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A Mixture of Morpholinium-Based Ionic Liquid Exhibits an Additive Effect on Ionic Liquid Mediated Decrease in Local, Structural, and Global Thermal Stability of Horse Myoglobin

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Abstract—*Ionic liquids (ILs) are low melting point organic salts typically consisting of organic cations and organic/inorganic anions. Using several spectroscopic (UV-visible, near UV-CD) and calorimetric (differential scanning calorimetry (DSC)) techniques, the work determines how the mixture of morpholinium-based ILs such as N-ethyl-N-methylmorpholinium bromide ([Mor1,2]Br) and N-hexyl-N-methylmorpholinium bromide ([Mor1,6]Br) modulate the thermal stability of horse myoglobin (Mb) at pH 7.4. Analysis of the effect of individual ILs ([Mor1,2]Br and [Mor1,6]Br) and their mixture [Mor1,2]Br+ [Mor1,6]Br on the thermal stability of Mb (based on absorbance at 409 nm, CD at 274 nm, and DSC scans) provided several important information; (i) individually morpholinium-based ILs decrease the thermal denaturation mid-point, Tm (based on absorbance (409 nm), ellipticity (274 nm) and DSC of Mb, suggesting these ILs decrease the local (heme-globin interaction), structural (tertiary structure) and global thermal stability of Mb, (ii) the local, structural and global thermal stability of Mb were decreased more for the larger alkyl chain containing ILs than the smaller one, which suggests that the hydrophobic interactions modulate ILs-mediated decrease of thermal stability of Mb, and (iii) the mixture of morpholinium based ILs ([Mor1,2]Br+ [Mor1,6]Br) exhibit an additive effect on the ILs-mediated decrease of the local, structural and global thermal stability of Mb.*

1. INTRODUCTION

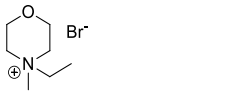
Proteins are the most imperative biomacromolecules of living beings. *In-vivo*, the cellular helper proteins such as molecular chaperones assist folding, while *in-vitro* the different molecular interactions are accountable for proper folding and stability of proteins. These molecular interactions can be destabilized or disrupted through pH denaturation, thermal and cold denaturations, pressure denaturation, and chemical denaturation [1]. Several chemical denaturants such as urea, guanidinium chloride, and alcohols, are generally used to denature and unfold the proteins. An increasing number of studies revealed that the ionic liquids (ILs) comprising organic cations and organic/inorganic anions can also destabilize the protein structure and decrease the thermal or thermodynamic stability of proteins [2, 3]. However, the molecular mechanism by different ILs modulates the thermal or thermodynamic stability of proteins is poorly understood.

Several in-vitro experimental investigations have depicted that environmentally benign ILs can be utilized for the extraction, purification, and preservation of protein [4-6]. Because of their tunable properties, the ILs can serve as a potential green designer solvent and, therefore can be used in many applications of pharmaceutical, biomedical, and biotechnological research fields [7-9]. The individual effects of ILs on the structure and stability of proteins have been extensively studied[10-12]. Still, the effect of the mixture of ILs on the stability, folding, and dynamics of proteins is not clearly understood. Several investigators have evaluated the effect of the alkyl chain length of different morpholinium-based ILs on the stability, folding, and refolding of proteins; however, how the mixture of different alkyl chain lengths containing morpholinium-based ILs alter the thermal or thermodynamic stability of proteins at the molecular level is not established properly. Using different spectroscopic (UV-visible, near UV-CD) and calorimetric (DSC) techniques and computational approaches, this research paperwork evaluated the effect of a mixture of different alkyl chain lengths containing morpholinium-based ILs (([$Mor_{1,2}$]Br) and ([$Mor_{1,6}$]Br)) on the thermal stability of horse myoglobin (Mb). Morpholinium-based ILs are considered as less toxic which makes it more biocompatible[13]. Mb is a model heme protein that is typically present in muscle tissue. It generally binds with oxygen with heme iron. It is a small-sized (16 kD, 153 amino acids) helical protein (8 helix). The heme is coordinated by the nitrogen atom of the histidine residue, and dioxygen binds to the heme iron in the opposite position of the bound histidine[14-16]. The crystal structure of Mb is shown below (PDB ID: 1YMB).

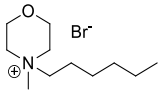
2. MATERIALS AND METHODS

Horse heart Mb and salts of buffer (monobasic and dibasic sodium phosphate) were acquired from Sigma-Merck. The [Mor1,2]Br and [Mor1,6]Br were synthesized by a well-established method as described earlier[17]. All the experiments were completed in 50 mM phosphate buffer at pH 7.4.

Structures of the synthesized ILs



N-ethyl-N-methylmorpholiniumbromide



N-hexyl-N-methylmorpholiniumbromide

2.2 Collection of visible absorption spectra of Mb

To investigate the effect of a mixture of morpholinium-based ILs on the structural properties of Mb, the visible absorption spectra (380 nm-700nm) of Mb were collected in buffer only and with ~0.5 M ([$Mor_{1,2}$]Br), ([$Mor_{1,6}$]Br) and their mixture (([$Mor_{1,2}$]Br +([$Mor_{1,6}$]Br)) at pH 7.4. The visible spectra were collected in a 10 mm path-length quartz cuvette on UV-visible spectrophotometer (UV-2600). The final concentration of protein was ~10 µM.

2.3 Thermal unfolding equilibrium experiments of Mb

2.3.1 Collection of visible absorbance (409 nm) based thermal melting curves of Mb for estimating the ILs mixture effect on local thermal stability of heme-globin interactions

To determine the effect of a mixture of ILs containing different alkyl chain lengths on the local thermal stability of Mb (heme-globin interaction), the thermal melting curves of Mb were collected by measuring the change in OD at 409 nm with temperature in different concentrations of [$Mor_{1,2}$]Br, [$Mor_{1,6}$]Br and in a mixture of equal concentrations of [$Mor_{1,2}$]Br+ [$Mor_{1,6}$]Br at pH 7.4. The melting curves were performed with a heating rate of 1 °C/minute on the S-1700 temperature controller system that is linked with the Shimadzu UV-2600 spectrophotometer. The final concentration of protein was ~10 µM in the reaction medium. The variation in OD at 409 nm was converted to $\Delta\varepsilon_{409nm}$ and the fraction unfolded with temperature as described earlier [18]. Thermal melting curves of Mb were analyzed using a transformed form of the van't Hoff equation (1)[19].

$$Y^{*} = \frac{\alpha_N + \beta_U exp(\frac{\Delta H_m}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right))}{1 + exp\,(\frac{\Delta H_m}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right))} \quad (1)$$

where the terms α_N and β_U are native and denatured state base-lines of Mb, respectively, and ΔH_m is described as the change in van't Hoff enthalpy at melting temperature, T_m.

2.3.2 Collection of near-UV CD (274 nm) based thermal melting curves of Mb for estimating the ILs mixture effect on structural thermal stability of protein

To know how the mixture of different alkyl chain length containing morpholinium-based ILs alter the tertiary structure thermal stability of Mb. The melting curves were performed on a JASCO-815 spectropolarimeter with a heating rate of 1 °C/minute on a *PTC 510 system coupled with a CD system*. The thermal melting curves of Mb were collected by estimating the change in CD at 274 nm ($[\theta]_{274\ nm}$) with temperature in buffer only and with 0.5 M [$Mor_{1,2}$]Br, 0.5 M [$Mor_{1,6}$]Br and 0.25 M [$Mor_{1,2}$]Br + 0.25 M [$Mor_{1,6}$]Br at pH 7.4. The protein concentration in CD experiments was 60 µM. melting curves were normalized and analyzed using equation (1) as described earlier [18, 19].

2.3.3 Collection of DSC scans of Mb for estimating the ILs mixture effect on global thermal stability of protein

To estimate the effect of a mixture of different alkyl chain lengths containing morpholinium-based ILs on the global thermal stability of Mb, the DSC scans of Mb were performed by determining the change in heat capacity with temperature in different concentrations of [$Mor_{1,2}$]Br, [$Mor_{1,6}$]Br and in a mixture of equal concentrations of [$Mor_{1,2}$]Br and [$Mor_{1,6}$]Br at pH 7.4. The DSC scans were performed with a heating rate of 1 °C/minute on a high-sensitivity MICROCAL PEAQ-DSC. The reference solvents containing different concentrations of ILs and the sample solution with a protein concentration of ~40 µM were prepared in 50 mM phosphate buffer. The protein solution was heated from 20 °C-100 °C. The values of T_m, ΔH_{cal}, and ΔH_m were calculated using non two-state model.

3. RESULTS AND DISCUSSION

3.1 Mixture of morpholinium-based ILs modulates the heme-globin interaction of Mb

The visible absorption spectrum of native-folded Mb in buffer only at pH 7.4, 25 °C exhibits a sharp *Soret peak* at 409 nm (Fig. 1), which is a characteristic of the well-defined heme-globin interaction in the native-folded state of Mb. Upon inclusion of individual ILs to reaction buffer such as 0.5 M [$Mor_{1,2}$]Br or 0.5 M [$Mor_{1,6}$]Br, the absorbance of the Soret band at 409 nm is significantly decreased and this decreased is more pronounced for [$Mor_{1,6}$]Br than the [$Mor_{1,2}$]Br (Fig. 1), which depict that the larger alkyl chain containing morpholinium based ILs decreased the heme-globin interaction of Mb more efficiently than the smaller alkyl chain containing species one. However, the inclusion of mixture of

ILs such as 0.25 M [$Mor_{1,2}$]Br + 0.25 M [$Mor_{1,6}$]Br to reaction buffer decreased the absorbance of Soret band at 409 nm between the 0.5 M [$Mor_{1,2}$]Br and 0.5 M [$Mor_{1,6}$]Br (Fig. 1), suggesting that mixture of different alkyl chain containing morpholinium based ILs shows an additive effect to decrease heme-globin interaction of Mb.

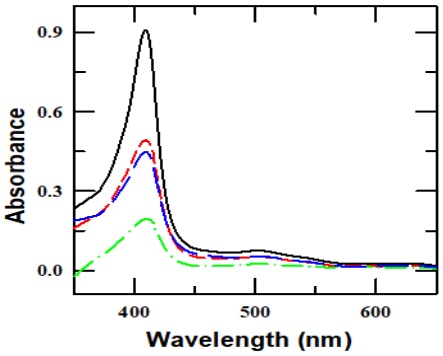


Fig.1 Visible-spectra of Mb in buffer only (native Mb (black solid line)) and with 0.5 M [$Mor_{1,2}$]Br (red short dash line), 0.5 M ([$Mor_{1,6}$]Br) (green dash-dot line) and mixture of 0.25 M [$Mor_{1,2}$]Br + 0.25 M [$Mor_{1,6}$]Br (blue long dash line) at pH 7.4, 25°C.

3.2 A mixture of morpholinium-based ILs exhibits an additive effect to decrease the local (heme-globin interaction), structural (tertiary structure), and global thermal stability Mb

To examine how the mixture of different alkyl chains containing morpholinium-based ILs alter the local (heme-globin interaction), structural (tertiary structure), and global thermal stability Mb, the spectroscopic (absorbance at 409 and CD at 274 nm) and calorimetric (DSC) based thermal melting curves were measured under different concentrations of [$Mor_{1,2}$]Br, [$Mor_{1,6}$]Br and in mixture of equal concentrations of [$Mor_{1,2}$]Br and [$Mor_{1,6}$]Br at pH 7.4. Fig. 2a and Fig. 2b depict the representative thermal melts of Mb performed by a change in absorbance at 409 nm and CD signals at 274 with temperature in buffer only and with 0.5 M [$Mor_{1,2}$]Br, 0.5 M [$Mor_{1,6}$]Br and 0.25 M [$Mor_{1,2}$]Br + 0.25 M [$Mor_{1,6}$]Br at pH 7.4. The variation in fraction unfolded of Mb (based CD at 274) with temperature and representative DSC scans of Mb in buffer only and with 0.5 M [$Mor_{1,2}$]Br, 0.5 M [$Mor_{1,6}$]Br, and 0.25 M [$Mor_{1,2}$]Br + 0.25 M [$Mor_{1,6}$]Br at pH 7.4 are shown in Fig. 2c and Fig. 2d respectively. The spectroscopic-based thermal melting data were analyzed using equation (1) and the calculated values of T_m and ΔH_m are given in Table 1. The DSC-based thermal data were analyzed by a non-two-state model, and the calculated values of T_m, ΔH_{cal}, and ΔH_m are summarized in Table 2. Fig. 3a and Fig 3b present the variations of T_m with [ILs] (based on absorbance at 409 (Fig. 3a) nm and DSC (Fig. 3b)) for varying individual concentrations of [$Mor_{1,2}$]Br or [$Mor_{1,6}$]Br and mixture of equal concentrations of [$Mor_{1,2}$]Br and [$Mor_{1,6}$]Br. Data in Fig. 3 and Tables 1-2 provided several important information, (i) as

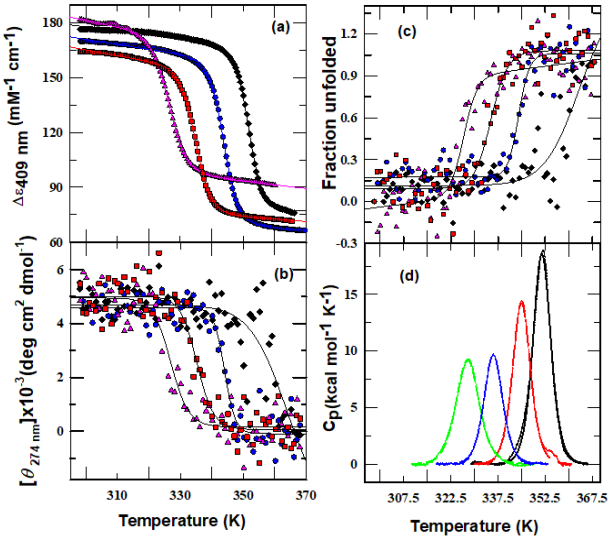


Fig.2 (a), (b), and (c) depict the variations in molar extinction coefficient ($\Delta\varepsilon_{409\ nm}$), ellipticity ($[\theta]_{274\ nm}$) and fraction unfolded (based on CD 274 nm) of Mb with temperature, respectively, at 0.0 M IL (♦) and 0.5 M [$Mor_{1,2}$]Br (●), 0.5 M[$Mor_{1,6}$]Br (▲) and mixture of (0.25 M $Mor_{1,2}$ + 0.25 M $Mor_{1,6}$) (■)) at pH 7.4. The black solid lines show the best fit of data to equation 1. Fig. 2d depicts the DSC scan of Mb in buffer only (solid black line) and with 0.5 M of [$Mor_{1,2}$]Br (long dash red line), 0.5 M of [$Mor_{1,6}$]Br (short dash green line) and mixture (0.25 M [$Mor_{1,2}$]Br + 0.25 M [$Mor_{1,6}$]Br) (dash-dot blue line) at pH 7.4.

Table 1 Visible spectroscopy (409 nm) and Near-UV CD (274 nm) thermodynamics parameters Tm and ΔH_m of thermal unfolding of Mb in the presence of (ILs)

Visible spectroscopy			Near-UV CD	
[$Mor_{1,2}$]Br (M)	T_m (K)	ΔH_m (kcal mol^{-1})	T_m (K)	ΔH_m (kcal mol^{-1})
0.0	351.85	111.30	364.23	43.60
0.25	347.12	107.09	--	--
0.5	343.87	102.23	344.36	143.41
0.75	340.50	95.16	--	--
0.85	339.52	95.49	--	--
[$Mor_{1,6}$]Br (M)				
0.25	336.81	90.97	--	--
0.5	326.50	75.87	326.29	74.96
0.75	315.95	66.93	--	--
0.85	311.79	56.95	--	--
([$Mor_{1,2}$]Br + [$Mor_{1,6}$]Br) (M)				
0.25	341.42	95.60	--	--
0.5	334.91	90.94	335.96	84.22
0.75	328.85	70.30	--	--
0.85	325.02	72.24	--	--

[ILs] is increased, the magnitude of T_m (based on absorbance at 409 nm, CD at 274 nm, and DSC is decreased, which suggests that the morpholinium based ILs decreased the local (heme-globin interaction), structural (tertiary), and global thermal stability of Mb, (ii) the ILs-mediated decreased in T_m is more pronounced for [$Mor_{1,6}$]Br than the [$Mor_{1,2}$]Br, which reveals that the alkyl chain length of cation or hydrophobicity of it controls the morpholinium based ILs-mediated decreased in the local (heme-globin interaction), structural (tertiary), and global thermal stability of Mb(iii) the comparisons of variations in the magnitude of T_m for individual [IL] [$Mor_{1,2}$]Br or [$Mor_{1,6}$]Br) and a mixture of equal concentration of each IL [$Mor_{1,2}$]Br +[$Mor_{1,6}$]Br) revealed that the mixture of morpholinium based ILs exhibit an additive effect to decrease in local (heme-globin interactions), structural (tertiary) and global thermal stability of Mb, and (iv) the ratio of $\Delta H_m/\Delta H_{cal}$ is nearly equal to 1, which indicates that thermal denaturation of Mb in presence of individual and mixture of ILs occurs by two state mechanism without any intermediate formation.

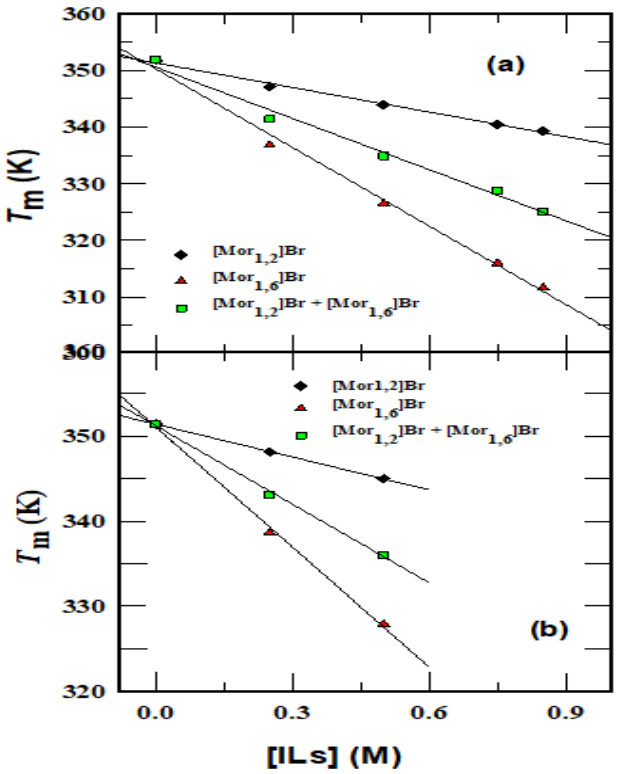


Fig. 3 (a) and (b) depict the variation of T_m of Mb as a function of [ILs] based on absorbance (409 nm) and DSC respectively, in the presence of [$Mor_{1,2}$]Br (♦) and [$Mor_{1,6}$]Br (▲) and mixture of ([$Mor_{1,2}$]Br + [$Mor_{1,6}$]Br) (■). The fitted black solid lines show the best linear fit of the data.

Table 2 Calorimetric DSC thermodynamic parameters T*m* and ΔH_m and ΔH_{cal} thermal unfolding of Mb in the presence of ILs

[ILs] (M)	T_m (K)	ΔH_m (kcal mol^{-1})	ΔH_{cal} (kcal mol^{-1})	$\Delta H_m/\Delta H_{cal}$
Buffer only	352.78	131	139	0.94
0.25 M [$Mor_{1,2}$]Br	348.17	128	122	1.05
0.5 M [$Mor_{1,2}$]Br	345.0	125	108	1.16
0.25 M [$Mor_{1,6}$]Br	338.63	113	103	1.09
0.5 M [$Mor_{1,6}$]Br	327.88	87.8	89.5	0.98
0.25 M ([$Mor_{1,2}$]Br +[$Mor_{1,6}$]Br	343.13	115	122	0.94
0.5 M ([$Mor_{1,2}$]Br +[$Mor_{1,6}$]Br	336.04	115	75.7	0.94

4. CONCLUSION

The morpholinium-based ILs decreased the local, structural, and global thermal stability of Mb at pH 7.4. Alkyl chain length or hydrophobicity of ILs controls the morpholinium-based ILs-mediated decrease in local, structural, and global thermal stability of Mb. Furthermore, the mixture of morpholinium based ILs ([$Mor_{1,2}$]Br+[$Mor_{1,6}$]Br) exhibits an additive effect to ILs-mediated decrease in local, structural, and global thermal stability of Mb.

5. ACKNOWLEDGEMENT

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6. AUTHOR CONTRIBUTIONS

Jayanti Rawat and Rajesh Kumar invented the ideas and planned the research. Jayanti Rawat carryout the research experiments. Jayanti Rawat and Manisha Yadav have been analyzed the experimental data. R Rajesh Kumar, Jayanti Rawat, and Manisha Yadav composed and wrote the manuscript.

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