Antibody catalysis of peptide bond formation

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ABSTRACT An antibody generated against a neutral phosphonate diester transition-state (TS) analog catalyzes the formation of an amide bond between a phenylalanine amino group and an acyl azide derived from L-alanine. The antibody is selective for L- vs. D-alanine and does not catalyze the hydrolysis of the acyl azide to an appreciable degree. A rate acceleration of 10,000-fold relative to the uncatalyzed reaction is observed. The antibody may achieve its catalytic efficiency both by acting as an entropy trap and by stabilizing the deprotonated form of the amine nucleophile. These experiments constitute a first step toward a general strategy for the generation of sequence-specific peptide ligases.

The formation of amide bonds is a key step in both the chemical and biological synthesis of proteins. Solid-phase peptide synthesis has made great strides in recent years but is still generally limited to peptides and proteins of fewer than 100 amino acids (1). The ability to specifically join peptide segments in the absence of side-chain protecting groups is, therefore, an area of increasing interest. The generation of peptide ligases from proteases and lipases has been investigated by site-directed mutagenesis (2, 3) and through the use of organic solvents to favor peptide bond formation over hydrolysis (4). In addition, specific chemical methods for peptide ligation have been developed (5, 6).

We wished to investigate the requirements for catalysis of peptide bond formation using a catalytic antibody. Previously reported studies in this area focused on condensation reactions involving activated esters and aryl amines (7, 8). We have applied a design strategy that was found to be highly successful for bimolecular ester formation (9). Antibodies were generated against the neutral phosphonate diester 1 that contains elements of the acyl donor 5, acceptor 7, and leaving group in a tetrahedral geometry mimicking that of the presumed transition state (Fig. 1a). One antibody was isolated that accelerated the formation of peptide 8 from the corresponding substrates and was characterized with respect to its substrate specificity and reaction kinetics.

MATERIALS

Diphenyl 1-(N-carboxyphenoxy)ethylphosphonate (10). To a solution of phosgene in toluene (31 ml, 1.9 M) at 0°C with added phenethyl alcohol (6.0 ml, 50 mmol). N,N-Dimethylaniline (6.4 ml, 50 mmol) was added dropwise over a 10-min interval, and after an additional 15 min, 8 ml of methylene chloride was added, and the reaction was warmed to room temperature. After 4 h residual phosgene was removed under reduced pressure, and the crude chloroformate was treated with 2.9 M aqueous ammonia (70 ml) and stirred rapidly for 30 min. The product was extracted into 50 ml of methylene chloride. The organic layer was dried (MgSO₄) and cooled to 0°C for 12 h to yield colorless crystals of phenethyl carbamate that were isolated by vacuum filtra-

![FIG. 1. Substrates, products, and haptens.](image)

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Abbreviations: FAB, fast atom bombardment; RSA, bovine serum albumin.

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8.30; 7.55-7.75

3.27-3.49 Hz), 4.03 (t, 2H, J = 8.6 Hz), 2.56 (br d, 1H, J = 3.6 Hz), 2.90 (dd, 1H, J = 14.0 Hz, 8.6 Hz), 3.27-3.49 (m, 6H), 4.38 (d, 1H, J = 8.4 Hz), 6.95 (br t, 1H, J = 6.4 Hz). 7.24-7.34 (m, 5H), 7.95 (br t, 1H, J = 6.4 Hz), 8.08 (d, 2H, J = 9.0 Hz). 8.30 (d, 2H, J = 9.0 Hz); mass spectrum (FAB\(^+\)), \(m/z\) 224 (MH\(^+\)).

Exact mass: Calcld for (C\(_{37}\)H\(_{39}\)N\(_4\)O\(_7\)PS)H\(^+\): 224.1035. Found: 224.1036.

**N-(4-Nitrobenzoyl)-N'-l-phenylalanyl-1,3-diaminopropanone (7).** This compoud was prepared in protected form from \(N\)-t-Boc-l-phenylalanine, where Boc is butoxycarbonyl, by a procedure similar to that described for the preparation of 12. The protected amine (0.32 g, 1.1 mmol) was dissolved in 5.0 ml of methylene chloride and treated with 5.0 ml of trifluoroacetic acid. After 1 h the reaction was judged to be complete by TLC and concentrated in vacuo, and the product was purified by preparative HPLC using a 2.5 x 25 cm C\(_{18}\) Microsorb column (Rainin Instruments) and a gradient of 20-70% (vol/vol) acetonitrile in 0.1% aqueous trifluoroacetic acid over 27 min (product retention time = 19.3 min) to give 0.16 g (29%) of the trifluoroacetic acid salt of 7 as a white powder, mp >100°C; \(^{1}H\) NMR (100 MHz, MeOD-d\(_4\)) \(\delta\) 1.62-1.78 (m, 2H), 3.62-3.75 (m, 6H), 4.03 (t, 1H, J = 6.5 Hz), 7.22-7.38 (m, 1H), 8.02 (d, 2H, J = 8.8 Hz), 7.28 (d, 2H, J = 8.8 Hz); mass spectrum (FAB\(^+\)), \(m/z\) 371 (MH\(^+\)). Exact mass: Calcld for (C\(_{30}\)H\(_{32}\)N\(_4\)O\(_7\))H\(^+\): 371.1719. Found: 371.1713.

**N-Carbobenzyloxy-L-alanine, Phenyl Estor (3).** To 0.51 g of N-carbobenzyloxy-L-alanine (13) (2.1 mmol) in 10 ml of ethyl acetate, phenol (30.0 g, 0.32 mmol), dicyclohexy carbodiimide (0.46 g, 2.4 mmol), and 4-dimethylaminopyridine (0.14 g, 1.1 mmol) were added. The reaction mixture was stirred at room temperature for 5 h. Precipitate was removed by filtration, and the filtrate was chromatographed (15% ethyl acetate in hexanes, \(R_t = 0.26\)) to yield 0.57 g (85%) of a colorless oil that solidified on standing, mp 77-78°C; \(^{1}H\)
RESULTS AND DISCUSSION

Catalysis of peptide bond formation in aqueous solution presents several challenges. Activated carbonyl groups are necessary for thermodynamically favorable reactions, but these groups are susceptible to hydrolysis. Consequently, effective catalysts must selectively catalyze the reaction with an amine nucleophile in the presence of 55 M water. Catalysts must also be selective for the α-amino group to avoid acylation of other primary amino groups in the peptide fragments. The α-amino group must be unprotonated to act as a nucleophile, but alkyl amines have pKₐ values of ~10 and are, therefore, present almost exclusively in the protonated form at physiological pH. The α-amino group of a peptide fragment may have a pKₐ that is as many as 3 units lower, but in the majority of cases this group is still predominantly in the protonated form at pH 7.0.

We anticipated that antibodies complementary to hapten 1 would bind to both amine nucleophile 7 and an activated carbonyl acyl derivative, 3, 4, 5, or 6, and position these substrates in an orientation suitable for reaction, thereby reducing the entropy of activation for the reaction. The tetrahedral geometry at phosphorus and the PO=O dipole reflect the geometry and electronic character of the expected transition state. The neutral phosphonate group might be expected to elicit a binding pocket that would stabilize the corresponding uncharged amine nucleophile relative to its protonated form. While the corresponding phosphonomatride characterizes the developing ionic character of the α-amino group in the transition state, it was not considered as a potential hapten due to concerns about its stability in serum.

The synthesis of hapten 1 is outlined in Fig. 1b. Hapten 1 was coupled to carrier proteins BSA and keyhole limpet hemocyanin by using a thiourea linkage, and the keyhole limpet hemocyanin hapten conjugate was used to immunize Swiss Webster mice. Standard protocols were used to isolate 28 clonal cell lines that produced antibodies specific for hapten 1 (15). Ascertains glucose was produced in irradiated Swiss Webster mice for 20 cell lines and purified by protein A/G affinity chromatography. Antibodies were judged to be >95% pure by polyacrylamide gel electrophoresis.

Antibodies were screened for catalysis of the reaction of L- and D-alanine phenyl esters 3 and 4 with phenylalanine amine 7 to form the corresponding amides 8 and 9. Substrates and antibodies were combined in 15 mM sodium phosphate (pH 7.4) containing 150 mM sodium chloride and 4 mM sodium azide, and product formation was assayed by HPLC. Reactions were carried out in the presence of sodium azide to provide the more reactive acyl azides 5 and 6 in situ as substrates for the antibody (16). Two antibodies were found to accelerate the reaction; both selectively catalyze the reaction of the L-alanine-derived substrate 5 in preference to its enantiomer 6. No product could be detected after 24 h in the absence of sodium azide (detection limit 0.2 nM). An enhanced reactivity of the acyl azide may be due to the greater leaving group ability of azide relative to phenol. An alternative hypothesis is that the azide group participates in the proton transfer steps required to form the tetrahedral intermediate (16). Attempts to examine the reaction of amine 7 with a cyanomethyl ester analog of 3 were complicated by a competing reaction of the amine with the decomposition products of the ester leaving group. Antibody 9B5.1 was characterized in d 20H 0.2 (m, 9H), 3.45-3.51 (m, 6H), 4.03-4.25 (m, 3H), 4.59-4.65 (m, 1H), 5.03 (br s, 1H), 6.52 (br d, 1H, J = 7.2 Hz), 6.77 (br s, 1H), 7.16-7.34 (m, 10H), 7.81 (br s, 1H), 8.07 (d, 2H, J = 8.8 Hz), 8.29 (d, 2H, J = 8.8 Hz); mass spectrum (FAB +), m/z 590 (M + H). Exact mass: Calcd for (C₁₇H₂₂NO₄)⁺ H⁺: 590.2615. Found: 590.2621.

N-Carboxyphenylalanyl-D-alanyl-phenylalanine-(N'-4-nitrobenzoyl)-1,3-diaminopropane (9). This compound was prepared from amine 7 and N-carboxyphenylalanyl-D-alanine using the conditions analogous to those described for the preparation of ester 3. The product was obtained in 65% yield as a white solid, m.p. > 180°C decomposes; 1H NMR (400 MHz, CDCl₃) δ 8.10 (d, 3H, J = 7.1 Hz), 1.00-1.81 (m, 2H), 2.88 (t, 2H, J = 6.9 Hz), 3.14-3.4 (m, 6H), 4.03-4.25 (m, 3H), 4.59-4.65 (m, 1H), 5.03 (br s, 1H), 6.52 (br d, 1H, J = 7.2 Hz), 6.77 (br s, 1H), 7.16-7.34 (m, 10H), 7.81 (br s, 1H), 8.07 (d, 2H, J = 8.8 Hz), 8.29 (d, 2H, J = 8.8 Hz); mass spectrum (FAB +), m/z 590 (M + H). Exact mass: Calcd for (C₁₇H₂₂NO₄)⁺ H⁺: 590.2615. Found: 590.2625.
presence of sodium azide during the reaction. The steady-state concentrations of azide 5 were determined by HPLC. The azide peak was monitored under the reaction conditions, and concentrations were determined by calibration against authentic material by using 6-nitroquinoline as an internal standard. The azide concentration was found to vary by <8% over the course of the reaction.

Initial rates were determined by HPLC for a range of concentrations of acyl azide 5 and amine 7. Reaction rates in the absence of antibody were measured under identical conditions, and the catalyzed rates were determined by subtraction of these background rates. Families of Lineweaver–Burk plots were constructed by holding one substrate concentration constant while varying the catalytic rate of the other (Fig. 2a). The slopes and y-axis intercepts obtained from these analyses were replotted as functions of inverse substrate concentration to give the true maximum rate, V_max, as 1.9 μM·min⁻¹, which corresponds to a rate constant, k_cat, of 0.059 min⁻¹ per antibody binding site (Fig. 2b). The Michaelis constant, K_M, is 15 μM for acyl azide 5 and 1.5 mM for amine 7. The apparent second-order rate constant k_cat/K_M is equal to 3900 M⁻²·min⁻¹ for the azide and 40 M⁻²·min⁻¹ for the amine. Analysis of the uncatalyzed rates gives the second-order rate constant k_nocat = 0.37 M⁻¹·min⁻¹, affording a rate acceleration (k_cat/K_M)/(k_nocat) of 1.0 × 10⁴. The family of plots in Fig. 2a intersects on the horizontal axis, indicating that the antibody exhibits sequential binding. This result is similar to that described previously for an antibody generated against another phosphonate diester (9). No significant product inhibition was observed; this is consistent with the different hybridization states of the amide product 8 and phosphonate hapten 1.

The antibody-catalyzed reaction was monitored at several pH values (pH 5.8, 6.5, 7.3, and 8.0). The maximal rate for the antibody-catalyzed reaction occurs at pH ~7.3. In contrast, the rate of the uncatalyzed reaction increases steadily with increasing pH. The shift in the pH optimum of the antibody-catalyzed reaction to lower pH relative to the uncatalyzed reaction suggests that the antibody may stabilize the unprotonated form of amine 7. This presents the possibility of using an antibody to specifically activate a single amino group in a peptide, obviating the need for side-chain protecting groups. The decrease in rate of the antibody-catalyzed reaction at higher pH may reflect an ionizable active site group or a competing hydrolysis pathway.

The antibody-catalyzed reaction is completely inhibited by addition of 63 μM hapten 2. The binding of hapten 1 to antibody 9B5.1 was assayed by measuring the enhancement of hapten fluorescence upon binding to the antibody. Titration of hapten with antibody followed by Scatchard analysis afforded the dissociation constant K_d for the antibody–hapten complex as 19 nM (Fig. 3). The differential binding of the antibody to the transition-state TS⁺ analog relative to the substrates (8, 9) is not as great as that observed for the

FIG. 2. Kinetic data for antibody 9B5.1. (a) Lineweaver–Burk plot with azide 5 held at four fixed concentrations while amine 7 was varied from 143 to 952 μM. Concentrations of azide 5: A, 11 μM; •, 21 μM; ◦, 29 μM; ◼, 36 μM. V. Velocity. Reaction mixtures contained 16 μM antibody 9B5.1, 48 mM sodium azide, and 86 mM Mes (pH 5.8) with 10% acetonitrile as the cosolvent. Initial reaction rates were determined by reversed-phase HPLC (Rainin Microsorb 25-cm C18 column; gradient, 40–90% acetonitrile in 50 mM sodium acetate [pH 5.0]). Reaction components were detected by UV absorbance at 272 nm. Products 8 and 9 were identified by coincision of authentic samples and by mass spectrometry and quantitated against an internal standard of 6-nitroquinoline. Initial rates were determined by linear fit of four or more points ranging from 1 to 16 h. (b) Slopes (•) and y-axis intercepts (○) of Lineweaver–Burk plots replotted as a function of 1/[S]. Analogous plots were constructed to give the kinetic constants for amine 7.

FIG. 3. Scatchard plot of fluorescence titration data for binding of antibody 9B5.1 to 300 nM hapten 1. R is the fraction of hapten that is bound and [Sites]bound and [Hapten]total are the total (bound plus free) concentrations of antibody combining sites and hapten, respectively. Similar plots were obtained for hapten concentrations of 250 nM, 200 nM, 175 nM, and 125 nM. Fluorescence of aminobenzamide hapten 1 is enhanced ~3-fold upon binding to antibody 9B5.1. A series of fluorescence titrations were performed using a Perkin-Elmer LS-5B luminescence spectrometer with samples of hapten 1 in 100 mM Mes (pH 6.0) containing 1% methanol. These samples were titrated with antibody 9B5.1 over a range of concentrations up to ~10-fold higher than the concentration of hapten. Fluorescence was measured at 360 nm with excitation at 305 nm. Fluorescence was corrected for volume change over the course of the titration and for fluorescence due to added antibody, and the fraction of the hapten bound was calculated based on the titration end points. Because hapten 1 was obtained as a mixture of four diastereomers, the relevant concentration of hapten is assumed to be one-fourth of the total concentration. Data corresponding to ~20–80% saturation of hapten were analyzed using Scatchard analysis (17); [Sites]bound/(1/R) = 1 was plotted as a function of (1 – R/[Hapten]total) to give the binding constant, K_d, as the y intercept. Values for five concentrations of hapten were averaged.
previously characterized transesterification reaction (9). This may account for the lower catalytic efficiency of antibody 9B5.1. Alternatively, the antibody may not effectively accommodate the developing positive charge on the amino group in the transition state of this reaction.

The specificity of the antibody-catalyzed reaction was characterized with respect to the nature of the acyl acceptor and the chirality of the acyl donor. The ability of antibody 9B5.1 to accelerate the hydrolysis of ester 3 under the reaction conditions was assayed in the absence of amine 7. A small rate acceleration was observed ($k_{cat}/k_{uncat} \approx 15$). This low hydrolytic activity is similar to that observed for the previously reported ester formation reaction and suggests that the antibody may accommodate the trigonal ground state of the acyl donor 5 in the absence of amine 7 and activate the carbonyl for attack by nucleophiles only when the binding site is fully occupied. Ester formation from phenylactic acid derivative 12 was investigated under the reaction conditions used for amide formation assays. No ester formation could be detected using ester 3. By using the cyanomethyl ester analog of ester 3, a background reaction could be detected, and antibody 9B5.1 provided no acceleration of this reaction. Finally, the substrate specificity of the antibody was characterized with respect to the chirality of the acyl donor. Acyl azide 6, derived from D-alanine, was examined as an alternative substrate. Comparison of initial rates at 1 mM amine 7 and 400 μM esters 3 and 4 under the standard reaction conditions showed a 7.3-fold smaller acceleration of the reaction with 6 relative to the L-alanine-derived azide 5.

We have demonstrated selective catalysis of amide bond formation by an antibody. This may be a first step toward a general strategy for the generation of peptide ligases with tailored specificities. The extension of our phosphonate diester design from ester formation to amide formation highlights the greater complexity of amide formation and the need for further refinements in hapten structure.

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