to have a significant effect on particulate size (1,6). There are no reports of particulates occurring in vivo; however, they are of high importance in the food processing and manufacturing industries (7–11).

We, among others, have previously proposed mechanisms of particle formation whereby partial unfolding of the protein is considered to precede aggregation (12–17). The unfolding of β-lactoglobulin is a complex and pH-dependent process, starting in a noncooperative transition above 30°C (18–21). After a more cooperative transition between ~70°C and ~90°C, the protein completely unfolds around 130°C (18–21). It is the transition between ~70°C and ~90°C that is associated with the formation of the spherical aggregates (1). In the mechanism recently proposed, protein conformations that are populated in this temperature range form nuclei that grow via the addition of additional partially and/or fully unfolded monomers to give rise to the particles (1). The strong correlation found between the experimentally observed trends in particle size with thermal history and numerical simulations based on this mechanism strongly support the mechanism's accuracy (1).

Since our proposed mechanism, like other mechanisms, does not incorporate details specific to the physicochemical properties of β-lactoglobulin, it might be expected that other proteins would be equally capable of forming comparable spherical aggregates. This expectation was recently shown to be true: seven different and unrelated proteins (β-lactoglobulin, hen lysozyme, bovine insulin, human transthyretin, human α-synuclein, bovine serum albumin, and horse heart myoglobin) were all found to form indistinguishable spherical particles at their respective isoelectric points and under partially denaturing conditions (22). These observations suggest that particulate formation is broadly accessible to polypeptide chains and that this assembly does not require any primary sequence-specific information. Further, these results indicate that upon partial unfolding of protein native conformations, generic polymer properties of the polypeptide chain emerge, allowing for the formation of aggregates with almost identical properties (22,23).

Submitted October 8, 2008, and accepted for publication March 17, 2009.

*Correspondence: amd3@cam.ac.uk

Glyn L. Devlin's present address is Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia.

Editor: Heinrich Roder.

0006-3495/09/06/5013/7 $2.00

doi: 10.1016/j.bpj.2009.03.028

The apparent generic ability of proteins to form spherical aggregates near their pI is mirrored by their well-documented generic ability to form amyloid fibrils away from the isoelectric point (24). Amyloid fibrils are fibrillar protein aggregates that are implicated in a variety of human diseases such as Alzheimer's disease, the transmissible spongiform encephalopathies, and type 2 diabetes (1–3,24–27). As with the particulate aggregates, it has been suggested that the generic polymer properties of the polypeptide chain appear to underlie the formation of amyloid fibrils (24,28). A further similarity between the spherical aggregates and amyloid fibrils is that both appear to form from a wide variety of partially folded proteins via a nucleation and growth-based mechanism (24,28). Indeed, the same seven proteins mentioned above, which form particulates at their respective isoelectric points, also form amyloid fibrils under conditions of higher net charge on the polypeptide chains (22). It would therefore appear that the extent of net charge on the polypeptide chain can govern the nature of intermolecular interactions and dictate the form of the final aggregated structure. Indeed, the importance of electrostatic effects in determining the nature of protein aggregation is gaining increasing prominence in the literature overall (29–32).

In this work we study the internal structure of the spherical particles using Fourier transform infrared (FTIR) spectroscopy, thioflavin-T dye binding, and x-ray diffraction. Taken together with data from small-angle x-ray scattering (SAXS) experiments (which will be presented in detail elsewhere), the results indicate that the particulate β-sheet structure with the particles shares properties with the cross-β structural motif observed in all amyloid fibrils. The observation of this structure in aggregates formed in solution conditions both at and away from the isoelectric point of the protein, as well as the subtle differences between the β-sheet structures formed under these conditions, allows us to speculate on the role of this type of secondary structure in protein aggregation in general.

MATERIALS AND METHODS

Proteins and solutions

All chemicals used were bought from Sigma-Aldrich (St. Louis, MO) in the highest available purity. β-Lactoglobulin was purchased as a mixture of genetic types A and B, and used as such. Most protein solutions were made by dissolving 30 mg of protein in 1 mL of deionized demineralized water containing the appropriate concentration of NaCl. The resulting solutions had protein concentrations of 3% w/v (30 mg/mL or ~1.6 mM) unless noted otherwise. The pH of the solution was adjusted to the desired value using 1 M NaOH or 1 M HCl solutions.

Differential scanning calorimetry

Aliquots of 30 μL from protein solutions were taken and placed in 40 μL differential scanning calorimetry (DSC) pans. The pans were subsequently sealed and placed in either a Perkin-Elmer (Waltham, MA) DSC7 or Pyris 1 DSC. DSC was used to ensure carefully controlled thermal histories. Furthermore, given the temporal overlap of unfolding and aggregation, the DSC data were neither saved nor analyzed. After initial equilibration at 20°C for 1 min, the samples were heated to a chosen temperature at a chosen heating rate and held for a chosen amount of time. After heating, the solutions were cooled down to 20°C at 25°C/min and held at 20°C for 1 min.

Environmental scanning electron microscopy of particulates

The particulates were imaged with the use of environmental scanning electron microscopy (ESEM) because that method does not require drying, fixing, or staining of the sample (33). Heated DSC pans were opened and their contents placed on aluminum stubs. These were placed inside the microscope chamber of an XL30-FEG ESEM (FEI UK, Cambridge, UK) and the chamber was pumped down to 1 Torr pressure. The chamber was flooded five times with water vapor and pumped back down to 1 Torr, ensuring that the only gas present was water vapor. Imaging was performed using an acceleration voltage of 10 keV. For each sample, four micrographs were taken at random locations. In each micrograph, at least 30 particulates were visible. The diameters of at least 30 particulates were measured using the software package Scion Image (version Alpha 4.0.3.2). In the text and figures, the average radius and standard deviation on the radius are shown. Polydispersity was calculated as the ratio of standard deviation over average size. No significant size changes were observed when identical heating conditions were repeated using different samples.

Transmission electron microscopy of particulate solutions

For transmission electron microscopy (TEM) of particulate solutions, protein solutions were diluted 100× with water at the same pH as the protein solution. Aliquots (3.5 μL) from this solution were placed on formvar- and carbon-coated copper grids. After 1 min, the solution was wicked off and 2 × 15 μL of a 2% (w/v) uranyl acetate solution was placed on the grid. This was removed after 30 s and the grids were left to dry. The grids were studied in a Tecnai 20 microscope (FEI UK, Cambridge, UK), usually at 26,000× magnification.

Fluorescence spectroscopy

Solutions of particulates were diluted to 980 μL with a 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4 PBS buffer. To this was added 20 μL of a 2.5 mM thioflavin-T solution in PBS, filtered through a 0.2 μm syringe filter. The resulting solution was placed in a plastic disposable cuvette and its fluorescence was measured on a Cary Eclipse fluorimeter with excitation at 440 nm and emission measured at 482 nm. Slit widths were 5 nm and 10 nm, respectively. Although particulates settled at the bottom of the cuvettes, this occurred over a length of time significantly longer than the few minutes necessary to collect the fluorescence of each sample.

Fluorescence microscopy

Solutions prepared in the same way as those for the fluorescence measurements were placed on microscope slides and a coverslip was placed over them. They were imaged on a Motic AE31 inverted fluorescence microscope using a mercury lamp and a fluorescence cube with excitation centered on 480 nm, ~40 nm bandwidth, and emission centered on 535 nm, ~40 nm bandwidth.

Attenuated total reflection Fourier transform infrared spectroscopy

For attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, aliquots from heated and unheated solutions were placed in a Bruker BioATRCell II inside a Bruker Equinox 55 FTIR spectrometer fitted with a liquid nitrogen-cooled MCT detector and silicon internal

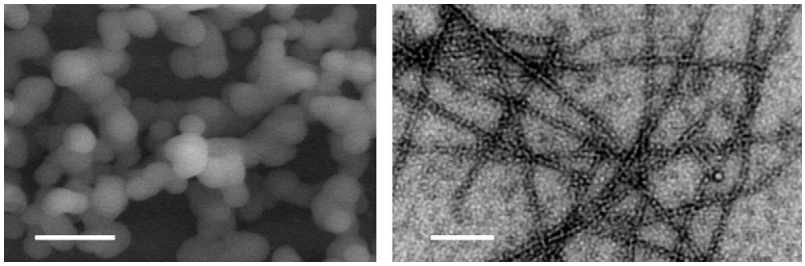

FIGURE 1 ESEM and TEM micrographs of particulates and amyloid fibrils formed by β-lactoglobulin. (*A*) Particulates were formed from 30 mg/mL (3% or ~1.6 mM) pH 5.3 β-lactoglobulin solutions heated to 80°C at 100°C/min and held for 10 min. Scale bar represents 2 μm. (*B*) Amyloid fibrils formed by heating a 4% solution of β-lactoglobulin at pH 2.0 for 48 h. Scale bar represents 200 nm.

reflection element. For each sample, 256 interferograms were collected at 2 cm^{-1} resolution and co-added. A water background spectrum was collected independently and subtracted from each sample. The spectra were analyzed by Fourier self-deconvolution with the Bruker Opus software.

X-ray diffraction

Particulate solutions were prepared in the presence and absence of 100 mM NaCl as described above. Aliquots from the solutions were placed onto mica sheets held on copper rings. Scattering patterns of the particulate and background solutions were collected after drops of each had dried on the mica sheet. The copper rings were mounted and irradiated with 1.54 Å x rays generated by a rotating copper anode. The beam was collimated and focused with Osmic Max-flux optics, and images were acquired on a Marr image plate. These experiments were done at the Biochemistry Department at Cambridge University. The 2D images were radially averaged using the program Fit2D (34) and the buffer background was subtracted from the particulate spectra.

RESULTS AND DISCUSSION

Solutions of β-lactoglobulin heated near their isoelectric point at pH 5.3 were found to contain many spherical particles upon investigation by ESEM (Fig. 1 *A*). The particle size can be reproducibly controlled by changing the solution conditions (1,6), but for the purpose of this study only one set of conditions was chosen: 3% (30 mg/mL or ~1.6 mM) β-lactoglobulin, pH 5.3, a brief hold at 20°C for 1 min, then heating from 20°C to 80°C at 100°C/min, holding for 10 min at that temperature, followed by cooling down to 20°C at 25°C/min and holding for 1 min at 20°C. In contrast, when 3% solutions at pH 2.0 were heated to 80°C for 48 h, fibrillar structures formed that were microns long but only 10–20 nm wide (Fig. 1 *B*). These structures were previously characterized as amyloid fibrils (1–3).

For the first stage of this investigation of the internal structure of the spherical particles, we used ATR-FTIR spectroscopy. Fig. 2 shows the spectra in the amide I region (1600–1700 cm^{-1}), which predominantly corresponds to the amide carbonyl stretching vibration. All spectra show a major peak in the 1620–1630 cm^{-1} region, corresponding to β-sheet structure (35,36). Deconvolution of the spectra indicates an increase in β-sheet content in the particulate form relative to the native form of the protein, as well as a shift in the β-sheet band from ~1629 cm^{-1} to ~1625 cm^{-1} (Fig. 2 and the Supporting Material), consistent with earlier work (14,22,37,38). It was previously shown that such red-shifting of this β-sheet band in protein FTIR spectra is consistent with the presence of intermolecular (rather than intramolecular) β-sheet structure, and is often observed in the spectra of amyloid fibrils relative to the spectra observed for their constituent proteins when natively folded (39). This led us to speculate that the structure of the particles at the molecular level may be that of an amyloid fibril: the cross β-sheet (40). Different tests for the presence of amyloid fibrils are available, each of which has its own strengths and weaknesses (41). We therefore performed several further tests and used a point scoring system to determine whether the particles did indeed contain amyloid fibrils (41).

The first such test examined the ability of the particles to bind thioflavin-T. This dye is considered specific to amyloid fibrils, since its fluorescence emission increases greatly when bound to amyloid fibrils (42,43). From other experiments it is known that diffusion of dye molecules into the particles is fast and not a limiting factor (44). In the presence of native β-lactoglobulin, fluorescence from thioflavin-T is very low. The emission intensity is greatly increased in the presence of particulates (Fig. 3 *A*). This further demonstrates the presence of amyloid-like structure, although a spectroscopic examination alone of the solution does not reveal whether the fluorescence emanates from the particles or from other

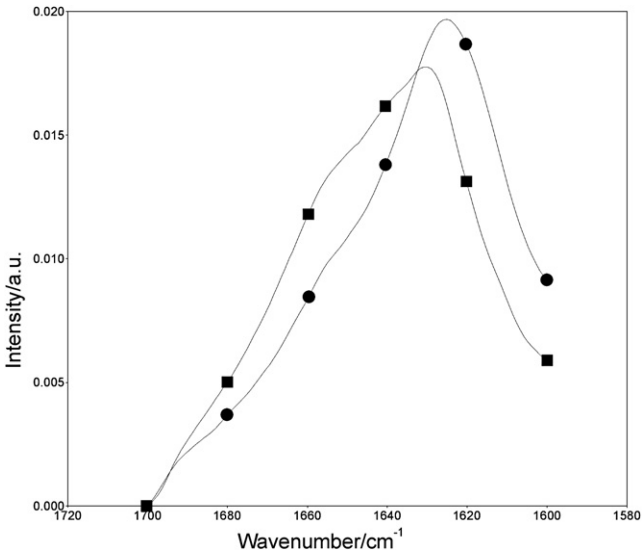

FIGURE 2 ATR-FTIR study of native and particulate β-lactoglobulin. Shown are the spectra of native β-lactoglobulin (■) and particulate β-lactoglobulin solutions formed by heating 3% w/v solutions of β-lactoglobulin at pH 5.3 at 100°C/min to 80°C and holding at that temperature for 10 min (●).

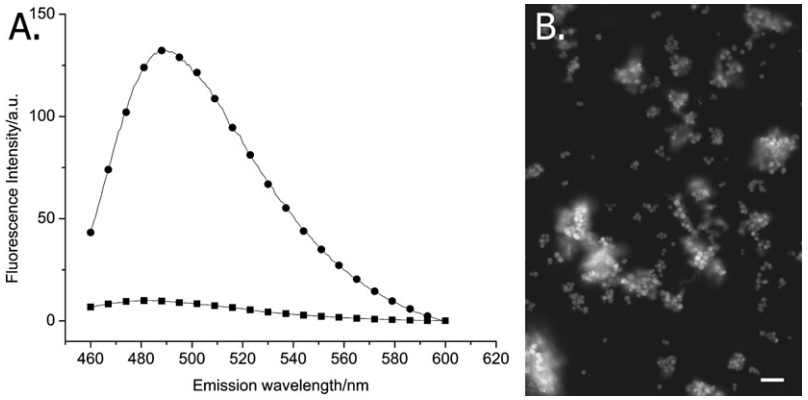

FIGURE 3 Thioflavin-T studies of particulates. (*A*) Fluorescence emission from thioflavin-T excited at 440 nm and in the presence of unheated β-lactoglobulin (■) or β-lactoglobulin particulates (●). (*B*) Fluorescence microscopy image of β-lactoglobulin particulates in the presence of thioflavin-T; scale bar represents 20 μm.

aggregates floating in the solution. Fluorescence microscopy was used to determine the source of the fluorescence. Images taken using thioflavin-T as the fluorescent dye showed that binding was localized to the particles (Fig. 3 *B*). This was also confirmed by confocal imaging (not shown). According to the point scoring system, thioflavin-T binding and β-sheet structure by FTIR together give rise to a score of 4, enough to classify the aggregates as containing amyloid structure (41). Given the lack of obvious fibrillar structure, however, we performed further tests to strengthen that conclusion.

Wide-angle x-ray diffraction was carried out on the particles as a further diagnostic test for the presence of the amyloid structural motif, as it can reveal spacings at the molecular level. The amyloid cross-β sheet is characterized by two distances: the 4.7 Å distance arising from the separation between β-strands, and the 9–12 Å distance arising from separation between the β-sheets (40). In samples of unaligned amyloid fibrils these two reflections appear as isotropic rings; in aligned samples, however, the reflections are anisotropic and oriented perpendicular to each other. Given the spherical nature of the particles, it is impossible to induce alignment in the sample. As such, the diffraction was carried out with the particles randomly oriented on a mica surface. The scattering pattern from the particulates revealed two isotropic reflections. Radial integration of the pattern revealed the presence of peaks at ~4.7 Å and~10.6 Å (Fig. 4), which are strongly indicative of the presence of the cross-β motif (40). Indeed, amyloid fibrils produced under acidic conditions by β-lactoglobulin were previously shown to yield almost identical wide-angle x-ray scattering reflections (45). All three tests performed to detect the presence of amyloid fibrils showed positive results. Since the wide-angle x-ray diffraction showed the presence of β-sheet again, the total points score was 6, confirming the presence of amyloid fibrils in the particles.

Given this unexpected result and the nonfibrillar morphology of the particles by electron microscopy, a further test was performed. Investigating the particles using SAXS gave rise to scattering patterns consistent with the presence of amyloid fibrils (46). When we investigated the internal structure of the particles by SAXS, the data revealed that at distances of a few molecular diameters, linear structure dominated (46). The current data suggest that these are amyloid fibrils. At longer length scales, corresponding to hundreds of molecules, SAXS revealed that the packing was space-filling and dense (M. R. H. Krebs and A. M. Donald, unpublished). Taken together, the data suggest that the amyloid fibrils are short and randomly packed, resulting in almost completely dense particles.

All of the data collected so far (i.e., the FTIR spectra, the binding of thioflavin-T, wide-angle x-ray scattering, and SAXS) are all positive for the presence of amyloid fibril-like structure in the spherical particles. Based on the scoring system (41), this leads us to suggest that short stretches of amyloid fibrils are indeed the core structure of these particles. The short nature of these fibrils and the random packing of these short fibrils lead to the appearance of spherical structures under electron and optical microscopes.

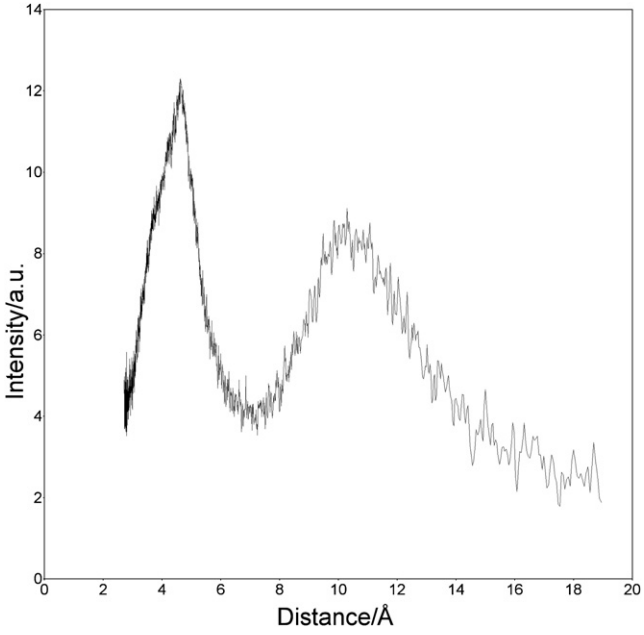

FIGURE 4 X-ray diffraction study of particulate β-lactoglobulin. Solutions of 3% w/v β-lactoglobulin at pH 5.3 were heated at 100°C/min to 80°C and held at that temperature for 10 min. The x-ray pattern was radially averaged and the mica scatter pattern background was subtracted.

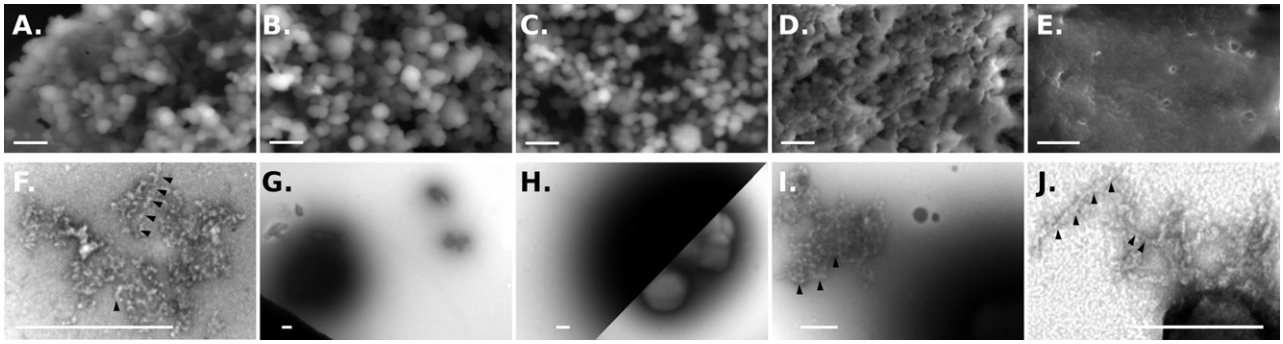

FIGURE 5 TEM/ESEM studies of particulate-containing solutions. All solutions consisted of 30 mg/mL β-lactoglobulin, in the absence of NaCl, heated to 80°C at 100°C/min and held for 60 min. The pH values of the solutions were 4.7 (*A* and *F*), 5.0 (*B* and *G*), 5.3 (*C* and *H*), 5.6 (*D* and *I*), and 5.9 (*E* and *J*). The first row (*A–E*) contains ESEM images, scale bar 2 μm; the second row (*F–J*) contains TEM images, scale bar 500 nm (*F–I*) or 200 nm (*J*). In image H, both a brightfield (*top half*) and darkfield (*bottom half*) image of the same aggregate is shown. Fibril-like aggregates or precursors are indicated with arrowheads in the images.

It is known that β-lactoglobulin forms amyloid fibrils of micron length away from the isoelectric point. Here we have shown that near the isoelectric point, short fibrils form and assemble into spherical particles. To examine more closely the solution conditions that promote the formation of spherical aggregates, rather than linear ones, we incubated the solutions at a range of pH values, from 4.7 to 5.9, in steps of 0.3 pH units. The pI of β-lactoglobulin varies depending on the source and the state of the protein (2,47), but it is usually taken as around pH 5.1. Based on this and published pK_a values (48), we can assume that the net charge on the protein ranges from ~+12 at pH 4.7 to ~−4 at pH 5.9. To ensure substantial aggregation, samples were held at 80°C for 60 min, rather than 10 min. Incubated samples were subsequently investigated by ESEM to detect the particles, and by TEM to detect amyloid-like fibrillar species or their precursors (Fig. 5). All images show the presence of spherical particles, although further away from the pI there is an increasing presence of apparently irregular material interspersed between the particulates (Fig. 5, *A–E*). In the TEM, particulates appear as large dark globules in brightfield mode: their density and size were such that the electron beam could not penetrate them. In darkfield imaging mode, however, where scattered (rather than undeflected) electrons are used for imaging, the particles could be resolved (Fig. 5 *H*). At pH values away from the pI on both sides of it, the presence of fibrillar material, not dissimilar to that often seen before the complete formation of amyloid fibrils, was detected by TEM (Fig. 5, *F–J*) (49–53).

Taken together, the data indicate that the spherical particles are composed, at least in part, of amyloid-like β-sheet stretches that are short enough to arrange in a random, close-packed fashion, resulting in the observed spherical particles. Across the pH range examined, therefore, β-lactoglobulin has a high propensity to assembly into aggregates with a cross-β-like structure. We propose that this structural motif may underlie multiple aggregates types, even those with no externally discernible fibrillar morphology. The high stability of the amyloid fibrils due their extensive hydrogen-bonding network may underlie this propensity. Furthermore, the net charge on the protein molecule appears to play an important role in influencing the morphology of the aggregate. As the net charge on the protein molecule is increased, the aggregation becomes more regular and fibrils appear in addition to spherical particles. Beyond a certain net charge, regular amyloid fibrils would be the only observed aggregate (G. L. Devlin, unpublished results). It seems likely that as the charge is further increased, this trend would continue, ultimately resulting in the formation of amyloid fibrils only. It is known that fibrils form more slowly than particles (minutes for particles versus hours or days for fibrils) (1,45). It would seem, therefore, that the aggregation rate and the structural regularity of the resultant aggregates are related, as mediated by the charge on the molecule. This notion was previously proposed in the limiting case of amyloid fibril formation by the SH3 domain of the α-subunit of bovine phosphatidylinositol-3′, where controlled growth under near-equilibrium conditions resulted in the formation of more regular fibrillar structures (54).

These findings allow us to draw a further analogy between the aggregation of proteins and soft condensed matter more generally. The aggregation of colloidal particles is to a large extent controlled by the charge on the particles: high charge will result in regular aggregation, whereas low charge or highly screened charges give rise to more disordered aggregation (55). The data presented here demonstrate that a similar effect is observed for protein aggregation. As the charge on an aggregating protein molecule is decreased, an increasingly disordered structure is formed. At high charge, β-lactoglobulin forms typical amyloid fibrils (1–3). These structures are known to be highly regular and contain only very few structural ''errors''—one in every several thousand molecules (56). If the net charge is decreased by incubating the protein nearer its isoelectric point, β-sheet-rich amyloid-like structures will still form; however, they will possess a much reduced level of structural order such that they can

randomly coalesce into the spherical structures discussed in this work. Further supporting evidence for this framework will be reported in a subsequent study on the effects on aggregation from the addition of salt.

CONCLUSIONS

We have shown that the spherical aggregates that form when protein solutions are heated at the isoelectric point possess structural characteristics consistent with the presence of amyloid-like cross-β structure, as determined by x-ray diffraction, thioflavin-T dye binding, and FTIR spectroscopy. Rather than full-length fibrils, however, the building blocks of particulates appear to be short fibrillar stretches that aggregate and pack into a spherical structure at larger distances.

More importantly, given that both amyloid fibrils and the spherical aggregates can be formed by apparently any protein, these experiments give us a profound insight into the nature of protein aggregation. It is clearly demonstrated that the formation of β-sheet structure is favored under all conditions, probably due to intermolecular hydrogen bonds that lie at the heart of its structure, resulting in great stability and ready self-proliferation. The structural integrity of this β-sheet, and hence the external morphological appearance of the aggregate are highly sensitive to the amount of charge on the aggregating protein molecule. A higher charge leads to a more regular aggregate in the form of amyloid fibrils; a lower or more screened charge will give rise to shorter fibrillar structures that can pack into spherical structures. Lastly, since the side chains only contribute to this process by a generic physicochemical property (in this case, charge), we see that under conditions for which the native globular structure has been destabilized, proteins show a behavior that is generic and may be well described from a soft condensed matter viewpoint.

SUPPORTING MATERIAL

A figure, a table, materials and methods, and references are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)00764-4.

We thank Dima Chirgadze (Biochemistry Department, University of Cambridge) for assistance with the x-ray diffraction experiments and subsequent data treatment.

M.R.H.K. and A.M.D. received funding from the Engineering and Physical Sciences Research Council.

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