The Influence of Hapten Size and Hydrophobicity on the Catalytic Activity of Elicited Polyclonal Antibodies

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According to the classic approach, catalytic antibodies are elicited with haptens designed to mimic presumed transition states of desired reactions.[1−5] Attention is usually focused on that portion of the hapten that is meant to resemble a charge distribution and/or geometry unique to the transition state in question. Little is known about how the rest of the hapten structure and properties influence catalytic activity. Fundamental questions that have not been addressed previously concern the importance of overall hapten size and hydrophobicity. Herein is reported the first systematic investigation of hapten size and hydrophobicity in the context of the same catalytic reaction. A series of haptens were synthesized that contained aromatic groups of increasing size attached to a phosphate moiety. Samples of polyclonal antibodies elicited in mice revealed that only the two smaller and less hydrophobic haptens produced antibodies capable of catalyzing the hydrolysis of directly analogous carbonate substrates. These results emphasize that general parameters such as hapten size and hydrophobicity can have a profound and nonrandom influence on the catalytic activity of elicited antibodies.

We[6−9] and others[10−13] have been studying the catalytic activity of polyclonal antibodies isolated directly from the serum of immunized animals. Polyclonal antibodies are composed of the entire distribution of IgG antibodies present in an animal, so they provide the most comprehensive view of general trends associated with the overall catalytic immune response. Additionally, polyclonal antibodies offer significant technical advantages in terms of the time, cost, and ease of preparation.[9]

The haptens **1**−**5** (Figure 1) were synthesized and used to prepare bovine serum albumin (BSA) conjugates.[14] Both the haptens and the presumed rate-limiting transition states for the base-promoted hydrolysis of corresponding carbonate substrates share a similar negative charge and tetrahedral geometry. Consequently, antibodies specific for this motif in the haptens

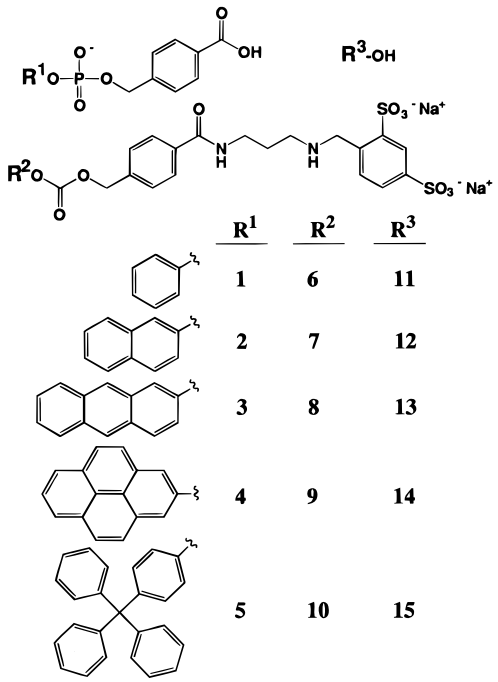

Figure 1. Structures of the phosphate haptens **1**−**5** and water-soluble carbonate substrates **6**−**10** used in the present study.

are expected to effect the catalytic hydrolysis of carbonate substrates using primarily transition state stabilization.[7−13] Each hapten−BSA conjugate was used to immunize a different set of 10 BALB/cJ mice a total of five times at 21-day intervals.[16] The use of 10 mice per hapten minimizes the risk of obtaining results that are overly biased by any individual animal. As a control, polyclonal antibodies were also isolated from 10 mice immunized five times with underivatized BSA.

The different polyclonal samples were tested for their ability to catalyze the hydrolysis of each the water-soluble carbonate substrates **6**−**10** Figure 1, (Table 1). For the catalytic samples, standard Lineweaver-Burk analyses were used to determine apparent K_M and V_{max} values. The fraction of high-affinity, hapten-specific antibodies present was measured by carrying out quantitative inhibition studies with hapten. These fractions were used as an estimate of catalyst concentration to calculate values of apparent k_{cat} and apparent k_{cat}/k_{uncat} for the different samples.[17] Only the smallest hapten **1**, and to a lesser extent the next-smallest hapten **2**, elicited active polyclonal catalysts. The larger haptens **3**−**5** failed to elicit any detectable catalytic activity. The apparent k_{cat}/k_{uncat} values of 4300 and 1000 seen for the anti-**1** and anti-**2** antibodies reacting with the directly analogous substrates **6** and **7**, respectively, are in line with the values of 10^3−10^4 observed with catalytic murine monoclonal antibodies raised against phosphate or phosphonate haptens of similar size.[18−21] A lower level of catalytic activity was also

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(14) Hapten−BSA ratios were determined to be in the range of 11:1 to 13:1 for the five conjugates.[15]
(15) These values were determined spectrophotometrically using the method described in the following: Habeeb, A. F. S. A. *Anal. Biochem.* **1966**, *14*, 328−332.
(16) Ten five-week old, male BALB/cJ mice were used for each hapten. The serum was removed from each mouse 10 days after the final immunization. The serum from each set of 10 mice immunized with the same hapten was pooled, and the polyclonal antibodies were purified using a previously described, protein G-based procedure.[7−9] Ten mice were used for each hapten to ensure that enough polyclonal antibody, ∼10 mg, was generated to carry out the required number of catalytic assays.
(17) A number of pieces of evidence verified that the observed catalysis was due to the elicited antibodies and not contaminating enzymes. First, the control polyclonal antibodies isolated after five immunizations of 10 mice with underivatized BSA displayed no catalytic activity with any of the substrates. In addition, the observed substrate selectivity is consistent with the expected programmability of antibody catalysis. Finally, the catalytic activity that was observed was quantitatively inhibited in a *specific* way with hapten. Haptens **1** and **2** were analyzed for their ability to inhibit catalysis by both the anti-**1** and anti-**2** polyclonal samples reacting with their directly analogous substrates. Only the same hapten used to elicit the antibodies effectively inhibited the reaction when as much as a 5-fold excess was used.

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Table 1. Immunological and Catalytic Parameters Determined for the Polyclonal Antibodies Isolated from Immunized Mice[a]

	hapten used to elicit polyclonal antibodies[a]				
	1[h]	**2**[i]	**3**[j]	**4**[k]	**5**[l]
serum titer[b]	1:4000	1:4800	1:1500	1:3200	1:1800
apparent hapten K_d (μM)[c]	6.9	13	0.048	0.0057	1.9
apparent substrate K_d (μM)[c]	nm[m]	nm[m]	22	2.1	>500[n]
apparent alcohol K_d (μM)[c]	>10000[o]	>5000[o]	200	6.4	nm[p]
apparent V_{max} (μM/min)[d]	0.53	0.32	nd	nd	nd
apparent K_M (μM)[d]	89	61	nd	nd	nd
hapten-specific antibody[e] total IgG	0.18	0.20	nd	nd	nd
apparent k_{cat} (min^{-1})[f]	0.21	0.090	nd	nd	nd
apparent k_{cat}/k_{uncat}[g]	4300	1000	nd	nd	nd

[a] nm, not measured; nd, none detected. [b] Measured by standard ELISA. [c] Measured by competition ELISA.[23] [d] Determined by Lineweaver-Burk analysis. [e] Determined by quantitative inhibition studies.[6−8] [f] Calculated as apparent V_{max} divided by the amount of high-affinity, hapten-specific antibody binding sites in the reactions. [g] The values for k_{uncat} were determined under identical buffer conditions in the absence of added antibody. The k_{uncat} values for **6** and **7** were 4.9×10^{-5} and 8.7×10^{-5} min^{-1}, respectively. [h] The catalytic parameters listed are for catalytic reactions using the carbonate substrate **6**, and affinity measurements used hapten **1** and alcohol **11**, respectively. [i] The catalytic parameters listed are for catalytic reactions using the carbonate substrate **7**, and affinity measurements used hapten **2** and alcohol **12**. [j] The affinity measurements listed utilized hapten **3,** substrate **8**, and alcohol **13**. [k] The affinity measurements listed utilized hapten **4**, substrate **9**, and alcohol **14**. [l] The affinity measurements listed utilized hapten **5**, substrate **10**, and alcohol **15**. [m] These substrate affinities were not measured by competition ELISA because of presumed interference by the catalytic reaction. [n] The dissociation constant for substrate **10** was at least higher than 500 μM but could not be measured exactly due to the limit of solubility (~500 μM) of this molecule in aqueous solution. [o] There was no observed competition up to the highest concentration of alcohol examined, 10 and 5 mM, respectively. [l] The alcohol **15** has such limited solubility in aqueous solution that no meaningful competition measurement was feasible.

detected when the phenyl substrate **6** reacted with the antibodies elicited by the naphthyl hapten **2**, exhibiting apparent K_M and apparent k_{cat}/k_{uncat} values of 140 μM and 160, respectively. No other combination of substrate and antibodies exhibited catalytic activity.

The results of this study indicate that there is a nonrandom decrease in the catalytic activity of elicited antibodies as the size of the hapten is increased. If it is assumed that the observed antibody catalysis is derived from transition state stabilization, then the catalytic activity should primarily result from antibody binding interactions elicited by the phosphate moiety of the hapten.[20,22] A reasonable hypothesis that explains the catalytic data in Table 1 is that for haptens **3**−**5**, the antibody binding energy is focused too much on the aromatic portions of the hapten and not enough on the phosphate groups. In the case of haptens **1** and **2** that produced catalytic activity, the antibody binding energy must be split more evenly between the phosphate and aromatic groups, with the larger hapten **2** eliciting antibodies with generally lower phosphate binding.

The hypothesis that binding energy is focused on the aromatic groups of the larger haptens is supported by the affinity data in Table 1.[23] Importantly, significant hapten affinities were elicited by all the haptens, so the absence of catalytic activity in the samples elicited by **3**−**5** cannot be ascribed to a lack of immunogenicity. Affinity for hapten increased along with the size of the planar aromatic group in the series of antibodies elicited by the haptens **1**−**4**. The antibodies elicited by the hapten with the nonplanar aromatic group **5** also showed an affinity for hapten within this range. Indeed, the noncatalytic polyclonal samples elicited by **3** and **4** can even bind their respective substrates **8** and **9** with high affinity (Table 1), but there is simply no catalysis. This substrate binding by the noncatalytic samples does appear to be largely focused on the large aromatic groups, since high affinities for the directly analogous alcohols **13** and **14** were also observed[24] (Table 1). In contrast, the catalytic polyclonal samples elicited by **1** and **2** exhibited no strong binding to the directly analogous alcohols **11** and **12**, respectively.

There are two attributes of the aromatic groups in **3**−**5** that may be operating alone or in concert to dominate binding interactions in the elicited antibodies: size and hydrophobicity. It is possible that the dimensions of **3**−**5** have exceeded some inherent size limit of catalytic antibody binding pockets, even though crystallographic studies indicate that antibody binding pockets should be generally large enough (30 Å × 20 Å × 10 Å) to accommodate all of the haptens in at least some orientation.[25] Another reasonable explanation for the catalytic results is that the immune responses to **3**−**5** diverted binding energy away from the phosphate group because of the increasing hydrophobicity of the larger haptens. Within the available immunological repertoire, perhaps a higher overall affinity could be achieved by removing the larger hydrophobic aromatic groups from aqueous solution and binding them in a completely hydrophobic pocket.[26]

The present study has verified by experiment that structural features of a hapten not associated with resemblance to a presumed transition state can impact the catalytic activity of elicited antibodies. In particular, the larger, more hydrophobic haptens elicited antibodies that focused more binding energy on the large hydrophobic groups, thereby presumably precluding transition state stabilization and thus catalysis. Studies of this type can provide important details about how the murine immune system responds to complex haptens. Such information will be important in the design of future haptens intended to elicit better catalytic antibodies. In addition, the present results serve to illustrate the value of comparative polyclonal studies for uncovering general trends in the catalytic immune response. Future efforts will be directed toward systematically probing other aspects of hapten structure, such as hydrophilicity, conformational flexibility, and stereochemistry.

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Supporting Information Available: Synthetic and experimental details; Lineweaver-Burk and quantitative inhibition plots (13 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(21) In fact, the vast majority of catalytic antibody studies have utilized haptens that fall in the size range represented by **1** and **2**.

(22) Similar ideas have been expressed in work involving minimal or attenuated haptens or substrates. See, for example, ref 20 above and the following: Janda, K. J.; Benkovic, S. J.; McLeod, D. A.; Schloeder, D. M.; Lerner, R. A. *Tetrahedron* **1991**, *47*, 2503−2506.

(23) Absolute affinities can sometimes be underestimated by the competition ELISA method used here. On the other hand, the relative order of determined affinities is accurate. Butler, J. E. In *The Structure of Antigens, Vol. I*; Van Regenmortel, M. H. V., Ed.; CRC Press: Boca Raton, FL, 1992; pp 209−259.

(24) Analysis of the antibodies elicited with hapten **5** was complicated by the lower solubilities of substrate **10** and especially the alcohol **15**. Nevertheless, it is significant that the antibodies elicited by hapten **5** failed to exhibit catalytic activity, so these data were included in the table.

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(26) Of course, other size-dependent molecular properties of the haptens, such as conformational differences, cannot be ruled out as contributing to the catalytic activity, or lack thereof, of elicited antibodies.