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Protein–Ion Interactions

Protein Denaturation by Ionic Liquids and the Hofmeister Series: A Case Study of Aqueous Solutions of Ribonuclease A**

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Biocatalytic reactions provide highly useful tools for synthesis in laboratory and industry. Whereas nature has optimized biocatalysts in an aqueous environment, alternative solvents may improve properties such as the selectivity of a reaction or the stability of substrates. In this context, room-temperature molten salts, usually referred to as "ionic liquids" (ILs), have become of interest as co-solvents for water,[1] in biphasic systems,[1] and as neat solvents.[1,2] ILs comprise organic cations, such as alkyl-substituted imidazolium, pyrrolidinium, and tetraalkylammonium ions, combined with a variety of anions defined later in this paper. For the major families of cations and anions see a review by Forsyth et al.[3]

In view of the growing importance of ILs, it is desirable to systematize the ion effects on enzyme properties such as stability, activity, and enantioselectivity. In aqueous solutions, salt effects have been of a long-standing debate because biological processes often occur at fairly high salt concentrations. Phenomenologically, these salt effects are systematized in the Hofmeister series, going back to Hofmeister's early study of the salt-induced precipitation of hen egg white proteins.[4] This series reflects the ability of ions to stabilize/destabilize the native state of proteins,[5] but other criteria, such as the ability to promote enzyme activity, were also used,[6,7] and the concept has found widespread applications in other areas such as colloid, polymer, and surface chemistry.[6] In some cases there seem to be specific interactions between the ions and proteins, but similar correlations for a broad range of phenomena point toward a more universal basis.[6] A possible rationale is founded in ion-induced changes in the water structure, which may promote the binding of ions to the protein interface or favor their exclusion from the interface.[8]

In the past, Hofmeister effects on enzyme behavior have been studied for many inorganic salts. For organic ions the information is essentially limited to tetraalkylammonium and guanidinium salts.[6,9] The growing importance of ILs in biocatalysis renders the characterization and understanding of Hofmeister effects generated by more complex ions to be mandatory because correlations may allow to predict IL-induced effects on many enzyme properties. Until now rudimentary data are availably on Hofmeister effects caused by the ions of ILs.[7]

In this study, we use differential scanning calorimetry (DSC) to systematically characterize the effect of ILs on the thermal denaturation of ribonuclease A (RNase A) near 60 °C. Our experiments fulfill two important criteria for a representative case study: first, protein stability is perhaps the most widely used probe for Hofmeister effects.[6] Second, in many studies of protein stability and protein hydration, RNase A was used as a prototypical model compound.[6,8,9] In particular, RNase A has been the subject of a pioneering study of salt effects upon the thermal stability of enzymes by von Hippel and Wong.[9] Simple salts were found to progressively shift the denaturation transition towards higher or lower temperatures, thus increasing or decreasing the thermally stable range of native RNase A. Effects of cations and anions were found to be practically additive, thus making it possible to establish mutually independent cation and anion series. When the transition temperatures from reference [9] are shifted by 2 °C, they can be linked to the data reported here.

Because notable shifts of T_m required salt concentrations above 0.1 M, we used salts that are completely miscible with water. In addition to simple inorganic ions, the following ions were considered: 1-ethyl-3-methylimidazolium ([emim]$^+$), 1-butyl-3-methylimidazolium, ([bmim]$^+$), 1-hexyl-3-methylimidazolium ([C_6mim]$^+$), 1-butyl-1-methylpyrrolidinium ([bmpyrr]$^+$), tetraalkylammonium ([$C_{i,j,k,l}$N]$^+$), and guanidinium ([guan]$^+$). The complex anions used are thiocyanate ([SCN]$^-$), methylsulfate ([$MeOSO_3$]$^-$), ethylsulfate ([$EtOSO_3$]$^-$), trifluoromethanesulfonate ([TfO]$^-$), bis(trifluoromethanesulfonyl)imide ([Tf_2N]$^-$), and dicyanimide ([$N(CN)_2$]$^-$).

Figure 1 shows some typical DSC traces. In the IL-free sample (5.0 mg mL^{-1} bovine RNase A, 10 mM Na_2HPO_4 buffer, pH 7), denaturation sets on near 53 °C and is essentially complete near 73 °C. Repetitive DSC runs of RNase A in IL solutions showed that a small fraction of protein unfolded irreversibly, which became less at higher salt concentration. Nevertheless, a two-state transition model was used to fit the DSC traces, leading to a good match of theoretical and experimental curves and yielding the T_m value. The DSC traces were characterized only by the peak for the protein unfolding at different temperatures, with the exception of [bmim][BF_4], which gave rise to a second peak near 100 °C, probably related to hydrolysis of [BF_4]$^{-[1a]}$ at elevated temperatures.

Figure 2 shows results for T_m at concentrations typically up to $c = 1.5$ M obtained for salts with Br^- and Cl^- as common anions, respectively. In contrast to many inorganic salts, all

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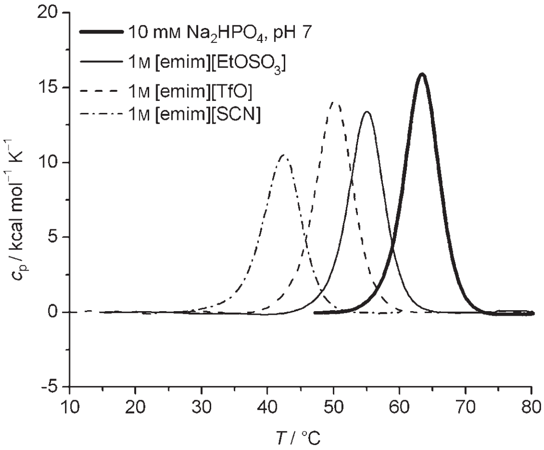

Figure 1. Examples for base-line-corrected DSC profiles of aqueous solutions of RNase A with added salts.

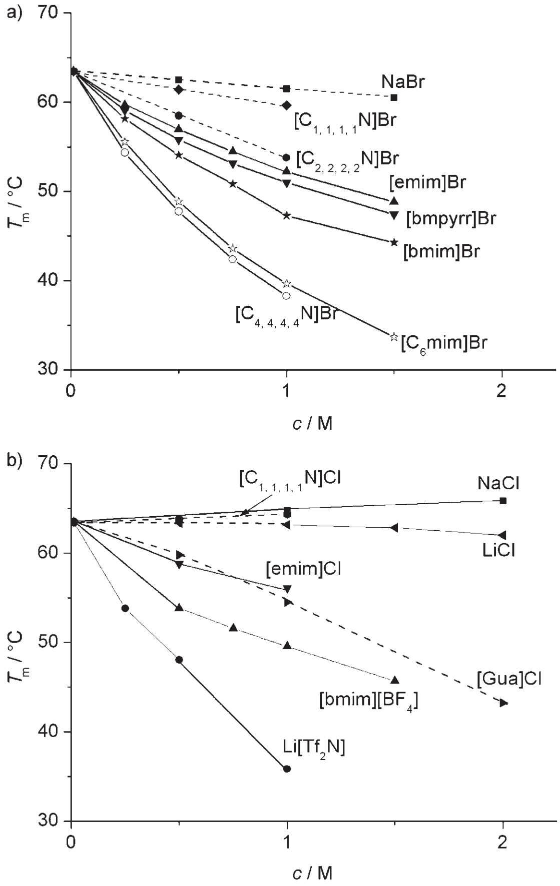

Figure 2. Transition temperatures T_m for the thermal denaturation of RNase A as a function of the concentration c of added ILs with a) Br$^-$ and b) Cl$^-$ as a common anion. Data for some other salts are also given. Dashed lines refer to data from Ref. [9].

organic salts (except for [C$_{1,1,1,1}$N]Cl[9]) destabilize RNase A, at least for the anions under test. In terms of decreasing T_m values, the resulting cation series reads: K$^+$ > Na$^+$ > [C$_{1,1,1,1}$N]$^+$ > Li$^+$ > [C$_{2,2,2,2}$N]$^+$ ≈ [emim]$^+$ > [bmpyrr]$^+$ > [bmim]$^+$ ≈ [C$_{3,3,3,3}$N]$^+$ > [C$_6$mim]$^+$ ≈ [C$_{4,4,4,4}$N]$^+$ Thus, the more hydrophobic the cation, the stronger the decrease in T_m. Literature data for the effect of three imidazolium chlorides on the thermal stability of lysozyme[10] confirm this picture. The different behavior of [bmpyrr]$^+$ and [bmim]$^+$ indicates a small difference in the hydrophobicity of structurally similar pyrrolidinium and imidazolium salts. This is possibly a result of the positive charge concentrated at the nitrogen in [bmpyrr], whereas in [bmim]$^+$ it is delocalized over the ring.

Figure 3 shows results for ILs with [emim]$^+$ as a common cation. Because organic salts comprising the widely used [Tf$_2$N]$^-$ ion are not miscible with water, we resorted for comparison to Li[Tf$_2$N] (Figure 2b) for ranking the [Tf$_2$N]$^-$ anion in the series. In terms of decreasing T_m values, the anion series reads:
[SO$_4$]$^{2-}$ > [HPO$_4$]$^{2-}$ > Cl$^-$ > [EtOSO$_3$]$^-$ > [BF$_4$]$^-$ ≈ Br$^-$ > [MeOSO$_3$]$^-$ > [TfO]$^-$ > [SCN]$^-$ ≈ [N(CN)$_2$]$^-$ > [Tf$_2$N]$^-$

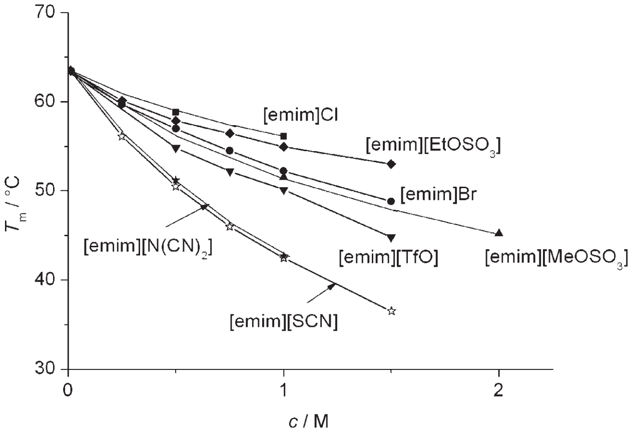

Figure 3. Transition temperature T_m for the thermal denaturation of RNase A as a function of the concentration c of added ILs with [emim] as a common cation.

Weak hydration as well as an increase in hydrophobicity of the anion has a destabilizing effect on the native state of the protein, but it is difficult to rank the anions by hand-waving arguments about ion size, surface charge density, hydrophobicity, etc., because they are not interrelated by homologous series. Even for chemically related ions, there are pecularities, for example, in the position of [MeOSO$_3$]$^-$ relative to >[EtOSO$_3$]$^-$. Zhao et al.[11] found effects in enzyme kinetics to reflect the series [EtOSO$_3$]$^-$ > [CF$_3$SO$_3$]$^-$ > Br$^-$ > [BF$_4$]$^-$, which corresponds to the trend reported by us but differs slightly in the position of neighboring ions.

In a widely used classification, cations and anions are divided into kosmotropic and chaotropic ions. Kosmotropes are said to promote the water structure, chaotropes are assumed to destroy the water structure. Based on this classification, it is commonly stated that kosmotropic anions stabilize the native structure of an enzyme, while kosmotropic cations destabilize the native structure. Thus, anions and cations show opposite correlations with the hydration strength.[6] However, when this picture is extended to complex anions, there are many exceptions to this rule, and the widely adopted picture of kosmotropy and chaotropy is too simplistic. In general, anion variations may have larger consequences on protein stability than cation variations have, as often also observed for enzyme activity.[7]

Although it is a considerable challenge to understand the Hofmeister series per se, the effects seem to be sufficiently general to provide a valuable guide for relating the stability and activity phenomena that underlie biological events in aqueous solutions, ranging from enzyme activity and biocatalysis,[1] to possible correlations affecting the cytotoxicity of ILs.[12] This does not exclude that some proteins exhibit opposite behavior which may be due to the protein's net charge and specific interactions.[13] Moreover, some biocatalyzed reactions are conducted in "neat" ILs as solvents (with little or no water). In these cases the effect of ions on the stability/activity of enzymes seems to be much more complicated[7] and does not follow the Hofmeister series.[14] As an example, we note that in water the hydrophobic ion $[Tf_2N]^-$ was found to be the most destabilizing of all the anions tested, whereas in neat $[Tf_2N]^-$-based salts enzyme stabilization was observed.[15]

Experimental Section

All ionic liquids were obtained from IoLiTec (Denzlingen, Germany), and they were dried for 24 h under vacuum at 60 °C. Bovine ribonuclease A was obtained from Sigma-Aldrich. DSC traces were obtained at a rate of 60 K h^{-1} by VP-DSC instrument (MicroCal, Northampton,USA). Two or three scans were recorded for each thermogram.

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[1] a) *Ionic Liquids* (Eds. P. Wasserscheid, T. Welton), Wiley-VCH, Weinheim, **2003**; b) U. Kragl, B. Eckstein, N. Kaftik, *Curr. Opin. Biotechnol.* **2002**, *13*, 565; c) F. van Rantwijk, R. M. Lau, R. A. Sheldon, *Trends Biotechnol.* **2003**, *21*, 131.
[2] a) S. N. Baker, T. M. McClaskey, S. Pandey, G. A. Baker, *Chem. Commun.* **2004**, 940; b) K. Fujita, M. Forsyth, D. R. McFarlane, R.-W. Reid, G. D. Elliot, *Biotechnol. Bioeng.* **2006**, *94*, 1209.
[3] S. A. Forsyth, J. M. Pringle, D. R. MacFarlane, *Aust. J. Chem.* **2004**, *57*, 113.
[4] T. Hofmeister, *Arch. Exp. Pathol. Pharmakol.* **1888**, *24*, 247.
[5] P. H. von Hippel, K.-Y. Wong, *Science* **1964**, *145*, 577.
[6] a) K. D. Collins, M. W. Washabaugh, *Q. Rev. Biophys.* **1985**, *18*, 323–422; b) W. Kunz, P. Lo Nostro, B. W. Ninham, *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 1.
[7] H. Zhao, *J. Mol. Catal. B* **2005**, *37*, 16.
[8] a) R. Ravindra, R. Winter, *Z. Phys. Chem.* **2003**, *217*, 1221; b) S. N. Timasheff, *Biochemistry* **2002**, *41*, 13473.
[9] P. H. von Hippel, K.-Y. Wong, *J. Biol. Chem.* **1965**, *240*, 3909.
[10] C. Lange, G. Patil, R. Rudolph, *Protein Sci.* **2005**, *14*, 2693.
[11] H. Zhao, S. M. Campbell, L. Jackson, Z. Song, O. Olubajo, *Tetrahedron: Asymmetry* **2006**, *17*, 377.
[12] See, for example, a) J. Ranke, A. Müller, U. Bottin-Weber, F. Stock, S. Stolte, J. Arning, R. Störmann, *Ecotoxicol. Environ. Safety* **2007**, *67*, 430; b) S. Stolte, J. Arning, U. Bottin-Weber, F. Stock, K. Thiele, M. Uerdingen, U. Welz-Biermann, B. Jastorff, J. Ranke, *Green Chem.* **2006**, *8*, 621.
[13] M. Boström, F. W. Tavares, S. Finet, F. Skouri-Panet, A. Tardieu, B. W. Ninham, *Biophys. Chem.* **2005**, *117*, 217.
[14] R. M. Lau, M. J. Sorgetrager, G. Carrea, F. van Rantwijk, F. Secundo, R. A. Sheldon, *Green Chem.* **2004**, *6*, 483.
[15] T. de Diego, P. Lozano, M. Gmouh, M. Voltier, I. M. Iborra, *Biomacromolecules* **2005**, *6*, 1457.