

Formation and dissolution of hen egg white lysozyme amyloid fibrils in protic ionic liquids†

Nolene Byrne* and C. Austen Angell

Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287, USA.

E-mail: nolene.byrne@asu.edu; Fax: +1 (0)480 965 2747; Tel: +1 (0)480 965 7217

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The formation of amyloid fibrils from non-disease-related proteins demonstrates that any protein can adopt this “rogue” form; we show that it is possible to use protic ionic liquids to fibrilize hen egg white lysozyme, and then subsequently to dissolve the fibrils with up to 72% restoration of enzymatic activity.

Given the common knowledge that concentrated saline environments are hostile to life because of biomolecule instability, “ionic liquids (ILs)” (in the sense of ambient temperature liquids consisting entirely of ions) would seem one of the least likely environments for successful studies of biomolecule behavior. Yet ionic liquids are finding increasing application in the biochemical field. Reports to date include enhancement of catalytic reactivity,¹ increased solubility,² thermal stability^{3,4} and lifetime in ambient temperature solutions.⁵ Ionic liquids are also being applied as solvents for protein crystallization^{6,7} and for the study of protein folding.^{8–10}

Many of the above studies have involved the protic subclass of ionic liquids, *i.e.* those formed by acid-to-base proton transfer. Designated as pILs, these have a chemical tunability that makes them particularly useful for studies of solutes, in a similar manner to proteins, whose conformations in aqueous solutions are sensitive to pH. The tunability derives from the state of the transferred proton and it provides a way to control the proton activity, or “effective acidity”, of the ionic liquid. We recently demonstrated how this can be monitored by NMR spectroscopy of the transferred proton, and used as an analog of pH for controlling protein unfolding–refolding behavior in a range of different pILs.⁸ Here we use it in controlled studies of protein *mis*folding and fibrilization, and finally for the study of fibril dissolution.

Protein fibrils are formed when globular protein molecules change their normal pliable structures, based on helical and ribbon components (*e.g.* Fig. 1a) to favor sheet structures that can then stack to form insoluble fibrous materials (see ESI†). These can have very high mechanical moduli^{11,12} due to the large number of hydrogen bonds that glue the sheets together. They present a new avenue for biotechnology that is attracting considerable attention,¹³ since the Young’s moduli of fibrils are estimated to be comparable to that of beta sheet silk.¹⁴

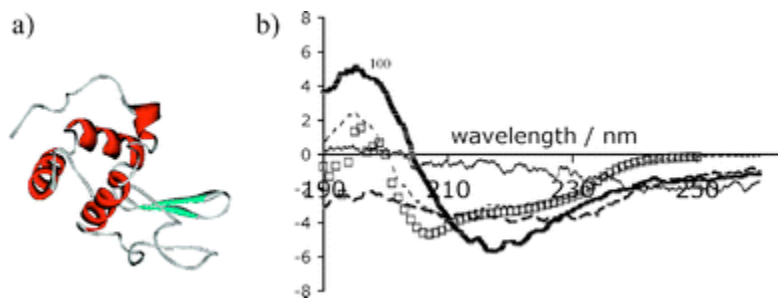


Fig. 1 (a) Ribbon structure of HWL, showing that in the native state alpha helices and beta sheet are present (taken from [ref. 22](#)) and (b) far-UV spectra of native HWL in water (thin dotted line), in the hydrated pIL TEAMS (squares), in 80 wt% NH_4HSO_4 –20 wt% H_2O at 25 °C (thin line) and the spectrum of HWL after heating (thick solid line). CD spectra were recorded on a Jasco J150, path length 0.01 cm, ten scans HWL concentration of 80 mg ml^{-1} .

Until recently, protein fibrilization was studied mainly for its role in debilitating diseases, for instance, Jacob–Kreutz, Huntington’s, Alzheimer’s and type II diabetes.¹⁵ It is best known for its role in “mad cow” disease. Now¹⁶ it is known that fibril formation is free energy-favored for *all* native proteins, and it happens rapidly when proteins are held near their denaturation temperatures or are acidified (which lowers the denaturation temperature^{17–19}). The understanding of how fibrilization occurs and how the process might be controlled is clearly important in both medical and materials sciences.

In this communication we describe the fibrilization process of the globular protein hen egg white lysozyme (HWL) as observed in pILs of the simple amine base, variable acid, type described earlier ([ref. 8](#) and ESI†). We use the proton activity variable to select an acid pIL and use temperature to reduce the lag time for fibrilization. We then show how the fibrils formed by this process, and also by two other methods, can be redissolved using appropriate ILs, and we test the redissolved lysozyme for bioactivity.

HWL is a 14 kDa protein that contains both alpha helices and beta sheet when in the native conformation ([Fig. 1a](#)). The secondary structure of proteins is commonly characterized by the far-UV circular dichroism (CD) spectrum of the dissolved protein. The thin dotted curve in [Fig. 1b](#) is characteristic of native HWL in a pH 7 aqueous solution. A very similar spectrum is obtained for HWL when dissolved in triethylammonium mesylate, TEAMS, (squares). We have previously shown that HWL in this pIL refolds 97% after thermal denaturation.⁵ This normal globular HWL spectrum is very different from the structureless spectrum (thin line) that is obtained when HWL is dissolved in the acidic pIL medium provided by hydrated ammonium bisulfate ($80\text{NH}_4\text{HSO}_4\cdot 20\text{H}_2\text{O}$ by weight). The lack of any characteristic alpha or beta signature implies that in this solution the protein is in the denatured form.

When this latter solution is heated to ~60 °C a structural rearrangement occurs resulting in the spectrum represented by the thick solid curve in [Fig. 2b](#). This spectrum is characteristic of beta

sheet structure in proteins, showing only a single pronounced minimum occurring $\sim 215 \text{ nm}^{-1}$. This spectrum is almost identical with that of the fibrillized HWL (ref. 18, 20 and 21), which we will reproduce later. Our initially clear solution giving the thick line spectrum of Fig. 2b becomes opaque white when maintained at 70°C for two hours, as fibrils grow to the micron dimensions that scatter visible light. The spectrum is not dependent on the fibril dimension.

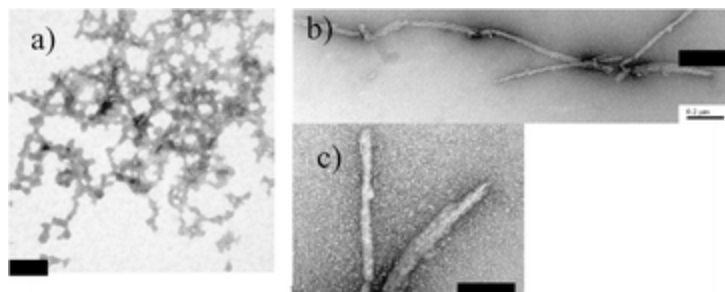


Fig. 2 Scanning electron micrographs: (a) the self-assembly stage of fibrillization, (b) the mature fibril, (c) magnification of the fibril. Scale bar 200 nm for (a) and (b), 50 nm for (c).

Held at room temperature, however, the fibrilization process takes weeks, and electron micrographs taken at different times after the initial heating (see Fig. 2), show the growth of fibrils following the same pattern of development reported by Dobson.²³ We may assume that the mechanism of fibrilization is similar in each case. Fig. 2 provides clear evidence that the HWL has fibrillized rather than merely aggregated. Further proof of the fibrillar structure was obtained using congo red assay and ThT fluorescence (see below).

We now turn our attention to the *dissolution* of fibrils—of which very few instances have been reported. Goto *et al.* reported that amyloid fibrils of β_2 -microglobulin can be dissolved using the common solvent DMSO.^{24,25} Redissolution of HWL has also been reported using high concentrations of GdnHCl.²⁶ Indeed GdnHCl at 6 M has some of the characteristics of the pILs that we study, as we will be demonstrating by NMR elsewhere. We find that dissolution of lysozyme fibrils is extremely facile in some pILs but sluggish and incomplete in others. To test the generality of our dissolution observations for HWL, dry fibrils were prepared by three separate methods, two from the literature and the one described above (which we now call Method 1). In Method 2¹⁸ the protein is fibrillized by additions of pure ethanol to the aqueous protein, while in Method 3²⁷ the pH of the aqueous solution is adjusted to 2.0, using 1 M HCl, and mild heat applied.

Confirmation that amyloid fibrils formed in each case is provided in Fig. 3 using far-UV (CD) spectra for beta sheet structure (Fig. 3a, cf. Fig. 1b) and congo red (CR) fluorescence spectra for fibrils²⁸ (Fig. 3b). CR offers a fast assay for fibrils, as the CR dye molecule binds specifically to the stacked β -sheets causing a spectral shift, and yielding yellow–green fluorescence under polarized light.

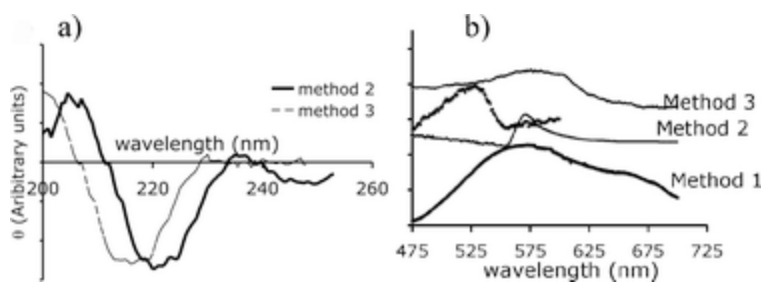


Fig. 3 (a) Far-UV CD spectra of fibrils from Methods 2 and 3, showing the single minimum at 215–220 nm characteristic of beta sheet secondary structure. (b) CR binding assay showing the spectral shift in the presence of lysozyme fibrils, from each preparation method. Thick curve is for the CR solution alone.

In the investigation of fibril *dissolution*, fibrils from each preparation were centrifuged from the solution, washed and vacuum-dried at 25 °C. Weighed samples (1 mg) were placed in vials and an aliquot (1 ml) of anhydrous pIL was added. After 20 min at ambient temperature the solutions were centrifuged for 3 min, decanted, washed, dried and weighed, and % dissolved is given in [Table 1](#).

Table 1 Percentage of fibrils dissolved in selected pIL

Solvent (fibrils prepared by)	% Dissolved
EAN (Method 1)	72 ± 3
EAN (Method 2)	100
EAN (Method 3)	100
TEATf (Method 2)	80 ± 4
TEATf (Method 3)	Solution not 100% clear but cannot separate with centrifuge
TEAMS (Method 2)	73 ± 6
TEAMS (Method 3)	100

With solvent ethylammonium nitrate, EAN, dissolution was complete and immediate (clear within one minute). However bioactivity essays (see below) could detect differences between fibril preparations. Dissolution results for EAN are compared with those for two other anhydrous pILs, TEAMS—selected for the ability of HWL to refold after thermal denaturation in this solution⁵ and triethylammonium triflate, TEATf, for its higher effective acidity.⁸ Only 50% of the protein refolds in this solution (at 200 mg ml⁻¹) after thermal denaturation.

ThT binding fluorescence intensity³⁰ is sensitive to beta sheet structure and in particular it is thought that the binding occurs when the aggregation has proceeded to the organized oligomeric stage.²⁹ When native HWL is dissolved in either EAN or TEATf containing 20 wt% H₂O, no fluorescence is observed at all (see ESI†).

The fluorescence intensities for fibrilized solutions from different sources are summarized in [Table 2](#). The ThT fluorescence intensity has been set to 100 for the hydrated NH_4HSO_4 (Method 1) fibril sample (#1). The intensity of the absorbance for the Method 2 (EtOH) fibrils (#2) is lower, which suggests immature fibrils (this is correlated with a higher restorable bioactivity, see below).

Table 2 ThT fluorescence intensities (see ESI⁴) for various samples normalized to that of sample 8, before and after redissolution in pILs

Sample #	Description (numbers in wt%)	ThT intensity
1	Method 1 prepared fibrils	100
2	Method 2 prepared fibrils	69
3	Method 3 prepared fibrils	100
4	Fibrils from Method 2 redissolved in EAN	0
5	Fibrils from Method 3 redissolved in EAN	0
6	Fibrils from Method 2 redissolved in TEAMS	12
7	Fibrils from Method 2 redissolved in TEATf	24

Entries 4 and 5, by contrast, confirm the complete disappearance of fibrillar character from samples in which fibrils were dissolved in EAN. Some native systems with similar null intensities are listed in ESI.[†] Some further cases (3, 6 and 7) are listed in [Table 2](#) without further comment.

A natural question regarding the redissolved HWL concerns its biological activity. To answer this critical question, standard biological assay tests (using the lysing of *Micrococcus lysodeikticus* cells observed at 450 nm³¹ as described in standard catalogs) were performed on the solutions after dilution in water. The results are given in [Table 3](#). A useful comparison can be made with the activity of lysozyme that has never been fibrilized but has instead been dissolved directly in anhydrous pIL and then diluted to biological concentrations for the bioactivity test. HWL is denatured when dissolved in the anhydrous pIL and refolds during dilution to different extents depending on the pIL in which it was dissolved. Such values are given in the first three lines of [Table 3](#).

Table 3 Enzymatic assay of lysozyme.³¹ Values are activities relative to that of native lysozyme in pH 7 phosphate buffer

Sample and treatment ^a	Activity \pm 5%
HWL in EAN, dissolved in neat pIL (denat.)	80%
HWL in TEATf, dissolved in neat (denat.)	73%
HWL in TEAMS, dissolved neat pIL with denat.	53%
Fibrils from Method 1, redissolved in EAN	46%

^a

All samples diluted to normal aqueous solution state for assay. Results are averages of three independent assays.

Sample and treatment ^a	Activity \pm 5%
Fibrils from Method 2, redissolved in EAN	72%
Fibrils from Method 3, redissolved in EAN	22%
Fibrils from Method 1, redissolved in TEATf	20%
Fibrils from Method 2, redissolved in TEATf	60%
Fibrils from Method 3, redissolved in TEATf	5%
Fibrils from Method 1, redissolved in TEAMS	0
Fibrils from Method 2, redissolved in TEAMS	10%
Fibrils from Method 2, redissolved in TEAMS	0

^a

All samples diluted to normal aqueous solution state for assay. Results are averages of three independent assays.

The most remarkable result is the one for the clear solution obtained from the very rapid redissolution in EAN of fibrils formed by Method 2 from the EtOH solution. The bioactivity level, 72% of aqueous HWL, is little different from the case of HWL dissolved in neat EAN and then diluted *i.e.* the case *which had never been fibrilized* (Table 2, sample 3 no ThT intensity observed). In stark contrast is the bioactivity result for the clear solution obtained from dissolution of the Method 3 fibrils in EAN. This solution registered no ThT fluorescence, hence was free of oligomeric or beta sheet aggregates, but yielded a *low* bioactivity, only 22% of aqueous lysozyme. This is much less than the 60% obtained in solutions of Method 2 fibrils redissolved in TEATf, notwithstanding the residual ThT fluorescence intensity shown by those solutions (Table 2).

In each pIL studied, the Method 2 (EtOH) fibrils were those that redissolved most, and gave greatest bioactivity restoration, EAN being the most successful solvent in each case. To explain this we note that the CR spectrum of the fibrils for the Method 2 case (see Fig. 3) is relatively sharp, suggesting a more regular structure. Also, according to the TEM study of ref. 14, the fibrils produced by this method are “protofilaments” *i.e.* are “less mature”. These features might be explained by a reduced extent of antiparallel beta sheet stacking, and thus a more easily reversed condition for the filaments. Method 3 fibrils were the least soluble and yielded least restored bioactivity with each solvent, TEAMS (the least acidic of the pILs studied⁸) being the least successful solvent in each case. In depth pursuit of understanding here will demand the development of stable phosphonium cation pILs so that ¹⁵N NMR (HQ5C) studies of the fibril structure can be carried out without interference from the solvent nitrogens. While full appreciation of the solubility–bioactivity relationships will require a lot more work, the possibility of facile fibril dissolution and, in some cases considerable bioactivity restoration, has been clearly established by this study (see also ref. 32, *asp67his* variant).

Further experiments will be aimed at understanding whether or not this reactivation behavior is unique to lysozyme (four disulfide bonds need correct location), but preliminary indications are that it is not. If restoration of bioactivity could be made reliable, then fibrilization could offer a novel, and very unexpected, approach to long term storage of proteins.

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Footnote

† Electronic supplementary information (ESI) available: (a) Formation, structure and importance of fibrils; (b) character of ionic liquids employed; (c) additional details on Th T tests of solutions; (d) bioactivity test details. See DOI: [10.1039/b817590j](https://doi.org/10.1039/b817590j)

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