Antibody-Catalyzed Hydrolysis of an Unsubstituted Amide

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Abstract: The generation of antibodies capable of catalyzing the unassisted hydrolysis of unactivated amides has been an enduring goal of research in the field. Antidialkylphosphinate **1** monoclonal antibodies were screened for their ability to catalyze the hydrolysis of four methyl esters and four primary amides at pH 5.0, 7.0, and 9.0. One of 68 antibodies, 13D11, enantiospecifically hydrolyzed the C-terminal carboxamide of a dansyl-alkylated derivative of (*R*)-phenylalaninamide (**2b**). At pH 9.0, 13D11 catalyzed amide hydrolysis with a k_{cat} of 1.65×10^{-7} s^{-1} and a K_m of 432 μM and was stereospecifically inhibited by hapten with a K_i of 14.0 μM. A shorter, acetylated derivative **3b** was not hydrolyzed by 13D11, demonstrating that dansyl–alkyl binding interactions are essential for catalysis. Equally active antibody preparations were obtained from two separate batches of ascites. The antibody Fab′ fragment was prepared, purified, and found to retain full activity. Amidolytic activity was not abolished by any of nine inhibitors of natural proteolytic enzymes. The rate of uncatalyzed amide hydrolysis was experimentally determined to be 1.25×10^{-9} s^{-1}, indicating an antibody catalytic rate enhancement (k_{cat}/k_{uncat}) of 132.

Introduction

One of the most frequently cited goals of catalytic antibody research is the generation of antibodies capable of efficient, specific hydrolysis of amide bonds.[1] This goal partly results from the scientific challenge of generating an antibody to catalyze a relatively kinetically inert reaction and partly from the potentially vast range of practical applications that proteolytic antibodies would have. Until now, no haptens have been designed which have demonstrably elicited antibodies that hydrolyze unactivated amides without cofactor assistance. In addition to peptide bonds, primary amides are attractive targets for catalytic antibody hydrolysis (Scheme 1). Antibodies capable of hydrolyzing the terminal amides present in asparagine and glutamine side chains and in the C-termini of many critical peptide hormones may be therapeutically[2] and industrially[3] valuable. Here we describe the first hapten-elicited catalytic antibody capable of catalyzing the cofactor-independent hydrolysis of a primary amide bond. Extensive screening of 68 anti-**1** (Figure 1) monoclonal antibodies against four primary amide (Figure 1, **2a,b** and **3a,b**) and four methyl ester (Figure 1, **2c,d** and **3c,d**) substrates yielded one antibody that catalyzed the hydrolysis of the dansyl-alkylated derivative of (*R*)-phenylalaninamide **2b**. Because the Gibbs free energies of activation for hydrolysis are similar for both primary amide and peptide bonds,[4,5] these results demonstrate the general feasibility of unassisted antibody-catalyzed hydrolysis of amide bonds.

Scheme 1

Experimental Section

Organic Synthesis. General Procedures. Melting points were taken on a Haake Buchler melting point apparatus and were uncorrected. A GE-300 NMR spectrometer was used for ^1H and ^{13}C NMR spectra. A Varian XL-300 (300 MHz) NMR spectrometer was used for ^{31}P NMR, operating at 120 MHz and using an internal standard of 80% H_3PO_4. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN). Mass spectra were acquired on a Finnigan 4600 quadrupole mass spectrometer (for chemical ionization (CI)) and a VG ZAB-25E mass spectrometer (for fast atom bombardment (FAB)). Samples analyzed by CI used ammonia by the direct exposure probe method (DEP). Optical rotations were measured with a Perkin-Elmer 241 polarimeter at ambient temperatures (22 ± 2 °C). High-pressure liquid chromatography (HPLC) was carried out on either a Perkin-Elmer Series 410 LC or a Waters HPLC System, both equipped with Vydac C18 columns (5 μm, 10 × 250 mm, The Separations Group, Hesparia, CA).

Tetrahydrofuran (Fischer, reagent grade) was predried over CaH_2 and distilled from benzophenone ketyl under argon just prior to use. Methylene chloride was distilled over phosphorus pentoxide. Triethylamine was distilled from KOH. Dimethylformamide was Aldrich Sure Seal. Preparative thin-layer chromatography (TLC) was carried out with 500-μm precoated silica gel plates with a fluorescent indicator (E. Merck).

Synthesis of [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]phosphinic Acid (5). To a solution of **4** (12.95 g, 70 mmol) in dioxane and aqueous 1 M NaOH (75 mL) at 0 °C was added dropwise benzyl chloroformate (20 mL). The pH of this solution was kept at 8–9 by the addition of aqueous 1 M NaOH (75 mL). The reaction was stirred for an additional 16 h and then was concentrated *in vacuo* to half-volume. The mixture was washed with diethyl ether (2 × 75 mL) and acidified to pH 1 with concentrated aqueous HCl. Following extraction with ethyl acetate (3 × 80 mL), the combined organic phases were washed with water (1 × 20 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated *in vacuo* to give after crystallization from ethyl acetate–light petroleum 15.44 g (69%) of [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]phosphinic acid (**5**), mp 136–137 °C (lit.[6] mp 137 °C). Confirmation was obtained spectroscopically: ^1H NMR (300 MHz, CD_3OD) δ 2.76 (m, 1 H), 3.11 (m, 1 H), 3.95 (m, 1 H), 4.91 (d, 1 H, J = 12.7), 4.97 (d, 1 H, J = 12.7),

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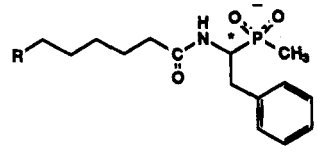
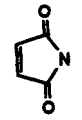
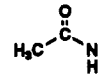
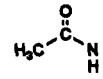
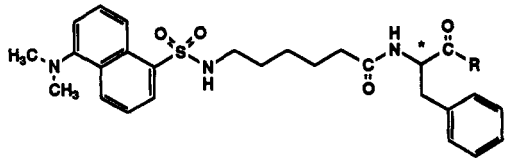
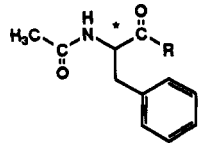


Compound	R	*Configuration (R or S) or Optical Rotation
1a		R/S mixture
1b		$[\alpha]_D^{24}$ -33.8° (c = 0.157, MeOH)
1c		$[\alpha]_D^{24}$ +33.5° (c = 0.155, MeOH)



Compound	R	*Configuration
2a	NH_2	S
2b	NH_2	R
2c	OCH_3	S
2d	OCH_3	R



Compound	R	*Configuration
3a	NH_2	S
3b	NH_2	R
3c	OCH_3	S
3d	OCH_3	R

Figure 1. Structures of the hapten (**1a**), hapten-based inhibitors (**1b,c**), dansyl-alkylated substrates (**2a–d**), and acetylated substrates (**3a–d**).

6.93 (d, 1 H, $J \approx 540$), 7.10 (m, 10 H); ^{13}C NMR (75 MHz, CD_3OD) δ 33.83 (d, ^2J(P–C) = 2.9), 53.81 (d, J(P–C) = 105.21), 67.53, 127.55, 128.50, 128.82, 129.38, 130.22, 138.13, 138.74, 138.93, 158.54; FAB MS 342 (M + Na)$^+$, 320 (M + 1)$^+$. Elemental Analysis. Calcd for $C_{16}H_{18}O_4NP$: C, 60.19; H, 5.68; N, 4.39. Found: C, 60.10; H, 5.77; N, 4.47.

Preparation of Methyl [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]methylphosphinate (6). [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]phosphinic acid (**5**) (510 mg, 1.60 mmol) was dissolved in methylene chloride (5 mL) and methanol (1 mL), and a solution of (trimethylsilyl)diazomethane (10% by weight in hexane) was added until the yellow color persisted. Glacial acetic acid was then added until the yellow color dissappeared. The solution was evaporated *in vacuo* to dryness. The residue was redissolved in dry THF (2 mL) and the solution transferred to a suspension of sodium hydride (37 mg, 1.53 mmol) in THF (2 mL), which was cooled to 0 °C. The reaction mixture was stirred at room temperature for 30 min, after which iodomethane (2.25 mmol) was added. After a further 3 h of stirring, the reaction was quenched by the addition of saturated aqueous NH_4Cl solution (35 mL) and extracted with ethyl acetate (75 mL). The organic phase was washed with water (5 mL), dried over anhydrous Na_2SO_4, filtered, concentrated *in vacuo*, and chromatographed on silica gel using methanol:methylene chloride (8:92) which gave 0.190 g (34%) of **6**. This was confirmed as a mixture of diastereomers by spectroscopy: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (d, 3 H, J = 13.5), 2.86 (m, 1 H), 3.25 (m, 1 H), 3.67 (d, 3 H for one diastereomer, J = 10.4), 3.74 (d, 3 H for one diastereomer, J = 10.5), 4.28 (m, 1 H), 4.99 (s, 2 H for one diastereomer), 5.00 (s, 2 H for one diastereomer), 5.20 (d, 1 H for one diastereomer, J = 10.1), 5.52 (d, 1 H for one diastereomer, J = 9.8), 7.24 (m, 10 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.28 (d, J(P–C) = 90.6), 11.64 (d, J(P–C) = 89.7), 33.96, 34.49, 51.23 (d, J(P–C) = 108.8), 51.51, 51.79 (d, J(P–C) = 104.3), 66.91, 126.76, 126.79, 127.65, 127.84, 127.99, 128.09, 128.46, 129.05, 129.14, 136.18, 136.44, 136.58, 155.76, 155.98; ^{31}P NMR (120 MHz, CD_3OD) δ 53.76, 53.98; FAB MS 348 (M + 1)$^+$. Elemental Analysis. Calcd for $C_{18}H_{22}O_4NP$: C, 62.24; H, 6.38; N, 4.03. Found: C, 62.53; H, 6.46; N, 3.85.

Preparation of [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]methylphosphinic Acid (7). Compound **6** (178 mg, 0.530 mmol) was dissolved in dioxane (0.55 mL) and water (0.50 mL). To this solution was added aqueous 2 M LiOH (0.55 mL), and the mixture was vigorously stirred at room temperature for 48 h. Water (25 mL) was added, the aqueous solution was washed with ethyl acetate (1 × 25 mL) and acidified to pH 0 with concentrated HCl, and the aqueous phase was extracted with ethyl acetate (2 × 40 mL). The organic extracts were combined, dried over anhydrous Na_2SO_4, filtered, and evaporated *in vacuo* to give after crystallization from ethyl acetate 0.153 g (87%) of **7**, mp 153–154 °C. The identity of **7** was confirmed spectroscopically: ^{1}H NMR (300 MHz, CD_3OD) δ 1.46 (d, 3 H, J = 13.8), 2.76 (ddd, 1 H, J = 19.2, 12.4, 6.8), 3.21 (dt, 1 H, J = 14.2, 3.7), 4.09 (ddd, 1 H, J = 15.3, 10.19, 3.1), 4.91 (d, 1 H, J = 12.7), 4.99 (d, 1 H, J = 12.7), 7.22 (m, 5 H); ^{13}C NMR (75 MHz, CD_3OD) δ 12.50 (d, J(P–C) = 91.3), 34.46, 53.89 (d, J(P–C) = 108.2), 67.47, 127.61, 128.42, 128.81, 129.40, 130.14, 138.22, 138.85, 139.03, 158.44; ^{31}P NMR (120 MHz, CD_3OD) δ 52.48; FAB MS 334 (M + 1)$^+$. Elemental Analysis. Calcd for $C_{17}H_{20}O_4NP$: C, 61.26; H, 6.05; N, 4.20. Found: C, 60.34; H, 6.02; N, 4.16.

Preparation of [1(*R*/*S*)-((6-(*N*-Maleimidyl)hexanoyl)amino)-2-phenylethyl]methylphosphinic Acid (1a). A mixture of 10% Pd–C (30 mg) and **7** (103 mg, 0.31 mmol) in methanol (30 mL) was stirred at room temperature under an atmosphere of hydrogen until the starting material was consumed as observed by TLC (4 h). The catalyst was filtered off through Celite, and the mixture was washed with methanol (30 mL) and water:methanol (1:9, 10 mL). Solvents were combined and evaporated *in vacuo* to give a solid (40 mg).

In a separate flask, 6-maleimidocaproic acid[7] (207 g, 9.8 mmol) was

dissolved in methylene chloride (10 mL) and DCC (0.845 g, 4.10 mmol) was added. After 3 h of stirring at room temperature, the reaction mixture was filtered and the filtrate was evaporated *in vacuo* to give 1.8 g (96%) of **8**.

The symmetric anhydride of 6-maleimidocaproic acid **8** (78 mg, 0.20 mmol) was dissolved in DMF (4 mL) and transferred to the above solid (40 mg), followed by the addition of triethylamine (0.10 mL). The reaction was stirred for 16 h, then quenched by the addition of aqueous 1 M potassium phosphate, pH 6 (2 mL). The product, 12 mg (10%) of **1a**, was isolated using reverse-phase C18 HPLC. A 30-min gradient of 2–30% mobile phase B was used where mobile phase A was water and B was acetonitrile:water (95:5). The product was confirmed by spectroscopy: ^{1}H NMR (300 MHz, CD_3CN–D_2O; 1:1 v/v) δ 0.85 (m, 2 H), 1.19 (d, 3 H, J = 13.6), 1.2 (m, 2 H), 1.32 (m, 2 H), 1.96 (t, 2 H, J = 2.5), 2.60 (dt, 1 H, J = 13.3, 6.5), 3.13 (bd, 1 H, J = 14.3), 3.29 (t, 2 H, J = 7.4), 4.17 (m, 1 H), 6.75 (s, 2 H), 7.20 (m, 5 H); ^{13}C NMR (75 MHz, CD_3-CN–D_2O; 1:1 v/v) δ 13.62 (d, J(P–C) = 92), 25.78, 26.23, 28.47, 33.86, 36.43, 38.16, 51.91 (d, J(P–C) = 103.3), 127.24, 129.17, 130.05, 135.20, 139.26 (d, ^3J(P–C) = 12.3), 173.30, 175.58; FAB MS 431 $(M + K)^+$, 415 $(M + Na)^+$, 393 $(M + 1)^+$.

Preparation of Compounds 1b,c. Compound **5** was resolved into its two enantiomers through crystallization of its (–)-(*S*)-α-methylbenzylamine and (+)-(*R*)-α-methylbenzylamine salts as described.[6] Each enantiomer of **5** was separately transformed to **7** as described above. The pure enantiomer of **7** (0.227 g, 0.683 mmol) derived from the (+)-(*R*)-α-methylbenzylamine salt of **5** was treated with 10% Pd–C (100 mg) at room temperature under an atmosphere of hydrogen for 3 h. The catalyst was filtered off through a pad of Celite, and the mixture was washed with methanol (10 mL) and water:methanol (1:9, 5 mL).

In a separate flask, 6-(*N*-acetylamino)-*n*-caproic acid (0.70 g, 4.05 mmol) was dissolved in a mixture of methylene chloride (25 mL) and DMF (3 mL). To this solution was added DCC (0.412 g, 2 mmol) in methylene chloride (10 mL). After 1 h of stirring at room temperature, the reaction mixture was evaporated *in vacuo* to a small volume (approximately 3 mL). The solution was transferred to the above solid, and triethylamine (0.42 mL, 3 mmol) was added to the mixture. The reaction mixture was stirred at room temperature for 3 h, to it was then added water:methanol (1:2, v/v, 20 mL), and the mixture was evaporated *in vacuo*. Methanol (3 mL) was added, the mixture was filtered, and the filtrate was collected. Compound **1b** was purified from the filtrate by preparative silica TLC (1 mm) using methanol:methylene chloride (20:80) which gave 0.083 g (34%) of **1b** ($[\alpha]_D^{24}$ –33.8° (c = 0.157, MeOH)). The synthesis of **1c** follows the above procedure except that the pure isomer of **7** was derived from the (–)-(*S*)-α-methylbenzylamine salt of **5** to give 0.083 g (34%) of **1c** ($[\alpha]_D^{24}$ +33.5° (c = 0.155, MeOH)). The identities of **1b,c** were confirmed spectroscopically: ^{1}H NMR (300 MHz, CD_3OD) δ 1.18 (d, 3 H, J = 13.4), 1.34 (m, 2 H), 1.49 (m, 2 H), 1.60 (m, 2 H), 1.90 (s, 3 H), 2.15 (t, 2 H, J = 7.6), 2.63 (dt, 1 H, J = 13.8, 5.9), 3.14 (t, 2 H, J = 7.0), 3.25 (m, 1 H), 3.87 (m, 1 H), 7.20 (m, 5 H); ^{13}C NMR (75 MHz, CD_3OD) δ 14.67 (d, J(P–C) = 92.4), 22.53, 26.56, 27.32, 29.93, 34.53, 37.02, 40.32, 52.76 (d, J(P–C) = 103.2), 127.16, 129.15, 130.19, 140.39 (d, ^3J(P–C) = 12.1), 173.10, 175.17; CI NH_3 MS 377 $(M + Na)^+$.

Monoclonal Antibody Generation and Purification. Hybridoma cell lines were generated from mice immunized with **1a** conjugated to keyhole limpet hemocyanin (KLH). The hapten **1a** was coupled to iminothiolane-modified KLH and bovine serum albumin (BSA) via the maleimide group, resulting in hapten densities of 15 and 23 per KLH and BSA molecule, respectively (using a molecular weight of 64 000 for both proteins). The amounts (in mol) of free sulfhydryls remaining on the iminothiolane-treated protein before and after coupling to the hapten were determined with Ellman's reagent and used to calculate the hapten conjugation ratio. The splenocytes of BALB/c mice immunized with the KLH conjugate were fused with SP2/0 myelomas. Over 4000 hybridomas were assayed for production of hapten-specific antibodies by ELISA. A total of 68 antibodies were selected on the basis of hapten–BSA binding and screened for catalytic activity by the early screening method described below.

Monoclonal antibodies were purified from ascites using standard procedures of lipid extraction and ammonium sulfate precipitation followed by column chromatography on protein A, DEAE, Mono Q, and finally Alkyl Superose (Pharmacia, Piscataway, NJ). Antibody 13D11 was prepared from two separate batches of ascites, yielding preparations with indistinguishable specific activities. The antibody was judged to be >99% pure by SDS-PAGE.

Fab′ fragments were generated by insoluble pepsin–agarose digestion of purified 13D11 at pH 4.2 and 37 °C for 15 h.[8] The reaction was stopped by adjusting the pH to 7.5. Following centrifugation and filtration to remove the insoluble pepsin, the $F(ab')_2$ was converted to Fab′ by stepwise reduction and alkylation. The fragments were purified by gel filtration on a Superose 12 HR10/30 column (Pharmacia LKB, Uppsala, Sweden).

Identification of Catalytic Antibodies. Initially, the hybridoma culture supernatants containing low levels of antibodies (typically 1–10 μg/mL) were subjected to the following screening protocol. Antibodies were concentrated by immobilization on anti-mouse immunoglobulin affinity gel (Calbiochem, La Jolla, CA). Immobilized antibodies were then individually transferred to wells of Millititer GV 96-well filtration plates (Millipore, Bedford, MA) and thoroughly washed by aspiration, first with phosphate-buffered saline (PBS) containing 0.05% Tween-20, then with PBS. After rapid washing with the buffer to be used in the reaction, appropriately buffered substrate solutions were added. To economize on experimentation, enantiomers of the same substrate were mixed and coincubated. Antibodies were incubated in the plates at pH 5.0, 7.0, and 9.0 with eight potential substrates (**2a–d** and **3a–d**) at room temperature for a time (3–24 h) dependent on the substrate and pH under consideration. Buffers during the screening contained 140 mM NaCl, 0.01% NaN_3, and either 25 mM Mes (pH 5.0), 25 mM Hepes (pH 7.0), or 25 mM Tris (pH 9.0). The same antibody-coated beads could be used for multiple reactions with intervening washes to change buffer and substrate. Product formation was detected by either TLC (used with fluorescent dansyl substrates) or HPLC (used with acetylated substrates). Dansylated substrates and products were separated on HPTLC Silica Gel 60 plates (E. Merck, Darmstadt, Germany) using a solvent system of acetone–methanol–triethylamine (90:8:2, v/v/v/). Fluorescence was visualized under UV illumination. Acetylated substrates and products were separated and detected by absorbance at 260 and 328 nm using a Waters HPLC system (Marlborough, MA) equipped with a Waters 490E multiwavelength spectral detector and a Vydac C18 analytical column (5μm, 4.6 × 250 mm). A 20-min linear gradient from 85% A/15% B to 28% A/72% B was used in which solvents A and B were water and acetonitrile, respectively, both containing 0.1% TFA. Antibodies deemed potentially catalytic in the early screen were purified from ascites and rescreened with individual substrates.

Kinetic Characterization. Antibody was routinely assayed at pH 9.0 and 37.0 °C in MTEN buffer (50 mM MES, 25 mM Tris, 25 mM ethanolamine, and 100 mM NaCl)[9] containing 0.01% NaN_3. The effect of temperature on the pH of this buffer was taken into consideration.

Hydrolysis of the dansylated substrate **2b** was quantitated by HPLC as described above except solvent A was 25 mM sodium acetate (pH 6.5)–acetonitrile–methanol (70:20:10, v/v/v) and solvent B was 25 mM sodium acetate (pH 6.5)–acetonitrile–methanol (10:45:45, v/v/v). Elution consisted of two linear gradients: from 0–50% B (0–20 min) and 50–100% B (20–27 min), followed by 100% B (27–32 min).

Kinetic parameters of 13D11 hydrolysis of **2b** were determined by fitting initial substrate concentrations (56–1000 μM) and velocities (generally after 6 days) to a form of the Michaelis–Menten equation adapted for non-negligible catalyst concentrations: $V/[E_t] = (k_{cat}[S])/([S] + [E_t] + K_m)$.[10] The antibody combining site concentration was generally 13 μM. All reported kinetic parameters have been corrected to account for buffer-catalyzed substrate hydrolysis.

Hapten K_i was determined by quantitating the effect of the concentration of **1b** or **1c** on the velocity of 13D11 hydrolysis of **2b**. Rates obtained at fixed antibody and substrate concentrations were fitted to the equation $V_i/V_0 = (2[Ab] - [I] - K_i' + \{([I] + K_i' - 2[Ab])^2 + 4K_i'2[Ab]\}^{0.5})/4[Ab]$, where V_i and V_0 are, respectively, the velocities in the presence and absence of hapten, 2[Ab] is the total concentration of antibody combining sites, [I] is hapten concentration, $K_i' = K_i/(1 + [S]/K_m)$, and K_i is the inhibition constant.[11]

Results and Discussion

Hapten **1a**, a dialkylphosphinate transition-state analog (TSA) of phenylalaninamide hydrolysis, was prepared as an enantiomeric mixture as outlined in Scheme 2. Synthesis of [1(*R*/*S*)-amino-2-phenylethyl]phosphinic acid (**4**) was carried out as previously

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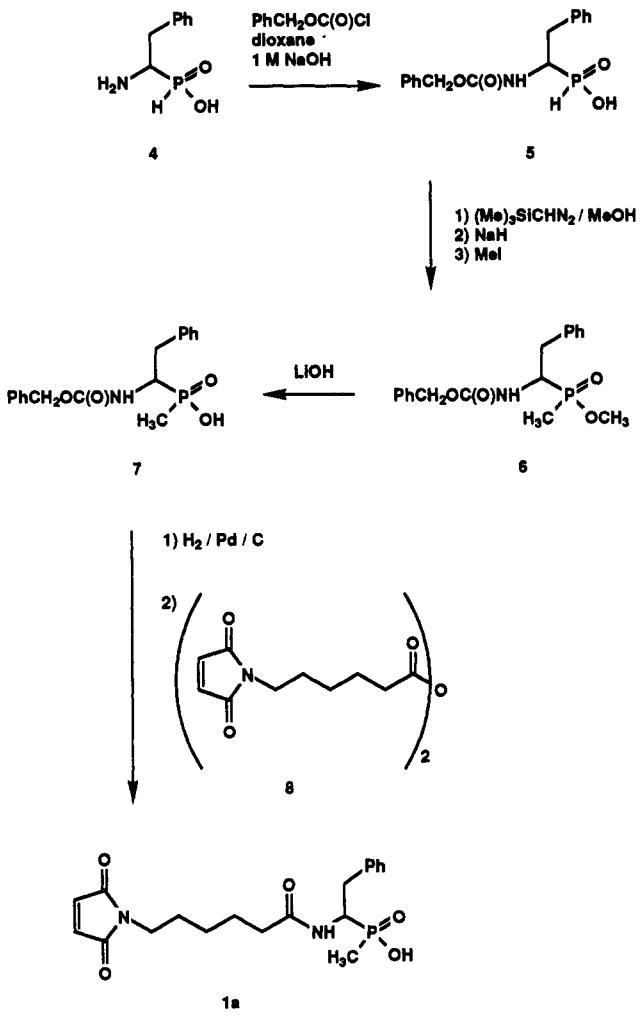
Scheme 2



described.[6] Reaction with benzyl chloroformate gave [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]phosphinic acid (**5**). Subsequent O-methylation with (trimethylsilyl)diazomethane and methanol followed by P-methylation using sodium hydride and methyl iodide afforded compound **6**. Hydrolysis of **6** with lithium hydroxide gave **7**. Catalytic hydrogenation of **7** and subsequent acylation by the symmetrical anhydride of 6-maleimidocaproic acid (**8**) yielded **1a**.

The haptenic inhibitor **1c** was prepared by first resolving [1(*R*/*S*)-(*N*-CBZ-amino)-2-phenylethyl]phosphinic acid (**5**) through recrystallization of its (–)-(*S*)-α-methylbenzylamine salt as previously described.[6] After acidification, the pure enantiomer was converted to **1c** using the same steps in Scheme 2 except that the symmetrical anhydride of 6-(*N*-acetylamino)-*n*-caproic acid was used instead of 6-maleimidocaproic acid. Compound **1b** was prepared as described for **1c** except that the recrystallization was done using (+)-(*R*)-α-methylbenzylamine. Because the chiral center substituents differ in atomic number, the (*S*)-hapten structurally corresponds to the (*R*)-substrate.

In order to facilitate the screening of large numbers of monoclonal antibodies for catalysis, we developed an early screen protocol in which the low levels of antibodies present in hybridoma culture supernatants can be adequately concentrated and purified for preliminary screening. Hybridoma supernatants containing anti-**1** antibodies were concentrated by immobilization on anti-mouse affinity gel and extensively washed in individual wells of a 96-well filter plates. Appropriately buffered substrates were added to the wells and later removed for analysis. Because the antibodies remained immobilized in the wells, the same antibody molecules could be screened with multiple substrates and pH values with appropriate intervening washes. A total of 68 antibodies were tested at pH 5.0, 7.0, and 9.0 against the four primary amide (**2a,b** and **3a,b**) and four methyl ester (**2c,d** and **3c,d**) substrates, giving a total of 1632 chances for catalysis.

A small number of potentially catalytic antibodies were selected from the early screen for large-scale production in ascites. Rescreening of highly purified antibodies from ascites yielded one antibody, 13D11, that hydrolyzed the dansylated (*R*)-amide **2b** at pH 9.0. The methyl ester **2d** corresponding to the amide substrate **2b** was not hydrolyzed by 13D11 at a rate above the background. The lack of detectable esterolytic activity suggests that either the rate-limiting step during hydrolysis is slower than the background rate of ester hydrolysis but faster than the background rate of amide hydrolysis or that the size of methyl ester, being larger than the corresponding group in the hapten or the amide substrate, hindered ester substrate binding.

Kinetic characterization showed that 13D11-catalyzed hydrolysis of **2b** has a pH optimum of 9.5 (data not shown). At higher pH values, 13D11 may have denatured over the relatively long reaction times (typically 3–8 days). Selwyn's test[12] showed no antibody inactivation over at least 6 days in pH 9.0 MTEN buffer at 37.0 °C. A study of the effect of substrate concentration on the rate of hydrolysis at pH 9.0 showed 13D11 to have a k_{cat} = 1.65 (±0.24) × 10^{-7} s^{-1} and a K_m = 432 (±132) μM. Although less than one turnover of substrate was generally measured in reactions, prolonged incubations demonstrated that 13D11 was indeed catalytic.

Control experiments verified that the hydrolytic activity was not due to a contaminating protease. Only one of the hapten enantiomers **1c**, presumably the (*S*)-isomer, inhibited 13D11. (The (*S*)-hapten spatially corresponds to the (*R*)-amide substrate.) The inhibition constant, K_i, was determined by an HPLC assay to be 14.0 (± 1.6) μM. The 13D11 Fab' fragment was prepared and found to have activity indistinguishable from that of the whole IgG. Equally active 13D11 was purified from two different batches of ascites. Finally, nine common inhibitors of known proteases were tested for their effect on the antibody-catalyzed reaction. Each inhibitor was used at a concentration sufficient to completely abolish the activity of its target protease. Amidolytic activity was essentially unaffected by PMSF, EDTA, aprotinin, antipain, leupeptin, and phosphoramidon. Partial inhibition was seen with zinc aminopeptidase inhibitor bestatin, the aspartic protease inhibitor pepstatin, and the irreversible inhibitor of cysteine proteases E-64, due presumably to nonspecific interactions with the antibody combining site.

Two additional observations indicated that the catalytic activity associated with 13D11 was not due to a contaminating protease. The stereospecificity of 13D11 was opposite of known naturally occurring primary amide-hydrolyzing enzymes; the (*R*)-amide **2b**, which spatially corresponds to a D-amino acid, was hydrolyzed but the (*S*)-amide **2a** was not. Secondly, the acetylated (*R*)-amide **3b** was not accepted as a substrate, suggesting that the five-carbon alkyl linker of the substrate **2b**, which was present in **1a** during immunization, is recognized by the antibody. However, we cannot rule out the possibility that the antibody makes nonspecific binding interactions with the dansyl group of **2b**, which was not present during immunization.[13]

Under the normal assay conditions, slow hydrolysis of **2b** was observed in the absence of antibody. Incubation of **2b** (100 μM) in various dilutions of the assay buffer showed that the rate of background hydrolysis was linearly dependent on buffer concentration and virtually disappeared as the buffer concentration approached zero (Figure 2, lower line). This indicates that background hydrolysis is due almost entirely to the buffer solutes rather than to inherent instability of **2b** at pH 9.0 and 37.0 °C. Buffer hydrolysis had no effect on antibody catalysis; when antibody (13 μM) and substrate (100 μM) were reacted in a

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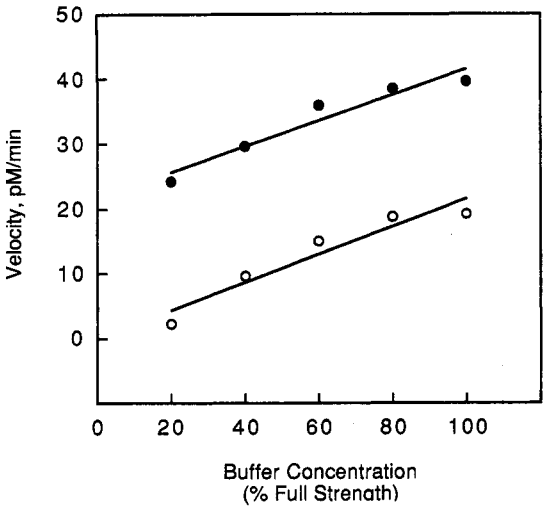

Figure 2. Effect of buffer concentration on hydrolysis of **2b** (100 μM) in the absence (open circles) and presence (closed circles) of antibody 13D11 (13 μM). The reactions were carried out for 7.1 days at pH 9.0 and 37 °C in the MTEN mixed buffer system (50 mM MES, 25 mM Tris, 25 mM ethanolamine, and 100 mM NaCl) containing 0.01% NaN_3. The product was quantitated by absorbance detection during HPLC.

range of buffer dilutions, a resulting plot of buffer dilution versus product formation gave a line parallel to that obtained with substrate alone (Figure 2, upper line). The observation that the buffer can catalyze substrate hydrolysis but does not affect antibody catalysis shows that the rate-limiting step in the mechanism of antibody catalysis cannot be accelerated by the buffer acting as a nucleophile or a proton donor.

A more rigorous study of the linear dependence of **2b** hydrolysis on buffer concentration was carried out to determine the uncatalyzed hydrolysis rate at zero buffer concentration. A constant substrate concentration (100 μM) was incubated in a series of seven different buffer dilutions, and the amount of product formed was determined after 9.0 days. Linear extrapolation of the rates to zero buffer concentration (data not shown) gave an uncatalyzed rate of 1.25 ($\pm$0.23) $\times$ 10^{-9} s^{-1} at pH 9.0 and 37.0 °C, indicating that the catalytic rate enhancement of 13D11 (k_{cat}/k_{uncat}) is 132. The half-life of the primary amide of **2b** is thus reduced from 17 years when free in solution to 42 days when bound to antibody 13D11. The uncatalyzed rate of the corresponding methyl ester **2d** was similarly measured and found to be 1.06 ($\pm$0.06) $\times$ 10^{-5} s^{-1} at pH 9.0 and 37 °C. The difference between the measured rates of uncatalyzed hydrolysis of **2b,d** are reasonable and consistent with the predicted differences between the base hydrolysis rates of methyl acetate[14] and acetamide[15] extrapolated to pH 9.0 and 37 °C.

Because there are two amide bonds in **2b**, appreciable hydrolysis of the internal amide bond or of both amide bonds could conceivably change the apparent rate of formation of the desired product. However, the described kinetic experiments involved hydrolysis of only a small percentage of substrate and little or no depletion of the desired product HPLC peak area occurred due to double hydrolysis. Indeed, no evidence of cleavage at the alternate amide bond was observed by HPLC.

The Gibbs free energies of activation for hydrolysis of primary amides and secondary amides such as those found in typical peptide bonds are similar, although structure–reactivity correlations have shown that primary amides may be somewhat more reactive than internal peptide bonds for steric reasons.[5] Our experimental determination of k_{uncat} for **2b** gives a hydrolysis rate very close to previous estimates and determinations of peptide bond hydrolysis[16] at 25 °C and pH 7.0.

Despite similarities in their inherent reactivities, primary amides and peptide bonds may not be similarly amenable to hydrolysis by catalytic antibodies. An immunogen designed as a tetrahedral transition-state analog of primary amide hydrolysis will display the tetrahedral group on the solvent-exposed tip of the hapten whereas an immunogen designed to elicit peptide bond-hydrolyzing antibodies will have a more "buried" transition-state analog flanked by bulky substituents. Because of greater exposure, the critical transition-state-mimicking features in a primary amide analog may be more thoroughly engulfed by elicited antibodies than the corresponding features in a peptide transition-state analog. If higher accessibility generally results in tighter binding, it may be a general phenomenon that highly solvent-exposed transition-state analogs (e.g., primary amide analogs) may elicit more catalytically efficient antibodies than analogs with lower solvent exposure (e.g., peptide transition-state analogs). An opposing possibility is that it may be more difficult for an antibody to hydrolyze a primary amide than a substituted amide because primary amides have a smaller leaving group (ammonia) than do substituted amides (e.g., peptides) such that less catalytic activity can be garnered from ground-state destabilization of the amino leaving group.[17] The relative contributions of these and other features of antibody-catalyzed amide hydrolysis remain to be determined.

It is not clear why this effort was successful in generating an amide-hydrolyzing antibody while other similar attempts have failed. As discussed above, one possibility relates to the highly solvent-exposed nature of the transition-state analog portion of the hapten. Another possibility is that our chances of finding a catalytic antibody were greater than in previous attempts because we employed a new screening method that allowed us to extensively screen a large number of antibodies for catalysis under various conditions. A third possibility is that the phosphinate hapten **1a** may be a better transition-state mimic of amide hydrolysis than other previously-tried tetrahedral phosphorous-based haptens (e.g., phosphonate of phosphonamidate).

Conclusions

Extensive screening of 68 anti-dialkylphosphinate monoclonal antibodies led to the discovery of a catalytic antibody able to catalyze the hydrolysis of the primary amide of a derivative of phenylalaninamide. The antibody stereospecifically hydrolyzed the (*R*)-substrate (the opposite enantiomer of most natural amino acids), required a dansylated aliphatic linker in the substrate, and had an apparent alkaline pH optimum. Kinetic characterization demonstrated that at pH 9.0 and 37 °C the antibody has a k_{cat} = 1.65 $\times$ 10^{-7} s^{-1} (k_{cat}/k_{uncat} = 132) and a K_m = 432 μM. Control experiments showed that the antibody had identical specific activity when purified from two separate batches of ascites fluid and that the Fab′ fragment retained full activity. In addition, the catalytic activity was inhibited by only one hapten enantiomer (K_i = 14.0 μM) and was not abolished by any of nine inhibitors of common proteolytic enzymes. This is the first hapten-elicited monoclonal antibody shown to catalyze the unassisted hydrolysis of an unactivated amide bond.

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Supplementary Material Available: Figures showing spectra (^1H, ^{13}C, and ^{31}P NMR and MS) and HPLC chromatograms (21 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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