Chemical Engineering Journal 147 (2009) 6–12

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Small-angle neutron scattering studies of model protein denaturation in aqueous solutions of the ionic liquid 1-butyl-3-methylimidazolium chloride

Gary A. Baker[a,*], William T. Heller[a,b,*,1]

[a] Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
[b] Center for Structural Molecular Biology, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

ARTICLE INFO

Keywords:
Ionic liquids
Protein aggregation
Small-angle scattering
Chaotropes
Cytochrome *c*
Human serum albumin
Hofmeister series

ABSTRACT

As we advance our understanding, ionic liquids (ILs) are finding ever broader scope within the chemical sciences including, most recently, pharmaceutical, enzymatic, and bioanalytical applications. With examples of enzymatic activity reported in both neat ILs and in IL/water mixtures, enzymes are frequently assumed to adopt a quasi-native conformation, even if little work has been carried out to date toward characterizing the conformation, dynamics, active-site perturbation, cooperativity of unfolding transitions, free energy of stabilization, or aggregation/oligomerization state of enzymes in the presence of an IL solvent component. In this study, human serum albumin and equine heart cytochrome *c* were characterized in aqueous solutions of the fully water-miscible IL 1-butyl-3-methylimidazolium chloride, [bmim]Cl, by small-angle neutron and X-ray scattering. At [bmim]Cl concentrations up to 25 vol.%, these two proteins were found to largely retain their higher-order structures whereas both proteins become highly denatured at the highest IL concentration studied here (i.e., 50 vol.% [bmim]Cl). The response of these proteins to [bmim]Cl is analogous to their behavior in the widely studied denaturants guanidine hydrochloride and urea which similarly lead to random coil conformations at excessive molar concentrations. Interestingly, human serum albumin dimerizes in response to [bmim]Cl, whereas cytochrome *c* remains predominantly in monomeric form. These results have important implications for enzymatic studies in aqueous IL media, as they suggest a facile pathway through which biocatalytic activity can be altered in these nascent and potentially green electrolyte systems.

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1. Introduction

The implementation of biomolecules in ionic liquid (IL)-based media has sparked immense interest for applications ranging from applied biocatalysis for hydrogen production to bioanalysis and cellulose processing [1–3], resulting in several reviews on the subject [3–12]. Lipases, most notably *Candida antarctica* lipase B (CALB), have received the most attention [13–29], due primarily to their broad industrial utility as well as for exceptional tolerance of non-aqueous environments; much of the research to date has focused solely on enzymatic activity. Other proteins have also been characterized for their catalytic activity, including α-chymotrypsin [30,31], horseradish peroxidase [32–34], papain [35,36], cytochromes [37–39] and tyrosinase [40], among others. Most recently, the first examples of homogeneous and heterogeneous bioreceptor-based immunoassay were described in which an antibody was found to retain its high-affinity haptenic recognition in aqueous buffer containing 75 vol.% [bmim][BF_4] as well as in a number of water-free [bmim][A] ILs where A = BF_4^-, NTf_2^-, TfO^-, or PF_6^- [41]. The excitement about possible application of enzymatic transformations and clean biotechnological processes by means of IL-based media continues to drive research in this area; in this regard, ILs may be employed as co-solvents for water, in biphasic systems, or as "neat" solvents containing little or no water. The effects of various ILs on enzymatic reactions, however, vary wildly and unpredictably, sometimes with seemingly conflicting reports. Clearly, a profusion of systematic studies are mandatory in order to establish a general understanding of structure–function relationships for enzymes in IL media.

Information on protein structure, dynamics, and solvation is crucial for understanding protein function in ILs. Regardless, relatively few biophysical characterizations of protein molecules in systems containing ILs exist in the literature. In particular, the influence of ILs on enzyme structure has only recently become a focus of study. In the original report on protein spectroscopy conducted within an IL, it was shown, using fluorescence from the single tryptophan residue in the sweet protein monellin, that the use of the IL 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide,

* Corresponding author. Tel.: +1 865 241 9361; fax: +1 865 576 5235.
E-mail addresses: bakerga1@ornl.gov (G.A. Baker), hellerwt@ornl.gov (W.T. Heller).
[1] Tel.: +1 865 241 0093; fax: +1 865 574 6268.

1385-8947/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.cej.2008.11.033

[bmpy][NTf_2], containing a modest amount of water (2 vol.%) yielded an astonishing entropically driven stabilization in opposition to thermal unfolding (i.e., the unfolding entropy, ΔS°, was determined to be 250 and 136 $J K^{-1} mol^{-1}$ for monellin in water and [bmpy][NTf_2] containing 2 vol.% water, respectively), consistent with the more rigid solvation present within wet [bmpy][NTf_2] [42]. Second-derivative single-pass attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy in the amide I region was employed by Lou et al. [36] to reveal the impact of the presence of 15 vol.% 1-alkyl-3-methylimidazolium, [$C_n$mim]$^+$, ILs combined with various anions on the structure of papain in buffer, and to correlate these conformational changes with the observed catalytic activity and selectivity of the enzyme. Contributing to the prevailing outlook in this area, their results demonstrate that in aqueous IL systems involving anions with pronounced hydrogen bond basicity (i.e., $[Cl]^-$, $[NO_3]^-$, $[CH_3CO_2]^-$, and especially $[HSO_4]^-$), a non-native amide I spectrum was observed as a result of disruption of internal hydrogen bonding, this perturbation being accompanied by a proportionate loss of activity. Meanwhile, for the more enzyme-suited anion $[BF_4]^-$, which exhibits a lower nucleophilicity, comparable or higher activity and enantioselectivity was exhibited by papain in comparison with aqueous phosphate buffer. Additionally, it is notable that although losses in α-helical content were apparent for papain in aqueous [$C_n$mim][BF_4] (n = 2–6), the contribution of β-turns and sheets increased, this evolution in secondary structure ostensibly leading to a more compact and stable enzyme configuration. Similarly, Iborra and co-workers [43] reported that the stabilization of CALB observed both in 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, [emim][NTf_2], and butyltrimethylammonium bis(trifluoromethylsulfonyl)imide, [N_{4111}][NTf_2], at 2 vol.% water content and 50 °C, was associated with a partial preservation of native α-helicity with simultaneous enhancement in β-strand content (as determined by circular dichroism, CD), again presumably resulting in a more condensed enzyme conformation displaying catalytic activity. In a prior study, the same authors showed that [emim][NTf_2] containing 12.5 vol.% 1-propanol bestowed excellent thermal stability to α-chymotrypsin, with an unfolding enthalpy triple that determined in water [44]; once more, the degree of stabilization appeared to be linked to the prevalence of β-strands in the presence of IL. Using small-angle neutron scattering (SANS) and dynamic light scattering (DLS), Sate et al. [45] showed that CALB dissolved in [emim][A] ILs undergoes substantial and inhomogeneous agglutination leading to a curtailed activity (A = $EtOSO_3^-$ and NO_3^-) or complete deactivation (A = $N(CN)_2^-$), lending credence to the notion that highly coordinating anions do not support enzyme action in ILs. In a key study, Weingärtner used differential scanning calorimetry (DSC) to systematically elucidate the impact of various cation/anion pairings on the thermal stability of ribonuclease A in aqueous systems containing a range of [emim]$^+$ or bromide-based ILs based on salt-induced shifts of the transition temperature [46]. During this research, the authors learned that protein–IL interactions within aqueous solutions can be loosely interpreted within a Hofmeister framework and, consistent with enzyme activity results from Zhao [47], anion variations generally appear to have larger consequences than cation variations. Incidentally, it is likely that this rule of thumb represents something of an oversimplification, particularly as ILs including wider cation types become available [48]. Most recently, fluorescence spectroscopic examination of singly-labeled defatted human serum albumin (HSA), prepared by site-specific conjugation of the solvent-responsive fluorescent reporter acrylodan to Cys-34, revealed that the thermal unfolding process of HSA in three different 1-butyl-3-methylimidazolium-based ILs ([bmim][A]; A = NTf_2^-, BF_4^-, and PF_6^-), as well as the protein's dynamical motions, deviated markedly from the mechanism followed in aqueous buffer [49]. The Angell group [50] also recently reported a remarkable multi-year stabilization against aggregation losses for near-saturation solutions of lysozyme in IL-rich, ice-avoiding solvent systems also containing sucrose and water. The protic IL ethylammonium nitrate itself was found to confer protection against aggregation; indeed, ~97% retention in refoldable protein sample per denaturing cycle was established, as judged by the declining area under the unfolding endotherm in successive DSC upscans to 100 °C. The vast array of physicochemical properties afforded by ILs necessitates continued research in this area to build a more complete picture of the influence of IL ion pairing on protein structure and aggregation and to correlate this behavior with enzyme activity.

Small-angle scattering methods, using neutrons or X-rays (SANS and SAXS, respectively), are ideally suited to the study of the impact of ILs on the structures of soluble proteins. These methods probe the global conformations of particles in solution, and have been used extensively to study biological macromolecules in solution; a review emphasizing this topic appeared recently by Svergun and Koch [51]. SANS and SAXS are excellent probes of solution-phase protein structural modification (e.g., denaturation, aggregation, oligomerization, ligand complexation), because these techniques are inherently sensitive to changes in the shape, size, and compactness of the scattering particle. In particular, a fair number of experimental studies have appeared in which small-angle scattering methods provide key information concerning the solution conformation of model proteins like the serum albumins [52–55] or equine heart cytochrome *c* (cyt *c*) [56–60] under denaturing conditions driven by chemical agents, or extremes of pH or temperature.

In the current work, we present the details of our investigations into the behavior of two of the most thoroughly characterized proteins within aqueous mixtures of the entirely water-miscible IL 1-butyl-3-methylimidazolium chloride, [bmim]Cl, using small-angle scattering and CD spectroscopy. The first model protein we selected for study, equine heart cyt *c*, is among the most well-studied metalloproteins. A class I *c*-type cytochrome involved in electron transfer in the mitochondrial respiratory chain, equine heart cyt *c* consists of a single polypeptide chain containing 104 amino acid residues that is covalently anchored by two thioether bonds at Cys-14 and Cys-17 to an iron-containing heme. The second archetypical protein we chose, HSA, is larger and a bit more complex, consisting of a single polypeptide chain of 585 amino acid residues and having a heart-shaped structure divided into three major and fairly distinct domains stabilized by 17 disulfide bonds. The most abundant protein found within human plasma, not surprisingly, HSA is involved in many physiological functions, including blood pH and pressure maintenance. Both cyt *c* and HSA were found to denature in high concentrations of [bmim]Cl, but the impact of the IL on the structure was not particularly pronounced until the aqueous solution reached 50 vol.% [bmim]Cl. SANS demonstrates a transition of the polypeptide chains from compact structures to highly denatured states; SAXS results corroborate this view. Visible CD spectroscopy in the Soret region of cyt *c* suggests that the environment of the heme is considerably perturbed at 25 vol.% [bmim]Cl before adopting a state consistent with a urea-denatured state [61] at the highest IL content studied (50 vol.%).

2. Experimental

2.1. Materials

Essential fatty acid-free human serum albumin (HSA) and cyt *c* from equine heart were obtained as lyophilized powders from Sigma (St. Louis, MO, USA) and used without further purification. High-purity [bmim]Cl was synthesized according to established lit-