Chapter 4

Structural Stability of Lysozyme in the Presence of Morpholinium Ionic Liquids

In this chapter, we have studied the effect of [Mor1,2][Br] and [Mor1,4][Br] which differ in the alkyl chain length of cation, on lysozyme in its native and chemically denatured states employing primarily the fluorescence correlation spectroscopy (FCS) technique. Fluorescence signal of Alexa488-labeled lysozyme (A488-Lysz) has been used to determine the changes in hydrodynamic radius of protein in the presence of additives. The results reveal a conformational dynamics of lysozyme with a time constant of 56 ± 10 µs in its native state. It is observed, when in its native state, both the morpholinium ILs induce structural changes of lysozyme. However, when in its unfolded state, [Mor1,4][Br] at low concentration compacts the protein, but at higher concentration it stabilizes the unfolded state, unlike [Mor1,2][Br], which compacts lysozyme both at low and high concentrations. A comparison of the effect of these salts and arginine, a protein stabilizer, on lysozyme indicates that [Mor1,2][Br] is a superior compacting agent for the unfolded state of the protein compared to arginine.

4.1. Introduction

Enzyme catalysis in organic solvents has been an important and interesting area of research in biotechnology and biochemistry since the last two decades.1-4 The stability of the proteins and solubility of the substrates are important considerations for catalysis in such media. While the enzymes are active in water and non-polar solvents, they show hardly any activity in most of the polar organic solvents.5 Hence, enzyme catalyzed reactions cannot be carried out for substrates that are soluble only in polar organic solvents. Search is on for alternative media to overcome this problem and the ionic liquids (ILs) can be
seriously considered as possible alternative in this regard. However, considering the fact that structure of a protein determines its activity, it is absolutely essential that prior to utilization of the ILs as media for catalytic reaction, the effect of the IL on the structural stability of the protein is investigated. This perhaps explains the recent interests in studies aimed at understanding the protein-IL interactions.\textsuperscript{6,7} We take note in this context that ILs are also being employed for the extraction, purification and preservation of proteins and as substitute media in biocatalysis.\textsuperscript{8} The ILs are also being explored as refolding agents and to improve the enzyme activity and selectivity in bio-transformations.\textsuperscript{9}

Yamamoto et.al studied the effect of N-alkylpyridinium chlorides and N-alkyl-N-methyl pyrrolidinium chlorides on hen egg white lysozyme and found that ionic liquid with short chain length enhances the refolding of lysozyme. They also found that ILs with increasing chain length destabilizes lysozyme.\textsuperscript{10} Recently, with the help of small angle X-ray scattering and FTIR studies Takekiyo and co-workers have shown that lysozyme unfolds in the presence of 6 M [bmim][NO\textsubscript{3}], whereas it exists in partially globular state when the concentration of the IL exceeds 10 M.\textsuperscript{11} Mann et al. used near-UV CD measurements to understand the effect of alkyl ammonium formate ILs on the thermal stability and activity of lysozyme.\textsuperscript{5} Horseradish peroxidase (HRP) activity was increased in [bmim][BF\textsubscript{4}], whereas a 50% loss in activity was observed in the presence of [bmpy][BF\textsubscript{4}] indicating that cation variation influences the stability of the enzyme.\textsuperscript{9} Noritomi et al. studied the thermal stability and activity of lysozyme in presence of [emim][BF\textsubscript{4}], [emim][Tf\textsubscript{2}N], and [emim][Cl]. A much lower activity of the protein was observed in the presence of [emim][Cl] when compared with that in [emim][BF\textsubscript{4}] and [emim][Tf\textsubscript{2}N].\textsuperscript{12} Recent NMR, CD and fluorescence experiments have shown that protic ILs can refold urea-denatured chymotripsin
and thus can attenuate the action of urea on it.\textsuperscript{13} Very recently, Bhattacharyya and co-workers have studied the effect of [pmim][Br] on human serum albumin and cytochrome c by using fluorescence correlation spectroscopy.\textsuperscript{14,15} To the best of our knowledge, the last two studies are the only ones where fluorescence correlation spectroscopy technique has been employed to understand the effect of ionic liquids on proteins.

The recent studies exploring ILs as the denaturants and refolding agents clearly show that the influence of ILs on enzymes is dependent on the cation-anion combination of the ILs.\textsuperscript{16} However, as the protein-IL interaction is yet to be understood it is necessary to extend the studies to a wider variety ILs and proteins. To obtain an improved understanding of the protein-IL interactions, we have chosen two morpholinium ILs, differing in alkyl chain length of cation, [Mor1,2][Br] and [Mor1,4][Br] studied their influence on lysozyme in its native and unfolded states using the fluorescence correlation spectroscopy. This technique allows monitoring of the size of the protein in the presence of high concentration of co-solutes\textsuperscript{17} and is shown to be ideally suited for the study of conformational dynamics of proteins, protein-protein and protein-lipid interactions.\textsuperscript{18-21}

Herein, the FCS measurements have been performed on Alexa488-labeled chicken egg white lysozyme. The influence of the morpholinium ILs both on the native and unfolded states of the protein has been monitored by a combination of FCS and near-UV CD measurements by following the change of the hydrodynamic radii of the protein and the spectral changes, respectively. The conformational dynamics of lysozyme is also studied by utilizing the Alexa488 dye interaction with the protein amino acids (tryptophan). The effect of a protein
stabilizer L-arginine on lysozyme is also studied and compared with the stabilizing effect of the morpholinium ILs on the same.

4.2. Results and discussion

4.2.1. Size and conformational dynamics of native lysozyme

The fluorescence correlation data of Alexa488-labeled lysozyme (A488-Lysz) is shown in Figure 4.1. The correlation data was fit to both equation 2.1 (simple diffusion) and equation 2.3 (simple diffusion with conformational fluctuation). As is evident from the quality of the fits (depicted in Figure 4.1), equation 2.3 clearly is a better model for this correlation data and hence, we have used this model for subsequent analysis.

Figure 4.1. Correlation data of A488-Lysz in phosphate buffered solution (pH 7.4) along with the fit to (a) single-component diffusion (equation 2.1) and (b) single-component diffusion along with conformational dynamics (equation 2.3) models. The quality of the fits is illustrated by the plots of the residuals as well.
We have also analyzed the correlation data using two component diffusion model but the diffusion time obtained for the second component from this model is not matching with the diffusion time of free dye (53 ± 2 μs). Hence, the presence of free dye in solution and its contribution to the faster component is ruled out. The fact that the correlation data of free Alexa488 dye is very well described by equation 2.1 (Figure 4.2. clearly indicates that under experimental conditions (excitation power of 4 μW) the fluctuation of the fluorescence intensity of the dye due to intersystem crossing is negligible. Majima et. al\textsuperscript{22} have also shown that the contribution from intersystem crossing of free Alexa488 dye is negligible at an excitation power of 45 μW which is much higher than the power used in the present study.

Considering these aspects, a better description of the correlation data of A488-Lysz in terms of equation 2.3 can be rationalized as follows. When bound to a protein, the fluorescence intensity of Alexa488 is known to be influenced by its interactions with the intrinsic amino acids (tryptophan and tyrosine) of the protein.\textsuperscript{23} As the conformational dynamics of protein can affect the interaction between Alexa488 and the amino acids leading to fluctuations of the fluorescence intensity, an additional term in the correlation function apart from the simple diffusion term explain the data better. The hydrodynamic radius (\(R_h\)) of lysozyme estimated from the FCS data is 2.1 ± 0.1 nm, which is in agreement with the literature.\textsuperscript{24} Wilkins et.al\textsuperscript{25} proposed an empirical formula, \(R_h = 4.75N^{0.29}\) Å, where \(N\) is the number of amino acid residues in the protein, for the calculation of the \(R_h\) value of a native protein. The \(R_h\) value of native lysozyme using this formula is estimated to be 1.9 nm, which agrees reasonably with the value estimated from our FCS experiment. It clearly suggests that after conjugation with dye, the structure of protein is not effected much which was shown earlier
by Melo et.al. The time constant of the conformational dynamics is measured to be $56 \pm 10$ µs and can be attributed to the chain dynamics of the protein.

![Correlation curve of free Alexa488 in phosphate buffer solution (pH 7.4) along with the fit to equation 2.1.](image)

**Figure 4.2.** Correlation curve of free Alexa488 in phosphate buffer solution (pH 7.4) along with the fit to equation 2.1.

### 4.2.2. Effect of additives on native lysozyme

#### 4.2.2.1. GdnHCl

The variation of the hydrodynamic radius ($R_h$) of native lysozyme with increasing amounts of guanidine hydrochloride (GdnHCl), a common denaturant, is shown in Figure 4.3. As can be seen, the $R_h$ value increases steadily and it reaches a value of $3.3 \pm 0.2$ nm for 6 M GdnHCl. The $R_h$ value for the unfolded state of lysozyme calculated using the empirical formula ($R_h = 2.21N^{0.57}$ Å) of Wilkins et.al. is 3.51 nm. A comparison of this value with the one measured by us from the FCS studies clearly suggests complete unfolding of lysozyme in the presence of 6 M GdnHCl. The disappearance of the CD signal in the presence of...
6 M GdnHCl, as can be seen from the near-UV CD spectra of lysozyme with and without GdnHCl, shown in Figure 4.4, also confirms the unfolding of lysozyme.

**Figure 4.3.** Variation of the measured $R_h$ value of lysozyme with increasing concentration of GdnHCl.

We carefully monitored variation of the relaxation time as a function of the concentration of GdnHCl (and other additives). The results for GdnHCl shown in Figure 4.5 indicate near constancy of the relaxation time with variation of the amount of GdnHCl. A similar trend is observed for the other additives (arginine and both the morpholinium ILs). While this observation is similar to those made earlier by Chattopadhyay and coworkers in their study of pH-induced unfolding of IFABP protein\(^{27}\) and by Sherman et al.\(^{28}\) for protein-L, Bhattacharyya et al found an increase in the relaxation time with unfolding of cytochrome c.\(^{15}\)
Figure 4.4. Near-UV CD spectra of lysozyme (90 μM) in aqueous solution without and with 6 M GdnHCl.

We now attempt to find out the reason for this near constancy of the conformational relaxation time despite denaturation of the protein. Considering that lysozyme consists of six lysine residues, all located on the surface of lysozyme,\textsuperscript{29} the highest chemical reactivity for Lys\textsuperscript{97} (47%) and Lys\textsuperscript{33} (40%)\textsuperscript{30} and the labeling ratio of close to unity (1.2) in our A488-Lysz conjugate, we can assume that Alexa488 dye is attached covalently to Lys\textsuperscript{97} or Lys\textsuperscript{33}. We also take note of the fact that lysozyme contains six tryptophan residues, four of which are exposed to the solvent and two are buried into the hydrophobic interior of the protein. Under these circumstances, one can conclude that the four tryptophan residues, which are exposed to water, interact with the Alexa488 dye and contribute to the fluctuations. As the tryptophan residues and the dye moiety are already on the surface of the A488-Lysz conjugate, the average distance between the probe and tryptophans may not change much during the unfolding process. Another possibility is that the effect due to change in distance is compensated by a faster motion of the protein in the unfolded state.
4.2.2.2. [Mor1,2][Br] and [Mor1,4][Br]

The effect of morpholinium ILs on the near-UV CD spectra of lysozyme is shown in Figure 4.6. A shift (1-2 nm) of the 295 nm peak is observed which can be attributed to the change in the microenvironment of the aromatic amino acid residues of lysozyme upon addition of the these ILs.\textsuperscript{31} It is however clearly evident from the decrease in the CD signal that in the concentration range of 0-1 M these ILs induce changes in the tertiary structure of lysozyme, but do not unfold it completely. The variation of the $R_h$ value of lysozyme, as estimated from the FCS studies, with increasing concentration of the two ILs are shown in Figure 4.7. Small change of the values observed in the presence of both ILs is in accordance with the CD results. It also appears from both CD signal and $R_h$ values that the changes induced by [Mor1,4][Br] are somewhat more compared to [Mor1,2][Br], indicating that [Mor1,4][Br] comprising a higher alkyl chain length destabilizes the protein structure more than the one with a shorter chain. This finding is in accordance with the observation made earlier in the case of imidazolium ILs.\textsuperscript{32}
Figure 4.6. Near-UV CD spectra of lysozyme (120 μM) with increasing concentration of (a) [Mor1,2][Br] and (b) [Mor1,4][Br].

Figure 4.7. Dependence of the measured R_h value of native lysozyme on the concentration of [Mor1,2][Br] and [Mor1,4][Br].
4.2.2.3. Arginine

Arginine, an α-amino acid, is a versatile additive in biological studies. It serves as a refolding agent for proteins,\textsuperscript{33} it can compact the native protein,\textsuperscript{34} and is also known to destabilize the protein because of its structural similarity with GdnHCl.\textsuperscript{33} The effect of arginine on the native state of lysozyme is best captured in FCS measurements by the change in the $R_h$ value of the protein with increasing arginine concentrations, shown in Figure 4.8. It can be seen that the $R_h$ value of native lysozyme decreases by nearly 30% in the presence of 0.5 M arginine, indicating that arginine compacts native lysozyme. In the case of cytochrome c and bovine serum albumin similar observation was made earlier by Chattopadhyay et.al.\textsuperscript{33,35} The near-UV CD spectra shown in Figure 4.9, also confirms a more compact structure of native lysozyme in the presence of arginine.

![Figure 4.8](image)

**Figure 4.8.** Plot of the $R_h$ value of lysozyme, estimated from the FCS measurements, against the concentration of arginine.
4.2.3. Effect of additives on unfolded lysozyme

4.2.3.1. [Mor1,2][Br] and [Mor1,4][Br]

We have shown earlier that lysozyme is denatured completely in the presence of 6 M GdnHCl, when its $R_h$ value increases to 3.3 nm. Under this condition, addition of [Mor1,2][Br] and [Mor1,4][Br] leads to a decrease of the $R_h$ value of the unfolded protein (Figure 4.10) indicating a significantly compact state of the protein in the presence of the morpholinium ILs. The $R_h$ value of lysozyme comes down to 2.1 nm for 0.3 M [Mor1,2][Br] and it remains there for concentration up to 0.7 M. In the presence of [Mor1,4][Br], the size of the denatured protein also decreases initially by ~25% for a concentration of 0.3 M. Interestingly, with further increase in concentration of [Mor1,4][Br], the size was found to be closer to that of the unfolded state. Thus [Mor1,4][Br] compacts the unfolded protein at low concentration, but at higher concentration it favors the
unfolded state. Observation of this kind was made earlier during the investigation of thermal stability and activity of lysozyme in the presence of propyl alkyl ammonium formate\textsuperscript{5} and [bmim][Cl]\textsuperscript{32}. We attribute the effect to more favorable interactions between the unfolded protein and [Mor1,4][Br] at high concentration.

![Graph](image)

**Figure 4.10.** Dependence of the $R_h$ value of unfolded lysozyme (in the presence of 6M GdnHCl) on the concentrations of [Mor1,2][Br], [Mor1,4][Br] and arginine.

4.2.3.2. Arginine

A steady decrease of the $R_h$ value of unfolded lysozyme is observed with increase in concentration of added arginine (Figure 4.10.). The $R_h$ value reaches 2.1 nm, which is close to that of the native state, in the presence of 0.7 M arginine suggesting that it also compacts the unfolded lysozyme. However, it is evident that a compact state of the protein that is reached using 0.7 M arginine can be achieved at a much lower concentration (0.3 M) of [Mor1,2][Br] indicating that this salt is more effective in compacting the denatured lysozyme compared to arginine. Hence, like the imidazolium ILs,\textsuperscript{32} these morpholinium ILs are also efficient refolding enhancers (with respect to size) and on occasion, the latter can be more efficient than arginine. We also note that like in the case of GdnHCl, the conformational dynamics of lysozyme is hardly affected in the presence of the
morpholinium ILs and arginine. Hence, the explanation offered earlier for the invariance of the time constant of the process is applicable here as well.

4.3. Conclusions

Effect of morpholinium ILs on the native and unfolded states of lysozyme is studied using fluorescence correlation spectroscopy and near-UV CD spectral measurements. A conformational dynamics of lysozyme with a time constant of 56 μs, which was not reported earlier, has been detected. It is found that both \([\text{Mor}1,2][\text{Br}]\) and \([\text{Mor}1,4][\text{Br}]\) destabilize the native state of lysozyme. However, the prominence of the effect in case of \([\text{Mor}1,4][\text{Br}]\) suggests possible role of the hydrophobic effect in the process. As far as the effect of morpholinium ILs on unfolded lysozyme is concerned, we found that \([\text{Mor}1,2][\text{Br}]\) compacts the protein, but the \([\text{Mor}1,4][\text{Br}]\), with a cation having longer alkyl chain length, compacts the protein at low concentration and stabilizes the unfolded state at high concentration. This study also reveals that arginine compacts both the native and unfolded states of lysozyme, but, it is significantly less effective compared to \([\text{Mor}1,2][\text{Br}]\) in compacting the unfolded state.
References

(8) Wei, W.; Danielson, N. D. Biomacromolecules 2011, 12, 290.