Activity and Thermal Stability of Lysozyme in Alkylammonium Formate Ionic Liquids – Influence of Cation Modification.

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The stability and activity of hen's egg white lysozyme in the presence of four protic room temperature ionic liquids (ethylammonium formate (EAF), propylammonium formate (PAF), 2-methoxyethylammonium formate (MOEAF) and ethanolammonium formate (EtAF)) have been

10 investigated. Near UV CD experiments have been used to determine protein structure in aqueous solutions of 25 wt%, 50 wt% and 75 wt% ionic liquid, and to assess the proteins ability to refold after heating to 90°C. It was determined that EAF and MOEAF are similarly effective refolding additives, while PAF is more effective at promoting refolding at concentrations up to ~ 62.5 wt%, but at higher PAF concentrations the protein spontaneously denatures. Both of these effects are

15 attributed to the increased hydrophobicity of the cation. EtAF is shown to stabilise lysozyme against unfolding at high temperature, and renaturing appears to be near complete upon cooling. Studies of enzyme kinetics reveal increased protein activity in the presence of all ionic liquids examined, but the most significant increase is noted for EtAF, where rates are six times higher than those determined for lysozyme in buffered water.

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Introduction

Enzymes are proteins that accelerate chemical reactions. They are highly substrate specific and have extraordinary catalytic power; a typical enzyme catalysed reaction proceeds

25 millions of times faster than that of comparable uncatalysed reactions.[1] Enzymes are synthesised in cells through DNA transcription and translation,[2] then fold into the functional conformation, either spontaneously or with the assistance of chaperon proteins. Folding is driven and reinforced by the

30 formation of a variety of bonds (hydrogen bonding, disulfide bridging, ionic attractions and hydrophobic attraction), by localised conditions, and the requirement to minimise unfavourable hydrophobic and hydrophilic interactions between parts of the protein chain.[2] These bonds are

35 influenced by solvent temperature, pH, ionic strength and polarity. Enzyme structure is described on four levels: primary (ordering of amino acid residues in the peptide chain), secondary (the formation of structures such as alpha helixes and beta sheets via inter-residue hydrogen bonds),

40 tertiary (folding into the active three dimensional morphology), and quaternary (combination of two or more protein chains).

For many years the use of enzymes was restricted to dilute aqueous solution. The use of organic solvents as enzyme

45 reaction media was pioneered by Klibanov.[3] Surprisingly, enzymes often retained their active conformation and in many cases enzymes demonstrated increased stability and activity. Water insoluble reaction precursors could now be dissolved in enzymatic solution, vastly increasing the range of enzyme

50 catalysed reactions, and product recovery was simpler. Today both aqueous and organic solvents are considered typical enzyme reaction media.

In 1994 it was found that enzymatic activity in organic solvents was dependant upon the presence of trace water.[4]

55 Many enzymes (lipases, hydrolases and proteases) work well in organic solvents, but for optimal performance require a certain degree of hydration that is dependent on water activity. This biological water is associated with the enzymes surface and helps maintain crucial three-dimensional

60 structure. Hydrophobic solvents do not strip this water from the enzyme surface,[5] but hydrophilic organic solvents do and this leads to protein denaturing.[6] As a result, enzymes are active in water and in non-polar organic solvents, but generally inactive in protic and polar solvents. This creates a

65 miscibility gap for enzyme catalysed reactions, as substrates strictly soluble in protic or hydrophilic solvents cannot be dissolved in solution with the active form of the enzyme.

Over the last decade, a considerable amount of attention has been focused on the development of novel solvents to help

70 breach this miscibility gap. Room temperature ionic liquids (ILs) are at the forefront of this research.[7-9] ILs are typically organic salts that are liquid at temperatures below 100°C. They often have negligible vapour pressure and high thermal stability, but their key feature is their 'tuneable' nature,

75 whereby important physical parameters (polarity, viscosity, Lewis acidity, etc) can be controlled by selection of appropriate cation and anion types in the first instance, with fine control facilitated by subtle variation in molecular structure of the individual IL components. These 'designer'

80 characteristics, combined with their ability to solubilise

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unusual combinations of chemical species, has led to a vast number of research articles in which ILs are employed as solvents for chemical synthesis.[10-12] Notwithstanding this, comparatively little is known about the relationship between cation and anion molecular structure and physical properties (especially for protic ILs),[13, 14] and even less concerning the nature of interactions with dissolved proteins.

In aqueous solutions, the influence of inorganic electrolytes on enzyme stability was first systematised by Hofmeister.[15] The Hofmeister series has since found widespread applications in other areas including colloid, polymer, and surface chemistry.[16] While in some cases there seems to be specific interactions between enzymes and ions, the similarity of results obtained in a wide variety of systems suggests a more universal mechanism.[16] One possibility is that ion-induced changes in water structure either promotes or hinders the binding of ions to the protein interface, leading to changes in stability. The Hofmeister series potentially provides a starting point for our understanding of the interactions of ILs with proteins, and an article has recently appeared to this end.[7] However, the complexity of IL ions, and particularly their hydrophobicity,[14] means that specific interactions with the protein are much more likely than for inorganic salts. Indeed, Constantinescu *et al.* state 'some biocatalysed reactions are conducted in "neat" ILs as solvents (little or no water).[7] In these cases the effect of ions on the stability/activity of enzymes seems to be much more complicated and does not follow the Hofmeister series'.

In hydrophobic ILs (for example, those including a PF_6^- or $(CF_3SO_2)_2N^-$ anion), enzymes often have high stability,[8, 17] attributed to the presence of biological water as per hydrophobic organic solvents. In these systems the protein is not actually dissolved, but present as a finely divided dispersion acting as a heterogeneous catalyst.[17] Increased stability in hydrophilic ILs (either the pure IL or as a water co-solvent) is more difficult to explain.

Summers and Flowers first demonstrated that the use of hydrophilic ILs ($R_4N^+NO_3^-$) as aqueous co-solvents suppressed enzyme aggregation in addition to increasing reaction yields.[18] It was suspect that adsorption of the IL cation to hydrophobic regions of the protein exposed upon heating created electrostatic repulsions that prevented protein aggregation, while columbic attractions between the ions and the charged portions of the enzyme stabilise secondary structure. Byrne *et al.* obtained similar results a few years later,[19] and also reported previously unheard-of multi-year stabilisation of lysozyme in the presence of ethylammonium nitrate (EAN). Differential scanning calorimetry (DSC) experiments revealed that while the temperature of the endotherm maximum was the same as for aqueous buffered samples, the protein was able to unfold and re-fold over repeated heating-cooling cycles. In pure aqueous solutions of lysozyme at equivalent concentrations 70% protein is lost after one heating cycle. In subsequent experiments the IL structure was varied and it was concluded that the availability of the N-H proton was the critical factor.[20] A measure of the N-H availability was obtained using nuclear magnetic resonance spectroscopy and it was found that maximal refolding occurred for NH chemical shifts between 7 and 9 ppm. Outside this region, enzyme unfolding was irreversible.

The precise reason for increased enzyme re-naturing and thermostability in the presence of some ILs is unclear,[21] with changes in hydration levels, structure compaction, free volume contributions, salt bridges, confinement effects and the bulk structure of the IL[22] all mooted as potential contributors. Similarly, there are several possible reasons for protein deactivation in other ILs including stripping of biological water,[21] interactions between the proteins charged groups and the IL[8] and the breaking of intra-protein hydrogen bonds,[8] all of which could lead to loss of tertiary structure. In this article we present results demonstrating that IL concentration, and subtle variation in the cation structure, can dramatically influence lysozyme's stability and activity.

Results and Discussion.

CD experiments.

CD measures the absorption difference between left-handed and right-handed polarised light arising from structural asymmetry.[28] The absence of regular structure produces zero CD single, while ordered structures produce a spectrum containing both positive and negative outputs. The near UV CD spectral region (250-350 nm) is sensitive to certain aspects of an enzymes tertiary structure.[28] The signals in this region are associated with phenylalanine (254, 256, 262 and 267 nm), tyrosine, (276 and 283 nm) tryptophan (280 - 300 nm) and disulfide chromophores, which give rise to broad, but weak signals. The signals in this region are sensitive to the chromophores' local environment and, consequently, the enzymes tertiary structure. As such, near UV CD spectra provide a 'fingerprint' of protein morphology, but little quantitative structural information can be derived from this region.[28] Secondary structural content can be calculated from the far UV CD spectrum, but some IL absorption bands interfere with the far UV, particularly those based on the nitrate anion. For this reason, formate based ILs, that do not interfere with this region of the spectrum, are the focus of this study. CD measurements are primarily used to ascertain whether the protein is in its folded or unfolded state as temperature is increased, and its ability to refold upon cooling.

The aromatic amino acid residues of lysozyme (phenylalanine, tyrosine and tryptophan) and disulfide chromophores produce the protein's near UV CD spectrum.[29, 30] The positive triplet-like signal in the 280 to 300 nm range dominates the spectrum, and is indicative of lysozyme's active conformation (Figure 1a).[27] In particular, the weak negative band at 295 nm has been attributed to tryptophan 108, which is associated with the active site (boxed region Figure 1a).[29] This provides direct insight into the immediate environment of the active zone.
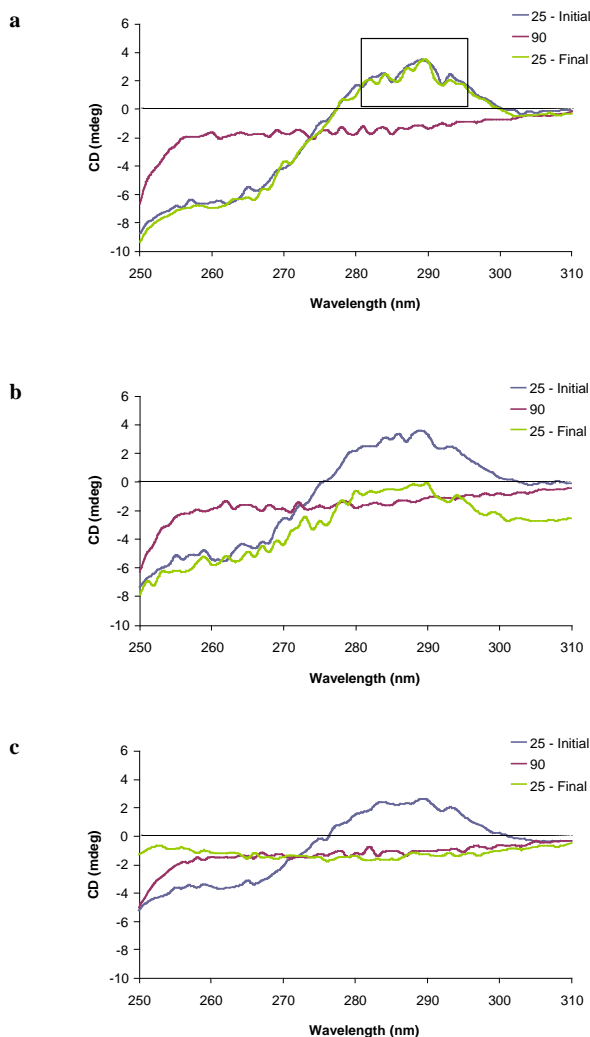
Fig. 1. Near UV CD spectrum of aqueous lysozyme (3.4×10^{-4} mol.dm^{-3}) in water with EAF loadings of (a) 25 wt%, (b) 50 wt% and (c) 75 wt%.

As the cell temperature is increased, lysozyme begins to denature with the increased thermal energy breaking the bonds that maintain lysozyme's active conformation. Lysozyme unfolds, exposing the internalised hydrophobic core[31, 32] to the surrounding medium. Attraction between exposed hydrophobic cores, or electrostatic interactions between oppositely charged residues (or a combination of both), potentially leads to aggregation or misfolding, whereupon the enzyme is irreversibly denatured. While renaturating of lysozyme is possible at low concentrations in pure water, refolding is known to be problematic at concentrations greater than ~ 1 mg.mL^{-1} due to aggregation.[18] Our CD based evaluations of lysozyme stability in ILs uses concentrations of 5 mg.mL^{-1}. Irreversible denaturing is the baseline result in water.

The near UV CD spectra of 3.4×10^{-4} mol.dm^{-3} (5 mg.ml^{-1}) lysozyme in water with EAF loadings of 25 wt%, 50 wt% and 75 wt% for the 25 – 90 – 25 ºC heating and cooling cycle ae presented in Figure 1. These relatively high IL loadings have been selected to elucidate the effect of IL concentration on temperature stability and protein activity for formate based ILs. It is worth noting that similar pure ILs can possess

nanoscale order,[33, 34] and experiments monitoring conductivity as pure IL is added to water suggest non-ideal behaviour.[35] It is possible that short alkyl group cations form self assembled aggregates at these concentrations in water in the absence of lysozyme.

The CD signal for the lysozyme / 75wt% water / 25 wt% EAF system increases from -8 mdeg at 250 nm to -6.5 mdeg at 255 nm. The signal plateaus at this value up to 265 nm, after which it increases to 0 at 277 nm. In this region (up to 276 nm) the spectral features are primarily due to phenylalanine residues. At wavelengths greater than 276 nm the CD signal, produced by asymmetric tyrosine and tryptophan groups, continues to increase reaching a maximum of 3.7 mdeg at the apex of the primary peak of the triplet feature (289 nm). This data is essentially consistent with results obtained for lysozyme in buffered water (see supplementary data Figure A), the primary difference being that the peak at ~290 nm is slightly weaker and broader due the presence of IL ions in the vicinity of trytophan residues, which alters the local environment (c.f. Figure 1a and 1b). Examination of lysosyme's activity (below) confirms maintenance of a constitutively active protein.

Upon heating to 90°C the CD spectrum changes dramatically. The signal now increases rapidly from -6 mdeg at 250 nm to -2 mdeg at 255 nm. This value is essentially constant up to 295 nm, after which it gently increases to ~ 0 at 310 nm. This comparatively featureless spectrum indicates that the protein has unfolded. The non-zero CD signal is due to disulfide bonds which are known to remain intact at elevated temperatures.[31, 32] This high temperature spectrum is essentially the same as that obtained for reduced lysozyme concentrations at 90°C in the absence of IL (see supplementary data Figure A). Upon cooling to 25 °C the CD spectrum is broadly consistent with that recorded prior to heating, suggesting that most of the lysozyme has refolded correctly.

When the EAF concentration is increased to 50 wt% the fine structure of the triplet feature is not as well resolved more resembling the triplet observed with 25% EAF after cooling from 90 to 25ºC (Fig. 1a and 1b) and the ability of lysozyme to refold after heating is reduced. The diminution of this characteristic triplet signal is more pronounced at 75 wt% EAF with a reduction in intensity also noted, 2.4 mdeg (75 wt% EAF) at 288 nm versus 3.6 mdeg (25 & 50 wt% EAF). At this high concentration the triplet feature has almost disappeared and cycling to lower temperatures does not initiate protein refolding, lysozyme has irreversibly denatured.

Summers and Flowers postulated that when unfolded, lysozyme's exposed hydrophobic core favourably interacts with the hydrophobic alkyl side chain of the ethylamonium cation (EA$^+$). Cation adsorption results in acquisition of a net positive charge preventing aggregation via electrostatic repulsion. It was also suggested that the protein's secondary structure was stabilized by co-ordination of cations and anions to charge residues. Combined, these interactions prevent aggregation and other denaturation mechanisms. Upon cooling, EA$^+$ must desorb from the hydrophobic surface of the core and from lysozyme's charged residues if the protein is to properly refold (re-nature). For this to occur the energy of

refolding must be sufficient to overcome the cation adsorption energy.

Our data clearly suggests that the protein refolding energy is greater than that of the adsorbed cations and refolding 280 occurs at 25 wt% EAF. This is consistent with previous reports.[18, 19] However, with increasing IL concentration, the cation chemical potential also increases, favouring adsorption to the protein. At these higher concentrations (and especially at 75 wt%) the energy associated with refolding is insufficient 285 to drive the cation from favourable (hydrophobic and/or electrostatic) interactions such that desorption does not occur. As a result the protein does not refold, but aggregation is prevented due to the presence of adsorbed IL, consistent with the absence of any cloudy appearance in post-analysed 290 samples.

The effect of increasing cation hydrophobicity was examined using PAF, which has a C_3 alkyl group. CD spectrums recorded at PAF loadings of 25 wt%, 50 wt%, and 75 wt% are presented in Figure 2. An additional sample 62.5 295 wt% was also prepared for this IL as the marked change in behaviour for this IL occurs between 50 wt%, and 75 wt%. For all IL concentrations, the spectra at 25°C show clear deviation form that obtained in pure water (and EAF), particularly between 285 nm and 295 nm where the triplet 300 feature is no longer apparent. This demonstrates that the environment of the tryptophan residues (at least) is markedly different in the presence of PAF compared to EAF and buffered aqueous samples. Logically, as the only difference between EAF and PAF is the size of the alkyl group, the 305 increased hydrophobic interactions are responsible for this difference.

The CD data indicate that lysozyme is able to refold into a structure similar to its original conformation for PAF loadings up to 50 wt% (Figure 2b), and remains constitutively active 310 (data presented below). At a PAF loading of 62.5 wt% the lysozyme partially refolds (Figure 2c), but the differences between the both the initial and final CD spectrum and that of lysozyme in pure water are marked, indicating non-native structure. At 75 wt% PAF the protein is denatured even 315 before heating (Figure 2d). This is likely due to the hydrophobicity of PAF creating a liquid environment agreeable for the hydrophobic core, and the protein unfolds. This suggests that increasingly hydrophobic ILs destabilise lysozyme, so lowering the hydrophobic nature of the IL 320 should increase stability, demonstrated below using EtAF and MOEAF.

The ability of the protein to refold at concentrations up to 62.5 wt% PAF compared to between 25 wt% to 50 wt% for EAF is surprising given that the refolding mechanism 325 proposed above requires desorption of IL cations. Intuitively, one would expect that the strength of electrostatic interactions between the cation and the protein would be the same for both EA^+ and PA^+, but that hydrophobic interactions would be significantly enhanced for PA^+ due to its longer alkyl group. 330 This means that the PA^+ would be more strongly bound to lysozymes hydrophobic core, which should prevent refolding, but in fact the opposite situation occurs. Most likely, self-aggregation of PAF in water is greater than for EAF due to the cation's increased hydrophobicity which lowers the energetic

335 cost of desorbing PA^+ compared to EA^+. As a result, the protein is able to refold at higher PAF concentrations.
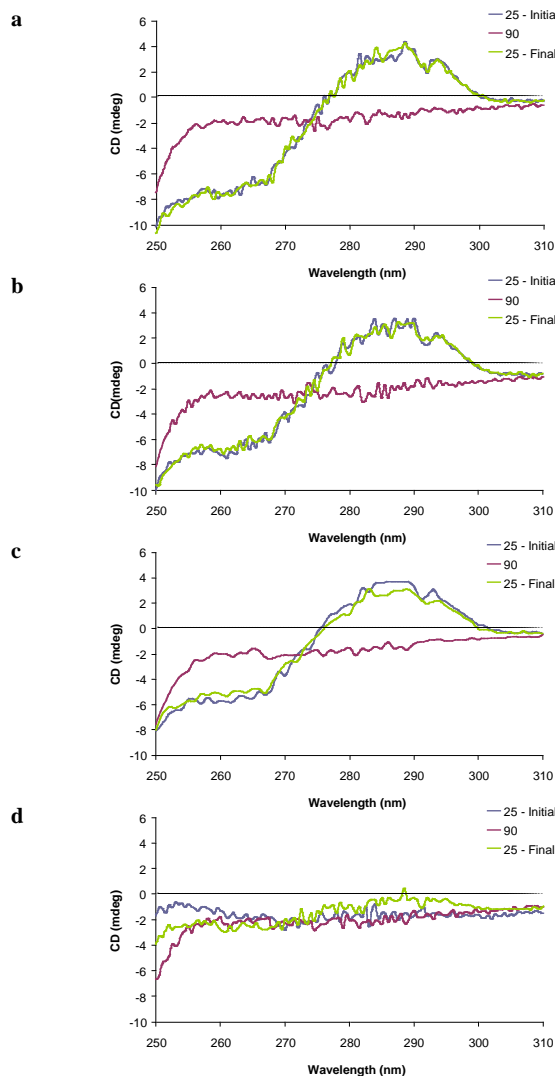
Fig. 2. Near UV CD spectrum of aqueous lysozyme (3.4×10^{-4} mol.dm^{-3}) at PAF loadings of (a) 25 wt%, (b) 50 wt%, (c) 62.5 wt% and (d) 75 340 wt%..

The effect of increasing the length of the cation alkyl chain without substantially changing hydrophobicity (relative to EAF) was investigated using MOEAF. MOEAF has a slightly 345 longer aliphatic backbone then PAF due to the ether group two carbons from the amine, but this ether group also disrupts hydrophobic interactions, and MOEAF is slightly more polar than EAF. The CD spectra obtained for 25 wt%, 50 wt% and 75 wt% MOEAF are presented in Figure 3. The results 350 obtained are similar those for EAF at equivalent concentrations, the key differences being that the characteristic triplet feature disappears at lower IL loadings, and that re-naturing is more efficient for MOEAF than for EAF at 50 wt% IL. The first observation suggests that the 355 immediate environment of tryptophan differs for the two ILs. The second observation is consistent with decreased strength of hydrophobic interactions between $MOEA^+$ and lysozyme's hydrophobic core, permitting refolding at higher IL concentrations according to the stated mechanism.

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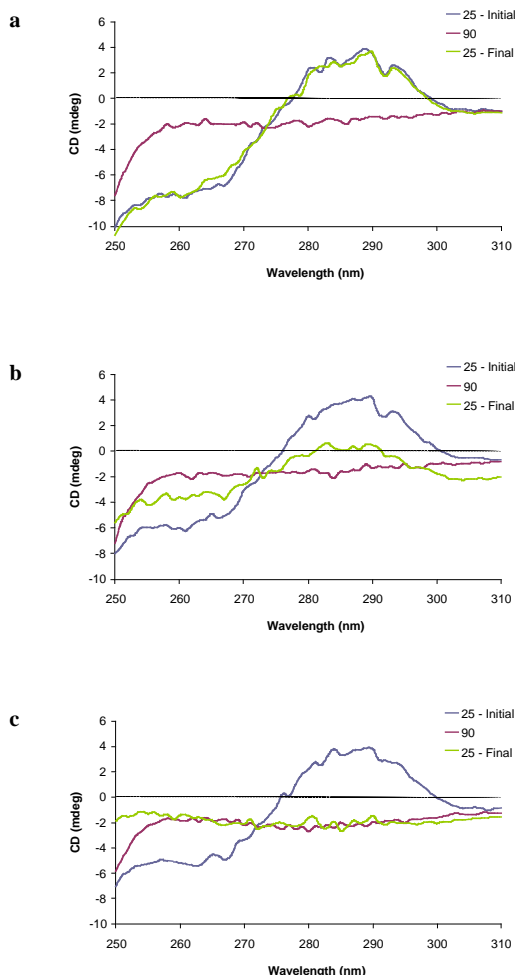
Fig. 3. Near UV CD spectrum of aqueous lysozyme (3.4×10^{-4} mol.dm^{-3})
365 at MOEAF loadings of (a) 25 wt%, (b) 50 wt%, (c) 75 wt%.

Given the enhanced stability noted for MOEAF, subsequent
experiments examined the effect of demethylation to the free
alcohol moiety affording EtAF, which is more hydrophilic
370 than the ether group, and thus more disruptive to hydrophobic
interactions. Alcohol substituted cations will interact less
strongly with the hydrophobic core of lysozyme, but more
favourably with the hydrophilic regions which are normally
exposed in aqueous environments even when the protein is
375 folded. Alcohols groups have a significantly stronger electron
withdrawing effect compared to unsubstituted aliphatic
chains, which significantly decreases the N-H proton
availability.[19]
CD spectra for EtAF for 25 wt%, 50 wt% and 75 wt% are
380 shown in Figure 4. The characteristic triplet feature is of
reduced intensity but activity data (below) clearly shows that
the protein is active. The striking feature is increased
temperature stability. On the timescale of the experiment,
protein unfolding is clearly reduced for 25 wt% EtAF as the
385 temperature is cycled from 25°C to 90°C and back to 25°C,
and refolding into the initial state appears to be complete,
although this will undoubtedly but partially due to the fact

that the protein does not completely denature. Increased
temperature stability is even more pronounced at 50 wt%
390 EtAF, then decreases slightly for 75 wt% EtAF. It should be
noted that the viscosity of EtAF is approximately 8 times
greater than EAF[36] but viscosity decreases rapidly as water is
added and the temperature increases, such that for the
different ILs investigated here the viscosity difference is less
395 pronounced.[37] The fact that stability is only slightly reduced
for the highest EtAF loading shows that temperature stability
is not purely due to viscosity.
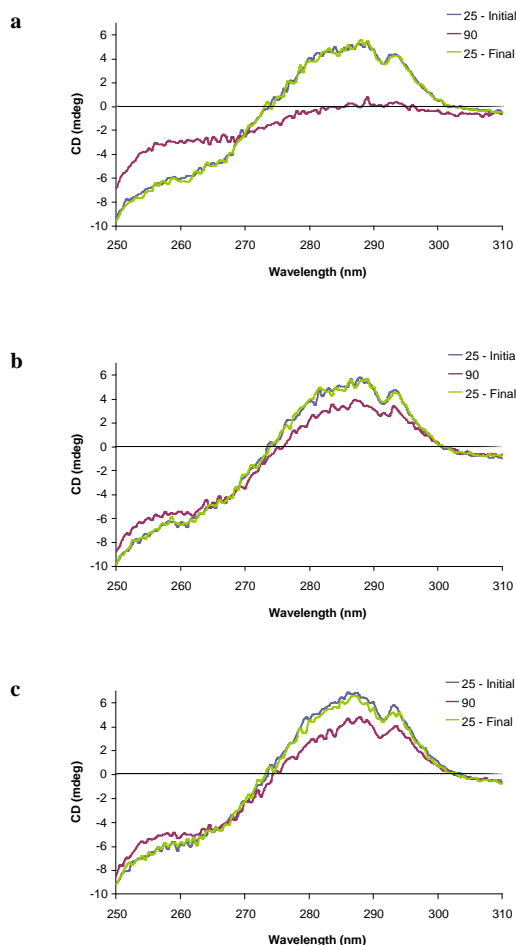
400
Fig. 4. Near UV CD spectrum of aqueous lysozyme (3.4×10^{-4} mol.dm^{-3})
at EtAF loadings of (a) 25 wt%, (b) 50 wt%, (c) 75 wt%.

The CD signal at 288 nm (the primary peak) for lysozyme
405 immersed in 75 wt% EtAF and 25 wt% water at 90°C (15 °C
higher than lysozymes reported melting temperature[18]) as a
function of time is presented in Figure 5. For comparison
lysozyme in buffer solution is also shown. In the absence of
IL the protein is unfolded by the time the first CD
410 measurement is completed, but with added EtAF
approximately 1 hour passes before lysozyme unfolding is
complete.
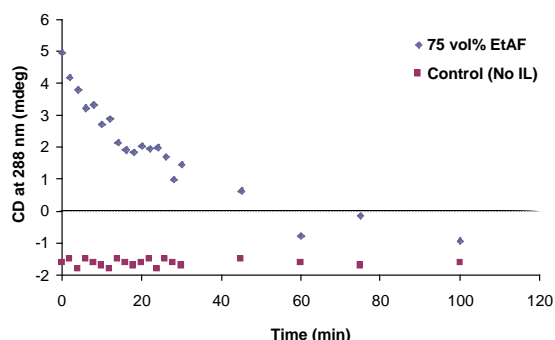
Fig 5. CD signal at 288 nm for lysozyme immersed in 75 wt% EtAF and 25 wt% water at 90°C as a function of time. A control experiment of lysozyme in water is shown for comparison.

The reason for enhanced temperature stability in the presence of EtAF compared to ILs that do not contain an alcohol group is not immediately obvious. We believe EA^+ interacts with negatively charged residues electrostatically, hydrogen bond to the protein via amine protons, and interacts with hydrophobic regions via its alkyl tail. While electrostatic interactions between EtA^+ and lysozyme are expected to be about the same as for EA^+, the alcohol group of EtA^+ provides and extra hydrogen bonding donor/acceptor site and will markedly reduce the strength of hydrophobic interactions with the protein. The fact that enhanced temperature stability is not observed for MEOAF, where the ether oxygen can act as a hydrogen bond acceptor, suggests an additional hydrogen bond donor group is pivotal to the observed increase in stability. Therefore, our results suggest that cations with multiple hydrogen bond donor sites co-ordinate to the protein more effectively than cations with a single hydrogen bond donor/acceptor group and non-alcohol containing alkyl chain, and thereby stabilise structure more effectively.

Activity Measurements.

The rate at which lysozyme hydrolyses 1,4-β-linkages in gram-positive cells (between *N*-acetylmuramic acid (NAM) and *N*-acetyl-D-glucosamine (NAG) residues in peptidoglycans and between *N*-acetyl-D-glucosamine residues in chitodextrins) is strongly influenced by preparative methods and the cell substrate conditions (whether freeze dried or fresh). As a result there is no accepted 'rate' of lysozyme action, and kinetics are examined by comparison to a control sample.[18, 27] The cell suspension is initially opaque, but as lysozyme hydrolyses the cell turbidity decreases, eventually yielding a transparent solution. Lysozyme activity measurements were performed on suspended *Micrococcus lysodeikticusce* cells for each of the four protic ILs at 25 wt%, 50 wt% and 75 wt%, c.f. Figure 6, except for 75 wt% PAF as the protein is immediately denatured in this solution. Solution turbidity was monitored using UV-Vis spectroscopy and the initial rate determined from a tangent drawn to the first few data points. The turbidity of blank solutions containing suspended cells and IL but no lysozyme was invariant over the timescale of the experiment, approximately 1 hour.
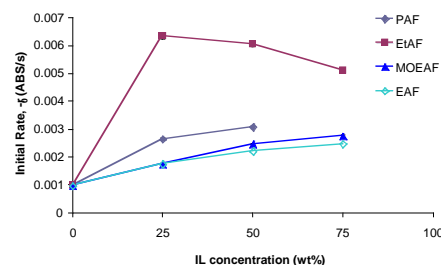
Fig 6. Rate of hydrolysis from lysozyme (2.6×10^{-8} mol.dm^{-3}) in 0.0014 % (w/v) *Micrococcus lysodeikticusce* at varying PIL concentrations.

The first and perhaps most important result is that lysozyme is active in the presence of all four protic ILs at 25°C. This shows that the structural changes that produce differences in CD spectra as a function of IL species and concentration have not deactivated the protein. It should be noted that activity measurements were also completed on lysozyme in the presence of 25 wt% IL following a temperature denaturing cycle. The activity was essentially equal to that determined for the lysozyme which had not been subjected to a temperature denaturing cycle.

The activity as a function of concentration for EAF, PAF, and MOEAF (ILs that do not possess alcohol moieties) follow similar trends; activity increases with IL concentration. The results for EAF and MOEAF are indistinguishable within error, with rates about twice that measured for lysozyme in buffered water obtained at 50 wt% and 75 wt% IL. Lysozyme is considerably more active in PAF at all concentrations, with rates about three times greater than that measured in buffered water obtained at higher IL loadings.

Lysozyme is even more active in the presence of EtAF, with rates six times faster than those determined for buffered water determined for 25 wt% IL. This activity decreases slightly at higher IL loadings to about five times faster than for buffered water at 75 wt% EtAF. As the viscosity of pure EtAF is several times higher than the viscosity of the other ILs investigated, the EtAF solutions will be the most viscous at equivalent concentrations, so increased activity for this IL will not be a mass transport phenomenon.

In general terms, the activity measurements follow trends observed in the protein denaturing – renaturing CD experiments. The greatest activity and most complete refolding was determined for EtAF, followed by PAF, while EAF and MOEAF were about equally effective in terms of activity enhancement and refolding. At the present moment it is unclear how these two sets of results are related, but it may be that the ILs re-enforce protein structure such that it is able to perform its lysing function more vigorously. Further experiments are required to test this hypothesis, but it would explain the correlations between protein activity, stability and IL species.

Conclusions

CD experiments have been used to probe lysozyme structure in aqueous solutions of 25 wt%, 50 wt% and 75 wt% IL (EAF, PAF, MOEAF, EtAF), and to assess the protein's ability to refold after heating to 90°C. EAF and MOEAF are

similarly effective refolding additives, while PAF is more effective at concentrations up to ~ 62.5 wt%. At higher PAF concentrations lysozyme denatures spontaneously, as adsorption of PA^+ to the proteins hydrophobic core effectively protects it from the bulk hydrophilic solvent. EtAF is stabilises lysozyme against unfolding at high temperature, and renaturing appears to be near complete upon cooling. This could be a consequence of differences in the way this alcohol containing IL interacts with lysozyme compared to ILs that do not contain alcohols. Electrostatic interactions between EtA^+ and lysozyme are expected to be about the same as for the other ILs, but the alcohol group of EtA^+ provides an extra hydrogen bonding donor/acceptor site which also reduces the strength of hydrophobic interactions with the protein. As enhanced temperature stability is not noted for MEOAF, where the ether oxygen can act only as a hydrogen bond acceptor, our results suggest that an additional hydrogen bond donor group is key. The temperature stability trends correlate well with activity data. Both EAF and MEOAF moderately increase lysozyme activity, with increased activity noted in the presence of PAF, and activity several times greater noted in the presence of EtAF. It is possible that the ILs re-enforce protein structure such that it is able to perform its lysing function more readily, which would account for activity being highest for EtAF, the IL which is the most effective stabilising agent.

Materials and Methods.

Lysozyme from chicken egg white (lyophilized powder, Protein $\geq$ 90 %, $\geq$ 40,000 units/mg protein) and *Micrococcus lysodeikticusce* cells (ATCC No. 4698) were purchased from Sigma and used without further purification. Ethylamine (70%), propylamine (70%), 2-methoxyethylamine (99%), ethanolamine (>99%), propanolamine (>99%), and formic acid (>98%) were also obtained from Sigma-Aldrich and used without further purification.

The protic room temperature ILs ethylammonium formate (EAF), propylammonium formate (PAF), ethanolammonium formate (EtOAF), and 2-methoxyethylammonium formate (MOEAF) were prepared by reacting equimolar amounts of the amine and concentrated formic acid to produce an aqueous solution.[23-25] Water was then removed by rotary evaporation at 25 ^oC, the last vestiges of water was removed by lyophilization affording ILs with water contents less than 0.5% (Karl Fischer titration). IL purity and identity was verified using ^{1}H-NMR spectroscopy. Formate based ILs are known to form amides via condensation reactions, e.g. after 50 months at room temperature 25% of EAF has been converted to amide.[26] In light of this, all experiments performed here used freshly prepared ILs. NMR revealed that during a heating – cooling cycle not more than 1% of IL reacted to form amide, and frequently conversion was much less.

CD measurements were performed on an Applied Photophysics Chirascan Spectrometer. Lysozyme concentrations of 3.4 $x10^{-4}$ mol.dm^{-3} were examined in the near UV region (310 to 250 nm) in a 0.1 cm path length quartz cell at a bandwidth and step size of 1 and 0.5 nm respectively.

Solutions containing 25, 50 and 75 wt% IL were prepared approximately one hour prior to measurements. Each sample was subject to a heating cycle of 25-90-25 °C at a rate of 20 °C/min (total analysis time = 6.5 min). Data was obtained in the form of CD (mdeg) versus wavelength (nm) and used without further modification.

Lysozyme assays were performed on a Shimadzu UV-1700 UV/Vis Spectrometer. Cell suspensions containing 0.0014 % (w/v) *Micrococcus lysodeikticusce* cells were prepared to give an initial absorbance of 1.4 at 450 nm and 25 ^oC. Stock solutions of 6.8 $x10^{-6}$ mol.dm^{-3} lysozyme in water - IL mixtures (25, 50 and 75 wt% IL, along with 62.5 wt % for PAF) were prepared and chilled to 4 ^oC prior to measurement. 2.9 cm^3 of the cell suspension was placed into a 1 cm path length quartz cell and allowed to equilibrate to instrument's operating conditions (25 ^oC and 1 atm). 0.1 cm^3 of the lysozyme solution was then added to the cell suspension and quickly inverted five times prior to measurement. The absorbance was then measured at 450 nm and 25 ^oC.[18, 27] Control solutions of the cell suspension and IL but no lysozyme produced a constant absorption signal for several hours.

Acknowledgements

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Activity and Thermal Stability of Lysozyme in Alkylammonium Formate Ionic Liquids – Influence of Cation Modification.

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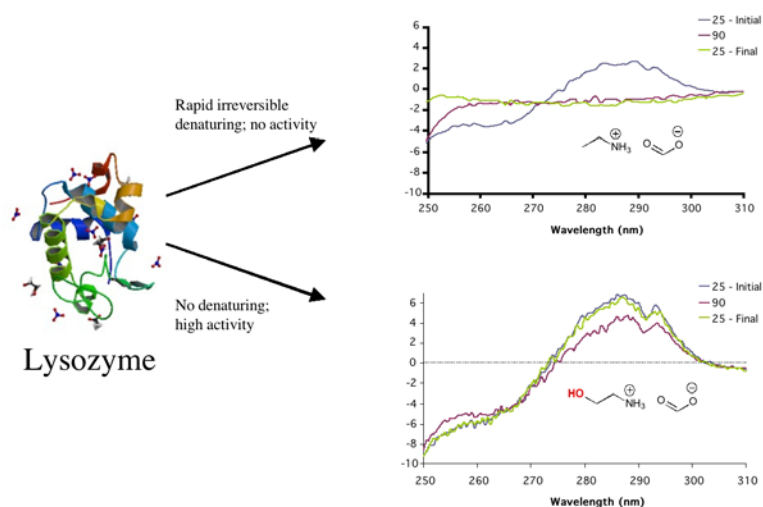
Variation in the cation molecular structure of protic room temperature ionic liquids produces
670 remarkable differences in the thermal stability and activity of lysozyme. Results suggest specific
interactions between the cation and enzyme.