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CATALYSIS OF THE KEMP ELIMINATION BY ANTIBODIES ELICITED AGAINST A CATIONIC HAPTEN

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Abstract: Rather efficient catalysis of the decomposition of 5-nitro-benzisoxazole to the cyanophenol was observed with antibodies elicited against a cationic hapten structurally unrelated to the benzisoxazole substrate. The rate enhancement by the most active antibody is better than 10^4 and the reaction is catalyzed by a carboxylate group associated with a hydrophobic binding site. © 1997 Elsevier Science Ltd.

The concept of transition state stabilization has been the basis for the successful generation of numerous catalytic antibodies.[1] The strategy relies on the design of haptens to mimic the transition state of a target reaction, which are used to elicit monoclonal antibodies. A complementary approach relies on haptens designed to elicit catalytic groups such as nucleophiles, electrophiles, general acids or general bases into the antibody binding sites. For example, charge complementarity has been used successfully to elicit a general base for an antibody-catalyzed β-elimination[2], and a general acid for the catalysis of acetal hydrolysis[3]. Recently Thorn *et al.*[4] reported that an antibody generated against a rationally designed hapten catalyzed the Kemp elimination[5] (Scheme 1) exceptionally efficiently, with an active site carboxylate acting as the general base. The same reaction is catalyzed, at similar rates, by several serum albumins, using lysine amino-groups associated with known hydrophobic binding sites.[6]

This result raised questions about the factors that contribute to the efficiency of proton transfer catalysis in enzymes. Intramolecular general acid/base catalysis is in most cases notably inefficient,[7] but recent work suggests that precise positioning of the reacting groups allows the proton transfer to take place with relatively high efficiency within a developing strong hydrogen bond.[8] The necessary precise positioning of catalytic and

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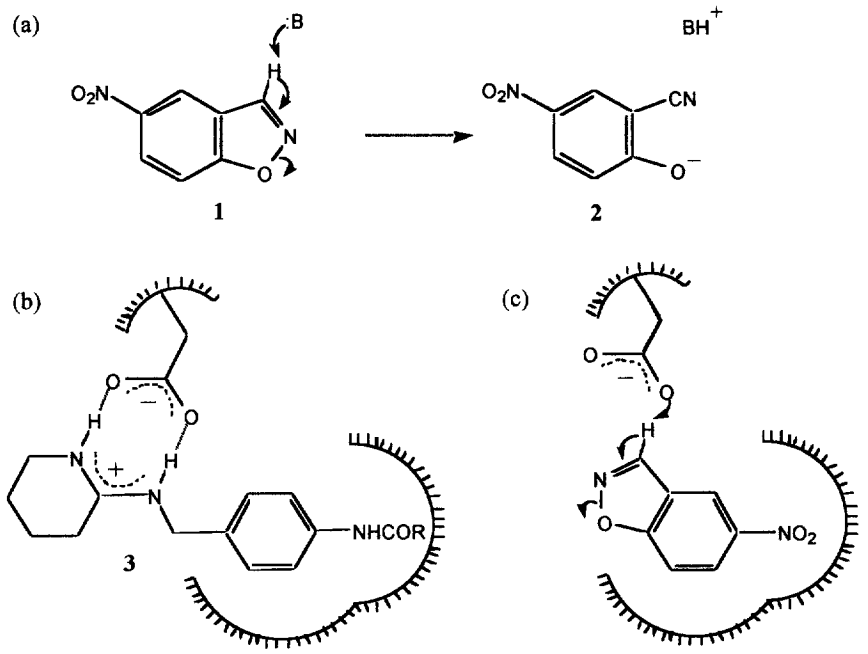
substrate groups just might be a feature of a carefully designed antibody: it is not likely to be achieved in reactions catalyzed by a random selection of albumins. So the interim conclusion was that medium effects[5] associated with the hydrophobic binding site make a major contribution to catalysis in the antibody-catalyzed reaction of Thorn *et al.*[4] The carboxylate general base plays an essential catalytic role, but does not need to be very precisely positioned. Hence results with another, unrelated protein with an "active site" carboxylate would be of particular interest.



Scheme 1

(a) The Kemp elimination catalysed by a general base B.

(b) The hapten used to elicit the antibodies in a schematic binding site.
3a, R = $(CH_2)_3CO$-KLH; **3b**, R = CH_3.

(c) The substrate for the Kemp elimination reacting in the same binding site.

We reasoned that the cyclic amidinium salt **3** would induce a complementary charged group, presumably carboxylate, in a hydrophobic antibody binding site. This hapten has been previously used by us [9] and by others [10] in attempts to generate antibodies with glycosidase activity, but no catalytic antibodies were detected (probably the planar structure of the amidine-based hapten does not permit favorable axial leaving group geometry in the aryloxytetrahydropyran substrate). However, several excellent hapten-binding monoclonal antibodies [11] (K_d = 10^{-8}-10^{-10} M)[12] were identified, with binding properties consistent with the presence of a hydrophobic binding site with a negative charge in close proximity. We therefore screened twenty hybridoma

supernatants for catalytic activity in the Kemp elimination at pH 6.[13] (Under the conditions background catalysis due to albumins present as tissue culture additives is very low. [14]) Six of the twenty proved to be active. Of these, two of the best hapten-binders, 4B2 and 6C2, exhibited the highest catalytic activity and were selected for detailed characterization.

The antibody-catalyzed ring-opening reactions (Scheme 1) of 5-nitrobenzisoxazole **1** followed Michaelis-Menten kinetics (Table 1). In contrast to the reaction catalyzed by BSA, no product inhibition was detected and multiple turnover (over 100-fold) was observed.

Table 1. Kinetic parameters for the antibody-catalyzed Kemp elimination, at pH 7.1, 30°C. [a]

Catalyst	% CH_3CN	K_M (mM)	k_{cat} ($\times 10^2\ s^{-1}$)	k_{cat}/K_M ($M^{-1}.s^{-1}$)	k_{cat}/k_{uncat} [b]
4B2	0.25	n.d.	n.d.	53	
	1	1.2 ± 0.4	3.5 ± 0.8	29.1	1.8×10^4
	5	2.1 ± 1.0	2.2 ± 0.7	10.1	6.3×10^3
	10	n.d.	n.d.	5.9	
6C2	1	n.d.	n.d.	1.4	

[a] Initial velocities were determined at 30° C with **1** (0.1 - 0.6 mM in 1% CH_3CN; 0.2 - 1.25 mM in 5% CH_3CN) and 4B2 (1.5 μM), and corrected for the rate of the background reaction under the same buffer conditions (Phosphate 40mM, NaCl 100mM, pH 7.1). The low solubility of **1** in water prevented measurements at substrate concentration above K_M ; standard errors are calculated from the statistical curve fits: hence true uncertainties may be larger than those indicated.
[b] k_{uncat} is based on an extrapolation to zero buffer concentration at pH 7.1 for each concentration of acetonitrile.

Complete inhibition of the antibody catalyzed reaction was observed upon addition of hapten **3b**, which behaved as a tight binding inhibitor. The calculated concentration of functional catalytic sites matched the experimental concentration of antibody combining sites (Figure 1): an evidence that the antibodies were kinetically homogeneous and that catalysis ocurred within the expected binding sites.
The pH dependence of k_{cat}/K_M (Figure 2) indicates that in the free antibody a base with a pK_a of 5.8 is involved in the reactions catalyzed by 4B2 (*cf.* the value near 4 expected for an aspartate or glutamate residue). Complete inactivation of the antibody was observed by selective modification of carboxylate functional groups.[15] Inactivation of 4B2 was partially prevented (50% activity remained) in the presence of hapten **3b** (3 mM). All these facts are consistent with the catalytic base being a carboxylate group, with its basicity increased in the apolar active-site micro-environment of the antibody.

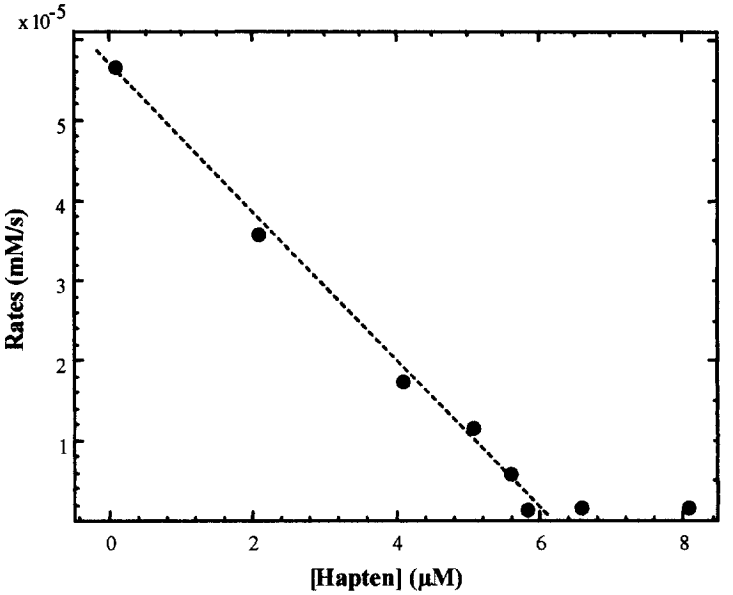


Figure 1: Active site titration of 4B2 by hapten **3b**. Rates were determined using 3 μM 4B2 and 0.4 mM **1**, at 30°C in 20 mM phosphate buffer containing 50 mM NaCl (pH 7).

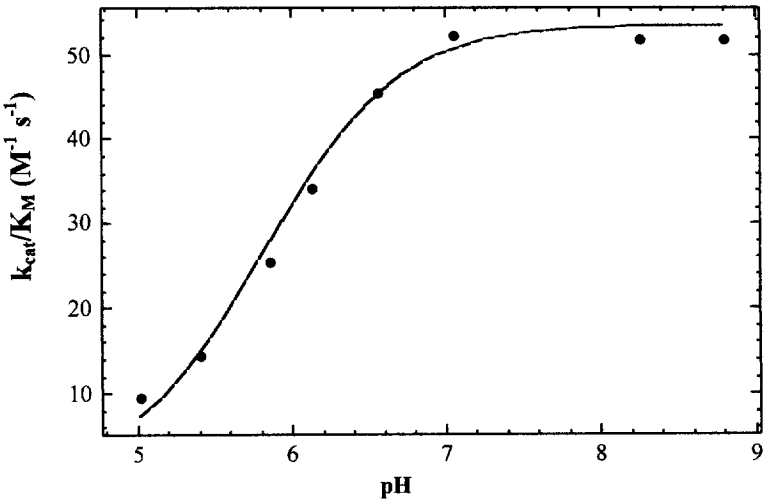


Figure 2: Plot of k_{cat}/K_M *vs* pH for the conversion of **1** to **2** catalyzed by 4B2. Kinetics were performed under pseudo-first order conditions (S_0 < 0.15 mM) at 30°C in the following buffers (40mM, containing 100mM NaCl and 0.25% CH_3CN): sodium acetate (pH <6); sodium phosphate (6<pH<7.5); sodium borate (pH>7.5). Data were fit to the equation $k_{cat}/K_M = (k_{cat}/K_M)^{max}/(1 + 10^{pK_{EH}-pH})$, giving $(k_{cat}/K_M)^{max} = 53.4 \pm 1.2$ $M^{-1}s^{-1}$ and $pK_{EH} = 5.79 \pm 0.05$.

Catalysis by 4B2 is relatively efficient, with k_{cat}/k_{uncat} up to 18,000 (Table 1). The effective molarity, k_{cat}/k_{AcO-} = 660 M at 30°C, is some 50 times lower [16] than for the "remarkably efficient" reaction reported by Thorn et *al.* [4] for their best catalytic antibody 34E4 (at 20°C). Both reactions are catalyzed by carboxylate groups, and we consider that the hydrophobic microenvironment of the binding site makes a major contribution to catalysis in both cases.[5c,6] This may involve further enhancement of the basicity of the catalytic carboxylate and stabilization of the developing delocalised system in the bound transition state. Comparison of the rate of 4B2-catalyzed reaction with the rate constant for the reaction catalyzed by a common general base (Table 2) indicates that catalytic efficiency is not much different for the less reactive 6-nitro- and unsubstituted benzisoxazoles. We conclude that transition state stabilization by 4B2 changes very little for various benzisoxazole substrates. This is not unexpected in view of the poor structural analogy between the original hapten and the transition state of the catalyzed reaction. It also indicates that the antibodiy pocket is relatively open in the vicinity of the substituents and makes no steric demands on the substrate in this region.

Table 2 : Kinetic constants for the reaction of benzisoxazoles with 4B2 or a general base in water.

Benzisoxazole substrate	k_{cat}/K_M [a] ($M^{-1}.s^{-1}$)	k_{amine} [b] ($M^{-1}.s^{-1}$)	ratio
5-NO_2	39	$8.2\ 10^{-1}$	48
6-NO_2	6	$1.6\ 10^{-1}$	38
H	0.6	$1.85\ 10^{-2}$	32

[a] Rate constants were measured at 30°C in phosphate buffer pH 7.1, 0.5 % CH_3CN
[b] Data from ref. 5b with triethylamine at 30°C in 0.1 M KCl.

Interestingly, k_{cat}/K_M is very sensitive to the small amounts of acetonitrile present (some is needed to dissolve the substrate) (Table 1). k_{cat}/K_M is reduced by 90% in 10% acetonitrile, and halved by just 1% of organic cosolvent. This cannot be a bulk medium effect: the pK_a of acetic acid and the rate of the acetate-catalyzed Kemp elimination show only small changes at low concentrations of added acetonitrile.[17] A likely explanation involves solvent sorting, with the hydrophobic binding site occupied preferentially by molecules of the organic cosolvent.

We know that the design of transition state analogues has practical limitations, and that catalytic antibodies rationally designed to catalyze one reaction can in some cases catalyze another reaction also.[18] This work demonstrates that "orphan" antibodies - good natural binders rejected because they do not catalyze their specific target reaction - may yet find a catalytic role if introduced to the right reaction.

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