Catalytic Antibodies Generated via Homologous and Heterologous Immunization†

Takeshi Tsumuraya,‡ Hiroaki Suga,§,⊥ Shinichi Meguro,‡ Astuko Tsunakawa,‡ and Satoru Masamune*,§

Contribution from Kao Institute for Fundamental Research, Tochigi, Japan, and the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received May 22, 1995⊗

Abstract: Two different immunization protocols using haptens **1** and **2** have been employed for the generation of catalytic antibodies capable of hydrolyzing ester **3**. Three successive injections with one hapten **1** or **2**, a protocol referred to as homologous immunization, provided hydrolytic antibodies with a rate acceleration in the range of 10^3-10^4. These antibodies exhibited inhibitory activity only by the hapten used for the immunization. On the other hand, two successive injections with hapten **1** followed by a boost with hapten **2**, a protocol referred to as heterologous immunization, induced catalytic antibodies with a rate acceleration up to 1.5×10^5. The majority of these catalytic antibodies possessed cross-reactivities to haptens **1** and **2**, and the catalytic activities were effectively inhibited by both haptens. Control experiments have suggested that catalytic antibodies via heterologous immunization are derived through the unique stimulation of **1**-primed memory B-cells by the secondary hapten **2**, but not through the primary response of virgin B-cells by **2**. Two catalytic antibodies H2-23 and H5H2-42, generated via homologous immunization with **2** and heterologous immunization with haptens **1** and **2**, respectively, were selected for the detailed kinetic studies. Antibody H2-23 showed burst kinetic behavior and the burst phase was eliminated by the addition of *p*-nitrophenolate **5**. Antibody H5H2-42 has no burst phase and exhibited high multiple turnover activity. The pH-dependent kinetic characterization of H5H2-42 suggested that bifunctional catalytic residues in the antibody combining site likely exist in the active site. These results imply that the heterologous immunization strategy offers a potential means of introducing multiple catalytic residues into antibody combining sites without recourse to complicated synthesis of multifunctional haptens.

Introduction

Antibody catalysis represents one of the most exciting, rapidly growing interdisciplinary fields. Immunization of animals with appropriately designed haptens induces antibodies capable of catalyzing a selected reaction. The haptens utilized are analogous to the putative transition state of the reaction in terms of charge distribution and geometry.[1] While major successes have been recorded in antibody catalyzed esterolysis,[2-5] rate accelerations, especially for the cleavage of the amide bond, have been modest compared with natural enzymes.[4] Clearly, further rate enhancement is an important and challenging task in order for catalytic antibodies to be of practical use.

Our preliminary work on the generation of esterolytic antibodies has revealed that efficient catalysis can be achieved with the aid of acidic and basic amino acid residues appropriately disposed in the antibody combining site, in a manner similar to that of aspartic proteases.[6] One possible strategy to generate both catalytic residues might be the use of a zwitterionic hapten incorporating both positively and negatively charged groups. Unfortunately, the design and synthesis of such a bifunctional zwitterionic hapten are often not a simple task and obviously more difficult than those of monofunctional haptens generally used for the preparation of catalytic antibodies. As an alternative, we have devised a new strategy called heterologous immunization wherein an animal is immunized in succession with two different but structurally related haptens.[7] Upon heterologous immunization, the host may respond cross-

† Taken in part from the Ph.D. dissertation of Hiroaki Suga, 1994, Massachusetts Institute of Technology.

‡ Kao Institute for Fundamental Research.

§ Massachusetts Institute of Technology.

⊥ Present address: Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114.

⊗ Abstract published in *Advance ACS Abstracts*, November 1, 1995.

(1) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659.

(2) (a) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1556. (b) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, *234*, 1570.

(3) (a) Tramontano, A.; Amman, A. A.; Lerner, L. A. *J. Am. Chem. Soc.* **1988**, *110*, 2282. (b) Jacobs, J. W.; Schultz, P. G.; Sugasawara, R.; Powell, M. *J. Am. Chem. Soc.* **1987**, *109*, 2174. (c) Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961.

(4) (a) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science*, **1988**, *241*, 1188. (b) Benkovic, S. J.; Adams, J. A.; Borders, C. L., Jr.; Janda, K. D.; Lerner, R. A. *Science* **1990**, *250*, 1135. (c) Gibbs, R. A.; Benkovic, P. A.; Janda, K. D.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1992**, *114*, 3528. (d) Jack Chen, Y.-C.; Danon, T.; Sastry, L.; Mubaraki, M.; Janda, K. D.; Lerner, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 357. (e) Martin, M. T.; Angeles, T. S.; Sugasawara, R.; Aman, N. I.; Napper, A. D.; Darsley, M. J.; Sanchez, R. I.; Booth, P.; Titmas, R. C. *J. Am. Chem. Soc.* **1994**, *116*, 6508.

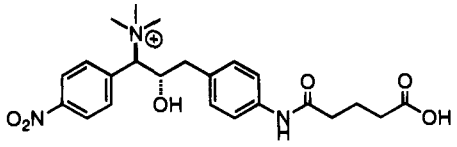
(5) (a) Martin, M. T.; Napper, A. D.; Schultz, P. G.; Ress, A. R. *Biochemistry* **1991**, *30*, 9757. (b) Landry, D. W.; Zhao, K.; Yang, G. X.-Q.; Glickman, M.; Georgiadis, T. M. *Science* **1993**, *259*, 1899. (c) Janda, K. D.; Ashley, J. A.; Jones, T. M.; McLeod, D. A.; Schloeder, D. M.; Weinhouse, M. I.; Lerner, R. A.; Gibbs, R. A.; Benkovic, P. A.; Hilhorst, R.; Benkovic, S. J. *J. Am. Chem. Soc.* **1991**, *113*, 291. (d) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science* **1989**, *244*, 437. (e) Tawfik, D. S.; Zemel, R. R.; Arad-Yellin, R.; Green, B. S.; Chap, R.; Sela, M.; Eshhar, Z. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 373. (f) Miyashita, H.; Karaki, Y.; Kikuchi, M.; Fujii, I. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5337. (g) Iwabuchi, Y.; Miyashita, H.; Tanimura, R.; Kinoshita, K.; Kikuchi, M.; Fujii, I. *J. Am. Chem. Soc.* **1994**, *116*, 771. (h) Miyashita, H.; Hara, T.; Tanimura, R.; Tanaka, F.; Kikuchi, M.; Fujii, I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6045. (i) Fujii, I.; Tanaka, F.; Miyashita, H.; Tanimura, R.; Kinoshita, K. *J. Am. Chem. Soc.* **1995**, *117*, 6199. (j) Zhou, G. W.; Guo, J.; Huang, W.; Fletterick, R. J.; Scanlan, T. S. *Science* **1994**, *265*, 1059.

(6) Suga, H.; Ersoy, O.; Tsumuraya, T.; Lee, J.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 487.

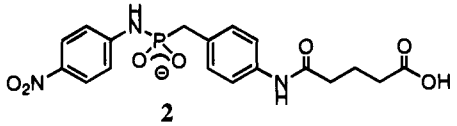
(7) (a) Suga, H.; Ersoy, O.; Williams, S. F.; Tsumuraya, T.; Margolies, M. N.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 6025. (b) Suga, H.; Ersoy, O.; Williams, S. F.; Tsumuraya, T.; Margolies, M. N.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 8432.

0002-7863/95/1517-11390$09.00/0

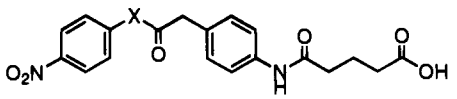
Chart 1



1



2



3 X = O
4 X = NH

reactively to the secondary hapten and produce antibodies which have affinity for the primary hapten, the secondary hapten, or both haptens.[8] When two haptens individually contain either a positive or a negative charge incorporated in the structure of a transition-state analog, the heterologous immunization of this hapten pair provides an opportunity to simultaneously generate an acidic and a basic catalytic residue in the antibody combining site. This article summarizes the outcome of this strategy utilizing a pair of haptens **1** and **2** (Chart 1), as compared with that of the standard homologous immunization. The catalyzed reactions are the hydrolysis of **3** (and **4**) as shown in reaction 1, and pertinent kinetic and thermodynamic data are critically evaluated.

Results and Discussion

Hapten Design and Synthesis. In the context of heterologous immunization to generate multiple catalytic residues in the antibody combining sites, the design of two functionally different haptens is of prime importance. These haptens must share a common chemical structure to cross-reactively induce the immune response, but each must also have a positive or negative haptenic charge. An appropriate isosteric tetrahedral geometry (in lieu of the carboxylic ester/amide moiety to be hydrolyzed) is an additional requirement. We have selected two structurally related haptens **1** and **2** with the goal of generating antibody catalysts to hydrolyze ester **3** and amide **4**. Both haptens share common structural features with **3** and **4** differing only in the moiety containing the scissile bond present in these substrates. Therefore, analogous pairs of haptens may be widely applicable to the hydrolytic cleavage of many other esters and amides.

Hapten **1** contains a 1,2-amino alcohol functionality and was designed to have the following two features: (1) the quaternized ammonium ion to generate a basic residue in the antibody combining site and also to create an empty cavity for the incoming water molecule, and (2) the alcohol moiety to represent a tetrahedral geometry in analogy to the transition state.[6] The phosphonamidate hapten **2** was originally designed by Lerner *et al.* and applied to generate catalytic antibodies for the hydrolysis of **3** and **4**.[4] The negative charge of the phosphonamidate provides an opportunity to induce an acidic residue in the antibody capable of stabilizing the oxyanionic transition state. The combination of these haptenic features by means of heterologous immunization would likely introduce both basic and acidic catalytic residues into the antibody combining site without recourse to a complex bifunctional hapten synthesis.

Syntheses of **1** and **2** have been described in the literature.[4a,6] A conventional *N*-hyroxysuccinimide (NHS) activated ester was chosen to couple the glutaric acid linker of haptens **1** and **2** with lysine residues of a carrier protein.[9]

Homologous and Heterologous Immunization. The NHS esters of haptens **1** and **2** were individually coupled with bovine serum albumin (BSA) and bovine plasma fibrinogen (Fib). The protein conjugates BSA-**1** and BSA-**2** were used for immunization, and Fib-**1** and Fib-**2** were used for enzyme-linked immunosorbent assay (ELISA).

Two different immunization protocols have been followed. In the first, an animal was immunized with three successive injections of BSA-**1** or BSA-**2** (homologous immunization). In the second, an animal was immunized with two injections of BSA-**1** followed by an injection of BSA-**2** (heterologous immunization). Comparison of the antibodies obtained by these two immunization protocols should reveal distinctive effects of these immunization strategies upon the generation of catalysts.

Four Balb/c mice each received a subcutaneous injection of 100 μg of BSA-**1** emulsified in RIBI adjuvant (MPL and TDM emulsion), and the same injection of BSA-**1** was given 2 weeks later. Seven days following the second injection, the induction of immunogloburin G (IgG) in anti-serum was analyzed by ELISA. In each case high titers to Fib-**1** were observed (>12800:1) whereas the IgGs in the anti-serum were poorly cross-reactive to Fib-**2** (800:1). Similarly, the same protocol employing BSA-**2** induced a strong immune response, and the anti-serum titers indicated that IgGs were specific to only Fib-**2** (>12800:1) but not Fib-**1** (background level). These results indicated that the immunization with a single hapten **1** or **2** was only capable of producing antibodies specific to the hapten used for immunization.

In the case of homologous immunization, 3 weeks after the second injection, a mouse was boosted with the same antigen as used for the 1st and 2nd immunizations (BSA-**1** or BSA-**2**) using the same procedure. In the case of heterologous immunization, seven weeks after the second injection with BSA-**1** a mouse received a subcutaneous injection of 100 μg of BSA-**2** emulsified in RIBI adjuvant.

Antibody Production and Purification. Three days following the last injection, the spleens were harvested and the cells were fused with 5×10^7 P3X63-Ag8.653 myeloma cells to prepare hybridomas.[10] After 2 weeks, the hybridomas that produced IgGs binding to Fib-**1** and Fib-**2** were selected by means of ELISA. These hybridomas were subcloned two or three times to obtain monoclonal cell lines. Homologous immunization with hapten **1** provided 48 individual hybridomas, and homologous immunization with hapten **2** also gave 48 hybridomas. In both cases of the homologous immunization, monoclonal antibodies produced from the individual hybridomas were specific to only the hapten used for the immunization (*vide infra*). Heterologous immunization afforded 50 monoclonal hybridomas, producing antibodies with varied affinities for **1** and/or **2** (*vide infra*). All cell lines were individually injected into pristane-primed Balb/c mice to generate ascites, and the

(8) Our idea of heterologous immunization was inspired by the following papers concerning the "original antigenic sin". (a) Eisen, J.; Little, R. J.; Steiner, L. A.; Simms, E. S.; Gray, W. *Isr. J. Med. Sci.* **1969**, *5*, 338. (b) Fazekas de St. Groth, S.; Webser, R. G. *J. Exp. Med.* **1966**, *124*, 347. (c) Davenport, F. M.; Hennesy, A. V.; Francis, T. *J. Exp. Med.* **1953**, *98*, 641. (d) Levin, H. A.; Herrman, J.; Levine, H.; Schlaossman, S. F. *J. Immunol.* **1972**, *108*, 538. (e) Klinman, N. R.; Press, J. L.; Segal, G. P. *J. Exp. Med.* **1973**, *138*, 1276. (f) Dentsch, S.; Bussard, A. E. *Eur. J. Immunol.* **1972**, *2*, 374. (g) Deutsh, S.; Vinit, M.-A.; Bussard, A. E. *Eur. J. Immunol.* **1973**, *3*, 1235. (h) Fish, S.; Senowich, E.; Fleming, M.; Manser, T. *J. Exp. Med.* **1989**, *170*, 1191.

(9) Erlanger, B. F. *Methods Enzymol.* **1980**, *70*, 85.
(10) Köhler, G.; Milstein, C. *Nature* **1975**, *256*, 495.

harvested ascitic fluid was treated with a saturated ammonium sulfate (AS) solution to give a final concentration of 45% AS. The precipitated antibodies were purified by anion exchange and Protein-G affinity chromatography as previously described.[6,7]

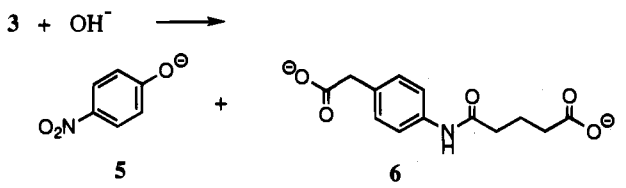
Control Experiments. To confirm the reproducibility of the heterologous immunization protocol, the protocol identical to that described above was followed with two additional mice and their anti-serum titers to haptens **1** and **2** seven days after the last (third) injection with BSA-**2** were measured. In both cases, high titers to both Fib-**1** (>12800:1) and Fib-**2** (>12800:1) were observed. These results demonstrated that the cross-immune response elicited by the heterologous immunization protocol and the generation of cross-reactive antibodies in antisera were reproducible.

Subsequently, the production of monoclonal antibodies was examined by following the *single* immunization protocol with **2**, where an animal was immunized with BSA-**2** and at day 3 hybridomas were prepared (two mice were immunized). This experiment was designed to reveal whether or not the antibody production observed in heterologous immunization was simply the result of the primary immune response by **2**. A total of 11 hybridomas producing positive binders to Fib-**2** were cloned. All of the antibodies exhibited very weak affinity to **2** and were found to be IgMs, the class typically observed in the primary immune response. This result was expected and contrasts with the observation that a large number of IgGs were obtained in heterologous immunization. These experiments indicate that the production of antibodies via heterologous immunization is not likely the result of the stimulation of naive B-cells by hapten **2**, but probably results from the unique stimulation of **1**-primed memory B-cells to produce a large population of IgG molecules binding strongly to one or both haptens **1** and **2**.[8h,11]

In addition, heterologous immunization was carried out reversing the order of haptens, where a mouse was first immunized with BSA-**2** twice followed by BSA-**1**. Although five mice were immunized and spleen cells were harvested to fuse with myeloma cells, only two fusions successfully provided a total of 8 monoclones producing positive binders to the haptens. Interestingly, all antibodies were found to be IgMs, indicating that the production of the antibodies was a result of the primary immune response to BSA-**1**. Thus, mice cross-reactively responded to haptens **1** and **2** only when the heterologous immunization was executed in the specific sequence of **1**, **1**, **2** but not that of **2**, **2**, **1**. It is not clear at the present time why the order of hapten injections is so critical. However, we tentatively assume that the size of the *secondary* hapten may play a role in the induction of the cross-immune response. While hapten **1**, which contains the sterically large trimethylammonium group and is used as the secondary hapten, may fail to stimulate the **2**-primed memory B-cells, the memory B-cells induced to hapten **1** may be able to accept the *sterically smaller* hapten **2** as a cross-reactive hapten. Further studies of heterologous immunization with various haptens carrying different functional and steric groups will increase our understanding of the factors for the induction of cross-immune response.

Thermodynamic and Kinetic Properties of Antibodies. To survey the general trend of antibody binding specificity, the dissociation constants (K_d) of all monoclonal antibodies generated were determined by competition ELISA[6,12,13] (Figure 1, Panel I). As expected, the antibodies induced by homologous immunization with hapten **1** have strong affinities to **1** while only five antibodies among them bind weakly to **2** (Figure 1A, Panel I). The antibodies homologously induced to hapten **2** were found to be exclusively specific to **2**; none of them showed affinity to **1** (Figure 1B, Panel I). On the other hand, antibodies raised by heterologous immunization exhibited diverse binding behavior to haptens **1** and **2** (Figure 1C, Panel I).[14] This result indicates that the heterologous immunization protocol provides a potential means of expanding the repertoire of antibody combining sites and the capability of generating more than one functionality.

The antibodies ([Ab] = 5.0 or 10 μM) were screened for their ability to hydrolyze the ester substrate **3** ([**3**] = 1.0 or 2.0 mM) in a buffered solution at three different pH's (50 mM Mes, 80 μM NaCl, pH 6.2; 50 mM KPi or 50 mM Pipes, 80 μM NaCl, pH 7.0; 50 mM Tris·HCl or 50 mM Hepes, 80 μM NaCl, pH 7.8) containing 5% organic cosolvent (4.8% acetonitrile and 0.2% dimethyl sulfoxide). The production of the *p*-nitrophenolate ion **5** (absorbance observed at 405 nm) was monitored.



The number of catalytic antibodies was 7 out of 48 monoclonal antibodies raised by homologous immunization with **1**, 17 out of 48 antibodies raised by homologous immunization with **2**, and 19 out of 50 antibodies raised by heterologous immunization. It is significant that, although the catalytic antibodies generated by homologous immunization of hapten **1** or **2** are highly specific only to the hapten used for immunization (Figure 1A and 1B, Panel II), the majority of catalytic antibodies obtained by heterologous immunization possessed cross-reactivities to both haptens **1** and **2** (Figure 1C, Panel II).

The Michaelis-Menten parameters (k_{cat}, catalytic rate constant; K_m, Michaelis−Menten constant) of representative catalytic antibodies obtained by three different immunization protocols are shown in Table 1. The rate accelerations (k_{cat}/k_{un}[15] where k_{un} is the zero-buffer background hydrolysis rate constant of ester **3**) of catalytic antibodies obtained by homologous immunization were found to be 10^3-10^4. All of these catalytic antibodies were strongly inhibited (the observed K_i's were less than 5 μM) by the addition of the hapten used in immunization whereas none of the catalysts were inhibited by the addition of the other hapten.[16] Although no significant product inhibition was observed in catalytic antibodies obtained by homologous immunization with hapten **1**, 12 out of 17 catalytic antibodies

(11) (a) Berek, C.; Milstein, C. *Immunol. Rev.* **1987**, *96*, 23. (b) Berek, C.; Milstein, C. *Immunol. Rev.* **1988**, *105*, 5.

(12) A slightly modified procedure of competition ELISA was used to determine the K_d's. For the original report of competition ELISA, see: Fringuet, B.; Chaffotle, A. F.; Djaudi-okaniance, C.; Goldberg, M. E. *Immunol. Methods* **1985**, *77*, 305. See also Experimental Section.

(13) In our procedure, the observed K_d is not necessarily the absolute K_d of an antibody with an inhibitor because of the short equilibration time (30 min, see Experimental Section). However, K_d values of most antibodies determined for the respective inhibitors after equilibration for 1 h were similar to those obtained after 30 min. Since the K_d's were measured under identical conditions in all experiments, these values should be useful for comparison.

(14) The distributions of monoclonal antibodies in Figure 1 might not necessarily represent those of antibodies originally induced *in vivo*.

(15) Under the conditions for the initial screening ([Ab] = 5.0 or 10 μM and [**3**] = 1.0 or 2.0 mM), it could be assumed that the active site of antibodies was saturated with substrate **3**. The observed catalytic constant k_{obs} ($=V_{obs}$ /[Ab]) then should approximate the kinetic catalytic constant k_{cat}, giving the rate acceleration (k_{cat}/k_{un}) by k_{obs}/k_{un}.

(16) Since the kinetic experiments to determine the K_i's for these antibodies were carried out under the conditions that the antibody concentration was near K_i values, the determined values might not be accurate. However, these values are useful to compare with those obtained for the antibodies H5H2-42 and H5H2-187.

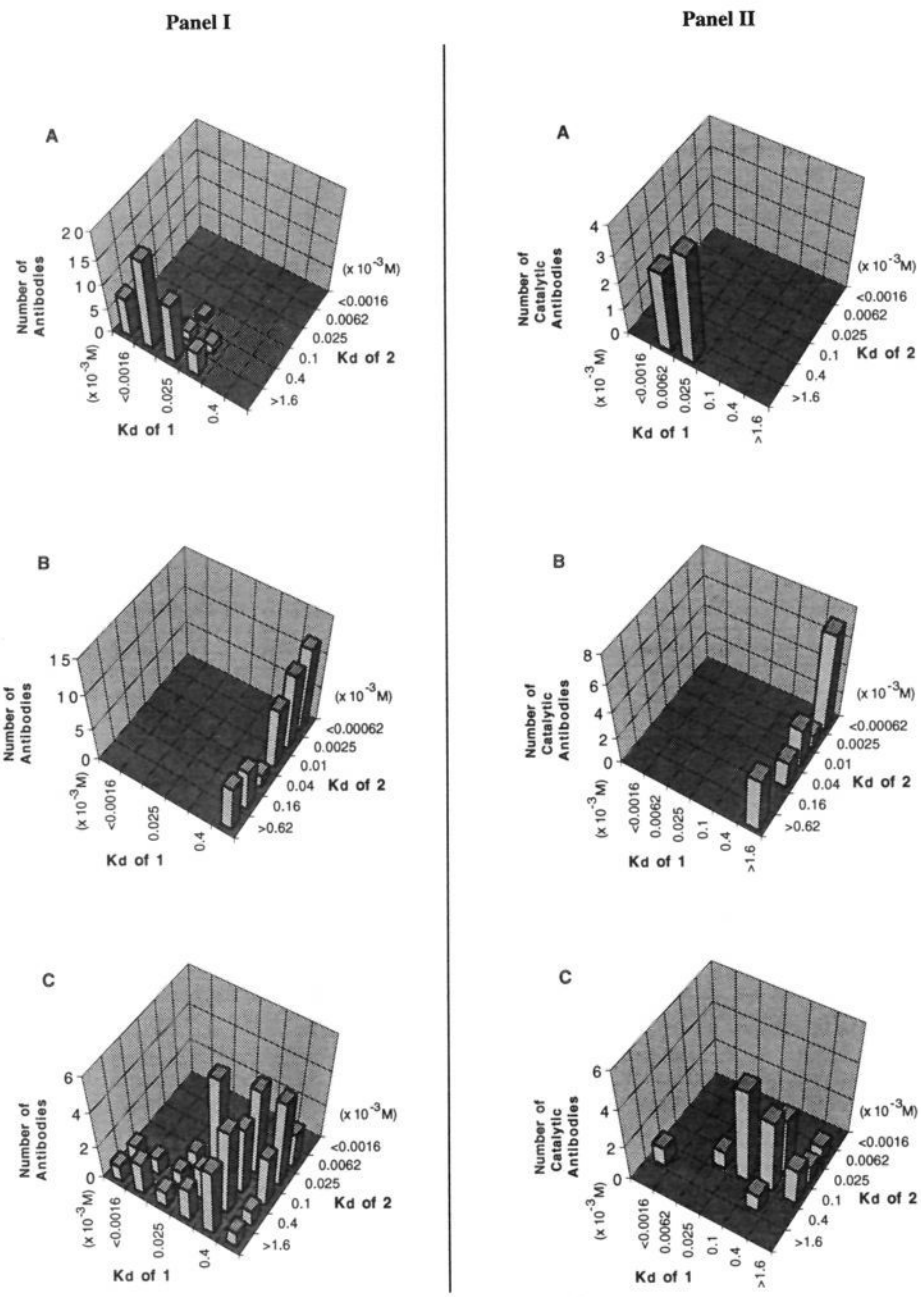


Figure 1. Distributions of antibodies (Panel I) and catalytic antibodies (Panel II), generated by homologous immunization with **1** (A), homologous immunization with 2 (B), and heterologous immunization with **1** and **2** (C), versus the dissociation constants (K_d) for haptens **1** and **2**. The K_d for each hapten was determined by competition ELISA.

induced homologously to hapten **2** showed strong product inhibition by *p*-nitrophenolate **5** (see also the following section).

In the case of heterologous immunization, 9 out of 19 catalytic antibodies showed significantly higher catalytic activities ($k_{cat}/k_{un} > 10^4$)[15] than those derived from the homologous immunization protocol. Interestingly, the catalytic activities of these antibodies were effectively inhibited by both haptens indicating that all of the significant catalysts are cross-reactive to the haptens, although the K_i's are not as small as those observed in catalytic antibodies generated via homologous immunization. Furthermore, despite differences in catalytic activity between the antibodies derived by homologous immunization with **1** and those derived by heterologous immunization with **1** and **2** (Table 1), the dissociation constants of the amide substrate **4** and the products **5** and **6** were similar (data not shown).[7] These observations imply that heterologous immunization did not alter the ability of the antibodies to recognize the substrate and its components (ground state molecules), but instead greatly enhanced their catalytic efficiencies, presumably by the participation of additional functional residues. It is also noted that none of the cross-reactive catalytic antibodies exhibited product inhibition in contrast to the catalysts isolated by homologous immunization with **2**, indicating that catalytic functions of the former antibodies could be different from the latter antibodies (see also the following section).

Further Kinetic Characterization of Antibodies. We further studied two significant catalytic antibodies, H2-23 and H5H2-42, generated via homologous immunization with hapten **2** and heterologous immunization with **1** and **2**, respectively.

H2-23. A large population of catalytic antibodies (70% of the total catalytic antibodies) induced homologously to hapten **2** showed burst kinetic behavior. One of such antibodies with high catalytic activity, H2-23, was investigated in detail. Antibody H2-23 exhibited a characteristic multiple-turnover pre-

Table 1. Dissociation Constants and Michaels–Menten Parameters of Catalytic Antibodies[a]

catalytic antibody	haptens	immunization	K_d for **1** (10^{-6} M)	K_d for **2** (10^{-6} M)	k_{cat} (min^{-1})	K_m (μM)	K_i for **1** (μM)	K_i for **2** (μM)	k_{cat}/k_{un}[d] (10^3)	product inhibition
H5-67	**1**	homologous	16	>2000	0.48 (0.11[b])	970	4[e]	nd[c]	1.6 (1.5[b])	no
H5-25	**1**		1.7	>2000	0.79	1430	5[e]	nd[c]	2.7	no
H2-23	**2**	homologous	>2000	0.16	3.2 (0.58[b])	160	nd[c]	4.6[e]	11 (7.8[b])	yes
H2-21	**2**		>2000	0.10	0.46	14	nd[c]	0.3[e]	1.5	no
H5H2-42	**1/1/2**	heterologous	350	31	12.5 (11.0[b])	240	300[f]	22[f]	68(149[b])	no
H5H2-187	**1/1/2**		120	85	3.5	820	322[f]	150[f]	19	no

[a] The K_d's for all compounds were determined by competition ELISA in 50 mM Pipes, 80 μM NaCl, pH 7.0.[13] The Michaelis–Menten kinetic parameters for H5-67 and H5-25 ([Ab] = 5.0 μM) were determined in 50 mM Tris·HCl, 80 μM NaCl, pH 7.8 at 25 °C, those for H2-21 and H2-23 ([Ab] = 5.0 μM) were determined in 50 mM Hepes, 80 μM NaCl, pH 7.8 at 25 °C, and those for H5H2-42 ([Ab] = 0.6 μM) and H5H2-187 ([Ab] = 2.5 μM) were determined in 50 mM Pipes, 80 μM NaCl, pH 7.0 at 25 °C unless otherwise noted. All assays were performed in triplicate. See Experimental Section for the details. [b] These data were obtained in 50 mM Pipes, 80 μM NaCl, pH 6.6 at 25 °C. [c] The abbreviation nd denotes that inhibition in the presence of 1.0 mM of inhibitor was not detected. [d] The background hydrolysis rate constants (k_{un}) of 3 at pH 7.8, 7.0, and 6.6 were determined to be 3.0×10^{-3}, 1.8×10^{-4} and 7.4×10^{-5} min^{-1}, respectively. [e] The K_i's were determined by Dixon plot.[19] [f] The K_i's were determined by Dixon plot and Lineweaver–Burk plot. Both methods gave approximately the same values.

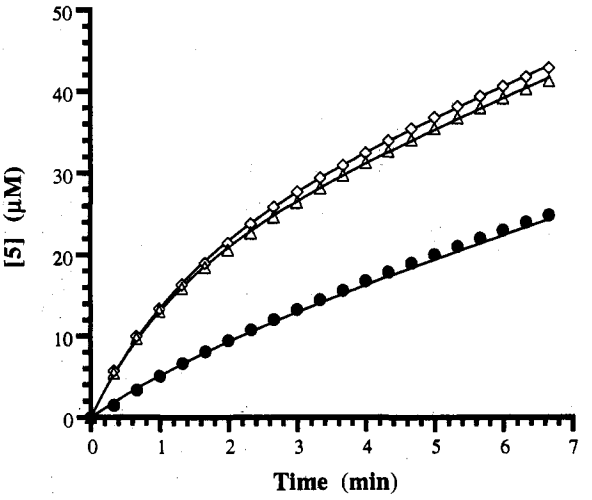


Figure 2. The formation of p-nitrophenolate ion 5 during the hydrolysis of ester 3 (500 μM) catalyzed by antibody H2-23 (5.0 μM) at pH 7.8. The reaction was initiated in the absence of products (◇), in the presence of 30 μM **6** (△), and in the presence of 30 μM 5 (●). The curves indicate the fit of data to eq 2 by nonlinear regression.

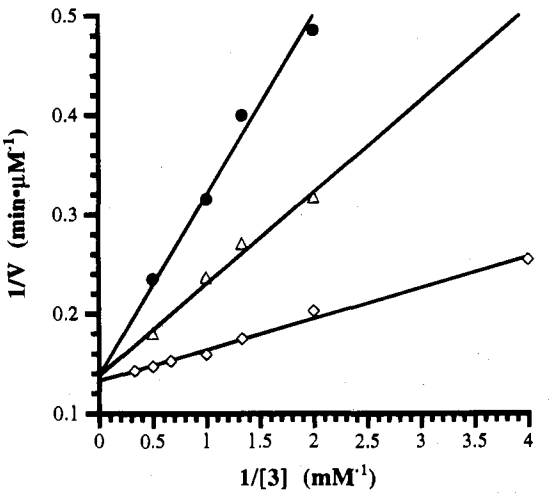


Figure 3. Lineweaver–Burk plot for the hydrolysis of ester **3** by 0.6 μM antibody H5H2-42 in the absence of inhibitors (◇), in the presence of 0.5 mM 1 (△), and in the presence of 0.1 mM 2 (●) at pH 7.0.

steady-state kinetic burst (Figure 2).[5a] The decrease of the rate of the catalysis was found to be a pseudo-first-order phenomenon as provided by the following equation:

$$V_t = (V_0 - V_t)\exp(-kt) + V_f \quad (1)$$

where k is the observed apparent first-order rate constant of the formation of deactivated catalysis, V_0 is the initial rate, V_t is the rate at time t, and V_f is the steady-state rate.[17] The progress curves were fitted well to eq 2, which is the integrated form of eq 1:

$$[P] = V_f t + [(V_0 - V_f)(1 - \exp(-kt))]/k \quad (2)$$

Both initial and steady-state rates follow Michaelis–Menten kinetics and Michaelis–Menten parameters were determined for both V_0 and V_f. For the initial rate V_0, $k_{cat} = 3.3$ min^{-1} and $K_m = 160$ μM, and for the steady-state rate V_f, $k_{cat} = 0.96$ min^{-1}, $K_m = 220$ μM were determined. The above kinetic experiments were also applied over the pH range of 6.2 to 8.5 (data not shown). The pH profile for the steady-state and initial rates revealed that both rates are dependent on the hydroxide ion concentration above pH 7, wherein the values of k_{cat} increase when the hydroxide ion concentration is increased. This behavior appears to be similar to that observed for other catalytic antibodies generated by phosphonate derivatives as reported in the literature.[2–5]

(17) Frieden, C. *J. Biol. Chem.* **1970**, *245*, 5788.

The catalytic activity of H2-23 was strongly inhibited by the addition of hapten **2** ($K_i = 4.6$ μM), while hapten **1** showed no inhibitory activity to the antibody. The burst behavior of H2-23 was eliminated by the addition of 30 μM of *p*-nitrophenolate **5**, and the apparent inhibition constant for **5** was estimated as 8 μM (Figure 2). On the other hand, the addition of acid **6** had no effect on the burst. It should be noted that this product inhibition behavior of H2-23 resulted in the loss of approximately one-third of the catalytic activity after 5 to 6 turnovers. Thus, we conclude that the burst behavior of H2-23 is partially due to the product inhibition of *p*-nitrophenolate **5**, although other possible mechanisms for the burst behavior such as the formation of an acyl intermediate are not ruled out.[4b,5a]

H5H2-42. Antibody H5H2-42, the best catalyst obtained by heterologous immunization, was selected for further study. The saturation behavior of antibody H5H2-42 is consistent with Michaelis–Menten kinetics, and a Lineweaver–Burk analysis at pH 7.0 (50 mM Pipes, 80 μM NaCl) gave apparent values for k_{cat} and K_m of 12.5 min^{-1} and 240 μM, respectively (Figure 3). The observed rate acceleration (k_{cat}/k_{un}, where $k_{un} = 1.8 \times 10^{-4}$ min^{-1}) at pH 7.0 was 6.8×10^4. It is noted that a k_{cat} of 11.0 min^{-1} was observed at pH 6.6 ($k_{un} = 7.4 \times 10^{-5}$ min^{-1}), achieving a rate acceleration of $k_{cat}/k_{un} = 1.5 \times 10^5$. This rate acceleration is approximately two orders of magnitude greater than that observed for the representative catalytic antibody H5-67 derived from the homologous immunization of **1** (Table 1).[6]

The catalytic reaction was competitively inhibited by the addition of hapten **1** or **2**, and the inhibition constants (K_i) of each hapten were determined at pH 7.0. Lineweaver–Burk plot

analysis resulted in K_i's of 300 and 25 μM, respectively (Figure 3). While the antibody binds more tightly to secondary hapten **2** rather than to primary hapten **1**, these results strongly suggest that catalytic residues in the active site of antibody H5H2-42 interact with the functionalities of both haptens. It should be noted that unlike some catalytic antibodies raised against **2** alone no significant product inhibition was observed during the hydrolysis of **3**, resulting in *actual turnovers greater than 500.* The lack of product inhibition is consistent with high dissociation constants measured with **5** ($K_d = 4.8 \times 10^{-2}$ M) and the product acid **6** ($K_d > 2 \times 10^{-2}$ M).

The dependence of the kinetic parameters (k_{cat} and K_m) upon the nature of the buffer was also studied. Apparent values of k_{cat} and K_m in 50 mM Pipes and 80 μM NaCl at pH 7.0 were determined to be 12.5 min^{-1} and 240 μM, respectively, while in 32.5 mM Pipes and 80 μM NaCl pH 7.0 these values were 12.3 min^{-1} and 109 μM, respectively. In the three-component buffer MDH (12.5 mM Mes, 12.5 mM Hepes, and 25 mM bis-(2-hydroxyethyl)amine), used for pH-dependent kinetics (*vide infra*), these values were determined to be 12.2 min^{-1} and 83 μM, respectively. Thus, the parameter k_{cat} did not depend upon the nature of buffer species but K_m was lower when the buffer concentration was decreased.

We have examined Michaelis–Menten kinetics over the pH range 6.2–8.5 and determined the first- and second-order rate constants, k_{cat} and k_{cat}/K_m. The pH dependence of k_{cat} and k_{cat}/K_m should follow the pK_a (ionization) of functional residues in the antibody–substrate complex (K^{ES}) and in the free antibody[18] (K^E), respectively.[19] The plots of pH vs log k_{cat} and pH vs log(k_{cat}/K_m) are illustrated in Figure 4. The former plot appeared to be a bell-shaped profile, indicating that the antibody in the state of complex with the substrate could have two ionizable residues with pK_a's of pK_a^{ES1} = 5.6 and pK_a^{ES2} = 8.7.[20] This observation is in contrast to that observed for H2-23 and H5-67, in which no reflection responsible for the pK_a of the catalytic residue was seen in this pH range. On the other hand, the latter plot for H5H2-42 reveals only one ionizable residue with pK_a^{E2} of 6.9 in this pH range. However, we believe that the kinetic experiments at pH's below 6 may be able to detect another essential residue (pK_a^{E1}) existing in the antibody catalytic site.[21] Although antibody H5H2-42 catalyzed hydrolysis of ester **3** with a remarkable rate acceleration (1.5×10^5 at pH 6.6), the cleavage of the amide bond in **4** was unfortunately not seen with an appreciable rate acceleration at the optimum pH (pH 6.6–7.0). Further screening of the available catalytic repertoire of antibodies generated via heterologous immunization will be required to isolate catalysts with amidase activity.

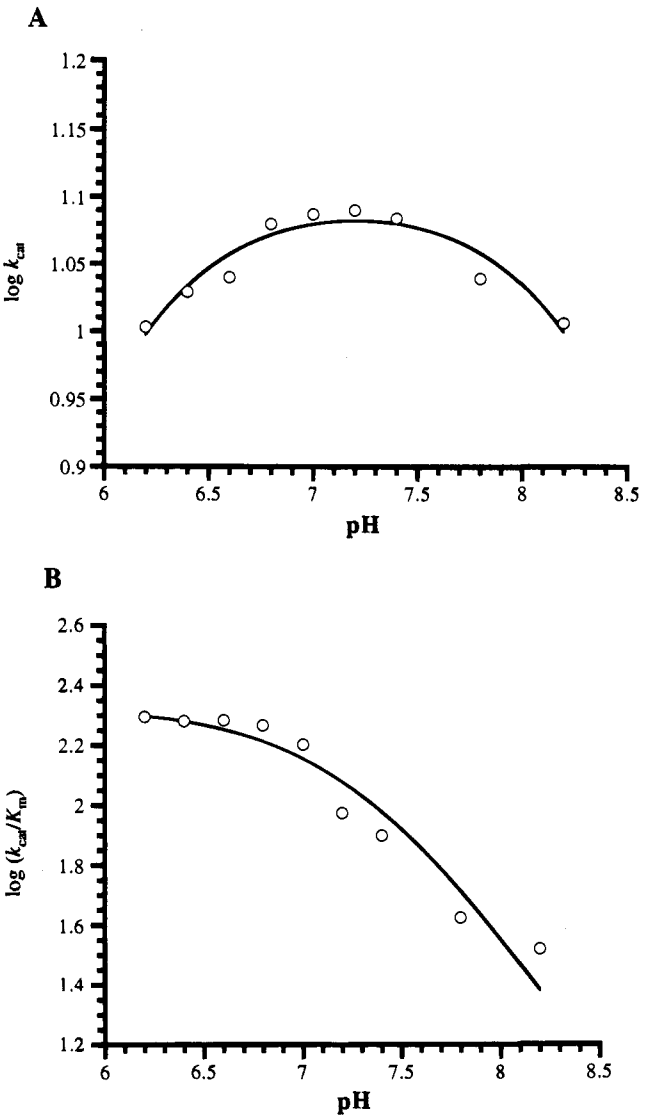


Figure 4. pH dependent steady-state kinetics for hydrolysis of ester 3 by antibody H5H2-42. (A) pH vs log k_{cat} and (B) pH vs log (k_{cat}/K_m). Assays were performed in MDH buffer (12.5 mM Mes, 12.5 mM Hepes, 25 mM bis(2-hydroxyethyl)amine for the entire pH range, and the curves indicate the fit of data to eqs 3 and 4 by nonlinear regression.

Conclusion

We have demonstrated that heterologous immunization by the use of two structurally related haptens **1** and **2** is capable of inducing catalytic antibodies with significant rate acceleration and multiple turnover activity in the hydrolytic reaction of ester **3** compared to those obtained by the standard homologous immunization using either one of the haptens. The heterologous immunization strategy will offer two distinctive advantages over the standard homologous immunization protocol: (i) the ability to expand the repertoire of available antibody combining site generated by transition state analog haptens and (ii) a potential means of introducing more than one functional residue in the antibody combining site without recourse to tedious hapten synthesis. This heterologous immunization strategy also has general applicability to various chemical reactions that involve general acid and base catalysis. Extensive investigations of this approach are currently in progress in our laboratory.

Experimental Section

General Procedure of Homologous Immunization. Balb/c mice each received a subcutaneous injection of 100 μg of hapten (**1** or **2**) conjugated with BSA (BSA-hapten) and emulsified in RIBI adjuvant (MPL and TDM emulsion). The same injection of BSA-hapten was

(18) Although pH vs log(k_{cat}/K_m) also follows the ionization of substrate (K^s), the carboxylate group of **3** remains ionized in this pH range.

(19) (a) Fersht, A. *Enzyme Structure and Mechanisms*; W. H. Freeman: New York, 1985. (b) Tipton, K. F.; Dixon, H. B. F. *Methods Enzymol.* **1979**, *63*, 183.

(20) The data of pH vs log k_{cat} and pH vs log k_{cat}/K_m were fitted to the following equations, giving the respective pK_a's.

$$\log k_{cat} = \log k_{cat}^{max} - \log (1 + 10^{-pH}/10^{-pKES1} + 10^{-pKES2}/10^{-pH}) \quad (3)$$

$$\log (k_{cat}/K_m) = \log (k_{cat}/K_m)^{max} - \log(1 + 10^{-pKE}/10^{-pH}) \quad (4)$$

(21) We are concerned about these pK_a^{ES1} and pK_a^{ES2} values which are not apparent in Figure 4 covering the limited pH range 6.2–8.5. Further studies are required to secure these pK_a^{ES} values. However, the values of K_m at high pH above 8.5 appeared to be too large (>1 mM) to accurately measure the catalytic rate constants. Also at pH below 6, a method for kinetic measurement other than monitoring absorbance change of *p*-nitrophenolate ion at 405 nm should be applied to determine the Michaelis–Menten parameters because the *p*-nitrophenolate ion is no longer detectable below pH 6.

given 2 weeks later. Seven days following the second injection, serum titers were analyzed by ELISA. Three weeks after the second injection, mice showing high titer (titers > 12800:1) were again immunized with the same BSA-hapten conjugate using the same procedure. Three days following the last injection, the spleen was removed from the mouse to prepare hybridomas.

Heterologous Immunization with Hapten 1 followed by Hapten 2. Four Balb/c mice each received a subcutaneous injection of 100 μg of BSA-**1** emulsified in RIBI adjuvant, and the same injection of BSA-**1** was given 2 weeks later. Seven days following the second injection serum titers were analyzed by ELISA, and in each case strong immune responses were observed (titers >12800:1). Seven weeks after the second injection, a mouse received a subcutaneous injection of 100 μg of BSA-**2** emulsified in RIBI adjuvant. Three days following the last injection, the spleen was removed to prepare hybridomas.

Heterologous Immunization with Hapten 2 followed by Hapten 1. Five Balb/c mice each received a subcutaneous injection of 100 μg of BSA-**2** emulsified in RIBI adjuvant, and the same injection of BSA-**2** was given 2 weeks later. Seven days following the second injection serum titers were analyzed by ELISA, and in each case strong immune responses were observed (titers >12800:1). Between three and seven weeks after the second injection, these mice each received a subcutaneous injection of 100 μg of BSA-**1** emulsified in RIBI adjuvant. Three days following the last injection, the spleen was removed to prepare hybridomas.

Single Immunization with Hapten 2. Two mice each received a subcutaneous injection of 100 μg of BSA-**2** emulsified in RIBI adjuvant, and three days after the injection the spleen was removed to prepare hybridomas.

General Procedure for Preparation of Monoclonal Hybridomas. The harvested spleen cells were fused with 5 x10^7 P3X63-Ag8.653 myeloma cells by standard protocols. Hybridoma cells were plated into six (homologous and single immunization) or eight (heterologous immunization) 96-well plates; each well contained 100 μL of HAT-DMEM or HAT-RPMI 1640 with 20% fetal bovine serum and 1% hybridoma enhancing supplement (Sigma) or 5% Briclone (BioResearch Ireland). After two weeks, the plates were analyzed by ELISA for binding to Fib-**1** and Fib-**2** conjugates. All positive colonies were subcloned two or three times according to the standard protocols.[6,10]

General Procedure for Preparation of Monoclonal Antibodies. All cell lines remaining active after subcloning were individually injected into pristane-primed Balb/c mice to generate ascites. The harvested ascites were treated with a saturated ammonium sulfate (AS) solution to give a final concentration of 45% AS. The precipitated antibodies were dissolved in 50 mM Tris·HCl, pH 7.8, and dialyzed against the same buffer prior to chromatographic purification. DEAE-Sephacel anion-exchange chromatography was performed by stepwise salt gradient (from 0 to 500 mM NaCl in 50 mM Tris•HCl, pH 7.8). The fractions containing antibody, confirmed by SDS-polyacrylamide gel electrophoresis, were then subjected to protein G-Sepharose affinity chromatography (loaded on in 20 mM potassium phosphate, pH 7.2, and eluted with 100 mM glycine·HCl, pH 2.7) to yield purified antibody (>95% by SDS-polyacrylamide gel electrophoresis). If necessary, antibody was further purified by mono-Q chromatography (Pharmacia).

Antibody Affinity Measurement and Class Determination. Dissociation Constants (K_d) were determined by the modified procedure of competition ELISA as described in ref 12. Prior to carrying out the competition ELISA, the optimum antibody concentration and the incubation time on the ELISA plate (precoated with 2 μg/mL of the protein−hapten conjugate and then blocked with BSA) were determined to establish a reproducible titration curve. This is critical for detecting a weak antibody affinity to an inhibitor. The minimum antibody concentration is defined as the concentration at which ca. 70% of antibody (reflecting on the absorbance) binds to protein−hapten conjugate. In our experiments, the concentration and the incubation time were generally in the range of 0.25−1.0 μg/mL and 10−20 min, respectively. Competition ELISA experiments were performed as follows. A 60-μL solution of each inhibitor (in 50 mM Pipes, 80 μM NaCl, pH 7.0) was serially diluted 2-fold across a BSA-blocked ELISA plate (from well A1 to A12), containing 60 μL of buffer per well. To each well was then added 60 μL of an antibody solution (the final concentration was pre-adjusted) and the mixture was equilibrated for 30 min at 25 °C. The resulting antibody-inhibitor solution (100 μL) from each well was transferred to the corresponding well of the ELISA plate with a multipipet and incubated for the pre-adjusted time (10−20 min). After the plate was washed, standard[6] ELISA protocol was followed and a titration curve (the sigmoidal plot of the log of the antibody concentration vs absorbance) was obtained. The results were analyzed by a Klotz plot (a double reciprocal plot of the inhibitor-concentration vs the ratio of inhibitor-bound antibody to the total added antibody), the slope of the line yielding the K_d for the inhibitor. All assays were repeated three times.

The class of each antibody was determined by using a monoclonal isotyping kit purchased from Pierce (37501G) and Amersham (RPN29).

Kinetic Measurements. Each purified monoclonal antibody solution was dialyzed against the assay buffer, and the concentration was determined by measurement of the absorbance at 280 nm. Assays were performed in a microtiter plate reader (Bio-Tek Instruments) equipped with the Delta Soft II computer program (Biometalics, Inc.) or Beckman DU 640 UV spectrometer. All assays were performed at least in duplicate.

Initial screening for catalytic antibodies was performed at 25 °C in buffer solution (for homologous immunization with **2**−50 mM Mes, 80 μM NaCl, pH 6.2; 50 mM Pipes, 80 μM NaCl, pH 7.0; 50 mM Hepes, 80 μM NaCl, pH 7.8; and for heterologous immunization−50 mM Mes, 80 μM NaCl, pH 6.2; 50 mM Pipes, 80 μM NaCl, pH 7.0; 50 mM Tris·HCl, 80 μM NaCl, pH 7.8), with 5% of organic cosolvents (4.8% acetonitrile and 0.2% dimethyl sulfoxide) containing 1 mM ester **3** and 5.0 or 10 μM antibody. The rates were determined by measuring the initial change in absorbance at 405 nm reflecting on *p*-nitrophenolate release. The background rate hydrolysis of the substrate (in the absence of antibody) was also measured each time. For the antibodies showing product inhibition, the progressive curves were fitted to eq 2 and initial rates (V_0) and the steady-state rates (V_f) were determined. The V_0 and V_f were analyzed by use of the standard Michaelis−Menten equation.

Antibodies found to be capable of catalyzing the hydrolysis were further studied under the conditions described in the text and Table 1. For antibody H2-23, the antibody-catalyzed hydrolysis rates with six different substrate concentrations from 0.02 to 0.5 mM were measured in the presence of 5.0 μM of the antibody concentration, and V_0 and V_f were determined according to eq 2. The analysis of the V_0 and V_f by the standard Michaelis−Menten equation gave Michaelis−Menten parameters (k_{cat} and K_m). For antibody H5H2-42, the antibody-catalyzed hydrolysis rates with eight different substrate concentrations from 0.25 to 3.0 mM (the solubility limit of substrate **3**) were measured in the presence of 0.60 μM of the antibody concentration. The Michaelis−Menten parameters were determined by standard methods.

Buffer-Dependent and pH-Dependent Kinetics of H5H2-42. The assays for buffer-dependent kinetics were performed under the conditions described in the text. The assays for pH-dependent kinetics were performed in MDH buffer (12.5 mM Mes, 12.5 mM Hepes, 25 mM bis(2-hydroxyethyl)amine) for the entire pH range between 6.2 and 8.5. The Michaelis−Menten parameters were determined as described above.

Acknowledgment. We thank Dr. Chad C. Huval for critically reading this manuscript. We also acknowledge Mr. Oguz Ersoy, Drs. Simon F. Williams, and Anthony J. Sinskey for early contributions to this work. The work at M.I.T. was generously supported by a grant from National Science Foundation (CHE-9424283) awarded to S.M.

JA9516571