

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

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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 fibrillize hen egg white lysozyme, and then subsequently to dissolve the fibrils with up to 72% restoration of enzymatic activity.

Given the general knowledge that saline environments are hostile to life because of biomolecule instability, "ionic liquids" (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 (ILs) are finding increasing application in the biochemical field. Reports to date include enhancement of catalytic reactivity¹, increased solubility², thermal stability^{3,4} and lifetime in ambient temperature solutions⁵. Ionic liquids are also being applied as solvents for protein crystallisation^{6,7} and for the study of protein folding⁸⁻¹⁰.

Many of the above studies have involved the protic subclass of ionic liquids which are those formed by acid-to-base proton transfer. Designated pILs, these have a chemical tunability which makes them particularly useful for studies of solutes, like 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 of controlling 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 pILs⁸. Here we use it in controlled studies of protein misfolding and fibrillization, and finally for study of fibril dissolution.

Protein fibrillization is intensely studied because of its role in some of humankind's most debilitating diseases, for instance, Jacob-Kreutz, Huntington's, Alzheimer's and type II diabetes¹¹. It is best known for its role in "mad cow" disease. The fibrillization process is now recognized as free energy-favored for all native proteins¹², and kinetically probable when deliberately destabilized by sustained elevation of temperature (to values near denaturation), or by increase of acidity (which lowers the denaturation temperature^{13,15}).

Recently, some positive aspects of fibrillization have been recognized^{16,17}. Amyloid fibrils present a new avenue for biotechnology¹⁸, due to an extraordinary stability against both temperature and pH changes. The Young's modulus is estimated to be comparable to that of beta sheet silk¹⁹. The importance of understanding how the protein fibrillizes and the bio-processing of these materials may therefore be far-reaching.

In this communication we describe the fibrillization process of the globular protein hen egg white lysozyme (HWL), as observed in pILs. We use the proton activity variable to select an acid pIL and use temperature to reduce the lag time for

fibrillization. We then show how the fibrils formed by this process, and also by two other methods, can be redissolved using appropriate ionic liquids, and we test the redissolved lysozyme for bio activity.

Formation of amyloid fibrils

HWL is a 14kDa protein that contains both alpha helices and beta sheet when in the native conformation (Figure 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 of Fig. 1(b) is characteristic of native HWL in a pH7 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 denaturation⁸. 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 bisulphate (80NH₄H₂SO₄·20H₂O by weight). The lack of any characteristic alpha or beta signatures 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 ~215 nm⁻¹. This spectrum is almost identical with that for the fibrillized HWL of^{14,20,21} which we will reproduce later. Our initially clear solution giving the thick line spectrum of Fig. 2b quickly becomes opaque white when maintained at 60°C, as fibrils grow to the micron dimensions that scatter visible light. The spectrum is not dependent on the fibril dimension.

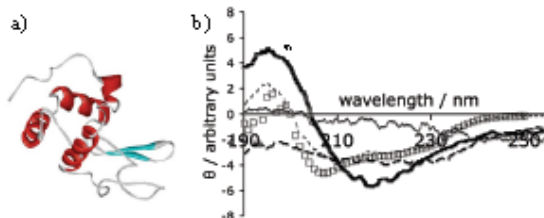


Fig. 1 (a) Ribbon structure of HWL, showing that in the native state alpha helices and beta sheet are present (taken from²²) (b) Far-UV spectra of native HWL in water, (thin dotted line), in the hydrated pIL TEAMS (squares), in 80wt% NH₄H₂SO₄·20wt% H₂O at 25°C, (thin line) and the spectrum of HWL after heating (thick solid line). CD spectra were recorded on a Jasco J150, path length 0.01cm, scans HWL concentration of 80µg/ml.

Held at room temperature, however, the fibrillization process takes weeks, and electron micrographs taken at different times after the initial heating (see Figure 2), show the growth of fibrils following the same pattern of development reported in the

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... Dobson group studies²³. We may assume that the mechanism of fibrillization is similar in each case. Figure 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).

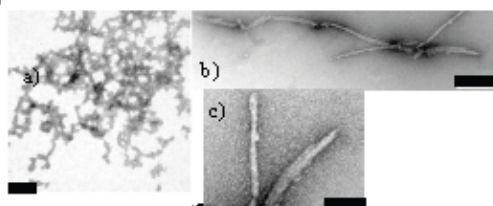


Fig 2: Scanning electron micrographs a) the self-assembly stage of fibrillization, b) The mature fibril, c) magnification of the fibril. Scale bar 200nm for a) and b) , 50nm for c)

... Dissolution of amyloid fibrils

We now turn 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 6M has some of the character 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 2¹⁴ 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 1M HCl, and then mildly heated. the solution →

Confirmation that amyloid fibrils formed in each case is provided in Figure 3 using far UV (CD) spectra for β -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 beta plated sheets causing a spectral shift, and yielding yellow-green fluorescence under polarised light.

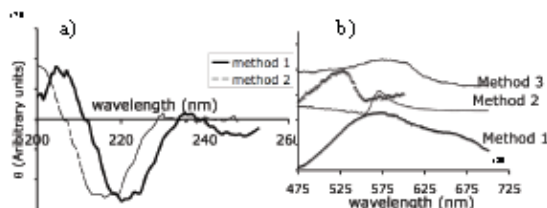


Fig 3: a) Far-UV CD spectra of fibrils from Methods 2 and 3, showing the single minimum at 215-220nm characteristic of β -sheet secondary structure. b) CR binding assay showing the spectral shift in the presence of lysozyme fibrils, from each preparation method. Thick curve is for the CR solution alone).

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

... washed, dried and weighed, and the % 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
TEATY (Method 2)	80 ± 4
TEATY (Method 3)	***solution not 100% clear but cant 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 assays (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 solution³ and triethylammonium triflate, TEATY, because of its higher effective acidity². Only 50% of the protein refolds in this solution (at 200mg/ml) after thermal denaturation.

ThT binding fluorescence intensity³⁰ is sensitive to β sheet structure and in particular it is thought that the binding occurs when the aggregation has proceeded to the organised oligomeric stage²⁹. When native HWL is dissolved in either EAN and TEATY containing 20wt% H₂O, no fluorescence is observed at all.

... The fluorescence intensities for native HWL solutions, and for fibrillized solutions from different sources, are summarized in Table 2. The ThT fluorescence intensity has been set to 100 for the hydrated NH₄HSO₄ (Method 1) fibril sample (#6). The intensity of the absorbance for the Method 2 (EtOH) fibrils (#7) is lower which suggests immature fibrils. (This is correlated with a higher restorable bioactivity, see below).

Entries 9 and 10, by contrast, confirm the complete disappearance of fibrillar character from samples in which fibrils were dissolved in EAN. Other cases are also shown in Table 2.

Table 2: ThT fluorescence intensities for various samples normalized to that of sample 8, before and after redissolution in pILs.

Sample #	Description (numbers in wt.%)	ThT intensity
1	Native HWL diss. in 80EAN20H ₂ O	0
2	Native HWL diss. in 80EAN20H ₂ O after thermal denaturation	0
3	Native HWL diss. in neat EAN	0
4	Native HWL diss. in 80 TEATY20H ₂ O	0
5	Native HWL diss. in 80 TEATY20H ₂ O after thermal denaturation	0
6	Method 1 prepared fibrils	100
7	Method 2 prepared fibrils	69
8	Method 3 prepared fibrils	100
9	Fibrils from Method 2 rediss. in EAN	0
10	Fibrils from Method 3 rediss. in EAN	0
11	Fibrils from Method 2 rediss. in TEAMS	12
12	Fibrils from Method 2 rediss. in TEATY	24

... a ThT binding was recorded on a R551 fluorescence spectrophotometer. The excitation was set at 450nm and the emission recorded at 485nm. 20 μ M Thioflavin T was added to each sample³¹. Protein concentration 10mg/ml. Results are the average of 5 measurements.

230 Bioactivity of lysozyme from redissolved fibrils

A natural question regarding the redissolved HWL concerns its biological activity. Have the four disulfide bonds - which must have been at least transiently ruptured to permit the β -sheet structure in the fibril to form - been able to reform to the extent that HWL regains its normal biological activity? To answer this critical question, standard biological assay tests (using *Micrococcus lysodeikticus* cells observed at 450nm³¹) were performed on the solutions after dilution in water to perform the assay. The results are given in Table 3. A useful comparison can be made with the activity of lysozyme which has never been fibrillized but has instead been dissolved in anhydrous pIL before dilution 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.

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, 73% 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 fibrillized (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". Method 3 fibrils were the least soluble and yielded least bioactivity with each solvent, TEAMS (the least acidic of the pILs studied³ being the least successful solvent in each case. The solubility/bioactivity relationships will require a lot more study, however, the possibility of facile fibril dissolution and, in some cases, considerable bioactivity restoration, has been clearly established by this study.

235 **Table 3:** Enzymatic Assay of Lysozyme³¹. Values are activities relative to that of native lysozyme in pH 7 phosphate buffer.

Sample and treatment ^a	Activity as %
HWL in EAN, diss in neat pIL (denat.)	80%
HWL in TEATf, diss in neat (denat.)	73%
HWL in TEAMS, diss neat in pIL (denat.)	53%
Fibrils from Method 1 rediss. in EAN	46%
Fibrils from Method 2 rediss. in EAN	72%
Fibrils from Method 3 rediss. in EAN	22%
Fibrils from Method 1 rediss. in TEATf	20%
Fibrils from Method 2 rediss. in TEATf	60%
Fibrils from Method 3 rediss. in TEATf	5%
Fibrils from Method 1 rediss. in TEAMS	0
Fibrils from Method 2 rediss. in TEAMS	10%
Fibrils from Method 3 rediss. in TEAMS	0

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

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 fibrillization could offer a novel, and very unexpected, approach to long term storage of proteins.

We are currently exploring the possibility of pIL solvent tuning to conditions where fibrils can be grown reversibly, a possibility which has already been suggested by observations on concentration manipulation of the protic salt guanidinium chloride³⁶. For materials science purposes, such control of the fibrillization process will be imperative.

References

1. Kragl, U., Eckstein, M., and Kafkik, N. *Curr. Opin. Biotechnol.*, **13**, 2002, 565-580.
2. Swatoski, R.P., Spear, S.K., Holbery, J.D. and Rogers, R.D. *J. Am. Chem. Soc.*, **124**, 2002, 4974-4975.
3. Fujita, K., MacFarlane, D.R., and Forsyth, M. *Chem. Commun.* 2005, 38, 4804-4806.
4. Baker, S.N., McCleskey, T.M., Pandey, S. and Baker, G.A. *Chem. Commun.* 2004, 8, 940-941.
5. Byrne, N., Wang, L.-M., Belieres, J.-P. and Angell, C.A. *Chem. Commun.* 2007, 2714-2716.
6. Pusey, M.L., Paley, M.S., Turner, M.B. and R.D., Rogers. *Crystal Growth & Design*, **7**(4), 2007, 787-793.
7. Helmst, D., Habel, D., Sebastian, J., Schmidt, M. and Weuster-Botz, D. *Biotech Let.*, **29**(11), 2007, 1703-1711.
8. Byrne, N. and Angell, C.A. *J. Mol. Biol.*, doi:10.1016/j.jmb.2008.02.050, 2008.
9. Lange, C., Patil, G. and Rudolph, R. *Protein Sci.*, **14**(10), 2005, 2693-2701.
10. Summers, C.A. and Flowers, R.A. *Protein Sci.*, **9**, 2000, 2001.
11. Dobson, C.M. *Trends Biochem. Sci.*, **24**, 1999, 329.
12. Fändrich, M., Fletcher, M.A. and Dobson, C.M. *Nature*, **410**, 2001, 165-166.
13. Buck, M., Radford, S.E. and Dobson, C.M. *Biochemistry*, **32**, 1993, 669-673.
14. Goda, S. et al. *Protein Sci.*, **2**, 2000, 369-375.
15. Sasahara, K., Yagi, H., Naki, H. and Goto, Y. *J. Mol. Biol.*, **372**, 2007, 961-991.
16. Arnold, C. *Chem. & Eng. News*, **86**(29), 2008, 48-50.
17. Gras, S.L. *Aust. J. Chem.*, **60**(5), 2007, 333.
18. Gras, S.L., Tickler, A. K., Squires, A. M., Devlin, G. L., Horton, M. A., Dobson, C.M. and MacRhee, C.E. *Biomaterials*, **29**(11), 2008, 1553-1562.
19. Greene, M.E. *Materials Today*, **9**(12), 2006, 21.
20. Fasman, G.D. *Circular dichroism and the conformational analysis of biomolecules*. 1 ed. 1996, New York: Springer-Verlag, 738.
21. *Circular Dichroism: Principles and Applications*. 2 ed, ed. N. Berova, K. Nakanishi, and R.W. Woody. Vol. 877, 2000, New York: Wiley-VCH.
22. <http://lysozyme.co.uk/lysozyme-structure.php>
23. Dobson, C.M. *Nature*, **426**, 2003, 884-886.
24. Hirota-Nakaoka, N., Hasegawa, K., Naki, H. and Goto, Y. *J. Biochem.*, **134**(1), 2003, 159-164.
25. Hoshino, M., Kanou, H., Hagiwara, Y., Hasegawa, K., Hironobu, N. and Goto, Y. *Nature Stru Biol.*, **9**, 2002, 332-336.
26. Vamaglia, B.A., Huang, J., and Clark, E.D. *Biomacromol.*, **5**, 2004, 1362.
27. Arnaudov, L.N. and De Vries, R. *Biophysical J.*, **88**, 2005, 515-526.
28. Klumk, W., E. Pettigrew, J. W. and Abraham, D. J. *J. Histochem and Cytochem.*, **37**(8), 1989, 1293-1297.
29. LeVine, H. *Protein Sci.*, **2**, 1993, 404-410.
30. LeVine, H. *Methods Enzymol.*, **309**, 1999, 274-285.
31. Szugar, D. *Biochimica et Biophysica Acta.*, **8**, 1952, 302-309.