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The effects of high concentrations of ionic liquid on GB1 protein structure and dynamics probed by high-resolution magic-angle-spinning NMR spectroscopy

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ABSTRACT

Ionic liquids have great potential in biological applications and biocatalysis, as some ionic liquids can stabilize proteins and enhance enzyme activity, while others have the opposite effect. However, on the molecular level, probing ionic liquid interactions with proteins, especially in solutions containing high concentrations of ionic liquids, has been challenging. In the present work the ^{13}C, ^{15}N-enriched GB1 model protein was used to demonstrate applicability of high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy to investigate ionic liquid–protein interactions. Effect of an ionic liquid (1-butyl-3-methylimidazolium bromide, [C_4-mim]Br) on GB1was studied over a wide range of the ionic liquid concentrations (0.6–3.5 M, which corresponds to 10–60% v/v). Interactions between GB1 and [C_4-mim]Br were observed from changes in the chemical shifts of the protein backbone as well as the changes in ^{15}N ps-ns dynamics and rotational correlation times. Site-specific interactions between the protein and [C_4-mim]Br were assigned using 3D methods under HR-MAS conditions. Thus, HR-MAS NMR is a viable tool that could aid in elucidation of molecular mechanisms of ionic liquid–protein interactions.

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1. Introduction

Ionic liquids are customizable materials that are composed entirely of ions and have phase transitions at or below room temperature. Ionic liquids provide a unique chemical environment and have drawn considerable attention in recent years, with numerous applications as media for chemical and biocatalytic transformations [1,2], preparation of materials [3–5], energy-related processes [6–8], as well as several environmental [9–11] and analytical [12,13] systems. Notably, many recent reports describe the ability of ionic liquids to modulate inter- and intramolecular interactions of small molecules [14–16].

The microenvironment surrounding a protein can greatly effect its folded state (including secondary and tertiary structure), stability, and function. Ionic liquids offer unique environments that can be tuned to alter the structural and biophysical properties of biomacromolecules. Thus, understanding the effect of ionic liquids on the native structure of biomacromolecules is a critical step in the advancement of many areas, including enzymology, biocatalysis, and bioengineering.

While it is evident that ionic liquids can alter the stability and function of proteins, the current mechanistic understanding of protein stability and enzyme activity in ionic liquid-rich environments requires clarification. For example, some ionic liquids were noted to increase the stability of proteins by serving as an anti-aggregation/unfolding media for lysozyme over an extended period of time [17]. However, it was also shown that certain proteins, for example the redox active form of cytochrome *c*, could be denatured by imidazolium-based ionic liquids [18,19], yet choline-based ionic liquids were recently shown to improve the redox activity of cytochrome *c* [20]. While stability and enzymatic activity of a few proteins in ionic liquid-containing aqueous media correlate with the Hofmeister's series [21,22] others do not [23,24]. Using simulations, it was also suggested that ionic liquids could influence xylanase activity by disturbing the dynamic motion of the protein in addition to affecting protein structure [25].

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Protein function is intimately linked to structure and dynamics, thus a molecular-based understanding of ionic liquid-protein interactions is vital for developing efficient applications. Recent studies [26,27] have demonstrated that solution NMR spectroscopy can be utilized to probe direct interactions between ionic liquids and proteins using NMR chemical shift perturbations, which are sensitive reporters of changes in the chemical environment, including protein structural changes. Kaar and coworkers [27] demonstrated interactions between [C_4-mim]Cl and lipase A at up to 0.29 M (*i.e.*, 5% v/v) ionic liquid, and Cabrita and coworkers [26] probed the interaction of various ionic liquids (up to 1 M concentration) with the protein Im. The results from these groups established that chemical shift perturbations in the 2D ^{1}H-^{15}N HSQC spectra could be used to monitor ionic liquid-induced structural changes in the proteins. It was suggested that both electrostatic as well as hydrophobic interactions occurred between proteins and ionic liquids, as a number of charged and nonpolar residues experienced chemical shift perturbations.

Although traditional solution state NMR techniques are applicable for relatively low ($\leq$ 1 M) concentrations of ionic liquids [26,27], the viscosity of the aqueous ionic liquid solutions above concentrations of 1 M are high enough to slow the tumbling of most proteins, thus sufficiently broadening NMR signals and limiting the use of solution state NMR. Importantly, several industrial and biomedical applications require high concentrations of ionic liquids. For example, ionic liquid-based pretreatment of biomass is being explored for removing lignin and hemicellulose under milder conditions than conventional acid or steam pretreatments [28], however the concentration of ionic liquids are fairly high (above 20% v/v) [28,29]. Another significant application of ionic liquids is to extend the life and quality of protein storage and formulation in the pharmaceutical industry; in these cases even higher concentrations of ionic liquids have been suggested for maintaining long-term maximal protein stability [17,30,31]. Thus, in order to probe ionic liquid–protein interactions at high ionic liquid concentration, pertinent to industrial applications, alternative NMR approaches that are not limited by slow tumbling are required.

High-resolution magic-angle-spinning (HR-MAS) NMR is particularly applicable for analyzing viscous or semi-solid samples using solution NMR methods while spinning at the magic angle in order to remove line-broadening effects. HR-MAS NMR has been shown to be a useful tool in monitoring chemical reactions [32] and in establishing the structure of small (e.g., $\leq$ 5 amino acids) peptides [33,34] in ionic liquids. HR-MAS reduces the line broadening caused by differences in magnetic susceptibly of the sample and also decreases the dipolar interaction and chemical shift anisotropy, although these effects are less significant in heterogeneous quasi-liquid samples [35].

Here, we demonstrate, for the first time, that a protein with $>$ 50 residues could be efficiently studied at atomic scale resolution in solution with high concentrations of ionic liquids using HR-MAS NMR. Specifically, structural and dynamical changes of the model 56-residue protein, immunoglobulin binding domain B1 of streptococcal protein G (GB1) [36] induced by a high concentration of [C_4-mim]Br (up to 3.5 M, which corresponds to 60%, v/v) were monitored by using 2D ^{1}H-^{15}N HSQC, 3D HNCA, and ^{15}N relaxation spectra of GB1. Significantly, the use of HR-MAS NMR spectroscopy surmounted the problem of line broadening due to the high viscosity of the ionic liquid-containing systems, and thus this technique could provide unique and precise information about site-specific ionic liquid–protein interactions. Arguably, this work provides an important foundation for probing protein secondary structure in ionic liquid-rich media.

2. Experimental

[C_4-mim]Br [37,38] and GB1 [39] were prepared as previously described. NMR samples were prepared by mixing a 4.4 mM GB1 stock solution in buffer (50 mM sodium phosphate, pH 5.50), D_2O, and neat [C_4-mim]Br in a pre-determined ratio to a 1.3 mM or 0.9 mM concentration of GB1 and a 10–50% v/v or 60% v/v final concentration of [C_4-mim]Br, respectively. Other samples included GB1 in 50% v/v glycerol/aqueous solution and 1.3 mM GB1 in the presence of 2.3 M KBr. A control GB1 sample was prepared without the addition of [C_4-mim]Br, KBr, or glycerol (referred to in the text as 0% v/v [C_4-mim]Br sample). HR-MAS NMR spectra were acquired on a 600 MHz Avance III Bruker NMR spectrometer equipped with a 4 mm HR-MAS probe at 27 °C and 5 kHz MAS frequency. Additional details are given in the SI.

3. Results and discussion

3.1. Selection of protein, ionic liquid, and experimental method

We probed the effects of [C_4-mim]Br on the protein GB1 to gain a better understanding of how ionic liquids impact protein structure, stability, and dynamics. The GB1 structure, folding pathway, and dynamics have been previously well characterized by NMR spectroscopy [40–44], which makes it an ideal model protein for assessment by HR-MAS spectroscopy. GB1 is a 56-residue stable protein comprised of one α-helix packed against a four-stranded β-sheet. 1-butyl-3-methylimidazolium bromide, [C_4-mim]Br, was chosen as a model ionic liquid, as it is a common, readily available, water-soluble, and widely used ionic liquid.

GB1 samples were prepared with varying concentrations of [C_4-mim]Br in NMR buffer: 0%, 10% (0.59 M), 25% (1.47 M), 40% (2.36 M), 50% (2.95 M), and 60% v/v (3.53 M) [C_4-mim]Br. To test the effects of high viscosity and high salt concentration media, control samples were also prepared in NMR buffer: GB1 in the presence of 50% glycerol and in the presence of 2.29 M KBr. A table describing the composition of each sample is given in Table S1. The HR-MAS NMR probe was configured for MAS and with a Z-axis gradient aligned along the magic angle to provide access to a wide range of solution NMR experiments. Significantly, using an HR-MAS probe allowed us to use deuterium lock and solvent suppression of the water signal using standard solution NMR pulse sequences. Although MAS induces large pressure especially on the sample near the inner wall of rotor, the low spinning frequency of 5 kHz in a 4 mm rotor did not destabilize GB1. A concentrated aqueous solution of GB1 was mixed with neat [C_4-mim]Br and D_2O, thus the ionic liquid concentration was limited by the minimum volumes of D_2O and protein solution. We acquired 1D ^{1}H, 1D ^{13}C, 2D ^{1}H-^{15}N HSQC, 3D HNCA, and ^{15}N relaxation spectra for GB1 utilizing HRMAS NMR. Although the VT inlet temperature was 27 °C under HRMAS condition, we estimated that the actual sample temperature was approximately 30–31 °C (due mostly to frictional heating) based on comparison of GB1 2D ^{1}H-^{15}N HSQC spectra under HR-MAS conditions to conventional solution GB1 2D ^{1}H-^{15}N HSQC spectra at various temperatures (Fig S1).

3.2. The effect of [C_4-mim]Br on the structure of GB1

1D ^{1}H spectra were not practical to detect changes in protein secondary structure due to the large excess of [C_4-mim]Br in solution (data not shown). Since GB1 was uniformly ^{15}N-, ^{13}C-enriched and [C_4-mim]Br was not, 1D ^{13}C spectra (Fig. S2) were utilized to monitor large changes in protein secondary structure in spite of the significant signal overlap, as discussed below. The [C_4-mim]Br signals were sharp, and their intensity