

Figure 5. Energy diagrams for hydrolyses catalyzed by chymotrypsin in aqueous solution (solid line) and in 50% DMF solution (dashed line). See Tables II and III for parameters.

chymotrypsin-catalyzed hydrolyses of ester and amide substrates along with aminolysis studies, we begin to understand why the active-site-methylated serine protease is an effective catalyst for peptide synthesis. When the enzyme is methylated, the transition-state energy required for ester hydrolysis increases more than the energy required for aminolysis. The acyl intermediate of methylchymotrypsin is relatively less stable than that of the native enzyme, as calculated from the k_{cat} values of ester hydrolysis and the known transition-state energy change. The energy barrier for the formation of acyl intermediate-nucleophile complex for aminolysis is therefore lower. In addition, the affinity of the peptide product to the methylated enzyme increases for charged or hydrophilic products (data not shown). These two factors cause the peptide formation to be a more irreversible process and account for the favorable aminolysis vs hydrolysis in the methylenzyme-catalyzed aminolysis. Energy diagrams (Figure 4)⁴² for hydrolysis

of activated substrates (Tables II and III) catalyzed by both the native and methylated chymotrypsin provide some insights into the effect of methylation on catalysis and binding. It should be noted that since synthetic peptides are not substrates for methylchymotrypsin, the $\Delta\Delta G$ for aminolysis was obtained from the hydrolysis of an activated amide. This energy difference for aminolysis, therefore, may not reflect the real value in aminolytic peptide synthesis. Similar situations were observed in the case of organic cosolvent mediated catalysis. As indicated in Figure 5, addition of DMF destabilized the acyl intermediate and enhanced the aminolysis reaction.

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(42) With serine proteases it has been established that with ester substrates the rate-limiting chemical step is deacylation⁴³ of the acyl enzyme (ES'), or k_3 . By use of k_3 and the k_{cat} determined for the amide substrate, k_2 (from ES complex to the acyl intermediate) can be determined based on $1/k_2 = 1/k_{cat}(\text{amide}) + 1/k_3$. Once k_2 is calculated, K_2 , the ES dissociation constant is determined by dividing k_2 by the second-order rate constant, k_{cat}/K_M (for the amide substrate). All the first-order rate constants were converted to free energies of activation by using the Eyring equation or transition-state theory.⁴⁴ The second-order rate constants and equilibrium constants were normalized to a 1 μM standard state before relative energies were calculated.⁴⁵ The equilibrium constants were converted to calories by using the thermodynamic relationship, $\Delta G^\circ = -RT \ln K$. The relative energy difference for ES', as compared to the free enzyme, can be estimated⁴⁶ by subtracting the free energy of activation for the ester k_{cat}/K_M [$\Delta G^\circ(k_{cat}/K_M)$] from the free energy of activation for k_{cat} [$\Delta G^\circ(k_{cat})$], that is, $\Delta\Delta G^\circ(\text{ES}') = \Delta\Delta G^\circ(k_{cat}/K_M) - \Delta\Delta G^\circ(k_{cat})$.

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Selective Cleavage of Trityl Protecting Groups Catalyzed by an Antibody[†]

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Abstract: Monoclonal antibodies have been produced that catalyze the cleavage of trityl protecting groups at neutral pH. These antibodies were induced by immunizing mice with a positively charged tris(4-methoxyphenyl)phosphonium hapten. With a 4,4',4''-trimethoxytrityl ether substrate, a rate enhancement (k_{cat}/k_{uncat}) of 270 was observed. Several other trityl ether derivatives and even a trityl thioether compound were accepted as substrate. The pH-rate profile of the reaction suggests that the observed rate acceleration is not the result of general-acid catalysis in the antibody binding site, but probably derives from electrostatic stabilization of a positively charged transition state.

A relatively large number of different chemical transformations have now been catalyzed in an antibody binding pocket.¹⁻¹¹ Exquisite and predictable substrate specificity, including stereospecificity, has been observed consistent with the structure of the original immunizing hapten. We now report the production of an antibody capable of catalyzing the cleavage of triphenylmethyl (trityl) protecting groups^{12,13} at neutral pH. This work represents the first examples of ether and thioether cleavage catalyzed by an antibody. In theory, a set of chemically similar yet structurally

distinct protecting groups can be developed, each one cleavable by a specific antibody at neutral pH. This "recognition-based

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Table I. Michaelis–Menten Parameters Determined in 25 mM Phosphate Buffer, pH 6.0, for a Variety of Substrates with Monoclonal Antibody 37C4^a

cmpd	R ₁	R ₂	dR ₃	X	R ₄	k _{cat} , min ⁻¹	K _m , μM	K _{cat} /K _{uncat}
2	OCH ₃	OCH ₃	OCH ₃	O		0.10	31	270
3	OCH ₃	OCH ₃	H	O		0.072	30	200
4	OCH ₃	H	H	O		4.2 × 10 ⁻³	30	230
5	OCH ₃	OCH ₃	OCH ₃	S		2.2 × 10 ⁻³	33	78
6	H	H	H	N		no significant rate acceleration		
7	OCH ₃	OCH ₃	H	O		no significant rate acceleration		

^a Reactions were run as detailed in the Experimental Section.

selectivity” of protecting group cleavage could be useful during the synthesis of chemically sensitive compounds not stable to currently used deprotection conditions.

A tris(4-methoxyphenyl)phosphonium compound **1** was used as hapten to elicit the catalytic antibodies. It was anticipated that the triarylphosphonium group would induce antibody binding pockets capable of accommodating an analogous substrate trityl protecting group. Furthermore, the positive charge of the phosphonium hapten was expected to resemble the growing positive charge in the trityl substrate, which presumably occurs during the acid-catalyzed detritylation reaction. Charge stabilization by the antibody might therefore lead to catalysis of the trityl cleavage reaction.

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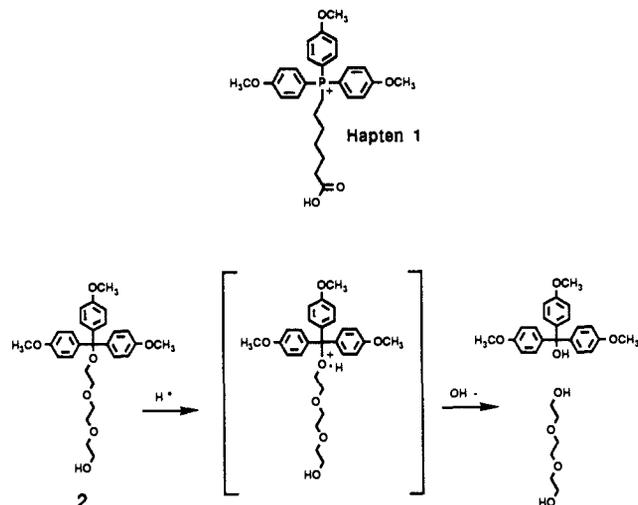


Figure 1. Structure of phosphonium hapten **1** and reaction catalyzed by monoclonal antibody 37C4.

Results and Discussion

The hapten **1** was synthesized from tris(4-methoxyphenyl)-phosphine and 7-bromoheptanoic acid. This was covalently linked to the carrier protein keyhole limpet hemocyanin (KLH) via the carboxylate terminus.¹⁴ The hapten–KLH conjugate was used to immunize 129GIX⁺ mice in a standard immunization regimen.¹⁵ By use of modified protocols,¹⁶ a total of 11 monoclonal antibodies

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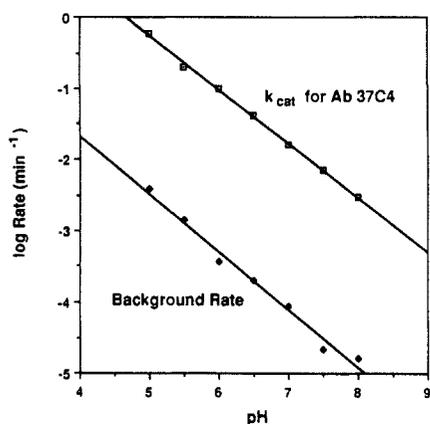


Figure 2. Logarithmic plot of the pH-rate profiles for the reaction of **2** with (as k_{cat} , upper trace) and without (lower trace) monoclonal antibody 37C4. The drawn lines are the result of the best linear fit of all points. Reactions were run with 25 mM phosphate buffer at the given pH; everything else was as described in the Experimental Section. Note the ionic strength was not constant throughout the pH range.

were produced and purified that specifically bound hapten **1** as determined by enzyme-linked immunosorbent assay (ELISA). The antibodies were initially screened for catalytic activity by using the substrate **2**. The polyether "tail" of **2** was necessary to provide adequate water solubility.

Eight of the 11 antibodies showed significant catalytic activity with **2** as determined by an HPLC assay. One antibody, 37C4, was analyzed in detail. For the catalytic reaction of 37C4 with substrate **2** in 25 mM phosphate buffer, pH 6.0, a k_{cat} of 0.10 min^{-1} and a K_m of $31 \mu\text{M}$ were measured. The background rate for the reaction under these conditions is 0.00037 min^{-1} , so the rate enhancement due to antibody ($k_{\text{cat}}/k_{\text{uncat}}$) is 270. Even after 21 turnovers per binding site (the most investigated), the antibody showed no loss of catalytic activity.¹⁷

Substrate Specificity. Significant rate enhancements were also seen with the 4,4'-dimethoxytrityl and 4-monomethoxytrityl ether substrates **3** and **4** as well as the 4,4',4''-trimethoxytrityl thioether substrate **5**. All of the compounds accepted as substrate by antibody 37C4 displayed saturation kinetics and the Michaelis-Menten parameters were determined as listed in Table I. As expected, hapten **1** was an extremely potent inhibitor of the catalytic reactions. A stoichiometric¹⁸ amount of **1** added to the reaction mixture virtually eliminated catalytic activity, offering persuasive evidence that the reactions indeed occur in the antibody combining site. An estimated dissociation constant of 25 nM for **1** with 37C4 was determined by competition ELISA.¹⁹ By virtue of comparison to authentic samples (UV spectrum, HPLC retention times), the trityl products of the antibody-catalyzed reactions were determined to be the trityl alcohols as illustrated in Figure 1. Product inhibition of the catalytic reaction was observed and an estimated dissociation constant for 4,4',4''-trimethoxytrityl alcohol of $80 \mu\text{M}$ with 37C4 was determined by competition ELISA.

As listed in Table I, all of the compounds accepted as substrate displayed a similar K_m value near $30 \mu\text{M}$. In as much as this value approximates an apparent binding constant for antibody and substrate, it appears that the 4-monomethoxytrityl substrate **4** bound with similar affinity to 37C4 as did the 4,4',4''-trimeth-

oxytrityl substrates **2** and **5**. Furthermore, the compound with no methoxy substituents (**6**) was neither a substrate for 37C4 nor an inhibitor of its reaction with **2**. Thus, even though a tris(4-methoxyphenyl)phosphonium hapten was used, a single 4-methoxy group on a trityl substrate seems sufficient and necessary for binding to 37C4, which in turn leads to the catalytic reaction. Antibody 37C4 displayed further substrate selectivity by not catalyzing the detritylation of the sterically hindered 5'-(4,4'-dimethoxytrityl)nucleoside derivative **7**.

pH Dependence. The pH-rate profiles of the reaction with **2** catalyzed by 37C4 as well as the background reaction were determined and the results are plotted in Figure 2. The similar logarithmic increase in rate with decreasing pH for the two traces suggests that the proton involved in both the background and antibody-catalyzed reactions is derived from the aqueous medium, and also that no ionizable side chain with a $\text{p}K_a$ between 5 and 8 is essential to the catalytic reaction. These findings show that the observed rate enhancement with 37C4 is not due to general-acid catalysis inside the antibody binding pocket. Previous studies of catalytic antibodies derived from positively charged ammonium or pyridinium haptens displayed a pH dependence consistent with direct participation of ionizable residues in the antibody.^{2,9}

Crystallographic studies have revealed an almost ideal tetrahedral geometry of both trityl groups²⁰ and triarylphosphonium compounds.²¹ Since these types of compounds bind tightly to 37C4, the antibody binding pocket is probably most complementary to tetrahedral structures. On the other hand, the presumed high-energy intermediate trityl cation is trigonal planar.²² Thus, the rate enhancement observed with 37C4 is probably not occurring via drastic geometric distortion of the trityl group in the binding site to help stabilize the planar trityl cation. However, subtle distortion not discerned from the structural data cannot be ruled out as contributing to catalysis.

The acid-catalyzed trityl cleavage reaction most likely results from an initial protonation step and then heterolytic bond breaking with formation of a trityl cation. In the absence of general-acid catalysis or geometric distortion, the most likely explanation for the observed rate enhancement with 37C4 is stabilization of a positive charge inside the antibody. The phosphonium hapten (**1**) possesses a positive charge; so it is reasonable to assume the induced antibody binding pocket accommodates this charge with some degree of complementary negative charge. Once the neutral trityl substrate is bound by the antibody, the energy of a positively charged transition state should be less than in bulk solution by virtue of this charge complementarity²³ thereby leading to the observed rate acceleration. The electrostatic interaction could be the result of an ionized Glu or Asp carboxylate (with a $\text{p}K_a$ less than 5.0) or perhaps specific alignment of dipoles acting in concert. Since both protonation and trityl cation formation involve a positive charge, either or both of these events could be assisted by the antibody.

The relatively slow catalytic rate enhancement and product inhibition observed with 37C4 must be improved before these antibodies will be of general synthetic utility. Modification of the antibody combining site or screening larger numbers of antibodies may be ways to overcome such difficulties. The advent of facile systems for the cloning, mutagenesis, and expression of the diverse repertoire of antibody combining sites in microorganisms²⁴ makes such antibody catalyst engineering²⁵ and

(17) The positively charged trityl cation presumably produced during the reaction could have irreversibly alkylated the antibody, thus shutting down the catalysis. The observed multiple turnovers indicate this alkylation does not occur to a significant extent, and more work is needed to see how many turnovers are possible with 37C4.

(18) Stoichiometric with respect to antibody combining sites.

(19) In these experiments, the antibody was preincubated with various concentrations of the compound of interest. The solution was then placed on a microtiter plate coated with a hapten 1-bovine serum albumin conjugate and incubated for 15 min. The rest was carried out just like a normal ELISA. The reported estimated dissociation constant was the concentration of compound required to prevent 50% of the antibody from binding to the plate.

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(25) Site-directed mutagenesis of an antibody combining site with an increase in catalytic rate has recently been reported: Baldwin, E.; Schultz, P. G. *Science* **1989**, *245*, 1104.

screening feasible, and we are now investigating these possibilities.

Conclusions. With a (4,4',4''-trimethoxytriphenyl)phosphonium compound (**1**) as hapten, monoclonal antibodies were produced capable of catalyzing the selective cleavage of trityl protecting groups at neutral pH. This represents the first examples of ether and thioether cleavage catalyzed by an antibody. For one of these antibodies investigated in detail, 37C4, catalysis was observed with the ether and thioether substrates **2–5** containing three, two, or one methoxy group on the trityl moieties. Compounds containing no methoxy substituents (**6**) or increased steric hindrance (**7**) were not accepted as substrates. We would like to combine this observed substrate specificity with more effective differentiation of trityl groups. Future designs of haptens will make allowances for the entire structure of the intended substrate (nucleoside, sugar, etc.) as well as utilize groups on the phenyl rings very different from methoxy in charge and shape. We hope that these differences will program the resulting antibodies with an increased ability to distinguish between corresponding trityl substrates, thus allowing selective removal of structurally distinct yet chemically similar protecting groups at neutral pH.

The pH-rate profile for the reaction with **2** indicated that the catalytic rate enhancement does not result from general-acid catalysis, but probably derives from electrostatic stabilization of a positively charged transition state inside the antibody binding site. We are currently investigating other reactions thought to involve carbocations that might be accelerated by an antibody catalyst.

Experimental Section

General Procedures. HPLC analysis was carried out on a Hewlett-Packard 1090M chromatograph equipped with diode array detection. Purification of hapten and antibodies utilized a standard FPLC (Pharmacia). ^1H NMR (300-MHz) spectra were recorded on a Bruker AM 300 spectrometer. Mass spectral analysis was performed at the Midwest Center for Mass Spectroscopy at Lincoln, NE; a National Science Foundation Regional Instrumentation Facility (Grant CHE 8211164). All trityl substrates were prepared according to published procedures.¹³ All chemicals were purchased from Aldrich Chemical Co. unless stated otherwise and used without further purification. Water was doubly distilled, and all aqueous solutions used with the catalytic reactions were sterilized by autoclaving.

HPLC Analysis of Catalytic Reactions. The reaction sample was injected onto a 4.6 mm \times 100 mm Propyl column (Synchrom, Inc.) with precolumn (Uptight, Upchurch Scientific, Inc.) and eluted isocratically with either 83% water–17% acetonitrile (substrate **2**) or 80% 10 mM ammonium carbonate (pH 8.0)–20% acetonitrile (substrates **3–7**) at a flow rate of 1 mL/min and monitored at 225 nm. Starting materials eluted between 1.3 and 2.0 min. The product trityl alcohols eluted between 3.0 and 3.2 min, and these were identified by coinjection with authentic materials as well as comparison of UV spectra.

Tris(4-methoxyphenyl)-(6-carboxyhexyl)phosphonium Bromide (1**).** Tris(4-methoxyphenyl)phosphine (1.0 g, 2.8 mmol) and 7-bromoheptanoic acid (0.71 g, 3.4 mmol) were placed in 10 mL of dry benzene and heated at reflux for 15 h. The benzene solution was washed with 0.1 M sodium bicarbonate solution, the aqueous layer was kept, and the product was purified by reverse-phase chromatography (Pharmacia HR 10/10 PEP RPC column on FPLC eluted with a 20-min linear gradient of 0–30% acetonitrile in water, flow rate 2.0 mL/min; product eluted as a broad peak near 10% acetonitrile) to yield 0.95 g (71%) pure material: ^1H NMR (300 MHz, D_2O) δ 7.42–7.35 (m, 6 H, Ar), 6.97–6.94 (m, 6 H, Ar), 3.67 (s, 9 H, CH_3O), 2.90–2.80 (m, 2 H, PCH_2), 2.85 (t, 2 H, $\text{CH}_2\text{CO}_2\text{H}$, $J = 7.3$ Hz), 1.39–1.37 (m, 2 H, CH_2), 1.23–1.20 (m, 4 H, CH_2), 1.07–1.03 (m, 2 H, CH_2); MS (HRFAB) m/z calculated for $\text{C}_{28}\text{H}_{34}\text{O}_5\text{P}$ 481.2144, found 481.2123.

Preparation of Protein Conjugates. Phosphonium compound **1** (2.0 mg, 4.0 μmol) and 4.0 mg of keyhole limpet hemocyanin (Sigma Chemical Co., dialyzed into water just prior to use) were placed in 0.85 mL total volume of water; 50 μL of 0.1 M *N*-hydroxysulfosuccinimide (Pierce Chemical Co.) and 100 μL of 1 M [1-ethyl-3-(dimethylamino)propyl]-carbodiimide were added for final concentrations of 5 and 100 mM, respectively.¹⁴ The pH was adjusted to 7.5–7.8 with 0.1 N NaOH via syringe. After 20 min at room temperature, 3.0 mL of 50 mM glycine, pH 7.2, was added and this solution was used for immunizations. A similar conjugate was prepared with bovine serum albumin by an identical coupling procedure and then purified on a Pharmacia PD-10 column; this was utilized for identifying antibody binding with ELISA. Use of significantly more phosphonium compound **1** (10–20 mg) in the coupling reaction produced conjugates that were not immunogenic.

Immunization and Monoclonal Antibody Purification. The hapten **1**-keyhole limpet hemocyanin conjugate was used to immunize 129GIX⁺ mice according to published protocols.¹⁵ Monoclonal antibodies were produced by slightly modified methods.¹⁶ The monoclonal antibodies were purified from the crude ascites fluid by the following procedures. To the crude ascites was added 83% by volume of saturated ammonium sulfate solution. After sitting on ice for at least 30 min, the mixture was placed in a centrifuge at 9000 rpm for 20 min at 4 $^\circ\text{C}$. The pellet was dialyzed against 50 mM phosphate–10 mM sodium chloride, pH 7.8, and then 50 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.5. The dialyzed solution was placed on a DEAE-Sepharose column (Pharmacia, 10 mL of dry beads to 100 mg of crude antibody) and the column was washed with 50 mM Tris, pH 7.5 (100 mL). The antibody was eluted with 50 mM Tris, pH 7.5, containing 50 mM NaCl (100 mL), 100 mM NaCl (200 mL), 150 mM NaCl (200 mL), 250 mM NaCl (100 mL), and 500 mM NaCl (100 mL). The antibody usually eluted in the 100–150 mM NaCl fractions. The antibody-containing fractions were concentrated to 15 mL and loaded onto a FPLC HR 10/30 column containing protein G–Superose (Pharmacia). After being loaded, the column was washed with 50 mL of 87.2 mM disodium hydrogen phosphate–6.4 mM citric acid, and the antibody was eluted with 10.8 mM disodium hydrogen phosphate–44.6 mM citric acid. During the entire purification ELISA was used to locate the antibody-containing fractions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PhastSystem, Pharmacia) was used to assess purity of the final antibody, and antibody was virtually the only protein present in these preparations judging from the gels stained with Coomassie blue. The purified antibody was dialyzed against 1 mM NaCl in 5 mM phosphate, pH 7.0, and frozen in solutions containing ~ 10 mg/mL antibody. The solutions were thawed just prior to use.

Catalytic Reactions. In a typical reaction, 1–10 μM antibody was placed with 50–500 μM substrate (from an acetonitrile stock solution) in 25 mM phosphate buffer at the desired pH. The final volume of acetonitrile in the reactions never exceeded 1%. The reaction mixture was immediately divided into 40- μL aliquots in microcentrifuge tubes. At the desired time, the reactions were quenched by the addition of 40 μL of acetonitrile to precipitate the antibody, which could interfere with HPLC. The product trityl alcohols were also very soluble in this mixture, so any precipitated product was recovered. A 20- μL aliquot of the supernatant was injected onto the HPLC for analysis. Kinetic parameters for the catalytic reaction were determined by integrating the amount of trityl product observed and using standard Lineweaver–Burk analysis.

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