A Kinetic Investigation for Substrate Specificity of a Hydrolytic Abzyme

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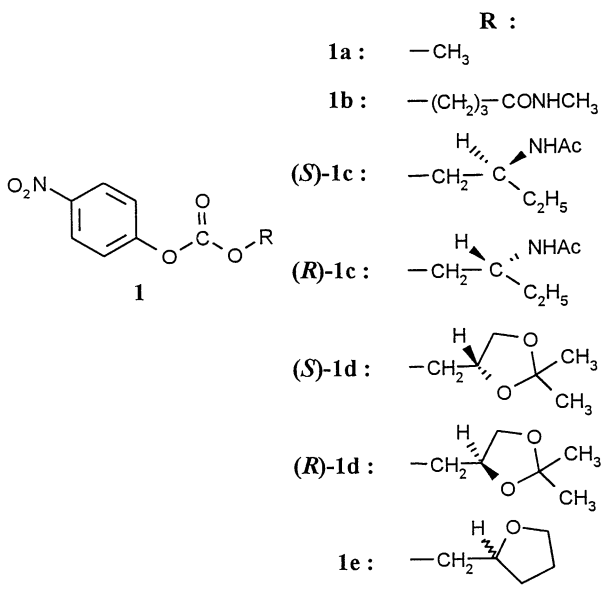
Catalytic antibody 4A1 showed a significant rate acceleration against substrates which are different from the structure of the inducing hapten in the carrier-proximal region. Kinetic analysis of Km and kcat values as well as the K_D values of the corresponding transition-state analogues indicated that the rate enhancement resulted from stabilization of the transition-state in the cleavage reaction.

Development of efficient hydrolytic catalytic antibodies (abzymes)[1] with broad substrate tolerance offers potential applications as reagents for chemical synthesis.[2] However, it is generally thought that a strict homology of the substrate to the inducing hapten / transition-state analogue must be maintained for catalysis to take place.[3] Abzymes are thought, therefore, to permit little latitude in choice of potential substrates.[4] Recently, Janda and Lerner et al.[5,6] demonstrated two successful approaches toward improving catalytic efficiency (kcat/kuncat) with substrate tolerance. One is to create abzyme-substrate destabilization via weaker apparent substrate binding, manifested by an increase in Km.[5] The other is to introduce a nonspecificity element with a strong immunogenic moiety in the hapten molecule.[6] A substrate structure for abzymes can be divided into carrier-distal and carrier-proximal regions according to the corresponding hapten structure. Herein we describe a hydrolytic abzyme which shows a significant rate acceleration of hydrolysis of substrates differing from the hapten in structure around the carrier-proximal region, without significant increase in Km or introduction of any nonspecificity element into the inducing hapten.



Phosphonate **2a** was coupled to the carrier protein KLH by its COOH group, using the method of Schultz,[7] and 34 monoclonal IgG antibodies were generated. Antibodies were purified from ascites fluid by Protein A-Sepharose 4B affinity chromatography eluted by pH gradient. The homogeneity of the antibodies was about 95%. Antibody 4A1 and two others (1G2 and 5H2) were found to have catalytic activities for carbonate **1a** with values of kcat/kuncat of 3.0×10^3, 4.5×10^2 and 4.4×10^2, respectively, at pH 8.5. All exhibited saturation kinetics and were completely inhibited by the addition of phosphate **2b**.[8]

Table 1. Kinetic constants for antibody 4A1-catalyzed reaction[a]

Substrate	Km (μM)	kcat (min^{-1})	kuncat ($x10^{-3} min^{-1}$)	kcat/kuncat ($x10^3$)	kcat/Km ($min^{-1} \mu M^{-1}$)
1a	641	13	4.4	3.0	0.02
1b	238	13	3.5	3.7	0.05
(*S*)-1c	812	23	3.8	6.0	0.03
(*R*)-1c	607	9	3.5	2.4	0.01
(*S*)-1d	586	224	3.5	64.0	0.38
(*R*)-1d	363	50	3.5	14.0	0.14
1e	547	30	3.8	8.0	0.05

[a] [4A1] = 4.4 μM, determined at 30 °C in 98% tris buffer (10 mM, pH 8.5) and 2% THF in the presence or absence of antibody.

Interestingly, in a study of effect of carrier-proximal region in the substrate,[9] one of the antibodies, 4A1, unlike the others, showed a significant rate acceleration for the racemic substrates **1d** and **1e**, which have five membered heterocyclic substituents spacially extended from the inducing hapten structure, with values of kcat/kuncat of 3.0×10^4 and 8.0×10^3 at pH 8.5, respectively.

Furthermore, an additional characteristic of this antibody was revealed when 4A1 was tested for enantioselectivity in separate experiments with chiral substrates **(*S*)-**, **(*R*)-1c** and **(*S*)-**, **(*R*)-1d**.[10] As shown in Table 1, 4A1 catalyzed hydrolysis of **(*S*)-1c** (kcat/kuncat = 6.0×10^3) and **(*S*)-1d** (kcat/kuncat = 6.4×10^4), greater than the corresponding **(*R*)-1c** and **(*R*)-1d**, although the enantioselectivity is relatively low ($k^S cat / k^R cat$ = 2.5 and 4.5, respectively). The present rate differential observed between the enantiomers is an indication that the chiral environment of the 4A1 combining site[11] discriminates between the enantiomerically different two transition-states, even though the antibody was elicited by a hapten without any chiral elements.

The extended structures of **1c**, **1d**, and **1e** do not increase Km, but rather increase kcat relative to **1a** and **1b**, which is structurally most congruent with the inducing hapten structure

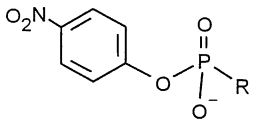
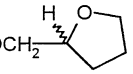
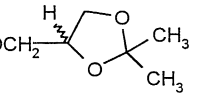


Table 2. Apparent binding constant (K_D) of antibody 4A1 with phosphonate and phosphates[b]

phosphonate/phosphates	K_D(M)
2a : R = —$(CH_2)_4$—CO_2H	2.5×10^{-5}
2b : R = —OCH_3	8.9×10^{-5}
2c : R = 	2.0×10^{-5}
2d : R = 	6.2×10^{-7}

The apparent binding constants (K_D) for the antibody 4A1 and various transition-state analogs were determined by competitive ELISA procedure using BSA-hapten conjugates.
[b] Binding determined at 30 °C in 10 mM Tris/HCl buffer, pH 8.5.

2a. As a result, the specificity constant (kcat/Km) for **1c**, **1d** and **1e** is increased (Table 1). Furthermore, thermodynamic study indicated that 4A1 had a stronger affinity for phosphate **2d**, the transition-state analog of **1d**, and the apparent K_D values[12] were determined to be 6.2×10^{-7} M as shown in Table 2. These results suggest that the rate acceleration for **1d** and **1e** is associated with a decrease in the activation energy due to stabilization of the transition-state in the cleavage reaction. Thus there are strong affinity contacts between the carrier-proximal portion of the reaction transition-state and hydrolytic abzyme 4A1. It should be noted that abzyme 4A1 has a 20-fold higher specific constant (kcat/Km) as well as hydrolysis rate for the alternate substrate, while most modifications of substrate resulted in lowering the specific constant in other reported abzymes. In addition, abzyme 4A1 can distinguish enantiomers with a single chiral center in the carrier-proximal region of the substrate, suggesting that the observation of accelerated hydrolysis is not due to an indirect effect by modifying the region.

In conclusion, these results show that the substrate specificity of an abzyme can be influenced, in part, by the portion of the substrate corresponding to the carrier-proximal region of the inducing hapten.[13] This observation allows new avenues for the design of more potent substrates for hydrolytic abzymes. Efforts are underway in our laboratory to elucidate further the unique character of the 4A1 antibody.

References and Notes

1 P. G. Schultz, *Angew. Chem., Int. Ed. Engl.*, **10**, 1283 (1989); P. G. Schultz, R. A. Lerner, and S. J. Benkovic, *Chem. Eng. News*, 1990, May 28; P. G. Schultz and R. A. Lerner, *Acc. Chem. Res.*, **26**, 391 (1993); I. Fujii *J. Synth. Org. Chem.*, **52**, 850 (1994); K. D. Janda and J. Y-C. Chen, in "The Pharmacology of Monoclonal Antibodies," ed by M. Rosenberg and G. P. Moore, Springer-Verlag, New York (1994), p. 209-242.

2 T. Li, K. D. Janda, J. A. Ashley, and R. A. Lerner, *Science*, **264**, 1289 (1994); L. C. Hsieh, S. Yonkovich, L. Kochersperger, and P. G. Schultz, *Science*, **260**, 337 (1993).

3 S. J. Pollack, J. W. Jacobs, and P. G. Schultz, *Science*, **234**, 1570 (1986).

4 S. Tramontano, A. A. Ammann, and R. A. Lerner, *J. Am. Chem. Soc.*, **110**, 2282 (1988); R. A. Gibbs, P. A. Benkovic, K. D. Janda, R. A. Lerner, and S. J. Benkovic, *J. Am. Chem. Soc.*, **114**, 3528 (1992).

5 K. D. Janda, S. J. Benkovic, D. A. Mcleod, D. M. Schloder, and R. A. Lerner, *Tetrahedron*, **47**, 2503 (1991); I. Fujii, R. A. Lerner, and K. D. Janda, *J. Am. Chem. Soc.*, **113**, 8528 (1991).

6 T Li, S. Hilton, and K. D. Janda, *J. Am. Chem. Soc.*, **117**, 2123 (1995).

7 J. Jacobs, P. G. Schultz, R. Sugasawara, and M. Powell, *J. Am. Chem. Soc.*, **109**, 2174 (1987).

8 The Ki for 1G2 and 5H2 were 15 μM and 40 μM at 30 °C, respectively. However, the plots for 4A1 obtained from the inhibition assay did not appear like the plots for competitive inhibitor. Since the antibody sample treated with phosphate **2b** only recovered 20% of its activity after 3 days of extensive dialysis at 4 °C, the inhibition observed for 4A1 should be considered irreversible.

9 All compounds were prepared by analogy to the reported procedure and gave satisfactory spectroscopic and combustion analyses.

10 Commercially available optically active fragments were used for the preparation of compound **1c** and **1d**. (***S***)-**1c**: $[\alpha]_D$ = -35.4 (c 1.0, $CHCl_3$); (***R***)-**1c**: $[\alpha]_D$ = +35.2 (c 1.0, $CHCl_3$); (***S***)-**1d**: $[\alpha]_D$ = +9.5 (c 0.87, $CHCl_3$); (***R***)-**1d**: $[\alpha]_D$ = -9.5 (c 0.87, $CHCl_3$).

11 For a similar observation on bimolecular amide formation see: K. D. Janda, R. A. Lerner, and A. Tramontano, *J. Am. Chem. Soc.*, **110**, 4835 (1988).

12 K. D. Janda, M. I. Weinhouse, T. Danon, K. A. Pacelli, and D. M. Schloder, *J. Am. Chem. Soc.*, **113**, 5427 (1991).

13 Prof. Schultz has revealed that antibodies isolated by Schultz's group specific for phosphate **2a** are relatively insensitive to substitutions in the aliphatic linker. G. R. Nakayama and P. G. Schultz, *J. Am. Chem. Soc.*, **114**, 780 (1992); B. Gong, S. A. Lesley and P. G. Schultz, *J. Am. Chem. Soc.*, **114**, 1486 (1992).