Phosphate versus Phosphorothioate Haptens for the Production of Catalytic Polyclonal Antibodies

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Catalytic activity has recently been produced and characterized in polyclonal antibodies isolated from the serum of immunized animals.[1-5] Compared with the widely used monoclonal approach,[6] using polyclonal antibodies has certain advantages for catalytic studies such as being significantly faster, technically easier, and dramatically less costly. Polyclonal antibodies are also composed of the complete distribution of antibodies elicited by a hapten; thus, catalytic results obtained with polyclonals are representative of an animal's entire catalytic immune response. Studying catalysis of polyclonal antibodies is therefore well-suited to comparative studies in which general trends are sought in the catalytic activities of the entire immune responses elicited by related haptens. Herein we report a polyclonal catalytic antibody study in which the catalytic activities produced by a phosphate hapten and a phosphorothioate hapten are compared.

Numerous acyl-transfer reactions have been catalyzed by monoclonal antibodies, and in general, aryl phosphonate and phosphonamidate haptens have been used to produce these antibodies.[6] We are interested in phosphate haptens because they offer significant synthetic advantages compared with phosphonates or phosphonamidates. Previously, a myeloma-derived IgA that binds (*p*-nitrophenyl)phosphocholine was found to catalyze the hydrolysis of *p*-nitrophenyl carbonate.[7] More recently, a *p*-nitrophenyl phosphate hapten was used to produce polyclonal antibodies in sheep that catalyze *p*-nitrophenyl carbonate and *p*-nitrophenyl anilide hydrolysis.[1-3]

It has been suggested that replacing an oxygen atom with sulfur in tetrahedral haptens such as phosphates might lead to catalytic antibodies with greater catalytic activity.[8] The phosphorus–sulfur bond should be 0.5 Å longer than an analogous phosphorus–oxygen bond, possibly providing a better mimic for a more expanded transition state.

The phosphate and phosphorothioate haptens **1** and **2** were synthesized[9] in order to investigate further the catalytic activity produced by a phosphate hapten, as well the catalytic effect of a sulfur for oxygen atom substitution in related haptens. The haptens were coupled to keyhole limpet hemocyanin (KLH),[5] and each conjugate was used to immunize a different male New Zealand white rabbit. After five immunizations each 20 days apart, the polyclonal IgG antibodies were purified from sera as described.[5]

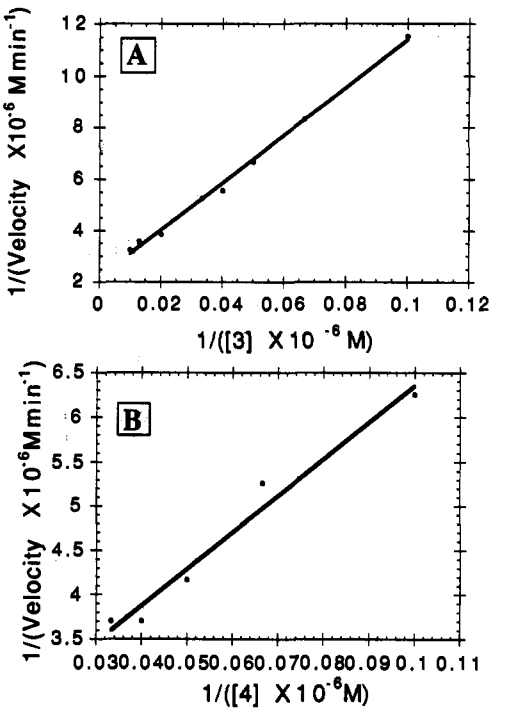
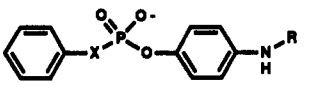
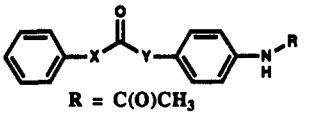


Figure 1. Representative Lineweaver–Burk plots for catalytic reactions using polyclonal antibodies elicited by haptens **1** and **2**. In these cases, total polyclonal IgG concentrations were 23 and 26 μM, respectively, and the reactions were carried out in 10 mM Tris buffer, pH 8.0, at 25 °C for 60 min and then analyzed with an HPLC assay. Fivefold lower total IgG concentrations were used in the carbonate hydrolysis experiments. Each data point is the average of two independent runs, and estimated errors are ±5%. The identity of each reaction product was verified by coinjection and spectral comparison with authentic materials. (A) Hydrolysis of ester substrate **3** catalyzed by the polyclonal antibodies raised against the phosphate hapten **1**. (B) Hydrolysis of the thioester substrate **4** catalyzed by the polyclonal antibodies raised against the phosphorothioate hapten **2**.



R = -C(O)(CH$_2$)$_4$CO$_2^-$

1 X = O
2 X = S



R = C(O)CH$_3$

3 X = O, Y = CH$_2$
4 X = S, Y = CH$_2$
5 X = O, Y = O
6 X = NH, Y = CH$_2$

The purified polyclonal antibodies were analyzed for their ability to catalyze the hydrolysis of the ester, thioester, carbonate, and amide substrates **3–6**, respectively, using an HPLC assay.[5] Initial experiments confirmed that both the phosphate-specific and phosphorothioate-specific polyclonal antibodies catalyzed the hydrolysis of substrates **3–5**, but not the amide substrate **6**. A detailed analysis was carried out with substrates **3–5**, yielding a series of linear Lineweaver–Burk plots (Figure 1). The plot linearity indicates that the catalytic behavior of each purified polyclonal antibody sample is consistent with Michaelis–Menten kinetics, despite the presumably heterogeneous distribution of catalysts present. This interesting result has been seen previously with polyclonal catalytic antibodies.[1-5]

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(9) Briefly, the phosphate (**1**) and phosphorothioate (**2**) haptens were synthesized by treatment of phosphoryl chloride with *N*-(4-hydroxyphenyl)-6-(methoxycarbonyl)pentanamide and then phenol or thiophenol, respectively, followed by water. The methyl ester was cleaved via hydrolysis with LiOH. All compounds exhibited spectroscopic characteristics consistent with the structures indicated.
(10) The actual amount of catalyst present is presumably smaller than this estimate, since some of the hapten-specific antibodies that bind hapten with high affinity in the inhibition studies are probably not catalysts.

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Table 1

hapten	substrate	app K_m (μM^{-1})	app V_{max} (μM/min)	app k_{cat} (min^{-1})	app k_{cat}/k_{uncat}
1	3	43	0.46	0.10	500
	4	50	0.44	0.096	680
	5	16	0.95	1.0	1800
2	3	149	0.37	0.071	360
	4	19	0.45	0.086	620
	5	17	0.39	0.39	680

As expected, the catalytic activity was inhibited with hapten. Quantitative inhibition studies indicated that total inhibition was achieved when enough phosphate hapten was added to bind 10 ± 1% of the antibody binding sites in the anti-phosphate polyclonal sample, and similarly when enough phosphorothioate hapten was added to bind 10 ± 1% of the antibody binding sites in the anti-phosphorothioate polyclonal sample. These values provide an estimate for the amount of hapten-specific and/or catalytic antibody in the samples[10] that can be used to calculate apparent k_{cat} values and thus apparent catalytic rate enhancement (k_{cat}/k_{uncat}). The k_{uncat} values were determined under the same conditions in the absence of antibodies. The entries in Table 1 are referred to as "apparent" values because they characterize the entire sample, rather than any individual catalyst. For comparison, the apparent rate enhancement of 1800 observed for the carbonate hydrolysis catalyzed by our anti-phosphate polyclonal antibody samples falls within the range previously observed for hydrolysis of carbonate substrates (800–10000) by catalytic monoclonal antibodies[7,11,12] as well as the range estimated for other polyclonal samples.[1,3] Catalytic rate enhancements for ester substrate hydrolysis have exceeded 10^6 with some monolconal antibodies.[13,14]

Judging from the rate enhancements listed in Table 1, the phosphate hapten **1** produced polyclonal antibodies that are more effective at hydrolyzing both ester and carbonate substrates, while the phosphate and phosphorothioate haptens produced polyclonal antibodies that are about equally effective at catalyzing the hydrolysis of the thioester substrate. Interestingly, for each sample of polyclonal antibodies, the lowest apparent K_m values are seen when the largest apparent rate enhancements occur. Furthermore, this was observed with the substrates that are most homologous to each hapten in terms of the heteroatoms present. One possible explanation for these results is that the most homologous substrates have hydrolytic transition states that are on average most complementary to the antibody binding pockets, as are the substrates themselves, but to a lesser extent.

Four pieces of evidence verified that the observed catalytic activity was the result of antibody catalysis and not some adventitious enzyme impurity or nonspecific protein effect. First, the purified IgG isolated from sera removed from the rabbits prior to immunization showed exactly the same rate as background. Second, following immunizations, the two different samples showed distinct substrate specificities consistent with the structures of the haptens. Third, the catalytic activity increased during the immunization regimen (data not shown). Finally, the catalysis was quantitatively inhibited with hapten.

In conclusion, using the new polyclonal approach, we have found an easily synthesized aryl phosphate hapten to be versatile, capable of eliciting polyclonal antibodies that are effective at hydrolyzing an ester, a thioester, and especially a carbonate substrate. Furthermore, there appears to be no significant advantage in substituting sulfur for oxygen in a phosphate hapten. Possible explanations include C–O bond stretching not being important in the transition state of the rate-determining step inside these antibodies, or if it is, perhaps a sulfur atom is not an effective mimic.[15] We are currently using the polyclonal method to carry out other comparative studies as well as to investigate details of how polyclonal antibody catalytic activity develops during an immune response.

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(15) Of course other factors such as different preferred conformations for the two haptens cannot be ruled out.