Isoabzymes: Structurally and Mechanistically Similar Catalytic Antibodies from the Same Immunization†

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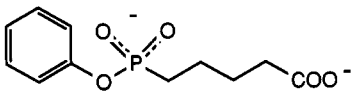
ABSTRACT: Mechanistic and structural comparisons of five catalytic monoclonal antibodies generated from the same hybridoma fusion indicated that all five hydrolyze phenyl acetate by subtle variations of the same mechanism. All of the antibodies showed a pre-steady-state multi-turnover burst in which k_{cat} and K_m declined but k_{cat}/K_m did not change. The burst of one of the antibodies, 20G9, has previously been found to result from inhibition by the product, phenol. Although all of the antibodies showed the burst, their individual values for k_{cat}, K_m, and hapten K_i differed substantially. Three of the antibodies that were investigated for the effect of pH on k_{cat} showed an acid limb pK of 9.5–9.6. Substrate inhibition was seen in four of the five antibodies. Variable region nucleotide sequencing of the heavy and light chains confirmed that all five antibodies were structurally similar and also revealed several potentially critical tyrosines. Despite their structural similarities, analysis of their sequences suggested that the antibodies are products of distinct, independent rearrangements of immunoglobulin gene segments that took place in different progenitor B cells. A plot of K_i for hapten inhibition vs K_m/k_{cat} for substrate hydrolysis for the mechanistically related antibodies ("isoabzymes") gave a linear relationship suggesting a catalytic role for transition-state complementarity. Taken together with previous work [Martin et al. (1991) *Biochemistry 30,* 9757–9761], the data conform to a mechanism in which the antibodies exploit both transition-state complementarity and an acyl-tyrosyl intermediate during phenyl acetate hydrolysis.

Despite the large number of possible antibody variable (V) region gene combinations, nucleotide sequence comparisons of genes from antibodies elicited to small haptens have revealed that the immune response to a defined epitope often consists of a very narrow set of V genes. This phenomenon has been demonstrated for a number of haptens including phosphorylcholine (Feeney & Thuerauf, 1989; Perlmutter et al., 1985), phenyloxazolone (Kaartinen et al., 1991; Rada et al., 1991), 4-hydroxy-3-nitrophenylacetic acid (NP) (Cumano & Rajewsky, 1985), progesterone (Deverson et al., 1987), and *p*-azophenylarsonate (Wysocki et al., 1987). As a consequence of their common gene usage, amino acid sequences of different monoclonal antibodies raised to the same hapten are often closely related. The low levels of sequence heterogeneity that are seen in these antibodies are generally attributable to junctional diversity during gene recombination and somatic hypermutation during the affinity maturation process. As might be expected, subtle differences in the geometries of the combining sites sometimes result in substantial variations in antibody hapten binding affinities.

On the basis of these and other similar observations with noncatalytic monoclonal antibodies, we suspected that different catalytic antibodies raised to a hapten and generated from the same hybridoma fusion may be structurally related but exhibit marked differences in both hapten binding affinity and kinetic efficiency. Comparative studies of structurally and mechanistically similar catalytic antibodies, herein termed "isoabzymes", could greatly assist mechanistic elucidation. Likewise, structural comparisons of catalytic antibodies with structurally related noncatalytic antibodies may also be mechanistically informative. Once a group of isoabzymes has been identified, the structural reasons for their kinetic differences may become apparent either by simple inspection of their deduced amino acid sequences or, more rigorously, by comparative molecular modeling, biophysical investigations, and detailed kinetic studies.

In this paper, we describe investigations of the kinetic and structural differences of five catalytic monoclonal antibodies raised against hapten **1** and generated in the same hybridoma



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fusion (Durfor et al., 1988). One of the antibodies, 20G9, is relatively well-characterized and known to be highly regulated by the binding of its substrate phenyl acetate and product phenol (Martin et al., 1991b; M. T. Martin and T. S. Angeles, unpublished work). In addition, 20G9 is postulated to hydrolyze phenyl acetate via an acetyl-tyrosyl intermediate (Martin et al., 1991a). The unique and remarkable kinetic features of this antibody were helpful since they facilitated mechanistic comparisons with potential isoabzymes. Mechanistic and sequence comparisons showed that all five antibodies are indeed isoabzymes.

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[1] Abbreviations: BSA, bovine serum albumin; CDR, complementarity determining region; DEAE, (diethylamino)ethyl; ELISA, enzyme-linked immunosorbent assay; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; TS, transition state; TSA, transition-state analog; V, variable; V_H, variable domain of the heavy chain; V_κ, variable domain of the light chain.

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MATERIALS AND METHODS

Antibodies and Chemicals. Monoclonal antibodies were generated as described (Durfor et al., 1988). Combined spleens of three immunized mice were used in the fusion. Antibodies were produced in ascites and purified by standard procedures involving lipid extraction, ammonium sulfate precipitation, and chromatography on protein A, then on DEAE Sephacel, and finally on Mono Q (Pharmacia, Piscataway, NJ) to a purity of >99% as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Phenyl phosphonate hapten **1** and BSA-coupled **1** were from earlier work (Durfor et al., 1988). All chemicals were of reagent or analytical grade.

Antibody Cloning and Sequencing. Total RNA was isolated from hybridoma cells by a single-step guanidinium thiocyanate/phenol–chloroform extraction procedure (Chomczynski & Sacchi, 1987). First-strand cDNA synthesis using M-MLV reverse transcriptase (BRL, Gaithersburg, MD) and random hexamer priming was performed using buffers and protocol provided by the enzyme manufacturer. PCR amplification of variable region heavy (V_H) and light (V_L) chains was done essentially as previously described (Chiang et al., 1989; Orlandi et al., 1989) using a set of 5′V_H and 5′V_κ consensus PCR primers designed based on the mouse immunoglobulin nucleotide database of Kabat et al. (1987). The 3′V_H and V_κ specific PCR primers were designed to be complementary to the known mouse J_H and J_κ nucleotide sequences (Kabat et al., 1987). Restriction sites incorporated into the above PCR primers were used to clone V_H and V_κ PCR products into the *Escherichia coli* plasmid vector pBluescript SK (Stratagene, LaJolla, CA). Following cloning, PCR products were sequenced by a dideoxy chain termination protocol using double-stranded plasmid DNA template and a Sequenase sequencing kit (USB, Cleveland, OH) following the provided protocol.

Antibody Nucleotide Sequence Analyses. Heavy and light chain sequences were compared from nucleotide 25 of the coding region to the end of the region encoded by V_H or V_κ. This excludes differences potentially introduced by the primers used in PCR amplification prior to cloning and differences due to junctional diversity in the recombination with J segments. The heavy and light chain sequences were separately aligned using the program DNASIS (Version 2.0, Hitachi Software Co.). To facilitate sequence comparisons, heavy and light chain consensus sequences were derived. In the consensus sequences, each position is represented by the nucleotide (or amino acid) that occurs most frequently at that position. The nucleotide consensus sequences were compared with the V_H and V_κ sequences in the GenBank (release 75.0 2/15/93) and EMBL Data Library (release 34.0, March 1993) databases. The most closely related sequences in the databases were extracted for closer comparison. Database comparisons were performed at the NCBI using the BLAST network service (Altschul et al., 1990).

Assays. Unless otherwise indicated, the antibodies (generally 1.0 μM) were assayed spectrophotometrically ($\Delta\epsilon_{270}$ = 1425 M^{-1} cm^{-1}; Martin et al., 1991a) for their ability to hydrolyze phenyl acetate at 25 °C in 10 mM Tris, pH 8.8, and 140 mM NaCl. Absorbance changes resulting from uncatalyzed substrate hydrolysis was corrected for by using a reference cuvette containing the same reaction solution but without the antibody. The minor decrease in substrate concentration due to uncatalyzed hydrolysis was taken into account during analyses. Reaction velocities were quantitated either by computer fitting of progress curves on an AT&T 6300 personal computer (Martin et al., 1991a) or by direct measurement of chart paper from portions of reactions that were essentially linear.

Data Analyses. The kinetic parameters were evaluated by fitting the data to the appropriate equations using Kaleidagraph, Version 2.1 (Abelbeck Software, Reading, PA) or the BASIC versions of the computer programs of Cleland (1979a).

pH Studies. A constant ionic strength buffer system composed of 50 mM Mes, 25 mM Tris, 25 mM ethanolamine, and 100 mM NaCl (MTEN) was used to cover the pH range from 6.0 to 11.0 (Morrison & Ellis, 1982). Data reported for pH values other than 8.8 have been corrected for pH-dependent changes in $\Delta\epsilon$ for the hydrolysis of phenyl acetate.

The apparent pK for hapten binding was determined by using a standard ELISA procedure in which various concentrations of antibody (50–0.024 μg/mL) at a range of pH values (6.0–10.5 in 0.5-unit increments) were added to 96-well plates containing BSA-immobilized **1**. Binding was detected and quantitated using goat anti-mouse IgG labeled with horseradish peroxidase. Control experiments showed that the antibodies remained fully catalytically active at pH 10.5 over the duration of the ELISA experiments.

All data to determine pK values were fitted to eq 1 or 2 where y is V_{max} or absorbance, C is the pH-independent parameter, and K_1 and K_2 are the dissociation constants of the groups on the acidic and basic side, respectively.

$$\log y = \log (C/1 + [H^+]/K_1) \quad (1)$$

$$\log y = \log (C/1 + K_2/[H^+]) \quad (2)$$

Inhibition Studies. Hapten inhibition assays were performed by varying the concentrations of **1** (0–5x[Ab]) at fixed levels of antibody and substrate. Apparent inhibition constants were obtained by fitting the data to eq 3, where 2[Ab] is the concentration of active sites (the antibodies are bivalent), [I] is the inhibitor concentration, $K_i' = K_i(1 + [S]/K_m)$, K_i is the inhibition constant, [S] is the fixed substrate concentration, and V_i and V_o are initial velocities in the presence and absence of hapten, respectively (Cha, 1975).

$$V_i/V_o = (2[Ab] - [I] - K_i' + \{([I] + K_i' - 2[Ab])^2 + 4K_i'2[Ab]\}^{0.5})/4[Ab] \quad (3)$$

Data for substrate inhibition were evaluated using eq 4, where K_{is} is the apparent substrate inhibition constant (Cleland, 1979b).

$$v = V[S]/[K_m + [S] + ([S]^2/K_{is})] \quad (4)$$

RESULTS

In these studies we compared the kinetic and structural properties of five catalytic monoclonal antibodies generated from the same hybridoma fusion. Antibodies 20G9, 45A11, 30F7, 18H4, and 7D4 were raised to hapten **1** designed to resemble the putative transition state of phenyl acetate hydrolysis (Durfor et al., 1988).

Kinetic Studies. Kinetic comparisons suggested that all five antibodies hydrolyze phenyl acetate by subtle variations of the same mechanism. Although they are obviously distinct antibodies, exhibiting different kinetic parameters for k_{cat}, K_m, and K_i for inhibition by **1** (Table I), they share an unusual

Table I: Kinetic Parameters of Phenyl Acetate Hydrolysis by Anti-1 Catalytic Antibodies[a]

antibody	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})	$(k_{cat}/K_m)_0/(k_{cat}/K_m)_f$	K_i (nM)
20G9 (V_o)[b]	4.9	300	1.6×10^4	1.1	2.2[c]
20G9 (V_f)	0.54	36	1.5×10^4		
7D4 (V_o)	0.91	514	1.8×10^3	0.86	166
7D4 (V_f)	0.38	183	2.1×10^3		
30F7 (V_o)	1.7	1420	1.2×10^3	0.75	118
30F7 (V_f)	1.1	667	1.6×10^3		
19H4 (V_o)	1.2	240	5.0×10^3	0.93	99
18H4 (V_f)	0.18	33	5.4×10^3		
45A11 (V_o)	0.40	400	1.0×10^3	1.5	1260
45A11 (V_f)	0.13	194	6.7×10^2		

[a] In 10 mM Tris, pH 8.8, 140 mM NaCl; 25 °C. [b] The subscripts o and f refer to the burst phase and steady-state phase, respectively. [c] Blackburn et al. (1990).

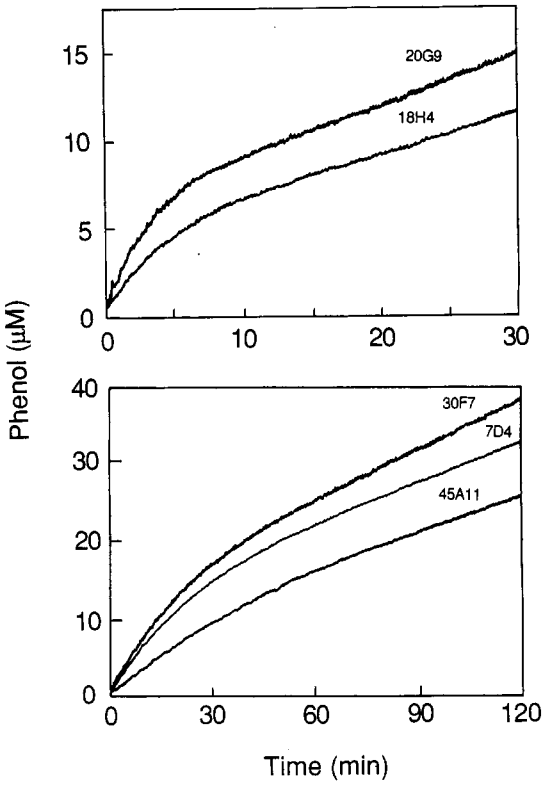

FIGURE 1: Pre-steady-state bursts during hydrolysis of phenyl acetate by 1.2 μM monoclonal catalytic antibody. Initial substrate concentrations were in all cases slightly above K_m (Table I); 350 (20G9), 1450 (30F7), 550 (7D4), 500 (45A11), and 250 μM (18H4). Reactions were carried out at pH 8.8 as described under Materials and Methods.

kinetic property. Figure 1 shows that all five antibodies have a characteristic multi-turnover pre-steady-state burst. As the bursts progress from the rapid initial velocity to the slower steady-state velocity, k_{cat} and K_m both decrease substantially, but to equal extents such that the ratio k_{cat}/K_m does not change (Table I).

The common burst suggested that all of the antibodies may employ variations of the same basic mechanism. To further investigate this possibility, activity pH profiles of three antibodies (20G9, 7D4, and 30F7) were compared. All three showed nearly identical acid limb pK's of 9.5 (7D4 and 30F7) or 9.6 (20G9) for k_{cat} (Figure 2), suggesting a catalytic role for a deprotonated tyrosyl residue in catalysis. Although the ethanolamine present in the buffer has a pK of 9.5 at 25 °C (Dawson et al., 1987), appropriate control experiments showed that the buffer did not participate in the catalysis. Thus, the

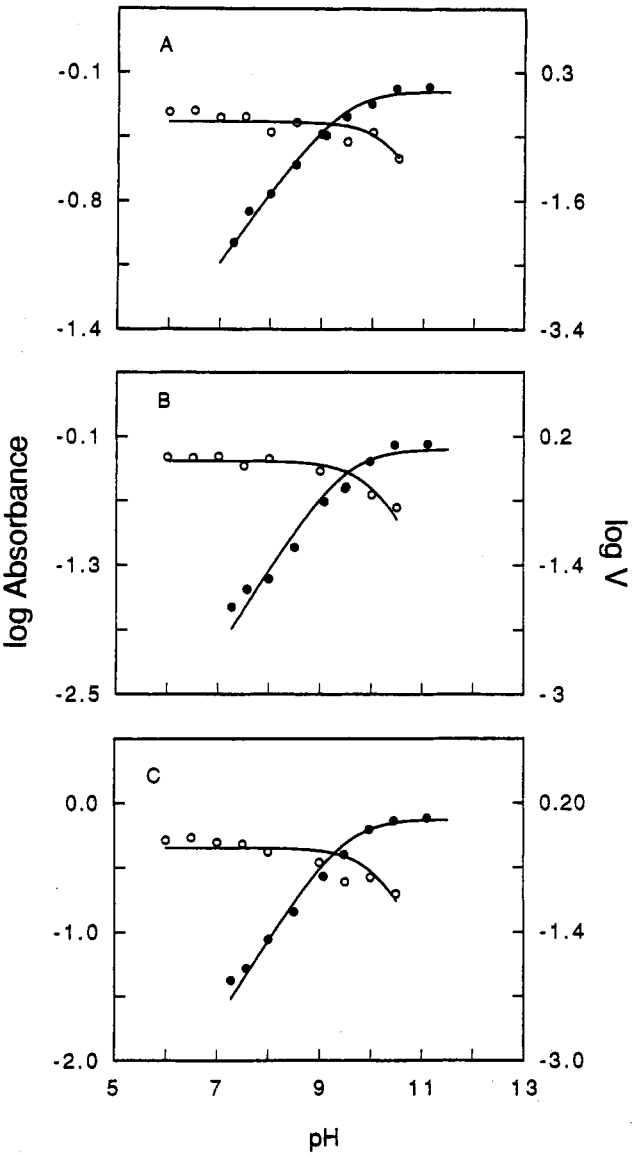

FIGURE 2: Effect of pH on antibody binding to BSA-conjugated **1** (○) and on antibody-catalyzed hydrolysis of phenyl acetate (●). Experiments were carried out at 25 °C in a constant ionic strength buffer system (50 mM Mes, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl). Relative binding of the hapten for the isoabzymes 20G9 (A), 30F7 (B), and 7D4 (C) was evaluated by ELISA as described under Materials and Methods. The curves represent data fitting to eqs 1 and 2 for steady-state velocities and hapten binding, respectively. The data points showing the effect of pH on the activity of 20G9 were from an earlier study (Martin et al., 1991a).

pH data for all antibodies investigated are in accord with the mechanism previously proposed for 20G9 involving an acyl-tyrosyl intermediate (Martin et al., 1991a).

The pH dependence of antibody–hapten binding was examined by a standard ELISA method using BSA-conjugated **1** (shown in Figure 2 for three of the antibodies). No major changes were seen over the range of pH values investigated, although minor loss of hapten binding was observed at the higher pH values, possibly indicating deprotonation of an antibody tyrosine, lysine or arginine. Although control experiments showed no loss of catalytic activity over the pH range shown in Figure 2, inactivation at higher pH values prevented us from confirming the existence of an alkaline limb. These results do clearly show, however, that the effects of pH on hapten binding and catalytic activity are different.

Substrate inhibition of four of the antibodies was observed. Substrate inhibition of antibody 18H4 is shown in Figure 3.

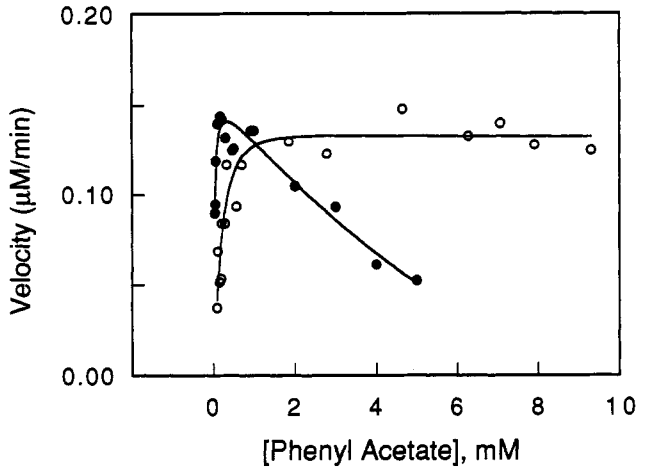

FIGURE 3: Effects of substrate concentration on the reaction rates catalyzed by the antibodies 18H4 (●) and 45A11 (○). Reactions were as described under Materials and Methods. Curves represent the best fit to eq 4 in the text.

Fitting the data to eq 4 gave an inhibition constant, K_{is}, of 2.5 mM for 18H4. Similarly, 20G9, 30F7, and 7D4 were inhibited by phenyl acetate with K_{is} values of 67 μM, 3.6 mM, and 1.6 mM, respectively (data not shown). In contrast, high concentrations of the substrate had no effect on the normal Michaelis–Menten hyperbolic behavior of 45A11 (Figure 3). Differences in substrate inhibition among these antibodies are not surprising in view of their other kinetic differences (Table I). It is curious to note that the most efficient of the catalytic antibodies (20G9) is the one that is most inhibited by the substrate, while the least efficient (45A11) is the only one that is not inhibited by the substrate at all.

Antibody Sequence Analysis. It can be inferred from these kinetic studies that the five catalytic antibodies share a common mechanism which may originate from highly homologous amino acid sequences. To investigate possible sequence homology, V region nucleotide sequencing was carried out and the sequences were compared. The most closely related light chain (V_κ) sequence in the databases was that of a member of the V_κ-21 family expressed in an IgM rheumatoid factor produced by hybridoma AM-15 (Accession No. MUSIGKC-SC; Schlomchik et al., 1987). Rheumatoid factors are IgM antibodies which have generally undergone a minimum of somatic mutation and are highly homologous to germ line sequences. The highest homology to the heavy chain (V_H) consensus sequence was to monoclonal antibody T8-3, which was derived from day 5 of a primary immune response to influenza virus and is specific for the hemaglutinin molecule (Accession No. MMIGHT83; Stark & Caton, 1991). This gene segment is a member of the J558 family, and the sequence expressed in T8-3 is likely to contain few, if any, somatic mutations as it is derived from the early phase of a primary response. The translated sequences of the V_H and V_κ regions of the five catalytic antibodies, the consensus amino acid sequences, and the best matches from the GenBank and EMBL databases are shown in Figure 4.

There is good evidence that all five V_κ sequences expressed in these antibodies were derived from the same germline sequence. The V_κ sequences expressed in these antibodies are between 96.3% and 98.2%, homologous to the consensus sequence (Table II). This sequence in turn differs in only 2 out of 273 positions to that of AM-15. All five V_κ sequences are more than 96% homologous to this sequence.

Performing a similar analysis for the V_H sequences gives a different result (Table II). The V_H consensus is identical in all but 4 out of 252 positions (98.4%) with a rearranged gene expressed in T8-3 (Stark & Caton, 1991). With the exception of 20G9, the other four antibodies are at least 94.8% homologous to this sequence and the consensus sequence and, therefore, are quite likely to be derived from a common V_H gene segment. The sequence of 20G9 on the other hand is only 87.5% homologous, differing in 34 out of 272 positions. When the sequence of the 20G9 V_H region was searched against the GenBank and EMBL databases, no sequence with a higher homology than this was found. There are two possible explanations for this; either 20G9 uses a different, and hitherto unidentified, V_H gene segment to the other antibodies or it has undergone a much higher rate of somatic mutation. The latter explanation is unlikely as the V_κ sequence expressed in 20G9 is very similar to the consensus sequence, indicating that it has not undergone an abnormally high rate of mutation. Thus, it seems most likely that antibodies 7D4, 45A11, 18H4, and 30F7 are derived from a single V_H segment and that 20G9 is derived from a different segment. However, the 88% homology between 20G9 and the other antibodies is sufficient to imply that these V_H segments are members of the same family.

When considering the relationship between antibody sequences, the role of somatic mutation, and its implications for the nature of the immune response, it is necessary to determine whether two or more of the antibodies are clonally related to each other. That is, were any of the hybridoma lines products of B cells which were derived from a common ancester? If they are clonally related, two antibodies must use the same V_κ, J_κ, V_H, D, and J_H gene segments, and the recombination events which brought them together during B cell development must have occurred in the same positions. The genetic rearrangements on the nonproductively rearranged chromosomes must also be identical, although this was not addressed for the hybridomas discussed here. On the basis of the assignments of the J_H and J_κ segments used by these hybridomas and the lengths and sequences of their D regions, we determined that it is unlikely that any of them are clonally related (data not shown).

The kinetic data described above and earlier (Martin et al., 1991a) suggested that the antibodies bind hapten and catalyze phenyl acetate hydrolysis using one or more critical tyrosine residues. The sequence analysis confirms that the combining sites of all five antibodies are rich in tyrosine residues. For example, 20G9, the prototype of this family of antibodies, has eight potentially essential tyrosines.

Transition-State Analysis. In an enzyme- or antibody-catalyzed reaction, the magnitude of the specificity constant (k_{cat}/K_m) is inversely related to the dissociation constant of the transition-state complex (K_{TS}) (Lienhard, 1973; Wolfenden & Frick, 1987; Kraut, 1988);

$$k_{cat}/K_s = k_{uncat}/K_{TS} \quad (5)$$

where k_{uncat} is the reaction rate in the absence of catalyst and $K_s \approx K_m$. If, in the case of an enzyme, an inhibitor is prepared that resembles the transition state (TS), the K_i of that inhibitor will approximate K_{TS} to a degree reflecting the extent of structural and electronic similarity between the inhibitor and the actual TS. This concept has been exploited for many years to provide evidence that an inhibitor is a transition-state analog (TSA) of an enzyme-catalyzed reaction (Westerlik & Wolfenden, 1972; Thompson, 1973; Thompson & Bauer, 1979). Classically, a type of inhibitor may be considered a TSA of an enzymic reaction if the magnitudes of the K_m/k_{cat} values of a series of substrates linearly correlates to the

Heavy Chain Sequences

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CONS.   1 E V Q L Q Q P G A E L V R P G A S V K L S C K A S G Y T F T S Y W M N W V K Q R    40
T8-3    1                           . . . . . . . . . . . . . . . . . . . . . . . . . . .    40
7D4     1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N . . . . . . . . .    40
18H4    1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . K .    40
20G9    1 . . . . H E . . . A . . . . . . . . M . P . R . . . . S . . . F . . . . . R . .    40
30F7    1 Q . K . . E S . . . . . . . . . . . M . . . . D . . . . . I . . . . . . . . . .    40
45A11   1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    40

CONS.  41 P D Q G L E W I G R I D P Y D S E T H Y N Q K F K D K A I L T V D K S S N T A Y    80
T8-3   41 . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . S . . .    80
7D4    41 . . . . . . . . . . . . . . . . . . R S . . . . . . . . V . . . . . . . S . . .    80
18H4   41 . . . . . . . L . . . . . . . . . . . . . . . . . . R . . . . . . . . . . I . .    80
20G9   41 . G . . . . . . . . . H . S . N R . R L . . . . . . . . T . . . . . . . S . . .    80
30F7   41 . . . . . . . . . . . . . . . . T I . . . . . . . . . . . . . . . . . . . S . .    80
45A11  41 . . . . . . . . . . . . . . . . . . . . . . . . . . R . . . . . . . . . . I . .    80

CONS.  81 M Q L S S L T S E D S A V Y Y C A R E G G A Y W G Q G T L V T V S A                114
T8-3   81 . . . . . . . . . . . . . . . . . S P F T T A V A M D Y W G Q G T S                114
7D4    81 I . . N . . . A . . . . . . . . . . . . . N . . . . . . . . . . . .                114
18H4   81 . . . . . . . . . . . . . . . . . . . . . L . . . R . . T L . . . S                114
20G9   81 . . . I . P . . . . . E . . . . . . . . . . . . . . . . . . . . . .                114
30F7   81 . H . I . . . . . . . . . . . . . . . . . - . . . . .                              107
45A11  81 . . . . . . . . . . . . . . . . . . . . . - . . . . . . T L . . . S                114
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Light Chain Sequences

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CONS.   1 D I V M T Q A A P S V P V T P G E S V S I S C R S S K S L L H S N G N T C L Y W    40
AM-15   1 . . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . Y . . .    40
7D4     1 . . . . . . . . . . . . . . . . . . . . . . . T . T . . . . . . S . . . H . . .    40
18H4    1 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N . . .    40
20G9    1 E . . L . . S P . . . I . . . . . . . . . . . . . . . . . . . . Y . . . Y . . .    40
30F7    1 . . . . . . . . . . I . . . . . . . . . . . . T . . . . . . . . . D Y . . . . .    40
45A11   1 . . . . . . . . . . . . . S . . . . . . . . . K . . . . . . . . . . I . . . . .    40

CONS.  41 F L Q R P G Q S P Q L L I Y R M S N L A S G V P D R F S G S G S G T A F T L R I    80
AM-15  41 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    80
7D4    41 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    80
18H4   41 . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    80
20G9   41 . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . .    80
30F7   41 . . . . . . . . . . . . L . . . . . . . . . . . . . . . . . . . . . . . . . . .    80
45A11  41 . . . . . . . . . . . . . . . K . . . . . . . . . . . . . . . . . . . . . . . .    80

CONS.  81 S R V E A E D V G V Y Y C M Q Y L E Y P Y T F G G G T K L E L K                    112
AM-15  81 . . . . . . . . . . . . . . . H . . . . . . . . . . . . . . I                      111
7D4    81 . . . . . . . . . . . . . . . . . . . . F . . . . . . . . . . .                    112
18H4   81 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .                    112
20G9   81 . . . . . D . . . . . . . . . . . . . . . . . . . . . . . . . .                    112
30F7   81 . . . . . . . . . . . . . . . . . . . . F . . . S . . . . . . .                    112
45A11  81 . . . A . . . . . . . . . . . . . . . . . . . . . . . . . . . .                    112
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FIGURE 4: Comparison of heavy and light chain *V* region amino acid sequences of antibodies 20G9, 45A11, 18H4, 7D4, and 30F7 and related antibodies.

magnitudes of the K_i values of their structurally-corresponding inhibitors (Bartlett & Marlowe, 1983; Hanson et al., 1989; Brady & Abeles, 1990). The magnitudes of the slopes may deviate from unity and quantitatively relate the structures of the inhibitors to the structures of the transition states (Brady & Abeles, 1990; Thompson, 1973; Westerlik & Wolfenden, 1972).

Many monoclonal antibodies, including those described in this work, have been raised to putative TSAs with the expectation that the antibodies may be catalytic by virtue of the theoretical relationship between TS affinity and catalytic efficiency shown in eq 5 (Janda et al., 1989, 1991; Pollack et al., 1989; Scanlan et al., 1991). If the hapten is a TSA of the antibody-catalyzed reaction, then some or all of the binding interactions between the hapten and the antibody (experimentally determined as the hapten K_i) will exist in the TS (manifested as K_{TS} but experimentally determined as k_{cat}/K_m). Using these concepts, it is a relatively straightforward experimental exercise to test whether catalytic antibodies that have been raised to the same hapten and utilize the same mechanism (i.e., isoabzymes) contain hapten-binding features that also stabilize the TS. A plot of the hapten K_i values versus the respective K_m/k_{cat} values with a single substrate will be linear if antibody–hapten binding interactions are also present in the TS and serve to accelerate the reaction rate. Figure 5 shows plots of K_i versus K_m/k_{cat} for the five isoabzymes

Table II: Comparison of Anti-1 Catalytic Antibody and Known Antibody Nucleotide Sequences with Consensus Sequence[a]

	differences to consensus sequence		homology to consensus (%)	
antibody	V_H	V_L	V_H	V_L
7D4	13/272	5/273	95.2	98.2
18H4	11/272	5/273	96.0	98.2
20G9	34/272	6/273	87.5	97.8
30F7	14/272	9/273	94.8	86.7
45A11	4/272	10/273	98.5	96.3
T8-3	4/256		98.4	
AM-15		2/273		99.3

[a] Nucleotide sequences were compared in the range of nucleotide number 25–296 of the coding region for V_H and in the range of nucleotide 25–296 for V_L. The sequence of T8-3 was only available in the nucleotide range 38–296.

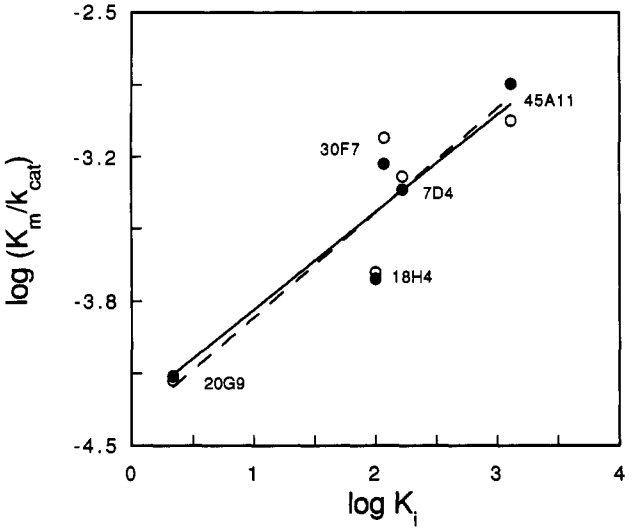

FIGURE 5: Plot of hapten (1) K_i values versus K_m/k_{cat} for antibodies 20G9, 45A11, 18H4, 7D4, and 30F7. A linear relationship was obtained for both the burst phase (○) and steady-state phase (●), in both cases giving a slope of approximately 0.5. Data represented in this figure were from Table I.

studied here. Linear relationships are apparent for both the burst (initial velocity) and steady-state phases of the reaction. The direction and magnitudes of the slopes (0.48 for the burst and 0.43 for the steady state) are consistent with theory and previous experimental results obtained with enzyme transition-state analogs (Brady & Abeles, 1990; Thompson, 1973; Westerlik & Wolfenden, 1972). Thus, the data provided evidence that some of the hapten-binding features in the antibodies also bind the TS and accelerate catalysis.

DISCUSSION

The kinetic and structural analyses presented here show that all five catalytic antibodies described hydrolyze phenyl acetate by variations of the same basic mechanism. The kinetic data implicate a critical deprotonated tyrosine for activity consistent with the previously proposed mechanism involving an acetyl-tyrosyl intermediate (Martin et al., 1991a).

Because all five of the catalytic antibodies appear to employ subtle variations of the same catalytic mechanism, we were able to study the effect of antibody–hapten complementarity (structure) on TS complementarity (function) by comparing the antibodies' kinetic properties. A linear relationship between hapten K_i values and phenyl acetate hydrolysis K_m/k_{cat} values (Figure 5) provided evidence that some interactions used by the antibodies in hapten binding are also used in TS binding to accelerate phenyl acetate hydrolysis.

The magnitude of the slopes of the lines in Figure 5 can provide information about the relationship between hapten binding affinity and TS binding affinity (Brady & Abeles, 1990; Bartlett & Marlowe, 1983). If a "perfect" hapten is prepared that exactly matches the TS structure of the uncatalyzed reaction and does not contain extraneous structural features that are not present in its corresponding substrate, that hapten will elicit catalytic antibodies with efficiencies that exactly match antibody–hapten affinity ($K_i = K_{TS}$). Consequently, any changes in antibody structure would have the same effect on hapten binding energy (K_i) as on TS binding energy (K_{TS}), and a plot such as that shown in Figure 5 would have a slope of 1.00. In Figure 5, the lines have slopes of about 0.5, demonstrating that a given change in antibody structure causes about a 2-fold greater change in hapten binding affinity than in TS binding affinity. Possible reasons for a slope deviating from unity comes from the fact that the hapten is not a perfect mimic of the TS. Because of the structural differences between the hapten and substrate-derived portion of the TS, alterations in the antibody combining site structures can be expected to produce different effects on hapten binding and TS binding. In addition, antibody–hapten contacts may involve specific interactions that are not present in the TS, such as interactions with the alkyl chain which was present in the hapten 1 during immunization but is not present in the substrate. Any changes in such contacts might be irrelevant to the magnitude of K_m/k_{cat} but have a significant effect on K_i. In conclusion, the linearity of the plots in Figure 5 indicate the importance of hapten affinity to TS stabilization, while the slopes of the plots may suggest the critical importance of designing haptens that closely resemble the true TS.

Because we did not set out in the beginning to demonstrate a correlation between K_i and K_m/k_{cat} for these antibodies, we were unable to test this correlation very rigorously. In this work, we compared the mechanisms and structures of only the most active catalytic antibodies generated from the fusion and, as a result, their kinetic constants covered a fairly narrow range of values (hapten K_i values varied at most by less than 600-fold) (Table I). Future, more rigorous substantiation of this concept with a larger number of antibodies with a wider range of antibody catalytic efficiencies and hapten binding affinities is warranted. Nevertheless, the correlation coefficients of the lines shown in Figure 5 (0.935 and 0.903, for the steady-state and burst phases, respectively) strongly indicate the statistical appropriateness of linear fitting, and the general magnitude of the slopes and the direction of the trends are in agreement with what would be expected from transition-state theory.

In combination with earlier work (Martin et al., 1991a), we have obtained evidence for a mechanism involving both an acyl-tyrosyl intermediate and transition-state complementarity. As illustrated in the proposed mechanism in Figure 6, these two elements are consistent with a mechanism similar to that used by serine proteases. In the upper left corner of Figure 6, the carbonyl group of the bound substrate is polarized by a putative oxyanion hole in the antibody (elicited by the negatively charged tetrahedral hapten). The polarized substrate is then attacked by the nucleophilic phenoxide of a tyrosine side chain to form (via a tetrahedral TS) an acetyl intermediate. This acetyl intermediate is then hydrolyzed, again via an antibody-stabilized transition state (lower right), to give free antibody. This mechanism satisfactorily conforms to the previous (Martin et al., 1991a) and current data.

Although the K_i vs K_m/k_{cat} correlation described in eq 5 was originally derived for a simple mechanism with only a

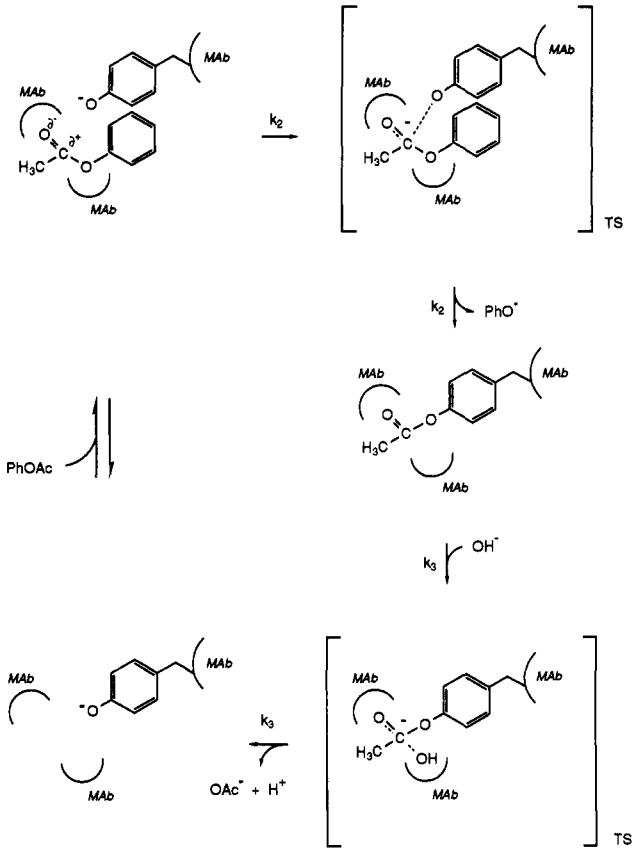

FIGURE 6: Proposed mechanism of phenyl acetate hydrolysis by the described isoabzymes involving both transition-state complementarity and an acetyl-tyrosyl intermediate. Hydroxide ion is shown as the hydrolyzing species because the hapten was designed as a transition-state analog of hydrolysis of phenyl acetate by hydroxide.

single transition state, we used it to evaluate transition-state complementarity of antibodies that are proposed to follow a multi-step, Ping Pong Bi Bi mechanism involving at least two transition states (Figures 5 and 6). A K_i vs K_m/k_{cat} correlation is also valid for a Ping Pong Bi Bi mechanism (Leinhard, 1973; M. T. Martin, unpublished work).

Because the antibodies appear to hydrolyze phenyl acetate with varying efficiencies but differ little in structure, it seemed possible that information regarding structure–function relationships could be obtained by a simple inspection of the sequences. Unfortunately, the individual differences between the antibodies tended to be unique so that we were unable to identify any obvious key amino acid residues with confidence. Kinetically, the antibody 45A11 was the least like the others; it had the lowest k_{cat}/K_m, the highest hapten K_i, and was the only antibody not inhibited by the substrate. In spite of these differences, there was nothing obviously remarkable about its amino acid sequence. It thus appears likely that the kinetic differences among these isoabzymes probably result from subtle structural changes.

This work may serve as a caveat for kinetic studies involving polyclonal mixtures of catalytic antibodies purified from serum. Unless one catalytic antibody in a polyclonal mixture is substantially more active or more concentrated than others that may be present, kinetic constants obtained with mixtures of catalytic antibodies will not have physical meaning.

As described above, it seems most likely that the κ chains of all five catalytic antibodies are derived from the same V_κ gene as AM-15. The heavy chains of 7D4, 18H4, 30F7, and 45A11 are probably derived from the same V_H gene as T8-3, with the heavy chain of 20G9 using a different member of the same V_H family. As found in previous well-characterized responses to defined haptens, the present work shows a restricted gene segment usage. The five isoabzymes described here arose independently in the immune system but are derived from the same V_κ gene segment and from two related V_H segments from the same family. In this study, we focused only on those antibodies derived from a single fusion which possessed catalytic activity toward phenyl acetate. In future studies, it would be interesting to analyze the sequences of antiodies, both catalytic and noncatalytic, raised against a single hapten to determine whether any structural differences or patterns of gene segment usage could be found which correlate with catalytic activity.

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