Chapter 4

The Role of Ionic Liquids in Protein Folding/Unfolding Studies

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Abstract

Ionic liquids (ILs) have emerged as novel solvent medium for several biotechnological processes *in vitro*. The use of ILs starts from protein extraction to catalysis to folding/unfolding studies. ILs are becoming the most favorite non-aqueous medium for protein studies due to their unique ionic combinations (cation + anion) and tunable physical properties. In this context, several research results have been published that use of pure or aqueous IL solutions as stabilizer for proteins. Hence, herein, in this chapter, we present a collection of research work that focuses on the importance of ILs (and their mixture) in protein stabilities. In addition, we have also reviewed the unique properties of ILs as counteracting solvents for cold-induced denaturation and also their refolding properties. This report will definitely generate a new understanding for the ILs, their importance and applicability in protein folding studies.

Keywords: ionic liquids (ILs), proteins, stability, biocompatibility, counteraction

1. Overview of the structure and importance of ILs

In recent years, various solvents have been used for numerous processes in academia and industries. Nevertheless, because of new environmental regulations, the challenges of using non-harmful solvents have prompted a great development of innovative products [1, 2]. In this regard, ionic liquids (ILs) emerged as new and novel class of solvents that are now considered to reduce both economic and environmental pollution [2–4]. The term ILs describe a popular class of organic salts that melt below ~100°C and have an appreciable liquid range [1–4]. IL is entirely composed of positive and negative ions [5, 6]. ILs typically consist of organic nitrogen-containing heterocyclic cations and inorganic anions [7]. Historically, the organic compound

that is now considered as the first IL is ethylammonium nitrate ($[C_2H_5NH_3][NO_3]$, EAN) and was prepared by Paul Walden in 1914 and has a melting point of 12°C [8]. After a long gap, the major studies of room temperature molten salts of pyridinium halides with aluminum chloride ($AlCl_3$) were made in the 1940s by a group led by Frank Hurley and Tom Weir at Rice University [9]. Later, alkylimidazolium salts $(C_nmim)^+$ were also reported in the early 1980s [10]. An excellent short history of the birth of ILs, which covers the crucial moments of this area, is presented by John S. Wilkes [11].

In its initial revolutionary stage, ILs were vastly considered in analytical chemistry based on their unique and tunable physical properties. Since then, synthesis of a large number of multifunctional ILs has been a prime interest for synthetic chemists [12, 13]. In this context, varieties of task-specific ILs have been synthesized and the advantages of their physical properties have been reported in an open literature [14]. Common ILs include ammonium, phosphonium, sulfonium, guanidinium, pyridinium, imidazolium and pyrrolidinium cations. The most common anions are chloride, bromide, tetrafluoroborate, hexafluorophosphate, trifluoromethanesulfonyl, bis(trifluoromethanesulfonyl)imide, dicyanamide and alkyl sulfate anions. These ILs have some unique properties such as negligible vapor pressure, good thermal stability, tunable viscosity and miscibility with water, a wide electrochemical window, high conductivity and high heat capacity [15–17]. These physical properties make IL a promising material in numerous fields, for example, their use in electrochemical devices and replacements for several organic reactions [18, 19]. Gordon [20], Parvulescu and Hardacre [21] and Crowhurst et al. [22] pointed out that there is an obvious advantage in performing many reactions in ILs due to the improvement in reaction activity, selectivity and yield. An in-depth literature survey reveals that there tremendously exist a large number of scholarly articles as well as elegant reviews that explicitly elucidate the various scientific applications of ILs [23–45].

2. Classification of ILs

ILs are composed solely of ions and their bulk and interfacial behavior is complex, governed by Coulombic, van der Waals, dipole-dipole, hydrogen-bonding and solvophobic forces [22, 46]. When an IL is formed by mixing a strong acid with a strong base, the proton is generally assumed to be located very strongly on the base. In this situation, the IL is most likely composed entirely of ions; however, ion complexation and aggregate formation may also occur [47]. A majority of the ILs with various combinations of the cation as well as the anions have been classified as protic ILs (PILs) and aprotic ILs (APILs) based on their respective physical properties to protonate/deprotonate in aqueous media [48]. The reason for this distinction is that PILs are volatile by their nature because the acidic proton can be abstracted by the basic anion at ambient temperature. The acid-base equilibrium for the abstraction reaction allows the formation of neutral molecular species that readily evaporate [49].

The potential environmental impact of PILs is expected to be smaller than the impact of APILs, due to their simpler structure. These PILs can be easily produced through the combination of a Brønsted base and a Brønsted acid [36]. A comparison with the APILs reveals that PILs often have higher conductivities and fluidities than the APILs. On the other hand, in PILs, the sizes

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of the ions are small and they also tend to melt at lower temperatures than their APIL analogs. The obvious difference of the PILs compared with APILs is the reversible hydrogen transfer between the acid and the base [50, 51]. This implies that for PILs where the transfer is weak, the properties are closer to the corresponding binary liquid, whereas the aprotic ILs keep their ionic character until decomposition [52].

3. Toxicity in ILs and its environmental impact

ILs in green chemistry mean that it should be applied to all the aspects of the product life cycle that begins from its invention to the disposal. Broadly speaking, it should be recycled easily from the environment [53]. Tremendous amount of recent research has focused on the physical properties of ILs and more recently relationship and cross-linking between the chemical properties, the toxicity and biological properties of ILs have been one of the most highly debated topics in this field [31, 54]. Pham et al. have reviewed the toxic effect of ILs on the environment and biological systems in a comprehensive way [55]. It was shown that with increasing hydrophobicity of the cation, the IL gets more toxic [56]. Literature reveals that increased alkyl chain length in the cation of ILs showed higher toxicities on biological systems [57].

4. Protein stability in the presence of ILs

The advantages of using ILs in enzymatic biocatalysis, as compared to volatile organic compounds (VOCs), are the enhancement in the solubility of substrates or products without inactivation of the enzymes, high conversion rates and high activity and stability [35, 53]. These unique properties of ILs make them very attractive nonaqueous solvents for protein stability studies. It is revealed from various studies that physicochemical properties of ILs can play a pivotal role in altering the structure, stability and activity of proteins/enzymes [44]. Moreover, ILs offer new possibilities of application of solvent engineering to enzymatic reactions. Biocatalysis with ILs as reaction medium was first showed in the beginning of 2000 [58–60]. Review on ILs as cosolvents in aqueous biocatalytic reactions reveals that these ILs help to dissolve nonpolar substrates while avoiding enzyme inactivation like water-miscible organic solvents, as dimethyl sulfoxide (DMSO) or acetonitrile [61]. During the last decade, ILs have increased their attention as reaction media for enzymes in aqueous media with some remarkable results [62, 63].

In the present situation, the stability of proteins in ILs has been an area for active research because of their biological and pharmaceutical applications. The first report on the protein stability in the presence of ILs came in the year 2000 by Summers and Flowers [64]. Later, in 2004, Iborra and coworkers [65] studied the stabilizing ability of 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide [Emim][NTf_2] on α-chymotrypsin (CT) and the stability of CT in this IL was compared with water, 3 M sorbitol and 1-propanol. Subsequently, lots of works have been reported on the stability of various proteins in various ILs. Among all the solvents, the IL was found to be a strong stabilizer for CT structure than with other solvent

media [65]. Studies carried out by various groups using CT suggest that the physical properties of ILs such as polarity and hydrophobicity play a major role in their stabilizing behavior toward CT [18, 52, 53]. Among various families of ILs, ammonium-based ILs are identified to display their wide range application in biochemical processes [44].

Many key studies related to ammonium-based IL interactions with lysozyme have been carried out, focusing on the role of these ILs. A study by Summers and Flowers [64] on lysozyme showed that ethylammonium nitrate (EAN) stabilized the lysozyme against irreversible thermal denaturation. Byrne et al. [66] reported the thermal refolding as well as extended period stabilization of lysozyme with concentration >200 mg/mL. To achieve the refolded fraction of the protein, EAN was used. Triethylammonium methanesulfonate [TEA][MS] was able to refold 97% of thermally denatured lysozyme [66]. Mann et al. [67] observed that ammonium-based ILs such as ethylammonium formate (EAF), propylammonium formate (PAF), 2-methoxyethylammonium formate (MOEAF) and ethanolammonium formate (EtAF) not only acted as good stabilizers for the lysozyme native structure but also protected the protein against thermal unfolding. Ammonium-based ILs such as EAN, triethylammonium triflate (TEATF) and triethylammonium mesylate (TEAMS) were observed to be acting as solvents for solubilizing the aggregates (amyloids) of denatured lysozyme structure. Interestingly, after solubilization in the ILs, the activity of aggregated lysozyme was observed to reappear up to 80% in the presence of EAN and more than 50% in the rest of the ILs [68]. Many studies, related to the role of ammonium-based ILs on lysozyme, suggest the role of ammonium family ILs as a refolding additive, fibrillizing agent, precipitating agent, additives for protein crystallization, prevention of aggregation and renaturing agent, as well as stabilizers against thermal unfolding [66–70]. Also, based on the above experimental results, EAN can be termed as a refolding additive, from the thermally as well as chemically denatured lysozyme. Furthermore, talking particularly about the cations having various hydrogen bond donor sites result in more effective coordination to the protein, thereby stabilizing the biomolecule structure in a more efficient manner. The stability of lysozyme in imidazolium-based ILs is an interesting aspect that helps us to understand the interactions that are responsible for stabilizing/destabilizing the protein structure in various ILs. The thermal stability of lysozyme crystals was obtained using imidazolium-based ILs such as 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), 1-butyl-3-methylimidazolium bromide ([Bmim][Br]) and 1,3-dimethylimidazolium iodine([Mmim][I]), as additives during lysozyme crystallization [71].

The stability of lysozyme in imidazolium-based IL is observed to vary with the concentrations of the IL. In this regard, Takekiyo et al. [72] observed the structural change of lysozyme in aqueous 1-butyl-3-methylimidazolium nitrate ([Bmim][NO_3]) solutions by using Fourier transform infrared (FTIR), circular dichroism (CD) spectra and small-angle X-ray scattering (SAXS) methods. The results illustrated that the structure of the protein significantly varied with changes in the structure and concentration of the ILs. In the first view, the authors observed that the increase in the [Bmim][NO_3] concentration completely disrupted the tertiary structure of lysozyme at 5 M of IL. Lange et al. [73] reported that the imidazolium-based ILs can also be considered as refolding agents. They tested the refolding of lysozyme in the set of imidazolium ILs, $[Emim]^+$, $[Bmim]^+$ and $[Hexmim]^+$ cations with a fixed anion Cl^-.

Interestingly, the stability of lysozyme in imidazolium-based ILs was observed to decrease significantly as the alkyl chain of the ILs increased from ethyl to butyl to hexyl. However, all ILs acted as refolding enhancers for the completely denatured lysozyme structure.

Interestingly, experimental results reveal that the partitioning of bovine serum albumin (BSA) is predominated by the hydrophobic interactions between the protein and imidazolium cation of the ILs in the aqueous system, which can be improved or modulated by changing the cation chain length of the ILs or modifying the surface of the BSA [74, 75]. The study indicated that the secondary structure of BSA was stabilized at low IL concentration (<3 mM) and the protein was denatured at higher IL concentration (>3 mM) [76]. Rawat and Bohidar [77, 78] reported that the interaction between imidazolium-based ILs and BSA is obviously dependent on the concentrations of the ILs. They observed that the BSA retained the secondary structure at low concentrations of 1-octyl-3-methylimidazolium chloride ([C_8mim][Cl]) of 0–0.2% w/v. Moving above this concentration range, the BSA structure was denatured significantly that is most likely caused due to intercalation of alkyl chain of the imidazolium cation of [C_8mim][Cl] IL into the hydrophobic interior of the protein [77].

5. Structure and stability of some other proteins in different ILs

In the earlier sections, the protein stabilization studies of most commonly used proteins have been delineated. Even though, the literature is still scattered, which deals with the stability of proteins in the presence of ILs. Hence, in this section, we have tried together all these research reports [79–114] under the same roof, so that it is easily available to the readers and also to expose its importance to the scientific world. An overview of the literature reveals stability studies of proteins such as amyloglucosidase [79], thyroglobulin [79], glutamate dehydrogenase [79], lactate dehydrogenase [79], glucose dehydrogenase [80], formate dehydrogenase [94], glycosidase (α and β) [81], monellin [82], β-galactosidase [83], glucose oxidase [106], lactate oxidase [109], oxidoreductases [107], subtilisin Carlsberg [84], Amano protease P6 [85], pepsin [86], papain [87, 100] esterases from *Bacillus subtilis* and *Bacillus stearothermophilus* [87], *Penicillium expansum* lipase [88], mushroom tyrosinase [88], chloroperoxidase [89], porcine pancreatic lipase [91], α-helical protein Im7 [92], pepsin [93], adenosine deaminase [95], α-amylases [96], xylanase II (GH11 enzyme, from *Trichoderma longibrachiatum* [97], lactoferrin [99], α-synuclein [101, 103], ribonuclease A [102], casein [104, 105], epoxide hydrolase [110], avidin [111], Abeta [1–40] peptide [112], zein [113] and firefly luciferase (*Photinus pyralis* luciferase) in the ILs. Among them some of the proteins have been stabilized [79–91], while some other proteins have shown to be destabilized in the presence of ILs [92–97].

Apart from the studies related to protein folding/unfolding, there are other research articles which have recognized the use of ILs as two-phase systems which are used for protein preservation, protein separation, purification, partitioning of proteins and many more purposes. For example, the extraction efficiency of papain was increased to 98.3% in the biphasic mixtures containing ILs [100]. On the other hand, Ebrahimi et al. [114] delineated the activity and stability of *P. pyralis* luciferase in two tetramethylguanidine-based [TMG][Lac] and [TMG][Pro]. The authors found that the luciferase activity increased up to 0.25 M of [TMG][Lac]

conversely the activity diminished in the presence of similar concentrations of [TMG][Pro]. Further, thermal stability studies show more stability of luciferase only in the presence of [TMG][Lac], whereas thermal stability was not improved in [TMG][Pro] [114]. Similar effects were observed in the stability of insulin in the presence of a set of imidazolium-based ILs such as [Bmim][Cl], [Bmim][Br], 1-butyl-3-methylimidazolium thiocyanate ([Bmim][SCN]), 1-butyl-3-methylimidazolium hydrogen sulfate ([Bmim][HSO_4]), 1-butyl-3-methylimidazolium acetate ([Bmim][CH_3COO]) and 1-butyl-3-methylimidazolium iodide ([Bmim][I]) [115]. The experimental findings reveal that [Bmim][Br] and [Bmim][Cl] ILs stabilized the native state of insulin, while the rest of the [Bmim] ILs with anions such as SCN^-, HSO_4^-, CH_3COO^- and I^- were destabilizers for the native form of insulin. Moreover, imidazolium-based ILs were also found to enhance the aggregated structure in insulin [115]. In support, Bae [101] and Hwang et al. [103] investigated the effect of imidazolium-based ILs on the aggregation properties of α-synuclein. Their results indicated the increase in the aggregated structure of the protein due to the ILs [101, 103].

6. Influence of mixture of ILs on the structure and stability of proteins

It is now obvious after examining the effect of various ILs (from different families of ILs) that some of the industrially important ILs acted as destabilizers for the proteins. Therefore, the search to offset the negative effects of ILs on proteins came into limelight. Therefore, the maintenance of ILs as the "green solvent medium" is a great challenge for a chemists or biochemists [116]. There are reports available in the literature that projects the negative effects of the ILs on the proteins. Klähn et al. [117, 118] reported the destabilization of *Candida antarctica* lipase B (CALB) in imidazolium- or guanidinium-based ILs through MD simulations. Further, very recently our research group has shown the destabilization of *heme* proteins in the presence of ammonium-based ILs [119]. We stress that all these results can be considered as an alarm for a chemist and biochemist to search for the novel method of counteraction for the denaturation action of ILs on the biomolecules.

In this context, the mixtures of ILs have been of continuing interest, of great fundamental practical importance and increasingly received a lot of attention from both academia and industry [120–123]. Recently, mixtures of ILs exhibit interesting and increased scope to the access of the properties, which are not readily apparent from those of the individual IL. Keeping this in mind, Lozano and coworkers [124] showed that [Bmim][Cl] behaved as a powerful enzyme-deactivating agent for cellulase. On the other hand, hydrophobic IL butyltrimethylammonium bis(trifluoromethylsulfonyl)imide ([N_{1114}][NTf_2]) clearly enhanced the enzyme thermal stability. Apparently, the mixture of [N_{1114}][NTf_2] and [Bmim][Cl] greatly improved the thermal stability of cellulase with respect to [Bmim][Cl] alone. By increasing the hydrophobic IL concentration, the deactivation effect of [Bmim][Cl] was reduced, which could be attributed to the preservation of the essential water molecules around the protein [124].

Similarly, the highest hydrolytic activity of immobilized lipase was obtained when the hydrophilic IL, 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF_4]), was used as an additive, while the highest stability of immobilized lipase was obtained by using hydrophobic IL,

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[C_{16}mim][Tf_2N] [125]. When Lee et al. [125] used the binary mixtures of these two ILs as additives, the optimal immobilized lipase showed both high activity and stability. The hydrolysis and esterification activities of lipase co-immobilized with the mixture of 1:1 at molar ratio of [Emim][BF_4] and [C_{16}mim][Tf_2N] were 10-fold and 14-fold greater than in silica gel without ILs, respectively. Therefore, the binary mixtures of these ILs as additives were used to obtain the optimal immobilized lipase which shows both high activity and stability [125].

Very recently, our group also showed the influence of mixture of two ILs on the structure and the stability of CT [126]. Evidently, the fluorescence and CD spectral results demonstrated that [Bmim][Br] alone acts as a stabilizer at low concentrations, while it acts as a destabilizer at high concentrations for the native structure of CT. On the other hand, [Bmim][I] is a destabilizer at all the concentrations. Nevertheless, the denaturing ability of [Bmim][I] was compensated by the [Bmim][Br]. Further, to offset the action of [Bmim][Br] on deleterious action of [Bmim][I] is more pronounced at lower concentration (0.025 M) than at higher concentrations. As shown in **Figure 1**, the fluorescence intensity maximum (I_{max}) for CT in buffer was 73.6 a.u., in 0.025 M [Bmim][Br] was 71.5 a.u. and in 0.2 M [Bmim][I] was 39.7 a.u. [126].

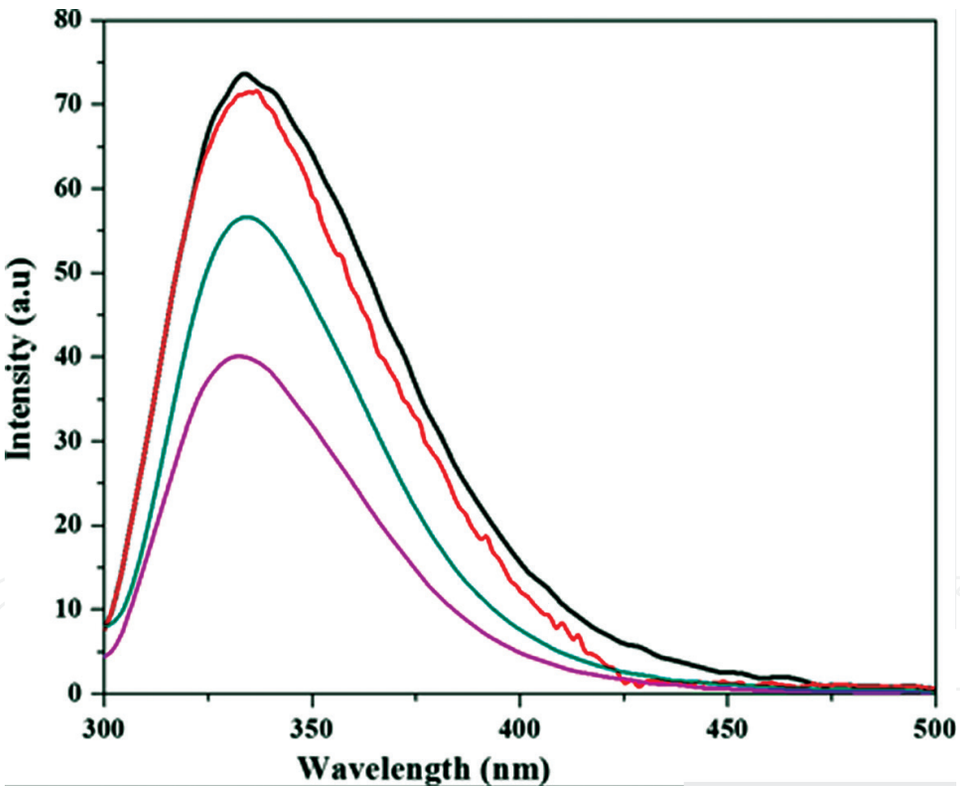
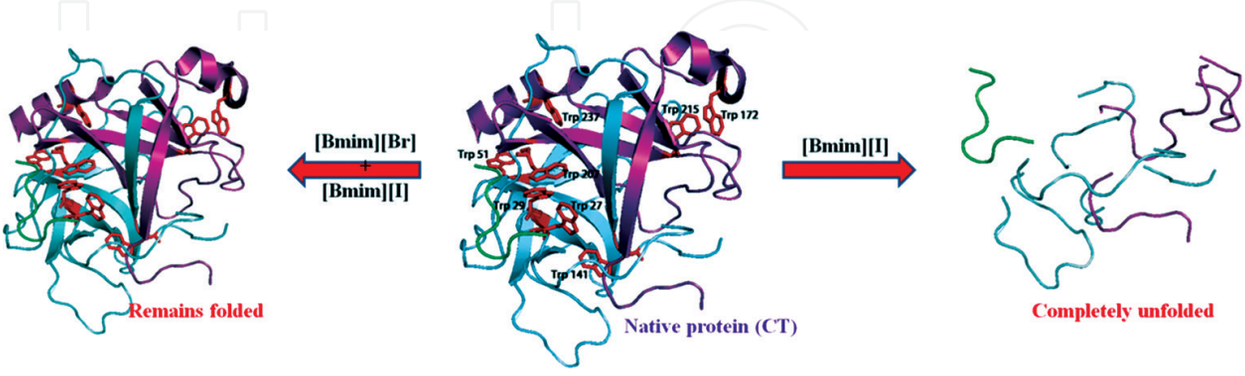


Figure 1. Fluorescence intensity changes for α-chymotrypsin (CT) in Tris-HCl buffer (black color line), 0.025 M [Bmim][Br] (red color line), 0.2 M [Bmim][I] (magenta color line) and 0.025 M [Bmim][Br] + 0.2 M [Bmim][I] mixture (dark cyan color) (Ref. [126]).

Interestingly, after addition of 0.025 M [Bmim][Br] to 0.2 M [Bmim][I], it is noticeable how the fluorescence spectra of the CT were clearly modified compared with the I_{max} for the CT in [Bmim][I], although the intensity was lower in the presence of [Bmim][Br] + [Bmim][I] than that of the native protein, which is, however, certainly larger than that of [Bmim][I].

The main reason behind the enhancement of intensity with the addition of [Bmim][Br] into the protein solution having [Bmim][I] is the movement of Trp toward a more hydrophobic environment and therefore high fluorescence intensity is observed due to higher quantum yield. Obviously, the mixture of the ILs may improve the stability of CT structure [126]. This phenomenon is schematically shown in **Scheme 1**.



Scheme 1. The presence of [Bmim][Br] counteracts the strong denaturation action of [Bmim][I] (Ref. [126]).

In 2006, the Lee group published a pioneering work [127] that showed that the effect of chloride impurity on the activity and stability of lipase in 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Omim][Tf_2N]) and [Omim][Cl] ILs. In result, the activity of enzyme exponentially decreased with increasing Cl^- content in [Omim][Tf_2N] and the activity of lipase in [Omim][Tf_2N] mixture containing 2% [Omim][Cl] was only about 2% of the activity in pure [Omim][Tf_2N]. The reason provided by the authors for the decrease in the activity of the enzyme in Cl^- ILs is due to the denaturation of the enzyme in these ILs. As mentioned before, the activity of the enzyme linearly decreased at about 5% for every 1% increase in [Omim][Cl] with there being no activity in [Omim][Tf_2N] containing about 20% [Omim][Cl] [127]. In another work of the Lee group [128], they systematically showed that the highest lipase activity was obtained in water-miscible [Bmim][TfO] which can dissolve a high concentration of glucose, while the highest stability of lipase was shown in hydrophobic [Bmim][Tf_2N]. The optimal activity and stability of lipase could be obtained in the mixture of [Bmim][TfO] and [Bmim][Tf_2N]. Therefore, the productivity obtained by using IL mixtures was higher than those in pure ILs [128]. A later study by this same research group reported that higher enzyme activity was achieved under ultrasound irradiation on lipase-catalyzed esterification of fructose in the mixture of [Bmim][TfO] and [Omim][Tf_2N] [129]. These results show that enzymatic reaction in ILs mixture under ultrasound irradiation is an effective method for enzyme activity and stability resulting in economic competitiveness of green process [129]. It is well documented that 100% conversion of cellulose to useful biochemical process in the presence of the mixture of ILs at low temperature, which overcomes the long intrinsic phase problem in the conversion of biomass to chemicals [130, 131]. Yao et al. [132] reported the activity and stability of *Candida rugosa* lipase in binary ILs.

Mixing of two different ILs, which show different physicochemical properties, can easily make new ILs because hydrophobic ILs and hydrophilic ILs are generally miscible. The mixing of

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different ILs may be a very useful method to make new IL solvent media for the structure and stability of biomolecules. These results improve knowledge of the excellent properties of IL mixtures as stabilizers for the native conformation of protein, since IL mixtures are able to stabilize enzymes and are suitable as reaction media for enzymatic biotransformations of industrial interest. Currently, these discoveries have opened new opportunities for obtaining better activity and improvement of stability of proteins in the mixtures of ILs.

7. The significant and specific contribution of ILs on protein stability

Practically, the native conformations of the globular proteins have adapted to environmental stresses that normally denature the proteins and the nature has provided a mechanism of adaptation that some of the cosolvents protect against denatured proteins [133, 134]. In fact, the protecting cosolvents stabilize the proteins against denaturing stresses and their presence in the cell does not alter protein functional activity [135]. The protein aggregation and its cold and thermal denaturation have been recognized as a major manifestation of instability that can severely affect a protein's functionality. As a group of novel green solvents, ILs have attracted extensive attention and gained popularity to overcome these physiological stresses. As discussed in earlier sections, ILs are potential cosolvent media for preservation of biomolecules because of their high stability and unusual solvent properties. We saw some of the ILs acted as efficient additives for the suppression of protein aggregation. Also, in the previous sections, we observed that the ILs behaved differently with various proteins. From the obtained results, we concluded that the stability of the proteins in ILs is completely dependent on the interactions of the ions of ILs with AA residues at the surface of the proteins.

In this section, we support the concept of novel behavior of ILs and explain the prevention of the self-aggregation of proteins, refolding of thermally and chemically perturbed proteins and also counteracting effects of ILs against the thermally and cold denaturation of proteins. Therefore, mechanistic insight into the effects of ILs on preventing all the deleterious effects on the proteins is desired for the understanding and designing of protein processes in biophysical chemistry and biotechnology.

As mention before, in 2000, Summers and Flowers [64] have observed that EAN has the ability to prevent lysozyme aggregation and is an efficient refolding additive for a completely denatured protein. Further, EAN has been utilized as a precipitating agent for the crystallization of lysozyme, providing crystals with good diffraction [135]. In another study, ammonium-based ILs such as EAF, PAF, 2-methoxyethylammonium formate (MOEAF) and Ethanolammonium formate (EtAF) not only acted as good stabilizers for the lysozyme native structure but also they protected the protein against thermal unfolding [67]. Subsequently, EAN IL not only stabilized the lysozyme native structure for long term rather it acted as a refolding agent preventing the lysozyme from aggregation. Bisht et al. [136] noticed that the presence of 1% v/v ammonium-based ILs can increase the activity of lysozyme up to 13% also refolded the urea-induced unfolded lysozyme structure. Interestingly, EAN has been observed to be possessing multicharacter in protein stability [64, 66, 67, 70]. We believe that information will surely help in understanding the microscopic mechanism existing between protein and ILs, particularly EAN.

Apparently, Lange et al. [73] explicitly elucidated that the set of imidazolium-based ILs such as $[Emim]^+$, $[Bmim]^+$ and $[Hexmim]^+$ cations with a fixed anion Cl^- can also be considered as refolding agents. The stability of lysozyme in these ILs was observed to decrease significantly as the alkyl chain of the ILs increased from ethyl, butyl to hexyl. Nonetheless, all ILs acted as refolding enhancers for the completely denatured lysozyme structure. A later study found that a series of the ILs such as [Mmim][Cl], [Emim][Cl], [Pmim][Cl], [Bmim][Cl], [Penmim][Cl], [Hexmim][Cl], [Hepmim][Cl], [Omim][Cl], [Ddmim][Cl], [i-Bmim][Cl] and [Bemim][[Cl] were applied to the denatured lysozyme structure and a significant refolding of the protein was observed. Among the ILs, in the presence of [Bmim][Cl], the refolding yield reached up to maximum of 84% [137]. The less hydrophobic ILs such as N-alkylpyridinium chlorides [EtPy][Cl], [BPy][Cl] and [HexPy][Cl] were effective in enhancing the refolding agents for the lysozyme structure and yielded up to 46–69% refolding [76]. As a consequence, the results conclude that more hydrophobic ILs behaved as a denaturants for lysozyme while the same ILs acted as refolding agents for the denatured lysozyme structure. In support to the above-mentioned facts, Takekiyo et al. [72] observed that the secondary structure of lysozyme was refolded in the 6–10 M concentration range of imidazolium-based IL, whereas the tertiary structure breaks down. Upon increase in the concentration more than 10 M of $[Bmim][NO_3]$, the secondary structure of the protein was still observed to be in a partially refolded state, while the tertiary structure was completely disrupted.

Very recently, at low concentration of [Bmim][Cl], the cyt c starts to unfold and again starts refolding with increasing concentration of the IL [138]. These results suggest a partial refolding of the secondary structure of cyt c in [Bmim][Cl]. The [Emim][Cl] was a very efficient in promoting refolding of the recombinant plasminogen activator (rPA) [139]. The delicate balance of favorable interactions with side chains and unfavorable interactions with the peptide backbone provides a molecular explanation of how this IL suppresses protein aggregation and simultaneously promotes refolding. Nonetheless, the protein denatured at high concentrations of [Emim][Cl] which indicates strong favorable interactions between AA side chains and ions of the IL [139].

The effect of EAN on renaturation of cyt c has been shown by Jaganathan et al. [140] and the results show that EAN in the range of 10^{-4} M helps in refolding of the protein from urea (8 M)-induced denaturation of cyt c. On the other hand, it was observed that at moderate concentrations (50–150 mM) $[Emim][CH_3COO]$ did not induce any significant effect over Mb structure, however, $[Bmim][BF_4]$, at the same concentrations significantly destabilized the Mb [141]. Further, there was minimal variation in the structure of Mb, when the mixture of 1.4 M GdnHCl and 150 mM of $[Emim][CH_3COO]$ IL was used as a cosolvent. That is, the protein is not completely unfolded. While in the presence of 150 mM $[Bmim][BF_4]^+$ 1 M of GdnHCl, the Mb completely unfolds. Thus, if compared, a combination of imidazolium cation with $[CH_3COO]$ anion in an IL is more biocompatible and giving protection against GdnHCl denaturation action on Mb native structure than those in ILs containing $[BF_4]$ anion. Moreover, it is interesting to note that some of the imidazolium-based ILs could be used as anticancer solvents [142].

IL such as triethylammonium acetate (TEAA) was found to be an efficient refolding additive for a thermally unfolded CT structure [143]. The refolding ability of TEAA against thermally denatured CT structure was monitored using ^1H NMR. In addition, the fluorescence, CD and

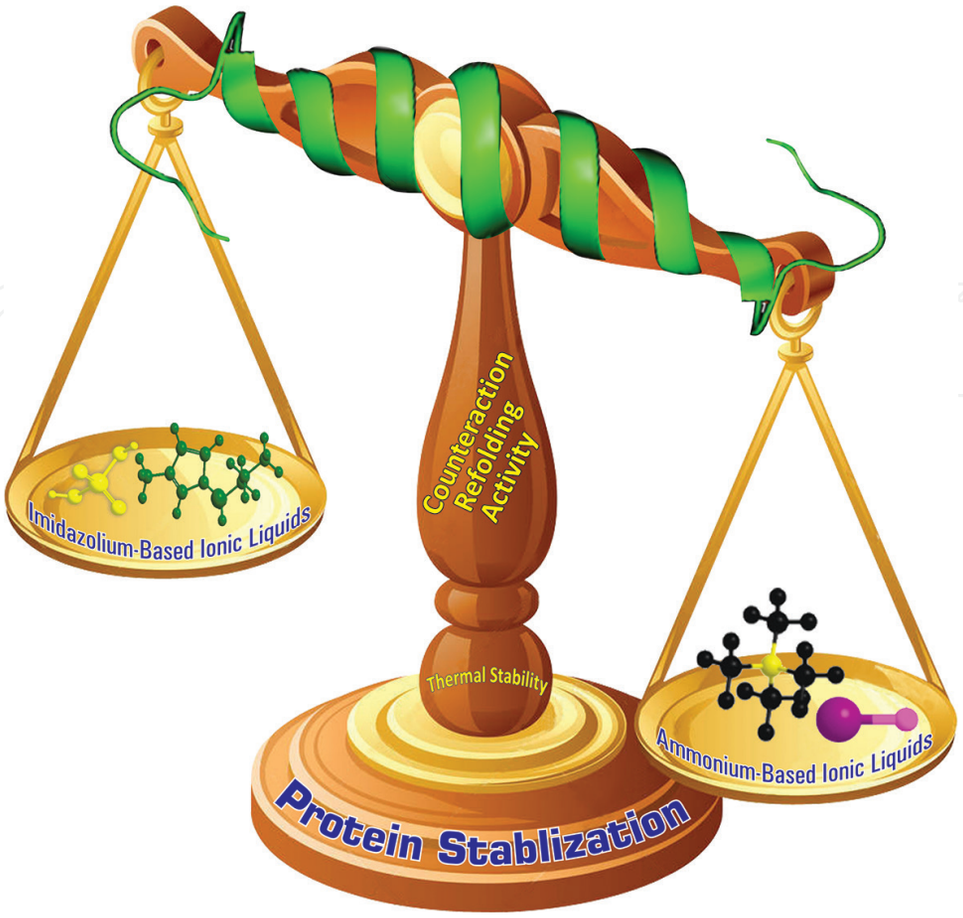
NMR results confirmed that TEAA strongly counteracted the deleterious actions of well-known denaturant, urea, on the CT structure [144]. It was demonstrated that TEAA and urea mixture substantially increased the T_m values which showed the counterbalance of the urea-induced denaturation of CT. Most importantly, deleterious action 5 M urea on CT was counteracted by only 1 M TEAA.

It has been already noted that a protic IL triethylammonium phosphate (TEAP) acted as a refolding additive for the urea-induced chemical denaturated state of the two enzymes, CT and succinylated Concanavalin A (S Con A) [22]. In one of the studies by Attri and Venkatesu, TEAP was shown to be acting as an efficient refolding agent for thermally denatured S Con A [145]. In 2013, Attri and Choi [146] showed that TEAP strongly attenuated the detrimental action of atmospheric pressure plasma jet (APPJ) on CT. This ammonium-based IL TEAP is able to maintain the structural integrity as well as activity of CT even after the exposure of APPJ [147]. The results show that one can use both enzyme and plasma simultaneously without affecting the enzyme structure and activity on the material surface, which can prove to be applicable in various fields.

Recent studies on ammonium-based ILs offer some valuable information to prevent the self-aggregation of the proteins. In this regard, Awanish and Venkatesu [148] for the first time showed ammonium-based ILs as a novel solvent for offsetting self-aggregation of insulin in the presence of TMAS, TEAS, TMAP, TEAP and TMAA. Therefore, the native structure of insulin was found to be stabilized in the presence of ammonium-ILs by unfavorable interactions with the surface of protein. The stability studies of insulin in ILs have opened a new way that can lead us to overcome the aggregation properties of insulin. This will not only increase the shelf life of insulin, whereas suitable formulations of insulin in biocompatible ILs can lead to safe and durable insulin formulations in pharmaceutical products. The ammonium-based ILs such as ethylammonium mesylate (EaM), diethylammonium mesylate (DeaM)-stabilized *tobacco mosaic virus*, whereas triethylammonium mesylate (TeaM) caused a change in the secondary structure of the virus [149].

From the literature and from our own experience, it can be suggested that ammonium-based ILs are more biocompatible as compared to the imidazolium-based ILs [44, 140–155]. Yu et al. [150] explored the stability of laccase in the presence of both ammonium- and imidazolium-based ILs such as [TMA][TfO], [Bmim][TfO] and [Bmpyr][TfO]. They found that only ammonium-based ILs [TMA][TfO] stabilized laccase, while [Bmim][TfO] and [Bmpyr][TfO] destabilized it. The contrasting nature of ammonium family ILs is also consistent with Rodrigues et al. [151], where among different families of ILs only ammonium-containing IL shows higher activity as compared to imidazolium-based ILs for *Thermomyces lanuginosus* lipase (TlL). **Scheme 2** shows the difference between the biocompability behaviors of ammonium- and imidazolium-based ILs.

On the other hand, Jha et al. [152] have explored the influence of a of imidazolium-based IL, 1-allyl-3-methylimidazolium chloride ([Amim][Cl]), on the stability of Hb. Unprecedented improvement in the stability of Hb in the presence of [Amim][Cl] at the lower concentration of [Amim][Cl] was observed by the authors [153]. Furthermore, the effect of [Amim][Cl] on bromelain stability and activity was investigated in another work. They observed that at low concentrations (0.01–0.10 M) of [Amim][Cl], there is ostensible only change in the stability and activity of BM.



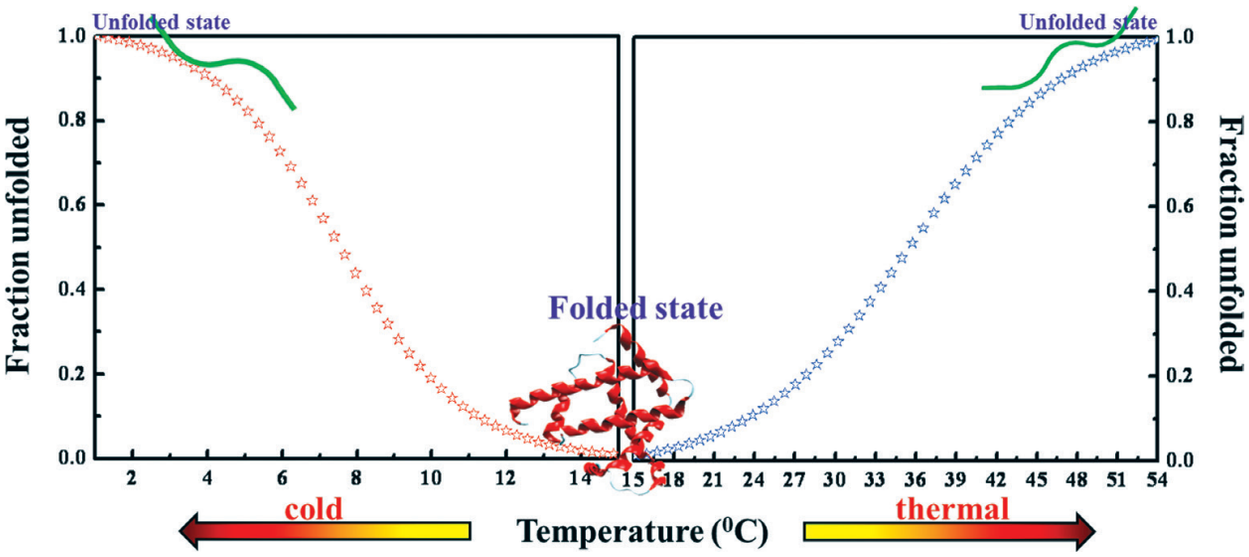
Scheme 2. The biocompatible behavior of ammonium-based ILs as compared to imidazolium-based ILs for proteins (Ref. [44]).

However, we cannot overlook the wide applications and uses of imidazolium-based ILs in various fields. Therefore, it is very important to emphasize the role of these ILs in biomedical applications, for example, counteraction of cold-induced unfolding of Mb and CT structures.

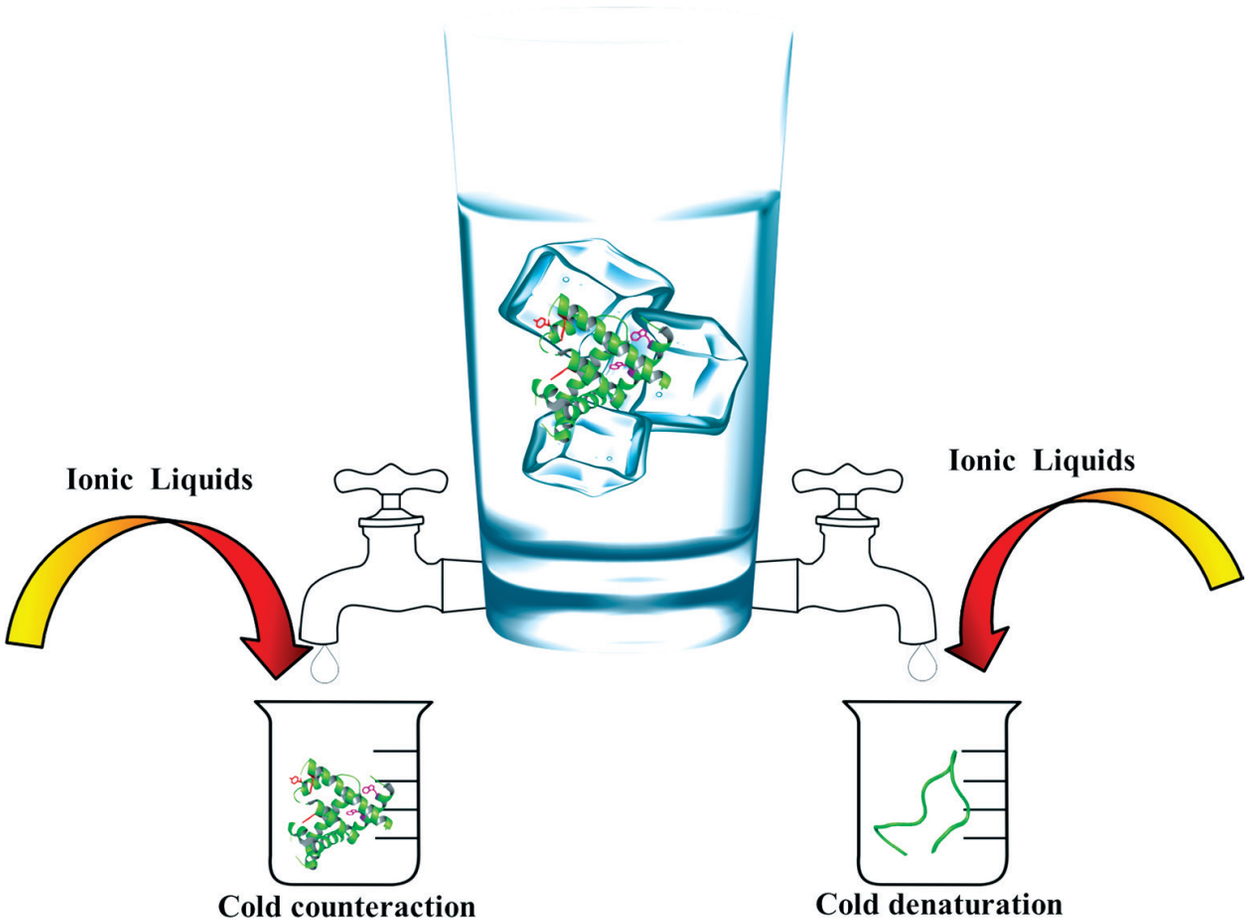
Cold denaturation is a fundamental fact in aqueous solutions where the native structure of globular protein disorders on extreme cooling [156]. Unlike thermal denaturation, whereby a native protein is disrupted at high temperature, cold denaturation is accompanied by decreases in both the system entropy and enthalpy [150]. As shown in **Scheme 3**, very recently we experimentally observed in one of our recent studies that the cold-induced unfolding of Mb and CT approaches closely a two-state folding mechanism similar to that experienced in the thermal denaturation of proteins [157].

Interestingly, for the first time, ILs having CH_3COO^- or Br^- with $[Bmim]^+$ proved to counteract the cold-induced unfolding of Mb and CT structures. Nevertheless, ILs containing Cl^-, HSO_4^- and SCN^- with $[Bmim]^+$ failed to prevent the Mb and CT structures against cold denaturation. These findings are concluded through **Scheme 4**.

http://dx.doi.org/10.5772/65924



Scheme 3. Schematic representation of two-state unfolding transitions in a protein with temperature (Ref. [157]).



Scheme 4. The ability of the ILs to offset the cold-induced unfolding of proteins.

An advantage of studying cold denaturation of globular proteins in ILs will certainly help us to encounter the reversible unfolding of proteins. Apparently, both cold and heat effects can lead to the unfolding of the proteins, which causes several human diseases. Evidently, the novel character of ILs offsets both the deleterious actions on protein keeping it in a proper folded conformation. It is hoped that the results obtained from these studies will be useful in recommending tailor-made ILs for various applications in biological systems as well as novel pharmaceutical applications.

Obviously, full access to the cold- and thermal-induced unfolding of proteins in ILs is still lacking. The interaction of the ions with protein surface is a complex result of the ability of the ions to enhance and disrupt water structure and internal protein residue interactions that contribute to the overall protein stability. Therefore, it is not necessary that only the IL is responsible for stabilization or destabilization of a protein under varying conditions, rather the solvent environment is equally responsible which creates variations in the interactions of the ILs with the protein surface. Apparently, ILs may stabilize or destabilize the proteins which are solely dependent on the molecular environment in the protein's surroundings. However, based on the literature survey and our experience, these studies might be inapplicable to all proteins in general (other than investigated proteins). Therefore, a lot of studies in this regard are essentially required to build a universal conclusion on ILs to protect the protein against various external stresses.

8. Conclusions

The extents of stabilization ability of ILs on the proteins vary and usually depend upon the combination of ions (both cation and anion). The activity and stability of protein in ILs that depend not only on the nature of the ions of IL but also on the functional groups of AAs sequences arrangement of the protein. Therefore, interactions of ions of ILs with proteins are important for understanding the effects shown by them on proteins whether in stabilization or destabilization. The results based on experiments revealed that the concentration of the ILs can play a major role in stabilizing/destabilizing a particular protein and also the alkyl chain length of the cation. Some of the novel characters in protein stability by ILs have been highlighted in this chapter. Prevention of self-aggregation and counteraction against extreme heat, cold and chemicals by ILs have been systematically presented. Cold counteraction is a new approach to stabilize proteins in ILs. This will help in increasing the stability of protein-based pharmaceutical products, which sometimes become inactive due to cold-induced denaturation of the proteins when stored at low temperature. Similarly, self-aggregation in proteins is also an issue which we believe can be controlled using biocompatible ILs. However, a very little amount of literature is available in this field of research.

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