An Unexpectedly Efficient Catalytic Antibody Operating by Ping-Pong and Induced Fit Mechanisms

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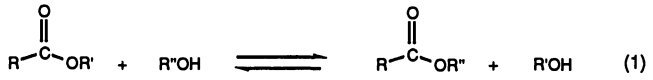
A transition state analogue was used to produce a mouse antibody that catalyzes transesterification in water. The antibody behaves as a highly efficient catalyst with a covalent intermediate and the characteristic of induced fit. While some features of the catalytic pathway were programmed when the hapten was designed and reflect favorable substrate-antibody interactions, other features are a manifestation of the chemical potential of antibody diversity. The fact that antibodies recapitulate mechanisms and pathways previously thought to be a characteristic of highly evolved enzymes suggests that once an appropriate binding cavity is achieved, reaction pathways commensurate with the intrinsic chemical potential of proteins ensue.

ENZYMES MAKE USE OF BINDING ENERGY IN A CONCERTED way to lower the activation barriers that appear along a reaction coordinate (*1*). The optimization of the interaction between an enzyme and its substrate presumably has evolved over a long period, often leading to enzymes that operate at rates limited only by diffusion (*2*). Catalytic antibodies offer promise for probing the nature of biological catalysis. The central question in antibody catalysis concerns the extent to which induction of a predetermined binding site coupled with the diversity of the immune response can reproduce refinements achieved by the genetic processes of mutation and selection that have endowed enzymes with their specificity and efficiency.

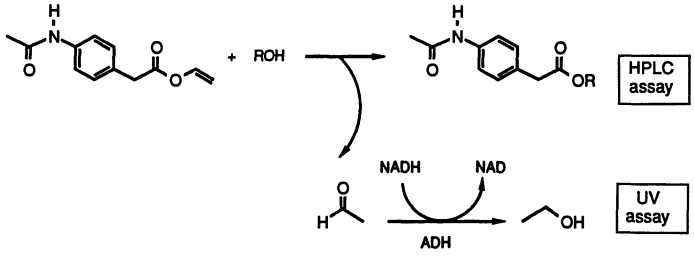
While seeking the catalysis of transesterification in water, we encountered an antibody with a group-transferase activity. This antibody appears to have achieved its efficiency through mechanisms thought to be restricted to highly evolved enzymes. Our experiments suggest that some reoccurring catalytic mechanisms may derive from the obligatory outcome of interaction between the intrinsic chemical potentials of protein molecules and the substrates to which they bind.

Transesterification in water. Transesterification is a difficult bimolecular reaction to run in water because water itself is present in vast excess and is preserved as a reactant. Thus any catalyst for transesterification in water must have enzyme-like specificity for the alcohol and the ester (Eq. 1).



(1)

Antibodies to phosphonate **1** (Fig. 1), which act as esterases (*3*), were studied as catalysts for ester synthesis with the use of the vinyl ester **3** and the alcohol **12** as the acyl donor and acceptor, respectively. This ester provided a good leaving group (*4*), and its modest size kept it within the steric constraints defined by the hapten. We monitored the release of acetaldehyde upon vinyl ester decomposition with a coupled enzyme assay (*5*) (Scheme 1).



Scheme 1

One antibody, monoclonal PCP21H3, catalyzed the reaction between **3** and **12** in a mixture of water and 10 percent dimethyl sulfoxide (DMSO). Our work had shown that this antibody is a slow (*S*)-specific esterase when **7** was used as a substrate.

Steady-state kinetics: A ping-pong pathway. In antibody catalysis, we anticipate that the substrates will fit snugly into a binding pocket as programmed by the hapten. Accordingly, for a two-substrate reaction, a random, sequential kinetic mechanism proceeding through a ternary complex might be expected. We were surprised to observe that the double reciprocal initial velocity patterns were families of parallel lines (Fig. 2). This is characteristic of enzymes that follow the ping-pong pathway (*6*).

The data were fit to Eq. 2, where A and B refer to the vinyl ester **3** and the alcohol **12**, as specified in the programs of Cleland (*7*).

$$v = \frac{V_{max}[A][B]}{K_a[B] + K_b[A] + [A][B]} \quad (2)$$

The kinetic constants are given in Table 1. The data are consistent with the ping-pong bi-bi pattern (product is released between addition of two substrates) involving a modified form of the enzyme

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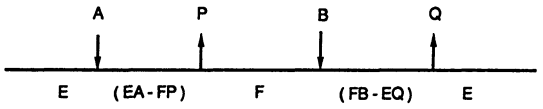
(antibody) as an intermediate. This is expressed in Eq. 3 in the notation of Cleland (*6*)



(3)

where A and B are as above and P and Q are acetaldehyde and the ester 7, respectively. Chemically, F is expected to be a covalent 4-acetamidophenylacetyl antibody. According to the steady-state rate equation, such a pathway is characterized by certain product inhibition behavior (*6, 8*). However, even at extremely high concentrations of acetaldehyde (150 to 200 mM) only a few percent decrease in the initial rate was seen, and our control experiments showed that this was a result of a decrease in the pH of the medium. It is unlikely that the acetaldehyde can effectively bind to F and compete with the alcohol. However, using the coupled assay (*9*) we examined the second product 7 (Q in Eq. 3) and showed the required noncompetitive inhibition with respect to the alcohol (Fig. 3A). Further evidence for the mechanism came in the form of substrate inhibition by **12** (Fig. 3B). Such inhibition, found and analyzed in enzymes that follow the basic ping-pong pathway, is considered to be characteristic and to support their assignments (*10*). The alcohol was inhibitory at rather low concentrations and especially at low concentrations of the vinyl ester. The inhibition results from combination with the wrong stable enzyme (antibody) form (B with E in Eq. 3). The strong inhibition appears reasonable because of the haptenic congruency of the alcohol, which also explains why no inhibition was encountered with **3** (A combining with F, Eq. 3).

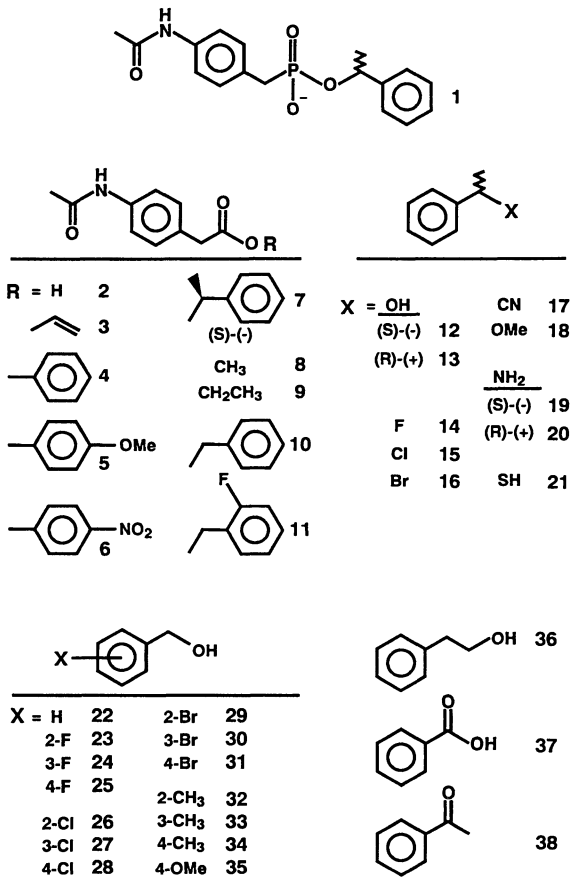


Fig 1. Structures and numerical assignments of compounds under discussion.

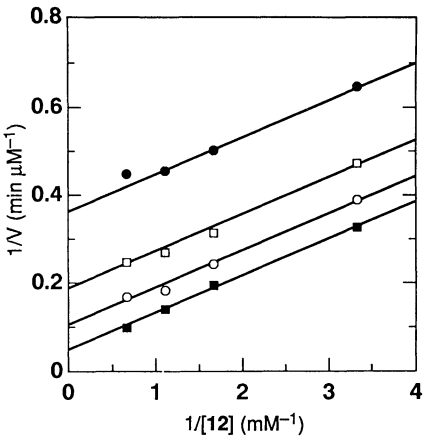


Fig 2. A representative example of PCP21H3 ping-pong kinetics. Double reciprocal plots of initial velocities of ester 7 formation at varying concentration of alcohol **12** and several fixed vinyl ester **3**. The reactions were carried out in I (0.1 ATE, pH 9.0, 10 percent DMSO) in the presence of 4 μM PCP21H3. The vinyl ester concentrations were: (●) 0.10 mM; (□) 0.20 mM; (○) 0.40 mM; (■) 1.2 mM.

We could not unequivocally rule out a sequential (ordered) mechanism (where $K_{ia} << K_a$) as a result of incomplete product inhibition data (*11*). Therefore, we sought acylating agents that would require the first product to be released before the binding of an acceptor. In catalytic antibody technology the binding pocket is induced experimentally, and therefore a rational approach to substrate design is often feasible. Thus, we anticipated that the geometry and reactivity of a phenyl ester linkage would be advantageous and, for steric reasons, the formation of a ternary complex would definitely be precluded. Indeed, with **4** and **12**, PCP21H3 displayed ping-pong kinetics with a similar overall velocity (Table 1). Much of the enhanced k_{cat}/K_M is manifested in the low K_M for the phenyl ester. The 4-methoxy derivative **5** also acted as an acyl donor (similar reactivity), but a kinetic study was not conducted. Most important was that the ester 7, the final product of the above reactions, transferred its acyl group to benzyl alcohol to form **10** in a ping-pong fashion (Table 1). In addition, with alcohol **23** as the acyl acceptor (see below) we showed that **12**, the first product released (P, Eq. 3), was a competitive inhibitor with **23** as the variable substrate (Fig. 4). If an ordered mechanism were operative, such inhibition would be noncompetitive. Finally, since the ester 7 is

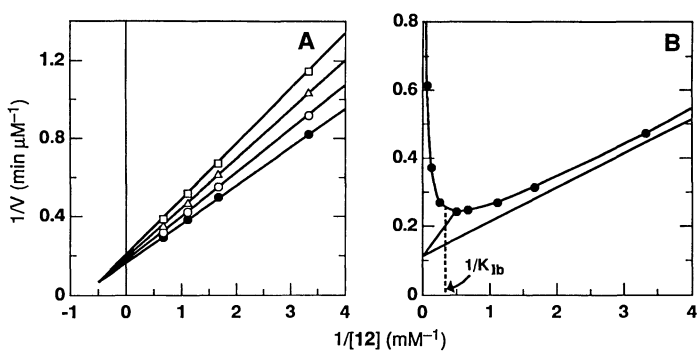


Fig 3. (**A**) Noncompetitive product inhibition by ester 7 with respect to alcohol **12** as the variable substrate. The reactions were performed in 100 mM Bicine, pH 8.5, 10 percent DMSO. The rates were measured by the coupled assay at the indicated concentrations of **12** and 1 mM **3** (vinyl ester). The concentrations of 7 were: (●) 0 mM; (○) 0.30 mM; (△) 0.65 mM; and (□) 1.0 mM. The K_{iq} = 0.39 ± 0.085 mM is an average value derived from expressions for apparent K_{is} and K_{ii} (and K_{ib} = 2.85 mM) (*11*). (**B**) A typical example of substrate inhibition by alcohol **12**. The reactions were performed in I (0.1 ATE, pH 9.0, 10 percent DMSO) in the presence of 4 μM PCP21H3. The rates were measured by the HPLC assay for ester 7 at the indicated concentration of **12** and 0.20 mM vinyl ester **3**. Under these conditions, apparent K_{ib} = 3.3 mM, K_b = 0.6 mM. The data were obtained as described by the method of Cleland (7).

congruent with the hapten, it secures the mechanistic assignment and also establishes that the lipase-like activity, elucidated earlier, proceeds through a covalent intermediate. There was no reaction detected when **8** and **9** were tried as substrates, an indication of the need for a balance of binding energy and reactivity.

The acyl intermediate. The well-known experiment of Hartley and Kilby demonstrated a rapid, stoichiometric release of *p*-nitrophenol from chymotrypsin when *p*-nitrophenyl ethyl carbonate (or *p*-nitrophenyl acetate) was used as a substrate (*12*). Examples of such "burst kinetics" have since been reported with other enzymes and, in several cases, the acyl enzyme has been characterized (*13*). This burst behavior substantiates the existence of a covalent intermediate. Our finding that phenyl esters were excellent group transfer substrates for PCP21H3 pointed to similar studies.

When the *p*-nitrophenyl ester **6** was added to varying concentrations of the antibody with rapid mixing, an equimolar amount of *p*-nitrophenol was quickly formed followed by a slower, steady-state release of this product which was also proportional to the catalyst concentration (Fig. 5A) (*14*). Despite the low ratio of substrate to antibody, the linearity was good with no evidence of product inhibition. The accurate correlation between the observed concentration of the highly purified antibody determined from the burst and its known value suggested that the pre-steady-state exponential phase was of the order of the mixing time (that is, a few seconds). This was confirmed by stopped-flow spectrophotometry. These experiments indicated that the half-life of free antibody was about 1 second (Fig. 5B) (*15*). Interestingly, when the cosolvent was changed from DMSO to DMF (dimethyl formamide) the rate of this phase became slower (Fig. 5A, inset). Other observations also showed that the rate of the transesterification reaction was decreased (about 35 percent) with DMF and might indicate conformational mobility in the active site region perhaps due to the effects of water structure. The ester **6** was also competent as a substrate in the transesterification reaction at pH 7 (Table 1) (*16*). While the k_{cat} obtained at this pH is in itself noteworthy, hypothetical extrapolation to pH 9 affords a value of ~30 min^{-1}. This is perhaps the upper limit of substrate turnover that can be realized by this catalyst.

In related experiments with vinyl ester **3** and high concentrations of PCP21H3, the detection of a burst was attempted with a coupled assay. In this case, when the acetaldehyde was reduced, the burst would appear as a rapid decrease in absorbance from the oxidation of an equivalent of NADH (reduced nicotinamide adenine dinucleotide). Antibody concentrations up to 40 μM (6.0 mg/ml; would give $\Delta A = 0.249$) and vinyl ester concentrations up to 3 mM did not reveal the stoichiometric accumulation of an intermediate. However, a drop in absorbance equivalent to about 30 percent of the expected amount occurred in the presence of protein but not in

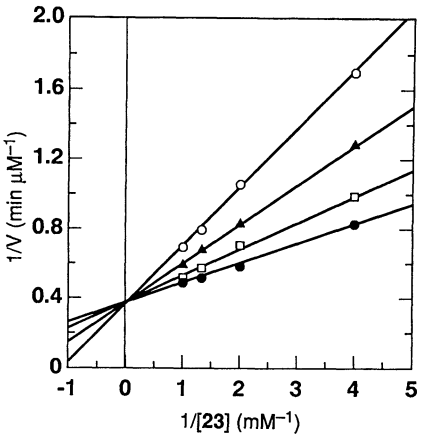


Fig 4. Competitive product inhibition by **12** with respect to **23** as the variable substrate. The reactions were performed in I at 0.1 ATE, pH 9.0, 10 percent DMSO. The rates were measured by the HPLC assay for ester **11** at the indicated concentration of **23** and 1.2 mM ester 7. The concentrations of **12** were: (●) 0 mM; (□) 0.10 mM; (▲) 0.20 mM; and (○) 0.40 mM. The apparent K_i = 0.20 mM (solubility limitations of 7 preclude the assignment of a K_{ip} and a K_{ia} was not determined) (*11*).

Table 1. Kinetic parameters for reactions catalyzed by monoclonal antibody PCP21H3. Initial velocities were determined by following the formation of ester product by high-performance liquid chromatography (HPLC) on an analytical reversed-phase C-18 column (C-18; VYDAC 218TP54) with various isocratic mobile phases of acetonitrile and water (0.1 percent trifluoroacetic acid) and a detector setting of 254 nm. All reactions (total volume, 1 ml) contained antibody at a final concentration of 4 μM; the antibody was stored in buffer and the concentration was determined from an ultraviolet absorbance assay (ϵ_{280} = 1.35 (mg/ml)$^{-1}$ cm^{-1} and an assumed molecular weight of 150,000 for the immunoglobulin G. The mixtures were equilibrated at 23°C, and reactions were initiated by the addition of varying amounts of substrates in DMSO stock solutions to give a final organic phase of 10 percent DMSO in buffer. All reactions were performed in I at 0.1 ATE, pH 9.0, except reaction B, which was done in 100 mM Bicine, pH 8.5, and reaction D which was in I at 0.1 ATE, pH 7.0, 9 percent DMSO, and 1 percent dioxane. Generally four 50-μl portions were removed at timed intervals within a period (all less than 20 minutes), in which no more than 5 percent of the substrate was converted to products; the reaction mixtures were quenched into 200 μl of external standard solution (fivefold dilution), vortexed, kept on ice, and then examined by HPLC. Known amounts of ester product had been calibrated (ratio of peak heights) against the external standard solution which consisted of benzophenone (15 or 30 μM) in the HPLC mobile phase used in the specific assay. The initial rates were obtained from a linear least-squares fitting of the data.

Reaction*	Substrates		K_M (mM)		k_{cat} (min^{-1})†
	Ester	Alcohol	Ester	Alcohol	
A	**3**	**12**	3.0	7.3	21
B	**3**	**12**	2.3	4.9	6.4
C	**4**	**12**	0.19	5.0	19
D	**6**	**12**	<0.040	13	0.33
E	7	**22**	8.5	10	4.3
F	7	**23**	6.9	2.0	4.5
G	**11**	**12**	9.9	1.7	4.7

*Mobile phases (CH_3CN:H_2O, 0.1 percent TFA) and flow rates used for assaying specific reactions above. For reactions A and B: (45:55), 1.5 ml/min; reaction C and D: (40:60), 1.5 ml/min; reactions E, F, and G: (36:64), 2.0 ml/min. †No correction was made for substrate inhibition. Solubility limitations also restricted the magnitudes of experimentally observable k_{cat} values which were decreased three- to fivefold. The calculated k_{cat}'s were used for comparisons.

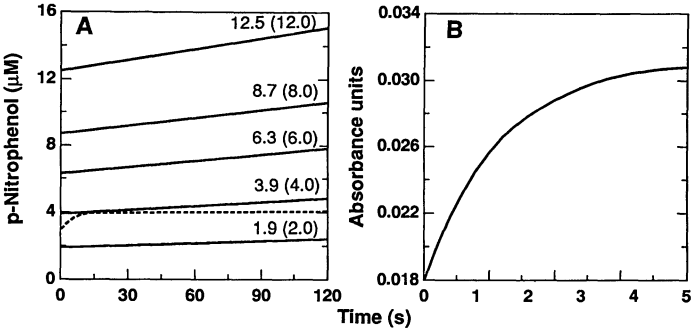


Fig 5. (**A**) The liberation of *p*-nitrophenol during the reaction of PCP21H3 with ester **6**. The reactions were carried out in I at 0.1 ATE, pH 7.0, 10 percent cosolvent (90 percent DMSO, 10 percent dioxane) in the presence of 0.10 mM **6** and varying concentrations of PCP21H3 (indicated by the values in parentheses). The values outside the parentheses correspond to the observed (*y*-intercept) concentration of protein from the "burst" formation of an equivalent of *p*-nitrophenol. Inset (dashed line): A reaction with 4 μM PCP21H3 in which the DMSO cosolvent was replaced with DMF showing the longer duration of the pre-steady-state phase. The steady-state rate is also decreased and can be extrapolated to 3.9 μM. (**B**) The pre-steady-state exponential phase of PCP21H3 acylation by ester **6**. The reaction was followed by measuring the release of *p*-nitrophenol with a stopped-flow spectrophotometer. The conditions were similar to those in (A) to give approximate final concentrations of 2 μM antibody and 0.10 mM **6**. The "burst" corresponds to 1.7 μM and proceeds with $k = 0.67 \pm 0.02$ s^{-1}.

Table 2. The apparent inhibition constants of compounds tested in the reaction between **3** and **12** catalyzed by PCP21H3. The K_i was obtained from a Dixon plot at 1 mM **3** and 2 mM **12** (saturating conditions for **12**) and is defined as the concentration that reduced the velocity to one-half of that in the absence of inhibitor. The inhibitions were assumed to be competitive. The K_i value is based on the expectation that only the (*S*)-enantiomer binds effectively where relevant. The reactions were done in 100 mM Bicine, pH 8.5, 10 percent DMSO in the presence of 4 μM PCP21H3. The rates were measured by the coupled assay.

Compound	K_i (mM)
1	2×10^{-3}*
2, 8, 9	>1†
13	>2†
14	0.65
15	2
17	0.20
18	3
19, 20	>7‡
32–35	>2§
37	0.19
38	1.4

*Determined by the HPLC assay. †Ten percent inhibition. ‡No inhibition detected. §Less than 10 percent inhibition detected.

the background reaction (*17*). Thus these results suggest that the intermediate species, although not completely accumulated, is present at some low steady state (*18*).

Further evidence for the covalent nature of the antibody intermediate was obtained by labeling. Using [^{14}C]acetamido-**6**, we observed that radioactivity remained with the protein after extensive dialysis (*19*).

Rate acceleration and energetics. It is difficult to quantify the magnitude of the rate acceleration afforded by an enzyme compared to that of the uncatalyzed reaction. There may be no measurable rate in the absence of the catalyst and the mechanisms of the nonenzymatic and enzymatic reactions can be entirely different. The analysis of antibody PCP21H3 catalytic efficiency is subject to these constraints.

Background formation of the product ester **7** (or similar esters) was not detectable during the assays. The most extensively investigated was vinyl ester **3**, which showed no reaction at its solubility limits under basic (pH 9.0 ATE, 10 percent DMSO, 0.1 M **12**, 1 hour) or acidic (pH 3.6, acetate buffer, 11 days or 1.25 M HCl, 7 hours; 0.1 M **12**) conditions at 23°C. However, a trace amount of ester was detected at 50°C in pH 9.0 ATE, 10 percent DMSO. A crude extrapolation provided a second-order rate constant for alcoholysis of **3** of k_{ROH} $\sim 10^{-4}$ to 10^{-5} M^{-1} min^{-1} at 23°C (on the basis that E_a = 25 kcal/mol). The low concentration of the required alkoxide is responsible for the virtually undetectable observed background rates (*20, 21*).

The antibody-catalyzed transesterification consists of two chemical steps linked by an acyl-antibody intermediate, while the reaction without antibody is a one-step, base-catalyzed process. Because the mechanisms in the two cases are different, no strict comparison can be made that yields a true effective molarity. However, an operational estimate can be made by comparing k_{cat} (min^{-1}) in the antibody-catalyzed transesterification of **3** by **12** under saturating conditions, to k_{ROH} (M^{-1} min^{-1}) the second-order rate constant for alcoholysis of **3** by **12**. Thus, to achieve the same rate of product synthesis (k_{cat} represents the number of moles of **7** produced per mole of antibody per minute), the concentration of **12** must approach 10^5 to 10^6 M (21 $min^{-1}/10^{-5}$ M^{-1} min^{-1}). Entropic advantages from intramolecularity of 10^5 and greater are well documented with values of 10^8 to 10^{11} generally being viewed as upper limits (*22*).

A particularly efficient mechanism for achieving a rate acceleration is through covalent catalysis. The opportunity for maximum entropy loss can be attained with a tight fixation of reacting groups facilitated by anchoring one of the reacting partners to the protein (*23*). In addition, if the antibody intermediate were an acyl tyrosine (*24*), the greatest catalytic gains would be realized by the alkyl esters where entropy loss in the acylation reaction generates a more reactive bond, paid for by the utilization of binding energy. The vinyl and phenyl esters do not benefit from this enthalpic component and therefore, relative to the uncatalyzed reactions, would show decreased effective molarities. If we assume that the hydrolysis rates for these esters are a rough index of their reactivity in transesterification, then the difference across this series should be $\sim 10^3$. Hence, reactions between esters such as **7** and alcohol **23** might be subject to rate accelerations of 10^7 to 10^8 M. The relative similarities of k_{cat} could signal a change in the rate-determining step. The reactions of the more labile esters are controlled predominantly by deacylation, whereas turnover of the alkyl esters is limited by the acylation step. This is reminiscent of comparisons between ester and amide substrates of the serine proteases (*26*). A similar k_{cat} in reactions of the vinyl ester and much less reactive alkyl ester could be a consequence of differences in transition state energies where the latter requires no further loss of entropy to reach the activated complex. The *sec*-phenethyl moiety is able to "pull" the ester into and orient it at the active site. This shows how binding energy can be expressed in the transition state and can provide catalysis even if it is not at the site of the reaction (*27*). The loss of entropy on substrate binding in conjunction with transition state stabilization affords an effective combination for obtaining rate acceleration.

Induced fit. The role of flexibility and induced fit in enzyme action has been described (*28*). The coupled assay for acetaldehyde was used to show that structures similar to *sec*-phenethyl alcohol, but devoid of an accepting hydroxyl, increased the hydrolytic turnover of **3**. The racemic compounds *sec*-phenethyl chloride and bromide, **15** and **16**, at 2 mM and 1.5 mM, respectively, increased the observed pseudo first-order rate constant (V/K) for antibody-catalyzed hydrolysis of **3** about tenfold. The activation effect showed a concentration dependence up to their solubility limits (2 mM, 1.5 mM). In the presence of 2 mM **15** (~ 0.5 K_i), the initial rate for 1 mM vinyl ester turnover was about 60 to 70 percent as fast as the rate observed with 0.50 mM alcohol **12** (~ 0.5 K_M) as the substrate. The compounds did not change the spontaneous rate of vinyl ester hydrolysis. Thus, these alcohol surrogates apparently activate the acyl antibody for hydrolytic cleavage at a rate roughly one-third slower than alcoholysis of the same species. The effect is quite subtle as indicated by the failure of **14** to enhance turnover. However, this compound and others were good inhibitors of transesterification (Table 2). Thus, a delicate balance of steric and electronic factors is required.

The enhancement of catalysis by substrate surrogates must be reconciled with the ping-pong and burst kinetics of the catalyst. The results suggest that binding by surrogate substrates allows an increase in the rate of hydrolysis of the acyl intermediate. The simplest interpretation is an induced fit model where, in the absence of activator, the antibody either does not have a conformation as accessible to water or one that does not complement the transition state that is achieved in the presence of activator or alcohol. The fact that the rate of acyl transfer to the water-activator surrogate is less than to the alcohol is probably a reflection on the differences in these transition states and additional interactions that might exist with the alcohol. These results have a precedent in natural enzymes. The increased rates of water reactions for phosphoglucomutase and hexokinase in the presence of incomplete substrate analogues has been ascribed to an induced fit of substrates (*29, 30*).

An estimate of the steady-state partitioning of the acyl antibody intermediate was obtained from the rates of formation of ester **7** and acid **2** with ester **6** as the acyl donor (*31*). The results indicate that, while the antibody catalyzes the formation of **2** in the absence of alcohol **12** as expected (that is, esterase activity), the rate of

Table 3. The initial velocities of compounds tested as alternative substrates for PCP21H3. The concentrations were 1 mM **3** and 2 mM **12** or alternative substrate. At these concentrations, **12** is saturating. This reaction was assigned a value of $v = 100$. The other compounds were not necessarily saturated at 2 mM; but in cases where saturation was examined, $v_{rel} < 100$. The reactions were performed in 100 mM Bicine, pH 8.5, 10 percent DMSO in the presence of 4 μM PCP21H3. The rates were measured by the coupled assay.

Compound	v_{rel}
12	100
13	2*
22	28†
23, 24, 25	98, 53, 83
26, 27, 28	25, 85, 85
29, 30, 31	ND, 29, 46
32, 33, 34	10
35	ND
36	10‡

*Also determined at 5 mM **3**, 8 mM **13**; by the HPLC assay. †v_{rel} ~50 at saturation. ‡v_{rel} ~25 at saturation. ND, none detected.

formation of acid is increased in the presence of **12** (16 mM, K_M). Hence, an induced fit of alcohol leads not only to transesterification, but also makes partitioning to water more favorable. A comparison of the two rates under identical conditions suggests that about 5 to 7 percent of the intermediate is lost by hydrolysis. The surrogate **15** also increased the rate of acid formation, but less effectively than the alcohol.

In our studies, the phosphonate hapten defined a "rigid" transition-state-like binding pocket within a flexible protein matrix. The covalent intermediate may incur changes disruptive to the conformation of the antibody congruent to the transition state. Also, the binding of the acceptor alcohol could induce movement to a structure that is more complementary to the transition state (*32*).

Specificity and active site mapping. Examination of several other compounds supplied information about the active site (Table 2). The amine **19**, expected to exist as the ammonium species at pH 8.5, was resistant to binding at concentrations up to 7 mM. The compounds benzoic acid and acetophenone (**37** and **38**) despite their geometric and steric similarity were disparate in binding affinity (benzoic acid being a much better inhibitor). The difference is attributable to the negative charge on the carboxylate. These observations, taken together, suggest that favorable electrostatic interactions are important in binding and that, with neutral substrates, such stabilization might be significant enroute to an anionic transition state. Our data also emphasize the potential contribution of desolvation to catalysis (*1*).

Compounds that could act as alternative substrates are shown in Table 3. The corresponding 4-acetamidophenylacetyl esters of the alcohols **22** to **28** displayed similar reactivity. The substrate specificity could be manifested in both the ground state and transition state. The stereospecificity of the reaction toward the (*S*)- rather than (*R*)-*sec*-phenethyl alcohol (**12** compared to **13**) is a discrimination in binding as revealed by the high K_i for **13**. Also, for **32** to **35**, unfavorable steric effects resulted in their low reactivity and poor inhibitory properties. With a smaller substrate such as **22**, which should have no difficulty binding but does have a reduced k_{cat} (twofold), decreased transition state interactions might occur. The binding energy of the benzylic methyl group (*34*) could "lock" the hydroxylic oxygen in the proper orientation in accord with the transition state model. The thiol **21**, which readily reacts with **3** in the absence of antibody, showed a minimal ability as a substrate. Although the specificity demonstrated by these compounds is not completely understood, it surely hinges on both steric and electronic effects imposed by the active site.

Enzyme-like reversibility. One hallmark of enzymes is their ability to readily catalyze both the forward and reverse directions of a specific reaction. The readiness of PCP21H3 to accept various substrates offered the opportunity for catalysis of a reaction and its exact reverse. Our initial attempt with **7** and **22** and its reverse (**10** and **12**) was not successful. The reverse reaction was too slow to obtain meaningful kinetic data. We turned to other substrates and found that reactions with esters **7** and **11**, and the corresponding alcohols (**12** and **23**), provided data that allowed the calculation of an equilibrium constant with the Haldane relationship (Eq. 4) (*35*).

$$K_{eq} = \left(\frac{V_f}{V_r}\right)^2 \frac{K_p K_q}{K_a K_b} \quad (4)$$

where V_f and V_r are the maximum velocities in the forward and reverse directions and the K's are the respective Michaelis constants. The value obtained was $K_{eq} = 1.1$. An equilibrium constant close to unity is expected for a transesterification reaction involving similar alcohols (*36*).

Abzymes as mimics of enzymes. The data and observations indicate that monoclonal antibody PCP21H3: (i) has both esterase and transferase activities; (ii) efficiently catalyzes transesterifications in aqueous media and is capable of reversibility; (iii) utilizes an acyl-antibody intermediate; (iv) demonstrates an induced fit of substrate probably as a consequence of the conformation of the acyl intermediate and the need to have the active site congruent to the transition state structure; and (v) operates under two general principles, entropy loss and transition state stabilization, which rely on the energy of protein-substrate interaction.

In displaying these effects, the antibody PCP21H3 has the qualities of both a highly evolved and a primitive catalyst. It exemplifies the remarkable utilization of binding energy to generate, through a covalent intermediate, an ester product in the presence of water. However, it cannot completely sequester it from water to decouple esterase and transferase activities. This is in marked contrast to most group transferase enzymes, which enforce the preservation of high energy substrates or intermediates. While induced fit occurs in the presence of substrate as a consequence of haptenic complementarity, it has not been invoked to promote the utmost specificity. A more highly evolved antibody would harmonize transition state stabilization, covalent catalysis, and induced fit with exclusion of water. Such concerted action may yet be found in the realm of antibody diversity and then naturally occurring catalysts will have been mimicked.

Our study may have implications regarding the evolution of catalytic efficiency in enzymes. Antibody induction is primarily a response to the antigen structure as dictated by the chemistry of protein molecules. However, a catalytic antibody, must catalyze a particular reaction. A preference for the reaction mechanism is implicit in the design of the hapten. In this case, the antibody favors the tetrahedral geometry and anionic nature of the transition state and the precise alignment of the two substrates. However, nothing in the design dictated the emergence of either covalent catalysis or the characteristic of induced fit. That these properties are in fact manifested by the antibody, suggests that their appearance is a consequence of coordinating the immunological property of tight binding with the additional requirement of efficient catalysis. The expression of this optimization is the emergence of a catalyst that exhibits the strategies of highly evolved enzymes.

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compatible with the assay], in the presence of 300 units of alcohol dehydrogenase (ADH), 300 μM β-NADH, and 4 μM PCP21H3 (for catalyzed reactions) at 25.0°C in 1-ml (1-cm) cuvettes. The rates were monitored from the decrease in absorbance at 340 nm resulting from the disappearance of β-NADH ($\epsilon = 6.22 \times 10^{-3}$ μM^{-1} cm^{-1}). A Shimadzu UV2100U spectrophotometer equipped with an automatic cell changer and Peltier temperature control was used. A typical preparation of reactions was as follows: A solution was prepared consisting of 100 mM Bicine, pH 8.5, solid ADH (1 μg/ml) (from baker's yeast; 305 units per milligram of protein, 270 units per milligram of solid; Sigma), and β-NADH (0.25 mg/ml) (Grade III, disodium salt; Sigma), which was kept on ice. An 825-μl portion of this solution was added to a cuvette and then 75 μl of PCP21H3 solution (stock at 8.0 mg/ml in 100 mM Bicine, pH 8.5; kept on ice). All organic compounds, except the vinyl ester, were then added as DMSO solutions (or just DMSO, where necessary) and the components were mixed (Mini-Mix; Precision Cells, Inc.). The solutions were then equilibrated for 15 minutes and the reaction was initiated with the vinyl ester in DMSO (usually as a 10-μl portion; 100 μl of total DMSO) and then mixed (Mini-Mix). The ADH activity varied with different lots of β-NADH and could be especially sensitive to β-NADH inhibitors under the assay conditions. An acceptable background initial rate of spontaneous vinyl ester decomposition at 1 mM was 7 ± 1 μM/min ($t_{1/2}$ = 141 ± 19 min^{-1}). The initial rates for experiments over the course of a day (with same solutions and other items) had a precision of 5 percent, but from one day to another could vary by 20 percent. Hence, a background (or control) reaction was run for each new solution. The background rate was subtracted from the rates of catalyzed reactions.

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15. The reactions were done under the same conditions as the steady-state experiments with a stopped-flow spectrophotometer; a 0.2-ml cell; 1-cm path length; 0.2-ml stop volume; 403 nm; filter time 10 to 33 ms. Syringe 1 was charged with 3 to 4 μM PCP21H3 in buffer. Syringe 2 was charged immediately before the addition with a solution of 0.2 mM **6** in buffer, 20 percent DMSO, 2 percent dioxane. An initial absorbance of 0.01 to 0.018 was usually observed.
16. The ester **6** was found to saturate at concentrations down to 0.040 mM. The k_{cat} was obtained at 0.10 mM **6** with **12** as the variable substrate.
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24. A plot of log V_{max} as a function of pH (pH 7 to 10) revealed an inflection point. The data were fit to the equation $\log V_{max} = \log (V_{lim}/(1 + [H^+]/K_a))$ and gave $pK_a = 9.4$, $V_{lim} = 103$ μM/min. A partial analysis of a V/K profile (2 mM **12**, varying **3**) showed no significant difference. While this kinetically determined pK_a is likely not indicative of the true pK_a because of the superimposition of equilibrium processes (*25*), a value in this range probably represents that of a tyrosine or cysteine residue. The catalytic stability of PCP21H3 (several weeks at pH 9.0 ATE, 8 to 9 mg/ml, 4°C with no loss of activity) makes it difficult to implicate the involvement of a cysteine in catalysis. The high proportion of tyrosine residues generally found in the variable region of antibodies makes it a more probable choice for the participatory amino acid.
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32. An Eyring plot of the dependence of k_{cat} on temperature (5° to 50°C) for the reaction between **3** and **12** was curvilinear. The nonlinearity was not a result of thermal inactivation or changes in fractional saturation of the antibody and could indicate that two or more steps contribute to rate determination. The activation energies (E_a) obtained from linear fits of the low- and high-temperature regions were E_a = 19.9 kcal/mol and E_a = 9.9 kcal/mol, respectively. Quinn, using isotope effects as a probe, ascribed curvilinear Eyring plots for acetylcholine esterase to rate determination by a virtual transition state involving protein conformational changes (induced fit) (*33*). We cannot state conclusively that our temperature dependence incorporates such changes, but the involvement of a covalent intermediate seems conducive to such behavior.
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30 January 1991; accepted 2 April 1991