Reversible folding–unfolding, aggregation protection, and multi-year stabilization, in high concentration protein solutions, using ionic liquids

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We report the reversible thermal unfolding/refolding, and long period stabilization against aggregation and hydrolysis, of >200 mg ml^{-1} solutions of lysozyme in ionic liquid-rich, ice-avoiding, solvents.

Studies of protein folding are normally carried out in buffered dilute aqueous solutions to avoid loss of protein to the aggregation phenomenon. In a trial study three years ago[1] we reported the observation of refolding of lysozyme, after denaturation, in samples of concentration up to 200 mg ml^{-1}. It must be regarded as remarkable that at this concentration not only was refolding observed, but that the process could be repeated many times with only small ($\approx$3%) losses of refoldable sample per cycle, as judged by the enthalpy absorbed during the denaturation process.

Such behavior deserves better documentation than was given in the original article, particularly as it has since been seen in other systems.[2] We therefore here report some details on these measurements, and discuss the nature of the solutions that render this phenomenon observable. We also demonstrate that solutions of this type preserve the folded state of the protein lysozyme at room temperature in the liquid state, with losses <20% per year for at least three years. The essential solution ingredient that is responsible for this behavior is an "ionic liquid". The term "ionic liquid" is currently applied to substances composed entirely of ions that remain in the liquid state below the boiling point of water, preferably down to room temperature. A great many of these exist[3–5] and they are currently the center of intense research activity, mainly as non-volatile solvents for chemical syntheses[4–9] but also as ambient temperature liquids for electrochemical devices, heat transfer, and other purposes.[10–16]

We note that an unexpected stability of the protein cytochrome c has recently been reported when the protein is supported in solutions of high ionic liquid content.[17] The (non-toxic) ionic liquid of this latter study was of the aprotic type, with T_m = 114 °C, while in our study a protic type,[18] namely ethylammonium nitrate, with T_m = 13 °C was used. Our solutions also contain a sugar, but the sugar does not by itself confer protection against aggregation in the liquid state, as we will document below. Other observations on the benign character of ILs with respect to dissolved biomolecules are cited by Baker *et al.*[19]

The solutions that provide the protection referred to above were developed for the particular purpose of providing a medium in which protein refolding could be studied at low temperatures in the absence of any interference from the crystallization of ice. Our purpose was not to eliminate water from the solution but only to eliminate the possibility of nucleation of ice crystals. Then the energy release during folding of the protein at low temperatures, after an initial quench had preserved the unfolded state, could be observed free of any exothermic energy releases due to ice formation.

Proteins are often stabilized in the glassy state by use of sugars, trehalose being found of special effectiveness.[20] Indeed, our initial attempts to create media in which to observe refolding of quenched denatured states, involved such solutions. However it was found that the fraction of unfolded protein that could be observed to refold during warm-up after initial quench was quite small, about 0.2. The refolding fraction was found to rise dramatically when an equal mass fraction of ethylammonium nitrate, [$CH_3CH_2NH_3^+$][NO_3^-], called hereafter EAN, was added to the solution. This addition, inspired by Summers and Flowers,[21] also greatly decreased the viscosity. A very satisfactory solution composition, which supported a lysozyme concentration of 200 mg ml^{-1} with about 97% protection against aggregation in a single unfold–refold cycle, was found to be one with the following weight percentages, sucrose 27 wt%, EAN 31 wt%, water 20 wt%, lysozyme 22 wt%.

The concentration of lysozyme in the above solution was sufficient to give a very clear quantifiable endotherm during upscans at 20 °C min^{-1} using a standard DSC, differential scanning calorimeter, TA 2920, or Perkin-Elmer DSC-7. Three successive denaturing cycles are shown in Fig. 1, where it is seen that the endotherms decrease in area by about 3% per cycle. For the above solution, the temperature of the endotherm maximum, 74 °C, and the enthalpy absorbed, are the same as reported for the normal *in-vitro* (aqueous buffer) process.[22] The denaturation temperature is sensitive to the sugar/EAN ratio, as we show below.

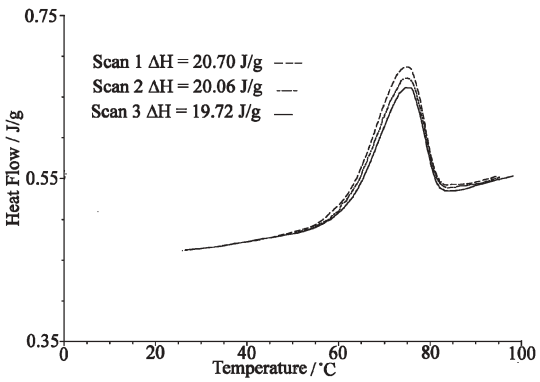

Fig. 1 Three successive denaturing scans with peak temperature at 74 °C, showing small (3% per cycle) loss of endotherm area from scan to scan.

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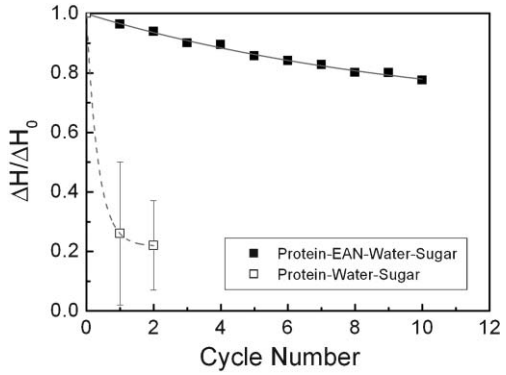

Fig. 2 Progressive loss of protein to aggregation over 10 cycles, assessed by endotherm area comparisons.

The quantitative assessment of the area under the endotherm is readily obtained with the instrument software which applies a standard baseline, and this was compared with that of the initial scan after each of ten cycles. Results are shown in Fig. 2. A systematic, decelerating, decrease is observed with successive cycles. The loss rate is strikingly reduced from that observed on cycling a solution that did not contain the EAN, shown by the dashed curve in Fig. 2. (Experimental uncertainty, indicated by vertical bar, was greater in this case.)

The solution in which these measurements were carried out does not have a high viscosity. We have measured the viscosities of some sugar–EAN–water solutions in the absence of the protein, and the viscosities are only about an order of magnitude greater than that of water. This is sufficiently low that the viscous retardation of molecular processes in the refolding of the protein is not a significant effect. The viscosity–temperature relations for some solutions of interest are compared with that for water in Fig. 3.

It is not necessary that the sugar be sucrose. Equally satisfactory results were obtained when the sugar used was glucose, which gives

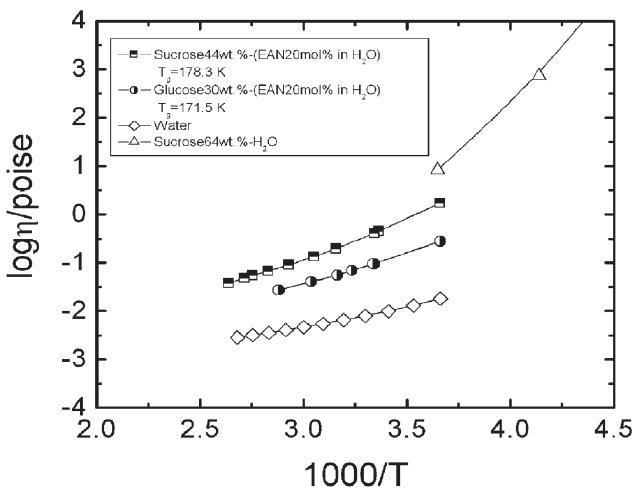

Fig. 3 Viscosity of 44% sucrose + 33% EAN + 22% water solvent (corresponding to 34.7% sucrose + 26.9% EAN + 17.8% water + 20.6% lysozyme solution) and 30% glucose + 42% EAN + 28% water solvent solutions, in the temperature range ambient to −100 °C. The viscosity of water (open diamond) is included for comparison.

a somewhat less viscous solution (Fig. 3). Trehalose provides equivalent protection but the presence, in trehalose, of a heat-absorbing anomeric exchange that appears at about the same temperature as the unfolding endotherm, renders trehalose as a unsatisfactory component (or at least complicated) for energy studies. In more recent work, see below, we have found that the sugar can be dispensed with completely, though in this case the long-term stability has not been established.

The manner in which the different energetic steps of the folding process of lysozyme can be followed, during warm-up from the glassy state obtained by quenching after denaturation, was shown in previous papers[1] and will not be repeated here. Rather, we turn attention to the extent to which the above solution stabilizes the lysozyme against deterioration. Samples from the studies of 2003 have now been subjected to rescan after storage in (a) a phial in a refrigerator, and (b) at room temperature in sealed aluminium DSC pans, during the intervening three years. The scans, taken using a TA instrument at 20 °C min^{-1}, are shown in Fig. 4 where the unfolding enthalpies per g protein are noted on the figure.

The unfolding enthalpy in the case of the refrigerated sample is quite unchanged from that of our present freshly prepared samples. The sample stored at room temperature has a smaller unfolding enthalpy, about 1/3 that of the fresh sample implying that 2/3 of the folded protein has been lost to aggregation or hydrolytic decomposition since encapsulation, *i.e.* about 22% per year. However, this is a worst-case number. This sample had been used in cycling experiments in 2003. The composition and sample mass are known but the number of cycles it experienced is not recorded. Based on the Fig. 2 evaluation, probably at least 20% of the sample was lost during the cycling experiments, but we do not include this in assessing the annual loss rate. Protein samples under normal laboratory study in buffered aqueous solutions are guarded against exposure to ambient temperatures even for hours, so it is clear that the ionic liquid has bestowed an extraordinary level of protection on the protein. Lysozyme is, reputedly, a fairly "tough" protein, and the preservation potential of EAN (and other ionic liquids currently under study) for more delicate proteins has yet to be established. It is not necessary for us to

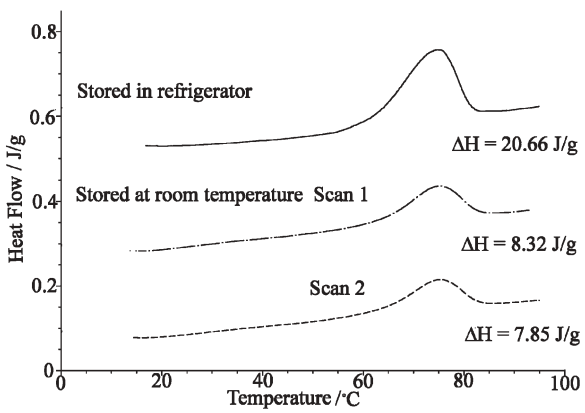

Fig. 4 DSC upscan of lysozyme solutions in EAN–sugar solutions after three years in (a) refrigerated sample, (b) DSC pan stored at room temperature. In the latter case a second scan is included to show that the smaller peak diminishes in size with unfold/fold cycling at a rate comparable to that of fresh samples. Much of the protein has survived over the years at ambient since the 2003 denaturation/renaturation studies.

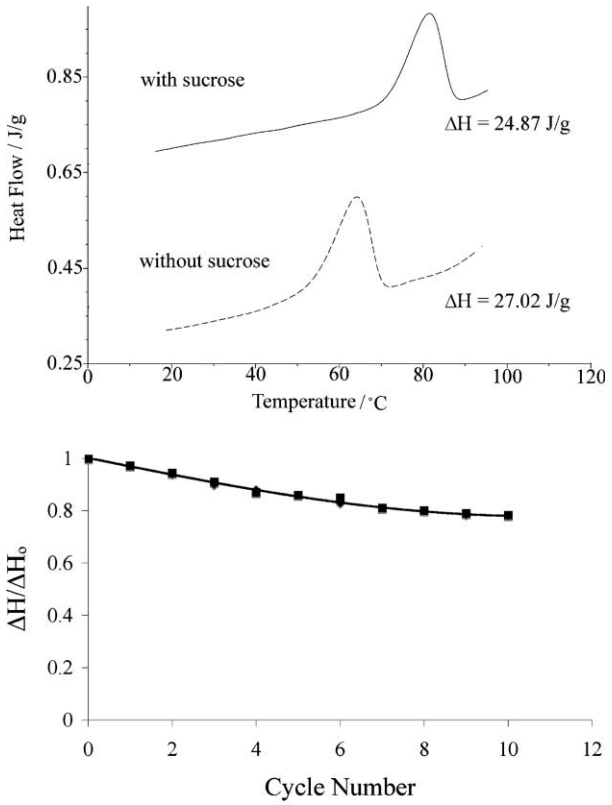

Fig. 5 (a) DSC upscans of lysozyme solutions containing TEAMS, with and without sucrose. Denaturation enthalpies are given in J per g protein. In the absence of sucrose, denaturation occurs below the temperature in aqueous buffer. Sucrose, and in particular trehalose, have been shown to increase the thermal stability (denaturation temperature) of proteins.[22,24] (b) Comparison of denaturation enthalpy with (■) and without (◆) sucrose in relation to cycle number. Behavior is comparable to that seen in Fig. 2. No sucrose effect on protein aggregation is seen, so protection against aggregation can be attributed to the 'ionic liquid'.

emphasize the potential benefits of long term ambient temperature stabilization of proteins, and biomolecules, in general.

It now becomes of interest to know if this protection against aggregation, and other types of deterioration, is unique to EAN or is a general property of ionic liquids. Results of refs. 17 and 23 certainly suggest that the bioprotection by ionic liquids may be common, though the previous examples have featured aprotic ILs.

Concerning refoldability, not all proteins behave as lysozyme in Fig. 1. While ribonuclease does, neither myoglobin nor cytochrome c refold after hot denaturation in our concentrated solutions (though apo-myoglobin does). Cytochrome c also did not refold in the ref. 17 study, though it can hot refold in pH 4 aqueous solutions when oxidised and dilute enough.[25] On the other hand, lysozyme hot refolds in solutions containing alternative ionic liquids of effective pH[26] similar to EAN, both with and without a sugar component. The sugar component is helpful only to enhance the glass-forming properties that are desirable for the energetics-of-refolding studies described earlier[1]—which will be the subject of separate papers.

In the final figure, Fig. 5, we demonstrate the aggregation resistance of lysozyme during repeated unfold–refold cycles in the two solvents, triethylammonium methane sulfonate (TEAMS) 29 wt% + sucrose 30 wt% + water 19 wt% + lysozyme 22 wt%, that differ only in the presence or otherwise of sucrose. TEAMS is low melting, T_m = 21.6 °C. Other data on these (and some ~100 others) protic ILs are available in ref. 27.

It is seen in Fig. 5(a) that the unfolding temperature in TEAMS, which has a slightly lower effective pH than the EAN-based solvent,[26] is a little higher than in biological solutions, and it is also a little higher in unfolding enthalpy. Both imply a more stable folded structure. It will be interesting to observe the effect of the increased stability on the refolding energy pathways. As seen in Fig. 5(a), when the sugar is removed from the solutions, the denaturation occurs at distinctly lower temperatures, however, as seen in Fig. 5(b) the resistance to aggregation is not affected. Again, the effect on the cold refolding energetics will be interesting to study, as will be the effect of larger changes in the effective pH that can be induced by alternative choices of ionic liquid.[26]

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