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Cofactor-dependent enzyme catalysis in functionalized ionic solvents†

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Functionalized, hydrogen-bonding ionic liquids have been successfully evaluated as media for the performance of cofactor-dependent enzyme catalysed oxidations; the effects of incorporating hydroxyl groups into both the cation and anion have been studied and the dependence of activity upon water content has been evaluated.

Extensive studies have been conducted into the performance of biocatalysis in non-aqueous solvents,[1–3] in order to circumvent the poor solubility and stability of many organic substrates and products in water. Certain enzymes exhibit increased stability *ex aqua*, the corollary usually being a drastic reduction in catalytic rate, caused by poor solubility and loss of conformational flexibility.[4] Practically the only enzymes found to exhibit satisfactory activity in pure organic solvents have been lyases and lipases, which have evolved to function at aqueous–organic interfaces. More complex systems, including cofactor-dependent enzymes, are essentially precluded from such media.

Interest has recently developed in the application of room temperature ionic liquids (RTILs) in biocatalysis.[5,6] Dialkylimidazolium RTILs, particularly 1-butyl-3-methylimidazolium hexafluorophosphate (BMIm PF_6, **1**‡), have been reported to facilitate the performance of enzyme-catalysed reactions.[7–9] The enzymes employed have been the same restricted range studied in molecular organic solvents, with the RTILs offering generally increased levels of retained activity. Almost all such investigations have utilized biphasic RTIL aqueous systems; being directly analogous to molecular organic solvent–water mixtures as biocatalytic media—the RTIL acting as a non-denaturing substrate reservoir and the catalysis occurring at the phase boundary or within the aqueous portion. The use of pure RTILs in biocatalysis has received comparatively little attention, materials such as **1** being far from optimized for such processes. Limited investigations into the effects of hydrophilic ionic liquids upon certain enzyme catalysed reactions have been discouraging, with substantial denaturation being elicited.[5,10,11] This has been attributed to hydrogen bonding between the enzymes and the component ions of the solvents; interaction of **1** with proteins would be expected to be highly limited, compared to analogous compounds exhibiting stronger hydrogen bonding. In order to examine this hypothesis, a range of hydrophilic ionic liquids was synthesized, using **1** as a template, but with incrementally increased H-bonding capabilities through the use of hydroxylated cations and more strongly nucleophilic anions.[12,13] These materials were evaluated using the $NADP^+$-dependent morphine dehydrogenase (MDH) from *Pseudomonas putida* M10, acting upon codeine,[14] with recycling of the cofactor using either alcohol dehydrogenase (ADH) from *Thermoanaerobium brockii* or glucose dehydrogenase (GDH) from *Cryptococcus uniguttulatus*. To establish the extent to which water was necessary for either the reactions themselves or for solvent-induced denaturation, RTILs with varying water contents were used and compared directly with **1**, two representative molecular organic solvents and aqueous buffer.

Compounds **1** and **3**‡ were prepared according to established procedures.[15] BMIm glycolate (**2**) was prepared from the chloride salt by metathesis, using potassium glycolate in acetone. 1-(3-Hydroxypropyl)-3-methylimidazolium salts **4–6**‡ were prepared by an analogous procedure.§ The water content of RTILs and organic solvents was determined by Karl Fischer titration, using a KEM MKS-500 volumetric instrument. Reactions were performed using lyophilized enzymes. Codeine was dissolved to a concentration of 8 mg mL^{-1} (26.7 mM) in 10 mL volumes of the solvents listed in Table 1. $NADP^+$ disodium salt (0.1 equiv.) and either acetone (20 equiv.) or D-gluconolactone (10 equiv.) were added and the solutions were flushed with argon. MDH (2 mg) and cofactor-recycling enzyme (500 μg) were added and the reaction vessels were shaken at 30 °C for 24 h. Primary analysis was performed by ^{1}H-NMR; the presence and identity of the ketones being confirmed by FT-IR spectrometry and HPLC. Reactions in **3** were conducted at 37 °C, in order to counter the higher melting point of this material in comparison to the other RTILs studied. Results are summarized in Table 1.

Major differences in enzyme activity were apparent, both between the ionic and molecular systems and between individual ionic liquids. Catalytic activity *in aqua* exceeded that in other solvents; however, product yield was severely depleted through hydrolysis. Negligible conversion occurred in the molecular organic solvents studied; by contrast, the ionic media generally permitted the retention of a limited level of enzyme activity, even at very low levels of water. Whilst the catalytic rates in these media did not approach those observed in water, product solvolysis was effectively suppressed. In certain cases, this permitted higher isolable yield than in water. Activity in the hydrophobic ionic liquids correlated closely with water content. The barely significant residual activity observed in **1** at <100 ppm H_2O was not replicated in **3**, which differed only in the absence of the acidic (and hydrogen-bonding) proton at the 2-position. Profound differences were observed between these solvents and the more strongly hydrogen-bonding, hydrophilic RTILs **2** and **4–6**.

The hydroxylated cation in **4** altered the water dependence of the catalytic activity, permitting its retention at far lower water levels than those possible in the hydrophobic RTILs or in molecular organic solvents. By contrast, the use of hydrogen-bonding anions was significantly less successful, the glycolate salt **2** being inferior to the PF_6 salt **4**. When the hydroxylated cation was employed, replacement of PF_6 with a more hydrophilic anion significantly impaired performance; the chloride **6** eliciting near-total abolition of activity. Furthermore, the presence of even a very small residual level of chloride in other RTILs was found to exert a detrimental effect on activity, as has been observed elsewhere.[10,11] Denaturation by both molecular organic solvents and high-salt aqueous solutions has been attributed to the disruption of the protein's aqueous shell, with subsequent unfolding,[4] whilst denaturation by strongly nucleophilic RTIL anions has been speculated to involve coordination to electropositive regions of the peptide backbone.[10] The activity trends at varying water levels in **2** suggested a combination of these factors to be involved, with a progressive decrease in activity with decreasing water content (*i.e.* increasing salt

† Electronic supplementary information (ESI) available: analytical data for novel ionic liquids; plots of catalytic activity *vs.* water content and product accumulation *vs.* time for all systems studied; spectroscopic data for products. See http://www.rsc.org/suppdata/cc/b4/b410467f/

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Table 1 Oxidation of codeine to codeinone by MDH in various solvents[a]

Primary solvent	Water content	Phases	Recycling enzyme	Net % codeinone @ hours (±0.5%)		
				1	4	24
1	40%	2	ADH	2	12	27
1	1%	2	ADH	small	2	13
1	<100 ppm	1	ADH	0	small	3
1	40%	2	GDH	small	7	19
1	1%	2	GDH	0	small	8
1	<100 ppm	1	GDH	0	0	2
2	40%	1	ADH	2	5	13
2	1%	1	ADH	0	small	small
2	<100 ppm	1	ADH	0	small	5
2	40%	1	GDH	3	8	19
2	1%	1	GDH	small	3	8
2	<100 ppm	1	GDH	0	5	12
3	40%	2	ADH	2	9	22
3	1%	2	ADH	0	small	3
3	<100 ppm	1	ADH	0	0	0
4	40%	1	GDH	4	12	23
4	1%	1	GDH	4	7	17
4	<100 ppm	1	GDH	2	9	20
5	40%	1	GDH	4	9	15
5	1%	1	GDH	small	5	12
5	<100 ppm	1	GDH	small	5	16
6	40%	1	GDH	0	0	small
6	<100 ppm	1	GDH	0	0	small
H_2O	—	1	ADH	4	15	25
H_2O	—	1	GDH	4	12	10
Hexane	1%	2	ADH	0	0	small
Hexane	<100 ppm	1	ADH	0	0	0
t-Butanol	1%	1	ADH	0	0	0
t-Butanol	<100 ppm	1	ADH	0	0	0
Me_2CO_3	1%	1	ADH	0	0	0
Me_2CO_3	<100 ppm	1	ADH	0	0	small

[a] Hydrolysis of hexafluorophosphate-based ionic liquids, liberating hydrogen fluoride, has been reported upon prolonged exposure to water under certain conditions.[16] No significant levels of fluoride ions were, however, detected over the experimental timescale under the described conditions, in any of solvents **1**, **3** or **4**. ADH was not used with hydroxycationic RTILs to avoid any risk of solvent oxidation. In the presence of water, codeinone exists in equilibrium with neopinone.[17] The combined NMR integrals of these isomers were thus used in quantification.

concentration). At very low (<0.1%) levels of water in hydrophilic RTILs, however, this trend underwent an apparent reversal—observed activity levels in **2** and **4** being higher when near-anhydrous than when acting as concentrated aqueous salt solutions. Higher activity levels were retained at 1% water content in **4**, indicating that high salt concentration was not solely responsible for the denaturing effects of **2**, **5** and **6**. Activity was thus significantly reduced with increasing anion H-bonding capability, yet benefited from an enhancement in the same property of the cation. Infra-red studies suggested stronger interaction between the protein and **2** than with **4**, particularly with respect to the amide II region, appearing to confirm the anion–protein interaction as critical.¶

The combination of a moderately hydrophilic cation with an hydrophobic anion in **4** was capable of supporting the activity of all enzyme systems studied, with the improvements over conventional RTILs or molecular organic solvents being most pronounced at very low water levels. The correct combination of solvent properties was crucial for enzyme activity, with simple variations in composition profoundly affecting behaviour towards enzymes. The physical properties of the anion appear to be the primary—but not exclusive—determinant of enzyme compatibility, yet the factors governing the correct choice of ionic liquid for a biocatalytic process are clearly more complex. The role of impurities—particularly water—requires extensive evaluation. We are currently studying the relationships between ionic liquid physical properties, water content and enzyme activity; it is envisaged that these studies will contribute towards an understanding of the extent to which water may be displaced as the solvent in biochemical systems, whilst providing a basis for the rational design of RTILs for biocatalytic procedures.

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Notes and references

‡ **1**: 1-Butyl-3-methylimidazolium hexafluorophosphate; **2**: 1-butyl-3-methylimidazolium glycolate; **3**: 1-butyl-2,3-dimethylimidazolium hexafluorophosphate; **4**: 1-(3-hydroxypropyl)-3-methylimidazolium hexafluorophosphate; **5**: 1-(3-hydroxypropyl)-3-methylimidazolium glycolate; **6**: 1-(3-hydroxypropyl)-3-methylimidazolium chloride.

§ 1-Methylimidazole and 3-chloro-1-propanol (1 equiv.) were heated to 70 °C for 48 h, with stirring. The product was cooled and washed with diethyl ether. Residual ether was removed *in vacuo* to give **6**. Compounds **4** and **5** were prepared from **6** by metathesis with the potassium salt of the requisite anion. Compound **6** was dissolved in dry acetone; anhydrous potassium hexafluorophosphate or potassium glycolate (1.05 equiv.) was added and the solution was gently refluxed for 48 h, with stirring. The mixture was cooled, filtered and the solvent was removed *in vacuo* to yield crude **4** or **5** respectively. Further purification was performed by dissolution in an equal volume of acetonitrile, followed by the addition of activated charcoal. The mixture was heated to 50 °C for 5 min, with agitation. The charcoal was removed by filtration and the filtrate was passed through three 20 cm columns, containing activated charcoal, alumina and 60 Å silica as the stationary phases. The filtrate was stripped of solvent and dried thoroughly *in vacuo*. Products were stored in a desiccated condition over molecular sieves.

¶ Infra-red studies of proteins in ionic liquids will form part of forthcoming publication.

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