Analytical and Bioanalytical Chemistry (2019) 411:4929–4935
https://doi.org/10.1007/s00216-019-01804-3

RESEARCH PAPER

Protein stability analysis in ionic liquids by ^{19}F NMR

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Received: 22 November 2018 / Revised: 24 February 2019 / Accepted: 25 March 2019 / Published online: 24 April 2019

Abstract

Ionic liquids have been extensively used as environmentally friendly solvents for enzymatic reactions and other biological systems. Understanding the mechanism of how ionic liquids affect protein stability is crucial for the biological reaction processes and protein storage using ionic liquids as solvents. Although effects of ionic liquids on protein stability have been studied, equilibrium thermodynamics of protein stability in ionic liquids has not been quantitatively measured. Herein, we utilized ^{19}F NMR to measure the equilibrium thermodynamics of protein stability in ionic liquid [C_4-mim]Br. Our results show that proteins are significantly destabilized in [C_4-mim]Br ionic liquids. Our results suggest that ^{19}F NMR provides a simple and effective way to study the thermodynamics of protein stability in ionic liquids. ^{19}F NMR will be applicable to facilitate the protein–protein interaction study in ionic liquids.

Keywords Ionic liquids · Protein stability quantification · ^{19}F NMR

Introduction

Ionic liquids (ILs) are molten salts at room temperature because of low melting point. ILs have many distinctive properties such as low vapor pressure, high ionic conductivity, wide liquid range, high thermal stability, and low toxicity. They are able to dissolve a variety of solutes. During the last two decades, ILs have emerged as biocompatible solvents for organic synthesis, biocatalysis, and other biological systems [1–12]. They have been widely used in protein separation, extraction, and purification due to their high thermal stability and excellent biocompatibility [13–17]. To evaluate the effect of ILs on the biological reaction processes, the structure, stability, and activity of proteins in ILs have been extensively investigated [18–32].

Protein stability can be tuned by the environment surrounding it. The ILs offer perfect environments that can be tuned to alter the structure and physicochemical property of biomacromolecules. Understanding the mechanism how ILs affect the protein stability is crucial for the biological reaction processes taking place in ILs, including enzymatic reactions, bioengineering, protein extraction, and purification. Although ILs can alter protein stability and function, the mechanistic understanding of protein stability in ILs requires to be clarified. The effects of ILs on protein stability have been extensively studied [33–49]. For instance, some ILs were reported to increase the stability of proteins [33, 34, 37, 38, 40, 47, 48]. Some ILs were shown to destabilize proteins [39, 43, 45, 49]. However, other ILs were investigated to stabilize or destabilize proteins depending on the concentrations, species, polarity, and hydrophobicity of ILs [35, 36, 41, 42, 44, 46]. The interactions between proteins and ILs including electrostatic interactions and hydrophobic interactions may play a role in the effects of ILs on protein stability.

NMR spectroscopy has been utilized to investigate direct interaction between ILs and proteins through NMR chemical shift perturbations in 2D ^{1}H-^{15}N HSQC spectra [39, 40, 50]. Cabrita and coworkers showed that the preferential binding of ionic liquids with Im7 is crucial to modulate its stability in ILs [39]. Kaar and coworkers studied the direct ion interactions between protein and ILs and its effect on protein stability in ILs [40]. Varga and coworkers used HR-MAS NMR to probe the effects of [C_4-mim]Br ILs on GB1 structure and dynamics [50].

Published in the topical collection *Young Investigators in (Bio-)Analytical Chemistry* with guest editors Erin Baker, Kerstin Leopold, Francesco Ricci, and Wei Wang.

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Although the effects of ILs on protein stability have been extensively studied, few studies concentrated on the equilibrium thermodynamics of protein stability in ILs. Quantifying the enthalpic and entropic changes of protein contributes to a better understanding of protein equilibrium thermodynamics in ILs. Here, we use two small globular proteins KH1 (MW 9.4 kDa and pI 6.8) and SH3 (MW 6.9 kDa and pI 4.6), a domain of human K-homology splicing regulator protein and a domain of *Drosophila* signal transduction protein drk, respectively. We have utilized ^{19}F NMR to examine the effects of [C_4-mim]Br ILs on the equilibrium thermodynamics of KH1 and SH3 stability. ^{19}F is a nucleus with almost 100% natural abundance and its intrinsic sensitivity is approximately 83% of the proton. The large chemical shift range and no background interference of ^{19}F NMR make it an ideal method to probe and monitor the stability and conformational transitions of protein in complex systems [51, 52].

The modified standard state Gibbs free energy of protein unfolding, $\Delta G^{0'}_{u,T}$, equals $-RT\ln(K_u)$, where R is the gas constant, T is the absolute temperature, and K_u is the equilibrium constant (K_u = [unfolded]/[folded]). The $\Delta G^{0'}_{u,T}$ can be separated into enthalpic and entropic components.

$$\Delta G^{o'}_{u,T}(T) = \Delta H^{o'}_{u}(T) - T\,\Delta S^{o'}_{u}(T) \quad (1)$$

$\Delta H^{o'}_{u}(T)$ and $\Delta S^{o'}_{u}(T)$ are the temperature-dependent modified standard-state enthalpy and entropy of unfolding, respectively.

$$\Delta H^{o'}_{u}(T) = \Delta H^{o'}_{u}(T_{ref}) + \int_{T_{ref}}^{T} \Delta C^{o'}_{p}\, dT \quad (2)$$

$$\Delta S^{0'}_{u}(T) = \Delta S^{o'}_{u}(T_{ref}) + \int_{T_{ref}}^{T} \frac{\Delta C^{o'}_{p}}{T}\, dT \quad (3)$$

T_{ref} is a reference temperature and $\Delta C^{o'}_{p}$ is the modified standard-state heat capacity of unfolding. $\Delta C^{o'}_{p}$ is assumed to be temperature-independent over the range studied. Combining Eqs. 1–3 gives Eq. 4.

$$\Delta G^{o'}_{u}(T) = \Delta H^{o'}_{u}(T_{ref}) - T\,\Delta S^{o'}_{u}(T_{ref}) + \Delta C^{o'}_{p}\left[T - T_{ref} - T\ln\frac{T}{T_{ref}}\right] \quad (4)$$

The temperature dependences of $\Delta G^{0'}_{u,T}$ for globular proteins are bell-shaped with a maximum at T_s, where denaturation is purely enthalpic, and $\Delta S^{o'}_{u}$ equals zero [53], whereas T_m is the melting temperature, which is the point where the $\Delta G^{0'}_{u,T}$ equals zero.

Material and methods

Protein expression and purification

The plasmids encoding the his-tagged KH1 domain protein and the N-terminal SH3 domain of drk (SH3) were transformed into *Escherichia coli* BL21 (DE3) competent cells. Expressions of S193A KH1 and SH3 were based on the described protocols [54, 55]. For 3-^{19}F-tyrosine-labeled S193A KH1, 70 mg of D,L-m-fluorotyrosine, 60 mg of L-phenylalanine, 60 mg of L-tryptophan, and 0.5 g glyphosate were added into 1 L of M9 minimal medium [56]. The inducer isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression. Cells were induced for 20 h at 20 °C and harvested by centrifugation. For 5-^{19}F-tryptophan-labeled SH3, 60 mg 5-fluoroindole was added into 1 L of M9 minimal medium [57]. Cells were induced by 1 mM IPTG for 2 h at 37 °C and harvested by centrifugation.

For S193A KH1, the cell pellet was resuspended in Ni-column buffer A (50 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0) for sonication. The supernatant was collected after centrifugation at 16000*g* at 4 °C for 30 min. KH1 was purified as reported [54] using Ni-affinity chromatography then followed by size exclusion chromatography. For SH3, the cell pellet was resuspended in buffer (50 mM Tris, pH 7.5) and protease inhibitors were added for sonication. Purification of SH3 was accomplished as the described protocols [55], which involved two chromatography steps using an anion exchange chromatography followed by size exclusion chromatography. Purified S193A KH1 and SH3 were applied to desalting columns, lyophilized for 24 h, and stored at −20 °C.

Ionic liquids

[C_4-mim]Br was purchased from Aladdin Ltd. and used without further purification. Solutions of various concentrations of ILs were prepared by dissolving [C_4-mim]Br in phosphate buffer (50 mM sodium phosphate buffer, pH 7.5). The ILs were then used to dissolve the lyophilized protein.

NMR spectroscopy

^{19}F spectra were acquired between 283 and 331 K on Bruker 600 MHz and Bruker 500 MHz spectrometers equipped with 5-mm H/F (C, N) triple resonance cryoprobes. ^{19}F spectra were acquired with spectral widths of 30 ppm with a duty cycle delay of 2.0 s.

Stability measurements

Topspin 3.2 (Bruker), MestRe-C, and Origin8.0 were utilized to process and analyze data. MestRe-c was used to process the

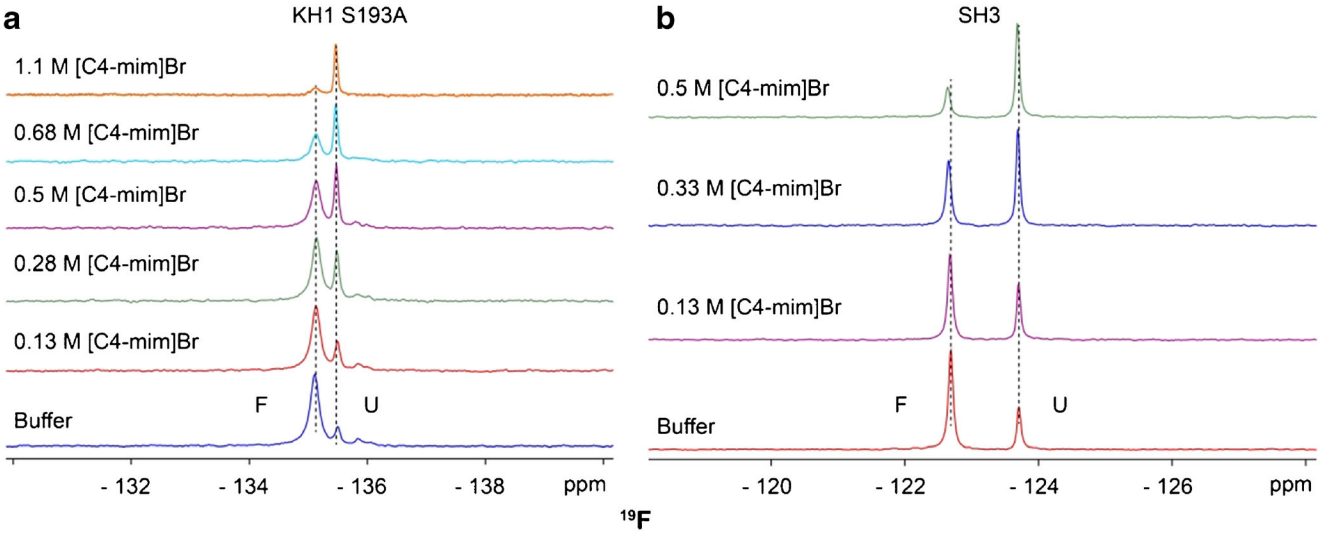

Fig. 1 Spectra of fluorine-labeled S193A KH1 (**a**) and SH3 (**b**) in buffer and various concentrations of [C_4-mim]Br ionic liquids at 298 K and 288 K, respectively

free induction decays and transform the decays into ASCII text files, which can be read by Origin8.0. Origin8.0 was applied to fit the peaks in ^{19}F spectra. Peak fitting and integration were accomplished by using Lorentzian functions in Origin8.0. Peak integrations were used to calculate the populations of folded state (F) and unfold ensembles (U). $\Delta G_u^{0'}$ was calculated from the integrals of the two peaks. Three measurements at one temperature were conducted to estimate the uncertainty expressed as the standard deviation (SD) of the mean. The uncertainties in $\Delta G_u^{0'}$ were utilized to calculate the uncertainties in T_S, $\Delta H_u^{0'}$ (T_s), T_m, $\Delta H_u^{0'}$ (T_m), and $\Delta C_p^{0'}$ via a weighted fit to Eq. 4.

Results and discussion

We labeled S193A KH1's sole tyrosine and SH3's sole tryptophan with fluorine. Fluorine labeling makes it easy to quantify the folded state (F) and unfolded ensemble (U) [55, 58]. ^{19}F NMR is a quantitative method with high sensitivity and no background interference. It is an ideal technique to probe protein stability and structure in complex environment [55]. The peak area under each ^{19}F resonance is proportional to its concentration. We acquired ^{19}F spectra (Fig. 1) of S193A KH1 and SH3 in buffer and various concentrations of ionic liquids at 298 K and 288 K, respectively. The ^{19}F spectrum of S193A KH1 in buffer at 298 K shows mainly one ^{19}F resonance,

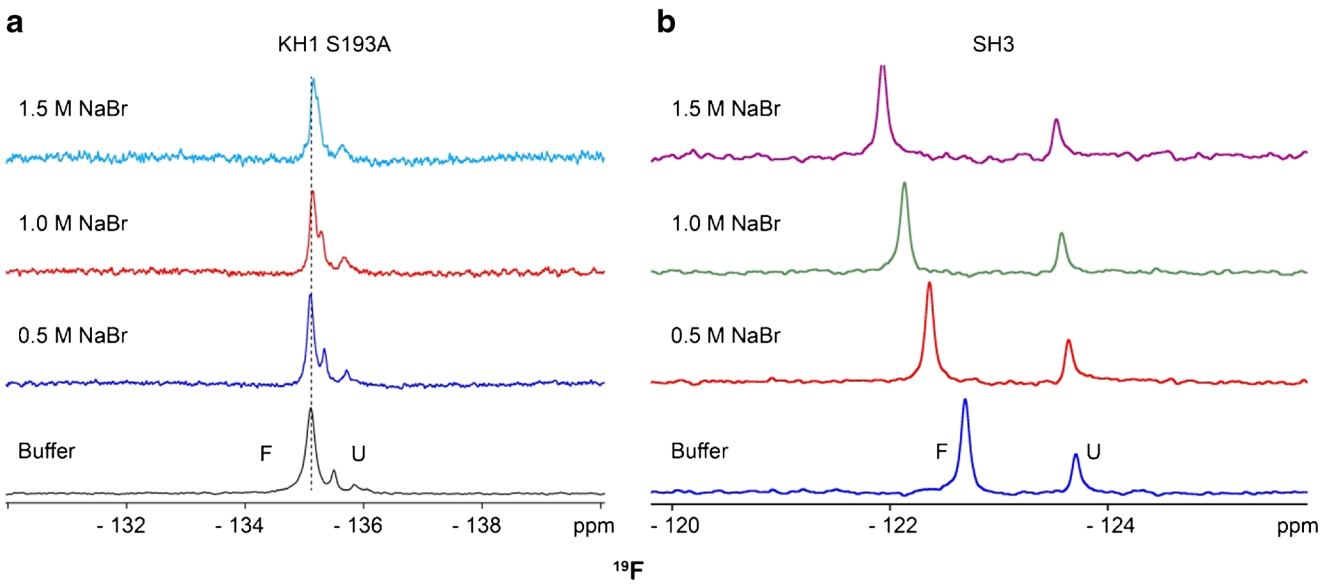

Fig. 2 Spectra of fluorine-labeled S193A KH1 (**a**) and SH3 (**b**) in buffer and various concentrations of NaBr solutions at 298 K and 283 K, respectively

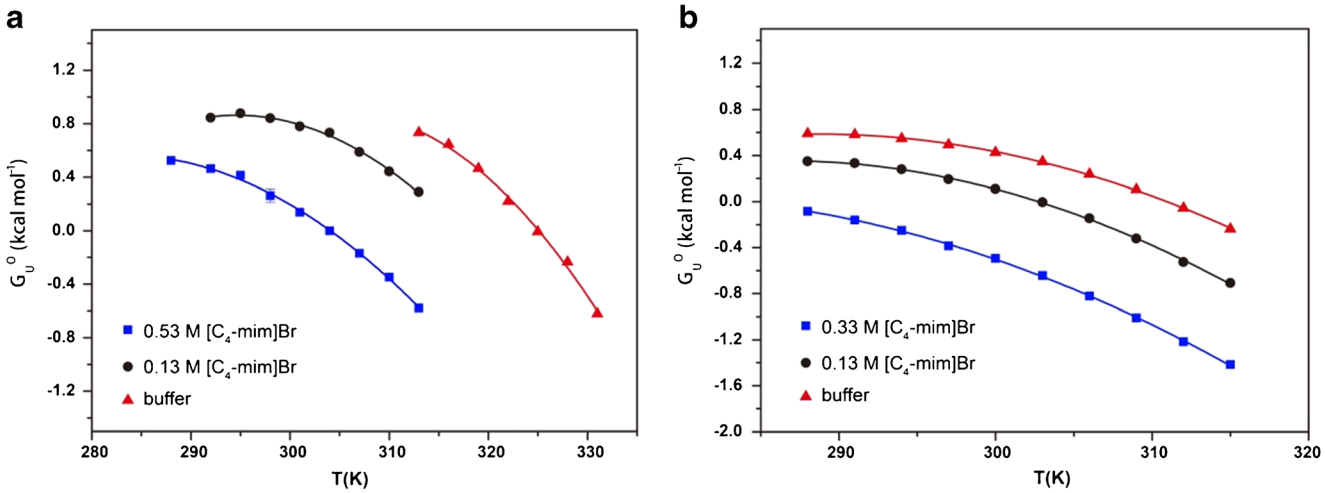

Fig. 3 Temperature dependence of S193A KH1 (**a**) and SH3 (**b**) stability in buffer and various concentrations of [C_4-mim]Br ionic liquids

which corresponds to the folded state (F). The ^{19}F spectrum of SH3 in buffer at 288 K shows two ^{19}F resonances, one from the folded state (F), the other from unfolded ensemble (U). The peak from U gradually increases as the concentrations of [C_4-mim]Br ILs are elevated for both S193A KH1 and SH3. These data indicate that S193A KH1 and SH3 are destabilized in ILs.

In order to assess the effect of ionic strengths on protein stability, we also acquired the ^{19}F spectra (Fig. 2) of S193A KH1 and SH3 in buffer and various concentrations of NaBr solutions at 298 K and 283 K, respectively. For S193A KH1, the peak intensity from U marginally increases as the concentrations of NaBr are elevated. The peak intensity from U in 0.5 M NaBr is significantly weaker than that in 0.5 M [C_4-mim]Br ILs. For SH3, the peak intensity from F marginally increases as the concentrations of NaBr are elevated, which means that SH3 are slightly stabilized in NaBr solutions. These results suggest that the effect of ionic strengths on protein stability does not dominate in the destabilizations of proteins by [C_4-mim]Br ILs.

To investigate the equilibrium thermodynamics of protein stability in ILs, we measured the temperature dependence of S193A KH1 and SH3 stability (Fig. 3) in buffer alone, 0.13 M, 0.33 M, and 0.53 M [C_4-mim]Br IL. The $\Delta G_u^{o'}$ values of S193A KH1 and SH3 in [C_4-mim]Br ILs are smaller than those in buffer, which means that the [C_4-mim]Br ILs destabilized S193A KH1 and SH3.

Equation 4 was applied to extract values of T_m, $\Delta H_u^{0'}$ (T_m), T_s, $\Delta H_u^{0'}$ (T_s), and $\Delta C_p^{0'}$ (Tables 1 and 2). T_m values were used to assess thermal stability. T_m of S193A KH1 and SH3 in 0.13 M [C_4-mim]Br ILs were 8.1 K and 8.3 K lower than those in buffer alone, respectively, which means proteins are significantly destabilized in ILs.

To quantify the enthalpic and entropic components, we assessed $\Delta H_u^{0'}$ and $-T\Delta S_u^{0'}$ at 308 K (Tables 3 and 4). For

Table 1 Thermodynamics parameters for S193A KH1 in buffer and ionic liquids

S193A	T_m, K	$\Delta H_u^{0'}$ (T_m), kcal/mol	T_s, K	$\Delta H_u^{0'}$ (T_s), kcal/mol	$\Delta C_p^{0'}$, kcal/mol/K
Buffer, pH 7.5	325.1 ± 0.2	29 ± 1	305.6 ± 2.8	0.87 ± 0.09	1.4 ± 0.2
0.13 M [C_4-mim]Br	317.0 ± 0.4	24 ± 1	294.6 ± 0.7	0.86 ± 0.01	1.0 ± 0.1
0.53 M [C_4-mim]Br	304.1 ± 0.2	16 ± 1	283.1 ± 1.4	0.56 ± 0.03	0.7 ± 0.1

Table 2 Thermodynamics parameters for SH3 in buffer and ionic liquids

SH3	T_m, K	$\Delta H_u^{0'}$ (T_m), kcal/mol	T_s, K	$\Delta H_u^{0'}$ (T_s), kcal/mol	$\Delta C_p^{0'}$, kcal/mol/K
Buffer, pH 7.5	311.0 ± 0.1	16.1 ± 0.1	288.7 ± 0.4	0.59 ± 0.01	0.70 ± 0.02
0.13 M [C_4-mim]Br	302.7 ± 0.1	13.1 ± 0.1	286.4 ± 0.6	0.35 ± 0.01	0.78 ± 0.03
0.33 M [C_4-mim]Br	283.5 ± 1.1	3.8 ± 1.1	277.1 ± 1.1	0.04 ± 0.02	0.59 ± 0.03

Table 3 Thermodynamics parameters for S193A KH1 in buffer and ionic liquids at 308 K

S193A	$\Delta H_u^{0'}$, kcal/mol	$-T\Delta S_u^{0'}$, kcal/mol
Buffer, pH 7.5	4.3 ± 3.6	− 3.4 ± 3.6
0.13 M [C_4-mim]Br	14.7 ± 0.5	− 14.2 ± 0.5
0.53 M [C_4-mim]Br	19.1 ± 0.5	− 19.4 ± 0.5

both S193A KH1 and SH3, the $\Delta H_u^{0'}$ values in ionic liquids are larger than those in buffer, showing that protein is enthalpically stabilized in ILs. The $-T\Delta S_u^{0'}$ values in ILs are smaller than those in buffer, indicating that protein is entropically destabilized in ILs. However, the enthalpic stabilization does not completely compensate the entropic destabilization in ILs, which contributes to an overall destabilization. The enthalpic stabilization and entropic destabilization of protein in ILs is consistent with the effect of trimethylamine *N*-oxide (TMAO) and other protective osmolytes on protein enthalpic and entropic changes [59–61]. However, the enthalpic stabilization compensates the entropic destabilization in TMAO and other protective osmolytes, which accounts for overall stabilization of protein in TMAO and other protective osmolytes.

The stability difference of SH3 in [C_4-mim]Br IL solutions and NaBr solutions indicates that the [C_4-mim]$^+$ dominates in the destabilization of proteins by ILs. At pH 7.5, both S193A KH1 (pI 6.8) and SH3 (pI 4.6) are negatively charged. The imidazolium cation will accumulate near the negatively charged residues of proteins. Cabrita and coworkers confirmed that the imidazolium cation would strongly bind to the non-polar amino acids [39]. The hydrophobic interactions between the [C_4-mim]$^+$ and protein hydrophobic residues will lead to the exposure of protein hydrophobic surface, which finally facilitates protein unfolding. Previous studies have demonstrated that the stability of protein in aqueous IL solutions does not necessarily obey the Hofmeister series [62]. Our data suggests that protein stability in ILs is the result of balance between hydrophobic interactions, electrostatic interactions, and hydration.

^{19}F NMR is a quantitative method to study the protein stability in ILs. The equilibrium thermodynamics of protein folding and unfolding in ILs can be readily investigated by ^{19}F NMR. Previous studies have shown that 2D ^{1}H-^{15}N HSQC spectra can be utilized to study the hydrophobic and electrostatic interaction between proteins and ILs through chemical shift perturbations. The combination of ^{19}F NMR with 2D ^{1}H-^{15}N HSQC spectra will give a quantitative analysis on how the interactions between proteins and ILs affect the protein stability

Table 4 Thermodynamics parameters for SH3 in buffer and ionic liquids at 308 K

SH3	$\Delta H_u^{0'}$, kcal/mol	$-T\Delta S_u^{0'}$, kcal/mol
Buffer, pH 7.5	14.1 ± 0.2	− 14.0 ± 0.2
0.13 M [C_4-mim]Br	17.1 ± 0.3	− 17.4 ± 0.3
0.33 M [C_4-mim]Br	18.3 ± 0.2	− 19.3 ± 0.2

Conclusions

For the first time, we used ^{19}F NMR to study the effects of [C_4-mim]Br ILs on equilibrium thermodynamics of protein. Two small globular proteins, S193A KH1 and SH3, are destabilized in ILs. Proteins are enthalpically stabilized and entropically destabilized in ILs compared to buffer. The enthalpic stabilization by ILs is counterbalanced by entropically destabilization, which leads to an overall destabilization. The preferential interactions between proteins and ILs are not strong enough to stabilize protein. Our results suggest that the preferential interactions between proteins and ILs are crucial to modulate protein stabilization in ILs. ^{19}F NMR provides a simple and convenient approach to quantitatively analysis of protein stability in ILs. Compared to other analysis methods such as CD spectroscopy, fluorescence, UV-vis and FT-IR spectra, ^{19}F NMR is able to study the structure and function of protein at atomic resolution with low background and facilitates the protein–protein interaction study in ILs.

Funding information This work is supported by Ministry of Science and Technology of China grant 2017YFA0505400, Innovation team of Hubei Province grant 2016CFA002, National Natural Sciences Foundation of China grant 21575156, and K.C. Wong Education Foundation and Chinese Academy of Sciences (QYZDJ-SSW-SLH027).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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