Kinetics and Mass Spectrometric Measurements of Myoglobin Unfolding in Aqueous Ionic Liquid Solutions

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ABSTRACT

Recent studies have characterized the effects of aqueous ionic liquids on myoglobin unfolding for the broader purposes of understanding their effects on protein structures, stabilities, and ultimately biocompatibilities for future applications. Here, we investigated the effects of four different ionic liquids (ILs) on the thermal stability, unfolding kinetics, and tertiary shape of myoglobin. We compared results for four different ILs: 1-butyl-3-methyl imidazolium tetrafluoroborate (BMIBF₄); 1-butyl-3-methyl pyrrolidinium tetrafluoroborate (PyrBF₄); 1-ethyl-3-methyl imidazolium acetate (EMIAc); and tetramethylguanidinium acetate (TMGAc). Results showed that ILs accelerate myoglobin unfolding kinetics both through aqueous solution ionic strength effects and ionic liquid-specific effects. Arrhenius plots of observed rate constants reveal that some ILs lower the energy barrier to unfolding, possibly by destabilizing the native protein state. The magnitude of these ionic liquid effects correlates with their effects on protein thermodynamic stabilities. Hydrogen-deuterium exchange (HDX) experiments using ESI-MS showed that myoglobin exhibits a more open, and presumably less stable, tertiary shape in aqueous IL solutions. Overall, BMIBF₄ and TMGAc exhibit the strongest effect on the myoglobin stability, unfolding rate, and tertiary structure while PyrBF₄ and EMIAc have weaker effects under our experimental conditions.

Keywords: Myoglobin; Ionic liquids; Protein unfolding

1. Introduction

The structures and stabilities of proteins in aqueous ionic liquid (IL) solutions have been the focus of numerous recent reports in the literature [1-15]. Such studies are motivated by the utilization of ILs in several different biomedical and biochemical techniques [5, 16-21], where ILs are present as solutes in aqueous solution. ILs can participate in enzyme reactions, and also can be used to inhibit, activate, or otherwise modulate enzyme kinetics [3, 4, 22-26]. They have been used as potent antimicrobial drugs and drug delivery systems [17], and can affect protein aggregation [1, 5, 15, 20], including amyloid fibril formation [6, 12]. They have also been used for controlling protein conformational transitions [27-29] and assisting in the preparation of cellulose and other biomass for dissolution and processing [19, 30-32].

Significant data exists to enable design of ILs for many non-biochemical applications [33], but researchers are just beginning to compile comprehensive IL/protein data for prediction and design of biochemical/biomedical applications. A number of groups have studied different ILs series and their effects on protein stability [11, 16, 34-36], IL-protein surface interactions [9, 26, 37, 38], and protein thermodynamic stability in aqueous IL solutions [5, 7]. Low-concentration ILs in aqueous solution often do not denature proteins, but can destabilize their structures as demonstrated by numerous thermodynamic studies [5, 7, 8, 10, 15, 39]. Several recent reports have focused on IL-protein interactions in α -helical proteins and their role in protein destabilization [2, 3, 8, 11, 40]. In this context myoglobin, an α -helical protein with a heme moiety, has received recent attention in both applied and fundamental studies [7, 11, 27, 39, 41-43]. Protein stabilization and/or destabilization by ILs in aqueous solution is often explained with the Hoffmeister anion series [44], but myoglobin may not exactly follow this series [35, 36, 39]. The effects of different ILs, especially new biocompatible ILs, on myoglobin's tertiary structure and dynamics are not yet completely understood and additional experiments can provide further insights.

While many reports have characterized the thermodynamic stabilities of proteins in aqueous IL solutions, relatively few have studied IL effects on protein unfolding kinetics. Myoglobin unfolding in high concentrations of urea denaturant proceeds slow enough to enable relatively facile kinetics

measurements [45, 46]. Such kinetics experiments have elucidated how hydrophobic and aromatic interactions with myoglobin residues near the heme group can destabilize the protein and increase unfolding rate constants [45, 46]. Kinetics measurements in the presence of aqueous ILs can thus offer insight into different IL effects on myoglobin, especially as hydrophobic/aromatic interactions have been identified as contributors to protein destabilization [8, 24, 36, 39, 47]. Ultimately, kinetics measurements can help elucidate IL effects on protein folding pathways and point to mechanisms of kinetic stabilization and protein/IL-selective unfolding.

Insights into protein-IL interactions and their effects on tertiary structures can also be obtained with mass spectrometry and hydrogen-deuterium exchange (HDX) experiments. In an HDX experiment, a protein in D₂O (heavy water) exchanges its H-atoms (typically from N-H groups) for D-atoms. Structural information is obtained due to exposed “surface” N-H groups exchanging faster than buried residues, and hence the number of D-atoms added to a protein during a given reaction time elucidates the structure as tightly folded (few D-atoms added), slightly loosened (more D-atoms added), or completely opened/unfolded (many D-atoms added) [48-50]. Nuclear magnetic resonance (NMR)-based HDX measurements have been used to characterize IL interactions with a short peptide [8], but for proteins mass spectrometry-based HDX measurements are more simple and can provide direct qualitative information regarding the extent of tertiary-level protein structural changes in aqueous IL solutions.

In this work, we investigate myoglobin in aqueous IL solutions using unfolding kinetics measurements and HDX experiments. We compare results for four different ILs: 1-butyl-3-methyl imidazolium tetrafluoroborate (BMIBF₄); 1-butyl-3-methyl pyrrolidinium tetrafluoroborate (Pyr⁺BF₄); 1-ethyl-3-methyl imidazolium acetate (EMIAc); and tetramethylguanidinium acetate (TMGAc). In a previous report [7], we quantified myoglobin thermodynamic stabilities in aqueous solutions of BMIBF₄ and EMIAc and showed that BMIBF₄ destabilizes myoglobin while EMIAc has minimal effect on the protein stability. The ILs are chosen to offer a broad array of ILs and to compare different cations and anions. BMIBF₄ and Pyr⁺BF₄ contain the same anion (BF₄⁻) and differ only in the aromaticity of the cation (imidazolium is aromatic while pyrrolidinium is not). EMIAc and TMGAc both contain the acetate anion (Ac⁻) and differ

in the cation. Notably, TMGAc has been designed as a biocompatible IL for protein purification [51] but has not previously been used with myoglobin.

The combination of routine protein stability studies in aqueous IL solutions with unfolding kinetics and HDX measurements offers broad insights into how destabilization by ILs affects protein shapes and unfolding transitions. In this work we present this combined analysis for the myoglobin protein. Unfolding kinetics measurements demonstrate how different ILs affect unfolding rates and unfolding energy barriers, and HDX measurements quantify the extent to which different ILs affect protein shapes.

2. Experimental Methods

2.1 Sample Preparation

Horse-skeletal myoglobin was obtained from Sigma-Aldrich and used without further purification. BMIBF₄, EMIAc, and Pyr⁺BF₄⁻ were obtained from Sigma-Aldrich ($\geq 98\%$ purity) and were also used without purification. TMGAc was synthesized using a reported procedure [51]. Tetra-methyl-guanidine (Alfa Aesar) and glacial acetic acid (Fisher Scientific) were mixed in a 1:1 molar ratio in 50 mL ethanol and stirred at room temperature overnight. The ethanol was removed by rotary evaporation at 40 °C. The identity and purity ($\geq 95\%$) of the TMGAc was verified by NMR spectroscopy.

For all protein studies, solutions were prepared containing constant myoglobin concentrations (0.2 mg/mL) in 20 mM pH-7 phosphate buffer. ILs were added from stock solutions of the ILs in aqueous solution and 20 mM pH-7 phosphate buffer. IL stock solutions were prepared fresh and used immediately prior to experiments to prevent effects of IL decomposition (e.g., hydrolysis of BMIBF₄ to generate HF). pH measurements showed that the ILs in aqueous solution had minimal effects on solution pH beyond expected ionic strength effects: the pH decreased to ~6.8-6.9 in the aqueous IL solutions.

2.2 Temperature Stability Studies

Preliminary thermodynamic stability studies were performed in a temperature-controlled Jasco J-810 circular dichroism (CD) spectrometer. For each sample, the absorbance and CD spectra were simultaneously measured from 300-500 nm at different temperatures from 25 to 93 °C. The sample was

placed in a sealed cuvette to prevent water evaporation. The heme moiety in myoglobin exhibits a strong absorbance band at 409 nm that also displays a strong positive CD signal [52]. While both the 409 nm absorbance and CD intensity can be used to quantify the fraction of the folded protein [52-54], we used the absorbance values for consistency throughout this work. The fraction of folded protein was computed from 409 nm absorbance following previous reports [7, 54]. Briefly, the 409 absorbance in the spectrum of native myoglobin in buffer (shown in Figure S1) is assumed to correspond to 100% folded protein and the 409 absorbance in the spectrum of myoglobin with 8.0 M urea (also shown in Figure S1) is assumed to correspond to 0% folded protein. The folded fraction was computed as

$$\text{Fraction Folded} = \frac{A_{409} - A_u}{A_f - A_u} \quad (1)$$

This method, in which A_{409} is the absorbance, A_u is the 409 nm absorbance of the unfolded protein, and A_f is the absorbance of the folded protein, corrects for the residual 409 nm absorbance of the unfolded protein and was used for all fraction folded calculations.

For reference, myoglobin stability studies were also performed using urea titrations in the presence of some ILs. For each experiment, ~10 solutions were prepared with increasing urea concentrations from 0 to 9.0 M, and for each experiment the solution contained a constant 150 mM concentration of aqueous IL. Urea (VWR) was added from a 10 M stock solution in 20 mM pH-7 phosphate buffer. As myoglobin unfolding in urea is slower than in GuHCl (*vide infra*), samples were incubated for two hours prior to measurement. Absorbance spectra were measured with a Perkin-Elmer Lambda-35 spectrometer with a solution of corresponding aqueous IL as blank.

2.3 Unfolding Kinetics Measurements

To measure unfolding kinetics, myoglobin samples were prepared in quartz cuvettes in aqueous IL solutions without urea. Appropriate aliquots of urea from the 10 M stock solution were then added and the absorbance at 409 nm (proportional to the folded protein concentration [53, 54]) was measured as a function of time after urea addition with continuous stirring. Reported urea and IL concentrations are the final values after urea addition. These experiments were performed using Vernier SpectroVis Plus

spectrometers placed on magnetic stirring plates. IL concentrations were varied from 0-400 mM and data sets were acquired with both 6 and 8 M urea. Because ionic strength is known to affect unfolding kinetics, NaCl control experiments were performed with the same concentrations (0-400 mM) [55].

Temperature-dependent kinetics were measured in the temperature-controlled Jasco J-810 CD spectrometer. Both the UV absorbance and CD signal at 409 nm were monitored versus time after urea addition at different temperatures with continuous stirring. Unfolding kinetics curves were generated based on the 409 absorbance.

2.4 HDX Experiments

Mass spectrometry experiments were performed using an Agilent 1100 Series LC/MSD trap with a needle voltage of 3.5 kV and a continuous LC flow rate of 100 μ L/min. Myoglobin samples were prepared at 1 mg/mL concentration in 20 mM pH-7 phosphate buffer in the presence of different concentrations of aqueous ILs. Proteins under various conditions, with or without ILs in buffer solution, can be rapidly separated from the other solution components (ILs, phosphate buffer salts, and denaturants) with size-exclusion or desalting LC columns [50]. To accomplish this, samples (8 μ L aliquots) were injected and passed through an Acclaim PolarAdvantage II desalting column to remove buffer and IL salts. Initially, the mobile phase was water with 0.1% formic acid to remove the salts, which were discarded into waste. The mobile phase was then switched to acetonitrile with 0.1% formic acid to elute the protein, which was directed into the ESI-MS instrument. The pH of the injected myoglobin solution after mixing with the 0.1% formic acid mobile phase was about 2.5, which was low enough to quench the H/D exchange immediately after injection.

Hydrogen-deuterium exchange (HDX) experiments were performed by adding 0.9 mL of D₂O (Cambridge Isotope Laboratories, 99.9%) to 0.1 mL of the myoglobin samples prepared above (90% D₂O present for all experiments). Samples were stirred vigorously for a fixed time length (~10-40 seconds) and quenched by adding buffer to lower the pH to less than 2.5. Samples were then injected immediately and

analyzed with the LC/ESI-MS system. One measurement was performed immediately prior to mixing with D₂O for t=0 data points.

3. Results and Discussion

3.1 Myoglobin Stability in Different Aqueous Ionic Liquid Solutions

We first discuss the effects of the aqueous ILs on the stability of myoglobin. These results supplement other thermal stability studies in the literature and help contextualize the kinetics and HDX experiments. We obtained protein thermal denaturing curves for myoglobin in different aqueous ionic liquid solutions by monitoring 409 nm absorbance values at increasing temperatures as described in Section 2. The results are summarized in Figure 1. In the absence of ILs (black squares in Figs. 1A-1D), myoglobin unfolds at ~80 °C. The rest of the data in Figure 1 yield clear and direct insight into the destabilizing effects of the aqueous ILs.

At 25 °C in pH-buffered solution, myoglobin remains folded in the presence of all the ILs at all concentrations up to 300 mM. This is consistent with previous reports [8, 16, 28, 39]. The results for EMIAc (Figure 1A) show that this IL does not have a detectable effect on the unfolding temperature. This is consistent with our previous report [7] that indicates EMIAc has only a minor effect on the myoglobin stability. However, the addition of some ILs clearly lowers the unfolding temperature, indicating protein destabilization. The TMGAc (Figure 1B) and PyrBF₄ (Figure 1C) lower the unfolding temperature by ~5-10 °C while the BMIBF₄ (Figure 1D) lowers the unfolding temperature by ~25 °C. Hence, EMIAc has negligible destabilizing effects on myoglobin, TMGAc and PyrBF₄ have minor destabilizing effects, and BMIBF₄ has major destabilizing effects.

3.2 Kinetics of Urea-Induced Unfolding in Aqueous Ionic Liquid Solutions

We used urea-based unfolding experiments to study how the IL destabilization demonstrated in Figure 1 affects the myoglobin unfolding kinetics. Equilibrium urea-denaturing measurements, shown in Figure 2, provide information about how urea affects the protein unfolding equilibrium in different aqueous IL

solutions. Results are shown only for the two extreme cases of EMIAC (little or no destabilization) and BMIBF₄ (major destabilization).

The results obtained in the absence of ILs (triangles) show that myoglobin unfolds with ~6.0 M urea (inflection point of denaturing curve). The presence of ILs (at 0.15 M concentration) causes the protein to unfold at lower urea concentrations in a manner consistent with the results of Figure 1. The unfolding midpoint is ~5.5 M urea with EMIAC (minor decrease from myoglobin without IL) and ~3.5 M with BMIBF₄ (major decrease from myoglobin without IL). Importantly, Figure 2 shows that in all cases myoglobin completely unfolds in 8.0 M urea. Time-based unfolding measurements in 8.0 M urea can therefore provide direct information regarding the IL effects on kinetics.

The results of the unfolding kinetics measurements are summarized in Figure 3. For all kinetics experiments, we compute the fraction of folded protein from the 409 nm absorbance by assuming that the fraction is 1 before addition of urea and 0 after unfolding is complete (consistent with Figure 2) to correct for the residual 409 nm absorbance values of unfolded myoglobin. A data set with error bars and the exponential fit is included in the Supplementary Material as Figure S2. For all ILs, increasing the IL concentration results in increased unfolding rates.

The unfolding decay curves can be fit to a single exponential decay function with an observed rate constant k_{OBS} . Under the two-state model of protein folding [8, 56], k_{OBS} is the sum of k_f and k_u , the folding and unfolding rate constants, respectively [57]:

$$k_{OBS} = k_f + k_u \quad (2)$$

In Equation (2), both k_f and k_u depend on the concentration of denaturant. The fundamental values of k_f and k_u in absence of denaturant can be measured by performing a chevron analysis, which requires measurements of k_{OBS} as a function of denaturant concentration [55, 58]. Due to limitations on protein and urea consumption, extensive concentration-dependent kinetics measurements were not performed; we only used 6.0 M and 8.0 M urea and focus our analysis on the 8.0 M data set. At 8.0 M urea

concentration, the k_f term in Equation (2) is very small and the value of k_{OBS} is dominated by the k_u term (which clearly depends on the urea concentration) [59].

From Figure 3, myoglobin unfolding is clearly accelerated in aqueous IL solutions, but it is well-known that protein unfolding kinetics are affected by solution ionic strength [55]. Thus, the increase in k_{OBS} values by the addition of electrolytic ILs into aqueous solution could in principle be due simply to increased ionic strength. Therefore we also performed urea-based unfolding experiments with NaCl, in which the increase in k_{OBS} is purely related to increased ionic strength.

The k_{OBS} values derived from the experimental data in Figure 3, including the NaCl-control values, are summarized in Figure 4. k_{OBS} rate constants were obtained by fitting both the raw data (409 nm absorbance vs time) and the folded fraction data (shown in Figure 3). No differences in rate constants were observed between the two data sets. These rate constants were obtained by fitting the Figure 3 data to the single-exponential decay function of Fraction Folded versus time using a function-fitting algorithm in Origin 8.5; the exponential decay constant is k_{OBS} . An example of the exponential fit is shown in Figure S2.

The rate constants in Figure 4 increase with increasing IL concentration (Fig. 4A). The increase in k_{OBS} from ionic strength effects alone should be proportional to $\sqrt{I}$ due to the electrostatic screening effect [55]. The unfolding data in the presence of NaCl and EMIAc appear to closely follow the $\sqrt{I}$ dependence. This is highlighted by plotting k_{OBS} versus $\sqrt{I}$ in Figure 4B. The data for NaCl and EMIAc clearly follow a linear trend. This immediately suggests that the effect of EMIAc on myoglobin unfolding is of a predominantly ionic strength nature. With PyrBF₄ and TMGAc, the k_{OBS} values appear to deviate from the NaCl trend at higher IL concentration and are ~15-20% higher than the values in NaCl at 300 and 400 mM. This suggests that the PyrBF₄ and TMGAc have some minor additional “IL” effect on the unfolding kinetics beyond the simple ionic strength effect. With BMIBF₄, the k_{OBS} values are significantly higher than the NaCl-control values, especially at IL concentrations above 200 mM. The influence of BMIBF₄ on the unfolding transition is clearly stronger than a simple ionic strength effect.

From Figure 4, some ILs have a significant effect on myoglobin unfolding kinetics while others appear to only increase the solution ionic strength. To further investigate these effects we performed temperature-dependent kinetics experiments as described in Section 2. Arrhenius plots of the k_{OBS} values measured from 25 to 55 °C are shown in Figure 5. For clarity, only the data with no additive, NaCl, and BMIBF₄ are shown. The complete data set is included in the Supplementary Material as Figure S3. From Figure 5, it is clear that the data with and without NaCl (red circles versus blue triangles) have different intercepts but essentially the same slopes. However, the data with BMIBF₄ (black squares) has a different slope from the other two data sets. Note that all the data sets in Figure S3 have different intercepts from the buffer-only data.

The slope of the Arrhenius plots in Figure 5 is not exactly related to the unfolding transition activation energy because k_{OBS} is not exactly the fundamental unfolding rate constant (i.e., k_u in absence of urea). Nevertheless, as k_{OBS} is dominated by the k_u value at 8.0 M urea, it is appropriate to discuss the slopes in terms of energy barriers to unfolding. The plots in Figure 5 were fit with a linear equation using Origin 8.5. The linear slope is $-E_A/R$, where E_A is the activation energy, or energy barrier. The energy barrier values derived from the Arrhenius plots in Figs. 5 and S3 are summarized in Table 1. The errors are computed from the errors in the Arrhenius plot fits. It appears that some ILs have negligible impact on the unfolding energy barrier while other ILs significantly lower the barrier. While the errors are rather large, it is clear that the BMIBF₄ has the most dramatic energy barrier-lowering effect.

Table 1. Unfolding energy barriers (kJ/mol) derived from the linear fits in Figure 5 and Figure S3.

200 mM IL	E_A (kJ/mol)	Error (kJ/mol)
No IL	115	± 14
NaCl	103	± 14
TMGAc	78	± 14
BMIBF ₄	71	± 13
PyrrBF ₄	108	± 14
EMIAc	91	± 6

A previous study has shown that sodium dodecyl sulfate (SDS) has a strong protein destabilizing effect and accelerates protein unfolding kinetics and lowers unfolding activation energies by ~40 kJ/mol at ~100 mM [55]. In this context the IL effects on myoglobin unfolding appear relatively weaker than surfactant effects. However, it does appear that there is a correlation between the destabilization effects of the different aqueous ILs (Figure 1) and the lowered unfolding energy barriers. 200 mM EMIAc and NaCl, which have no effects on the stability, lower the energy barrier by less than 20 kJ/mol, while 200 mM BMIBF₄, which clearly has the strongest destabilization effect, lowers the energy barrier by ~40 kJ/mol.

3.3 HDX-MS Results for Myoglobin in Ionic Liquid Solutions

The ILs in aqueous solution do not denature myoglobin but can clearly destabilize the protein structure and lower the energy barrier to unfolding. These results are consistent with previous reports showing that protein-IL interactions result in more open protein structures [1, 2, 8, 39, 60, 61] and suggest that the myoglobin structure is itself weakened by the IL molecules in solution. We investigated the extent of the protein structural changes induced by ILs using MS-based HDX experiments.

Briefly, the pH-buffered myoglobin-IL solution was mixed with an aliquot of D₂O and the resultant mass changes were measured as a function of time. If the protein tertiary shape changes, a greater fraction of easily-exchangeable hydrogen atoms are exposed to D₂O in solution allowing H-D exchange and resulting in an increase in the total mass of the protein. For example data, Figure 6 shows the ESI-MS spectra of myoglobin (+16 charge state) before and after HDX in the absence (Fig. 6A) and presence (Fig. 6B) of BMIBF₄. In both cases, the HDX results in an increase in mass of ~0.5-1.5 m/z unit, which corresponds to the addition of ~10-20 D-atoms. Note that additional peaks at 1066 and 1075 m/z in Fig. 6B can be attributed to myoglobin-BF₄ adducts. Comparing Figs. 6A and 6B, it appears that more D-atoms are added to the protein in the presence of the IL (larger mass shift after D₂O addition), which immediately suggests faster HDX exchange and further suggests that the protein tertiary structure is more open in the BMIBF₄ solution.

The results of the HDX measurements versus time are presented in Figure 7. We computed the average protein mass and standard deviation at each time-point by calculating the weighted-average mass of each charge state, multiplying this weighted-average mass by the protein charge, and then averaging together the different total protein mass values for the different charge states. To calculate the weighted-average mass for a given charge state, we defined a mass window of ± 3 amu around the maximum (e.g., 1060.5 m/z for the bottom trace in Fig. 6A) and within this window computed the average of the ion masses each weighted by their intensities. For each time point we used the same charge states from +9 to +22.

Prior to the addition of D₂O ($t = 0$), all samples exhibit protein masses within the measurement error of each other. After D₂O addition, the protein masses increase within the first ~10-20 seconds and become roughly constant after this time, suggesting that the fast-exchanging amides react in this time period. Using the 30-seconds time point as an approximation of equilibrium exchange, the mass increases by ~15 amu with no IL in solution, corresponding to the addition of ~15 D-atoms. With ILs present, the protein mass increases more significantly: ~25 D-atoms are added to myoglobin in the presence of EMIAC and TMGAc (1.5-fold greater H/D exchange compared to myoglobin without ILs) and ~35 D-atoms are added to myoglobin in the presence of BMIBF₄ (more than 2-fold greater H/D exchange compared to myoglobin without ILs). It appears that the HDX addition to myoglobin in the presence of PyrBF₄ is similar to myoglobin without ILs. While the error bars are large (due to low MS resolution), the differences between these data sets are significant.

The HDX-based mass increases in Figure 7 show that the amides exchange D-atoms faster and to a greater extent in the presence of ILs. This immediately suggests that the ILs in aqueous solution cause some structural change in myoglobin, resulting in looser, more open protein tertiary structures with more exposed peptide backbones. We performed control experiments with 200 mM NaCl, shown in Figure S4 in the Supplementary Material. The mass increase with NaCl is the same as without any IL, indicating that the greater H/D exchange is not due to increased ionic strength and that the data in Figure 7 indeed illustrate the effects of the different ILs on the protein structure. The degree of myoglobin structural “loosening” is strongest with BMIBF₄ and TMGAc and less pronounced with EMIAC and PyrBF₄, which

is clearly consistent with the previous thermodynamic (Figure 1) and kinetics (Figs. 3-5) results. Notably, the “protein-loosening” effect is almost as strong with TMGAc as it is with BMIBF₄, which is consistent with the results in Table 1 that show that TMGAc lowers the energy barrier to unfolding almost as much as BMIBF₄.

3.4 Discussion

The thermal stability analysis presented in Figure 1 offers quick insight into how the different ILs affect myoglobin. BMIBF₄ destabilizes myoglobin while EMIAc has minimal effect. Studies have shown that the aromatic imidazolium cations can bind to helical proteins, and the alkyl chains destabilize the protein secondary/tertiary structures [26, 40, 47]. Thus it makes sense that BMI⁺, with the longer butyl chain, destabilizes the protein more than EMI⁺ with its ethyl chain. Regarding the anion, Ac⁻ is in the middle of the Hoffmeister series [39, 44] and BF₄⁻ can bind directly to the protein [37]. Again, it makes sense that the Ac⁻-containing IL has less destabilizing effect than the BF₄⁻-containing IL.

The comparison in Figure 1 with the newly-studied ILs PyrBF₄ and TMGAc offers further insight. PyrBF₄ has a significantly reduced destabilization effect relative to BMIBF₄, immediately indicating that the aromaticity of the BMI⁺ cation is very influential in destabilizing myoglobin (likely through aromatic interactions with the protein). TMGAc has a somewhat stronger destabilization effect relative to EMIAc (though its effect is weaker than BMIBF₄), illustrating the importance of the TMG⁺ cation. TMGAc may affect the water hydrogen bond network [51] and may destabilize the protein structure in a similar manner to the well-known denaturant guanidinium hydrochloride [62, 63].

The nature of ILs in aqueous solution and their effects on proteins is of fundamental interest, and the kinetics study provides data and insights. ILs in water can dissociate as strong electrolytes, but may also be found as contact ion pairs or ion-cluster aggregates [64-66]. As aqueous electrolytes, ILs could have effects simply related to increased solution ionic strength. By comparing myoglobin unfolding kinetics in aqueous IL solutions with kinetics in aqueous NaCl solutions, it is clear that some ILs affect myoglobin primarily through increased ionic strength while other ILs have some extra IL-specific effect. This conclusion is supported by the observed unfolding rate constant, k_{OBS} , and its IL (or NaCl) concentration

dependence (Figure 4), and by the IL-specific energy barrier-lowering effects shown in Table 1. It is clear that BMIBF₄ and, to a lesser extent, TMGAc, lower the energy barrier to protein unfolding. This is consistent with the energy destabilization reported previously and shown in Figure 1. Decreased folded protein stability will result in a decreased barrier for unfolding even if the mechanism and transition state (and any intermediates, etc) are unaffected.

The results presented here and elsewhere consistently show that myoglobin remains folded in aqueous solutions of ILs but may be thermodynamically and kinetically destabilized [7, 11, 39]. The nature and extent of the native structure destabilization is elucidated by the HDX measurements. In aqueous IL solutions, myoglobin exhibits greater H/D exchange, and the degree of extra H/D exchange correlates with the IL-based decreases in thermodynamic stability (Figure 1) and energy barrier-lowering IL effects (Table 1). Based on the average protein mass measurements in Figure 7, we conclude that myoglobin adopts a more open structure in aqueous IL solutions. Importantly, the extent to which the myoglobin structure is “loosened” (based on the extent of H/D exchange) correlates with the extent to which the protein destabilizes the protein structure and lowers the energy barrier to unfolding (BMIBF₄>TMGAc>PyrrBF₄>EMIAc).

4. Conclusions

The effects of ILs on proteins in aqueous solution strongly depend on the specific IL molecular cation and anion. Recent studies investigating proteins in IL environments have focused on myoglobin to elucidate general and specific IL influences on α -helical protein characteristics. We have used thermal denaturing studies, urea-based unfolding kinetics measurements, and HDX experiments to investigate the effects of four different ILs on myoglobin in aqueous solution. The ILs have been chosen to compare destabilizing with non-destabilizing systems (BMIBF₄ vs EMIAc) and also compare ILs containing the same anion but different cations (BMIBF₄ vs PyrrBF₄ and EMIAc vs TMGAc). Thermal denaturing studies show that the BMI⁺ cation has a strong destabilizing effect on myoglobin and, based on the observation that PyrrBF₄ does not stabilize myoglobin as strongly as BMIBF₄, the aromaticity of the

imidazolium cation is important. Also noteworthy is the observation that TMGAc, a new biocompatible IL, has some destabilizing effect on myoglobin.

The key conclusions from this work concern the nature and extent of the IL effects on myoglobin. Unfolding kinetics measurements show that ILs accelerate protein unfolding, which would be expected for any electrolytic species as increased solution ionic strength screens electrostatic fields and increases unfolding rates. We can separate IL-specific effects from ionic strength effects by comparing unfolding rate constants in aqueous IL solutions to those in NaCl control solutions. The ILs that do not significantly destabilize myoglobin (EMIAC) appear to increase unfolding rates only due to the ionic strength effects, while the ILs that destabilize myoglobin have strong additional IL-specific effects. These IL-specific effects include lowering the energy barrier to unfolding, as demonstrated by the Arrhenius plots. These IL-specific effects likely include both protein-IL interactions and hydration structure alterations.

The extent of the IL effects on myoglobin's structure was further characterized using the HDX experiments, which utilize H/D exchange with exposed surface amide groups. The ILs in aqueous solution appear to result in a more open, less stable myoglobin structure with more exposed surface amide groups. The degree to which the ILs cause more H/D exchange correlates with their destabilization effects and the magnitudes of the IL-specific kinetics effect. This work demonstrates how ILs affect myoglobin unfolding kinetics by destabilizing the native protein and lowering the energy barrier to unfolding.

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Figure Captions

Figure 1. Thermal unfolding of 0.2 mg/mL myoglobin in pH-7 phosphate buffer in the presence of different ILs. The y-axis is the fraction of folded myoglobin computed from the initial and final 409 nm absorbance values. Each data set is an average of 3 experiments, and a representative data set with error bars is shown in Figure S2. A) Thermal unfolding in increasing concentrations of EMIAc; B) Thermal unfolding in increasing concentrations of TMGAc; C) Thermal unfolding in increasing concentrations of PyrBF₄; D) Thermal unfolding in increasing concentrations of BMIBF₄.

Figure 2. Unfolding of 0.2 mg/mL myoglobin in pH=7 phosphate buffer in the presence of different ionic liquids, each shown as a function of increasing urea concentration. The y-axis is the fraction of folded myoglobin computed from the absorbance at 409 nm based on initial and final absorbance values. Data shown is an average of two experiments.

Figure 3. Urea-induced myoglobin unfolding kinetics plots with 8.0 M urea in the presence of increasing IL concentration. The fraction unfolded values are computed by normalizing the 409 nm absorbance values to the value at time zero (which assumes the fraction is 1 before unfolding) and correcting for the remaining absorbance value after unfolding (which assumes the fraction is 0 after unfolding). Each data set is an average of 3 experiments, and a representative data set with error bars is shown in Figure S2. A) Unfolding in increasing concentrations of EMIAc; B) Unfolding in increasing concentrations of TMGAc; C) Unfolding in increasing concentrations of PyrBF₄; D) Unfolding in increasing concentrations of BMIBF₄.

Figure 4. Room-temperature k_{OBS} rate constants obtained by fitting the data in Figure 3. Error bars are computed based on errors in the exponential fits and are shown as ± 1 standard deviation. (A) Values shown as a function of increasing IL concentration; (B) Values shown as a function of $I^{1/2}$ to illustrate that some ILs have effects due to ionic strength (in which case k_{OBS} vs $I^{1/2}$ is linear) while other ILs have extra effects.

Figure 5. Arrhenius plots of the k_{OBS} values measured for myoglobin unfolding in 8.0 M urea at different temperatures from 25 to 55 °C. Only the plots for myoglobin in no IL, NaCl, and BMIBF₄ are shown; all plots are shown in Figure S2.

Figure 6. MS of myoglobin (+16 charge state) before and after deuterium exchange (HDX). A) 1 mg/mL myoglobin in pH-7 phosphate buffer; B) 1 mg/mL myoglobin in pH-7 phosphate buffer and 200 mM BMIBF₄.

Figure 7. Average protein mass after initiation of HDX experiment shown vs time after adding D₂O, for myoglobin in pH-7 phosphate buffer with different ILs present at 300 mM. The HDX reaction was quenched with low-pH buffer after specific mixing times immediately prior to LCMS injection. The measurement at t=0 seconds corresponds to the ESI-MS performed immediately prior to D₂O addition.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7