Published in final edited form as:

Chembiochem. 2015 November ; 16(17): 2456–2459. doi:10.1002/cbic.201500398.

Crystallographic Investigation of Imidazolium Ionic Liquid Effects on Enzyme Structure

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Abstract

We present the first crystallographic insight into the interactions of an ionic liquid (IL) with an enzyme, which has widespread implications for stabilizing enzymes in IL media for biocatalysis. Structures of *Bacillus subtilis* lipase A (lipA) and an IL stable variant (QM-lipA) were obtained in the presence of increasing concentrations of 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]). These studies revealed that the [BMIM] cation interacts to surface residues via hydrophobic and cation- π interactions. Of specific interest was the disruption of internal stacking interactions of aromatic side chains by [BMIM], which provide structural evidence for the mechanism of enzyme denaturation by ILs. The interaction with [BMIM] and Cl ions to lipA was reduced by the stabilizing mutations Y49E and G158E in QM-lipA. Ultimately, these findings present the molecular basis for stabilizing enzymes from IL-induced inactivation as well as the selection of ILs that are less denaturing.

Graphical Abstract

The nature of the interaction of ionic liquids with enzymes was investigated by determining the crystal structure of *Bacillus subtilis* lipase A and an ionic liquid stable variant in the presence of increasing concentrations of [BMIM][Cl]. Analysis of the binding modes of the ionic liquid offer unprecedented insight into the molecular basis for stabilizing enzymes from ionic liquid-induced denaturation.

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Keywords

Lipase; Ionic Liquids; 1-Butyl-3-methylimidazolium chloride; Biocatalysis; Crystallography

Understanding how enzyme structure and function are perturbed in non-natural or “extreme” environments has important implications in many fields, including, in particular, biocatalysis. Owing to their unique and diverse molecular features, one such environment in which enzymes have been extensively studied for biocatalysis is ionic liquids (ILs).^[1–5] The realization that enzymes may be used in neat as well as aqueous-IL mixtures has specifically generated widespread interest with the goal of broadening the scope of feasible biotransformations.^[6–9] Despite this immense focus, there remains a critical need to develop rational means to diminish the impact of ILs on enzyme activity and stability.

We have recently engineered a quadruple mutant (G158E/K44E/R57E/Y49E) of lipase A (QM-lipA) from *Bacillus subtilis* with enhanced stability towards the IL 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) via rationally mutating surface residues.^[10] In this work, regions on the enzyme surface that were structurally perturbed by the IL were identified by HSQC NMR and mutated to glutamic acid to inhibit interactions with the IL. Additionally, glutamic acid mutations were introduced on the surface of lipase A to inhibit electrostatic interactions with the anions that were undetectable by NMR. As a result of these mutations, a 7-fold improvement in $T_{1/2}$ in 50% v/v [BMIM][Cl] compared to wild-type lipase A (lipA) was observed. Lipase A was targeted in these studies because it is an industrially important enzyme, which is used in esterification and transesterification reactions, as well as a model enzyme for understanding the effects of ILs on enzyme structure.^[11–16]

To understand the molecular basis for the improved stability of QM-lipA, as well as, more generally, the nature of the interaction of ILs with enzymes, we have determined the crystal structure of an enzyme in the presence of ILs. Specifically, we have solved the structure of wild-type lipA and QM-lipA crystals soaked in aqueous solutions with different concentrations of [BMIM][Cl]. Of specific interest was determining the location and binding modes of the cation and anion of the IL in each structure. Additionally, we examined differences in the occupancy of the cations and anions both globally and at mutation sites

between the wild-type lipA and QM-lipA. Although not for the purpose of elucidating enzyme stability for biocatalysis, ILs have previously been used as additives and precipitating agents to facilitate protein crystallization.^[17,18] Analogous studies of crystal structures of chymotrypsin and subtilisin determined in anhydrous organic solvents have led to a profound understanding of the impact of solvent environment on enzyme function.^[19–21]

Wild-type lipA and QM-lipA readily crystallized in the absence of IL in the $P2_12_12_1$ space group with two (chain A and B) and one molecule in the asymmetric unit, respectively. For soaking, the enzyme crystals were incubated in mother liquor supplemented with 0, 5, 10, or 20% v/v [BMIM][Cl] for 5–10 min, which did not significantly impact the integrity of the crystals. The structures of the enzymes at the different soaking conditions were determined by molecular replacement and refined to 1.2–1.9 Å resolution (see Tables S1 and S2 in the supporting information for refinement statistics). Notably, for the 20% v/v soaking condition, incubation with the IL induced a space group transition from $P2_12_12_1$ to $P4_32_12$ for wild-type lipA. This transition, which indicates a change in crystal packing, was presumably due to increased interaction with the IL at this soaking condition. For all IL-soaked and unsoaked structures of wild-type lipA and QM-lipA, the coordinates and structure factors have been deposited in the Protein Data Bank (PDB codes: 5CRI, 5CT4, 5CT5, 5CT6, 5CT8, 5CT9, 5CTA, 5CUR)

The overall structures of wild-type lipA and QM-lipA in the absence of [BMIM][Cl] were virtually identical, indicating that the mutations in QM-lipA (G158E, K44E, R57E, Y49E) did not alter the structure of lipA (Figure S1). As reported previously, the structure of wild-type lipA and, in turn, that of QM-lipA consisted of an α/β hydrolase fold that is common among lipases.^[22,23] Upon soaking of wild-type lipA and QM-lipA crystals with [BMIM][Cl], only minor perturbations in structure were observed over the range of IL concentrations that were used. The lack of structural changes in wild-type lipA and QM-lipA was apparent by overlaying the models of lipA as well as of QM-lipA at each soaking condition, which showed only minimal differences (Figure S1).

Due to the resolution of the structures, we were able to directly observe the interaction of [BMIM] and Cl ions with wild-type lipA and QM-lipA. Only [BMIM] and Cl ions with complete or near complete electron density, which could be unambiguously modeled, were included in the structures. For both wild-type lipA and QM-lipA, the number of unique [BMIM] and Cl ions bound on the enzyme surface increased with [BMIM][Cl] concentration in the soaking solution (Figure 1). However, interestingly, more [BMIM] ions were observed bound to lipA than QM-lipA at all soaking conditions, providing evidence that the mutations in QM-lipA inhibit binding of the cation (Figure 1 and Table S3). Although there were differences in the extent of [BMIM] binding, the number of Cl ions bound to wild-type lipA and QM-lipA were the same for each concentration of IL. Accordingly, the increased stability of QM-lipA relative to wild-type lipA to [BMM][Cl], which we observed previously,^[10] may be explained by differences in cation binding. However, because the Cl binding sites between lipA and QM-lipA are not identical, namely at the 20% soaking condition, anion-induced destabilization of lipA cannot be altogether discarded.

Inspection of the location of the resolved [BMIM] ions found that most of the cations interacts with hydrophobic regions on the surface of wild-type lipA and QM-lipA. Notably, the binding of a [BMIM] ion in the substrate binding cleft (near residues 11-13 in chain A), which is largely hydrophobic and, unlike other lipases, does not have a lid, was apparent at all IL concentrations and mimicks the binding of natural fatty acid ester substrates. In general, the interaction of [BMIM] with hydrophobic regions of each enzyme involves the butyl tail of the [BMIM] ion and non-polar surface residues, as illustrated in Figure 2A in which [BMIM] ions are depicted as teal stick models. In other cases, [BMIM] binding appeared to be stabilized by cation- π interactions between the imidazolium ring of [BMIM], which has a permanent positive charge, and aromatic residues. In one such example, the interaction of the [BMIM] head group with W31 resulted in the displacement of H3, which stacks with W31 in the absence of [BMIM] (Figure 2B). In another example, a bound [BMIM] ion is stabilized through both cation- π and hydrophobic interactions, involving both the imidazolium ring and butyl tail (Figure 2C). Notably, the location of all of the [BMIM] as well as Cl ions in the crystal structures correlate with sites that were perturbed by [BMIM][Cl] in our previous NMR experiments.^[10]

To further characterize the nature of the observed binding modes of [BMIM], the residue type involved in interactions with the imidazolium ring and butyl tail were analyzed. Figure 3 shows the fraction of contacts with the imidazolium ring and butyl tail as a function of amino acid type for wild-type lipA and QM-lipA for the 20% soaking condition. Analysis of the contacting residues (i.e., residues within 4 Å) found that the imidazolium ring interacts mostly with aromatic residues for both forms of lipA followed by hydrophobic residues (Figure 3A). This further illustrates the prevalence of cation- π interactions while interactions with hydrophobic residues most likely involve the N-methyl group of the imidazolium ring. Interestingly, only a small fraction of the contacts made by the imidazolium ring were with negatively-charged amino acids. This suggests that the role of electrostatic interactions in stabilizing the binding of the imidazolium ring to wild-type lipA and QM-lipA is minor. Such interactions are presumably weak due to the delocalization of the positive charge of the imidazolium ring as well as the overall bulky size of [BMIM], which restricts ion packing.^[24,25] The high dielectric constant of the solvent containing the IL, particularly in the case of the 20% v/v soaking condition, may also screen electrostatic interactions between [BMIM] and the enzyme. In the case of the butyl tail, as expected, virtually all of the contacting residues have non-polar side chains (Figure 3B).

The analysis of the binding modes of [BMIM] ions to wild-type lipA and QM-lipA provides unprecedented insight into how imidazolium ILs may induce enzyme denaturation. Specifically, our results suggest that the interaction of [BMIM] with an enzyme may disrupt intramolecular hydrophobic as well as stacking interactions that are critical for stability. This is perhaps most clearly illustrated by the displacement of H3 and, in turn, the loss of the apparent stacking interaction between H3 and W31. Consistent with our findings, Constantinescu and coworkers^[26] previously showed that enzyme stability was inversely related to the hydrophobicity of the cation of imidazolium ILs. In this work, the authors found that the T_m of RNase A decreased with increasing length of the alkyl tail of N-substituted imidazolium ILs. Similarly, Figueiredo and co-workers^[27] reported that the [BMIM] cation was more destabilizing than 1-ethyl-3-methylimidazolium to the colicin E7

inhibitor protein Im7 with the same anion. Moreover, Silva et al.^[28] more recently showed via STD-NMR that hydrophobic interactions involving the alkyl tail of N-substituted imidazolium ILs were also involved in cation binding to human serum albumin, and that these interactions increased in strength, which correlated with a decrease in stability, with increasing alkyl tail length.

Given the enhanced stability of QM-lipA relative to wild-type lipA to [BMIM][Cl], it is particularly intriguing to consider how the mutations in QM-lipA alter interactions with the IL. Interestingly, around two of the mutation sites, we observed significant differences in the occupancy of [BMIM] and Cl ions, suggesting local changes in IL binding. The most striking difference is observed for the 20% v/v soaking condition in the vicinity of Y49, which was mutated to a glutamic acid in QM-lipA. In the structure of wild-type lipA, a well resolved [BMIM] ion appears to interact with the sidechain of Y49 via a cation- π interaction involving the imidazolium ring of the [BMIM] ion (Figure 4A). Binding of this [BMIM] ion, which was modeled in two orientations, is further stabilized via electrostatic interaction with a neighboring asparagine residue (N50) in one of the orientations. However, in the QM-lipA structure, there is no observable [BMIM] ion bound to this site (Figure 4B). The absence of [BMIM] binding to this site is presumably the result of the loss of the cation- π interaction upon removal of the ring of the Y49 sidechain. Notably, the electrostatic interaction with N50 and/or E49 in QM-lipA is evidently too weak to stabilize the [BMIM] ion in the absence of the cation- π interaction with Y49.

Differences in the occupancy of [BMIM] and Cl ions in lipA and QM-lipA were also observed in the vicinity of G158, which similarly was mutated to a glutamic acid in QM-lipA. For the 20% v/v soaking condition, a [BMIM] and Cl ion are present near G158 in lipA whereas, in QM-lipA, there is no evidence of [BMIM] binding in this region (Figure 5). The disruption of binding of [BMIM] near the mutation site is likely due to the displacement of the bound Cl ion through which the [BMIM] in the wild-type structure is stabilized. Displacement of the bound Cl ion, which is perturbed by 4.3 Å away from the mutation site, is likely due to electrostatic repulsion by E158 in QM-lipA. The lower occupancy of the IL near E158 is consistent with smaller chemical shift perturbations in this region relative to near G158 in lipA in NMR experiments, and may contribute to enhanced stability.^[10] In the case of the other two stabilizing mutations from our prior work (K44E and R57E, which resulted in 2.9 and 1.2-fold improvement in stability, respectively), we did not observe binding of [BMIM] or Cl near these sites in either wild-type lipA or QM-lipA at the [BMIM] concentrations used.

In summary, we have determined the first crystal structures of an enzyme in complex with an IL, which provides a detailed molecular picture of enzyme-IL interactions. Our results specifically uncover the role of hydrophobic and cation- π interactions in stabilizing the binding of [BMIM] to surface residues, which likely contribute to denaturation in solution. Moreover, we found that the stabilizing mutations in QM-lipA reduce the extent of binding of [BMIM] and Cl. Ultimately, these findings provide structural insight into the molecular basis for the stabilization of enzymes in IL media as well as the rational design of novel ILs that are less denaturing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NSF (CAREER 1454379 to JLK) as well as graduate research fellowships through the GAANN program (P200A120125 to EMN) and the NIH Pharmaceutical Biotechnology Training Program (5T32GM8732 to JGP). We also thank David McKay for assistance with crystallographic data collection.

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Figure 1.

Surface representation of the structure of wild-type lipA (left) and QM-lipA (right) soaked with 5, 10, and 20% v/v [BMIM][Cl] from top to bottom, showing the location of unique, surface bound [BMIM] (displayed as blue sticks) and Cl (displayed as red spheres). Protein structures are shown as orange models with hydrophobic residues (Ala, Val, Ile, Leu, Met, Phe, Tyr, Trp, Pro) colored white. Note, the [BMIM] and Cl ions that are shown include those resolved bound to both molecules in the asymmetric unit of wild-type lipA. For clarity, only one molecule of wild-type lipA is shown.

Figure 2.

Binding modes of [BMIM] (teal stick model) in complex with wild-type lipA and QM-lipA in non-mutated regions. (A) Example of hydrophobic interactions between the butyl tail of [BMIM] and non-polar residues in the active site of lipA for the 20% v/v soaking condition. (B) Example of cation- π stacking interaction between [BMIM] and W31 in QM-lipA at the 20% v/v soaking condition, which induces the displacement of H3. To illustrate the displacement of H3, the structure of QM-lipA in the absence of IL (i.e., for the 0% v/v soaking condition) is shown in yellow. (C) Example of a [BMIM] ion bound through both cation- π stacking and hydrophobic interactions, involving both the imidazolium ring and butyl tail.

Figure 3.

Fraction of contacts (all residues ≥ 4 Å) with the imidazolium head (A) and butyl tail (B) as a function of amino acid type for wild-type lipA and QM-lipA for the 20% v/v soaking condition. The fraction of contacting residues was normalized to the total number of contacts in each structure.

Figure 4. Comparison of the binding of [BMIM] (teal stick model) in the vicinity of Y49 in wild-type lipA (A) and E49 in QM-lipA (B) for the 20% v/v soaking condition. Residue 49 in wild-type lipA and QM-lipA is highlighted in yellow while the remainder of the residues in each structure are colored green. No observable [BMIM] ion was bound near E49 in QM-lipA.

Figure 5. Comparison of the binding of [BMIM] (teal stick model) and Cl⁻ (red sphere) in the vicinity of G158 in wild-type lipA (A) and E158 in QM-lipA (B) for the 20% v/v soaking condition. Residue 158 in wild-type lipA and QM-lipA is highlighted in yellow while the remainder of the residues in the each structure are colored green. The dashed line in (B) shows the distance (4.3 Å) that the Cl⁻ ion is displaced in QM-lipA relative to in wild-type lipA.