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Use of a hydrophobic dye to indirectly probe the structural organization and conformational plasticity of molecules in amorphous aggregates of carbonic anhydrase

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Abstract

Understanding protein aggregation may hold important clues to understanding what goes wrong with protein folding in neurodegenerative disorders and in bioreactors in which proteins are overexpressed. Unfortunately, aggregates tend to be intractable to most standard methods of biochemical investigation. Thus, relatively little is even now known about the micro- and macrostructural features of aggregates. To gain insights into the thermal aggregation of a model globular protein [bovine carbonic anhydrase (BCA)], we have used spectrofluorimetry to examine the binding of a hydrophobic dye, 8-anilinonaphthalene sulfonate (ANS), to hydrophobic clusters on the protein's surface both before and after heat-induced aggregation and upon cooling. Whereas native BCA shows no surface hydrophobicity, thermally aggregated BCA displays significant hydrophobicity both in the heated state and upon cooling. The timing of the addition of ANS in the course of aggregation makes no net difference to the ANS bound; we argue that this suggests that aggregates are essentially porous. Cooling of aggregates results in a dramatic, fully reversible increase in ANS binding that cannot be explained by the temperature dependence of fluorescence quantum yield alone; we argue that the enhancement of fluorescence upon cooling indicates possible structural consolidation of unfolded regions within aggregates (akin to refolding), with the required structural reorganization being facilitated by porosity. Finally, implications of porosity in aggregates are discussed, in particular, for the possible immobilization of enzymes through fusion with aggregation-prone protein domains.

Keywords: Protein aggregation; Thermal unfolding; Hydrophobic interactions; Aggregate structures

Globular proteins often aggregate in the course of protein unfolding, refolding, or de novo folding. In recent years, evidence has gathered in support of the presence of structured polypeptides within aggregates [1–3] and of selective, homomeric interactions within heterogenous mixtures of aggregating proteins. Such an evidence has lent credence [4–9] to the view that specific recognition-based interactions amongst partially folded molecules may play an important role in aggregation, with intermolecular interactions mimicking at least some native intramolecular interactions, and dominating over such interactions in a manner determined by the overall protein concentration (determining the mean molecular collision frequency in solution) and by the relative rates of folding and assembly of interacting substructures [10–12]. An emerging, alternative view that is still taking shape holds that polypeptides can associate through the total loss of native three-dimensional structure, followed by a reorganization of polypeptides (in a sequence-independent manner) into intermolecular, cross-beta sheet arrangements. Ultimately, it may well turn out to be the case that the aggregation of any particular protein involves a combination of both native-like and non-native intermolecular interactions, with the relative proportions of each type of interaction being determined by the overall fold and stability of the aggregating protein and also by the specific conditions that effect aggregation (e.g., change of temperature, change of pH, etc.). What is common to both views of aggregation is the significant realization that aggregation does not simply produce

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disordered coagulates, as was once believed to be the case. The urgency to try and understand how polypeptides are organized within aggregates is further heightened by the additional realization that if aggregation can be prevented from occurring, treatments could potentially be found for a number of diseases and the expression and handling of protein molecules too could be made less frustrating.

Here we specifically address two fundamental issues concerning aggregates, namely: (a) whether amorphous aggregates produced through heating are predominantly porous or solid-like, and (b) what happens within aggregates as the physical/chemical conditions effecting aggregation are themselves reversed, e.g., through cooling after heat-induced aggregation. Can protein chains undergo partial refolding in the aggregated state, or is such temperature-dependent structural consolidation forbidden on account of, e.g., a tight and optimized packing of sidechains within the aggregate and a network of stable intermolecular interactions based on hydrogen bonding and hydrophobicity?

It is worth considering that for any limited formation of structure to occur within an aggregate, a degree of (sub-microscopic; molecular-level) porosity could very well be required at the level of environments of individual molecules. Naturally occurring proteins are thought of as micro-particulate solids [13], with optimal packing of side chains ensuring the optimal usage of spaces within structures. It would be interesting to examine what fraction of molecular surfaces exposed upon partial, or complete, unfolding ultimately get buried through aggregation. It would also be interesting to examine how optimal the usage of space is within protein aggregates. The issue of porosity in aggregation has earlier been addressed in theoretical treatments [14,15]; however, no experimental studies have been carried out. The present study is an attempt to understand the physical nature of thermal aggregates of a model globular protein, through the application of spectrofluorimetry to the study of uniform, microparticulate suspensions of aggregated protein created through heating in solution with constant stirring; suitable controls are used and attention is paid to the possible vagaries of interpretation (see experimental section), especially because such studies have never been performed before. Our results indicate that thermal aggregates of BCA may be 'nanoporous' in nature, with porosity potentially extending down to the levels of environments of molecules.

Materials and methods

Materials. Bovine carbonic anhydrase (BCA) was obtained from Sigma. Ethylenediaminetetraacetic acid (EDTA), other chemicals and solvents were from either Merck or Glaxo and 8-anilino-1-naphthalenesulfonate (ANS) was obtained from Aldrich Chemical. ANS was dissolved in spectroscopic grade methanol to a stock concentration of 8.57 mM; this stock was further diluted to desired concentrations in aqueous solution containing proteins for all experiments performed.

Spectrofluorimetry. A Perkin–Elmer (LS-50B) spectrofluorimeter was used for all fluorescence measurements. For acquisition of spectra, excitation was carried out at 350 nm and scanning of emission was done between 400 and 500 nm, with 5 nm bandpasses used at both excitation and emission slits. Under the same conditions of excitation, the intensity of ANS fluorescence emission at 480 nm was also monitored as a function of time for control and other aggregation experiments. Two issues need to be clarified at the outset in respect of fluorescence measurements of ANS bound to aggregating or aggregated protein molecules: (1) there is no scope for direct contamination of emission measurements by scattering of photons used for fluorescence excitation since the difference between the wavelength settings of the excitation and emission monochromators (≈130 nm) ensures that neither the Rayleigh scattering component (peaking at the excitation wavelength of 350 nm) nor the Raman scattering component (peaking at about 385 nm) would pass through an emission slit of 5 nm bandpass, fixed to allow passage of only 480 nm light. (2) Micro-particulate aggregates forming in solution during heating (manifesting as a uniformly distributed, mild turbidity) remain suspended in the path of light from the excitation source throughout the experiment, aided by the constant vortexing motion of a magnetic bead rotating at the bottom of the cuvette. Thus, photons incident on the suspension excite fluorophores bound to molecules present either on the surfaces of aggregates, or within aggregates, just as they ordinarily excite fluorophores within folded, native protein molecules in any other fluorescence experiment. Any effect of the scattering of incident photons on the observed fluorescence quantum yield occurs to similar extents in experiments using indentical conditions, so this too does not affect interpretation of results. The experimental setup, considerations, and protocols used here are essentially identical to those reported previously in a study of the interaction of ANS with BCA, in which ANS was used at very high molar excesses over BCA (upto 150-fold molar excess and at concentrations ranging upto 750 μM ANS) to examine whether bound dye can inhibit the aggregation of BCA during thermal unfolding and refolding [16]. Here, of course, ANS is used at very low concentrations (~10 μM), as a probe of exposed hydrophobic surfaces, and adequate controls were done to confirm that at these concentrations the dye does not alter the saturation level of aggregation achieved; however, it must be mentioned that we have noted that the nonpolar solvent used to transfer the dye into BCA solution effects a marginal decrease in the rate at which aggregation occurs, without affecting the saturation level of light scattering.

Scattering measurements during thermal aggregation. All aggregation experiments were performed in the LS-50B spectrofluorimeter in a manner identical to that reported previously [16], with protein samples placed in standard fluorescence cuvettes. Scattering was monitored through measurement of the 90° Rayleigh scattering component of 650 nm light by the solution, using spectral bandpasses of 3 or 5 nm each for the excitation and emission slits.

Fluorimetry during thermal aggregation. Phosphate buffer (sodium phosphate, pH 7.6, 50 mM) was used to make protein solutions. All experiments used a protein concentration of 0.1 mg/ml (≈3 μM) and an ANS concentration of 10 μM where applicable. Thermal aggregation was carried out by placing protein solutions in a cuvette within the spectrofluorimeter's sample compartment, connected to a circulatory, thermostated water bath (Pharmacia Multitemp III). To avoid complications of interpretation arising from binding/release of Zn^{2+} ions, a known chelating agent EDTA was included at a final concentration of 1 mM in all experiments. ANS fluorescence was measured by excitation of the solution at 350 nm and observing the emission spectrum by scanning between 400 and 500 nm. For initial standardization experiments, aiming to determine optimum conditions for effecting aggregation, temperature within the cuvette was raised from 25 to 74 °C in increments of 5 °C. [While changes in temperature were effected at the circulatory water bath, actual temperatures within the cuvette were

measured with a digital Barnant thermocouple thermometer probe (accuracy of 0.1 °C)]. Protein solutions were incubated for 5 min at the desired temperatures within the cuvette before scattering measurements were made. Within the range of temperatures effecting protein melting/unfolding, changes in the level of light scattering were assumed to reflect differences in the kinetics of aggregation at different temperatures, since identical periods of incubation were used for all temperature points. The behavior observed was the basis for choosing a standard temperature of 64 °C for the performance of steady-state aggregation measurements in subsequent experiments (since aggregation appears to initiate at this temperature).

For experiments probing kinetics, cuvettes containing protein solutions were placed, as before, in the pre-heated sample compartment of the fluorimeter. Measurements of light scattering, or fluorescence, were made as a function of time, beginning with the attainment of a temperature of 60 °C (since protein aggregation commences with the attainment of this temperature and the final temperature of 64 °C is attained asymptotically). Plots presented represent measures of the final scattering or ANS fluorescence data obtained at the end of the incubation period in a heating experiment (i.e., after saturation of the rise in light scattering or ANS fluorescence) after suitable averaging over the length of observation and further averaging over a minimum of five independent experiments using identical conditions.

Use of ANS: background information and controls. The fluorescent hydrophobic dye, ANS, has been used widely as a probe for the presence of hydrophobic residue clusters on the surfaces of proteins [17,18]. ANS has also been used to examine the formation of partially structured intermediates in experiments monitoring protein folding in real-time [19–23]. Binding of ANS to partially structured BCA has previously been used as a means of monitoring the lifetime and kinetics of the formation of refolding intermediate(s) of BCA [24]. We have shown previously that ANS (at several 100-fold molar excess over BCA) binds to molten BCA in the course of thermal unfolding, to prevent aggregation [16]; in the course of that study, we also established conditions for rapid temperature-rise experiments through which ANS binding to molten BCA could be observed to attain saturation prior to the occurrence of detectable aggregation at protein concentrations of about 0.1 mg/ml.

The fluorescence quantum yield of ANS is known to be very poor in water and aqueous media, rising with the increasing nonpolarity of the solvent or with the hydrophobicity of the dye's environment [17,18]. Upon binding to hydrophobic residue clusters in proteins, there is a significant increase in the intensity of the dye's fluorescence, as well as often a blue-shifting of its wavelength of maximum emission [19–23,25]. A number of control experiments were performed prior to the use of ANS as a probe for thermal aggregation, as follows: (i) ANS fluorescence was measured in phosphate buffer (pH 7.6, 50 mM) at a variety of concentrations (1, 5, 10, 20, and 50 μM), to check for linearity of fluorescence intensity with increasing concentration and onset of inner filter effect. A concentration of 10 μM ANS was found to be free from the inner filter effect and standardized as a uniform concentration for use in subsequent experiments. (ii) Temperature dependence of fluorescence quantum yield is determined by both the nature of the fluorophore and its environment. To examine the quantum of the effect of temperature on the fluorescence intensity of ANS in polar (aqueous; poorly fluorescing) and nonpolar (isopropanolic; richly fluorescing) environments, 10 μM solutions of ANS were separately heated in phosphate buffer, and isopropanol, respectively, to a temperature of 64 °C, and cooled back to 25 °C. No significant effect of change in temperature on the fluorescence of the dye in an aqueous environment was observed. Significantly too, a reversible drop of only twelve percent in fluorescence intensity was observed upon heating of ANS in isopropanol up to 64 °C (data not shown). (iii) The scattering of light due to aggregation of BCA was monitored in the presence and absence of 10 μM ANS to examine the effect, if any, of ANS binding on the unfolding of BCA and eventually on the kinetics of BCA aggregation. However, no effect was seen at this concentration of the dye.

Results

Fig. 1 shows a summary of various experimental results obtained for binding of ANS to BCA (appropriate controls are described in the methods section). Bars B and D (Fig. 1) show the final ANS fluorescence observed in experiments in which ANS is allowed to bind to BCA as its aggregates are matched in intensity by the ANS fluorescence observed in experiments in which ANS is added to BCA samples (post-heating) immediately after plateauing of the light scattering occurring due to aggregation, following a rapid rise of temperature to 64 °C. Aggregates of BCA thus bind ANS when it is added following completion of aggregation to a level that is comparable to that observed when ANS is present from the very beginning of the aggregation reaction (i.e., where binding is observed to be completed before there is any detectable aggregation; see experimental section and also Kundu and Guptasarma [16]).

Upon cooling of solutions to room temperature, there is a dramatic rise in ANS fluorescence in samples containing ANS-bound aggregates generated in the presence [bar C] and absence [bar E] of ANS. That the ANS fluorescence is again the same in both solutions, upon cooling, further corroborates the inferences made above regarding the irrelevance of the timing of addition of ANS. Additional support comes from another experiment in which ANS was neither present from the beginning nor added at 64 °C. In this experiment, BCA was heated from 25 to 64 °C in a rapid temperature-rise

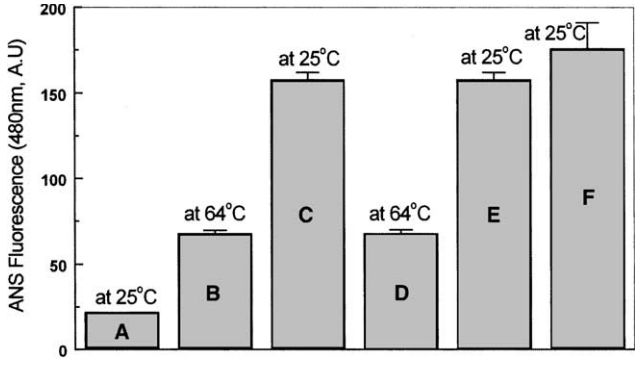

Fig. 1. Fluorescence of ANS bound to aggregated BCA. Bar A. Basal ANS fluorescence in the presence of native BCA (identical to basal fluorescence in the absence of BCA). Bar B. Fluorescence obtained with concomitant binding of ANS to BCA during heating, measured following saturation of the aggregation reaction, at 64 °C. Bar C. Fluorescence obtained through cooling (of the sample shown in bar B) to 25 °C. Bar D. Fluorescence obtained through addition of ANS to BCA that has already been allowed to aggregate, at 64 °C. Bar E. Fluorescence obtained through cooling (of the sample shown in bar D) to 25 °C. Bar F. Fluorescence obtained through subsequent addition of ANS, at 25 °C, to BCA aggregates already created at 64 °C and cooled to 25 °C in the absence of ANS. Each error bar shown is based on averaging of saturation ANS fluorescence data from five independent experiments.

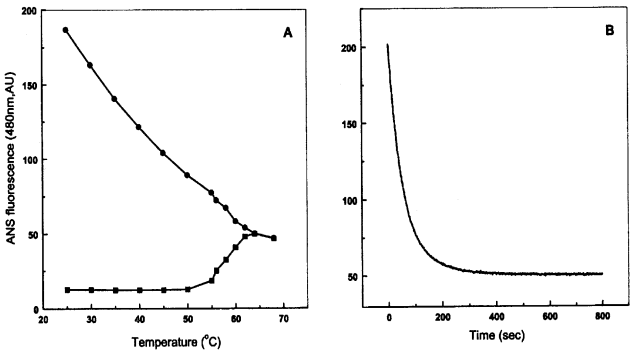

Fig. 2. Curve A: Fluorescence measurements of ANS bound to BCA, after 5 min incubation at each temperature. Fluorescence intensity is observed to increase gradually as BCA unfolds and aggregates with rising temperature. As suspended microparticulate aggregates are cooled, however, the ANS fluorescence intensity increases dramatically as a function of decreasing temperature. Curve B: Kinetic data for the observation shown in curve A. ANS emission intensity shows a decrease to original levels when the cooled aggregate is reheated by placing at 64 °C.

experiment and allowed to cool to room temperature, with ANS being added subsequently to this cooled aggregate, with the same final concentration used in earlier experiments. Here too, the fluorescence of ANS [bar F] turned out to be comparable in intensity to those of cooled forms of the samples in which ANS had either been included from the beginning [bar C] or had been added in the heated state, at 64 °C [bar E]. The dramatic increase in the fluorescence of ANS in the aggregate upon cooling was not accompanied by any change in the scattering of light by aggregated BCA samples, indicating that there was no disaggregation of aggregate particles (see methods section, and also discussions below). Fig. 2 shows that the change in ANS fluorescence with temperature is fully reversible; cooling of heated aggregates leads to intensification of ANS fluorescence and heating of cooled (ANS-bound) aggregates results in weakening of fluorescence to comparable degrees.

Discussion and perspectives

Given that molecules undergoing aggregation are commonly assumed not to be in rapid equilibrium with soluble, unimolecular forms of proteins, separate addition of ANS to BCA samples (prior to heating and following the completion of thermal aggregation) allowed us to examine whether or not molecules become buried away in inaccessible regions of aggregates following binding of the dye to hydrophobic surfaces not directly involved in aggregative interactions. If molecules were indeed buried away in this manner, hydrophobic surfaces on buried molecules could conceivably become unavailable for later binding by ANS molecules through addition of the dye to a sample already subjected to aggregation; thus, post-aggregation addition of ANS could be expected to result in a lower degree of binding of ANS than that obtained with samples heated in the presence of ANS. However, as shown in this paper, the levels of ANS binding observed are found not to depend on whether the dye is added prior to aggregation (in both hot and cold aggregates), or after aggregation has already occurred, suggesting that exposed hydrophobic surfaces on molecules within aggregates remain accessible to the solvent. From a previous experiment done as a control in a study examining a possible chaperoning role of ANS at high molar excesses [16], we already know that dye binding precedes the onset of detectable aggregation and that the kinetics of the two processes at very low concentrations of the dye does not suggest that any significant fraction of the dye bound by the aggregating protein gets squeezed out by associating surfaces. Taken together, these observations suggest that what is being monitored here is the ANS bound by all regions of aggregates at all surfaces that are accessible and available to the dye and that such surfaces (presumably left over from the association of partially unfolded molecules) remain accessible to the dye even after aggregation has occurred. In other words, BCA aggregates could be porous to dye at the level of the environments of molecules, i.e., a nanoscale level of porosity.

It is interesting that molecules remain stable in aggregated form with such poor satisfaction of the need to bury away hydrophobic surfaces through inter- or intra-polypeptide interactions. Since ANS binds only to clusters of hydrophobic residues and not to individual hydrophobic residues, it is clear that hydrophobic residues must be clustered into patches within either individual molecules in the aggregates or between neighboring molecules. Evidently therefore there is some tertiary structure within the aggregate, irrespective of the question of whether such a structure is native-like or otherwise. It is also clear that such hydrophobic patches (as remaining available for ANS binding) are unable to interact mutually and bury themselves away from the aqueous solvent through intra-aggregate interactions. One reason for such an inability could be that molecules are tethered to each other and not free to diffuse and interact. An axiomatic consideration emerges from such reasoning. Since all regions of all polypeptides in aggregated structures are unlikely to be structurally rigid and since some regions (particularly those lacking structure) can be expected to remain flexible even while the polypeptides that host such regions are 'tethered,' the inability of hydrophobic patches to interactively bury themselves away through intra- aggregate interactions suggests that such patches are separated by distances that are too large to be spanned under conditions that do not allow molecular dissociation and/or diffusion. In other words, large spaces may exist within aggregates.

The increase in fluorescence upon cooling of aggregates could have several different explanations that are not necessarily mutually exclusive: (1) Additional ANS from the solvent could bind to the cooled protein (aggregated) sample, due to an increase in its binding capacity, arising from an increase in available hydrophobic surface area. (2) There could be an increase in the fluorescence quantum yield of already-bound ANS brought about solely through the lowering of temperature (i.e., since raising of temperature ordinarily leads to reduced fluorescence quantum yields, cooling could potentially lead to enhanced fluorescence). (3) The same amount of hydrophobic surface area could remain in the aggregate upon cooling, with the same amount of ANS bound to the protein; however, the local environment of the bound ANS in the aggregate could have changed in a manner altering the fluorescence quantum yield of already-bound ANS molecules.

Of the above three mechanisms, the second can be ruled out based on the observations (see control experiments described in the methods section) that: (a) there is no significant change in ANS fluorescence accompanying changes in temperature, in aqueous solutions in which ANS fluoresces poorly and (b) there is only a relatively small change in the intensity of fluorescence of ANS in hydrophobic environments accompanying a similar change in temperature. [There is nearly a 300% rise in ANS fluorescence upon lowering of the temperature of the aggregate from 64 °C to 25 °C; in contrast, there is only a 12% change in the fluorescence intensity of ANS in a nonpolar solvent (isopropanol) over the same range of temperatures]. There is no detectable change in the level of scattering of light by the aggregate sample during cooling. While, of course, it is conceivable that a reduction in the sizes of individual light scattering elements happens to be perfectly balanced by an increase in the number of such elements in the light path (leading to no net change in the levels of light scattering), we consider this possibility to be extremely unlikely. Thus, we propose that the rise in ANS fluorescence upon cooling does not result from the 'making available' of more surfaces for ANS binding through the disaggregation of the aggregate. Consequently, only mechanisms 1 and 3 can be invoked to explain the change in fluorescence, i.e., creation of more surfaces for ANS binding within aggregates and/or changes in the environments of already-bound ANS.

A rationalization would be that the reduction in the strength of most noncovalent interactions brought about by increase in temperature tends to be reversed by cooling. This tendency could lead to 'partial refolding' of molecules within aggregates, through improved tendencies to allow hydrogen bonding, electrostatic interactions, etc., to create new clusters of hydrophobic residues, thus increasing the fluorescence quantum yield of ANS. It is also possible that existing clusters become more defined, leading to an improved ANS fluorescence.

Finally, since aggregation occurring in unimolecular fashion necessarily involves placement of chemically identical entities in different regions, the question naturally arises about how this can be effected without creating structural instability and affecting the growth of aggregates into the large entities that inevitably give rise to visible turbidity at the end of an aggregation experiment. If aggregation were to occur in such a manner that identical regions in identical polypeptide chains get effectively buried away to different extents in different molecules, it would become difficult to conceptualize how aggregates might grow to such large sizes. On the other hand, if aggregates were to be uniformly porous over their entire structure at the nanoscale, molecules in the interior and exterior would effectively exist in identical environments and adopt similar/identical partially folded structures, with unfolded regions of chains remaining loosely organized in the spaces between molecules. Thus the entire aggregate could be a 'nanoporous' structure that is uniformly awash with solvent, rather than a solid-packed structure, with molecules in the interiors and at the surfaces being 'buried' or 'exposed' to the solvent to identical extents.

We are aware that we may be pushing the interpretation of fluorescence data to an extreme in a system in which fluorescence has not been previously applied (except to the extent described in Kundu and Guptasarma [16]) but we do so deliberately to stimulate discussion and debate since such little information is available about the physical nature of amorphous protein aggregates that any information that is indicative of trends could be valuable. With this in mind, we have used the thermally induced, 'unfolding- associated' aggregation of a typical globular protein, BCA, as a model with which to conduct investigations. If indeed protein aggregates are porous, we feel that applications of such porosity may not be far away. For example, a genetic fusion of two different proteins that unfold and aggregate at different temperatures could facilitate the creation of spongy thermal aggregates in which a fusion partner that unfolds at the lower temperature is caused to undergo aggregation through controlled heating to incorporate within itself the folded and functional domains of the other fusion partner as a sort of 'immobilized enzyme'. Experiments to test this possibility are underway.

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