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# Protein Unfolding, and the “Tuning In” of Reversible Intermediate States, in Protic Ionic Liquid Media

**N. Byrne\* and C.A. Angell**

Department of Chemistry and  
Biochemistry, Arizona State  
University, Tempe,  
AZ 85281-1604, USA

Received 21 November 2007;  
received in revised form  
16 February 2008;  
accepted 25 February 2008

Protic ionic liquids (PILs) are currently being shown to be as interesting and valuable to chemical manipulations as the well-known aprotic ionic liquids (APIL). PILs have the additional advantage that the proton activity (PA) can be adjusted by the choice of Bronsted base and Bronsted acid used in their formation. In the absence of solvent, the PA plays the role of pH in ordinary solutions. Previously, we have shown that solution of proteins in ionic-liquid-rich solutions conveys surprising stabilization against hydrolysis and aggregation, permitting multiple unfold/refold cycles without loss to aggregation. Here, we show that the denaturing temperatures of both hen egg white lysozyme and ribonuclease A are sensitive to the PA of the PIL as much as they are to pH in aqueous solutions. A maximum stability for more basic solutions is found, and the unfolding process is well described by the two-state (cooperative) model. Finally, we show that, by PA tuning, the PILs can select folding pathways featuring the postulated intermediates so that they are fully populated during the unfolding process. The intermediates are themselves capable of multiple unfold/refold cycles with little loss per cycle to aggregation process.

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*Edited by K. Kuwajima*

**Keywords:** intermediate folding states; protic ionic liquids; protein unfolding; proton activity; effective pH

## Introduction

The use of differential scanning calorimetry (DSC) remains a powerful and direct means of analyzing the thermodynamic parameters that control noncovalent bond formation (and, thus, stability) in proteins and other macromolecules.<sup>1–4</sup> Thermodynamic information on the structure of the protein can be determined by integration of the system’s heat capacity as it undergoes the unfolding process. This yields a specific enthalpy change, which was first investigated in detail by Privalov *et al.* in the early 1970s, and their findings have been confirmed many times since.<sup>4–6</sup> The unfolding enthalpy is found to be unique to

the structure of the protein dissolved within a given environment (solvent). By comparing such directly measured enthalpy changes with an enthalpy derived within the van’t Hoff equation description of a two-state equilibrium (which requires a specific shape for the calorimetric signal of the protein unfolding event), information on the cooperative nature of the unfolding can be obtained. This, in turn, allows the protein under study to be evaluated as a simple “two-state” unfold, which seems to be the case for many small proteins, or otherwise.

Generally, protein DSC measurements have been conducted using microcalorimetric instruments because very low protein concentrations have been needed to avoid aggregation. This has tended to restrict this sort of study to specialist laboratories. The restriction can now be lifted thanks to the recent finding that protein solutions of high concentration resist aggregation when the solution contains a large mole fraction of a protic ionic liquid (PIL).<sup>7</sup> Thus, where previous studies have been obliged to report partial heat capacities, we will simply report the total system heat capacity and attribute the part in excess of a self-evident baseline, defined in Materials and Methods, to the protein mass fraction of our solution.

\*Corresponding author. E-mail address:  
[nolene.byme@asu.edu](mailto:nolene.byme@asu.edu).

Abbreviations used: PA, proton activity; PIL, protic ionic liquid; DSC, differential scanning calorimetry; TMS, tetramethylsilane; HWL, hen egg white lysozyme; RNase A, ribonuclease A; APIL, aprotic ionic liquid; TEATf, triethyl ammonium triflate; EAN, ethyl ammonium nitrate; TEATFAC, triethyl ammonium trifluoroacetate; EAFm, ethyl ammonium formate.

PILs, which constitute a subgroup of the ionic liquid family, are currently under intense study in a variety of applications, including fuel cells, where their ability to transport protons between electrodes has been a recent discovery.<sup>8</sup> The broader family of ionic liquids have proven successful in chemical synthesis,<sup>9,10</sup> enzyme catalysis,<sup>9,11</sup> and green chemistry generally,<sup>12,13</sup> due largely to their excellent stability, low volatility, and unusual solvent properties. Recently, ionic liquids have been shown to dissolve and stabilize proteins.<sup>7,14,15</sup> An attractive feature of ionic liquids is the possibility to “tune properties” by exploiting the great range of cation and anion choices. In this work, we will show that the protic subclass of ionic liquids is of special value in this context because of the extra variable provided by the proton activity (PA), the nature of which we now discuss.

A PIL is formed by transfer of a proton from a Bronsted acid to a Bronsted base. PILs all have a concentration of protonated species (the analog of the  $\text{H}_3\text{O}^+$  ion), in the range 2–10 M. However, this does not establish them as acid in character unless the base is water itself (or a base of similar Bronsted base strength). Indeed, many PILs can be strongly basic in character. The activity of the protons can vary enormously depending on the nature of the acid and base from which the PIL is formed and the consequent thermodynamic drive to place the protons firmly on the basic site of the proton acceptor species. The availability of the protons on PIL cations to relocate onto other acceptor species must be expected to play a role analogous to the pH-related acidity of aqueous solutions, but its determination is problematical due to the comparable concentration of protonated species in all PILs and the absence of any solvent to serve as a diluent. However, the existence of an “effective pH” is made obvious by the very different colors manifested by damp pH paper immersed in different PILs. The colors, which cover the whole familiar range, depend on the extent of protonation of weak acid dyestuffs.

Electrochemical methods for PA determination are still under development,<sup>16</sup> and in the present work, the PA will be characterized using NMR proton chemical shift measurements for the proton that has been transferred. Although transferred to the base, which is a nitrogenous species in all the cases we study, the proton is still being counter-attracted (hence, deshielded) by the conjugate base of the acid from which it was transferred. The more deshielded the proton is, the more downfield the transferred proton will appear on the NMR ppm scale when referenced to the external standard tetramethylsilane (TMS), and this downfield shift,  $\delta$  (N–H), will provide our measure of the PA. We should stress that in PILs, increasing downfield shift means increasing basic character, which is the opposite of the case for familiar aqueous solutions in which increasing downfield shift means increasing acidity. In this work,  $\delta$  (N–H) will be seen to vary over the range 7.05 ppm (most acid) to 9.15 ppm (most basic). To provide some sense of equivalence, we anticipate our results to note that, when  $\delta$  (N–H)=7.64, ribonuclease A (RNase A) unfolds at the same temperature,  $T_d=56^\circ\text{C}$  (peak value

of  $C_p$ ), as its maximum unfolding temperature in aqueous solutions ( $T_d=56.54^\circ\text{C}$ ), which is reached at pH 5.5.<sup>17</sup> For these conditions, the unfolding enthalpy, as defined in Materials and Methods, is 27.6 J/g (98 kcal/mol), which is very close to the value [30.3 J/g (106 kcal/mol)] reported in Ref. 17. However, in our solutions, this unfolding temperature is not the maximum value, and  $T_d$  continues to increase with increasing basicity, at least to the maximum basicity value explored in this study,  $\delta$  (N–H)=9.15).

Recently, we reported that the protein hen egg white lysozyme (HWL) has a particular PA range in which it can reversibly fold and unfold at high concentrations, with losses to aggregation or other processes of less than 3% per cycle.<sup>18</sup> It is this range that presumably will prove to be the most interesting for long-term storage and transport of biomolecules.

It is the purpose of the present article (i) to report, in greater detail, the correlation of folding reversibility with PA in ionic liquid solutions; (ii) to demonstrate that in the stable range, the unfolding and folding processes are well described as “all-or-nothing” processes; and (iii) to show how careful application of the PA variable can reveal the presence of intermediate folding states in a detail not previously attainable for lysozyme. We do not attempt to establish that these intermediate states that we observe so clearly are structurally identical with transient intermediate, possibly “wrong,” folding states transiently visited in the folding of lysozyme under physiological conditions.

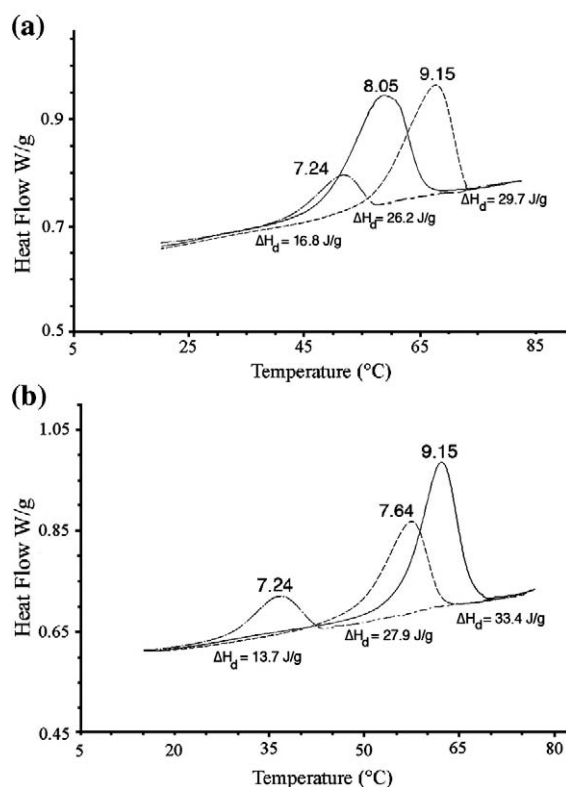
## Results and Discussion

Figure 1a shows the heat capacity *versus* temperature behavior of three HWL solutions that differ in PA, as indicated by the  $\delta$  (N–H) chemical shift of the PIL components of the solutions (see Materials and Methods). It is clear that the unfolding temperature is altered by changes in PA, in a manner similar to that induced by changes in pH in aqueous solution studies. The shape of the endotherm also changes as the PA of the ionic liquid solution is changed.

Figure 1b shows a similar trend for the protein RNase A. Protein stability is often described as dependent on a delicate balance between temperature and pH, such that sufficient changes in either of these variables would result in a loss of protein stability as manifested by the protein either completely or partially unfolding. Changes in the PA of the ionic liquid lead to similar changes in the protein stability. This has been clearly demonstrated in Fig. 1a and b, confirming that the PA of PILs is analogous to pH in aqueous solutions. In each of the proteins studied, an increase in thermal stability is observed as the  $\delta$  (N–H) shift becomes larger.

The similarities in the thermal unfolding of the two proteins studied are highlighted below:

- (1) An increase in temperature gives rise to a linear increase in heat capacity up to the temperature at which the accelerating heat absorption linked to the denaturing of the protein commences.



**Fig. 1.** DSC thermograms (heat flow in response to temperature) at scan rates of 20 K/min for (a) HWL and (b) RNase A, dissolved in PILs of three different PAs, as characterized by the N–H PMR chemical shift [ $\delta$  (N–H)] values, which are recorded next to the peaks. The heats of denaturation  $\Delta H_d$  are noted next to the respective endotherms. Conversion factors: 1.0 J/g = 3.47 kcal/mol (HWL) and 3.29 kcal/mol (RNase A). The PIL composition giving each value of  $\delta$  (N–H) is as follows:  $\delta$  (N–H) = 7.24, 1:1 molar ratio triethyl ammonium triflate (TEATf):ethyl ammonium nitrate (EAN);  $\delta$  (N–H) = 7.64, 1:1 molar ratio EAN:triethyl ammonium trifluoroacetate (TEATFAC);  $\delta$  (N–H) = 8.05, 1:1 molar ratio EAN:ethyl ammonium formate (EAFm);  $\delta$  (N–H) = 9.05, 1:3 molar ratio EAN:EAFm.

- (2) An increase in heat capacity of the protein solution, relative to that of the solution before denaturation commenced, is observed on denaturation, analogous to that which accompanies the melting of a solid or to that associated with passage through the glass transition.

The enthalpy change measured by calorimetric methods can be used to determine how cooperatively a protein unfolds. This can be done by comparing the measured enthalpy with the enthalpy obtained by the van't Hoff analysis.<sup>1,4,19</sup> The van't Hoff analysis can be used when the state of equilibrium between folded and unfolded states is changed by temperature. A protein that unfolds by a simple two-state process may be represented by the simple equilibrium



where N and U are the native and unfolded states, respectively.

Two-state folding implies that, at the molecular level, the process under study is an all-or-nothing process, like the melting of a pure crystal; that is, the heteropolymer protein molecule is either fully folded or not folded at all. The existence of intermediates in such cases can, however, sometimes be revealed by causing the folding process to occur under lower-temperature conditions as has, for instance, been shown by different chemical<sup>20–22</sup> and physical<sup>23</sup> approaches in the case of lysozyme. The final part of this article is devoted to a new and particularly clear demonstration of how intermediate states can be revealed, using the special character of the ionic liquid solvent, though whether or not these are the same intermediates that may be transiently populated in normal folding is not established.

Privalov *et al.*<sup>1,3–6</sup> have shown, by analysis of the thermodynamics of a simple equilibrium process in which the population of the unfolded state builds up with temperature according to the van't Hoff equation,

$$d \ln K_{eq} / d(1/T) = -\Delta H / 2.303 RT, \quad (1)$$

that the enthalpy change in the unfolding process,  $\Delta H_v$ , can be obtained in terms of the peak heat capacity,  $\Delta C_d$ , and the temperature at which it is reached,  $T_d$ , where these quantities have been defined in Fig. 7. The relationship is

$$\Delta H_v = 2R^{0.5} T_d M^{0.5} \Delta C_d^{0.5} \quad (2)$$

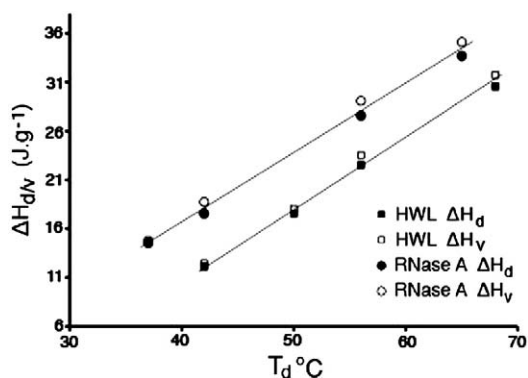
where  $M$  is the molar concentration of protein in the solution and  $R$  is the gas constant.

In Fig. 2, the values of  $\Delta H_v$  obtained using Eq. (2) are compared with those of  $\Delta H_d$ , the heat of denaturation obtained directly from the integration of the denaturing “apparent” heat capacity as shown by the hatching in Fig. 7, by plotting both as a function of  $T_d$  for experiments in which the PA represented by  $\delta$  (N–H) was varied.

It can be seen that the enthalpy of unfolding increases linearly with increases in  $T_d$  and that the values obtained for  $\Delta H_v$  and  $\Delta H_d$  almost superimpose, indicating that both RNase A and HWL can be described (within 6%) by the two-state model. This is the same finding made in the original Privalov and Khechinashvili study conducted in physiological solutions when using pH as the variable.<sup>1</sup>

### Stabilization of intermediates

Although the coincidence of  $\Delta H_v$  and  $\Delta H_d$  seen in Fig. 2 implies that HWL and RNase A can be described as two-state unfolders, the evidence from low-temperature folding studies<sup>23</sup> and the finding that a partial denaturation can be induced using trifluoroethanol<sup>20–22</sup> suggest that the process is intrinsically more complex and that under the right circumstances, the unfolding process, and its reverse, will be noncooperative. Such additional complexity is already well known in the structurally similar  $\alpha$ -lactalbumins<sup>24</sup> and other c-type lysozymes.<sup>22,25</sup> RNase A has also been shown to unfold via an intermediate



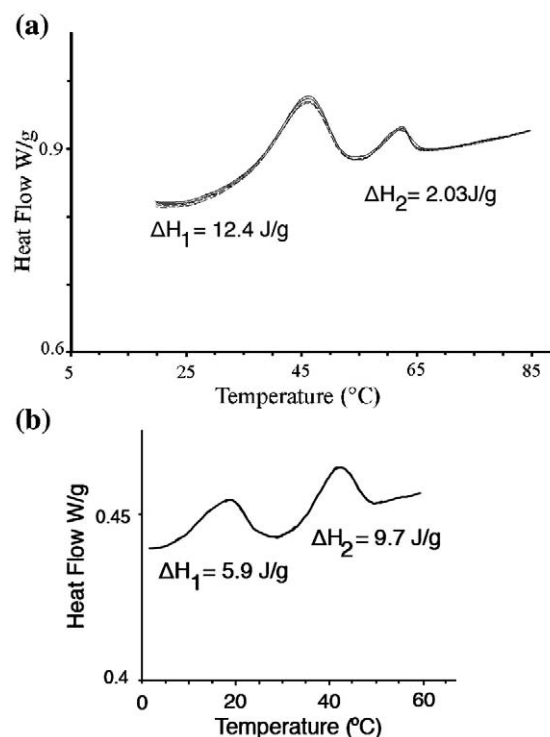
**Fig. 2.** Unfolding enthalpies assessed both from direct DSC measurements (endotherm area) ( $\Delta H_d$ ) and from Eq. (2) analysis ( $\Delta H_v$ ), in relation to the temperature of denaturation,  $T_d$  (conversion factors are given in Fig. 1 caption). Also, see Fig. 7 for the definition of  $\Delta H_d$ . Note the close accord of the two enthalpy values. For RNase A,  $T_d=37$  °C,  $\delta$  (N-H)=7.24 ppm, PIL of the solution is 1:1 molar ratio TEATf:EAN;  $T_d=42$  °C,  $\delta$  (N-H)=7.42 ppm, solvent EAN;  $T_d=56$  °C,  $\delta$  (N-H)=7.64 ppm, solvent is 1:1 molar ratio TEATfAc:EAN;  $T_d=65$  °C,  $\delta$  (N-H)=9.15 ppm, solvent is 1:3 molar ratio EAN:EAFm. For HWL,  $T_d=43$  °C,  $\delta$  (N-H)=7.19 ppm, solvent is ethyl ammonium perchlorate:TEATfAc;  $T_d=51$  °C,  $\delta$  (N-H)=7.24 ppm, solvent is 1:1 molar ratio TEATf:EAN;  $T_d=56$  °C,  $\delta$  (N-H)=7.42 ppm, solvent is EAN;  $T_d=68$  °C,  $\delta$  (N-H)=1:3 molar ratio EAN:EAFm.

state. This was achieved at a pH of 3.3 with 0.3 mM sodium dodecyl sulfate.<sup>26</sup> This intermediate state was identified by the presence of two successive thermal events. However, once thermally unfolded, RNase A remained denatured. We show, by exercising the PA degree of freedom in our ionic liquid milieu, that the complete unfolding process can be observed to resolve itself into two well-separated stages and that, in our case, the two-stage process is completely reversible.

The division of the unfold/refold process into two successive processes arises rather suddenly following a small increase in the PA [decrease in  $\delta$  (N-H)] beyond the value of 7.24 ppm displayed in Fig. 1a and b. The change from the single unfolding peak of Fig. 1 to the double peak of Fig. 3a follows from a change of solution composition in the mixed PIL+aprotic ionic liquid (APIL) binary EAN+1-butyl-3-methylimidazolium perchlorate, from a 1:1 to a 1:3 molar ratio, corresponding to a change of  $\delta$  (N-H) from 7.24 to 7.16 ppm (see Materials and Methods). A further change of  $\delta$  (N-H) to 7.05 (by a change of molar ratio to 15:85 in the same binary) causes the additional separation of the two stages seen in Fig. 3b. As in the case of streptokinase<sup>27</sup> and others previously mentioned,<sup>26</sup> the unfolding event has clearly been separated into two distinct processes, but in the present case, the whole process has become reversible. Figure 3a contains four superposed successive unfolding endotherms. For the process seen in Fig. 3b, the behavior was equally reversible, although only one endotherm is shown. However, with a further small change of  $\delta$  (N-H) to 6.97 (binary molar ratio, 10:90), reversibility is completely lost.

The unfolding endotherms of Fig. 3a are independent of scan rate up to 20 K/min. Thus, the folding kinetics are rather fast. Whether or not the upper component is to be described as the melting of a molten globule state is a matter for future discussion when the structures typical of the different stages have been elucidated.

This two-stage unfolding domain is one of great sensitivity to the PA as can be seen from Fig. 3b. The small increase in acidity, from  $\delta$  (N-H) 7.16 to 7.05, causes both a displacement in temperatures of maximum unfolding rate ( $d[N]/dT$ ) and an inversion of the unfolding enthalpies of the two stages. In order for the endotherms shown in Fig. 3b to be scan rate independent, it was necessary to reduce the scan rate below 6 K/min (suggesting the possible use of scan-rate-dependent calorimetry to study the folding kinetics). On cooling at these same rates, the curves are retraced, within the temperature calibration uncertainty. Since each of these unfolding stages has long-term stability in the ionic liquid milieu, it should be possible, using solution-based structural analyses, to arrive at some conclusions concerning the structures of the intermediate state under different PA conditions.



**Fig. 3.** (a) DSC thermograms (heat flow *versus*  $T$  at scan rate of 20 K/min) for HWL at PAs at the acid edge of the high refoldability domain, showing reversible intermediates. (a) Unfolding endotherms at  $\delta$  (N-H) value of 7.16 ppm, obtained using the PIL-APIL mixture EAN+1-butyl-3-methylimidazolium perchlorate at 1:3 molar ratio. Four successive upscans are shown to demonstrate the fact that the protein can return to this intermediate state repeatedly. (b) Effect on the two-stage process of increasing acidity from  $\delta$  (N-H)=7.16 to 7.05 ppm (achieved by altering the 1:3 molar ratio of the first mixture to 15:85 in the second).

The intermediate states observed at 55 and 30 °C, respectively, in Fig. 3 are presumably in dynamic equilibrium with the folded and fully unfolded states of the protein. The total unfolding enthalpies are comparable, 14.4 and 15.1 J/g, respectively, but the total unfolding entropies must differ more, with the entropies being greater in the more acid case. That these are well-defined free energy pathways is made clear by our ability to obtain multiple upscans showing the same unfolding pathway. There is little to no loss to aggregation processes, as shown by the barely detectable change in the unfolding energy with each successive upscan.

While the presence of equilibrium intermediates on the unfolding pathway is well established in a number of cases,<sup>28-30</sup> we have not been able to find cases in which the fully unfolded state has been able then to refold, regenerating the same intermediate state, in the way that evidently can be achieved (indeed repeatedly) in the present PIL media. Normally, under the conditions where the intermediate state is observed, the final unfolded state is too prone to aggregation for the reverse process to be manifested.<sup>26,27</sup> Refolding can, however, sometimes be achieved by addition of refolding agents. Our observation may be seen as another aspect of the ability of solutions containing large ionic liquid contents to isolate and preserve proteins over long periods.<sup>7,15</sup> We hope that this ability may prove useful in future investigations of states of residual secondary structure in the nominally fully unfolded states of proteins, as well as in the study of other biomolecules whose normal state is one of low order.

The presence of intermediate states, even at the PA where the analysis using Eq. (2) implies two-state behavior (evidenced in Fig. 3), was previously demonstrated by the quench- and cold-refold study of Angell and Wang.<sup>23</sup> This isocompositional study, in turn, confirmed the findings of the chemical renaturation studies.<sup>20,21</sup> A comparable quench- and cold-refold study of the solutions of Fig. 3 would be of much interest because, under cold-refold conditions, the intermediate state could probably be fully populated then trapped by recoiling to permit proper characterization by structural methods.

For the quench- and cold-refold study to be performed unambiguously, however, an additional component, a sugar, will need to be added to suppress the cold-unfolding process, which could otherwise invalidate the study. Cold unfolding can occur whenever there is a sufficiently large denaturation heat capacity increase (see Fig. 7) as explained succinctly in the papers of Franks *et al.*<sup>31,32</sup> Our systems give rather large unfolding heat capacity changes, and we have indeed been able to confirm the predicted occurrence of cold-unfolding events. These will be described in separate articles, but it is relevant to note here that we have found them, like the other processes in these PIL media, to be fully reversible. For the moment, we need to only point out that the addition of sugars, which are also desirable to enhancing the suppression of ice formation on slow reheating, completely represses the cold-unfolding

phenomenon so that the interesting question of protein folding intermediates would seem to be open to the quench- and cold-refold technique.

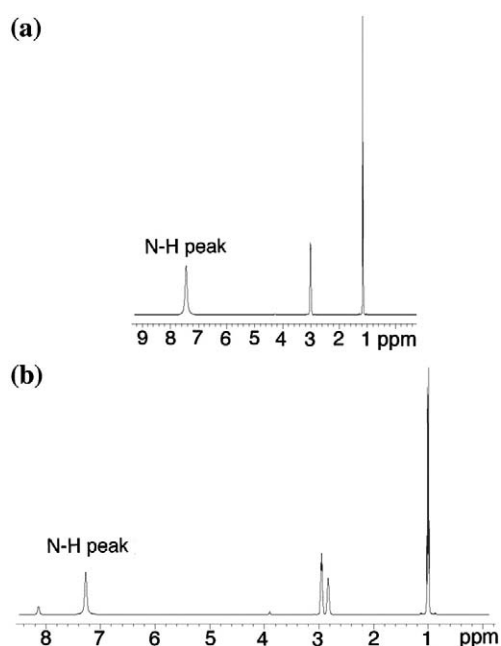
## Concluding Remarks

From the foregoing section, in particular from the results shown in Fig. 3, it seems that the use of protein solutions based on PILs and their mixtures, with or without APILs, can bring a new element to the study of the protein folding problem by establishing folding conditions that are unattainable in standard aqueous solutions. By subtly changing the protein-solvent interactions, while at the same time reducing the chance of disruptive aggregation, the PIL-based solution studies allow us to define the steps along the folding path more clearly than has previously been possible. It will clearly be of interest to study the enzymatic activity of proteins in these unusual and highly tunable solvent media.

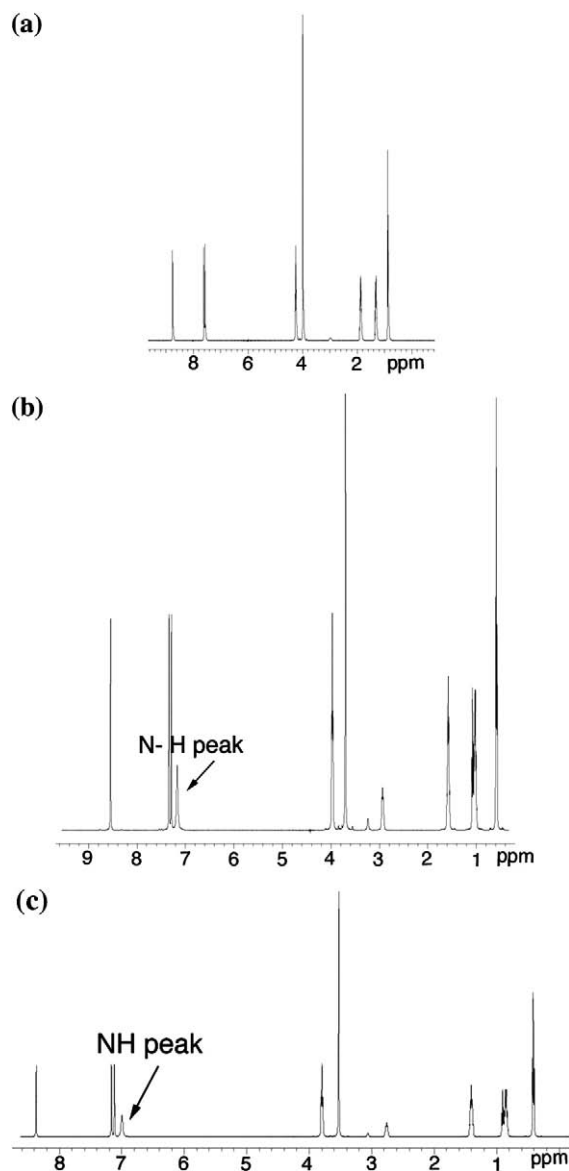
## Materials and Methods

### Preparation of PILs

The PILs of this study are generally simple to synthesize (from the reagent-grade acids and bases, anhydrous where possible). When aqueous routes are necessary, care must be exercised in the dehydration. For details on the preparation of PILs, the reader is referred to Ref. 33. The PIL mixtures of variable PA (characterized as described below) were prepared by weighing out the anhydrous components.



**Fig. 4.** (a)  $^1\text{H}$  NMR spectrum of EAN. The  $\delta$  (N-H) = 7.42 ppm with respect to TMS. (b)  $^1\text{H}$  NMR spectrum of a binary 1:1 molar ratio mixture of EAN and TEATf. The  $\delta$  (N-H) = 7.24 ppm with reference to TMS.



**Fig. 5.** (a)  $^1\text{H}$  NMR spectra of the APIL 1-butyl-3-methylimidazolium perchlorate. (b)  $^1\text{H}$  NMR spectrum of a binary 1:3 molar ratio mixture of EAN and 1-butyl-3-methylimidazolium perchlorate. The  $\delta$  (N-H)=7.16 ppm with reference to TMS. This mixture was used to stabilize the intermediate structures shown in Fig. 3a. (c)  $^1\text{H}$  NMR spectrum of a binary 10:90 molar ratio mixture of EAN and 1-butyl-3-methylimidazolium perchlorate. The  $\delta$  (N-H)=6.97 ppm referenced to TMS.

### Preparation of protein/PIL solutions

HWL and RNase A were purchased from Aldrich in lyophilized form and were used without further purification (molecular mass=14.5 and 13.7 kDa, respectively). Solutions were prepared by adding weighed amounts of water to the PA-characterized PIL or PIL mixture and then dissolving the lyophilized protein in the hydrated liquid in mass appropriate to yield a final concentration, in each case, of 14 mM protein and 20 wt.%  $\text{H}_2\text{O}$ . Note that the PA characterization described below was made before either the water or the protein was added.

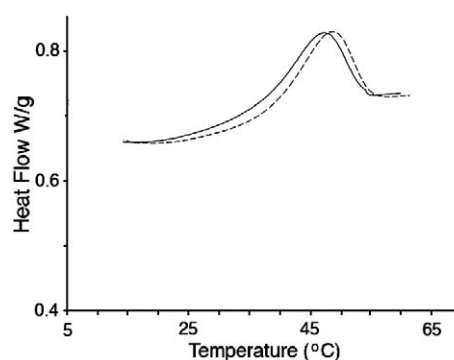
### Proton NMR chemical shift measurements

In this study, we use the NMR chemical shift of the N-H protons,  $\delta$  (N-H), as an internal probe, or metric, for the state of the proton, or the PA, while electrochemical methods of quantifying the PA are being refined (T. Tang, *et al.*, unpublished results). Because we have no solvent, we cannot use a single standard state as reference for other solutions, as in the usual solution studies. The value of  $\delta$  (N-H) used to characterize each solution is determined in the anhydrous PIL or APIL-PIL mixture, before the small amounts of water and protein components needed to complete the test sample have been included. As justification for this choice, we cite the consistency of refolding behavior that we observe, regardless of whether we establish the chemical shift using a single protic salt or a mixture of two or more with the same chemical shift (Fig. 4).

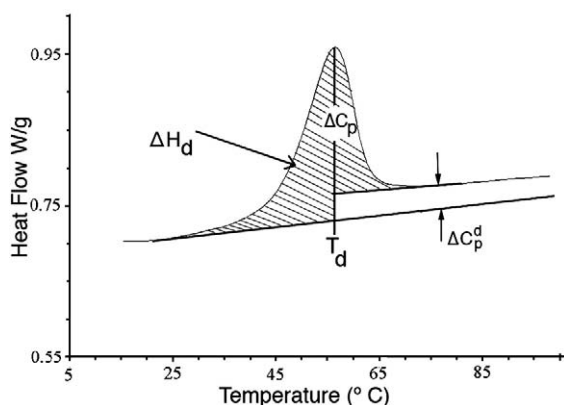
The  $\delta$  (N-H) was measured using a 500-MHz Varian spectrometer. Sample spectra identifying the N-H resonance and showing how the peak shifts with changes in the ionic liquid composition are shown in Fig. 5b and c, where the N-H peak-free spectrum of an APIL is shown for comparison (Fig. 5a).

The binary mixtures used in the solvents for this study need not be restricted to PILs. The mixing of APIL and PIL yields a  $\delta$  (N-H) peak that is sensitive to the solution composition; indeed, some of our most interesting results are obtained with such solutions. This is seen in the cases chosen for Fig. 5, which are binary mixtures of 1-butyl-3-methylimidazolium perchlorate and EAN at 1:3 molar ratio (Fig. 5b) and at 9:1 molar ratio (Fig. 5c). The solution becomes more acidic as the concentration of the 1-butyl-3-methylimidazolium perchlorate is increased, according to the position of the  $\delta$  (N-H) peak. The  $^1\text{H}$  spectrum of 1-butyl-3-methylimidazolium perchlorate, which contains no N-H peak, is provided for comparison in Fig. 5a.

To further validate the use of  $\delta$  (N-H) as a measure of the PA and an analog of pH in aqueous solutions, we show how similar the unfolding endotherms of HWL are when dissolved in solutions of quite different ions that have the same  $\delta$  (N-H). Figure 6 shows the unfolding endotherms of HWL dissolved in (a) the binary PIL EAN+TEATf at 1:1 molar ratio and (b) the protic+aprotic binary mixture EAN+1-butyl-3-



**Fig. 6.** Comparison of thermal unfolding of HWL in the 1:1 molar ratio mixture of the EAN and TEATf,  $\delta$  (N-H) of 7.24 ppm,  $T_d=48\pm 2$  °C, represented by the broken lines, and the thermal unfolding of HWL in the 1:1 molar ratio mixture of EAN and the APIL 1-butyl-3-methylimidazolium perchlorate,  $\delta$  (N-H)=7.24 ppm,  $T_d=47\pm 2$  °C (continuous line), to demonstrate the importance of  $\delta$  (N-H) over chemical constitution in fixing the protein unfolding energetics.



**Fig. 7.** DSC scan for HWL in solution with PIL of  $\delta$  (N-H)=7.42 ppm, (EAN solution) illustrating terms that are used in the text. The shading shows the area used in the unfolding enthalpy assessment, according to Privalov *et al.*<sup>1,4</sup>

methylimidazolium perchlorate at 1:1 molar ratio, both of which have a  $\delta$  (N-H) of 7.24 ppm. It is clear that the unfolding event for HWL is very similar both in peak temperature and in form (cooperative) when  $\delta$  (N-H) is the same. (A reviewer has pointed out that the residual difference could be due to differences in perchlorate *versus* triflate anion absorption onto the protein.)

### DSC measurements

Calorimetric measurements were performed using a standard DSC instrument (TA2920) rather than a microcalorimeter, thanks to the ability of PILs to dissolve high concentrations of proteins while protecting the solution against aggregation.

Solution samples, typically 5 mg, were hermetically sealed in aluminum DSC pans. All scans were conducted at 20 K/min unless otherwise stated.

Figure 7 shows a typical DSC scan of HWL in a PIL (in this case, HWL in EAN solution) from which the terms used in our analysis are defined. The enthalpy of denaturation,  $\Delta H_d$ , is assessed as indicated by the hatching in Fig. 7, to match with the definition introduced by Privalov and Khechinashvili in 1974.<sup>1</sup> The denaturation (sometimes “melting”) temperature,  $T_d$ , is taken at the peak maximum of the absorption curve.

### Acknowledgements

This work has been supported by the National Science Foundation, Chemistry Division under the Collaborative Research in Chemistry program, Grant No. 040714. We acknowledge stimulating discussions with our CRC colleagues P.G. Debenedetti, P. Rossky, and H. E. Stanley. The four protic ILs used in preparing the solutions of this work were kindly provided by Jean-Philippe Belieres (see Ref. 33). The aprotic IL was prepared in earlier work by Wu Xu (see Ref. 34). The DSC instrument was purchased with funds from the NSF CHE grant no. 0608581 (to J. L. Yarger).

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