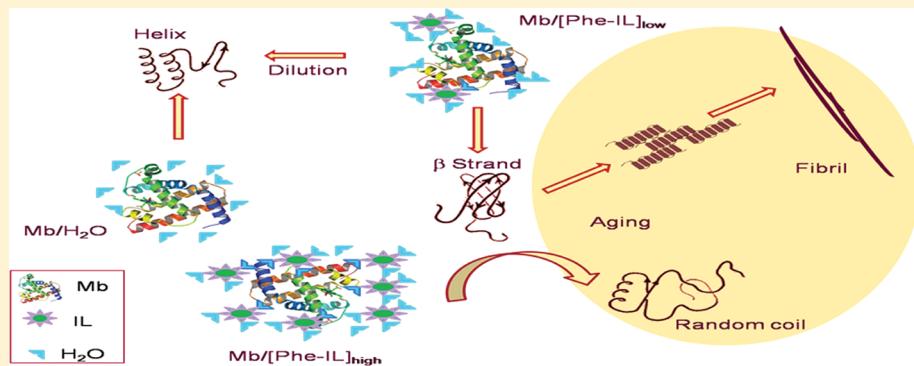


Reversible and Irreversible Conformational Transitions in Myoglobin: Role of Hydrated Amino Acid Ionic Liquid

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ABSTRACT: Hydrated phenylalanine ionic liquid (Phe-IL) has been used to solubilize myoglobin (Mb). Structural stability of Mb in Phe-IL analyzed using fluorescence and circular dichroism spectroscopy shows that for low levels of hydration of Phe-IL there is a large red shift in the fluorescence emission wavelength and the protein transforms to complete β sheet from its native helical conformation. Rehydration or dilution reverses the β sheet to an α helix which on aging organizes to micrometer-sized fibrils. At concentrations higher than $200 \mu\text{M}$, the protein changes from β to a more random coiled structure. Organization of the protein in Phe-IL in a Langmuir film at the air/water interface has been investigated using the surface pressure–molecular area isotherm and shows nearly the same surface tension for both pure Mb and Mb in Phe-IL. Scanning electron microscopy of the films of Mb in Phe-IL transferred using the Langmuir–Blodgett film technique show layered morphology. This study shows that the conformation of Mb is completely reversible going from $\beta \rightarrow$ helix \rightarrow β sheet up to $200 \mu\text{M}$ of Phe-IL. Similar surface tension values for Mb in water and in Phe-IL suggests that direct ion binding interactions with the protein coupled with the change in local viscosity from the IL seems to not only alter the secondary structure of individual proteins but also drives the self-assembly of the protein molecules leading finally to fibril formation.

INTRODUCTION

The study of the structure and dynamics of water in the context of solvation of proteins is one of the most exciting interdisciplinary research topics in science.^{1–3} The important functions of these solvated proteins are dependent on the folded and unfolded states. In many hydrated proteins, the proteins are capable of switching from the fully native folded state (N) to the unfolded denatured state (D) back and forth.⁴ This reversibility of protein folding, to a biologically relevant structure that is stable enough, occurs in a reasonable time for it to perform the function reliably. A number of research papers have appeared recently on the effect of low molecular weight additives that significantly enhance the yield of the refolding process.^{5–9} The refolding of a given protein may be promoted by stabilizing its native state, by accelerating the kinetics of the correct folding reaction, and by suppressing unspecific aggregation of the unfolded polypeptide and/or intermediates on the folding pathway.

Recent research on proteins in solutions has shown that protein stability in solution can be increased by improving electrostatic interactions among charged groups on the surface of the folded protein. Studies on different folded states of the

proteins in various solvents suggest that, even when the hydrophobic and hydrogen bonding interactions that stabilize the folded state are disrupted, charge–charge interactions on the exposed residues can reduce the net electrostatic interactions and even determine the stability of denatured state ensemble. The local interactions due to the asymmetry experienced by the water molecules at the protein/water interface together with the changes in hydrophobic and H-bonding interactions seem to drive such processes. In this context, models or experiments of organized assemblies of proteins at air/water interface, as in Langmuir films offer a good starting point to understand the factors that contribute to the stability of the protein and also relate it to the denatured state ensembles in the bulk.^{10,11}

The kinetics of the protein folding and its aggregate formation are some crucial steps which need to be addressed in modeling the denatured state ensemble. These steps can be regulated by changing the solvent environment or by adding

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ionic liquids (ILs). ILs have the desirable properties such as nonvolatility, thermal stability, low melting temperature, high decomposition temperature, low viscosity, and zero toxicity and can be also made functional by the choice of the right anion or cation. A number of studies have reported on the influence of ILs on organization and activity of enzymes.^{12–15}

K.R. Seddon et al. have indicated that ILs containing a strongly coordinating anion, such as nitrate or lactate, dissolved the enzyme *Candida antarctica* lipase B but also effected its (partially reversible) deactivation.¹⁶ A similar report on the inactive state of cellulase from *Trichoderma reesei* when dissolved in IL, like [BMIm][Cl] has been reported. The study was based on fluorescence which showed that the enzyme had denatured, presumably due to interactions with the—strongly coordinating—chloride ion.¹⁷ R. A. Sheldon et al. have shown that ILs that interact with the protein sufficiently strongly to effect its dissolution also induce structural changes that lead to loss of activity.¹⁸ C. M. Soares et al. have done a molecular dynamics simulation of the enzyme serine protease cutinase in the presence of two ILs, [BMIm][NO₃] and [BMIm][PF₆], and have shown that replacing the [PF₆][−] anion by [NO₃][−] promotes a significant perturbation on the enzyme structure and destabilizing effect on several enzymes.¹⁹ They have suggested that the strong interaction of the [NO₃][−] anion with the enzyme hydrogen donor groups, especially the amide group from the peptide bond in the α-helix and loop regions, is one of the main mechanisms of enzyme structure destabilization by this IL. These results suggest that the different ions in the ILs tend to compete with water. This may lead to different degrees of hydration in water IL mixtures as solvent medium for proteins.

Among the different factors that affect the different levels of hydration around a protein also known as the solvent fraction has been estimated for globular proteins such as myoglobin and BSA by A. Dhathathreyan et al.^{20,21}

Since amino acids (AA) contain both an amino group and a carboxylic acid residue in a single molecule, with various side groups and a chiral carbon atom, they are a good candidate to act as functional ILs. The advantages of using AAs are due to their low cost and also good biodegradability and biological activity suitable for biological applications.²²

In this study a hydrated amino acid IL has been prepared by adding defined amounts of water to synthesized hydrophilic phenylalanine IL (Phe-IL). Myoglobin (Mb), a water-soluble globular protein, has been dissolved in this IL for different levels of hydration. The change in the local solvated environment due to the presence of Phe-IL has been analyzed using UV-visible, fluorescence, and circular dichroism (CD) spectroscopic techniques. Its properties of organization at the air/water interface in Langmuir films have been studied. The effect of changes in local viscosity on the conformation of the protein has been studied using quartz crystal microbalance (QCM).

MATERIALS AND METHODS

Horse heart Mb was purchased from Sigma Aldrich chemicals, USA. All aqueous solutions were prepared with distilled water further purified with a four-stage Milli-Q water system (Millipore, resistivity greater than 18.2 MΩ).

Phe-IL was prepared according to the procedure of Ohno et al. and shown in Figure 1.²² 1-Ethyl-3-methylimidazolium as cation, [EMIm], is commonly used in preparing many ILs. After using an anion exchange resin the aqueous solution of

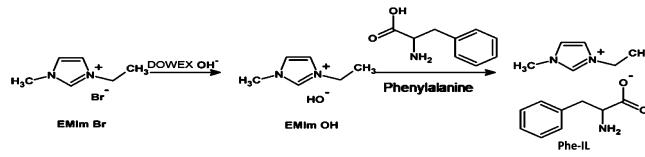


Figure 1. Synthesis of Phe-IL.

[EMIm]OH obtained is further treated with an equimolar amino acid (phenylalanine) solution to prepare Phe-IL. The product was dried in vacuo for 24 h at 80 °C. The obtained product was analyzed and confirmed using ¹H NMR spectroscopy and mass spectrometry. Myoglobin (0.4 mg/mL) was dissolved in various concentrations of hydrated Phe-IL (from 1 μM to 1000 μM), and the mixtures were stirred in a magnetic stirrer overnight to avoid phase separation. The resulting solution was clear and was used for all the experiments.

UV-VIS and Fluorescence Spectroscopy. The samples of Mb and Mb with Phe-IL were analyzed using UV-1800-Shimadzu spectrophotometer with quartz cells of 1 cm path length. The corresponding hydrated ILs were used as reference for the measurements. The steady state fluorescence spectroscopy was performed using Cary Eclipse Spectrometer with the λ_{ex} = 280 nm.

QCM Measurements. Cleaned gold-coated quartz substrates were stored in a desiccator. Before use, they were exposed to UV/ozone for 10 min. Measurements were performed in exchange mode, i.e., a volume of 50 μL of temperature-stabilized and degassed sample liquid was delivered to the chamber containing the sensor crystal to ensure a complete exchange of the liquid. In this way, processes of adsorption and surface adlayer changes can be followed in situ while subsequently exposing different solutions to the surface. All measurements were performed at a temperature of 24–25 °C.

QCM sensors quartz crystal microbalance, crystal holders, and polished gold AT cut 5 MHz gold crystals of 25 mm diameter crystals from Maxtek were used for the study. The oscillation frequency was measured using a Maxtek RQCM with phase lock oscillator and three independent crystal measurement channels. Data acquisition was performed using the Maxtek RQCM Data Logging software (v. 1.6.0) on a PC connected through an RS 232 serial interface. A sampling rate of 1/60 Hz was employed for all experiments. Any baseline drift was regulated using the coarse and fine capacitance adjustments. Upon interaction of (soft) matter with the surface of a sensor crystal, changes in the resonance frequency, Δf, are related to attached mass governed by the Sauerbray's equation

$$\Delta m = -(\mu\rho)^{1/2}(\Delta f)/(2f_0^2) \quad (1)$$

f₀ is the resonance frequency of the crystal (5 MHz at 25 °C), μ is the shear modulus of the quartz crystal (2.947×10^{11} g s^{−2} cm^{−1} at room temperature), and ρ is the density of quartz (2.648 g cm^{−3}).

Shear viscosity is then given by the equation

$$\eta = (19.627 \times (\Delta f)^2 \times 10^{-7})/\rho \quad (2)$$

CD Spectroscopy. The CD spectra of pure Mb and Mb dissolved in Phe-IL were carried out using a JASCO J-715 spectropolarimeter (JASCO Corp., Tokyo). The far-UV (260–180 nm) spectra of Mb in different levels of hydrated Phe-IL obtained using 0.1 cm path length quartz cells, were analyzed

using the Dichroweb fitting to three structural parameters: α -helix, β -sheet, and aperiodic.²³ The above solutions prepared with Mb and Mb/Phe-IL were aged for a week by storing the solutions under controlled conditions of temperature and humidity after which CD measurements were repeated.

Langmuir Film Balance Measurements. Langmuir films of Mb in Phe-IL were prepared in a NIMA 601 S thermostatted ($T = 22^\circ\text{C}$) single barrier trough ($32 \times 20 \times 0.5 \text{ cm}^3$) with a Wilhelmy balance for the surface pressure measurement (accuracy in surface pressure = 0.01 mN/m). The protein film from the stock was spread by pouring a small volume of protein solution along a glass rod implanted across the water/air interface^{24,25} on a subphase whose pH was adjusted to 7.5. After a waiting period of about 5 min, the film was compressed at a speed of 5 mm/min. Each experiment was repeated at least three times and checked for reproducible isotherms.

The glass slides used for the transfer of the films have been cleaned in a Plasma Cleaner (Harrick Plasma, USA). The films were transferred from Langmuir films of freshly prepared Mb and Mb/Phe-IL onto glass substrates using the Langmuir–Schafer film transfer technique at a constant surface pressure of $\pi = 20 \text{ mN/m}$. In another experiment films of Mb and Mb/Phe-IL after aging (details in experimental section) were prepared by the above procedure and characterized using scanning electron microscopy (SEM). A thin layer of gold (200 \AA) was sputtered on these samples, and SEM studies were undertaken using a Hitachi SU1510 model.

RESULTS AND DISCUSSION

Mb dissolved in hydrated Phe-IL was characterized using a UV-vis spectrophotometer. Figure 2 shows the absorbance at

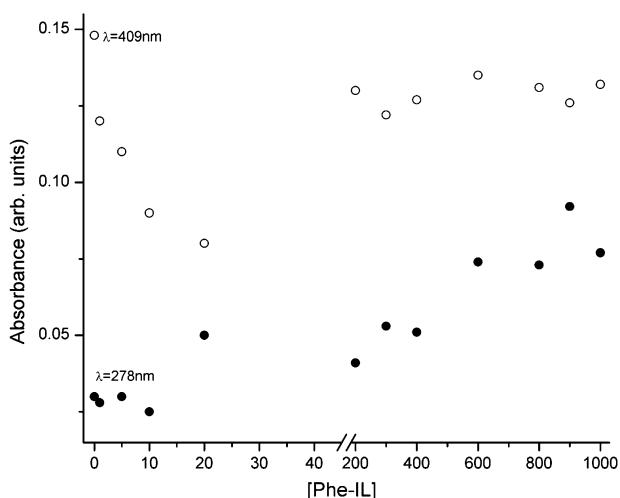


Figure 2. UV-visible Spectra of Mb: Absorbance at 278 and 409 nm for Mb in increasing concentrations of hydrated Phe-IL.

278 and 409 nm corresponding to the Trp residues and heme group of Mb for increasing concentrations of Phe-IL.

The initial decrease in absorbance at the 409 nm corresponding to the porphyrin moiety arises due to refolding of the protein with the heme group localized inside the hydrophobic pocket. At about $200 \mu\text{M}$ of the Phe-IL there is a sudden increase in the absorbance indicating that the porphyrin moiety moves away from the heme pocket. Change in Trp absorbance and that of porphyrin with Phe-IL concentration

are similar suggesting that the protein does not get denatured in the presence of Phe-IL.

The steady state fluorescence studies for the Mb in Phe-IL shows a weak emission at 330 nm for the native Mb. Figure 3a

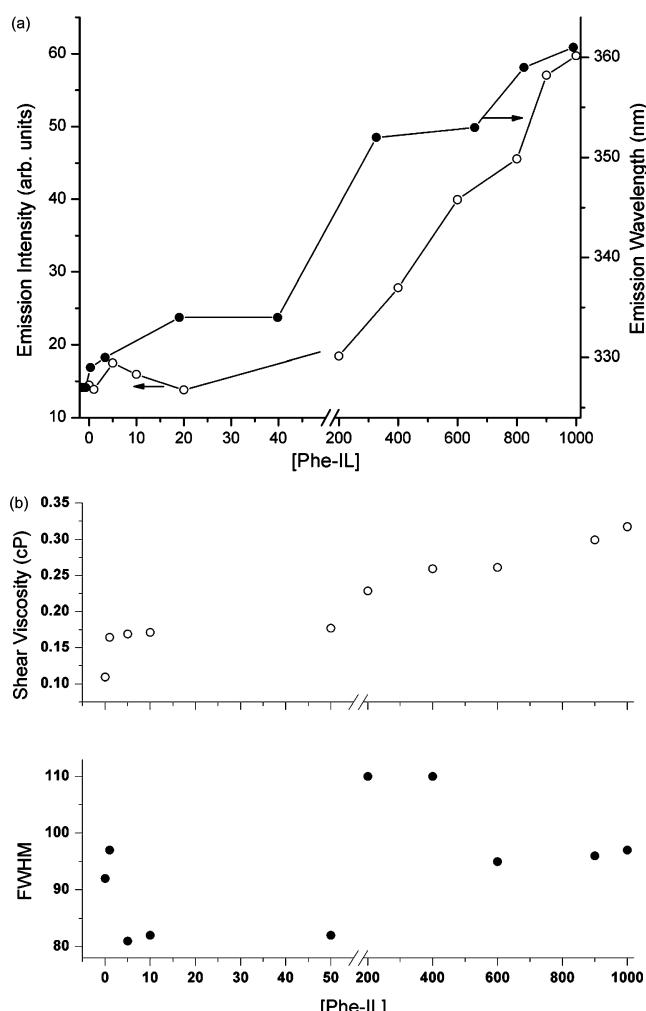


Figure 3. Steady-state fluorescence (a) emission intensity and emission maximum ($\lambda_{\text{ex}} = 280 \text{ nm}$) (b) FWHM from emission spectra and shear viscosity for Mb in increasing concentrations of hydrated Phe-IL.

shows increasing emission intensity as well as red shift in the emission maximum for increasing concentration of Phe-IL, possibly due to the exposure of the Trp to a polar environment. Such red shifts in the emission due to increase in the polarity of the localized medium have been reported for BSA interacting with long-chain imidazolium IL.²⁶ Such red shifts in λ_{em} have been often observed in low-temperature glasses, polymer matrixes, and organized assemblies such as micelles, vesicles, proteins, and membranes.^{27–29}

This “red edge effect” (REE) is now established to occur in organized assemblies, arising primarily because of the spatial heterogeneity of these assemblies. In the present study, the UV-vis spectra of Mb/Phe-IL show slight broadening in comparison to Mb. This may be due to multiple solvation sites involving the hydrophobic and hydrophilic pockets contributing to inhomogeneous broadening of the absorption spectra. Such broadening due to differently solvated sites have been reported earlier.²⁷

In addition, the presence of Phe-IL is expected to alter the local viscosity near the protein. Because of changes in the local viscosity, a distribution of ground-state molecules arising from the different networks of water, water-Phe-IL and subsequent interaction of Mb with the different hydrated Phe-IL differing in their interaction energies is possible. There could be a selective excitation of these different species and the corresponding fluorescence state is slow. Therefore REE is expected to occur. This also implies that the excited-state relaxation processes such as solvation and/or energy transfer are significantly slower in these media. To confirm the role of multiple solvation in REE here, a control experiment with Trp in the presence of varying concentrations of Phe-IL was carried out. The results showed that there is no red shift in the emission maximum. Vivian and Callis in their work on Trp fluorescence in proteins have analyzed several proteins and found that water may cause significant (10–20 nm) red shifts for Trps that are essentially buried and attributed this to be because of the collective action of regions of water up to 25 nm distant that are probably oriented by the charges and/or shape of the protein.³⁰ Therefore the large red shift seen in the present study could arise from a combination of protein electric field relative to the ground state dipole of Trp and the exposure of the Trp group to more polar environment.

The full width at half-maximum (FWHM) for emission bands of Mb given in Figure 3b increases with increasing concentrations of Phe-IL, which is indicative of reduced dynamics of the protein contributing to the delayed deactivation mechanism. The plot corresponding to the shear viscosity in Figure 3b measured using QCM agrees well with this model.

To check the stability of the protein in the hydrated ionic Phe-IL, CD spectra of Mb and Mb with different concentrations of Phe-IL are shown in Figure 4a. In agreement with the results of fluorescence, the unfolding and refolding of the protein is governed largely by the degree of hydrated IL in the medium. With increasing concentration of Phe-IL, the compact helical nature of the protein changes to beta structure after about 50 μM of the IL.

Figure 4b shows the percent secondary structures of Mb for all the concentrations of Phe-IL. It can be seen that for high levels of hydration or low concentrations of the IL the protein is stable in the helical state, whereas increasing concentrations of Phe-IL bring in drastic secondary structural changes. This could be due to the fact that the anions interact with protein sufficiently strongly to break the intermolecular bonds to such a degree that refolding takes place.

The reversibility of the conformational transition in the protein was checked by diluting Mb/Phe-IL at 200 μM . It was observed that the protein goes back to the helical conformation. On aging (1 week) however the protein reverts back to β sheet. At higher concentrations of Phe-IL, on dilution the protein does not revert back and on aging moves to a total random structure. This could be due to the fact that at low concentrations the IL exists as solvated cation or anion, whereas at higher concentrations, the IL anions and cations begin to interact with one another, either directly or mediated by water, increasing the degree of order in the liquids.³¹ Since the network of solvent around the protein at high concentrations of Phe-IL, is rigid, no more change in the secondary structure can be seen.

The Langmuir films of Mb and Mb with Phe-IL at air/water interface for [Phe-IL] = 100 μM is presented in Figure 5.

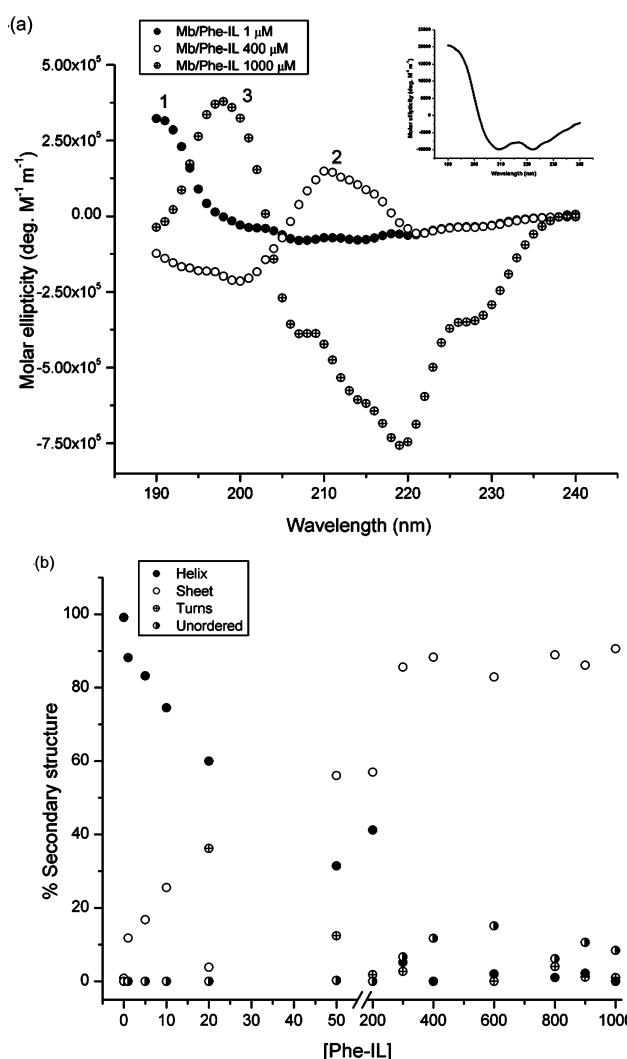


Figure 4. (a) CD spectra. (b) Percent secondary structures for Mb in increasing concentrations of hydrated Phe-IL (inset in part a is pure Mb).

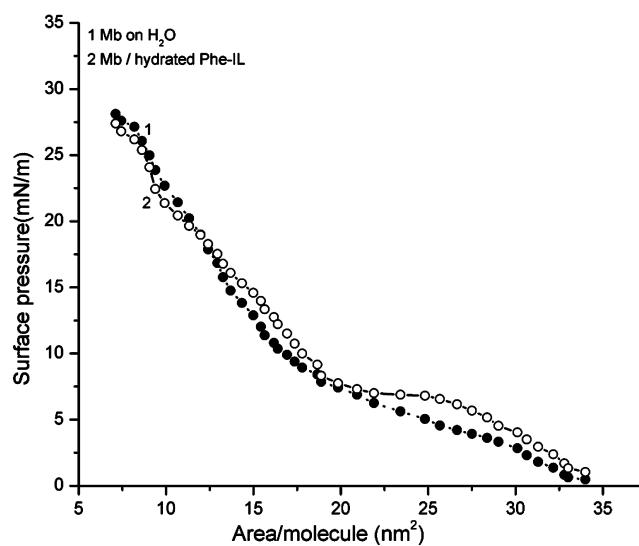


Figure 5. Surface pressure-molecular area isotherms of Mb and Mb in hydrated Phe-IL at the air/water interface.

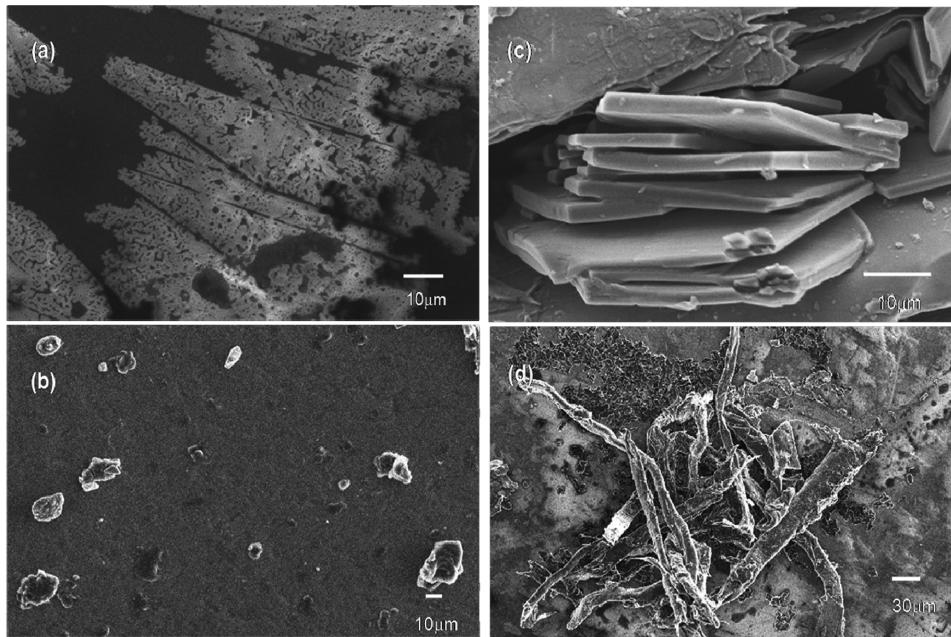


Figure 6. Morphology of LB films of (a) Phe-IL, (b) pure Mb, (c) Mb/Phe-IL (at low concentration), and (d) on aging.

Normally the compression modulus C_s^{-1} is used in the description of phase states of monolayers at air/water interface.³²

This is defined as

$$C_s^{-1} = -(A)(\partial\pi/\partial A) \quad (3)$$

Both Mb and Mb with Phe-IL show nearly the same type of isotherm. The isotherm of Mb in the presence of IL shows a plateau around $\pi = 7$ mN/m, suggestive of a first-order phase transition which may arise from the changes in the secondary structure of the protein possibly influencing the packing at low surface pressures. However on further compression the ensembles of the molecules with the different populations of secondary structural features are pushed together. At $\pi = 25$ mN/m both the isotherms show almost similar C_s values of 12.6 and 11.2 mN/m, respectively, indicative of "liquid expanded state". Thus a macroscopic measurement like the π - A isotherm is only an average of several different populations of protein molecules pushed together

In a control experiment, adsorption of the protein from the subphase of pure water and water with Phe-IL at the air/water interface was monitored through change in surface tension of water as a function of time. This showed nearly similar values for the protein (55.43 mN/m) and protein with Phe-IL (53.42 mN/m). This suggests that the IL may interact through a direct ion binding with the hydrophobic moieties of the protein and not through any restructuring of water. More experimental studies need to be carried out to confirm this behavior. Such effects have been reported for amino acids interacting with ILs. Results have shown that the magnitude and direction of the interaction are dependent on the nature and concentration of the cations, anions, and amino acids that are present in a given system.³³

For high levels of hydration of Phe-IL the films compressed and expanded showed no appreciable hysteresis. These films transferred to glass substrates were subjected to SEM. The morphology of Phe-IL, film of pure Mb, Mb/Phe-IL (at low concentration) and on aging is shown in Figure 6. The flow

pattern of Phe-IL seen in Figure 6a is due to partial dewetting which may be driven by its high viscosity. The protein at low concentration of Phe-IL shows stacked structures suggesting self-assembly driven by Phe-IL (Figure 6c). Since the transfer was done horizontally we assume that there is no drastic disturbance of the films at the interface. The increase in local viscosity leading to "shear" could cause the stacked structures. The Mb/Phe-IL on aging shows long threadlike particular aggregates (Figure 6d). The pure protein in the absence of Phe-IL on aging does not show this type of morphology.

Such stacking and assembly into threads from soft disklike structures under shear flow has been simulated.³⁴ Ordered fibrils have been reported for Mb, inspite of its so-called compact globular structure at low pH values.³⁵ ILs seem to prevent aggregation and also to help assemble the proteins in an organized manner. Protection offered by ILs against hydrolysis and aggregation in a number of enzymes has been analyzed by Byrne and Angell.³⁶ Direct ion binding interaction of Phe-IL with the protein coupled with the change in local viscosity from the IL seems to not only change the secondary structure of individual proteins but also drives the self-assembly of the protein molecules. With proper choice of the concentrations of protein, IL and pH it would be interesting to study if shorter nanometer-sized fibrillar structures are possible to be assembled.

CONCLUSIONS

This study demonstrates that amino acid IL can bring a new element to the study of the protein folding problem by establishing folding conditions that are unattainable in standard aqueous solutions. By fine-tuning the protein–solvent interactions, simultaneously reducing the disruptive aggregation, the Phe-IL-based solution studies allows us to study the steps along the folding path. Phe-IL interacts with the protein, and competes with some of the water molecules in promoting a polar environment. Small concentrations of Phe-IL stabilize native structure with high helicity, whereas higher concentrations lead to dramatic shift to beta structures. Morphology of

the Mb in Phe-IL using SEM shows that the protein can assemble to long thread like structures. The results suggest that assembly of proteins with specific changes in secondary structures can be tuned by alteration of the viscous environment.

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Notes

The authors declare no competing financial interest.

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