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UNDERSTANDING EFFECT OF IONIC LIQUID ON METALLOPROTEINS: LACCASE AND AZURIN

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by

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Abstract

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Interactions between ionic liquids and biomolecules have been of great interest due to the intrinsic properties of ionic liquids and the flexibility to mix and match cations and anions to create unique ionic liquids. A number of ionic liquid-biomolecule studies have focused on the interactions with proteins, including industrially relevant enzymes. One of these, laccase from *Trametes versicolor*, is a naturally derived enzyme used in the breakdown of phenolic compounds in a wide variety of industries, especially useful in breakdown of lignocellulosic materials. Here, a combination of experiments and molecular dynamics (MD) simulations were used to investigate the interactions of ionic liquids with laccase. Enzyme kinetics assays indicated that ionic liquids composed of tetramethylguanidine (TMG) and either serine or threonine caused significant reduction of enzymatic activity, while kinetics was not impacted by TMG-Asp or TMG-Glu ionic liquids. Similarly, intrinsic fluorescence of laccase in the presence of TMG-Ser and TMG-Thr exhibited a shift in spectral properties consistent with structural destabilization, but again TMG-Asp and TMG-Glu had no impact. MD simulations of laccase and ABTS with/without TMG-Ser ionic liquid provide insight into the deactivation mechanism of laccase. The simulations indicate that TMG-Ser disrupts the electron transfer mechanism in laccase.

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Introduction and Literature Review

1.1 Introduction

Proteins are long-chain polymers of amino acids connected by peptide bonds. These polypeptide chains are further interlinked with hydrogen bonds, van der waals interactions, and electrostatic interactions, which leads to formation of secondary structures in proteins and subsequent further organization of these secondary structure elements form tertiary structures [1]. Protein function is governed by the specific three-dimensional structure the protein adopts by arranging the appropriate functional groups in the proper orientation. Proteins are involved in multiple processes in the living cell and are located on the extracellular surface, intracellular region, and in the cell membrane [2]. Some examples of proteins that are commonly found in biological systems are hormones, antibodies, enzymes (biological catalysts), transporters, and receptors [3]. Because of these biological functions, proteins are also used as therapeutic agents using specific formulations and as components of industrial processes [4]. Industrial processes utilize a variety of proteins such as metalloproteases, laccases, cellulases, lipases, phosphatases, and amylases for numerous applications [5]. Therapeutically, proteins such as immunoglobulins, erythropoietin, interferons, insulin, and anti-clotting proteins are widely used in the clinic [5]. Depending on the structure of the protein they are only stable in specific physiochemical environments, and therefore it is important to evaluate effects of various physical and chemical conditions for developing a robust formulation [6].

In some cases, protein structures are associated with metal ions including, but not limited to, Ca^{2+}, Mg^{2+} Cu^{2+}, Fe^{2+}, and Zn^{2+}, and this class of proteins are referred to as

metalloproteins [7]. Nearly 50% of the existing proteins in nature are metalloproteins [7]. Metal ions within metalloproteins play a key functional role in many biological redox processes and can provide structural stability to the protein [8]. Metal ions within these proteins play important roles not only in catalyzing biological processes, but they are also involved in binding interactions with organic and inorganic molecules [9]. Examples of processes that metalloproteins are involved with include the process of neuronal signal transmission, oxygen transport to and from the lungs, control of numerous redox processes, and nitrogen fixation [10]. Well known examples of metalloproteins include many electron transfer proteins (cytochrome b5, azurin, and [4Fe4S]-ferrodoxin), oxygen binding proteins (myoglobin and hemoglobin), as well as multiple enzymes such as oxidases (methane monooxygenase, heme-coper oxidase, cytochrome P450, laccase), peroxidases (horseradish peroxidase), hydrolases (carbonic anhydrase), hydrogenases ([FeFe]-hydrogenase), and reductases (copper nirite reductase, nitric oxide reductase, sulfite reductase) [11-13]. In addition to the metals listed above, many proteins have been demonstrated to bind and utilize "trace" metals, or those that are not found in high concentrations in biological organisms. These trace metals, and the metalloproteins that utilize them, are an area of renewed interest as a result of improving methods to identify and characterize the metals and proteins [14].

The metals that bind to a protein are dependent on the metals available to the organism in general and the protein's ability to functionally adapt to the metals available. For example, many proteins in plants use the available iron from their environment, while organisms in the oceans often use copper instead of iron more frequently due to the scarcity of iron in the oceans [7] . Ion channels in the cell membrane are utilized to import these

environmentally derived ions into the cytoplasm for further use by the cell. These ion channels can be selective for one or two ion species, or can be more promiscuous allowing multiple different species through the channel [15]. Once acquired, the location of the metal within the protein is key as it should not preferentially interact with the surrounding environment [13]. The structure of a metalloprotein is partially dependent on the metal; however, this structure can often be slightly modulated to accept a variety of similarly sized and charged metal ions [16, 17]. This is a complex interplay between the folded protein and the binding pocket for the metal. The protein can often fold into a similar structure in the absence of the metal, referred to as the apo-form. With the metal present, there are additional intermolecular contacts formed that stabilize the structure, known as the holo-form. Importantly, metal atoms of similar size/charge/valence may interact with the same binding site, although the protein structure is usually most stable with native metal ion ligand. The native 3D structure of the metalloprotein allows interaction of amino acid side chains with the appropriate type and number of metal ligands. This orientation promotes the correct metal-amino acid geometry facilitating the functional role and reactivity of the metal ions [13]. Protein folding is important for protein stability, and each polypeptide can adopt different three-dimensional conformations depending on the microenvironment in which it is being held [18, 19]. Changes in the surrounding microenvironments may lead to the addition or removal of the metal ions from the protein, which can impact stability of the protein [13].

Importantly, proteins are not the only biomolecules that interact with metal ions in nature. Small organic molecules, carbohydrates, and nucleic acid interactions with metal ions have all been well established in the literature. Again, in these cases, the metal ions

can be structural and/or catalytic in functionality. Examples include the structural bridging of alginate chains by Ca^{2+} [20], Mg^{2+} bridging and charge stabilization of the bacterial lipopolysaccharides [21], stabilization and structural modification of DNA and RNA by numerous monovalent and divalent cations [21-23], and metal-mediated catalysis by nucleic acids [24-27].

1.1.1 Protein Folding/Unfolding

Protein folding is the process by which the primary amino acid chain adopts an active 3D structure that is capable of carrying out the evolved function. The investigation of how proteins fold and unfold along with the forces that govern these processes has been an area of intense study for >50 years [28, 29]. In nature, the folding process occurs in the cell and is often aided by chaperone proteins or very specialized local environments such as the interior of the transcolon. However, it can occur in vitro as well, which is more dependent on the specific protein sequence and the environment [30]. Protein folding is achieved by the 3D rearrangement of a linear polypeptide chain, driven through Van der Waals interactions, hydrogen bonding, hydrophobic burial, and electrostatic interactions. All of these interactions occur between protein moieties, ligands, cofactors, and solvent molecules [28, 29]. This allows for amino acid functional groups to be brought together enabling chemical processes to occur per the specific protein function [30, 31]. Most often, the key to proper folding of proteins in their amino acid sequence [32]. The initial steps of this process often involve the burial of hydrophobic groups in a collapsed form, followed by the formation of secondary structures driven through electrostatics and hydrogen bonding. Regardless of whether or not the protein can spontaneously fold, there are thermodynamic and kinetic constraints that govern the folding process. In terms of

thermodynamics, the protein must be able to fold into the native conformation that is stable under the environmental conditions where the protein must carry out the evolved function. In cells, proteins are often only marginally stable, which allows for effective degradation of these molecules when needed [33]. In terms of kinetics, a denatured or unfolded polypeptide chain must be able to achieve the native conformation state in a period of time that is reasonable within the constraints of cellular function [34]. The secondary structure, alpha helices and beta sheets, and the tertiary structure are also dependent on the primary sequence which are an integral part of the proper 3D structure allowing for proper 3D positioning of functional groups from the amino acid side chains [31, 32].

When a protein unfolds or denatures, it means that the protein has lost stability in the functional 3D-structure, resulting in the protein being more flexible. This process is driven by the disruption of the bonds that drive the protein to fold, such as hydrogen bonds or electrostatics. If a protein is in its native conformation, it requires some physical or chemical interactions to initiate the unfolding process, which is usually achieved by increasing temperature or adding a chemical denaturant [35] in order to understand the thermodynamics and stability of a protein in vitro [36]. There are four main denaturation techniques that can be utilized: chemical denaturants, temperature, pressure, and force. By utilizing these various denaturants to unfold the proteins, various aspects of protein stability can be elucidated [37]. There are numerous studies which have been performed to evaluate effect of physical stress conditions such temperature, pressure, agitation, and packing/container/closure surface, as well as effect of chemical change in terms of pH, surfactant, inorganic salts, ILs, and co-solvents, folding and unfolding of proteins [38-43].

Chemical denaturation is a widely used approach allowing greater understanding of protein stability. One of the most common denaturants is urea, which acts by causing the disruption of nearby water-water interactions and increasing hydrogen bonds between urea molecules and the backbone of the protein. These interactions allow for increased hydrophobic solvation, which furthers the process of unfolding [44]. Another common denaturant is guanidinium, most commonly used as a hydrochloride salt (GuHCl). The exact mechanism by which GuHCl denatures proteins is still a controversy in the field, but various work has indicated hydrogen bond disruption, water-interactions, hydrophobic interactions, or backbone contacts as possible mechanisms [45, 46]. There is also some evidence that GuHCl is more effective at denaturing β-sheets [46, 47]. In addition to these chemical denaturants, temperature or pH changes are also used to evaluate unfolding processes in the proteins [48]. Increasing temperature provides thermal energy to increase molecular motion, while pH changes will impact charge state of ionizable groups in the proteins, thus impacting electrostatic folding forces [49].

In one study, the C12 protein underwent protein unfolding at high temperatures, influencing the rate at which it unfolds [48]. C12 is a globular protein with one domain and has been considered to be a good model for studying protein unfolding. During the unfolding process, there were disruptions in the structure of the protein core that were caused by hydrogen bond disturbances. This experiment showed that the unfolding process is an activated process since the protein would first disrupt the core protein structure and then underwent sliding movements that caused it to unfold into its transition state. Following this, the protein fully denatured with no native structure present [48]. This represents only one example of numerous reports in the literature of protein denaturation.

Other well studied model systems include ribonuclease A, T4 lysozyme, myoglobin, and others [50-54].

In order to understand the unfolding process in a protein, several spectroscopic methods can be used, with the most common being fluorescence, absorbance, circular dichroism (CD), infrared (IR), and nuclear magnetic resonance. UV-Vis absorbance is a widely utilized analytical technique, although not all proteins have appropriate chromophores that exhibit spectroscopic changes upon unfolding [55-57]. One example where the UV absorbance was used was to follow the changes that happened after tyrosyl ionization; during the unfolding process of the protein in pH, the tyrosine residues, which are buried in the protein structure, are uncovered and UV absorbance can detect the tyrosyl ionization [58]. The UV spectroscopy technique used is dependent on the protein unfolding to reveal the buried aromatic residues and make these residues exposed to the solvent, showing an increased absorbance in the 280-310 nm range due to tyrosine ionization at high pH. UV spectroscopy revealed whether or not the protein was undergoing an unfolding transition event [59]. The experiment showed that the technique used on the UV measurements to analyze protein denaturation is viable and can be used to understand other proteins. However, Tyr ionization only occurs at high pH, and other UV-active amino acids generally do not exhibit significant changes in UV-absorbance upon protein denaturation. Alternatively, some proteins are associated with cofactors that absorb light in the UV or visible wavelength ranges, and many of these cofactors will exhibit changes in absorbance properties when the protein denatures [60-62]. Fluorescence spectroscopy is also commonly used to follow the denaturation of proteins, generally utilizing the intrinsic fluorescence of Trp residues. The emission spectrum of Trp is inherently environmentally

sensitive, exhibiting a red-shift in emission maximum when moving from a less polar environment to a more polar environment [60, 63, 64]. Trp residues buried in the hydrophobic core of a protein will exhibit such a change in environment upon protein denaturation. These emission shifts have been widely utilized to study denaturation in proteins such as myoglobin, glycoprotein E from dengue virus, β-lactoglobulin, and [65, 66]. Nuclear magnetic resonance spectroscopy is useful in determining the protein kinetics as well as the mechanism by which the protein folds and unfolds [67]. It can give information about the unfolding and folding process of proteins based on specific residues and labeled proteins. It can also provide information regarding chemical shifts, which can help us determine the state at which the protein exists, unfolded or folded. It reveals information regarding the dynamics of the protein when it is an unfolded conformation [68]. NMR can also be used to generate high resolution structures of proteins and peptides, including metalloproteins [69-73]. Similarly, electron paramagnetic resonance (EPR) spectroscopy which relies on the spin of unpaired electrons, is useful for the study of metalloproteins with magnetic metal centers[74-77]. CD can be used to observe the structural changes by monitoring the disappearance of specific spectral signatures associated with α–helices and β–sheets [78, 79]. It can also be used to analyze how proteins form ligand with specific molecules, such as substrates and cofactors [62, 80]. A variant of traditional CD spectroscopy known as magnetic CD (MCD). This method aligns the protein sample in a magnetic field during spectroscopic interrogation which allows for the study of energy levels in the metal. MCD has been applied to a variety of metalloproteins including nitrogenases, cytochrome c, and aminopeptidases [81-85]. Similarly, traditional IR spectroscopy has been widely utilized to study metalloprotein structures and

interactions. Standard Fourier transform IR (FTIR) can monitor secondary structures in the protein, while far-infrared spectroscopy (FIR) can be utilized to interrogate low frequency vibrations, such as those in metal complexes [86, 87]. These IR methods have been used to investigate the structure of numerous metalloproteins and peptides including EndoIII, azurin, bovine serum albumin, and natural and designed peptides [87-92]. All of these pieces of information can together provide information regarding the stability of a protein's structure [93].

1.1.2 Ionic Liquids (ILs)

ILs are organic salts with melting points below 100 °C. In 1992 the first IL which was stable in air and ambient moisture was reported [94]. After that, ILs have been developed as an alternative to organic solvents and used in many more applications. ILs are useful industrial and laboratory solvents. The molecular composition of ILs is a combination of different cations and anions that leads to countless potential ionic liquid species. ILs have a wide range of physicochemical properties including low vapor pressure, high thermal stability, high conductivity, non-flammability, and varying degrees of biocompatibility [95]. Therefore, they could be used as a reaction media for synthesis and can be recycled multiple times, which underpins the "green" reputation of these solvents [96]. ILs have the ability to act as a host and can interact with both host and guest molecules via a combination of electrostatic, hydrogen bonding, π- and van der Waals interactions [97]. The non-covalent interactions within IL are easily broken and therefore commonly used to dissolve recalcitrant materials [97]. ILs are currently being used in many different applications including electrochemistry, energy, organic synthesis and catalysis as well as in biotechnology [98-101].

1.1.3 Ionic Liquid Interactions with Biomolecules

In nature, biomolecules are surrounded by charged species including proteins, polysaccharides, nucleic acids, inorganic ions, and small organic molecules. Although proteins have evolved to function in these ion-rich environments, not all ionic species have identical effects on proteins. Specifically, there has been a significant amount of study regarding the ability of ionic species to stabilize or destabilize proteins in solution. This ranking of ions based on the effects on protein solubility, known as the Hofmeister series, is a core component of understanding protein behavior in complex ionic solutions [102-104]. Importantly, extensive study of the Hofmeister series has determined that the anionic component of the salt generally has a larger effect on protein solubility [102-104]. Mechanistically, the Hofmeister ions are thought to change the ordering and interactions in the bulk water around the protein rather than more direct protein interactions which then impacts protein hydration and stability[103, 105-107]. Numerous ILs have been studied from the context of the Hofmeister series, especially since many commercially available ILs have simple anions or cations as part of the IL pair [108, 109]. These studies include direct influences of ILs on biopolymers but also more fundamental studies of IL properties in solution including physicochemical parameters such as ion hydration number which appears to be an important factor in IL-biomolecule interactions [103, 108, 110-113]. When considering the descriptions of IL-protein interactions below, the IL composition and ion placement in the Hofmeister series, when known, should be considered in the reader's interpretations.

The unique properties of IL have made them very useful as potential solvents for protein preservation, media for enzymatic reactions, as well as applications in the field of bioconversion and protein production/purification [42, 114, 115]. ILs are also found to enhance solubility of certain proteins, mainly through prevention of aggregation [116-118]. Furthermore, enhanced solubility of proteins in ILs can also help achieve highly supersaturated solutions, which was successfully used as an additive in media to promote protein crystallization. ILs were shown to influence crystallization of multiple proteins as well as improving the size of the crystal formed (helping crystal growth), quality of crystals, and enhances the reproducibility of the crystallization process [119, 120]. In addition, IL/aqueous bi-phasic systems were also used for extraction of proteins from biological fluids [117, 121]. These are a few representative instances where ILs can enhance protein stability and activity. However not all ILs are compatible with proteins. Many ILs have been shown to destabilize protein structure and activity. The physicochemical properties of ILs such as polarity, alkyl chain length, hydrophobicity, and viscosity all have different effects on protein stability [42]. Therefore, a rational selection of IL for a specific protein under investigation is necessary before using it as a solvent for that application. Furthermore, there is only limited knowledge regarding the mechanism of protein stabilization or destabilization in presence of ILs and therefore research is still needed to understand based on the chemistry of ILs how they interact with protein [122].

There has been great interest in recent years to use ILs in various industries because of the beneficial properties and the desire to stabilize protein functionality over wider ranges of reaction conditions. Specifically, how these ILs interact with biomolecules and what cation-anion combinations may impact biomolecular functions is of great interest for

industrial applications. Numerous groups have studied the interactions of proteins with a wide variety of ILs, resulting in some ILs enhancing protein activity and stabilizing protein structures, with others disrupting protein structures [5, 123, 124]. The disruptive ILs are effectively a destabilizing agent, acting as a denaturant. Exploiting the ability of some ILs to enhance protein denaturation can yield greater insights into the protein-IL interactions. In one study, ribonuclease A was used to understand the effect of ILs on protein stability and aggregation. Ribonuclease A, a small enzyme, was examined in the presence of ILs such as choline dihydrogen phosphate ([Chol][Dhp]), 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][Dca]), 1-butyl-3-methylimidazolium bromide ([BMIM]Br), and choline chloride ([Chol][Cl]). From this study it was observed that [Chol][Dhp] promotes stability of the native state and increases the chances of refolding, which prevents protein aggregation [125]. In another study, human serum albumin (HSA), was studied in the presence of ILs, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF4]) and choline dihydrogen phosphate ([Chol][Dhp]). [BMIM][BF4] shown to induce swelling of HSA loop 1 causing it to be 0.6 nm wider compared to what it is in water although [Chol][Dhp] was not able to impart a similar effect [126]. While this is one example, there are numerous reports in the literature comparing numerous proteins with an even greater number of ILs.

1.2 Interaction of Ionic Liquids with Metalloproteins

Due to the sheer number of unique proteins found in nature, combined with the ever-increasing number of ILs, it is unlikely there will be a set of hard and fast rules that define all IL-protein interactions. As a result, it is important to begin to focus the interpretation and analysis by refining the types of molecules being investigated. This literature review

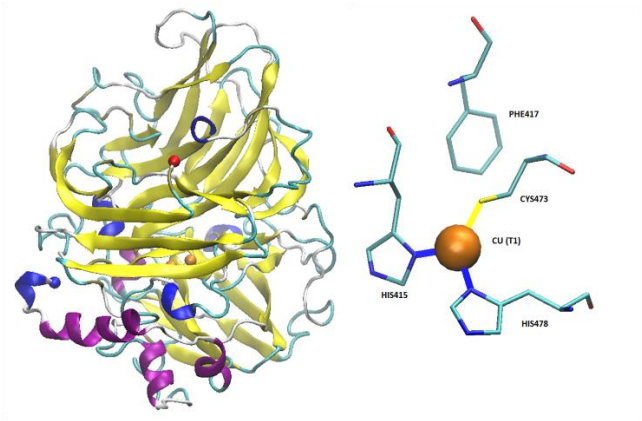
focuses on understanding the impact of various ILs on a specific metalloprotein enzyme called laccase.

1.2.1 Effects of Ionic Liquids on Laccase

Laccase is a metal containing protein containing four copper ions in its active center [127, 128]. Laccase was originally isolated from the Japanese lacquer tree *Rhus vernicifera*. After that laccases were also found in multiple different plant sources like *Rhus succedanea, Acer pseudoplatanus*, *Pinus taeda*, *Populus euramericana*, *Liriodendron tulipifera,* and *Nicotiana tabacum* [129-134]. Laccases found from these sources are monomeric proteins which have molecular weights between 90-130 kDa [54]. Notably, they are also highly glycosylated, with carbohydrate content between 22-45% [135, 136]. In addition to plant sources, fungi is a common source of laccase and most fungi produce different laccase isoforms and isoenzymes. One of the most commonly studied forms of laccase is derived from the *Trametes versicolor* fungus [137-140]. The *T. versicolor* laccase contains two copper sites, a mono-copper and a tri-copper site (Figure 1). The Cu^{2+} at the mono-copper site is coordinated by two His and one Cys residue, while the Cu^{2+} atoms at the tri-copper site involve coordination of at least 3 His residues and multiple carboxyl containing residues (Asp and Glu) [128, 141, 142]. Recent studies show laccase is also present in bacteria, although these proteins are less well studied [143-145].

Figure 1

Structure of Laccase From T. Versicolor



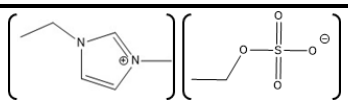
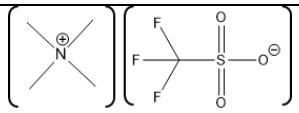
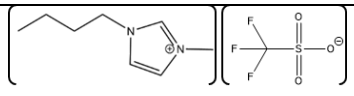
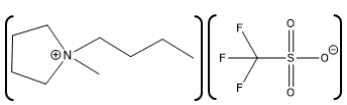
Note. The crystal structure was solved by Choinowski and coworkers (1GYC) [127, 146]. 3D structure of laccase (left) and structural geometry of the mono-copper site with chelating residues highlighted (right).

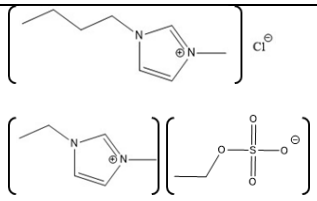
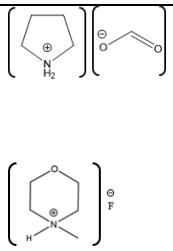
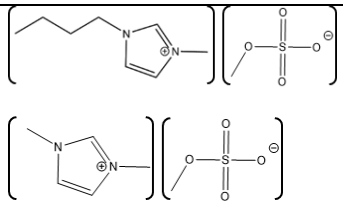
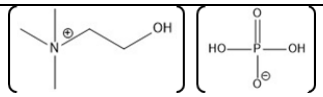
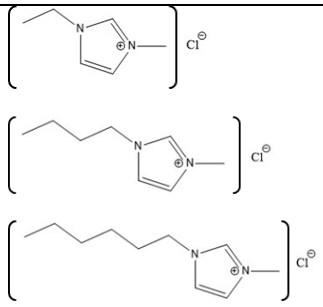
In nature, laccases typically oxidize phenolic compounds and reduce molecular oxygen into water after several rounds of catalysis [147]. This is typically involved in the synthesis or degradation of naturally occurring plant lignins [148]. Laccase has found utility in bioremediation of waste products from numerous industries, remediation of excess pesticides & herbicides, as well as cleaning of wastewater streams [149]. Additionally, many synthetic organic compounds can be substrates for laccase. Organic substrates of laccase are categorized in three groups: ortho-, meta-, or para- substituted compounds (all with a lone pair of electrons). In most cases of laccase, ortho-substituted compounds work as the better substrate over para- or meta-substituted compounds [145, 150]. One of the most useful synthetic laccase substrates is 2,2'-Azino-bis(3-

ethylbenzthiazoline-6-sulfonic acid) (ABTS), which is a colorimetric substrate allowing spectroscopic monitoring of laccase activity. ABTS was used in monitoring the oxidation of non-phenolic lignin structures which gave the impetus to find new laccase mediators [151, 152]. A particularly interesting application of laccase is in the detoxification of chlorophenol-containing wastewater which is achieved by laccase-mediated polymerization via radical coupling [153, 154]. The industrial applications of laccase coupled with the straightforward monitoring with ABTS have made it a very attractive system to study with ILs.

Table 1

Summary of Studies Done Pertaining to Monitoring Effect of ILs on Laccase

Laccase source	IL	Structure	Study done	Results	Ref
Aspergillus	1-ethyl-3-methylimidazolium ethylsulfate ([EMIM][$EtSO_4$]		Activity at different temperature in presence or absence of IL	([EMIM][$EtSO_4$] IL decrease the activity of laccase	[155]
Trametes versicolor	tetramethylammonium trifluoromethanesulfonate ([TMA][TfO]).		Enzyme kinetics, Time dependent fluorescence, CD analysis	[TMA][TfO] can stabilize laccase and keep its catalytic efficiency unchanged.	[156]
Trametes versicolor	1-butyl-3-methylimidazolium trifluoromethanesulfonate ([BMIM][TfO]), 1-butyl-1-methylpyrrolidinium trifluoromethanesulf	 	Enzyme kinetics, time dependent fluorescence, CD analysis	High level of [BMIM][TfO] or [BMPyr][TfO] destabilizes laccase and decrease its activity	[156]

Laccase source	IL	Structure	Study done	Results	Ref
	onate ([BMPyr][TfO]				
Trametes versicolor	1-butyl-3-methylimidazolium chloride, [BMIM]Cl; 1-ethyl-3-methylimidazolium ethylsulfate, [EMIM] [$EtSO_4$]		Enzymatic kinetic[spectrophotometric measurement of activity at 420 nm}	Inhibition of laccase activity	[157]
Trametes versicolor	Pyrrolidinium Formate ([Pyrr][F]) ; the Morpholinium F ([morph][F]), mb b ($C_5H_{11}NO_3$)		Fluorescence	red shift in λmax in presence of ILs	[158]
Trametes versicolor	1-butyl-3-methylimidazolium methyl sulfate, [BMIM][$MeSO_4$] and 1,3-dimethylimidazolium methyl sulfate, [MMIM][$MeSO_4$],		Effect of both water soluble ILs at different concentration was monitored using laccase activity assay	Laccase activity did not changed upto 25% IL concentration in both cases. However, at 35% both the IL increased the laccase activity about 1.7 times	[159]
Trametes versicolor,	Choline dihydrogen phosphate [Chol][H_2PO_4]		Fourier Transform Infra-Red spectroscopy (FT-IR)	Effective in increasing and stabilizing laccase activity	[123]
Bacillus HR03	1-ethyl-3-methyl imidazolium chloride [EMIM][Cl]; butyl-3-methyl imidazolium chloride [BMIM][Cl]; hexyl-3-methyl imidazolium chloride [HMIM][Cl]		Enzyme activity, fluorescence, CD analysis	As IL concentration increases, activity decreases. (Km increases)	[160]

Laccase enzymatic activity towards oxidation of ABTS was shown to increase when [MMIM][$MeSO_4$] and [BMIM][$MeSO_4$] were used as ILs at a concentration of 35% v/v Below this concentration ILs do not show much impact on laccase activity [159]. In another study, increased IL concentrations produce a red shift in λ_{max} for laccase fluorescence [158]. Specifically, researchers have shown that when laccase was combined with various volumetric fractions of pyrrolidinium formate ([Pyrr][F]) and morphilinium formate ([Morph][F]) ILs, they both showed a red shift in λ_{max} for laccase fluorescence [158]. However, the authors did not specifically investigate the mechanism of IL inhibition of enzymatic activity [158].

Solution pH is another parameter that is important to understand the stability of laccase in ILs. Isoelectric point (pI) of laccase is 4.6 [161, 162] and based on the nature of the IL it would affect its interaction with laccase. For example, the fluorescence intensity of laccase was found to decrease in presence of the IL [TMA][TfO] more at pH 3.6 than at pH 5 [156]. On the other hand, at pH 5.8, the fluorescence intensity of laccase was found to increase in the presence of [TMA][TfO]. At pH 3.6, there is greater contribution from $CF_3SO_3^-$ anion with respect to its interaction with the laccase interaction and as a chaotropic anion it has higher preference to bind with the protein-water interface and destabilize the enzyme (Hofmeister effect) [109, 163]. However, at pH>pI (pH 5.8) the cation $[TMA]^+$ is more active in terms of ordering the water structure surrounding enzyme and makes laccase more compact, resulting in increased fluorescence intensity from the greater shielding of buried Trp residues by the bulk polar aqueous milieu [156]. In another study, the effect of laccase activity in the presence of three 1-ethyl-3-methyl imidazolium ILs (with anions [$MDEGSO_4$], [$EtSO_4$] and [$MeSO_3$]) was determined at pH 5, 7, and 9.

The results show that at pH 7 and 9, the activity of laccase does not change with the addition of ILs. However, at pH 5 the laccase showed significantly reduced activity overall, but the IL-laccase samples showed a smaller loss of activity, that is, the laccase+IL mixtures performed better than laccase alone at pH 5 [164].

Above 75% (v/v) concentration of ILs like 1-ethyl-3-methyl imidazolium ILs (with anions [$MDEGSO_4$], [$EtSO_4$] and [$MeSO_3$]) laccase precipitated under most conditions [164]. In the case of 4-methyl-N-butylpyridinium tetrafluoroborate, [4-MBP][BF_4] laccase precipitated even at 50%(v/v) concentration. Precipitation occurs because salting out effects are promoted at high concentration. Novel formulations such as microemulsions made up of ILs, can also influence laccase activity. For example, when water-in-[BMIM][PF_6] was used as the IL, laccase activity was found to be higher for the water-in-IL microemulsion compared to pure IL or water-saturated IL [42].

In addition, ILs can impact the biocatalytic activity of the laccase. For instance, aqueous biphasic systems containing IL cholinium dihydrogen citrate ([Chol][DHCit]) have been shown to enhance the extraction efficiency of the enzyme and increase the biocatalytic activity by 50% [165].

Chapter 2

Effect of Ionic Liquids on Laccase

2.1 Introduction

Ionic liquids (ILs) have been an attractive and dynamic topic of investigation in the fields of analytical and electrochemistry over the past 15-20 years. This interest stems from the many unique and interesting properties of ionic liquids (aka room-temperature ionic liquids) including low to negligible vapor pressure, high conductivity, non-flammability, and high thermal stability [95]. These properties have made ILs of immediate interest to studies of energy storage and conduction, as well as a variety of other electrochemical applications [166-168]. More recently, groups have begun the more depth investigation into the biocompatibility of ILs as well as the mechanisms of how ILs interact with specific biomolecules.

Proteins, being one of the four major classes of biomolecules, is of obvious interest for investigating IL interactions. The biocompatibility of ILs with proteins is important for developing the scope of applications a given ionic liquid can be used in, that is, ionic liquids which are toxic to cells or destabilize/damage critical proteins cannot be considered for in vivo uses. Alternatively, the beneficial chemical properties of ILs may be useful in many industrial bioprocesses in which enzymes are used as biocatalysts for production or conversion applications [145, 150, 169-172]. Specifically, the conductivity of ILs make them ideal candidates for processes involving redox reactions while the low vapor pressure and thermal stability allow for improved recycling of these agents between processes [98-100, 171].

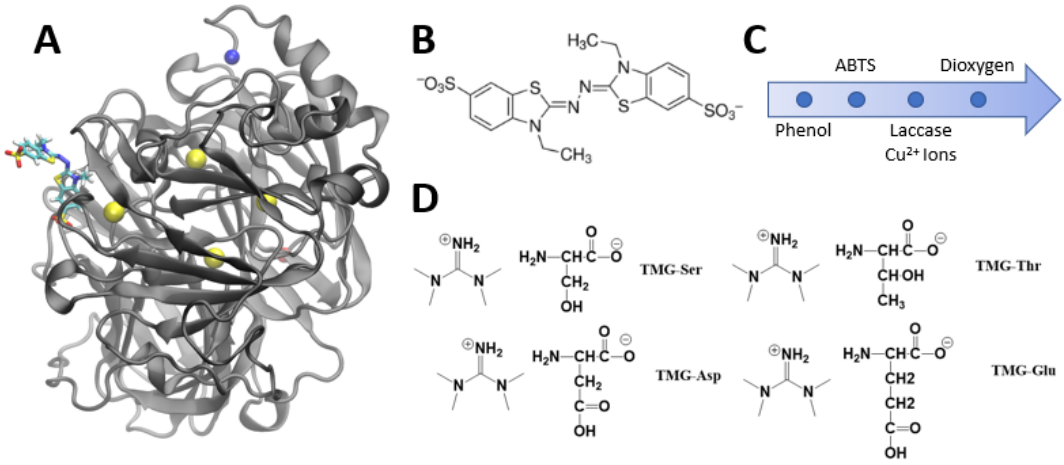
The ability for a protein to function in the expected manner is directly linked to the native 3D structure of the protein, and thus maintaining native structure is critical for proteins to be a useful part of any industrial or experimental process. Protein native structures formed through a complex interaction of hydrogen-bonding, hydrophobic forces, ionic interactions, and solvent interactions [28-32]. The protein folding process normally occurs in cells under native cytoplasmic conditions however, extensive investigation on protein denaturation or unfolding has been carried out in vitro and has yielded significant insights into the thermodynamics of protein structural stability. In the context of ionic liquids, more and more proteins are being investigated for interactions with ionic liquids [173]. This is a challenging problem as there are seemingly infinite combinations of ionic liquids and proteins to investigate. The interactions of proteins with ILs have been shown to follow the Hofmeister Series which relates ion identity to effects on protein solubility[108]. Interestingly, some ionic liquids have been shown to stabilize protein structures while others have been demonstrated to destabilize protein structure [60, 62, 122]. Thus, there is a critical need to increase the fundamental understanding of how ILs interact with proteins.

A widely utilized protein in industrial bioprocesses are laccases. Laccase was originally isolated from the Japanese lacquer tree *Rhus vernicifera*, but have since been identified in numerous plant and fungal species [129-134]. Laccases function in nature to oxidize phenolic compounds, utilizing multiple Cu^{2+} ions in the active site of the protein to facilitate electron transfer. Interestingly, naturally occurring laccases can often catalyze these redox transformations on a number of different substrates. This catalytic promiscuity has enabled laccases to find utility in the remediation of waste streams from a variety of

industries [149]. Laccase has also found widespread utility as a model system when studying the effects of ionic liquids on protein activity. The enzymatic activity of laccase can be monitored through the use of a chromogenic substrate, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), which undergoes a color change upon oxidation by laccase. The electron transfer chain has been proposed: electrons that are extracted from phenolic molecules are eventually transferred to dioxygens forming reduced water and oxidized phenols as end products through an internal pathway involving ABTS, T1 and T2/T3 Cu2+ ions as electron shuttles (Figure 2). This has also allowed a number of groups to investigate the impact of various ionic liquids on the function of laccase [158-160, 162-164, 169, 171].

Figure 2

Structure of Laccase, ABTS and Ionic Liquids and the Electron Transfer Chain of the Laccase Catalysis



Note. (A) Tertiary crystal structure of laccase (PDB ID: 1GYC). Protein backbone: Grey ribbons. ABTS ligand: Licorice tubes colored by element. Cu2+ ions: Yellow VDW balls. N-terminus: Red VDW ball. C-Terminus: Blue VDW ball. (B) Chemical structure of ABTS. (C) The putative electron transporting chain in the redox catalysis mechanism of laccase. Order of electron flow starts from left-to-right: an electron is passed from a phenol to ABTS and laccase Cu2+ ions before reducing dioxygen to water. (D) Chemical structures of the ionic liquids TMG-Ser, TMG-Thr, TMG-Asp and TMG-Glu.

In this study, the impact of two classes of ionic liquids on laccase activity was investigated. The widely studied imidazolium chloride ionic liquids were first investigated, followed by the more recently developed amino-acid based ILs. The imidazolium ILs exhibited variable disruption of laccase enzymatic activity but are incompatible with many spectroscopic studies on proteins due to the high absorptivity of the imidazolium ring in the low-UV region. The amino acid ionic liquids exhibited differential effects on the

enzymatic activity of the protein which was not present when free amino acids were used. Specifically, TMG-Ser and TMG-Thr cause significant reductions in enzymatic activity. (The nomenclature TMG-Ser, TMG-Thr, TMG-Asp, and TMG-Glu will refer to the ionic liquid species that is added to aqueous solution throughout the manuscript.) Fluorescence spectroscopy confirmed a change in spectral properties of laccase in the presence of TMG-Ser and TMG-Thr consistent with protein denaturation. MD simulations elucidate the mechanism of inactivation of laccase by TMG-Ser and likely TMG-Thr via disruption of its electron transfer catalysis reaction, likely due to charge-charge (electrostatic) interactions between TMG-ABTS and Ser-T1/T3 Cu^{2+} ions.

2.2 Materials and Methods

2.2.1 Materials

Laccase was purchased as a 70% pure lyophilized powder from Sigma (St. Louis MO, USA) and further purified as described below. Imidazolium ionic liquids were purchased from Alfa Aesar (Tewksbury, MA, USA) and used without further purification (99% purity or greater). All other reagents were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA), VWR (Radnor, Pennsylvania, USA), or Sigma-Aldrich (St. Louis, Missouri, USA). TMG-ILs were synthesized as described previously and contained ~5% residual water as determined by FTIR spectroscopy [174]. Ionic liquid abbreviations used in the manuscript are: TMG-Ser (1,1,3,3-tetramethylguanidine glutamate), TMG-Thr (1,1,3,3-tetramethylguanidine threonine), TMG-Asp (1,1,3,3-tetramethylguanidine aspartate), TMG-Glu (1,1,3,3-tetramethylguanidine glutamate), OMICl (1-octyl-3-methylimidazolium chloride,), HMICl (1-hexyl-3-methylimidazolium

chloride,), EMICl (1-ethyl-3-methylimidazolium chloride), and BMICl (1-butyl-3-methylimidazolium chloride).

2.2.2 Laccase Purification

Crude laccase was purified by ion exchange chromatography using a CM sepharose column with ammonium acetate buffer. Crude laccase was dissolved in 50 ammonium acetate buffer pH 4.1 and eluted using 50 mM ammonium acetate pH 5.1 buffer. Aliquots of each fraction were removed and checked for laccase activity using ABTS. Active fractions were pooled and dialyzed against 5 mM sodium phosphate pH=7.0 buffer buffer for ~24h. The sample was then collected and lyophilized. Laccase stock solutions were made fresh by dissolving the lyophilized powder in 2 mM sodium phosphate pH=7.0 and concentration was determined spectrophotometrically using $\varepsilon_{280} = 60{,}520$.

2.2.3 Enzymatic Assay

The laccase enzyme activity was also measured by using absorbance spectroscopy. Briefly, the 5 µM purified laccase was mixed with pH 5.1 sodium acetate buffer and added to a quartz cuvette. The solution was stirred in the cuvette using a micro-stir bar. The reaction was initiated by the addition of ABTS to a final concentration of 50 µM and the increase the absorbance at 450 nm was monitored as a function of time with auto sampling mode at room temperature. Substrate was added at t = 10 s in each experiment. Absorbance measurements were collected in 3 second increments. Data for replicate samples were averaged and standard deviations were calculated and reported as error bars.

2.2.4 Fluorescence Spectroscopy

Fluorescence emission spectra were recorded from laccase using a Horiba Fluoromax-4 fluorescence spectrometer (Horiba Ltd., Edison NJ USA). Excitation wavelength was 280nm while emission was collected from 300-400nm. In these assays, laccase concentration was 6.25 µM. Ionic liquid concentrations varied based on the experiment. Experiments were carried out in either sodium citrate buffer pH 5.1 or sodium phosphate buffer pH 7. Data presented are representative spectra.

2.2.5 Molecular Dynamics Simulations

MD simulations were performed to probe the deactivation mechanism of laccase enzyme protein in the presence of TMG-Ser. A total of two systems were built: 1.) Laccase and ABTS in water and 2.) Laccase and ABTS with TMG-Ser. Each system was constructed using the high-resolution structure of laccase (PDB ID: 1GYC) from the RCSB Database. The laccase protein was prepared using the Protein Preparation Wizard of Maestro program [175]. Pre-processing and optimization of the pH=7 protonated state and geometry optimization used default parameters for restricted minimization. The ff14SB force field was used to represent the prepared laccase protein and the disulfide bonds within it. Ions characterizing the ILs were prepared using AMBER16 software and the GAFF2 force field and manually added to the laccase system; the force fields of TMG and Ser molecules were taken from our previous work [174]. Each IL system contained enough ions to constitute 0.05 M concentration. IL molecules were randomly placed around the protein and a relaxation protocol further randomized the IL molecular positions. Each system was built using the TIP3P water solvent model. Each system is contained in a

solvent box of truncated octahedron using a 10 Å cut-off. Enough counter-ions were added to neutralize each system.

The simulation of each system was carried out using the AMBER 16 simulation package using standard simulation protocols. TMG-Ser IL-containing systems had a 1,000 ps pre-run at 500 K to ensure that the position and orientation of TMG and SER molecules was randomized before a production run at 300 K. Laccase's position remained fixed during this pre-run. A production run at 300 K included a short 1.0 ns MD using the NPT ensemble mode (constant pressure and temperature) to equilibrate the system's density, followed by 999 ns dynamics in the equivalent NVT ensemble mode (constant volume and temperature). All bonds interconnecting hydrogen atoms were treated with the SHAKE algorithm using a 2.0 fs time step in the simulations. Long-range electrostatic interactions were treated with the particle-mesh Ewald method under periodic boundary conditions (charge grid spacing of ~1.0 Å, the fourth order of the B-spline charge interpolation; and direct sum tolerance of 10–5) [176]. Short-range non-bonded interactions were defined at 10 Å and long-range van der Waals interactions were based on a uniform density approximation. To reduce computation time and expense, non-bonded forces were calculated using a two-stage RESPA approach [177]. Short-range forces were updated every step and long-range forces were updated every two steps. The temperature was controlled using the Langevin thermostat with a coupling constant of 2.0 ps. The trajectories were saved every 50.0 ps for analysis purposes.

Root mean-squared deviation (RMSD) values were calculated for the laccase protein (Cα atoms) and ABTS in each system after aligning the Cα atoms of laccase of the first snapshot by least square fitting. Root mean-squared fluctuation (RMSF) values were

calculated for all individual residues in the laccase protein in water and with TMG-Ser to characterize the entropic change in the protein conformation in each system.

Last snapshots of laccase and ABTS of each system were generated to quickly assess the structural insights from the MD simulations. Accounting for the stability of the laccase protein backbone in the binding process, the protein backbone of the stable complexes was aligned by a least square fitting. The Daura algorithm was used to cluster all aligned complexes into different structural families based on a 2 Å pair-wise RMSD cutoff of ABTS only without ligand fit. Centroid structures are defined as a structure with the largest number of neighbors in the structural family and is thus used to represent that structural family. Confirmed via visual analysis, super-families corresponding to major binding modes were formed by merging the centroid structures together. Atom contact plots were generated for Protein-TMG, Protein-Ser, Polar Protein-IL and Nonpolar Protein-IL interactions, with a cut-off of 2.5 Å. These plots highlight (1) TMG-protein contacts, (2) Ser-protein contacts, (3) protein polar side chain-IL interactions and (4) protein hydrophobic side chain-IL contacts.

2.3 Results

2.3.1 Protein Sequence, Structure, and Activity

Laccase from *Trametes versicolor* is a 519aa Cu^{2+}-containing protein originally isolated from a white-rot fungus and normally functions to break down lignins and other plant based materials. The three dimensional structures of laccase is shown in Figure 2A and the amino acid sequence and properties summary can be found in Figure 7. The enzymatic activity of the protein was characterized in the presence of imidazolium ILs EMI-Cl, BMI-Cl, HMI-Cl, OMI-Cl or the amino acid ILs TMG-Ser, TMG-Thr, TMG-Asp,

TMG-Glu (Figure 2D). The imidazolium ILs vary in the length of the alkyl group attached to the imidazole ring and have been shown to exert differing levels of protein destabilizing activity based on that alkyl chain length [60-62]. The amino acid ionic liquids are a newer class of ionic liquids and have been demonstrated to exert differential effects on protein stability based on amino acid R-group identity [174]. The amino acid ILs selected represent those with an alcohol in the side chain (Ser, Thr) and those with a carboxyl moiety in the side chain (Asp, Glu).

2.3.2 Enzymatic Activity

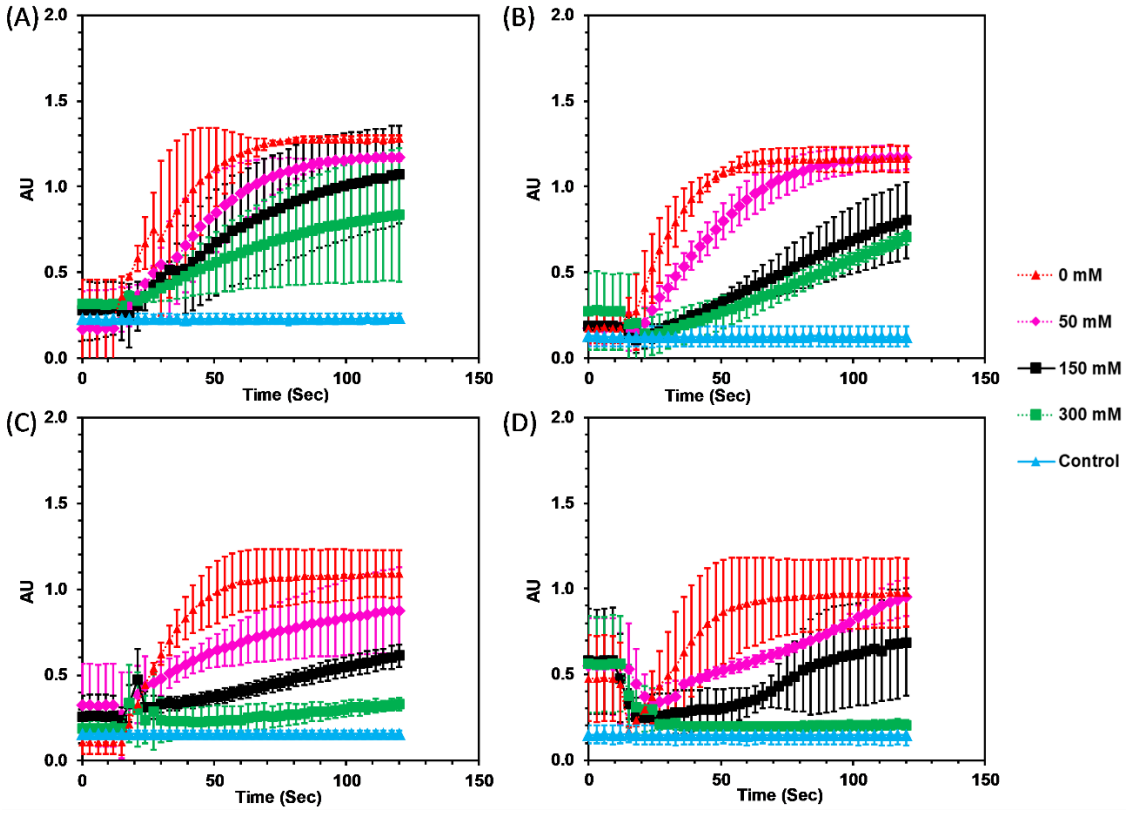
The enzymatic activity of laccase in the presence or absence of ILs was characterized by the conversion of the chromogenic substrate ABTS. The ABTS molecule is oxidized by laccase and converted into a cationic radical which has a different absorbance spectrum than the reduced form. The evolution of this absorbance at 450nm is measured as an indicator of laccase activity. In the absence of any ionic liquids, laccase exhibited a typical substrate concentration dependence (Figure 8).

2.3.2.1 Imidazolium Ionic Liquids. Laccase activity was carried out in the presence of differing concentrations of the four ILs EMI-Cl, BMI-Cl, HMI-Cl, OMI-Cl. These compounds only differ in the length of the alkyl chain attached to the imidazole ring, ranging from 2 carbons (EMI-Cl) to 8 carbons (OMI-Cl). The data from these experiments are shown in Figure 3. In each case, the laccase activity exhibited a clear dose-dependent decrease in activity as ionic liquids increased. The data shows that even at the lowest ionic liquid concentration tested (0.05M), there was a noticeable decrease in the conversion ABTS. Importantly, this decrease was more dramatic as the alkyl chain length increased. Similarly, the enzyme activity in the presence of the highest concentration of OMI-Cl

which contains the longest alkyl chain was completely abolished (Figure 3D). Interestingly, even the lower concentrations of OMI-Cl induced significant reductions in activity and yielded substrate conversion curves that were atypically shaped. These results may indicate that at lower concentrations the OMI-Cl is acting to destabilize the protein but may require longer to achieve the same level of effect.

Figure 3

Laccase Enzyme Activity with Imidazolium ILs



Note. The enzymatic conversion of ABTS by laccase is shown in the presence of differing concentrations of (A) EMICl, (B) BMICl, (C) HMICl, or (D) OMICl. Concentrations of IL are

shown in the legend, with the "control" sample being laccase and 300 mM IL in the absence of substrate. Substrate was added at t = 10s. In all samples, laccase concentration was 5 μM, ABTS concentration was 50 μM, and absorbance was monitored at 450 nm. All data are averages of 3-7 samples with error bars representing standard deviations.

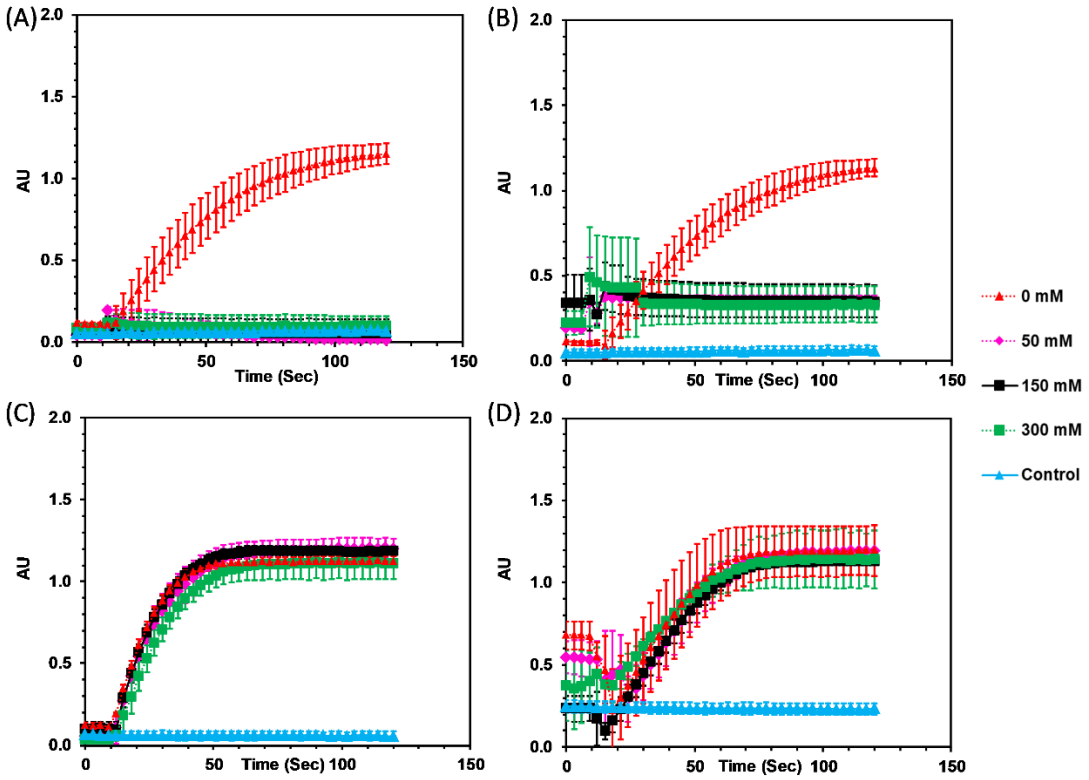
2.3.2.2 Amino Acid Ionic Liquids. Unfortunately, there is limited spectroscopic information that can be extracted from the laccase interactions with imidazolium ILs due to the high absorptivity of the imidazole ring in the low-UV region corresponding to wavelengths used in protein circular dichroism and fluorescence spectroscopy. Coupled with the desire to make biocompatible ionic liquids, the focus was shifted to amino acid based ILs. These complexes use a traditional amino acid in complex with another ion to create an ionic liquid. There is increasing interest in these ILs due to the likelihood of tolerance by cellular systems and the existing knowledge of amino acid chemistry and interactions.

Laccase activity was examined in the presence of four different amino acid ILs: TMG-Ser, TMG-Thr, TMG-Asp, and TMG-Glu (Figure 2D). These amino acid ILs share the TMG cation but vary the amino acid associated, with Asp & Glu being acidic amino acids containing a carboxyl functionality in the side chain and Ser & Thr containing a hydroxyl in the side chain. The laccase activity is shown in Figure 4. In the case of TMG-Ser and TMG-Thr, there was a complete loss of enzymatic activity under all conditions tested. Conversely, the TMG-Asp and TMG-Glu had effectively no impact on the laccase activity, exhibiting substrate conversion curves nearly indistinguishable from laccase in the absence of ILs. As a control experiment, laccase activity in the presence of free L-Serine

was also carried out and showed no inhibition of activity (Figure 9), indicating that the TMG-Ser ionic liquid is behaving differently than either of subcomponents.

Figure 4

Laccase Enzyme Activity with Amino Acid ILs



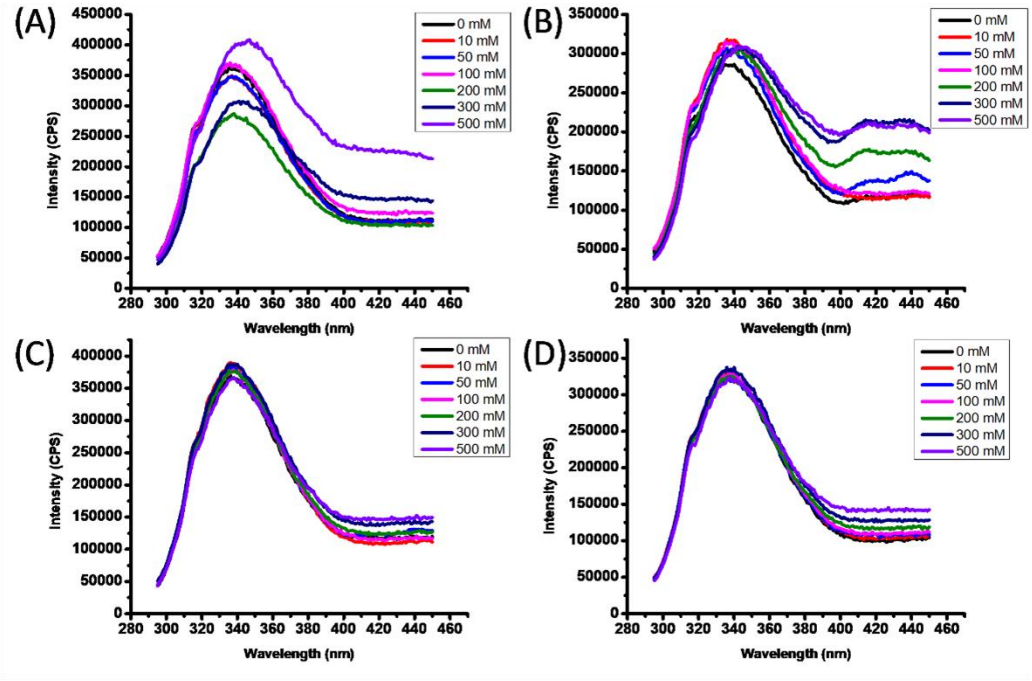
Note. The enzymatic conversion of ABTS by laccase is shown in the presence of differing concentrations of (A) TMG-Ser, (B) TMG-Thr, (C) TMG-Asp, or (D) TMG-Glu. Concentrations of IL are shown in the legend, with the "control" sample being laccase and 300 mM IL in the absence of substrate. Substrate was added at t = 10s. In all samples, laccase concentration was 5 µM, ABTS concentration was 50 µM, and absorbance was monitored at 450 nm. All data are averages of 3-7 samples with error bars representing standard deviations.

2.3.2.3 Laccase Intrinsic Fluorescence. The intrinsic Trp fluorescence of proteins is a useful tool in monitoring protein structure and folding/unfolding processes. The Trp residue is an environmentally sensitive fluorophore which exhibits a dramatic shift in emission wavelength when moving between environments of low polarity and high polarity [178, 179]. In the context of protein folding, Trp residues are often located at the interior of folded proteins where the side chain is shielded from the polar aqueous milieu, but as the protein denatures these Trp residues become more exposed to the aqueous environment resulting in a shift in fluorescence emission spectra [174].

The intrinsic Trp fluorescence from laccase was monitored in the absence and presence of varying concentrations of the amino acid ILs at pH 5.1, consistent with the conditions used for the enzyme kinetics assays. The emission spectra from these experiments are shown in Figure 5, while additional spectra collected at pH 7 are shown in Figure 10. The Trp emission spectra exhibited a concentration-dependent broadening and red shift as the concentration of TMG-Ser or TMG-Thr was added. The overall shift in the maximum emission wavelength (lmax) and spectral barycenter ranged between 5-10nm, depending on the conditions. However, the spectra did undergo significant broadening, as evidenced by changes in the ratio of intensities at 330 and 350 nm. The full set of λmax, barycenter, and 330/350 ratios can be found in Figure 11. In contrast, the spectra of laccase in the presence of TMG-Asp and TMG-Glu exhibited minimal shifting, regardless of IL concentration. Taken together, the fluorescence results are consistent with the observations in enzymatic behavior with the hydroxyl-containing amino acid ILs having significantly greater effect on the protein. The fluorescence results are indicative of protein denaturation or destabilization.

Figure 5

Laccase Fluorescence Emission Spectra At pH 5.1 In the Presence and Absence of Amino Acid Ionic Liquids



Note. In all panels, laccase concentration was 6.25 µM and amino acid IL concentrations are denoted in the legend. Fluorescence was measured in the presence of (A) TMG-Ser, (B) TMG-Thr, (C) TMG-Asp, and (D) TMG-Glu. Representative spectra are shown in each panel. Ionic liquid concentrations are shown in the legends

2.3.2.4 Molecular Dynamics Simulations. To probe the inhibition mechanism of laccase by TMG-Ser, we simulated laccase with ABTS with/without TMG-Ser. Because TMG-Thr has very similar properties to TMG-Ser and we expect our findings from TMG-Ser should be also applicable to TMG-Thr system. To check the convergence of the MD

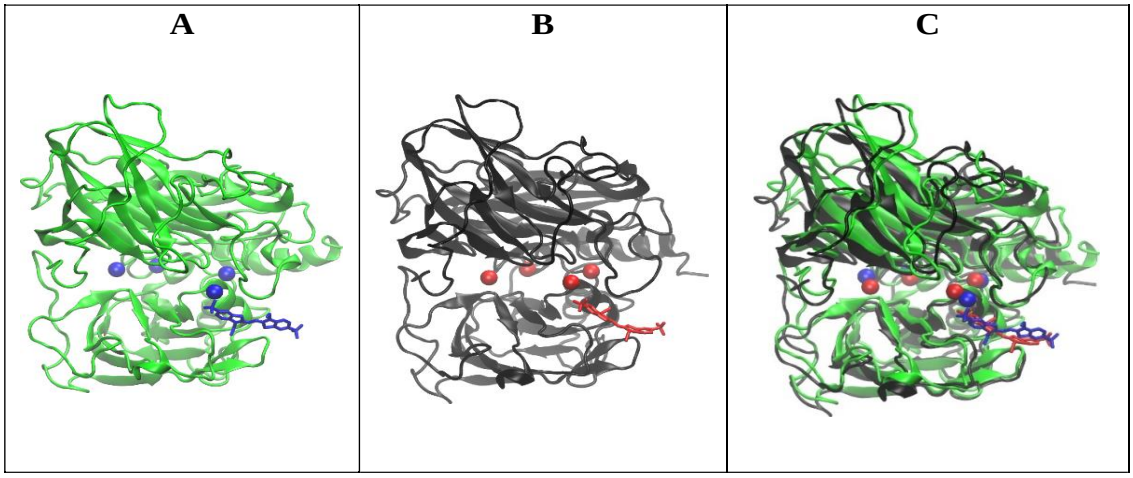
simulations, the average RMSD over three trajectories for 1000 ns in water and with TMG-Ser. We observe that laccase undergoes a conformational change in the presence of TMG-Ser, but binding of ABTS to the receptor binding pocket is generally not affected. Results showed that, there is virtually no difference in laccase RMSD in the water (1.8±0.8) and TMG-Ser systems (1.3±0.9) their values are relatively low (< 3 Å), which implies that the flexibility of laccase do not change significantly in the presence of TMG-Ser. Between the water and water/TMG-Ser systems, no significant changes were observed in the laccase protein conformation. Additionally, ABTS interaction with the substrate binding pocket occurred in all trajectories except trajectory 2 of the water/TMG-Ser system, where it has been pushed slightly out of the binding pocket and thus potentially reducing laccase activity. Interestingly, TMG cations are observed interacting with the solvent-exposed sulfonate group of ABTS as well as laccase. More specifically, Ser anions interact with the Cu^{2+} ions buried within laccase; trajectories 1 and 3 show Ser interacting with the same Cu^{2+} ion, whereas trajectory 2 show Ser interacting with another Cu^{2+} ion. These results motivated us to further study the interactions between TMG and Ser with ABTS and laccase Cu^{2+} ions to elucidate its ability to inactivate laccase.

To probe the effects of TMG-Ser on laccase protein conformation and ABTS ligand binding, clustering analysis was done for the three trajectories of each MD simulation system. Three clusters representing the major conformations of laccase and ABTS with or without TMG-Ser were generated for each system. The most abundant cluster of each system was picked and then superimposed on each other (Figure 6). The protein structure is not destabilized by TMG-Ser, thus it likely doesn't contribute to the loss in laccase activity. Interestingly, the binding of ABTS to the laccase substrate binding pocket is not

inhibited by TMG-Ser, as its orientation between the two systems is not significantly different. Therefore, a more subtle, local interaction mechanism likely explains the reduction in laccase activity by TMG-Ser. To probe the interaction mechanism between laccase protein in TMG-Ser IL, we obtained an average atom contact plot. We observed that the number of Protein-TMG and Protein-Ser contacts are not significantly different and that Polar Protein-IL contacts dominate over Nonpolar Protein-IL contacts. Protein-TMG contacts (14±4) are slightly higher than Protein-Ser contacts (12±3). This suggests that the nature of the interactions of TMG and Ser with laccase are very similar. Unsurprisingly, TMG and Ser are ionic molecules and thus would predictably form greater interactions with polar protein regions. To get detailed interaction between ILs and the protein, we plot the TMG and SER within 2 Å of Cu^{2+} ions and ABTS from the combined trajectories. Interestingly, TMG cations cluster around the two sulfonate groups of ABTS, one exposed to the solvent and the other adjacent to the T1 Cu^{2+} ion. The TMG-sulfonate interactions would likely disrupt electron transfer from a nearby phenol, and hence, the TMG-Ser likely upsets the electron flow mechanism of laccase and thus prevents the reduction of oxygen gas to water.

Figure 6

Superimposition of the Most Abundant Protein and Ligand Conformations from Each System



Note. (A) In water. (B) In TMG-Ser. (C) Superimposed image of Cluster (B) on Cluster (A).

2.4 Discussion

The enzyme laccase has garnered significant interest due to the applicability to industrial processes regarding biomaterials, biowaste, biofuels, and bioremediation [169, 171, 172]. However, the ability to extend the effective working conditions of the enzyme is of interest to become more flexible with regard to substrate preparation and reaction environment. Thus, developing ionic liquids that can maintain enzymatic activity while contributing beneficial solution properties to the samples is the ultimate goal.

The enzymatic activity of laccase is clearly impacted in the presence of some ionic liquids. The imidazolium ILs were used as a benchmark as these IL species are relatively well understood regarding protein-IL interactions. The destabilization of protein structure and activity by the imidazolium ILs has been correlated to the length of the alkyl chain

attached to the imidazole ring [60, 62, 160]. The results on laccase from *T. versicolor* are consistent with the prior findings in that enzymatic activity was reduced by increasing concentrations of IL with longer alkyl chain ILs having a more dramatic effect. The shortest chain IL, EMICl, had minimal effect on activity at 50mM solution concentration and still retained activity at 300mM concentration. The longest chain, OMICl, had dramatic effects on activity at all concentrations tested, including complete loss of activity at 300mM solution concentration. This is consistent with the protein structure being disrupted by the alkyl chains in a detergent-like manner.

The amino acid ILs were shown to disrupt activity according to side chain identity or functional groups. The enzymatic activity of laccase in the presence of TMG-Asp and TMG-Glu was effectively identical to the untreated samples. Conversely, the activity in the presence of TMG-Ser or TMG-Thr was completely eliminated at all concentrations tested. The control experiment showing no loss of activity in the presence of free serine (Figure 9) coupled with all amino acid IL species containing the TMG cation indicates there is a specific additive or synergistic interaction between TMG and the hydroxyl-containing amino acids in the deactivation of the enzyme.

Elucidating the underlying mechanism of IL-protein interactions can be a significant challenge due to physico-chemical challenges arising from the chemical nature of many ILs. In the case of imidazolium ILs, the inherent absorptivity of the imidazole ring in the low-mid UV region precludes many spectroscopic approaches traditionally used for investigating protein structure including circular dichroism and intrinsic Trp fluorescence spectroscopy [93, 180]. Similarly the amino acid ILs, due to their composition, prevent use of any spectroscopic approaches that rely on protein backbone signatures such as

circular dichroism or FTIR [181]. Thus, the approaches to investigate given IL-protein systems must be both flexible and robust as some confirmatory approaches will be inaccessible. That said, the case of laccase with amino acid ILs does allow the use of Trp fluorescence spectroscopy, a well characterized approach to monitoring protein denaturation [180, 182, 183]. The Trp fluorescence emissions spectrum typically exhibits a red-shift due to the side chain becoming more exposed to the polar, aqueous milieu upon protein denaturation. However, this environmental sensitivity can also be influenced by changes in the local environment around the side chain. Gierasch and coworkers were one of the first groups to report abnormally red-shifted Trp fluorescence despite location in a nonpolar environment, which was then confirmed to be a specific Trp-Asp interaction [184, 185]. Indeed, from the MD simulations, the laccase does not significantly change 3D structure in the presence of TMG-Ser (see discussion below). Instead, the Trp emission shift appears to be caused by the binding of Ser near the T3 copper ion. This location is in close proximity to two of the Trp residues in laccase (Trp65 and Trp107). This portion of the protein structure did exhibit some increased flexibility in the simulations with TMG-Ser as evidenced by the small changes in copper ion positions (see below). Thus, it appears the overall increase in local polarity and the potential interactions between the Trp side chains and the hydroxyl and/or carboxyl groups of Ser cause the emission shift.

From the MD simulations, we observed that laccase protein conformation doesn't change significantly and ABTS is still able to bind to the laccase catalytic site in the presence of TMG-Ser. Therefore, the change in laccase's global conformation induced by TMG-Ser does not explain its reduced activity. We attributed the inactivation of laccase to localized charge-charge interactions and hydrogen bonding interactions between TMG-

ABTS and Ser-T1 Cu^{2+} ion, and to some degree Ser-T3 Cu^{2+} ion, thereby disrupting its electron chain transfer mechanism. It's thus necessary to explain laccases electron transfer mechanism to understand the combined role of TMG and Ser in disrupting this process. First, a phenolic molecule (i.e. lignin) transfers an electron to a co-oxidizing agent and becomes oxidized (Phenol $\xrightarrow{e^-}$ ABTS). Upon binding to laccase's substrate binding pocket, reduced ABTS then transfers its electron to the T1 laccase Cu^{2+} ion (ABTS $\xrightarrow{e^-}$ T1 Cu^{2+}). T1 Cu^{2+} ion can then transfer its electron to a T3 or the T2 Cu^{2+} ions (T1 Cu^{2+} $\xrightarrow{e^-}$ T2/T3 Cu^{2+}). T2 and T3 Cu^{2+} ions contain binding sites for dioxygen, where the final electron transfer occurs to produce water (T2/T3 Cu^{2+} $\xrightarrow{e^-}$ O_2) [186, 187]. TMG-Ser likely reduces laccase activity by disrupting its electron transfer mechanism at two critical points: 1.) TMG: Electron transfer from phenols to ABTS and 2.) Ser: Electron transfer from ABTS to T1 Cu^{2+} ion. Interestingly, under physiological conditions, the sulfonate groups of ABTS, one exposed to the solvent and another close to the T1 Cu^{2+} ion, are deprotonated and become anions. TMG could potentially form a salt-bridge interaction with the solvent-exposed sulfonate and disrupts its ability to accept an electron from a phenol. Simultaneously, Ser could potentially form a hydrogen bond between its hydroxyl side chain and the deprotonated oxygen of the other sulfonate group, disrupting its electron transfer to the T1 Cu^{2+} ion. To some degree, Ser anions also clustered between the T1 and an adjacent T3 Cu^{2+} ion; again, the hydroxyl group of Ser may disrupt electron transfer between T1 and T3 Cu^{2+} ions.

Based on the results, TMG-amino acids with hydroxylated side chains seem quite critical for inactivating the redox catalysis reaction of laccase; conversely, TMG-amino

acids with carboxylated side chains are relatively ineffective. This might be an issue of the water solubility of each IL. Upon dissociation from TMG, Ser and Thr may likely form less interactions with water and allow them to interact with the T1/T3 Cu^{2+} ions. On the other hand, Asp and Glu may form too many interactions with water and thus prevent interaction with the T1/T3 Cu^{2+} ions.

The selective inhibition of laccase by TMG-Ser and TMG-Thr could potentially be extended to other biologically-important redox enzymes containing metal cofactors. One example is superoxide dismutase, which utilizes Cu^{2+} and Zn metals to convert superoxide radicals to hydrogen peroxide and water [refs]. More importantly, TMG-Ser and TMG-Thr could be used to treat infectious diseases, such as COVID-19. The protein RNA-dependent polymerase (RdRp) contains two Mg^{2+} ions to catalyze viral replication of SARS-CoV-2 [188-190]. The function of this RdRp is dependent on the presence and correct positioning of the Mg^{2+} ions. Thus, interactions between Ser and Thr with Mg^{2+} may inactivate RdRp and stop viral replication.

2.5 Conclusions

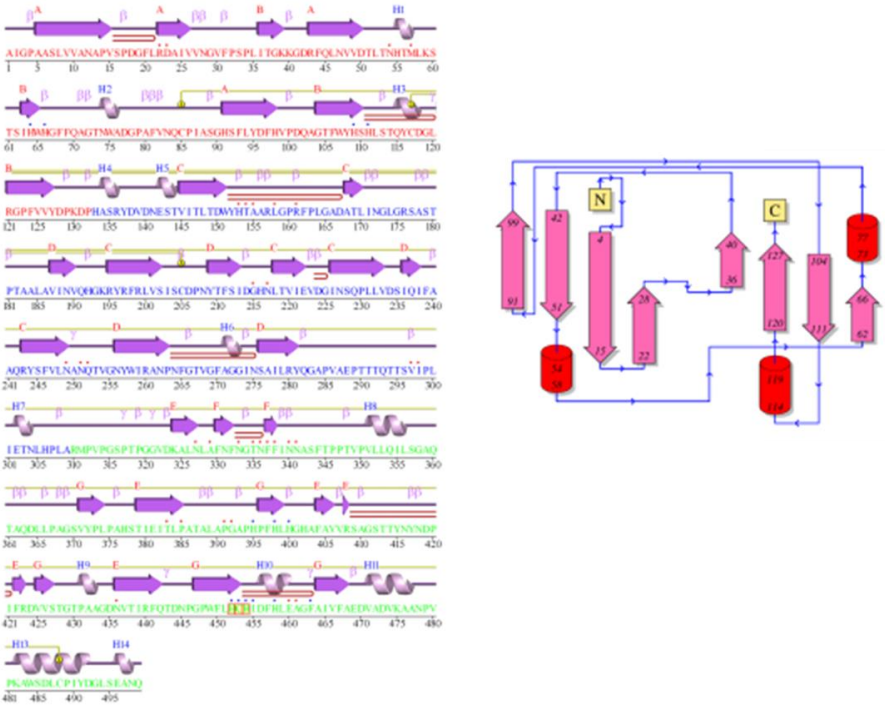
The combination of experiments and MD simulations of laccase with/without TMG-Ser elucidates how TMG-Ser causes inactivation of laccase. While fluorescence studies indicate there is some structural perturbation in the protein, simulations indicate the TMG-Ser interactions do not dramatically change the protein structure or the ligand binding conformation. Simulations show that TMG and Ser both largely interact with polar protein regions. TMG interacts with the sulfonate group of ABTS via ionic interactions while Ser interacts with the T1 and T3 Cu^{2+} ions via weaker hydrogen bonding interactions, leading to the conclusion that TMG-Ser disrupts the electron transfer mechanism of laccase and

thus reduces its activity. Taken together, the combination of simulations and experiments prove to be invaluable in the elucidation of the mechanism for amino acid ILs and, with more experiments and simulations on related systems, can be the basis of developing predictive models for IL-biomolecular interactions.

2.6 Supporting Figures

Figure 7

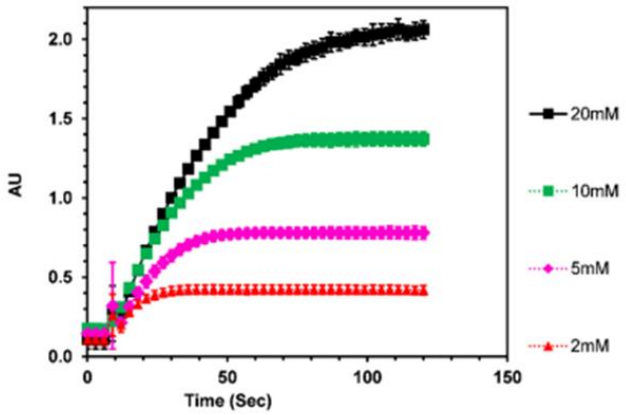
Primary Structure, Secondary Structure and Topology of T. Versicolor Laccase Protein



Note. Obtained from PDB Server (PDB ID: 1GYC)

Figure 8

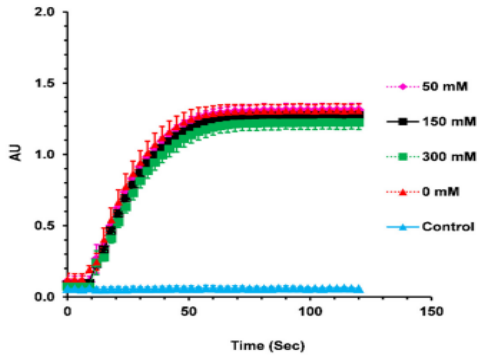
Laccase Enzymatic Activity with Variable Substrate Concentration



Note. Conversion of ABTS by laccase at pH 5.1 was monitored over time. ABTS concentrations are shown in the legend. Data points are the average of 3-7 replicates and error bars represent standard deviations.

Figure 9

Laccase Enzymatic Activity in the Presence of Free Serine

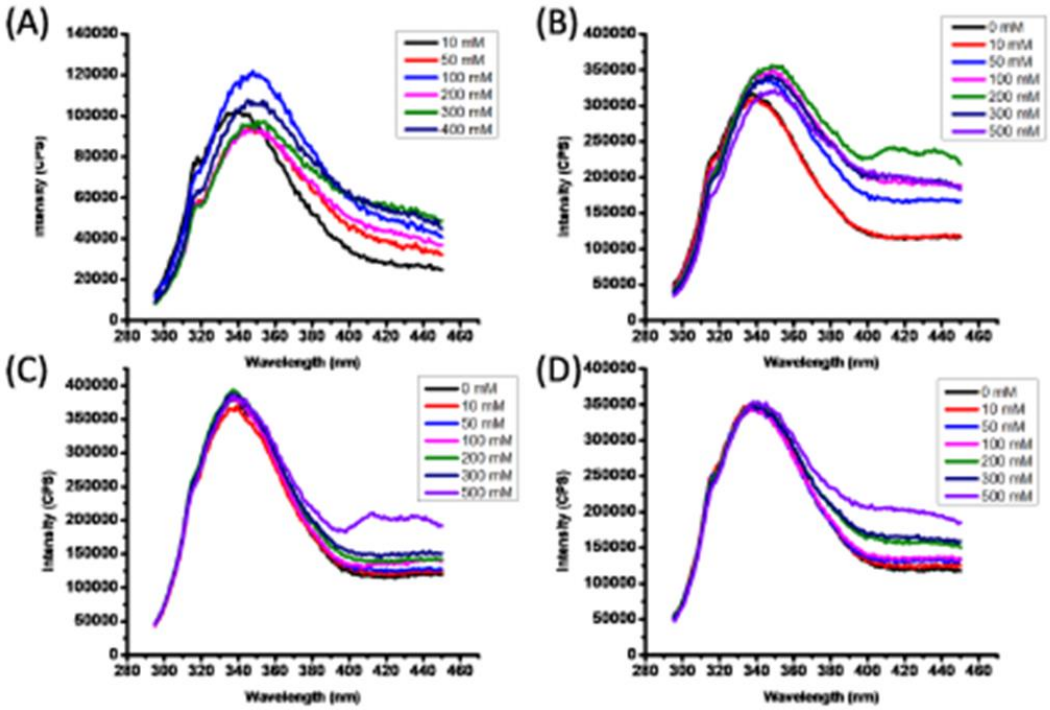


Note. Conversion of ABTS by laccase at pH 5.1 was monitored over time. ABTS concentrations were constant in all samples. Serine concentrations for each sample are

shown in the legend. The control data refers to samples that contained laccase and 300 mM serine but no ABTS. Data points are the average of 3-7 replicates and error bars represent standard deviations.

Figure 10

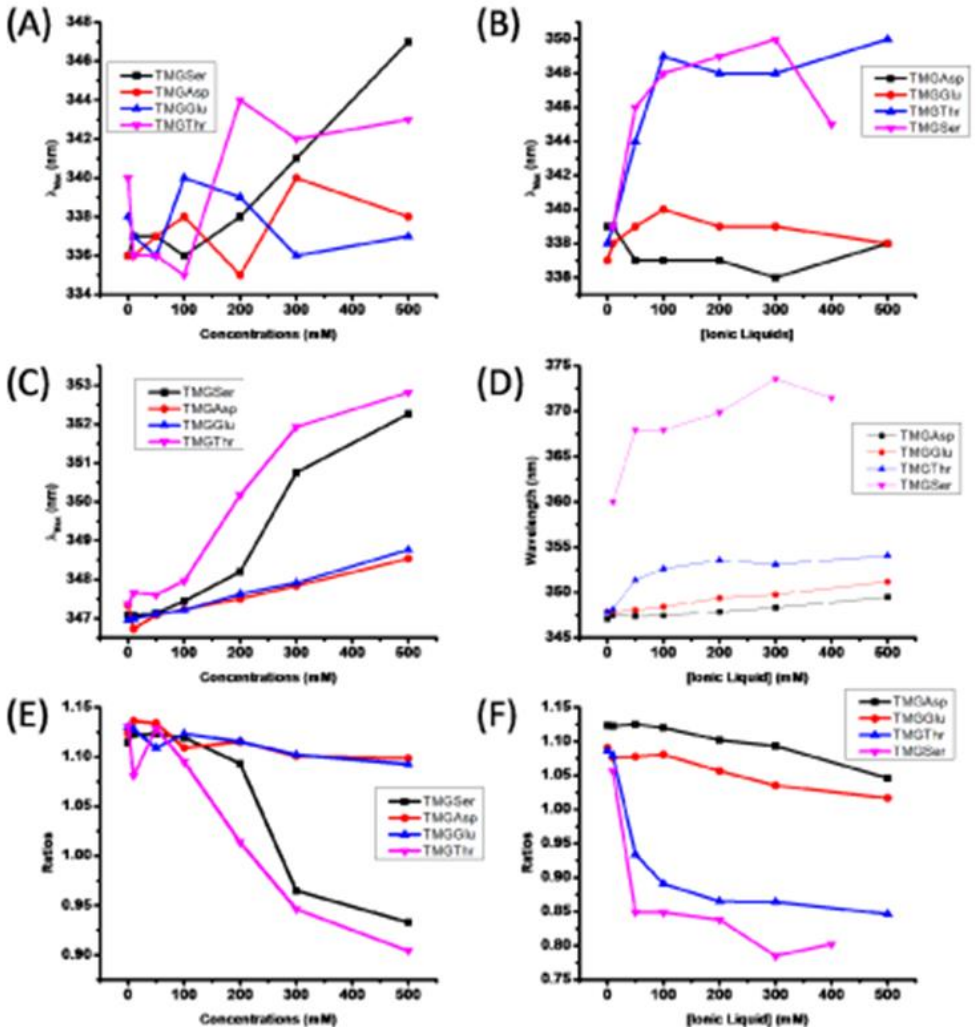
Laccase Fluorescence Emission Spectra at pH 7.1 in the Presence and Absence of Amino Acid Ionic Liquids



Note. In all panels, laccase concentration was 6.25 µM and amino acid IL concentrations are denoted in the legend. Fluorescence was measured in the presence of (A) TMG-Ser, (B) TMG-Thr, (C) TMG-Asp, and (D) TMG-Glu. Representative spectra are shown in each panel. Ionic liquid concentrations are shown in the legends

Figure 11

Laccase Fluorescence Emission Spectral Properties at pH 5.1 or 7.1 in the Presence and Absence of Amino Acid Ionic Liquids



Note. In all panels, laccase concentration was 6.25 mM and amino acid IL identities are denoted in the legends. Fluorescence properties measured were (A) λmax at pH 5.1, (B) λmax at pH 7.1, (C) spectral barycenter at pH 5.1, (D) spectral barycenter at pH 7.1, (E) I350 /I330 at pH 5.1, and (F) I350 / I330 at pH 7.1.

Chapter 3

Effects of IL on Other Metalloproteins

3.1 Introduction

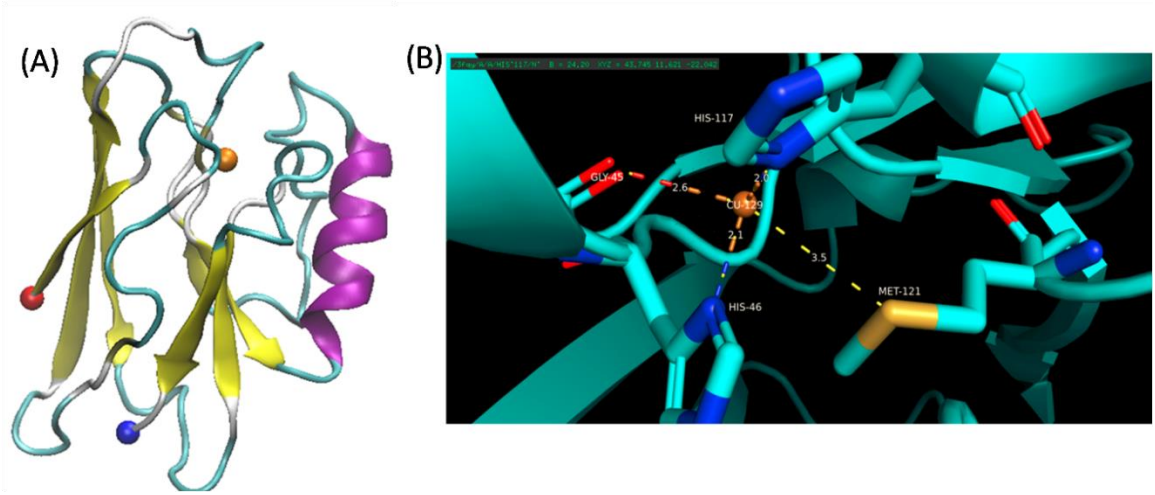
Apart from laccase, effect of ILs on many other metalloproteins have also been investigated. Because of protein specific interaction of ILs with metalloproteins, along with having varieties of ILs chemistry, it is unlikely there will be a set of hard and fast rules that define specific ILs interactions with metalloproteins. Therefore, this literature review focuses on understanding the impact of various ILs on azurin, myoglobin, alcohol dehydrogenase, and horseradish peroxidase (HRP). The primary purpose for these studies was to understand the how the ILs will influence folding and/or unfolding behavior of these proteins.

3.1.1 Effects of IL on Azurin

The blue copper protein, azurin, is part of the azurin-nitrate reductase redox protein complex. This protein is involved in denitrification metabolism in bacteria [92, 191]. The presence of copper is necessary for protein stability. It is a small protein that can be produced from two bacterial strains – *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* [192]. The azurin structure from *P. aeruginosa* consists of a hydrophobic alpha helix, six short beta sheets and a random-coil that allows for copper-binding [92, 193, 194] (Figure 12). Notably, azurin exhibits the most blue-shifted Trp emission spectrum from naturally derived proteins, arising from the single Trp residue at position 48 [195]. This is attributed to the very hydrophobic interior of the protein, which also includes the copper binding site. The Cu^{2+} is coordinated by Gly45, His46, Asn47, Cys112, Phe114, His117, and Met121 [195].

Figure 12

Structure of Azurin from P. Areuginosa



Note. The crystal structure was solved by Adman and Jensen; downloaded from rcsb.org (1AZU([194])[146] The structure was visualized using VMD. (A) 3D structure of azurin. The N- and C-termini are shown as red and blue spheres respectively while the copper is shown in orange (partially occluded in the structure). (B) Structural geometry of the copper shown in orange and chelating residues highlighted.

ILs can affect the protein structure and its stability based on several characteristics. As a protein with a mixed structure, azurin's stability is affected in the presence of ILs. Recently we demonstrated 1.0 M alkyl-imidazolium chloride ILs in aqueous solutions were seen to have a variable effect on azurin; the three ILs were [BMIM][Cl], [HMIM][Cl], and [OMIM][Cl]. The difference in these three ILs are the length of the alkyl chains and hydrophobicity. Due to less hydrophobicity, [BMIM][Cl] and [HMIM][Cl] have some interactions at the surface of the protein. Furthermore, these ILs denature the secondary

structure completely at a high temperature at 55˚C and the tertiary structure slowly at 65˚C. Thermodynamically, it can be observed that the ionic liquids affect that the destabilization in terms of entropy; there is an increase in entropy, as ILs increases the disorder of the unfolded protein. In general, all three ILs affect the structure of the protein by making it less rigid and flexible, while maintaining the secondary and tertiary components of the protein. [OMIM][Cl] destabilized azurin, due to a high ΔS_u compared to the ΔS_u of [BMIM]Cl and [HMIM]Cl, which was lower. Furthermore, [OMIM][Cl] was able to destabilize the protein much faster, proving that [OMIM][Cl] is stronger than the other ILs presented in this study, which were consisting of smaller alkyl chains and a decreased level of hydrophobicity [92]. It is important to note that at the concentrations tested, the [OMIM][Cl] has been shown to form micelles, These micelle structures likely impact the interactions with the protein, and can potentially form mixed structures with the protein. [92].

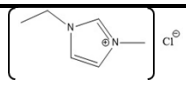
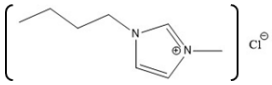
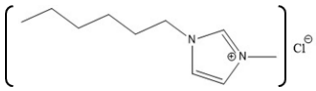
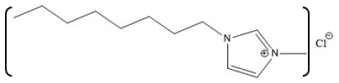
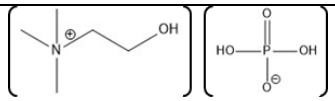
In a study by Fujita *et al.*, the interaction between the hydrated IL [Chol][Dhp] and several metalloproteins, such as azurin and pseudoazurin, was investigated. The study focused on the solubility and properties of the proteins dissolved in 70 wt% [Chol][Dhp]. Specifically, in [Chol][Dhp], it was found that these proteins, when dissolved, do not have any disturbance to the active sites found in the proteins. Notably, not all proteins tested were soluble under these conditions. Among those that were soluble, the retention of structural elements was supported by spectral signatures in Raman and CD spectra. Notably, resonance raman spectra showed the peaks near 260 cm^{-1} and 400cm^{-1} for Cu-N and Cu-S, respectively, which was consistent with the spectra for azurin in its native

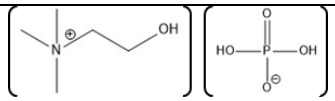
conformation. This indicated that the protein retained its structure and function when dissolved with the IL [196].

In another study the same IL, hydrated [Chol][Dhp], was studied to understand the interaction between the IL and azurin, specifically focusing on the redox reaction rate for azurin (dissolved in the IL) and the SAM-AuNP electrode. In the presence of this IIL, it was found that the proteins were able to maintain their structure, showing long term and thermal stability. Similar to the previous study explained above, it was found that the active site of the protein was maintained in the presence of the IL using Raman spectroscopy. It was also found that electron transfer rate constant (k_s) between azurin and the electrode in the IL (202 s^{-1}) was found to be larger than that of the ammonium acetate buffer solution (44 s^{-1}) and the reason for this difference could possibly be due to protein shrinkage. Both the buffer and the IL showed that electron transfer reactions were possible at a fast rate; this would mean that this fast rate would be much more stable over a broad range of temperature values and a longer time period for the IL [197].

Table 2

Summary of Studies Done Pertaining to Monitoring Effect of ILs on Azurin

Azurin source	IL	Structure	Study done	Results	ref
Azurin from P. *aeruginosa*	1-ethyl-3-methylimidazolium chloride, [EMIM][Cl] 1-butyl-3-methylimidazolium chloride, [BMIM][Cl] 1-hexyl-3-methylimidazolium chloride, [HMIM][Cl] 1-octyl-3-methylimidazolium chloride, [OMIM][Cl]	   	Temperature-Dependent Fluorescence Spectroscopy Temperature-Dependent IR Spectroscopy IR and VCD Spectroscopy Temperature Jump Kinetics	ILs affected the protein structure by destabilizing it; however, the degree to which the protein unfolded is dependent on the ionic liquid in terms of hydrophobicity and alkyl chain length.	[198]
Azurin II was purified and expressed from *Alcaligenes Xylosoxidans*	Hydrated choline dihydrogen phosphate, [Chol][Dhp].		CD Measurement Raman Spectroscopy Enzymatic Activity Assay	The protein and IL didn't have an interaction that caused a disturbance in the structure or function of the protein.	[124]

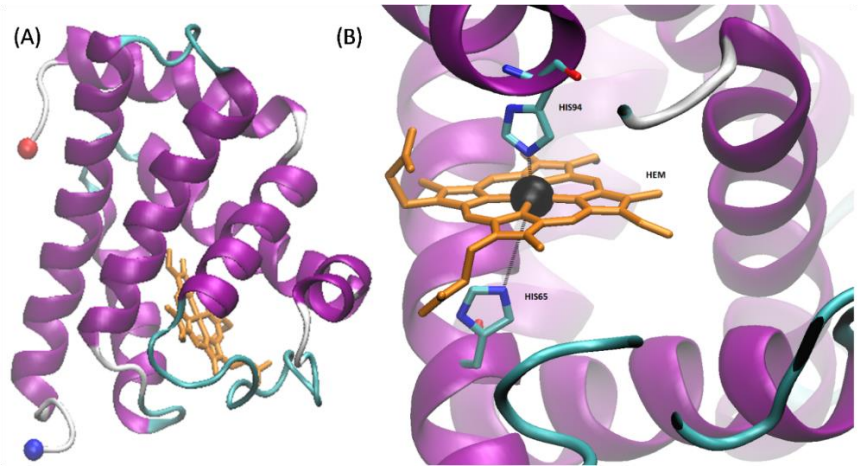
Azurin source	IL	Structure	Study done	Results	ref
(Az). Pseudo azurin was isolated from *Achromobacter cyclastes* IAM 1013 (Paz).					
Azurin from *P. aeruginosa*	Hydrated choline dihydrogen phosphate [Chol][Dhp].		Raman Spectroscopy Direct electrochemistry of azurin performed on SAM-AuNP Electrode	The protein maintained its structure and its active site in the presence of the IL. Fast and stable electron transfer reactions could occur over a range of temperature values at longer periods of time.	[199, 200]

3.1.2 Effect of ILs on Myoglobin

Myoglobin (Figure 13) is a water-soluble globular protein of 150 amino acids involved in transport and storage of oxygen found in mammalian muscle tissues [201, 202]. Like laccase, myoglobin is a metalloprotein having an iron atom incorporated in the heme group which together are involved in reversibly binding oxygen [203]. The heme binding site of the protein contains two His residues, one (proximal) is attached directly to the heme iron and the other (distal) is on the opposite face of the heme but does not bind the iron, instead being available for binding to O_2. The presence of this iron imparts a reddish-brown color to the protein and yields an intense absorption band at ~409 nm [204]. The heme group is buried under a hydrophobic pocket of the myoglobin in its native folded state, however, upon unfolding, the heme group is exposed to the aqueous environment, resulting in decrease in the absorption at ~409 nm [204]. Because of these easily interrogated absorbance properties, myoglobin has been widely used as model protein to understand folding and unfolding kinetics as a function of the varieties of conditions involving not only thermal, pH, and mechanical stress, but also wide range of denaturants such as detergents, organic solvents, and ILs [205-208].

Figure 13

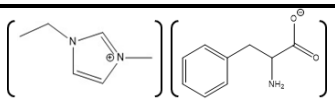
Structure of Myoglobin from Cardiac Muscle of E. Caballus

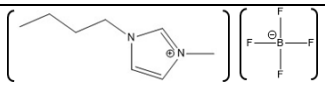
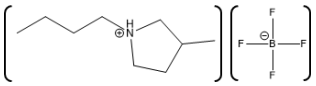
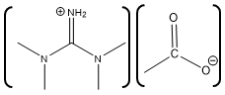


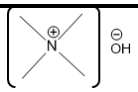
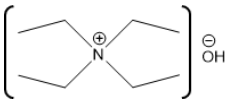
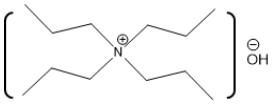
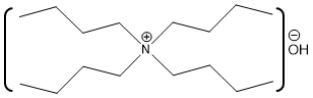
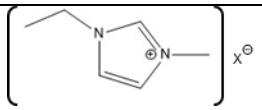
Note. The crystal structure was solved by Brayer and coworkers; downloaded from rcsb.org (1WLA) [146, 209]. The structure was visualized using VMD. (A) 3D structure of myoglobin. The N- and C-termini are shown as red and blue spheres respectively while the heme is shown in orange (partially occluded in the structure). (B) Structural geometry of the heme with the iron shown in black and chelating residues highlighted.

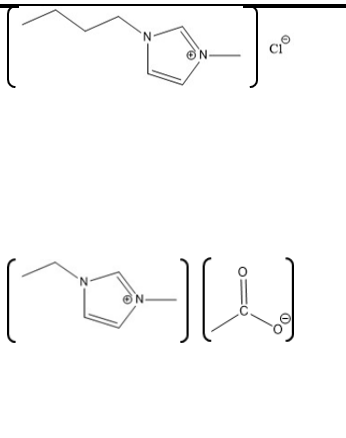
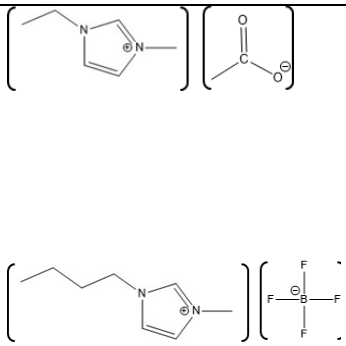
Table 3

Summary of Studies Done to Monitoring Effect of ILs on Myoglobin

Myoglobin source	**IL**	**Structure**	**Study done**	**Results**	**Ref**
Horse-heart myoglobin	1-ethyl-3-methylimidazolium phenylalanine		Fluorescence and circular dichroism spectroscopy	Small concentrations of increase helicity and stabilize protein, while higher	[198]

Myoglobin source	IL	Structure	Study done	Results	Ref
	[EMIM][Phe]			concentrations lead to increase in beta structures	
Horse-skeletal myoglobin	1-butyl-3-methyl imidazolium tetrafluoroborate ([BMIM][BF_4]);		Temperature stability studies, HDX experiments, Unfolding Kinetics Measurements	ILs enhances myoglobin unfolding kinetics	[124]
	1-butyl-3-methyl pyrrolidinium tetrafluoroborate ([Pyrr][BF_4]);				
	1-ethyl-3-methylimidazolium acetate [EMIM][Ac]); Tetramethyl guanidinium acetate [TMG][Ac])				

Myoglobin source	IL	Structure	Study done	Results	Ref
Horse-skeletal myoglobin	Tetramethyl ammonium hydroxide [TMA][OH];		Fluorescence and circular dichroism (CD)	Decreases thermal stability of myoglobin	[199]
	Tetraethyl ammonium hydroxide				
	[TEA][OH];				
	Tetrapropyl ammonium hydroxide				
	[TPA][OH];				
	Tetrabutyl ammonium hydroxide				
	[TBA][OH]				
Salt free myoglobin (Mb)	1-butyl-3-methylimidazolium cation [BMIM]+	 X = SCN^-, HSO_4^-, Cl^-, Br^-, CH_3COO^- and I^-	Uv vis spectroscopy, fluorescence spectroscopy, Circular Dichroism	A negative impact on the stability of Myoglobin, a sharp decrease in the transition temperature (Tm) of the myoglobin	[210]

Myoglobin source	IL	Structure	Study done	Results	Ref
Horse-skeletal myoglobin	1-butyl-3-methylimidazolium chloride ([BMIM]Cl); 1-ethyl-3-methylimidazolium acetate ([EMIM]Ac); 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4])		Detergent (N,N-dimethyl-N-dodecylglycine betaine) induced denaturation and heme-loss from myoglobin monitored by fluorescence and circular dichroism	ILs have no significant effect on heme dissociation as well as denaturation of myoglobin	[61]
Horse-skeletal myoglobin	Ethylmethylimidazoliu m acetate ([EMIM]Ac) and Butylmethylimidazolium boron tetrafluoride ([BMIM][BF_4])		Guanidin HCl induced myoglobin unfolding by combined absorption/fluorescence spectroscopic	[EMIM]Ac does not affect myoglobin unfolding (up to 150 mM), while [BMIM][BF_4] facilitated myoglobin unfolding	

In one study, the results suggested that ILs containing sulfate or phosphate ions and having higher viscosity such as diethylammonium sulfate ([DEA][SO_4]), triethylammonium sulfate ([TEA][SO_4]), dihydrogen phosphate ([DEA][P]), triethylammonium dihydrogen phosphate ([TEA][P]), Trimethylammonium dihydrogen sulfate ([TMA][SO_4]) and Trimethylammonium dihydrogen phosphate ([TMA][P]) improve the stability of the myoglobin [208]. On the other hand, they also reported that less viscous ILs having acetate anions such as diethylammonium acetate ([DEA][Ac]), triethylammonium acetate ([TEA][Ac]), diethylammonium and Trimethylammonium acetate ([TMA][Ac]) were shown to destabilize myoglobin structure. One hypothesis is that ILs affect the stability of a protein by altering the hydration later (i.e. layer of water molecules around the protein). Specifically, in this case, the authors postulated that phosphate-containing ILs significantly interact with the myoglobin polypeptide chain and hence are repelled from the protein. In addition, because of these repulsions this IL also helps to provide better structure to the hydration layer, improving the stability of the protein [208]. As the acetate ions have greater affinity toward the polypeptide chain of myoglobin, they penetrate deep inside the protein structure and interact with amino acids of the polypeptide. Therefore, acetate ions present in ILs also disturb the native hydrogen bonding pattern as well as interactions of the protein with the hydration layer, resulting in protein destabilization. Further, results have indicated that anionic variation in the ILs has greater impact on stability of myoglobin compared to the cationic variations [208].

Table 4

Effect of Various ILs on the T_m and Secondary Structure of Myoglobin

Sample	Fluorescence T_m (°C)	DSC T_m (°C)	α-Helix (%)	β-Strand (%)
Buffer	65.1	67.0	56.12	7.77
[TEA][P]	87.1	86.8	69.92	1.57
[DEA][P]	84.0	78.9	64.05	3.11
[TMA][P]	83.1	77.9	61.13	4.23
[TEA][SO_4]	76.0	75.8	60.13	5.23
[DEA][SO_4]	74.2	73.4	58.76	4.38
[TMA][SO_4]	73.0	75.8	57.12	6.77
[TEA][Ac]	56.3	62.4	53.52	8.87
[DEA][Ac]	54.2	56.7	32.42	22.82
[TMA][Ac]	52.0	54.5	30.62	25.96
Urea (1 M)	NA	NA	54.63	7.23

Note. NA=data not available; Effect of various ILs on the melting temperature from fluorescence and DSC along with secondary structure composition of myoglobin determined from Far-UV CD spectra (adapted from reference [208]).

In work from Zhang et al. it was demonstrated that variation in the cation can also influence the stability of myoglobin [211]. They demonstrated that GuHCl-induced denaturation midpoints of myoglobin was not altered when interacted with phosphate buffer having 150 mM of various ILs differing only in their anions such as BF_4^-, NO_3^-, Cl^-, and Br^- , while keeping the same cation 1-butyl-3-methylimidazolium ($BMIM^+$) [211]. Furthermore, they have shown that increasing length of alkyl chain of imidazolium cation in the ILs affects denaturation of the myoglobin and the denaturation midpoint were found

to be [HMIM][BF_4]<[BMIM][BF_4]<[EMIM][BF_4]<buffer. Additionally, hydroxy-substitution on the imidazolium cation also enhanced the denaturation of the myoglobin [211]. These differences in variation in the effect of various ILs on their capability to stabilize or destabilize the protein structure is still an unresolved question.

While some previous studies demonstrated positive or negative impact of ILs on the stability of the myoglobin, other studies demonstrated that some ILs are inert toward the stability of myoglobin. For instance, the effects of [BMIM][C]l, [EMIM][Ac], [Pyrr][BF_4] and [TMG][Ac] was investigated [124]. The results from this study indicated that these four ILs accelerate myoglobin unfolding kinetics not only due to changes in the aqueous solution ionic strength, but also due to IL-specific interactions [124]. While, in another study, [EMIM][Ac] did not impact myoglobin stability, but the IL [BMIM][BF_4]drastically reduced the free energy required for myoglobin unfolding and hence significantly destabilized the myoglobin structure [60].

In addition, impact of ILs on the detergent-mediated denaturation of myoglobin was also evaluated. According to one study, inclusion of a series of ILs such as 1-butyl-3-methylimidazolium chloride (BMICl), 1-ethyl-3-methylimidazolium acetate(EMIAc), and 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIBF_4$) in aqueous solution had negligible impact on the detergent N,N-dimethyl-N-dodecylglycine betaine induced denaturation and heme-loss from myoglobin [61]. In another study, the effect of alkylated imidazolium chlorides based ILs such as [EMIM][Cl], [BMIM][Cl], [HMIM][Cl], and [OMIM][Cl] was tested on unfolding of myoglobin in the presence of different detergents such as N,N-dimethyl-N-dodecylglycine betaine (zwitterionic; Empigen BB®, EBB), tetradecyltrimethylammonium bromide (cationic; TTAB), and sodium dodecyl sulfate

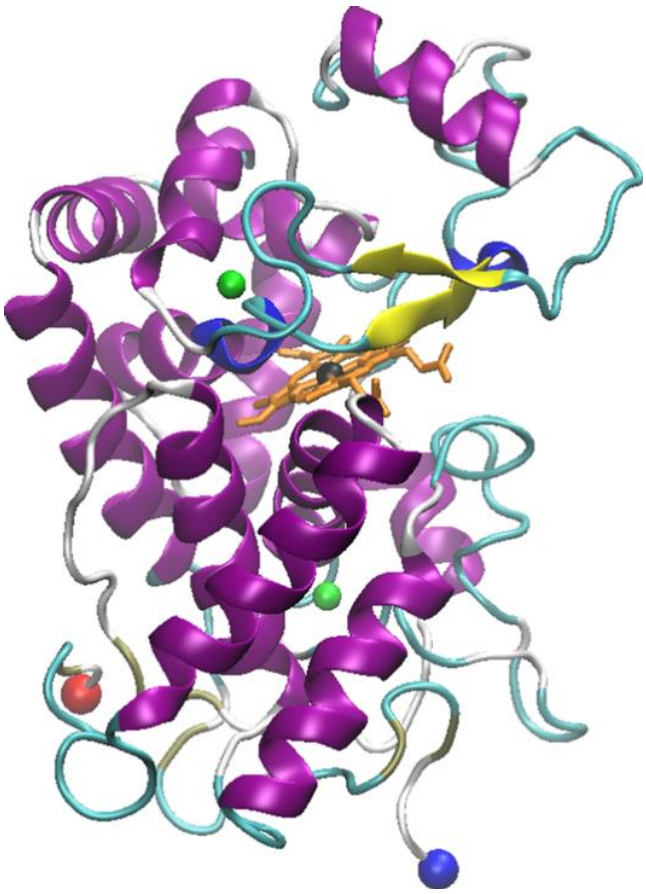
(anionic; SDS) [62]. It was observed that, presence of ILs does not affect the EBB- and TTAB-induced dissociation of heme, however, SDS-induced dissociation is affected by presence of ILs. Furthermore, it was found that, heme dissociation follow a cooperative process at low IL concentration, while at high IL concentration the heme dissociation occur via more complex pattern, which could be due to micellization of the ILs or their direct interactions with the myoglobin [62].

3.1.3 Effect of ILs on Horseradish Peroxidase (HRP)

Horseradish peroxidase (HRP) is an enzyme having two different metal ions namely, a ferrous ion incorporated in a heme group and a calcium ion (Figure 14). Notably, the heme-iron is directly involved in the catalytic reaction center, while the calcium is structural [212]. The effect of various ILs on activity of the HRP was evaluated using chromogenic substrates. In one study, the effect of various ILs as well as hemin and calcium cofactors were evaluated for effects on the refolding properties of HRP. This study used ILs with varying anions such as EMIM with Ac^-, $BF4^-$, Cl^-, ES^-, and TfO^-, as well as with different alkyl chain lengths such as $EMIM^+$, $BMIM^+$, $HMIM^+$, and $OMIM^+$ [213]. Among various tested anions, Cl^- based ILs showed highest enzyme activity, while, among various ILs having different alkyl chain lengths, EMIM showed highest enzyme activity [213]. Notably, in the presence of IL [BMIM][PF_6], the activity of HRP was also shown to be enhanced [213]. Moreover, HRP immobilized on a sol-gel matrix prepared from [BMIM][BF_4] and silica was shown to have 30-fold higher activity compared to that of the enzyme immobilized on only silica gel [214].

Figure 14

3D Structure of Horseradish Peroxidase from A. Rusticana



Note. The crystal structure was solved by Hajdu and coworkers; downloaded from rcsb.org (1W4Y)[146, 212]. The structure was visualized using VMD. The N- and C-termini are shown as red and blue spheres respectively while the calcium ions are shown in green, the heme in orange and the heme-iron in black.

A tailor-made IL specifically designed to work with HRP was also developed, which has the cation tetrakis(2-hydroxyethyl)ammonium 2 possible anions:Cl^- or $[CF_3SO_3]$ [215]. This tailor-made IL has a structure similar to TRIS (buffer), possessing four hydroxyethyl moieties. Improvement in the enzyme activity was observed with the tailor-made ILs

compared to that of the common, commercially available ILs such as [BMIM][Cl], [BMIM][CF_3CO_2], [BMIM][alanine], [BMIM][CF_3SO_3], [BMIM][BF_4], and a hydrophobic IL [BMIM][PF_6] [215]. In addition, the effect of [BMIM][Cl] and [BMIM][BF_4] on the thermal stability of the horseradish peroxidase was also evaluated. The results of the study showed that [BMIM][BF_4] is capable of improving the thermal stability of the horseradish peroxidase when used at a concentration of 5-10% (v/v) [216]. Furthermore, [BMIM][BF_4] is also capable of enhancing the reaction yield and purity for the reactions converting water insoluble phenolic compounds to a novel compound 4-phenylphenol ortho dimer [2,2′-bi-(4-phenylphenol)] [217]. However, the enzymatic catalysis was sensitive to solution pH with the best catalytic activity observed with [BMIM][BF_4] (90% v/v IL in water) at pH>9. The enzyme activity was found to decrease as the pH was shifted toward neutral and as pH decreases further, the [BMIM][BF_4] exerts inhibitory action on the HRP attributed to the tetrafluoroborate anion releasing fluoride ions which bind with the heme iron group [217].

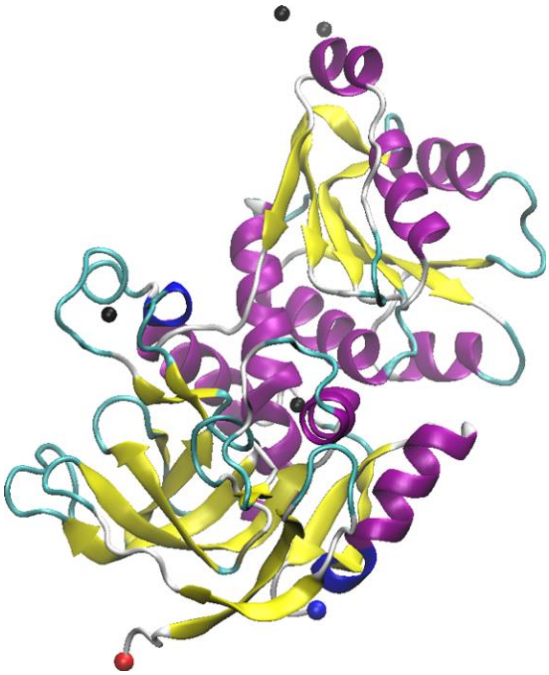
3.1.4 Effect of ILs on Alcohol Dehydrogenase

Alcohol dehydrogenase is another commonly studied metalloenzyme which has zinc ions in the active structure. The *S. cerevisiae* alcohol dehydrogenase has a homotetrameric structure with each subunit having a zinc ion in the catalytic center (Figure 15) [218]. The major function of this enzyme is to carry out oxidation of alcohols using the co-substrate β-nicotinamide adenine dinucleotide (NAD^+). This is a thoroughly studied model system that, in yeast, converts acealdehyde into ethanol along with formation of NADH and H^+. The active site contains the Zn^{2+} atoms coordinated by Cys, His, and Glu residues [219]. In one study, the activity and stability of the yeast alcohol dehydrogenase was evaluated in

solutions containing various ILs including 1-methylimidazolium chloride ([MIM][Cl]). The data showed that the order of activity enhancement was [BMIM][Cl] > [BMIM][BF_4] > [MIm][BF_4] ~ [MIM], while the order of stability was found to be [MIM][Cl] > [MIM][BF_4] > control (no ILs) > [BMIM][BF_4] > [BMIM][Cl]. The structural similarity of the cationic group of [MIM][Cl] with the adenine moiety of NAD^+ was proposed to allow interaction with the active site and hence stabilize the enzyme at higher temperature [220]. In another study, the effect of [BMIM][PF_6] on the yeast alcohol dehydrogenase was investigated and the data indicated a rapid decrease in the activity of the enzyme as a function of [BMIM][PF_6] concentration [221].

Figure 15

3D Structure of Alcohol Dehydrogenase From S. Cerevisiae



Note. The crystal structure was solved by Ramaswamy and coworkers; downloaded from rcsb.org (5ENV)([146, 218]. The structure was visualized using VMD. The N- and C-termini are shown as red and blue spheres respectively while the zinc ions are shown in black. The structure represents one monomer of a homotetramer.

The effect of variation of the anionic and cationic moieties in the ILs has also been investigated on the stability of the yeast alcohol dehydrogenases [222]. Regarding anion variation in the ILs, [EMIM] was used as a fixed cation with different anions forming [EMIM][Cl], [EMIM]Br, [EMIM][$EtOSO_3$], [EMIM][TfO], [EMIM][BF_4], [EMIM][dca], [EMIM][SCN], [EMIM][NTf_2] [222]. In the same study, [Cl] was used as fixed anion with different cations forming NaCl, [Me_4N][Cl], [Chol][Cl], [EMIM][Cl], [Et_4N][Cl], [Bu_4N][Cl], [Gdm][Cl], [BMIM][Cl]. The results of this study showed that [EMIM][Cl] and [Me_4N][Cl] have enzyme enhancing effects on the yeast alcohol dehydrogenase, while enzyme deactivating ILs are found to have anions in the order of $Br^- > [EtOSO_3]^- > [TfO]^- > [BF_4]^- > [dca]^- > [SCN]^-$ [222]. On the other hand, for variation in the cation, the enzyme deactivating order was found to be $[Chol]^+ > [EMIM]^+ > [Et_4N]^+ > [Bu_4N]^+ > [Gdm]^+ > [BMIM]^+$, while [EMIM][$NTf_2$] was found to have strongest deactivating effect [222]. In addition, the effect of ILs on a bacterial alcohol dehydrogenase obtained from *Thermoanaerobacter brockii* (TBADH) were also investigated. Specifically, the impact of ILs such as [BMIM][Cl], [BMIM][BF_4], [MIm][Cl] and [MIm][BF4] was monitored on the TBADH activity. The results showed compared to control and other ILs, the enzymatic activity and catalytic efficiency was enhanced in [BMIM][Cl] and [BMIM][BF_4]. This study also showed that in ILs with

similar anions, the activity depends on the alkyl chain length of imidazolium as well as structural similarity of cations to that of the substrate, because of this structure similarity these ILs to that of the enzyme subtract they act as an enzyme inhibitor [223]. As a result of the structural similarity of MIM ILs to that of substrate (NADP+), it was proposed that reduction in activity caused by this IL and the related [BMIM] were due to direct substrate competition rather than kosmotropic interactions with bulk water [223].

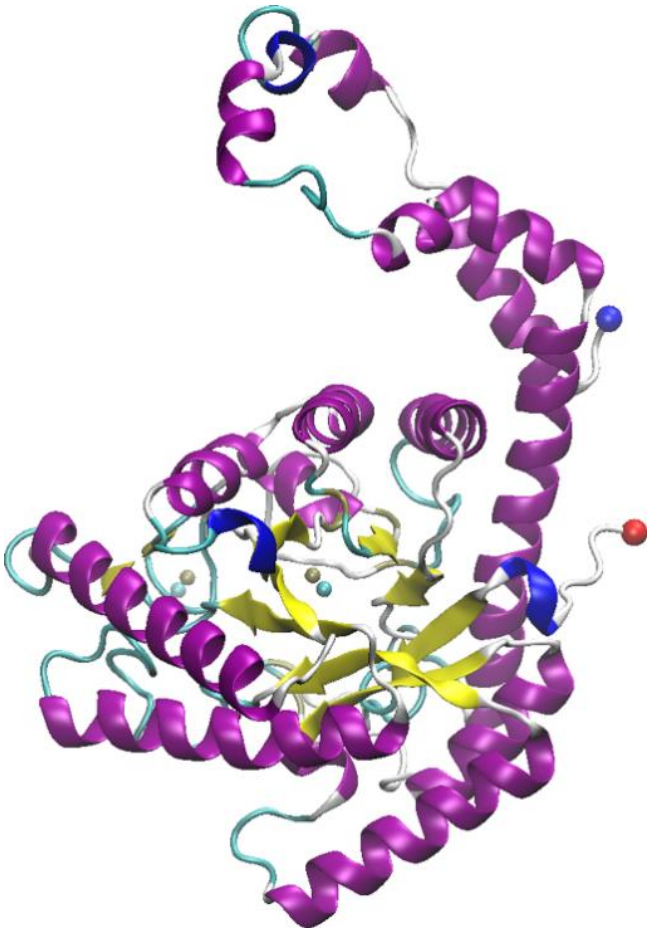
3.1.5 Effect of ILs on Glucose Isomerase

Glucose isomerase is a homotetrameric metalloenzyme with four catalytic centers and promiscuous functionality (Figure 16) [224]. The enzyme catalyzes reversible isomerizations of D-glucose to D-fructose as well as D-xylose to D-xylulose. Each of the catalytic centers has two subunits that form a pocket-like shape and have two divalent metal ion binding sites. Glucose isomerase is usually associated with metal ions like Mg^{2+}, Co^{2+}, or Mn^{2+}, or a combination of these [225]. The active site contains the metal ions and several critical carboxyl containing residues (Asp & Glu) as well as a His residue involved in proton transfer. Glucose isomerase is a very important industrial enzyme for petroleum and food applications as it is used for production of ethanol for fuel as well as high fructose corn syrup [225]. One study compared effects of various ILs on the activity of glucose isomerase toward converting glucose to fructose [226]. This study investigated the ILs [DMEA][F], [DMEA][Pr], [DMEA][De], [Choline][Pr], [DMBA][Pr], [MPIP][Ac], [DBEA][Oc], [Choline][Ac], [EMIM][Ac], [EMIM][Cl], [BMIM][Cl] and [BMIM][Ac]. Among these ILs [EMIM][Cl] and [BMIM][Cl] showed a deactivating effect on the glucose isomerase and no fructose production was observed. On the other hand, [DBEA][Oc] showed the highest fructose production (of about 52%) in comparison to

other ILs, when the final water content was kept at 21% w/w. In addition, [DBEA][Oc] was the only IL which was also able to produce mannose at 2% w/w, while all other ILs showed intermediate fructose production. These results indicate that the presence of ILs can significantly affect enzyme activity/stability and it is important to screen multiple ILs to find the one which provides optimum results [226].

Figure 16

3D Structure of Glucose Isomerase From Streptomyces Rubiginosus



Note. The crystal structure was solved by Dauter and coworkers; downloaded from rcsb.org (1OAD)[146].[224] The structure was visualized using VMD. The N- and C-termini are shown as

red and blue spheres respectively while the manganase ions are shown in tan and the magnesium ions shown in cyan. The structure represents one monomer of a homodimer.

ILs have also shown to impact the crystallization and X-ray diffraction resolution for glucose isomerase [227]. For instance, in a study by Judge *et al.*, glucose isomerase was crystallized in presence of ILs such as [EMIM][BF_4], [EMIM][Cl], [BMIM][Cl], [HMIM][Cl], triisobutyl (methyl) phosphonium p-toluenesulfonate, [n-BP][Cl]. Among all ILs the triisobutyl (methyl) phosphonium p-toluenesulfonate was shown to produce bigger crystals with a change in the morphology of glucose isomerase crystals compared to control samples without ILs [227]. However, proper optimization of the IL concentration during the crystallization is necessary because in some cases higher amounts of IL might negatively impact the crystal. For example, when crystallization of glucose isomerase was carried out with [BMIM][Cl] at 0 M, 0.2 M, and 0.4 M, plate-like crystals of glucose isomerase were obtained only with 0.2 M IL, while the samples with no IL gave salt precipitates and samples with 0.4 M IL did not yield any crystals or precipitates [228]. Furthermore, a synergistic effect was observed when ILs were combined with other techniques that also promote enzyme activity. For instance, the activity of immobilized glucose isomerase and reaction yield for glucose conversion to fructose was found to be highest when [EMIM][Cl] was used in combination of ultrasound irradiation, compared to use of only the IL or ultrasound irradiation individually [229].

3.2 Conclusions and Future Perspective

Depending on the physicochemical properties of ILs such as polarity, alkyl chain length in cation, anions in IL, hydrophobicity, and viscosity, ILs can have differential

effects on protein stability. Some ILs have been shown to improve the stability of proteins, some are inert, and others disruptive to protein structure and function. Because of these unique properties, ILs have applications in multiple fields such as chemistry/synthesis, biotech, pharmaceutical, and the electronics industries. Specifically, ILs that have been shown to stabilize proteins can potentially be beneficial in developing formulations of protein therapeutics or in industrial processes using biocatalysts.

As the protein stabilization or destabilization is very specific to the chemistry of ILs, a rational selection of IL for protein under investigation is necessary before using it as a solvent for improving protein stability or activity. There is only limited knowledge regarding the mechanism of protein stabilization or destabilization in the presence of ILs and therefore research is still needed to understand fundamental chemistry of ILs and how they interact with proteins. This is a crucial step before ILs can be effectively incorporated into protein production, purification, or biocatalytic processes. These experiments, in total, should aim to develop a predictive model for IL-biomolecule systems which varies both the cation and anion of the IL based on the properties and functional environment of the protein. This is a critical but challenging process because of the variability in IL compositions, ongoing development of new ILs, and the variability and complexity between different proteins.

One approach which has been recently described is instead of single entities, mixtures of different ILs have also been used for obtaining better protein stability [230]. In addition to experimental approaches for evaluating the effect on ILs on the protein stability, various *in silico* analysis have also been performed. For instance, a study using molecular dynamics simulation analysis indicated that in the presence of ILs the bovine serum

albumin does not destabilize the structure it adopts, which was also confirmed by experimental analysis [231]. These molecular dynamics simulations will undoubtedly help to narrow the field of potential IL candidates for specific protein and biomolecular applications.

Importantly, in the study of metalloproteins with ILs, there are still numerous questions regarding mechanism of IL-protein interactions. Most importantly, the majority of studies focus on the protein structure for obvious reasons. However, it leaves any direct interactions between ILs and the metal ions ambiguous. While in most cases it is clear from spectroscopic measurements that the metal ions are no longer properly coordinated in the protein structure, which was the initial driving force? Does IL interactions directly with the metal cause a destabilization in the protein or does destabilization of the protein cause a loss of the metal? While the latter is intuitive, there is only preliminary direct evidence. Additional studies that directly interrogate the metal sites such as vibrational methods and magnetic circular dichroism will help shed light on this question.

Another important aspect that must be considered when discussing IL-biomolecule applications is toxicity. The ability of a specific IL to stabilize a protein structure does not inherently mean it will be stabilizing to ALL proteins and may cause cytotoxic effects through other mechanisms. Similarly, there is no guarantee that because an IL is well tolerated by one organism that it will be equally biocompatible with all organisms. As such, the study of IL toxicity is an ongoing and rich area of research with numerous groups focused on this problem. Many studies have shown that some ILs can exhibit environmental toxicity or organismal cytotoxicity[232-234]. Alternatively, there are numerous examples in the literature of ILs that exhibit low levels of cytotoxicity,

encouraging the investigation of these formulations for biological and pharmaceutical applications [232, 233, 235-238]. Our own work has shown that the cytotoxicity of ILs with imidazolium-based cations is dependent on alkyl chain length but can be used synergistically with traditional antimicrobials well below the cytotoxicity window against human cells [239, 240]. These findings parallel that of many other groups which have shown a link between lipophilicity and cytotoxicity for ILs [241, 242]. However, in light of the vast number of IL species combined with the breadth of biological species, it is necessary to expand the throughput of screening IL toxicity. Many groups have employed computational QSAR approaches to build predictive models of IL toxicity to cells [241, 243-245]. These studies can potentially yield a great deal of insight for experimentalists in the design of IL formulations for specific applications.

Finally, the significance and importance of metalloproteins will continue to grow. Numerous industrial processes rely on metalloproteins for catalysis. These include enzymes such as metalloproteases, laccases, cellulases, lipases, phosphatases, and amylases [246, 247]. Further, some of the metalloproteins are involved in the progression of the cancer and other diseases [248]. Once suitable ILs are identified and their effects on a given protein have been thoroughly evaluated, they can successfully be used in combination with those targets to enhance or reduce activity. Because of having these beneficial properties, ILs have potential to serve as an ideal vehicle for protein therapeutics, a combinatorial therapeutic component, and an activity-enhancing additive in industrial processes in the near future.

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