Efficient Catalysis of Proton Transfer by Synzymes

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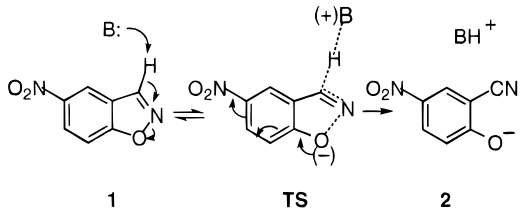
Enzyme catalysis depends on subtle combinations of effects that are difficult to separate and quantify.[1] Medium effects are a crucial part of this package, but the assignment of a local dielectric constant to the structured microenvironment of an active site and its effect on ground state destabilization[2] or transition state stabilization by electrostatic interactions[3] is experimentally impossible. We aim to mimic and thus begin to quantify such active site medium effects experimentally. As a test reaction, we use the eliminative cleavage of benzisoxazole (**1** → **2**, the Kemp elimination), known to be particularly sensitive to the effects of the medium.[4,5] We report that a subset



of several hundred water-soluble polymers prepared by alkylating polyethyleneimine (PEI) with different combinations of three contrasting alkyl groups catalyzes the Kemp elimination, in water, with rate accelerations as high as 10^6 and at least 1000 turnovers per basic site. Proton transfer from carbon is catalyzed by polymer amine groups with pK_a values as low as 5.7 and apparent effective molarities of the order of 1000 M.

Polyethyleneimine (PEI) is a highly branched polymer with primary, secondary, and tertiary amines linked by ethylene units. Klotz showed that PEIs modified by alkylation or acylation of their amine groups can act as enzyme-like catalysts ("synzymes"), with substantial rate accelerations, generally of the order of 10^3-10^4.[6]

The Kemp elimination is a well-characterized model for proton transfer from carbon—a key biological reaction—where other catalytic systems are available for comparison.[7−9] The rate is sensitive to solvent polarity primarily through activation of the catalytic base by desolvation, but also, to a smaller extent, through specific transition state stabilization by dispersion interactions.[5,8,9a] Desolvation−activation (measured as the rate increase upon transfer from aqueous solution to a polar, aprotic solvent) is large ($>10^7$) when the catalytic base is a carboxylate anion, but much smaller (∼10) with uncharged amines. Precise positioning of the catalytic group[7] is not expected in a system with loose, nonspecific binding. Therefore, PEI, with its range of amine bases, should allow us to focus on specific transition state stabilization.

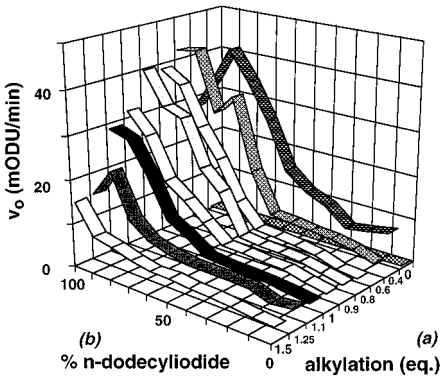


Figure 1. Initial assay of synzymes for the Kemp elimination. Initial rates for 64 polymers (of the 448 synthesized) plotted against (a) the degree of alkylation (in equivalents of benzyl bromide *plus n*-dodecyl iodide per monomer residue) and (b) the mole percent of dodecyl iodide in this alkylating mixture. This alkylation was followed by addition of methyl iodide (0.2 equiv in the subset illustrated here; 0−3 equiv over the seven analogous subsets of 64 polymers). Initial rates were measured in a microtiter plate (0.2 mL/well) at 25 °C in 70 mM BisTris, pH 7.12, [**1**] = 0.5 mM, [polymer] = 0.2 μM.

With the aim of "tuning" the microenvironment of these amine bases systematically to optimize specific transition state stabilization, we alkylated PEI with various combinations of dodecyl iodide, benzyl bromide, and methyl iodide. This should generate (i) a range of hydrophobic cavities or regions to drive substrate binding, in close proximity to (ii) a range of amine groups to serve as catalytic bases, embedded in (iii) a positively charged polymer framework, expected to stabilize the delocalized negatively charged transition state (**TS**). This simple synthesis, combined with an efficient screening protocol, made it possible to generate and assay hundreds of polymers in the search for high catalytic activity.

First results are summarized in Figure 1 and Table 1. Although low rate accelerations are the norm (e.g., with high total alkylation, or with over 60% of benzyl bromide in the alkylation mixture), many synzymes are efficient catalysts (Figure 1). We selected for detailed characterization four polymers combining high activity with sufficient solubility (Table 1). Saturation behavior was observed, and the data were analyzed following the Michaelis−Menten model. Turnover numbers (k_{cat}) range from 25 to 370 min^{-1} (at pH 5.86), and K_M values lie in the millimolar range. Figure 2 (inset) compares catalysis by two synzymes with the background buffer-catalyzed reaction: we see substantial rate accelerations and multiple turnovers. (Note that the polymer−catalyst concentration is 20 *nanomolar*.) Comparison with the buffer-catalyzed reaction for 7.D/2.2 indicates a minimum of 7000 turnovers (corresponding to >1000 turnovers *per site*; see below). The absence of significant product inhibition stands in contrast to a recent host−guest system[8] and to serum albumins,[7,9b] which also use amine bases to catalyze the Kemp elimination. Rates and turnover for our best polymers are the highest observed for this reaction.

The pH-dependence for catalysis by 7.D/2.1 is compared in Figure 2 with that for parent PEI. The pH rate profile confirms that the catalytic species is active in its basic form. The profile is not simply sigmoidal, showing that reaction is catalyzed by

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(1) *Transition States of Biological Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978.
(2) Jencks, W. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 219−410.
(3) Warshel, A. *Computer Modelling of Chemical Reactions in Enzymes and in Solutions*; Wiley: Chichester, 1991.
(4) Kemp, D. S.; Casey, M. L. *J. Am. Chem. Soc.* **1973**, *95*, 6670−6680.
(5) Kemp, D. S.; Cox, D. D.; Paul, K. G. *J. Am. Chem. Soc.* **1975**, *97*, 7312−7318.
(6) Klotz, I. In *Enzyme Mechanisms*; Williams, A., Page, M., Eds.; The Royal Society of Chemistry: London, 1987; pp 14−34. A more recent example of the "combinatorial approach to catalyst design" is reported: Menger, F. M.; Eliseev, A. V.; Migulin, V. A. *J. Org. Chem.*, **1995**, *60*, 6666−7.
(7) Hollfelder, F.; Kirby, A. J.; Tawfik, D. S. *Nature* **1996**, *383*, 60−63.
(8) Kennan, A. J.; Whitlock, H. W. *J. Am. Chem. Soc.* **1996**, *118*, 3027−3028.
(9) (a) Thorn, S. N.; Daniels, R. G.; Auditor, M.-T. M.; Hilvert, D. *Nature* **1995**, *373*, 228−230. (b) Kikuchi, K.; Thorn, S.; Hilvert, D. *J. Am. Chem. Soc.* **1996**, *118*, 8184−8185.
(10) Highbarger, L. A.; Gerlt, J. A.; Kenyon, G. L. *Biochemistry* **1996**, *35*, 41−46.
(11) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183−278.

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Table 1. Composition and Kinetic Parameters for Synzymes (25 °C, pH 5.86)

synzyme	$C_{12}H_{27}I$ (equiv)	C_7H_7Br (equiv)	MeI (equiv)	K_M[a] (mM)	$(k_{cat})^{total}$ [a] (min^{-1})	$(k_{cat})_H^{total}$ [b] (min^{-1})	pK_a[b]	$(k_{cat})^{site}$ [c] (min^{-1})	no of sites per synzyme[d]	$[(k_{cat})^{site}/k_{uncat}]$[e]	EM (M) in H_2O [f]	EM (M) in CH_3CN[g]
7.D/1.1	0.38	0.45	0.4	9.6 ± 4	370 ± 130	670 ± 235	5.7					
7.D/2.1	0.23	0.26	0.4	7.7 ± 4	120 ± 50	280 ± 115	6.2	1.4 ± 0.2	85	2.9×10^5	1260	100
7.D/2.2	0.23	0.26	0.2	4.2 ± 1	40 ± 8	135 ± 25	6.35	>5	<8	$>1.0 \times 10^6$	5055	390
8.B/8.2	0.36	0.04	2	3.3 ± 0.6	25 ± 5	150 ± 30	6.7	2.1 ± 0.7	12	4.4×10^5	2145	165

[a] Initial velocities were determined in a microtiter plate reader by monitoring the release of product **2** at 405 nm, at 0.2 μM synzyme and 0.15−1.8 mM substrate **1**. Data were analyzed according to standard methods (Lineweaver−Burk, Eadie−Hofstee, linear regression); errors represent standard deviations plus differences in values obtained by different analytical methods. Larger errors in k_{cat} and K_M cannot be excluded since measurements could only be made at $[S_o] < K_M$, due to limiting substrate solubility. (Note that substrate solubility is not a problem for the $(k_{cat})^{site}$ measurements, from which rate accelerations and EMs are derived.) [b] Derived from the fit to a single pK_a (Figure 2); $(k_{cat})_H^{total} = (k_{cat})^{total}$ [v_o^H/v_o (at pH 5.86)]. (v_o^H is the plateau rate at pH ≫ pK_a.) [c] Determined with 5 μM substrate and 0−50 μM synzyme in a 1 cm path length cell. [d] $(k_{cat})^{total}/(k_{cat})^{site}$. [e] $k_{uncat} = 4.8 \times 10^{-6}$ min^{-1} (at zero buffer concentration). [f] $EM = (k_{cat})_H^{site}/k_2^{amine}(H_2O)$; k_2^{amine} (the second-order rate constant for the 2-methoxyethylamine-catalyzed conversion of **1** to **2** in water [1.02 M^{-1} min^{-1}, ref 7] corrected to the synzyme pK_a) = 1.02 M^{-1} $min^{-1}/10^{\Delta pK_a\beta}$ using $\beta = 0.73$[4,5] and the difference between the pK_a of 2-methoxyethylamine (9.75) and the synzyme pK_a (column 8); $(k_{cat})_H^{site}$ is defined analogously to footnote *b*. [g] $EM = (k_{cat})_H^{site}/k_2^{amine}(MeCN)$ using $k_2^{amine}(MeCN)$ 13-fold higher than in H_2O (see text).

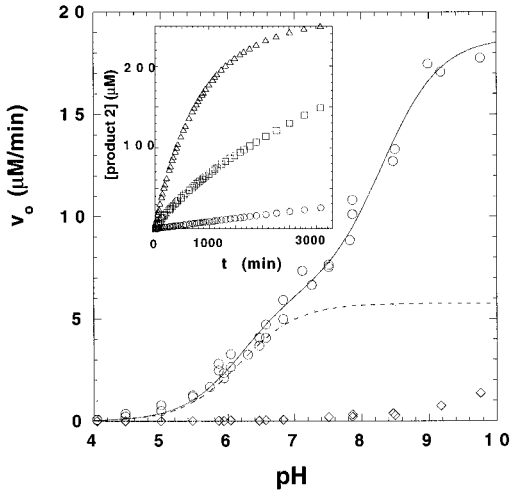

Figure 2. Plot of initial velocities v_o (under k_{cat}/K_M conditions) *vs* pH for the 7.D/2.1 (○) and PEI (◇)-catalyzed conversion of **1** → **2**. The lines drawn compare fits to the data with two (—, 6.2 and 8.3) and one (---, 6.2) pK_a values. The data could be fitted at least as well ($r \geq 0.996$) using more pK_a values. Conditions: [**1**] = 0.5 mM, [polymer] = 0.2 μM in 70 mM buffer, 25 °C. Inset: Product appearance *vs* time for the conversion **1** → **2** in buffer alone (○) and with 20 nM solutions of polymers 7.D/1.1 (△) and 7.D/2.2 (□). Active site concentration for 7.D/2.2 = 0.16 μM (Table 1). Conditions: [**1**] = 0.25 mM in 40 mM BisTris buffer, pH 5.86, 25 °C.

a different range of catalytic bases depending on the extent of protonation. Most interesting is the reaction at low pH. Here the catalytic sites appear to be relatively homogeneous, as shown by the rather good fit to a single ionization (dashed curve in Figure 2): the apparent pK_a of this new catalyst is lowered by 4−5 units compared with that of simple alkylamines (pK_a 10−11). These low pK_a values are ascribed primarily to electrostatic through-bond effects. An estimate of this contribution in unmodified PEI is provided by the pK_a values of ethylenediammonium dications, which are lowered by some 2.5 units compared with the protonation of the neutral diamine.[2] The further reduction in apparent pK_a (5.7−6.7) observed for these synzymes indicates an additional electrostatic through-space effect,[6] together with destabilization of charged ammonium species in hydrophobic microenvironments. Such large pK_a shifts are familiar for active site lysines of natural enzymes, allowing them to be active at pH 7.0 where an alkylamine is normally fully protonated and hence not available as a nucleophile or a general base. Similarly, our synzymes exhibit high rate accelerations at pH ≤ 6.0, where the background or buffer-catalyzed reaction is very slow.

Despite the inherent heterogeneity of the sample of active sites of the synzymes, it is possible to determine kinetic parameters to estimate apparent effective molarities (EM[12]) and catalytic proficiencies for single sites. Rate constants $(k_{cat})^{site}$ for single active sites were obtained as described by Klotz:[13] pseudo-first-order rate constants measured in the presence of a large excess of *synzyme* over substrate, analyzed according to a Michaelis−Menten model, gave $(k_{cat})^{site}$ values between 1 and 5 min^{-1} at pH 5.86 (corresponding to $(k_{cat})_H^{site}$ [see Table 1] of 3−17 min^{-1}). Rate accelerations *per site* over background are of the order of 10^5−10^6. We find EM values (given by $(k_{cat})^{site}/k_2^{amine}$) ranging from 1200 to 5100 M (after correcting for the difference in basicity). Allowing an additional factor of 13 for the maximum expected activation of the amine base by desolvation (measured by $k_2^{amine}[MeCN]/k_2^{amine}[H_2O]$, for $Me_2N(CH_2)_2N^+HMe_2$) gives values of 100−400 M as lower limits for the EM.

Comparison with other enzyme models[7,14] shows that low EMs are a crucial shortcoming of artificial catalysts. Even intramolecular model systems, which readily support high EMs for nucleophilic catalysis, rarely exhibit EMs larger than 10 M for general acid/base catalysis.[12] EMs of many hundreds are exceptionally high for an enzyme model, particularly where preassociation of catalyst and substrate is involved. These synzymes provide the most efficient artificial catalytic systems for proton transfer from carbon, second only to rigid intramolecular models,[12,14] so the origin of the high EMs is of particular interest. In contrast to effective molarities measured for intramolecular reactions, EMs in these systems provide an *overall* measure efficiency which includes contributions from more sources than are likely in bulk solvent. Thus, we consider that the efficient synzymes owe little to the precise positioning of the catalytic base,[7,14] but stabilize the negatively charged transition state **TS** primarily through a specific medium effect, involving dispersion interactions with the delocalized π-system with a further contribution from through-space electrostatic stabilization.[15] Negative charge density develops particularly at the in-plane oxygen atoms on the periphery of **TS** and would be effectively stabilized at a dynamic polar interface.

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Supporting Information Available: Preparation and screening of synzymes (1 page). See any current masthead page for ordering and Internet access instructions.

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(12) Kirby, A. J. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 707−724.
(13) Suh, J.; Scarpa, I. S.; Klotz, I. M. *J. Am. Chem. Soc.* **1976**, *98*, 7060−7064.
(14) Kirby, A. J. *Acc. Chem. Res.* **1997**, *30*, 290−296.
(15) Tobin, J. B.; Frey, P. A. *J. Am. Chem. Soc.* **1996**, *118*, 12253−12260.