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Catalysis of Carbamate Hydrolysis by an Antibody

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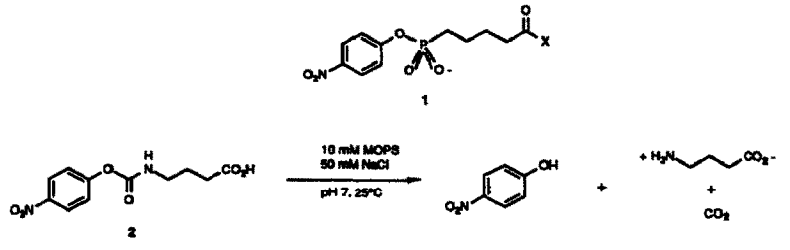
Abstract: We demonstrate that antibodies generated to a nitrophenylphosphonate hapten are able to catalyze hydrolysis of the corresponding ester 4 and carbamate 2, but not the alternative carbamate 5 or amide 6.

In order to better understand the requirements for antibody-catalyzed acyl transfer reactions antibodies generated against an aryl phosphonate transition state analog **1** were assayed for their ability to catalyze hydrolysis of the corresponding ester, carbamate and amide. The tetrahedral transition-state analog **1** has previously been shown to elicit antibodies capable of catalyzing the hydrolysis of esters and carbonates but not the energetically more demanding reaction of carbamate hydrolysis.[1] We have screened a set of 22 antibodies elicited to the KLH (keyhole limpet hemocyanin) conjugate of **1** for the ability to hydrolyze the corresponding carbamate **2** (Eq. 1).[2] Of the 22 antibodies screened, three showed the ability to catalyze the hydrolysis of carbamate 2; one of these antibodies, 33B4F11, was characterized further.



(1)

The IgG 33B4F11 was purified from ascites by affinity chromatography with protein A-sepharose. That the catalytic activity is not due to a contaminating enzyme from the ascites is supported by the fact that similarly purified antibodies from other cell lines were unable to catalyze hydrolysis of 2 and that further purification by anion exchange chromatography (Pharmacia mono-Q) led to no decrease in specific activity. In addition, antibody purified by anion exchange chromatography was shown to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining.

Hydrolysis of 2 by 640 nM 33B4F11 (10 mM MOPS, 50 mM NaCl, pH 7.0) at 25°C was shown to follow Michaelis-Menten kinetics. Reactions were initiated by the addition of 2 μL of a freshly prepared stock solution of carbamate in methanol to 0.498 mL of an antibody solution. Kinetic constants were determined by the method of initial rates by following the release of 4-nitrophenolate spectrophotometrically at 405 nm. The hydrolysis of the carbamate at pH 7 may be treated as a pseudo first order reaction with values of k_{cat} and K_m of 1.5 min^{-1} and 5.5 μM respectively (derived from Lineweaver-Burk analysis and non-linear regression). The pseudo-first order rate constant, k_{uncat}, for the background reaction (where $v = k[OH^-][S]$ and $k_{uncat} = k[OH^-]$) is 5.6×10^{-3} M^{-1} min^{-1}. Thus the rate acceleration for this reaction by IgG 33B4F11 is 260. No catalysis by the buffer was observed under similar conditions (10-40 mM MOPS, NaCl, Ionic Strength = 0.05, pH 7.0, 25°C).

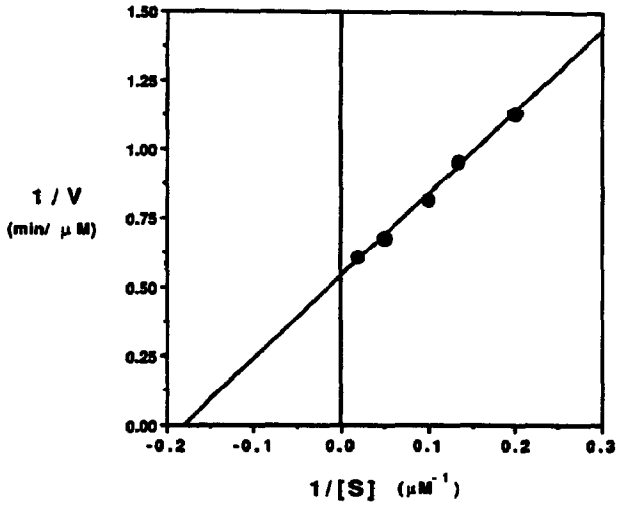


Fig. 1. Lineweaver-Burk Plot for hydrolysis of 2 by 33B4F11

Antibody 33B4F11 Hydrolysis of carbamate 2 by 33B4F11 is competitively inhibited by free hapten 1 (K_i = 100 nM) as determined by the method of Henderson.[3,4] While the reaction is inhibited effectively by the original hapten, the tetrahedral N-oxide (3, racemate) corresponding to 1 was shown to inhibit the catalytic activity of 33B4F11 only weakly (K_i not determined).[5] Antibody 33B4F11 also catalyzed the hydrolysis of 4-nitrophenyl

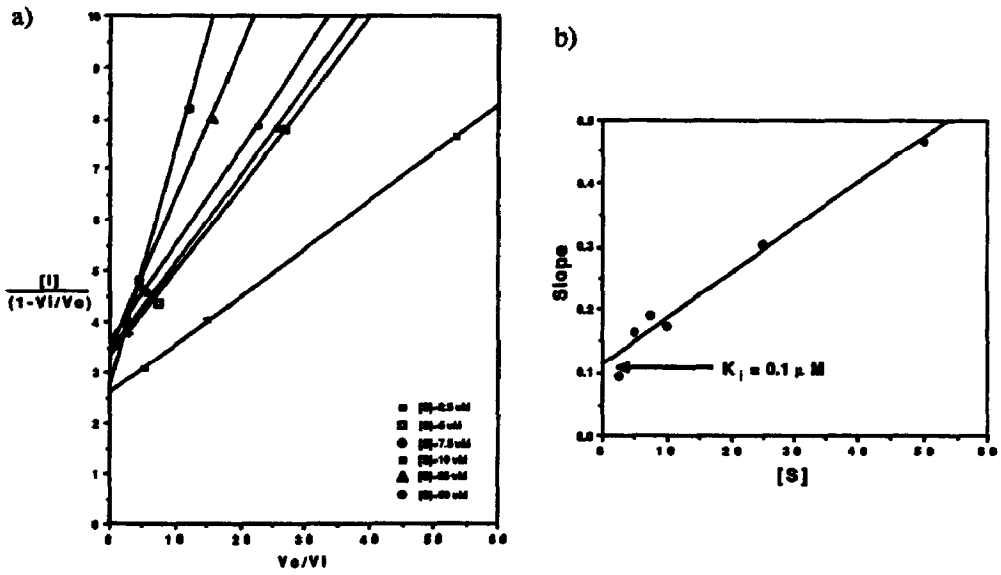


Fig. 2. Determination of K_i for free hapten 1. a) Henderson Plot, b) Replot of slopes.

acetate 4. Lineweaver-Burk analysis of initial rate data for the hydrolysis of ester 4 by 640 nM 33B4F11 (10 mM MOPS, 50 mM NaCl, pH 7.0) at 25°C gave k_{cat} = 0.4 min^{-1} and K_m = 38 μM. The rate acceleration for this reaction is 170 (Fig. 3).

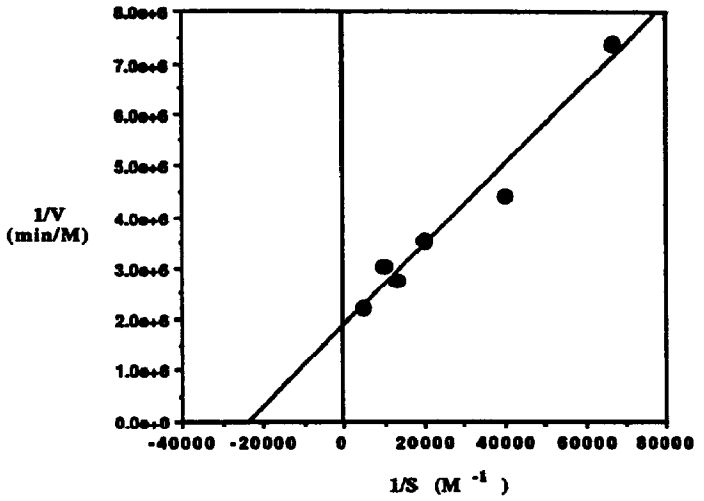
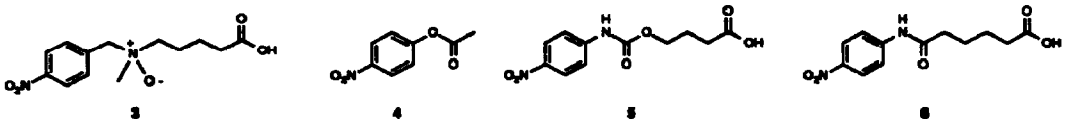


Fig. 3. Lineweaver-Burk Plot for hydrolysis of 4 by 33B4F11



3 4 5 6

From its ability to catalyze the hydrolysis of ester 4 and carbamate 2, it is clear that 33B4F11 allows expulsion of the nitrophenol leaving group (versus cleavage of the acyl-N linkage). We sought to determine whether catalytic acitivity is retained when the substrate phenolic oxygen is replaced with a nitrogen because the rate of hydrolysis is much slower for such substrates. The intrinsic hydrolytic rates for these substrates is in the order 4 ~ 2 >> 5 > 6; k_{OH} for these substrates is $2x10^{-3}$ min^{-1}, $1x10^{-3}$ min^{-1}, $1x10^{-7}$ min^{-1}, and $<<10^{-7}$ min^{-1}, respectively. When antibody 33B4F11 was assayed for its ability to catalyze the hydrolysis of the alternative carbamate 5 or the nitroanilide 6, no catalysis was observed.[6] Thus, when nitrophenol (pKa 7.14) is the leaving group catalysis is observed; when nitroanilide is the leaving group no catalysis is observed. These results suggest that the inability of antibody 33B4F11 to catalyze hydrolysis of substrate 5 and 6 may be due to the lack of a general acid to facilitate a productive breakdown of the tetrahedral intermediate. Alternatively a conformational restriction in carbamate 5 and amide 6, which is not present in the ester 4 or carbamate 2, may prevent catalysis by the antibody.

7 8

In an effort to generate antibodies which not only stabilize the tetrahedral transition state in amide bond hydrolysis, but also provide a general acid to facilitate expulsion of the leaving group, antibodies were generated against the KLH conjugate of hapten **7**.[7] The positively charged ammonium ion might be expected to generate a complementary carboxylate in the antibody binding site that could act as a general acid.[8] Of the nine monoclonal antibodies that were generated to the KLH-7 conjugate, none catalyzed the hydrolysis of the corresponding amide **8**. Although it has been reputed that one out of 44 antibodies raised against an aryl phosphonamidate hapten were found to catalyze the hydrolysis of the corresponding anilide, additional insight into the relationship between reaction mechanism, hapten structure and elicited antibodies will be required for the design of haptens which can reliably be used to elicit antibodies capable of the catalyzing the energetically demanding reaction of amide hydrolysis.[9]

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References and Notes

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2. Carbamate **2** was prepared in 7% yield by acylation of γ-aminobutyric acid under Schotten-Baumann conditions. IR (3M IR card) 3600(b), 1710(s)cm^{-1}. ^1H NMR (CD_3OD, 400 MHz) δ8.26(d, *J*=9.1Hz, 2H), 7.36(d, *J*= 9.1Hz, 2H), 3.24(t, *J*= 6.8Hz, 2H), 2.38(t, *J*= 7.4Hz, 2H), 1.85(m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ 176.9, 157.7, 155.6, 146.0, 126.0, 123.3, 41.4, 32.0, 25.9. LRMS (m/z) 269(90), 251(25), 176(30), 154(100). HRMS calcd. for $C_{11}H_{12}O_6N_2$: 269.0774. Found: 269.0776
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4. IgG concentrations were determined by from the absorbance at 280 nm. While this method was shown to be accurate by comparison with known standards (Pierce), the IgG concentration indicated from the Henderson plot (Fig. 2a) differs from that calculated from the absorbance at 280 nM. This has been observed by others: Benkovic, S.J.; Napper, A.D.; Lerner, R.A. *Proc. Nat. Acad. Sci.* **1988**, *85*, 5355.
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