Biphasic Biocatalysis

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Homogeneous Biocatalysis in both Fluorous Biphasic and Supercritical Carbon Dioxide Systems**

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Enzymes are now routinely used under non-aqueous conditions to catalyze amide and ester formation and for the processing of industrially important substrates that are only sparingly soluble in water.[1] The ability to produce active proteins that are soluble in fluorous solvents and supercritical fluids opens up a range of possibilities for innovative reaction design, simplified reaction workup, and new methods to incorporate proteins into materials. To produce homogeneous reaction systems with efficient mass transport properties, a number of methods of solubilizing enzymes in non-aqueous media have been reported including hydrophobic ion pairing (HIP).[2–5] Typically, HIP occurs between an anionic surfactant, such as sodium bis(2-ethylhexyl)sulfosuccinate (AOT), and the cationic residues (Lys, Arg, and His) on the surface of a protein dissolved in aqueous buffer below its isoelectric point, pI. The protein then precipitates or is extracted directly into a second, non-aqueous phase. Enhanced catalytic activity has been reported for HIP–enzyme complexes in organic solvents compared with suspended or immobilized enzymes.[3–5] Here, for the first time, we demonstrate extraction of proteins from an aqueous solution into a fluorous solvent, perfluoromethylcyclohexane (PFMC), by means of ion pairing with the perfluoropolyether carboxylate surfactants Krytox FSL 157 and KDP 4606 (Figure 1). The nature of the complex formed by cytochrome c (Cc) with Krytox and KDP in PFMC was examined using dynamic light scattering and circular dichroism. The complex formed with KDP and α-chymotrypsin is shown to be catalytically active in a fluorous biphasic system as well as in supercritical CO_2 ($scCO_2$), in which it forms a homogeneous solution.

Fluorous biphasic catalysis was first described by Horváth and Rábai in 1994.[6,7] It provided a new approach to performing homogeneous catalysis with simplified recovery of the catalyst and product as summarized in Scheme 1. For example, a 1:1 mixture of hexane and PFMC is monophasic at 20 °C, but then phase separates on cooling to about 0 °C.[7] Very few examples of biocatalysis in fluorous biphasic systems have been described,[8–12] and none, due to the insolubility of proteins in fluorous solvents, is able to fully exploit a key facet of this approach, namely, recycling of the catalyst retained in the fluorous phase at the end of the reaction.

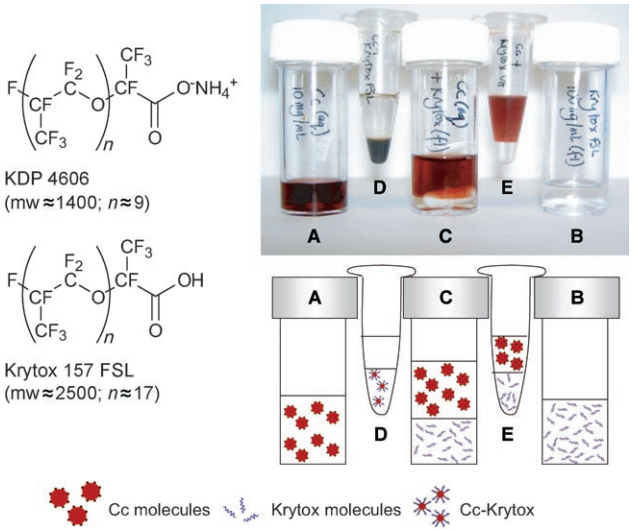

Figure 1. Hydrophobic ion pairing (HIP) of cytochrome c (Cc) with Krytox: A) Red aqueous solution of the heme protein, Cc. B) Krytox dissolved in PFMC. C) A biphasic system is initially observed with Cc in the aqueous (top) phase. D) On stirring, Cc is extracted into the lower fluorous phase as it forms ion pairs with Krytox molecules. The colorless aqueous phase then contains less than 1% of the starting amount of Cc (as determined by the bicinchoninic acid assay;[13] see Supporting Information). Up to 20 mg (Cc) mL^{-1} could be obtained in the fluorous phase (determined by absorbance at 280 nm). E) If Krytox alcohol (no acidic group) is used, ion pairing is not possible and Cc stays in the aqueous phase. Note: Cc and Krytox molecules are not drawn to scale. HIP complexes with only one Cc molecule surrounded by Krytox molecules are shown for clarity.

Beier and O'Hagan have demonstrated that *Candida rugosa* lipase can be used to resolve *rac*-α-methylpentanoic acid with a perfluorinated alcohol in a hexane/perfluorohexane system.[9] The majority of the *S*-fluorinated ester formed dissolves in the fluorous phase leaving the unreacted *R*-α-methyl acid in the organic phase. The insolubility of the enzyme in these solvents necessitated a filtration step for its separation and reuse. Fluorous bi- or triphasic solvent systems have also been employed to facilitate product separation from a biocatalytic reaction at the workup stage.[14,15]

Circular dichroism spectra of ion-paired Cc–KDP in PFMC indicate that the protein retains its α-helical secondary structure. Dynamic light scattering measurements show that Cc forms single-molecule particles of about 2 nm in diameter

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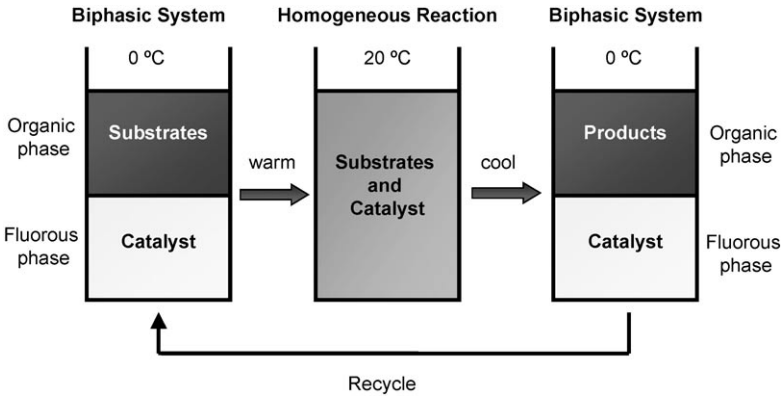

Scheme 1. Fluorous biphasic system: At low temperature, the fluorous and organic phases are immiscible. On warming above the consolute temperature, the two phases become miscible and a homogeneous phase forms allowing a reaction to occur. On cooling, the phases separate and the catalyst is retained in the fluorous phase, where it is easily separated from the product and recycled. The temperatures shown are for a 1:1 hexane/PFMC system.

in aqueous solution, whilst particles with an average diameter of 21.2 nm are detected for Cc–KDP in PFMC which suggests that these complexes contain small aggregates of Cc molecules surrounded by surfactant (see Supporting Information). A similar phenomenon has recently been observed in the direct solubilization process described by Akbar et al. for subtilisin Carlsberg and other enzyme–HIP complexes with AOT in a variety of organic solvents.[16]

To investigate biocatalysis in a fluorous biphasic system, the enzyme α-chymotrypsin (CMT) was extracted into PFMC by ion pairing with KDP, forming a transparent fluorous solution of up to 0.8 mg(protein) mL^{-1}. Dynamic light scattering indicated that CMT in aqueous solution formed particles 3.6 nm in diameter, that CMT–AOT in hexane formed particles 9.2 nm in diameter, and that CMT–KDP in PFMC formed particles 11.7 nm in diameter, again suggesting that the complexes formed contained small protein aggregates surrounded by surfactant.

CMT–KDP in PFMC can form a fluorous biphasic system on addition of substrates dissolved in hexane. This system is monophasic at 20 °C and biphasic on cooling to 0 °C. The catalytic activity of CMT–AOT in hexane and CMT–KDP in hexane/PFMC were studied on an analytical scale for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester with *n*-propanol including appropriate control reactions. We obtained promising results (Table 1) with both CMT–AOT and CMT–KDP. As expected, the control reactions (AOT, KDP) demonstrated negligible catalytic activity. Even a suspension of native CMT showed low catalytic activity, although the enzyme was highly aggregated in the solvent, and it has previously been shown that CMT is inactivated upon dimerization.[17]

Table 1: Testing of various catalysts for their activity for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester with *n*-propanol to give *N*-acetyl-L-phenylalanine *n*-propyl ester (APPE) at 40 °C.

Catalyst	Solvent system	APPE production [mM h^{-1} mg(catalyst)$^{-1}$]
native CMT	hexane	3.5×10^{-4}
AOT	hexane	1.5×10^{-3}
CMT–AOT	hexane	9.8
KDP	hexane/PFMC	1.4×10^{-4}
CMT–KDP	hexane/PFMC	4.5

We investigated the effect of the pH of the buffer on both the complexation efficiency (% protein extracted into PFMC) and the activity of CMT–KDP. On increasing the pH, the complexation efficiency increased dramatically between pH 7.0 and 7.3 and only slightly from pH 7.3 to 8.0. Simultaneously, the activity of CMT–KDP decreased gradually from pH 7.2 to 8.0. Therefore, pH 7.3 was identified as the optimum pH for a reasonable complexation efficiency (70%) and yield of APPE (17%).

It was also shown that CMT–KDP is able to retain its activity over four reaction cycles in a fluorous biphasic system (see Supporting Information). By comparison, native CMT demonstrates poor activity (< 1% yield) and was difficult to recycle because it was insoluble in and partitioned between the hexane and PFMC phases.

In contrast with biocatalysis in fluorous solvents, the investigation of enzyme-catalyzed reactions in supercritical fluids especially $scCO_2$ has been the focus of considerable research over the last two decades with both hetero- and homogeneous systems being described.[8] Supercritical CO_2 offers the advantage of being a non-toxic, non-flammable, and cheap solvent whose solvating properties can be tuned by changing the temperature and pressure. Of most relevance here are the "lipid-coated" enzymes formed using a neutral surfactant (didodecyl *N*-D-glucono-L-glutamate) that have been reported by Mori et al. to be dissolved in both $scCO_2$ and supercritical fluoroform.[18,19] While these complexes are catalytically active, a major limitation of this approach is that the concentration of protein in the supercritical fluid is only about 0.1 mg mL^{-1}. Ion pairing to solubilize metals and organometallic catalysts in $scCO_2$ has previously been explored using fluorinated anions such as tetrakis[3,5-di(trifluoromethyl)phenyl]borate (BARF),[20] while quaternary ammonium ions (dodecyltrimethylammonium) have been used for the solubilization of the indicator dye, Acid Red 57.[21]

Highly fluorinated substances are often soluble in $scCO_2$.[22] Thus, we studied the solubility of Cc–Krytox in CO_2 by using a small fixed-volume high-pressure cell in conjunction with UV/Vis spectroscopy. The solubility of the complex was shown to increase with increasing pressure and hence solvent density, up to a maximum protein solubility of 7 mg mL^{-1} at 40 °C and 27.5 MPa. This is two orders of magnitude higher than the concentration of "lipid-coated" enzymes reported by Mori et al. in $scCO_2$ and fluoroform.[18,19] A high-pressure variable-volume view cell[23] was used to observe the behavior of the Cc–Krytox complex at 40 °C. As the pressure increased up to 27.5 MPa, Cc–Krytox was seen to dissolve to give a red solution (Figure 2B). This process was

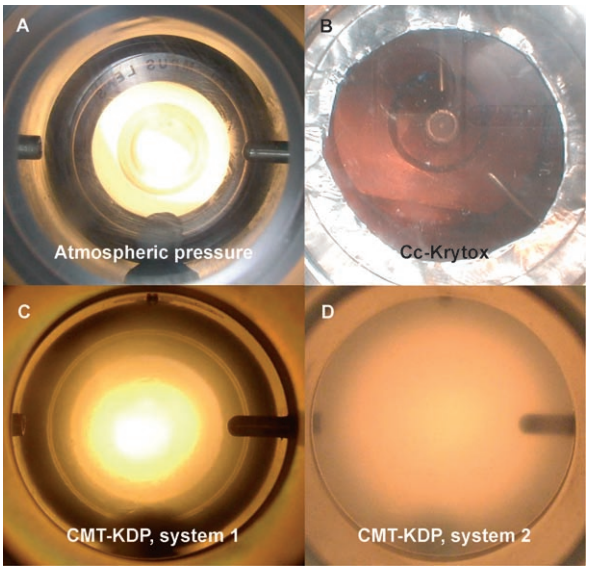

Figure 2. Experiments in a variable-volume view cell: A) Empty view of the cell at atmospheric pressure. B) With Cc–Krytox at 40 °C and 25.1 MPa $scCO_2$, the cell is clear red indicating full solubility/dispersion of the complex under these conditions. C) System 1: CMT–KDP in PFMC and CO_2 (0.02, 21.5, and 78.5 wt%, respectively) at 17.2 MPa and 40 °C. D) System 2: CMT–KDP in PFMC and CO_2 and APEE and *n*-propanol (0.02, 20.6, 78.5, 0.04, and 3.1 wt%, respectively) at 20.7 MPa and 40 °C.

reversible with the complex precipitating at about 18.6 MPa (for a video of this process, see http://www.nottingham.ac.uk/~pczmp, file name: chip.avi). CMT–KDP was also soluble in $scCO_2$ as shown in Figure 2C.

The reaction mixture in $scCO_2$ (CMT–KDP in PFMC and substrates; Figure 2D) appeared brown–red in color and, owing to the substrates, was very turbid between 6.9 and 34.5 MPa. The system was stirred at 40 °C and 20.7 MPa overnight. Following depressurization, the cell contents were analyzed by gas chromatography; the yield of APPE was approximately 11%.

In summary, by forming protein–surfactant complexes by hydrophobic ion pairing with a highly fluorinated anionic surfactant, cytochrome c can be solubilized up to 20 mg (protein) mL^{-1} in PFMC and up to 7 mg (protein) mL^{-1} in $scCO_2$. The secondary structure of the proteins within these ion-paired complexes has been shown to remain intact, and particle sizing indicates that small aggregates of protein molecules surrounded by surfactant molecules are formed. The presence of the KDP ion paired with CMT appears to enhance the catalytic activity of CMT compared to that of the native enzyme in a fluorous biphasic system. The facile recycling of the CMT–KDP complex in a fluorous biphasic system has been demonstrated with retention of enzyme activity over four reaction cycles. CMT–KDP was also found to dissolve and be catalytically active in $scCO_2$. To our knowledge, this is the first report demonstrating homogeneous enzyme catalysis in a fluorous biphasic system.

Experimental Section

Hydrophobic ion-pairing methodology: Cc (10 mg mL^{-1}) dissolved in buffer (10 mM sodium acetate, pH 3.8, 1.0 mL) was stirred (500 rpm) at room temperature for 30 s with a solution of Krytox 157 FSL (DuPont) in PFMC (80 mM, 0.5 mL). The mixture was centrifuged (13000 *g*, 2 min) to give a biphasic solution. The concentration of Cc in PFMC was determined by UV absorption of aromatic amino acids at 280 nm ($\varepsilon_{280} = 11600\,M^{-1}\,cm^{-1}$), and the concentration of Cc in the aqueous phase was determined by the bicinchoninic acid assay[13] (see Supporting Information for further details).

CMT (1.1 mg mL^{-1}, 1 mL) in aqueous solution (10 mM sodium phosphate buffer, pH 6.5) containing $CaCl_2$ (4 mM) was stirred (300 rpm) for 10 min at room temperature with a solution of AOT in hexane (2 mM, 1 mL) or a solution of KDP 4606 (DuPont) in PFMC (2 mM, 1 mL). The resulting solution was centrifuged (13000 *g*, 2 min) and the organic–fluorous phase collected. The concentration of CMT in PFMC was determined by UV absorption of aromatic amino acids at 280 nm ($\varepsilon_{280} = 51240\,M^{-1}\,cm^{-1}$). Complexation efficiency was calculated by dividing the mass of protein extracted by the original mass of protein.

Two buffers were used for the determination of the optimum complexation pH value, namely sodium acetate (10 mM, pH 4.0–5.5) and sodium phosphate (10 mM, pH 6.0–8.0). All complexations were carried out in triplicate.

CMT–KDP-catalyzed reaction: CMT–KDP in PFMC (1 mg mL^{-1}, 0.5 mL) was added to APEE (4.3 mM) and *n*-propanol (0.5 M) in hexane (0.5 mL) in a glass vial (5 mL) fitted with a rubber septum (Supelco), and the mixture was gently stirred for 1 h at 40 °C. An aliquot (100 μL) of this mixture was diluted in hexane (900 μL), and the products were analyzed on a Shimadzu GC-2010 GLC equipped with a RTX5-FAST column (Restex).

Recycling of CMT and CMT–KDP: After a reaction time of 1 h, the system was cooled to about 0 °C, the hexane phase was removed, and the products were analyzed by gas chromatography. A fresh batch of substrate solution was added to the remaining enzyme–surfactant complex in PFMC, and the reaction was carried out as before. Four cycles were completed in total.

The variable-volume view cell was constructed at the University of Nottingham.[23] The cell was pressurized with SCF-grade CO_2 (99.99%, ≈20 g) at room temperature, heated to the desired temperature, and stirred magnetically. When the working temperature had been obtained, the pressure was increased to approximately 34.5 MPa. The pressure was decreased slowly by increasing the cell volume, and visual observations were made.

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