On the Magnitude and Specificity of Medium Effects in Enzyme-like Catalysts for Proton Transfer

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Medium effects are normally studied by comparing the rates of reactions in different solvents. However, medium effects at the active site of enzymes differ dramatically from bulk solvents, both in their diversity (the presence of more than one type of "solvent") and in their spatial arrangement. We describe medium effects in a simple catalytic system, obtained by systematic alkylation of a polymeric scaffold bearing amine groups to give synzymes that catalyze the Kemp elimination of benzisoxazoles with remarkable efficiency. Our analysis indicates that catalysis by these synzymes is driven primarily by *specific*, localized enzyme-like medium effects, and these effects seem to differ dramatically from the *nonspecific* medium effects (i.e., desolvation activation) exhibited by solvents. Ligand-binding studies indicate that the synzyme active sites provide localized microenvironments affording a combination of hydrophobic and apolar regions on one hand and dipolar, protic, and positively charged on the other. Such localized microenivronments are not available in bulk solvents. A Brønsted (leaving group) analysis indicates that, in comparison to solvent catalysis, the efficiency of synzyme catalysis shows little sensitivity to leaving group pK_a. We show that enzyme-like medium effects alone, in the absence of efficient positioning of the catalytic amine base relative to the substrate, can give rise to rate accelerations as high as 10^5, for both activated and nonactivated substrates. Supported by the accidental identification of active sites on the surfaces of noncatalytic proteins and the promiscuous activities found in many enzymes, our findings suggest that the interfaces of protein surfaces and their hydrophobic cores provide a microenvironment that is intrinsically active and may serve as a basis for further evolutionary improvements to give proficient and selective enzymes.

The remarkable catalytic proficiency of enzymes is ascribed to a combination of effects, including medium effects. A specific role for medium effects was first proposed by Max Perutz in conjunction with the crystal structure of lysozyme—the very first enzyme structure to be solved:[1] "Organic solvents have the advantage over water of providing a medium of low dielectric constant, in which strong electrical interactions between the reactants can take place. The nonpolar interior of enzymes provides the living cell with the equivalent of the organic solvents used by chemists". This fundamental observation has since been reproduced in the crystal stuctures of many enzymes. But the interpretation and mechanistic implications of medium effects have evolved to become far more complex and sometimes matters of dispute.

It is now widely accepted that medium effects vary according to the nature of the transition state (TS) of the enzyme-catalyzed reaction and the interactions leading to its stabilization. A charged transition state is stabilized by solvation in an aqueous solvent and destabilized by a hydrophobic, aprotic environment resembling a "classical", aprotic organic solvent.[2] However, enzymes are much more versatile than ordinary solvents and may, at close but distinct sites, strip part of a substrate or TS of water molecules (thus resembling an "organic solvent") while simultaneously providing interactions with polar, charged, or protic groups, at least as efficiently as solvent water, at other parts. Thus, a fundamental difference between the "solvent" molecules constituting the active site of enzymes and bulk solvents used by organic chemists is in the potential for diversity and spatial localisation of their medium effects.[2−5]

Despite these refinements in our understanding of medium effects, there is still no general agreement regarding their actual contribution to enzyme catalysis. Jencks suggested that medium effects, manifested in the desolvation of active site functional groups, had the potential to explain the entire enzymic rate acceleration for certain reactions.[6] In an extreme position, Dewar calculated that medium effects alone can account for

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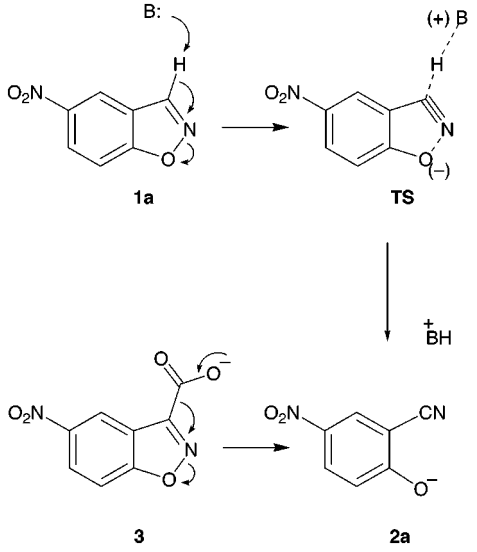
enzymic rate accelerations even for such demanding reactions as amide hydrolysis.[7] Others, however, regard medium effects as secondary and ascribe catalytic efficiency to more "specific" forces.[8,9] All these ideas have some support from theoretical studies and extrapolations from solution chemistry. They remain, however, untested experimentally. Separating medium effects from all other contributions to catalysis and quantifying their effect on the overall rate acceleration of an enzyme is not a trivial matter. Medium effects are not localized at a single active-site residue, and altering the active-site environment (using protein engineering, for example) without affecting substrate binding and the other parts of the catalytic machinery is essentially impossible. Thus, studying the behavior of reactions in different solvents, and specifically designed enzyme mimics, may provide insights into the role and magnitude of medium effects in enzyme catalysis.[10]

Reactions showing dramatic changes in rate upon transfer from water to organic solvents provided the first experimental support for Perutz's solvent hypothesis:[1] decarboxylations are classical examples. Thus, Kemp demonstrated solvent rate accelerations of over 10^8 for the decarboxylation of 3-carboxy benzisoxazoles (e.g., **3**) in water vs a dipolar, aprotic solvent such as acetonitrile.[11,12] This difference in rate results primarily from destabilization of the carboxylate ion in microenvironments deficient in hydrogen bond donors. Kemp's hypothesis was substantiated by the observation of similar high solvent rate accelerations in the elimination reactions of benzisoxazoles (e.g., **1a** → **2a**) when carboxylate is the catalytic base (e.g., B: = CH_3COO^-).[11]



Ascribing solvent rate accelerations primarily to *nonspecific* desolvation and activation of the catalytic base is also supported by the very small solvent effect on this reaction (**1a** → **2a**) catalyzed by amine general bases (10-fold vs 10^8 with carboxylate).[11] In fact, the rate in acetonitrile might be expected to be even lower than in water, as amines become less basic upon transfer to a dipolar aprotic medium (in which the protonated, charged amine is destabilized).[13] (Carboxylates, in which the unprotonated, charged form is destabilized, become more basic). The fact that there is a slightly positive solvent effect ($k_{MeCN}/k_{water} > 1$) suggests a possible secondary (*specific*) role for the solvent in stabilizing the TS of the reaction; however, any such contribution to the solvent rate acceleration must be minor.[11]

The decarboxylation and elimination reactions of benzisoxazoles have become the subject of much investigation. In particular, as intimated by Kemp,[11] they became attractive probe reactions for enzyme mimics and models. However, the rate accelerations exhibited by such catalysts are typically much lower than the exceptional solvent rate accelerations observed by Kemp.[14−17] Moreover, the distinct advantages of the *specific* and localized medium effects characteristic to enzymes, over *nonspecific* medium effects that dominate solvent rate accelerations, have not been shown thus far.

In this work, we demonstrate how medium effects can lead to highly active catalysts. We report evidence for the predominance of *specific* over *nonspecific* medium effects in catalysis by synzymes[18,19]—a range of polymeric, enzyme-like catalysts generated by the systematic modification of polyethyleneimine (PEI).[20] We compare their catalytic activity for the elimination reaction not only of **1a**—an activated substrate—but also for increasingly demanding eliminations, with higher activation energy barriers and poorer leaving groups.

Results

Synzymes were produced by the systematic derivatization of PEI with lauryl iodide, benzyl bromide, and methyl iodide (Scheme 1). A combinatorial derivatization protocol combined with high-throughput screening enabled us to search effcicently for successful reagent combinations. This protocol led to modified PEIs capable of catalyzing the Kemp elimination with rate accelerations above 10^7 and more than 7000 turnovers, at neutral pH and in water.[20] The combinatorial experiment indicated that the best catalysts are obtained by alkylation with 0.4−0.8 molar equiv of alkylating mixture (moles of dodecyliodide *plus* benzyl bromide per mole of PEI monomer residue) followed by methylation with 0.2−0.4 equiv of methyl iodide. The optimal ratios of dodecyliodide vs benzyl bromide were found to be 90:10−80:20. Although the addition of benzyl bromide did not necessarily improve the rates exhibited by the modified PEIs, it did significantly increase their water solubility.

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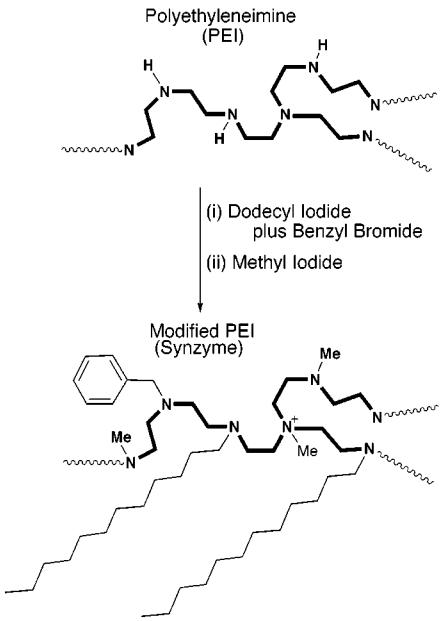
Scheme 1



We selected several compositions in the high efficiency range described above for larger-scale synthesis. The high activities were reproduced: overall rate enhancements (k_{cat}/k_{uncat}) surpass 10^7 (Table 1), though activity maxima were obtained under slightly different reagent compositions. The spectrum of catalytic activities of the synzymes was similar compared with the combinatorial preparation—the best and the worst synzymes in each preparation were within 10% of the original values. The differences in the reagent composition between these preparations can be ascribed to differences in the efficiency of PEI drying and variations in temperatures and reaction times (see the Experimental Section). The synzymes obtained in the large-scale preparations were characterized kinetically (Table 1) to gain insight into the mechanism of catalysis by these synzymes and in particular about the microenvironment of their active sites and its contribution to catalysis.

A comparative evaluation of the synzyme catalysts shows that their first-order rate comparisons (overall $k_{cat}/k_{uncat} \sim 10^7$) are well above those for a set of catalytic antibodies ($k_{cat}/k_{uncat} \sim 10^4$),[26] serum albumins ($k_{cat}/k_{uncat} \sim 10^3-10^4$),[13] orphan antibodies ($k_{cat}/k_{uncat} \sim 10^4$),[27] host−guest complexes ($k_{cat}/k_{uncat} \sim 10^3$),[15] coal ($k_{cat}/k_{obs} < 200$),[17] micelles ($k_{max}/k_w < 400$),[28] and vesicles ($k_{max}/k_w < 850$).[28]

Linear Free Energy Relationships (LFERs). LFERs have been widely used to monitor the change in charge of the transition state with respect to a given reference equilibrium. Such information is normally used to characterize the bond-making and -breaking process and to define the low energy pathway on the free energy surface. However, local charge development can be offset differently depending on the stabilizing or destabilizing interactions of solvent molecules or active-site groups surrounding the reaction TS. Thus, LFERs can be used to probe the microenvironment *around* the developing charge of a TS. LEFRs, in the form of Brønsted plots (logarithm of rate constant vs pK_a of the salicylonitrile leaving group), have been described for the Kemp elimination catalyzed by both amine and acetate, in both water and acetonitrile.[11,25] We have carried out a similar analysis for the synzyme-catalyzed Kemp elimination.

Rates of elimination for eight different benzisoxazole substrates were determined in the presence of several synzymes including 9.D/1.1 (Table 2, Figure 2A). The Brønsted plot for synzyme 9.D/1.1 gave a slope corresponding to a β_{LG} of −0.84, albeit with a poor fit (R = 0.92; Figure 2A). A much-improved fit is obtained when the least reactive benzisoxazole (5-methoxybenzisoxazole; $pK_a = 7.4$) is excluded ($R = 0.95$) giving a slope (β_{LG}) of −0.70 (see also the Discussion). Brønsted plots obtained with other synzymes at pH 6.77, and at higher pHs, all gave a similar β_{LG} and exhibited the same deviation from linearity observed with 9.D/1.1 where the least reactive benzisoxazole falls below the line.

Since amines (secondary and tertiary) act as the catalytic bases in the synzymes, we compared the synzyme-catalyzed rates with the second-order rates measured by Kemp and Casey[25] for the amine-catalyzed reaction in water. The data are shown in Figure 2A, where rate constants for the *N*-methylmorpholine (pK_a 7.73) catalyzed reaction, fitted to a single line ($R = 0.999$) with a slope corresponding to a β_{LG} of −0.63,[25] are compared with the corresponding plot for catalysis by a representative synzyme. (Data are available for amines with lower pK_a's, closer to the synzyme leaving group pK_a's (∼6), but only for the highly activated benzisoxazoles, with $pK_a^{LG} \leq 4.1$).

Inhibition of Synzyme Catalysis. We tested a large number of ligands, most of them known to be bound by serum albumins. In serum albumins, two sites (IIA and IIIA)[29] have been shown to be catalytically active, in the Kemp elimination[13,14] and in several other reactions.[30,31] These sites are characterized by a clustering of hydrophobic side chains and positively charged residues (e.g., the amino side chain of lysine). Hydrophobic ligands containing negatively charged groups (e.g., carboxylates) are bound at these sites with affinities (K_d) ranging from 1 to 100 μM.[29] These observations have been used to assign the catalytic sites in serum albumins. [13,32] The active sites of synzymes resemble the serum albumin sites in their hydrophobicity (clustering of dodecyl and benzyl groups) and in the presence of basic amino groups. Thus, it is not surprising that the synzyme catalysis of the Kemp elimination is also inhibited by many of these ligands (Figure 3).

The best inhibitor identified (data shown for synzyme 7.D/1.1) is sodium dodecyl sulfate (**6**), with an a IC_{50} of about 20 μM (well below the cmc for SDS). The high

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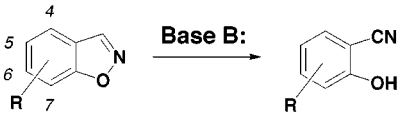
Table 1. Composition and Kinetic Parameters for the Synzymes Studied in This Work (pH 5.86, 70 mM BisTris, $[S_o] = 0-0.146$ mM, [Synzyme] = 0.2 μM, $T = 25$ °C)

synzyme	$C_{12}H_{27}I$[a] (equiv)	C_7H_7Br (equiv)	MeI (equiv)	K_M (mM)	$(k_{cat})^{total}$ (min^{-1})	$(k_{cat})^{site}$ (min^{-1})	no. of sites per synzyme	$[(k_{cat})^{total}/k_{uncat}]$[b]	$[(k_{cat})^{site}/k_{uncat}]$
9.B/2.1	0.66	0.09	0.4	12 ± 24	325 ± 58			6.8×10^7	
9.C/1.1	0.59	0.24	0.4	0.4 ± 0.1	139 ± 15	2.0 ± 0.6	70	2.9×10^7	4.2×10^5
9.C/4.1	0.35	0.14	0.4	0.7 ± 0.3	134 ± 25	2.7 ± 1.2	50	2.8×10^7	5.6×10^5
9.D/1.1	0.38	0.45	0.4	0.3 ± 0.1	181 ± 16	no satn		3.8×10^7	
9.D/3.1	0.41	0.49	0.4	0.6 ± 0.3	178 ± 40			3.7×10^7	
9.E/4.1	0.29	0.20	0.4	0.9 ± 0.3	139 ± 24			2.9×10^7	

[a] Equiv = molar equivalents of reagent per mole of PEI monomer residue (CH_2CH_2N). [b] Rate accelerations were calculated using $k_{uncat} = 4.8 \times 10^{-6}$ min^{-1} (see the Experimental Section).

Table 2. Comparison of Catalyzed and UnCatalyzed Reactions of Benzisoxazoles 1, with Varying Substituents



1 **2**

		B = NR_3			B = $RCOO^-$ (from ref 10)		
benzisoxazole **1** R =	pK_a (**2**)[a]	$k'^{synzyme}$[b] (M^{-1} min^{-1})	$k_2^{amine}(H_2O)$[c] (M^{-1} min^{-1})	$(k_{cat}/K_M/k_2)$	$k_2(H_2O)$ (M^{-1} min^{-1})	$k_2(CH_3CN)$ (M^{-1} min^{-1})	$k_2(CH_3CN)/k_2(H_2O)$
5,7-$(NO_2)_2$	0.6				1.2	3.1×10^7	2.6×10^7
5-NO_2 (**1a**)	4.1	109729	1.1×10^{-1}	1.0×10^6	8.4×10^{-3}	1.7×10^5	2×10^7
5, 7-Cl_2	4.3	116250	1.0×10^{-1}	1.2×10^6			
4,6-Cl_2	5.1	72544	3.3×10^{-2}	2.2×10^6			
6-NO_2	5.2	31063	2.7×10^{-2}	1.2×10^6			
5-Cl	6.4	8504	5.0×10^{-3}	1.7×10^6	5×10^{-4}	8.4×10^2	1.7×10^6
6-MeO	6.6	1640	3.8×10^{-3}	4.3×10^5			
unsubstituted	6.9	1517	2.2×10^{-3}	7.1×10^5	1.6×10^{-4}	90	5.6×10^5
5-MeO	7.4	97	9.3×10^{-4}	1.0×10^5	0.77×10^{-4}	21.6[d]	2.8×10^5[e]

[a] pK_a values are for the cyanophenol products (**2**); see the Experimental Section. [b] $k'^{synzyme}$ is the pseudo-first-order rate for the synzyme-catalyzed reaction; these were derived from the initial velocities measured with synzyme 9.D/1.1 at pH 6.77 with different benzisoxazoles (**1**) as described in the Experimental Section. Assuming that initial velocities with all the above benzisoxazoles were taken at $[S_o] < K_M$ and that the number of active sites per polymer molecule is the same with all substrates, the $(k')^{synzyme}$ values should reflect the relative k_{cat}/K_M values with the different benzisoxazoles. [c] $k_2^{amine}(H_2O)$ is the second-order rate constant for the coversion of **1** to **2** catalyzed in water by an amine with a pK_a of 6.77, the pH at which the rates in this table were measured. This value was extrapolated from the *N*-methylmorpholine catalyzed reaction (see ref 21) $k_2(N\text{-methylmorpholine})/10^{\Delta pK_a{}^*\beta}$ using $\beta_{nucleophile} = 0.72$ and the difference between the pK_a of *N*-methylmorpholine ($pK_a = 7.71$) and the reaction pH. [d] $k_2(CH_3CN)$ for 5-methoxybenzisoxazole was determined essentially as described in ref 10. [e] $k_2(H_2O)$ for 5-methoxybenzisoxazole was extrapolated from the rate of the acetate-catalyzed elimination of the other benzisoxazoles using a $\beta_{leaving\ group}$ of −0.63 (Figure 2B) as described in ref 10.

affinity seems to result from both the long alkyl chain (less hydrophobic sulfates or sulfonates, e.g., **4**, exhibit much lower affinities) and the strong acidity (carboxylic acids, even those with very long chains, e.g., **5**, exhibit lower affinities and may be bound at the assay pH of 5.86 as the neutral, COOH form). Aromatic, negatively charged ligands also exhibit high affinities: an example is triiodobenzoic acid (**9**), which is bound with moderate affinity (~20 μM) at both sites (IIA and IIIA) of serum albumins[29] and inhibits synzyme 7.D/1.1 (and many other synzymes tested) with an IC_{50} of 50 μM. However, several other aromatic, negatively charged ligands that are good inhibitors of catalysis by serum albumins ($IC_{50} \leq 50$ μM)[29]—examples are aspirin (**7**; $IC_{50} > 2000$ μM), iodophenoxy acetate (**8**; $IC_{50} \sim 200$ μM), and naproxen ($IC_{50} > 10000$ μM)—hardly affect the synzymes.

Discussion

Synzymes Are Bona Fide Enzyme Mimics. The rate accelerations (k_{cat}/k_{uncat}) exhibited by our synzymes approach 10^6 per site (Table 1). These values, although at the low end of the enzymatic efficiency scale, compare very favorably with other enzyme mimics, including catalytic antibodies.[33,34] In addition to the rate accelerations, the derivatized PEIs share a combination of features with real enzymes, as discussed below, which make them more faithful enzyme mimics than most described thus far (for the Kemp elimination or any other other reaction).

(1) Turnover. Efficient turnover is rarely observed in enzyme mimics, let alone in single-turnover enzyme models.[33,34] Thus, the challenge of differential recognition of substrate vs transition state (rate acceleration) and substrate vs product (product inhibition hindering multiple turnover) is largely unresolved. Synzyme active sites, however, can catalyze the elimination reactions of over 1000 substrate molecules (at pH 5.86, with 5-nitrobenzisoxazole, pK_a (leaving group) = 4.1; Table 1 and ref 20). This efficient turnover is particularly notable in view of the opposite charges of the cyanophenolate product and the synzyme catalyst. Although many negatively charged aromatic compounds (e.g., carboxylic acids **7**−**9**; Figure 3) inhibit catalysis efficiently, the product cyanophenol, **2**, and other phenols (e.g., 4-iodophenol) do not. It is noteworthy that serum albumins, which share

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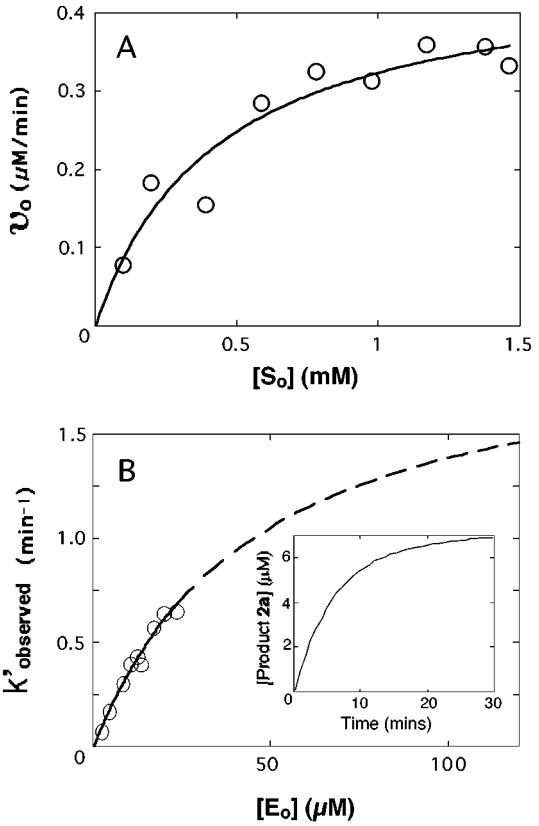

Figure 1. Determination of kinetic parameters for synzyme 9.C/1.1. **(**A**)** The initial rates of release of product **2a** were determined at different substrate (**1a**) concentrations. [conditions: $[S_o] = 0-1.46$ mM, $[E_o] = 0.2$ μM in 70 mM BisTris pH 5.86, $T = 25$ °C.]. Data were fitted to the Michaelis−Menten model ($v = [E_o]\ (k_{cat})^{total}\ [S_o]/([S_o] + K_M)$) to give a $(k_{cat})^{total}$ of 139 ± 15 min^{-1} and K_M of 0.4 ± 0.1 mM. **(**B**)** Rates of release of product **2a** were monitored under $[S_o] \ll [E_o]$ conditions, as shown, for example in inset [conditions: $[S_o] = 8$ μM, $[E_o] = 0.247$ mg/mL $= 4.9$ μM in 70 mM BisTris, pH 5.86, $T = 25$ °C] to give an apparent first-order rate constant, $k'_{obs} = 0.1747$ min^{-1}. The apparent first-order rate constants (k'_{obs}) were plotted against the polymer concentrations [E_o], and the saturation level of this curve (2.0 ± 0.6 min^{-1}; extrapolated from a fit to $k'_{obs} = (k_{cat})^{site}\ [E_o]/([E_o] + K_M)$) was taken as $(k_{cat})^{site}$ − the k_{cat} per active site. The number of active-sites per polymer molecule was obtained by dividing $(k_{cat})^{total}$ by $(k_{cat})^{site}$ to be ca. 70.

many of the features of these synzymes—catalyzing the Kemp elimination[13,29] and binding the same ligands (Figure 3)—do suffer from product inhibition and thus limited turnover (≤10).[14] Subtle differences in binding selectivity are an important feature of enzyme reactions: evidently our best synzymes are better at recognizing the TS for the Kemp elimination than the product it turns into.

(2) Effective pK_a of the Catalytic Base. The pK_a's of many of the amine general bases in these synzymes are remarkably low, falling between 5.7 and 6.7. These pK_a's are lower by 4−5 units than those of simple alkylamines, a consequence of through-bond and through-space electrostatic effects[20] as well as the apolar microenvironment of the active sites.[13] As a result, the synzymes show activity at low pHs (≥5), where the buffer-catalyzed elimination reaction (even of the relatively reactive **1a**) is barely detectable. Similar effects play a critical role in enabling enzymes to function at physiological pHs while using functional groups with pK_a's of 4−16.

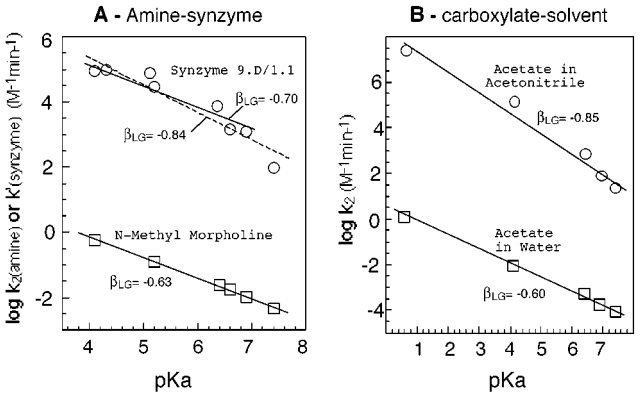

Figure 2. Brønsted plots for the amine and synzyme-catalyzed (A) and the acetate-catalyzed (B) Kemp eliminations. (A) Logarithmic dependence on the pK_a of salicylonitrile leaving group of the second-order rate constants for the Kemp elimination catalyzed by synzyme 9.D/1.1 (k′(synzyme); ○) and *N*-methyl morpholine (k_2(amine); □) (see also Table 2 and the Experimental Section). (B) Logarithmic dependence on the pK_a of salicylonitrile leaving group of the second-order rate constants for the acetate-catalyzed Kemp elimination in acetonitrile (○) and in water (□). (Data from ref 11; see also Table 2).

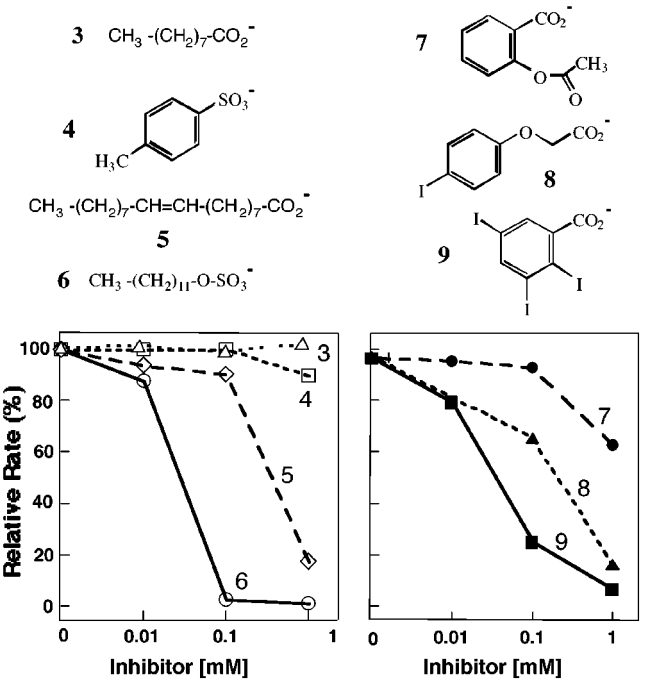

Figure 3. Titration of synzyme catalysis by various ligands. Synzyme 9.D/1.1 (at $[E_o] = 0.2$ μM, in 70 mM BisTris, pH 5.86) was incubated with ligands **3**−**9** at 0−1 mM concentrations for 3 h at 25 °C. The substrate (**2a** at $[S_o] = 0.5$ mM) was added, and rate of product release monitored. The relative rate is the ratio of initial rates of product release observed in the presence of an inhibitor vs that of the unihibited reaction.

(3) The *p*-Nitrophenyl Syndrome. Enzymes such as chymotrypsin catalyze the hydrolysis of *p*-nitrophenyl esters (pK_a^{LG}, the pK_a of the conjugate acid of the leaving group, ≈7), methyl esters (pK_a^{LG} ≈15) and amides (pK_a^{LG} >35) with comparable rate accelerations.[35] In contrast, enzyme models and mimics frequently fail to catalyze reactions with poor leaving groups. [This implies that

(35) Menger, F. M.; Ladika, M. *J. Am. Chem. Soc.* **1987**, *109*, 3145−3146.

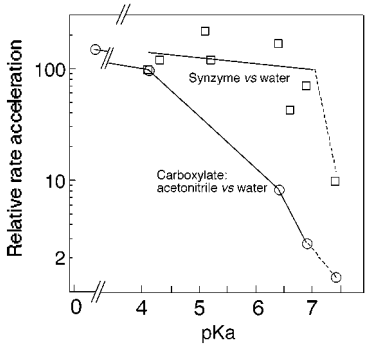

Figure 4. Relative rate accelerations for the synzyme-catalyzed and acetate-catalyzed Kemp eliminations. These are the ratios of second-order rate constants for the elimination reactions of different benzisoxazoles (Table 2), normalized on the basis of **1a** (pK_a^{LG} 4.1) = 100. For the "synzyme vs water" curve (□), the synzyme-catalyzed rate constant for a given substrate (k'(synzyme)) was divided by the rate constant (k_2-(amine)) for the elimination of the same substrate catalyzed with an amine of the same pK_a; for the "carboxylate acetonitrile vs water" curve (○), the acetate-catalyzed rates in acetonitrile ($k_2(CH_3CN)$) were divided by the rates in water (k_2 (H_2O)) (see Table 2). Notably, both curves show a region for which the extent of catalysis is independent of base pK_a (for carboxylate in water/acetonitrile, up to pK_a a 4.1; for synzyme amine, up to pK_a 6.5). At higher pK_a values, the extent of catalysis decreases as the reaction becomes more difficult. Based on the observation that catalysis is sustained longer at a high level for the synzyme shown, we postulate that this indicates a specific solvent effect, which effectively stabilizes charge development on the leaving group (see the text).

enzyme Brønsted plots are generally linear, whereas those of enzyme mimics are not. However, it should be noted that linearity is observed only when the "natural" substrate—the substrate for which the enzyme had evolved—is not activated (e.g., chymotrypsin's natural substrates are amides, not esters). An interesting exception is paraoxon hydrolase: the enzyme evolved to hydrolyze (activated) *p*-nitrophenyl phosphotriesters and exhibits a nonlinear Brønsted plot ($\beta^{LG} \approx 0$ for $pK_a^{LG} > 7.0$ and β^{LG} of -2.5 for $pK_a^{LG} < 7.0$). Despite the nonlinearity of its Brønsted plot, paraoxon hydrolase is one of the fastest enzymes known, exhibiting a turnover number of 10^4 s^{-1} and rate acceleration of over 10^{12}.[41] In contrast, the nonlinearity of Brønsted plots of enzyme mimics generally reflects their intrinsically low catalytic efficiency.] Menger, discussing an esterase mimic that exhibited high rate accelerations with activated phenyl esters ($pK_a^{LG} \leq 7$) but failed to enhance the rate of hydrolysis of esters with poorer leaving groups ($pK_a^{LG} > 10$), dubbed this phenomenon the "*p*-nitrophenyl syndrome".[35,36] In this context, it is notable that the rate accelerations exhibited by the synzymes vary over a very narrow range (factor of 5: Table 2 and Figure 4) as the pK_a of the leaving group changes from 4 to 7. In contrast, the rate of spontaneous elimination in buffer drops by more than 35-fold over this range.

(4) Origins of Rate Acceleration: Postioning vs Medium Effects. Our kinetic analysis indicates that, in comparison to bulk solvent, amine bases placed in a synzyme active site can be *at least* 100-fold more effective in catalyzing the elimination of 5-nitrobenzisoxazoles. This (conservative) estimate is based on effective molarities (EMs) obtained by dividing the rate constant for a single synzyme site at pH 5.68 (k_{cat}), by the second-order rate estimated for an amine of pK_a of 5.86 in acetonitrile (Table 1 in ref 20). A much higher EM—well above 1000—is obtained when the synzyme rates are compared to the second-order rates for an amine in water. The high EMs exhibited by the synzymes might suggest that their catalytic efficiency results from particularly effective positioning of the active site amine relative to the benzisoxazole substrate (in contrast to the reaction in solvent that relies on random collision). We consider that positioning contributes rather little to the rate accelerations exhibited by our synzymes. This conclusion is based on the consistently low EMs (<10) observed in numerous intramolecular models where a general-acid or base catalyst and a substrate are covalently linked: noncovalent and thus looser binding would be expected to result in still lower EMs.[15] High EMs have been achieved for general acid catalysis in a few cases, but these depend on a very precise (covalent) alignment of catalyst and substrate.[33,37] Enzyme models[33] and catalytic antibodies[13] overwhelmingly show low EMs, identifying precise positioning as a major hurdle in catalyst design.[13] Neither the structure nor the design of our synzymes is expected to yield precise positioning. We suggest that catalysis by these synzymes results from *specific* medium effects, stabilizing the TS of the reaction.

Linear Free Energy Relationships: Solvent vs Synzyme. The comparison of the acetate-catalyzed Kemp elimination in water vs acetonitrile (Figure 2B; data from ref 11) shows how the Bronsted plots diverge as the reaction becomes "more sensitive to solvent change as the benzisoxazole is more reactive".[11] This changing sensitivity to solvent is manifested not only in the more negative β^{LG} observed for acetonitrile (-0.85 vs -0.60 in water; Figure 2B) but more clearly when the solvent rate acceleration (relative second-order rate constant in acetonitrile over water, based on k_2^{MeCN}/k_2^{water}) is plotted against leaving group pK_a (Figure 4). We suggest that this behavior stems from the *nonspecific* manner by which the bulk solvent (acetonitrile) is acting on the catalytic base as well as on other parts of the TS. As the benzisoxazole becomes less reactive (i.e., as ring subsituents become less electron-withdrawing; Table 2), the developing negative charge in the (later) TS becomes more substantial. Consequently, the acetonitrile dipolar aprotic environment, which activates the carboxylate catalyst by desolvation of its negative charge, simultaneously destabilizes negative charge developing on the phenoxide (leaving group) oxygen in the TS. As the leaving group becomes poorer (less acidic) and π-delocalization of the developing negative charge less extensive, the second of these opposing medium effects predominates, resulting in the sharp drop in the solvent rate acceleration at pK_a^{LG} above 4 (Figure 4). This argument suggests that the rate acceleration will disappear when the basicities of the phenoxide leaving group and of the carboxylate base become similar ($pK_a^{LG} \geq 10$).

Synzyme catalysis, by contrast, shows little sensitivity to the pK_a of the leaving group. The rate acceleration (synzymes' rates under k_{cat}/K_M conditions at pH 6.77, compared to k_2 for an amine of pK_a 6.77) changes by a factor of only 5 over a range of almost 3 pK_a units (4.1—6.9; Figure 4). The solvent rate acceleration carboxylate-

(36) Breslow, R.; Czarniecki, M. F.; Emert, J.; H., H. *J. Am. Chem. Soc.* **1980**, *102*, 762—769.

(37) Kirby, A. J. *Acc. Chem. Res.* **1997**, *30*, 290—296.

catalyzed reaction drops 35-fold in the same pK_a range. Even when the least reactive substrate (5-methoxy; pK_a 7.4) is included in the analysis, the synzyme rate acceleration shows a drop of 10-fold in rate acceleration relative to the 5-nitro benzisoxazole (pK_a 4.1), whereas the solvent rate acceleration drops by 70-fold (Table 2).

Thus, a crucial difference between synzyme and solvent catalyis is whether the level of catalysis is sustained as the reaction gets more difficult (Figure 4): If the level of catalysis is supported with poorer leaving groups we expect a flat curve (constant rate acceleration with progessively more difficult reactions)—if not, a downward trend. Figure 4 shows that the point where catalysis breaks down occurs at higher leaving group pK_a's (>4.5 for solvent catalysis, >7 for synsymes). We interpret this phenomenon as evidence for a *specific* medium effect in synzyme catalysis—*specific* enough to stabilize negative charge on a leaving group.

Localized vs Homogeneous Media: Specific vs Nonspecific Medium Effects. This analysis is consistent with our hypothesis that synzyme catalysis is due primarily to *specific* medium effects leading to TS stabilization rather than effective positioning of amine general bases or *nonspecific* activation as with the acetate-catalyzed reaction in acetonitrile. Further, it implies a fundamental difference between the medium effects exerted by bulk solvent and those exhibited by the synzyme active-sites. This difference can be accounted for in terms of the nonhomogeneous microenvironment of the synzymes, where different groups can exert different effects. These groups are localized in the various active sites, where they can adopt favorable geometries relative to the substrate and the TS of the reaction.

The difference between the fixed dipoles of an enzyme and the random ones of a bulk solvent is a fundamental feature of enzyme catalysis.[2,4,5] Achieving high rate accelerations with poor as well as activated substrates requires the cooperation of different "solvent" effects at each stage of the reaction or even on each part of the TS. Efficient catalysis by synzymes may require the combination of several solvent or medium effects leading to the following: (i) substrate binding driven by hydrophobic interactions; (ii) activation of the catalytic base and/or adjusting its pK_a to the pH of the reaction; and (iii) stabilization of the developing negative charge of the TS through dispersion interactions or via hydrogen bonding. Each of these features could be achieved individually in bulk solvent in a *nonspecific* manner, but not in combination with the others. By localizing its medium effects, the synzyme's active site can achieve all three simultaneously.

The inhibition data (Figure 3) also support these ideas. To bind to a synzyme active-site and thereby inhibit its catalytic activity, a ligand needs to be hydrophobic on one hand and negatively charged on the other. Notably, polarizable groups (e.g., iodophenyl) make particularly effective inhibitors (matched only by C_{12}, or longer, alkyl chains). This is consistent with the synzyme active sites being heterogeneous, with positively charged amine groups and hydrogen-bond donors (protonated amines) placed in the vicinity of apolar, hydrophobic groups. Moreover, the lack of catalysis by inhibitor carboxylates supports our thesis of specific transition state stabilization. If the local environment medium were conducive to simple desolvation of any species (e.g., a bound carboxylate or sulfate as in **3**−**9**), this would be expected to show up as rate *acceleration* with increasing concentrations of compounds **3**−**9**.

The inhibition pattern shown in Figure 3 is similar to that observed with protein active sites that catalyze the Kemp elimination.[13,32] Such sites are known to have hydrophobic residues in close proximity to positively charged groups.[29,38] For example, the ϵ-amino-group of Lys222, present in the IIA site of BSA, has been identified as the primary source of its catalytic activity. In HSA, and many other serum albumins, the IIA site (presumably with Lys199 acting as a general base) also participates in catalysis (accounting for more than 50% of the catalytic activity) along with other sites (primarily IIIA).[13] The detailed structure of synzyme active sites is unknown, and most likely unknowable; but the structure of HSA gives an idea of the structural requirements of a catalytic site for the Kemp elimination. We presume that the active sites of synzymes exhibit similar structural features and that suitably disposed amine(s) and other dipoles, charges and apolar, hydrophobic groups hold the key to catalysis in both cases. Micellar and vesicular catalysis shares some structural features with synzymes, but the mechanism of catalysis is likely to be *nonspecific* activation−desolvation of the negatively charged hydroxide ion (and the magnitude of catalysis with at best 850-fold rate acceleration much smaller).

The observation that synzyme catalysis is 10^5-times stronger than nonspecific desolvation-activation of amines in aprotic acetonitrile (compared with water) lends further support for a specific catalytic effect.

Conclusions

Medium Effects and the Origins of Enzyme Catalysis. The microenvironment of the synzymes described here is obtained by simple chemical modification of a polymeric scaffold bearing amine groups. The active sites are ill-defined, heterogeneous, and "primitive" relative to enzyme active sites and deploy little chemical diversity. Nevertheless, in this microenvironment an amine group is at least 100-fold (probably more than 1000-fold) more active than in bulk solvent, be it acetonitrile or water. Moreover, this microenvironment is highly selective: first, in its ability to stabilize the TS to a much higher extent relative to the ground state (up to 10^6-fold higher, as indicated by the rate acceleration, k_{cat}/k_{uncat} per site); and second, in its ability to release the product and thereby exhibit turnover. These results suggest that medium effects alone can give rise to significant rate accelerations with both activated and nonactivated substrates, even in the absence of precise positioning of the catalyst relative to the substrate, or elaborate catalytic mechanisms of the type observed with enzymes, which may require the proper alignment of several different active-site residues. The microenvironments of protein active-sites, with their much greater chemical diversity and control of geometry, can undoubtedly exert medium effects of similar—and most likely higher—magnitude. Thus, the contribution of medium effects to enzyme catalysis might be generally underestimated in comparison with other more readily quantifiable effects, such as positioning of active-site catalytic groups.[8,9]

Finally, these results may shed some light on the very early stages of evolution of biocatalysts. Catalysis has

(38) Curry, S.; Mandelkov, H.; Brick, P.; Franks, N. *Nature Struct. Biol.* **1998**, *5*, 827−834.

been observed in other "primitive" microenvironments such as micelles, although generally for reactions of activated substrates. Synzymes—which resemble micelles structurally to some degree[18] but offer a higher degree of structural organization and chemical diversity—exhibit significant rate accelerations for the Kemp elimination as well as for many other reactions.[18,39] This suggests that on the surface of proteins, and other biopolymers including perhaps primordial molecules, potential active sites with similar properties are not uncommon. Such sites, using a catalytic side-chain functional group, cofactor, or even water molecules[2] placed in a suitable environment, might have served as "starting points" for the evolution of the highly efficient enzymes we know today. The discovery of such active sites on the surfaces of such proteins as serum albumins that have not evolved as enzymes (and certainly not for the Kemp elimination),[13] and the promiscuous activities found for many enzymes, i.e., with substrates and reactions that are not related to the one the enzyme had evolved to catalyse,[40] suggest not only that such "starting points" are reasonably common, but also, that, at the interfaces of proteins' surfaces and their hydrophobic core, the structural and chemical demands for such a "starting point" can be met with relative ease.[1]

Experimental Section

Synthesis of Synzymes. Commercial PEI (Sigma, 50% in water) was rendered anhydrous by repeated coevaporation with anhydrous ethanol and toluene followed by drying under vacuum for 24 h. The polymer and 2,6-lutidine (4 equiv per monomer (CH_2CH_2N) residue) were dissolved in DMF (by stirring and warming to 40 °C) to give a final PEI concentration of 13.75 mg/mL (0.3125 M in monomer residues). For derivatization, freshly prepared solutions of benzyl bromide and dodecyl iodide mixtures (0−2.5 M total concentration) were made up in DMF. These solutions (0.169 mL) were added to aliquots of 0.9 mL of the PEI/lutidine solution (to give alkylation ratios of 0−1.5 benzylbromide plus dodecyl iodide per monomer residue) in 96-well, 1 mL polypropylene plates (Matrix, Hudson, USA). The (homogeneous) reaction mixtures were incubated with occasional agitation for 5−7 days at ambient temperature. Freshly prepared solutions of methyl iodide in DMF (0−5 M) were then added (0.169 mL per reaction; to give methylation ratios of 0−3 equiv of methyl iodide per monomer residue), and incubation was continued for a further 4−9 days.

For the initial screening,[20] 10 μL aliquots of the synzyme reaction mixtures were diluted (1:20) into DMF and then into 50 mM NaOH (1:5) and allowed to stand for 20 h. The sodium hydroxide solutions were then diluted (1:5) into reaction buffer (70 mM BisTris pH 7.12) and incubated for an additional 20 h before they were assayed for catalytic activity (see below). Synzymes studied in detail were purified by dialysis, as follows. Crude reaction mixtures were transferred into dialysis tubes containing 20% DMF in 50 mM HCl (to a polymer concentration of 10 μM). The resulting solutions were dialyzed against the following buffers (each round ~24 h): 20% EtOH in 50 mM HCl; 10% EtOH in 50 mM HCl; 50 mM HCl (twice); water (twice); 50 mM NaOH (twice); water (three times). Dialyzed samples could be stored in the fridge, but slowly lost activity (approximately 25% of the original activity remained after one year). By contrast, reaction mixtures in DMF stored at −20 °C and worked up as above showed no loss of activity after 18 months.

The above procedure (synthesis and purification by dialysis) was also scaled up by a factor of 10, in 15 mL (polypropylene) Falcon tubes (but otherwise unchanged). Synzymes generated on the larger scale are identified by the prefix 9 (e.g., 9.X/Y.Z): prefixes 7 (e.g. 7.X/Y.Z) or 8 refer to products from small-scale synthesis.

Kinetic Measurements. For intial screening, a solution of 5-nitrobenzisoxazole **1a** in methanol was freshly diluted (1:10) into water and added (20 μL; to give a final concentration [**1a**] = 0.5 mM) to the diluted reaction mixtures (100 μL of 0.02 mg/mL [0.4 μM] polymer in 70 mM BisTris pH 7.12 plus 80 μL of the same BisTris buffer in microtiter plates). Initial velocities were determined by monitoring the release of product **2a** at 405 nm in a microtiter plate reader (Molecular Devices). Kinetic parameters (Table 1) were determined at 25 °C, pH 5.86, 97.5 mM BisTris, [**1a**] = 0−1.46 mM, and [synzyme] = 0.2 μM. The determination of k_{cat} and K_M is compromised by the limited solubility of substrate **1a** in water and errors larger than the statistical ones quoted in Table 1 are possible, in particular for the overall parameters $(k_{cat})^{total}$ and K_M. Higher substrate concentrations could be attained in acetonitrile/water mixtures, but the rise in substrate solubility corresponded to a rise in K_M (e.g. for 10% instead of 2% acetonitrile in water, K_M rises some 10-fold, to >4 mM).

To determine rate accelerations for a single active site $(k_{cat})^{site}$ and the number of sites per polymer molecule, experiments were performed at $[S_0] \ll$ [Synzyme], where the first-order rate of reaction of the active-site complexed **1a** could be determined under single-turnover conditions.[19,21,22] Polymer solutions were concentrated by incubating them in dialysis tubes immersed in poly(ethylene glycol) (M_w 8000). The volume was reduced by approximately 5-fold and the concentrated polymer solution dialyzed against water. Reactions were followed in the spectrophotometer at 405 nm at [**1a**] = 5 μM and [synzyme] = 0−32 μM in 70mM BisTris pH 5.86 and 25 °C. The appearance of product **2a** was fitted to an exponetial curve. For higher synzyme concentrations and the (fast) exponential curves were corrected for a (slow) linear drift (perhaps due to polymer bleaching or precipitation). The derived first-order rate constants (e.g., Figure 1, inset) were plotted against polymer concentration and extrapolated to infinite synzyme concentration to give $(k_{cat})^{site}$ (Figure 1).

Background Reactions. The buffer-catalyzed elimination of **1a** (0.125 mM) was monitored at pH 5.86 in 50−150 mM BisTris buffer at 25 °C. Data points were averaged for at least three measurements and the rates extrapolated to zero buffer concentration, yielding $k_{uncat} = 4.8 \times 10^{-6}$ min^{-1}. Second-order rate constants for the amine catalyzed elimination of **1a** were measured for tetramethyl ethylenediamine (TMEDA; pK_1 = 5.9).[23] Solutions of TMEDA cations were prepared in acetonitrile (by addition of 1 equiv of *p*-toluenesulfonic acid monohydrate) and in water (by addition of 1.5 equiv of hydrochloric acid, giving a 50% free base solution). Pseudo-first-order rate constants were plotted against the amine concentration. The slopes in these plots (in case of water multiplied by two) gave the second-order rate constant for the amine-catalyzed reaction k_2^{amine}.

Determination of Turnover. The appearance of reaction product **2a** was followed over 60 h ([synzyme] = 0.02 μM, [**1a**] = 0.25 mM in 40 mM BisTris, pH 5.86). The turnover limit was set to the point where the rate of the polymer-catalyzed reaction matched the rate of the background reaction, followed in parallel without polymer, and the concentration of product released divided by the concentration of active sites.

Synthesis of substituted benzisoxazoles was performed according to published procedures,[24] as briefly described here for 5,7-dichlorobenzisoxazole. Aqueous sodium acetate (1.2 mL of a 4 M solution) was added to a warm solution of 3,5-dichlorosalicylaldehyde (472 mg, 2.47 mmol) and hydroxylamine hydrochloride (350 mg, 5 mmol) in aqueous ethanol (80%, 6 mL). The clear solution was heated at reflux for 3 h, when water was added (1.2 mL), and the mixture was left at 4 °C. The solid product was filtered off, washed with water, and dried to yield the oxime as colorless needles 425 mg (84%). The oxime (410 mg, 2 mmol) was dissolved in THF (5 mL),

(39) Suh, J.; Hah, S. S. *J. Am. Chem. Soc.* **1998**, *120*, 10088−10093.
(40) O'Brien, P. J.; Herschlag, D. *Chem. Biol.* **1999**, *6*, R91−R105.
(41) Caldwell, S. R.; Newcomb, J. R.; Schlecht, K. A.; Raushel, F. M. *Biochemistry* **1991**, *30*, 7438−44.

and a solution of trichloroacetyl isocyanate in THF (2 M, 2 mL, 4 mmol) was added under anhydrous conditions. After the mixture was stirred for 10 min at room temperature, anhydrous potassium carbonate (286 mg, 2.7 mmol) was added and the mixture stirred for a further 30 min. Water (2 mL) and dilute hydrochloric acid (5 mL) were then added, and the solution was extracted with dichloromethane. The combined organic extracts were dried ($MgSO_4$), evaporated at reduced pressure, and purified by column chromatography (SiO_2, CH_2Cl_2) to give 5,7-dichlorobenzisoxazole (140 mg, 37%).

Linear Free Energy Relationship. The phenol products were generated by sodium hydroxide elimination of the corresponding benzisoxazoles and their extinction coefficients measured at the wavelengths indicated below. The pK_a value of the cyanodichlorophenol product of 5,7-dichlorobenzisoxazole was determined as 4.3 by measuring the inflection point of the absorbance at 340 nm with varying pH (in citric acid hydrogen phosphate buffers, pH 2.6−7.2). The other substituted benzisoxazoles employed, the pK_a values of the corresponding phenols[25] and the wavelengths at which the reaction was followed, were as follows: 5-methoxy (7.4, 340 nm), parent (6.9, 311 nm), 6-methoxy (6.6, 310 nm), 5-chloro (6.4, 340 nm), 6-nitro (5.2, 405 nm), and **1a** (4.1, 405 nm). Synzyme-catalyzed initial rates were determined at 70mM BisTris pH 6.77 and 25 °C, and divided by the benzisoxazole and synzyme concentrations (0.2 mM and 0.8 μM, respectively), to give the pseudo-first-order rate constants $k'_{synzyme}$ (Table 2).

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