Communication

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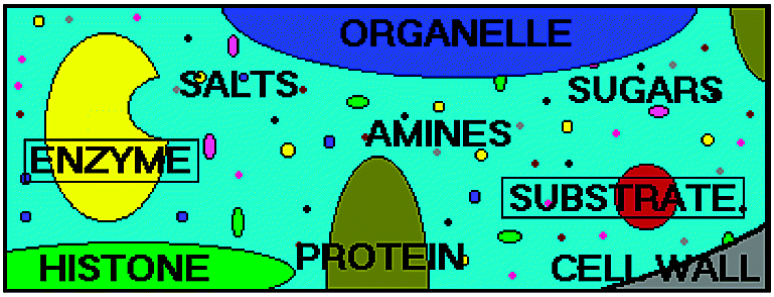

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Cytosol-Mimetic Chemistry: Kinetics of the Trypsin-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate upon Addition of Polyethylene Glycol and *N*-*tert*-Butyl Acetoacetamide

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The sensitivity of an enzyme to its environment has provoked much interest both for its immediate relevance to biochemistry and for its importance in the use of enzymes in chemical synthesis.[1] This study examines the effect of concentrated solutions of biomimetic compounds on enzyme reactivity.

The cytosol is an aqueous region, surrounded by a lipid bilayer membrane, crowded with macromolecules and solutes totaling several hundred grams per liter.[2] This environment is markedly different from the dilute aqueous solutions generally used as background comparisons for biochemical processes.[3] Indeed, in a recent book Ball asks: "*What is the structure of water in the cell? This is one of the most important unresolved issues in biology.*"[4] In the cellular environment the rate of diffusion, especially of large molecules, is reduced[5] and macromolecular crowding agents promote protein folding and self-association.[6] Our aim is to study biologically relevant chemistry in biologically relevant media—ultimately to mimic the environment found inside cells and to study its properties as a chemical medium.

It would be reasonable to assume that as long as the active site of an enzyme is shielded from the bulk solution, the catalytic rate constant (k_{cat}) would be unaffected by the addition of cosolutes or crowding agents, up until they cause denaturation or affect the diffusional rate-limit. The substrate binding constant (K_M) would be expected to be sensitive to solvent composition since it depends on the chemical potential of the substrate free in solution. Substrate binding also requires the dehydration of both the active site and substrate, hence might be dependent on water activity. Previous studies have highlighted the importance of water concentration,[7] water activity and cosolute nature,[8] and dielectric constant with simultaneous competitive inhibition;[9] however, no clear picture has emerged.

We selected the trypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate (pNPOAc) as a model enzyme reaction which can conveniently be followed by UV−visible spectroscopy.[10] Our initial choice of crowding agent and cytosol mimic was bovine serum albumin (BSA). However, we observed catalysis of the ester hydrolysis by BSA.[11] We therefore changed the additive to inert poly(ethylene glycol) (PEG, M_w 8000) which has been widely employed as a solubilizing agent and stabilizer in chemoenzymatic synthesis.[12] An additional solute, *N*-*tert*-butyl acetoacetamide (NBAA) was studied to assess whether the observed kinetics were a property of the PEG additive or a general response of the enzyme to changes in its environment. NBAA is chemically inert in our test system and contains the amide linkage (which is ubiquitous inside the cell) and a balance of polar and hydrophobic groups reflecting those found in biomolecules. As a relatively small molecule, it would also not be expected to produce the same effects on diffusion and association as PEGs.

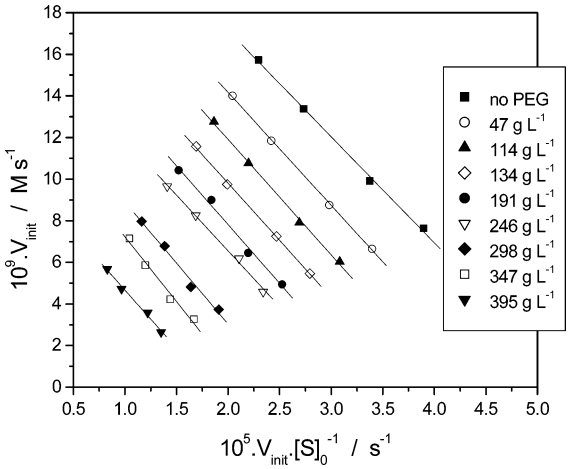

Figure 1. Eadie−Hofstee plots for the trypsin-catalyzed hydrolysis of pNPOAc at varying concentrations of PEG (M_w 8000), 25 °C, and pH 7.7.

Trypsin catalysis was measured for a series of solutions ranging from no added solute up to 395 g L^{-1} of PEG and up to 269 g L^{-1} of NBAA.[13] Monitoring the concentration of the product rather than the substrate allowed the use of four convenient initial substrate concentrations in the region of K_M, at the expense of having to calibrate against the extinction coefficient of *p*-nitrophenolate in each PEG and NBAA solution. Kinetic parameters k_{cat} and K_M were determined by the Eadie−Hofstee method (Figure 1).[14]

The observed Eadie−Hofstee plots are of the form classically expected for noncompetitive inhibition of an enzyme.[15] Substrate binding is unaffected (mean $K_M = 5.57 \times 10^{-4}$ M); hence, PEG does not compete for the active site and does not distort its structure. The added PEG does however cause a decrease in k_{cat} from 3.85 $\times$ 10^{-3} s^{-1} to 1.44 $\times$ 10^{-3} s^{-1} over the PEG concentration range studied. Interestingly, the decline in the catalytic rate constant is directly proportional to the concentration of added PEG (which varies linearly with the concentration of water) and the same linear dependence of k_{cat} on PEG concentration is observed when PEG (M_w 1500) is used (not shown). The same profile was obtained for NBAA, but with an even greater decline in k_{cat} (Figure 2). To allow for a fair comparison of the differently sized solutes, kinetic parameters are plotted as a function of molar water concentration, rather than solute concentration.

Addition of simple solutes to water affects both polarity and water activity. Polarity, as measured by Reichardt's solvatochromic E_T-(30) probe,[16] varies linearly with the concentrations of solute and water in binary mixtures. Hence, a property that varies linearly with water concentration may indicate a dependence on polarity. Water activity, as measured by vapor pressure osmometry, is nonlinear with concentration (Figure 3).

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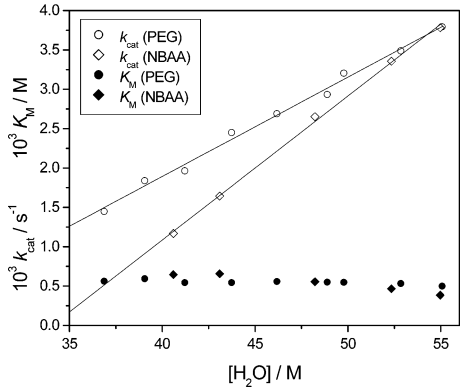

Figure 2. Variation of enzyme kinetic parameters with solute concentration at 25 °C and pH 7.7. The solid lines are linear fits to k_{cat}.

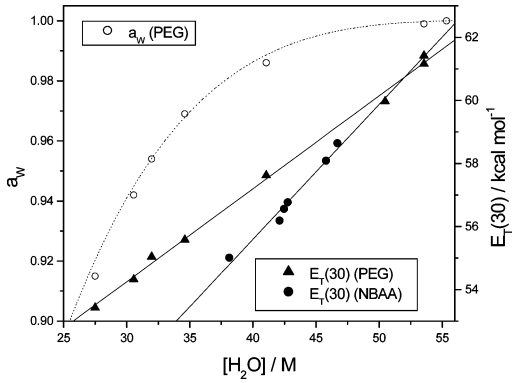

Figure 3. Dependence of $E_T(30)$ and water activity[17] on solute concentration at 25 °C.

It is inconceivable that PEG and NBAA have a common noncompetitive binding site on the enzyme and unlikely that binding of PEG and NBAA by trypsin at different sites remote from the catalytic center accounts for the observed kinetics. A crowding-induced change in enzyme conformation has previously been proposed to account for lowering of enzyme activity,[18] but unlike PEG, NBAA is not a crowding agent. The observed kinetics must therefore be attributable to an environmental effect on the enzyme, the substrate, or both.

It is possible that the effect of the solute is limited to a perturbation of the pK_a of one or more catalytically important groups on the enzyme. However, a study of the background nucleophilic attack of TRIS on the substrate in the absence of enzyme[19] revealed an analogous linear variation of the (second-order) rate constant with PEG concentration (not shown). Since isotopic substitution experiments have shown that the solution phase nucleophilic and chymotrypsin-catalyzed hydrolysis of pNPOAc proceed via effectively the same mechanism,[20] we propose that in this system the effect of the additive on solvent polarity is the dominant factor.

It appears therefore that the same effect of solvent polarity is observed in both the enzyme-catalyzed and the solution-phase reactions. This is reasonable since the active site of trypsin is relatively exposed to solvent.[21] The results presented here point to a correlation between k_{cat} and $E_T(30)$; however, there is insufficient data to propose a direct link. We have observed similar trends in a Diels−Alder reaction and an A1 acetal hydrolysis.[22]

In summary, the presence of large concentrations of inert solutes has only a modest effect on enzyme activity—less than an order of magnitude reduction in k_{cat}. Apparently, the catalytic process hardly reacts to the large reduction in the concentration of water in the model system. The enzyme probably retains its specific hydration state, and consequently it is difficult to pinpoint the exact reasons for the small reduction in k_{cat}. It is noteworthy that the decline in k_{cat} is linear with increasing additive concentration. Comparison to solvent polarity data and the nucleophilic reaction of the substrate with TRIS indicate that this appears to be a medium effect on enzyme chemistry and further attention should be paid to environmental effects upon enzyme catalysis in vivo.

Acknowledgment. We are grateful to the NRSCC for financial support of this work.

Supporting Information Available: Eadie−Hofstee plots for trypsin-catalyzed pNPOAc hydrolysis in the presence of NBAA. Plots of pseudo-first-order rate constant against TRIS concentration for buffer-catalyzed pNPOAc hydrolysis at different PEG concentrations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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