The pH-Rate Profile for the Hydrolysis of a Peptide Bond

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Abstract: The rate of hydrolysis of *N*-(phenylacetyl)glycyl-D-valine (PAGV), an acyclic penicillin G analogue, at pH 0, 1, 3, 5, 7, 9, 11, 13, and 14 has been measured at 37 °C and a pH-rate profile constructed. At each pH, hydrolysis of both the (phenylacetyl)glycyl amide bond and glycyl-D-valine peptide bond was monitored. At pH 3, 5, 7, 9, and 11, the hydrolysis products glycyl-D-valine and D-valine were derivatized with naphthalene-2,3-dialdehyde in the presence of cyanide; the resultant 1-cyano-2-substituted-benz[*f*]isoindole (CBI) derivatives, which are highly fluorescent, were then quantified using reverse-phase HPLC. The hydrolysis reactions were explicitly shown to be first-order in peptide concentration at pH 5 and 9, and all rates were shown to be independent of the buffer concentration. The rates at pH 0, 1, 13, and 14 were measured in 1 M DCl, 0.1 M DCl, 0.1 M NaOD, and 1 M NaOD, respectively, and the hydrolysis products were detected by ^{1}H NMR. The first-order rate constants obtained from the above reactions were fit to the general equation $k = k_{H_2O} + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-]$ to yield the following results: for hydrolysis of the (phenylacetyl)glycyl bond, $k_{H_2O} = (9.05 \pm 6.36) \times 10^{-11}$ s^{-1}, $k_{H_3O^+} = (1.60 \pm 1.04) \times 10^{-6}$ M^{-1} s^{-1}, and $k_{OH^-} = (1.11 \pm 0.73) \times 10^{-6}$ M^{-1} s^{-1}; and for hydrolysis of the glycyl-D-valine bond, $k_{H_2O} = (8.23 \pm 4.33) \times 10^{-11}$ s^{-1}, $k_{H_3O^+} = (1.67 \pm 0.80) \times 10^{-6}$ M^{-1} s^{-1}, and $k_{OH^-} = (1.16 \pm 0.56) \times 10^{-6}$ M^{-1} s^{-1}. At pH 7, the hydrolysis of both the (phenylacetyl)glycyl amide bond and glycyl-D-valine peptide bond is dominated by k_{H_2O}. The corresponding half-life for (phenylacetyl)glycyl bond hydrolysis is 243 years (with a range of 143−817 years within experimental error), while that for glycyl-D-valine bond hydrolysis is 267 years (with a range of 175−564 years).

Introduction

Except at extremes of pH and temperature, nonenzymatic peptide bond hydrolysis is a slow process that is difficult to quantify accurately. Recently, however, we described a homogeneous assay for the direct measurement of this rate under mild conditions.[1] (This work was stimulated by our interest in identifying antibodies with sequence-specific peptidase activity.[2]) In the assay, which is based upon that of Stobaugh and co-workers,[3] the newly generated α-amino group of each hydrolysis product is derivatized with naphthalene-2,3-dialdehyde in the presence of cyanide ion (NDA/CN).[4] The resultant 1-cyano-2-substituted-benz[*f*]isoindole (CBI) derivatives, which are highly fluorescent, are then quantified using reverse-phase HPLC. We now report the use of NDA/CN assay (at pH 3, 5, 7, 9, and 11), as well as ^{1}H NMR spectroscopy (at pH 0, 1, 13, and 14), to construct the pH-rate profile for the hydrolysis of *N*-(phenylacetyl)glycyl-D-valine, an acyclic analogue of penicillin G.

In 1988, Kahne and Still[5] published the pH-rate profile for the hydrolysis of the tetrapeptide (phenylalanyl)$_3$[^{14}C]glycine, linked via its amino terminus to a polyacrylamide resin; the release of radiolabeled products from the solid support was monitored. Only the carboxy-terminal phenylalanylglycine peptide bond was observed to undergo hydrolysis, and the absence of *endo*-peptide bond cleavage was attributed either to the conformational flexibility of glycine (and, thus, the increased likelihood of intramolecular anhydride formation) or to an artifact of the solid-phase system. Over the pH range 4−10, the rate of hydrolysis was found to vary by less than 1 order of magnitude; a first-order dependence on [H$^+$] was observed at pH < 3, and on [OH$^-$] at pH > 10. At pH 7 and 25 °C, the oft cited rate constant of 3×10^{-9} s^{-1} was determined, which corresponds to a half-life of approximately 7 years. That this value may be artificially high was noted by Kahne and Still, but a series of elegant control experiments suggested that the rate constant measured does indeed correspond to hydrolysis of the *endo*-phenylalanylglycine peptide bond.

In 1996, two additional reports on peptide bond hydrolysis appeared. Using the NDA/CN assay, our group[1] measured a rate constant of 1.3×10^{-10} s^{-1} at pH 9 and 25 °C for the hydrolysis of the peptide bond in hippurylphenylalanine, a carboxypeptidase A substrate. Radzicka and Wolfenden[6] studied the hydrolysis of acetylglycylglycine *N*-methylamide, acetylglycylglycine, and glycylglycine at temperatures ranging from 100 to 180 °C. Rate constants at pH 6.8 and 25 °C of 3.6×10^{-11} s^{-1}, 4.4×10^{-11} s^{-1}, and 6.3×10^{-11} s^{-1},

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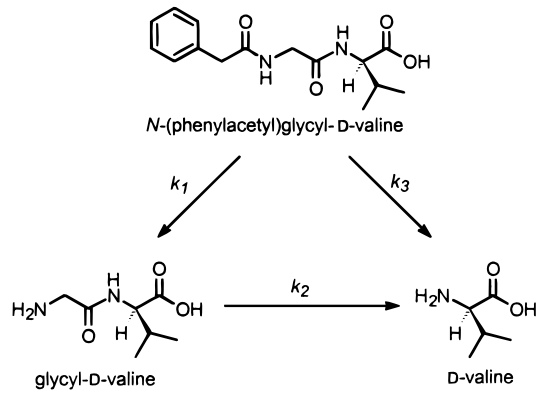

Figure 1. Reaction scheme for the hydrolysis of *N*-(phenylacetyl)-glycyl-D-valine (PAGV).

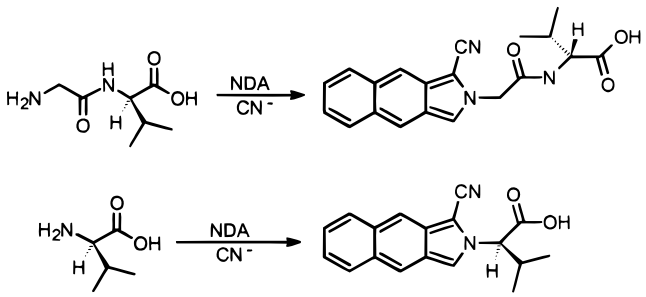

Figure 2. Reaction of glycyl-D-valine (top) and D-valine (bottom) with NDA/CN to yield the respective CBI derivatives.

respectively, for hydrolysis of the glycylglycine peptide bond in each of these derivatives were then calculated from Arrhenius plots. While these more recent determinations suggest that the half-life for peptide bond hydrolysis at neutrality is considerably longer than 7 years, Radzicka and Wolfenden did observe, in agreement with Kahne and Still's results, little dependence of the rate on pH over the range 4.2−7.8.

Results and Discussion

Here, we report the use of homogeneous, solution-phase assays to measure directly the rate of hydrolysis at 37 °C of the peptide *N*-(phenylacetyl)glycyl-D-valine (PAGV) as a function of pH; the production of both glycyl-D-valine and D-valine was monitored (Figure 1). At pH 3, 5, 7, 9, and 11, aliquots from buffered incubations of peptide were removed periodically, alanine was added as an internal standard, and the mixture was allowed to react with NDA/CN (Figure 2). The resultant CBI derivatives were then detected by reverse-phase HPLC and their concentrations quantified by integration of the chromatogram. Although the glycyl-D-valine formed can, in principle, cyclize to yield the diketopiperazine (a species unreactive with NDA/CN and thus invisible to the assay), the dipeptide itself is expected to predominate at 37 °C.[6] Since the overall extent of reaction was never more than 0.2% (and was far less at pH 5, 7, and 9), the further hydrolysis of the product glycyl-D-valine (k_2, Figure 1) was assumed to be negligible.

All reactions were run in duplicate, and rate constants were calculated using the method of initial rates; the values reported are the weighted average of the two replicates. The results from an incubation at pH 7 (48 mM PAGV, 200 mM imidazole) are plotted in Figure 3. After 28 days, approximately 2.7 μM of both D-valine and glycyl-D-valine had been generated, corresponding to reaction of only 0.006% of the PAGV initially present. At pH 5 and 9, three different peptide concentrations were employed; the rate constants determined assuming first-order kinetics are essentially identical (Table 1), indicative that the hydrolysis reaction is, in fact, first-order in peptide.

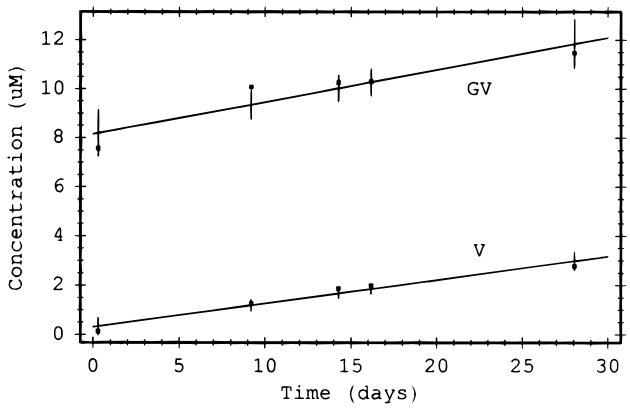

Figure 3. Reaction of 48 mM PAGV in 200 mM imidazole (pH 7). **GV**: production of glycyl-D-valine (the product present at time zero is due to residual contamination of the synthetic PAGV with glycyl-D-valine, see Experimental Section); **V**: production of D-valine. Error bars indicate the 95% confidence interval calculated from the predicted value of each point.

Table 1. Rate Constants for the Hydrolysis of the (Phenylacetyl)-glycyl Bond (k_1) and Glycyl-D-valyl Bond (k_3)[a]

pH	[PAGV] (mM)	k_1 (s^{-1})	k_3 (s^{-1})
5	9.5	$(7.41 \pm 3.19) \times 10^{-11}$	$(1.26 \pm 0.33) \times 10^{-10}$
5	19	$(8.90 \pm 5.10) \times 10^{-11}$	$(1.46 \pm 0.06) \times 10^{-10}$
5	38	$(5.45 \pm 2.26) \times 10^{-11}$	$(1.72 \pm 0.09) \times 10^{-10}$
9	6.0	$(3.88 \pm 0.26) \times 10^{-11}$	$(2.21 \pm 0.17) \times 10^{-10}$
9	12	$(3.97 \pm 0.16) \times 10^{-10}$	$(2.38 \pm 0.02) \times 10^{-10}$
9	24	$(3.62 \pm 0.14) \times 10^{-10}$	$(2.02 \pm 0.02) \times 10^{-10}$

[a] Hydrolysis measured at 37 °C and pH 5 (100 mM sodium acetate) or 9 (100 mM sodium tetraborate) as a function of PAGV concentration. The error limits indicated are ±1 standard error of the estimate.

Table 2. Rate Constants for the Hydrolysis of the Glycyl-D-valyl Bond (k_3)[a]

[buffer] (mM)	k_3, first replicate (s^{-1})	k_3, second replicate (s^{-1})
100	$(1.71 \pm 0.16) \times 10^{-9}$	$(1.35 \pm 0.12) \times 10^{-9}$
150	$(1.34 \pm 0.17) \times 10^{-9}$	$(1.36 \pm 0.16) \times 10^{-9}$
200	$(1.18 \pm 0.11) \times 10^{-9}$	$(1.50 \pm 0.09) \times 10^{-9}$

[a] Hydrolysis measured at 37 °C and pH 3 as a function of buffer (sodium malonate) concentration. The error limits indicated are ±1 standard error of the estimate.

In addition, three different buffer concentrations were employed at each pH. Typically, no discernible trend was apparent in the narrow range of rates observed, and the data were not extrapolated to zero buffer concentration. (The absence of buffer catalysis is in agreement with the earlier studies.[5,6]) Listed in Table 2, for example, are the data obtained at pH 3 for the hydrolysis of the glycyl-D-valyl bond (k_3). The final rate constants reported in Table 3 for hydrolysis at pH 3, 5, 7, 9, and 11 are, therefore, simply derived from the replicates measured in 150 mM buffer.

The rates at pH 0, 1, 13, and 14 were determined in unbuffered 1 M DCl, 0.1 M DCl, 0.1 M NaOD, and 1 M NaOD, respectively, and the progress of each reaction was monitored by ^{1}H NMR. A 1 M solution of DCl contains 1 M D_3O^+, and a 1 M solution of HCl contains 1 M H_3O^+. Hence, our designation of a 1 M solution of DCl as "pH" 0. The analogous argument holds for the pH 1, 13, and 14 solutions employed. (Although a solvent kinetic isotope effect, k_{H_2O}/k_{D_2O}, might be expected to skew the results, values reported for acid-catalyzed

Table 3. Rate Constants for the Hydrolysis of the (Phenylacetyl)-glycyl Bond (k_1) and Glycyl-D-valyl Bond (k_3) in PAGV at 37 °C as a Function of pH[a]

pH	k_1 (s^{-1})	k_3 (s^{-1})
0	$(2.52 \pm 0.01) \times 10^{-6}$	$(1.01 \pm 0.01) \times 10^{-6}$
1	$(9.30 \pm 1.30) \times 10^{-8}$	$(3.33 \pm 0.40) \times 10^{-7}$
3	$(2.11 \pm 0.09) \times 10^{-9}$	$(1.35 \pm 0.08) \times 10^{-9}$
5	$(4.91 \pm 1.07) \times 10^{-11}$	$(1.51 \pm 0.05) \times 10^{-10}$
7	$(4.65 \pm 0.44) \times 10^{-11}$	$(2.69 \pm 0.13) \times 10^{-11}$
9	$(4.31 \pm 0.05) \times 10^{-10}$	$(2.33 \pm 0.02) \times 10^{-10}$
11	$(1.72 \pm 0.07) \times 10^{-9}$	$(9.90 \pm 0.30) \times 10^{-10}$
13	$(2.32 \pm 0.94) \times 10^{-8}$	$(6.23 \pm 0.38) \times 10^{-8}$
14	$(3.23 \pm 0.06) \times 10^{-6}$	$(2.42 \pm 0.01) \times 10^{-6}$

[a] For pH 3, 5, 7, 9, 11, and 13, the error limits indicated are ±1 standard error of the estimate; for pH 0, 1, and 14, the error limits indicated represent the spread between the two independent determinations.

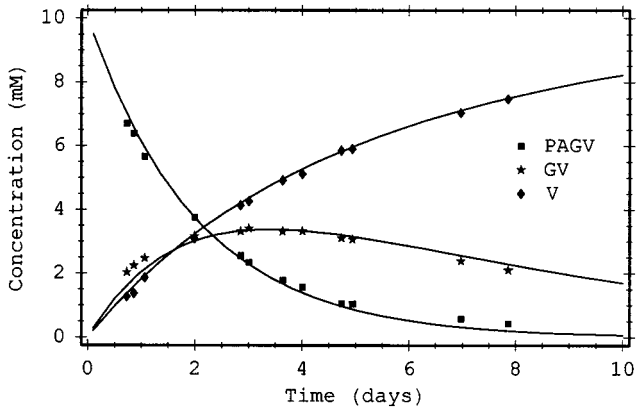

Figure 4. Reaction of 9.5 mM PAGV at pH 14. **PAGV**: *N*-(phenylacetyl)glycyl-D-valine; **GV**: glycyl-D-valine; **V**: D-valine.

anilide and base-catalyzed toluamide hydrolysis, reactions mechanistically similar to those studied here, are essentially unity.[7]) Signals for the diketopiperazine were not observed, as is expected at these extremes of pH.[6]

To analyze the data at pH 0, 1, and 14, exact analytic solutions were derived for the reaction kinetic scheme shown in Figure 1. The rate constants k_1 and k_3 were then calculated from nonlinear regression analysis, and the values reported in Table 3 for these pHs are the average of the two independent determinations. The data and fit for one of the pH 14 replicates are plotted in Figure 4. At pH 13, considerable overlap of the PAGV and glycyl-D-valine resonances was observed, and the data obtained could not be fit to the analytic equations. Thus, the rate constants shown in Table 3 for pH 13 were calculated from early time points using the method of initial rates.

At pH 0 and 14, the data were robust enough to yield reliable values for k_2, the rate constant for the hydrolysis of the initially formed glycyl-D-valine to free glycine and free D-valine. At pH 14, $k_2 = 1.81 \times 10^{-6}$ M^{-1} s^{-1}, a value nearly identical to k_1 and k_3 at this pH; at pH 0, $k_2 = 1.10 \times 10^{-7}$ M^{-1} s^{-1}, a value approximately 1 order of magnitude lower than k_1 and k_3. These results suggest that the rate of peptide bond hydrolysis is not particularly sensitive to substitution at or the protonation state of the flanking amino and carboxy functionalities.

To generate the pH-rate profiles shown in Figures 5 and 6, the data in Table 3 were analyzed according to the equation $k = k_{H_20} + k_{H_30^+}[H_3O^+] + k_{OH^-}[OH^-]$.[8] A nonlinear Levenberg−Marquardt fit of the data yielded the rate constants listed in Table 4. (Although the rate constants determined at basic pH deviate from the fit to a degree greater than those measured in acid, analyzing the data assuming a second-order dependence on $[OH^-]$ yielded a substantially poorer fit.) Three conclusions should be drawn from these results. One, at pH 7, the hydrolysis of both the (phenylacetyl)glycyl amide bond and glycyl-D-valine peptide bond is dominated by k_{H_2O}, the rate of direct water attack on peptide (or the kinetically indistinguishable attack of hydroxide on protonated peptide). The half-life corresponding to this rate constant is 243 years (with a range of 143−817 years within experimental error) for the hydrolysis of the (phenylacetyl)glycyl bond, while that for hydrolysis of the glycyl-D-valine bond is 267 years (with a range of 175−564 years). These values are in good agreement with the recent work of Radzicka and Wolfenden[6] (extrapolation of their pH 6.8 data to 37 °C yields half-lives of approximately 150 years). Two, the rate constants for specific-acid ($k_{H_3O^+}$) and specific-base (k_{OH^-}) catalyzed hydrolysis are almost identical, and they are comparable to the values previously measured for *N*,*N*-dimethylbenzamide.[9] That no deviation from linearity is seen at acid pH indicates further that $k_{H_3O^+}$ is independent of the protonation state of the terminal carboxylic acid functionality of PAGV. And three, the plateau in rate observed from pH 5−9 indicates that k_{H_2O} dominates the kinetics throughout this pH range. Intriguingly, a plateau is not observed at intermediate pH in the hydrolysis of ethyl acetate;[8] only a small plateau, if any, is observed in the hydrolysis of a variety of torsionally

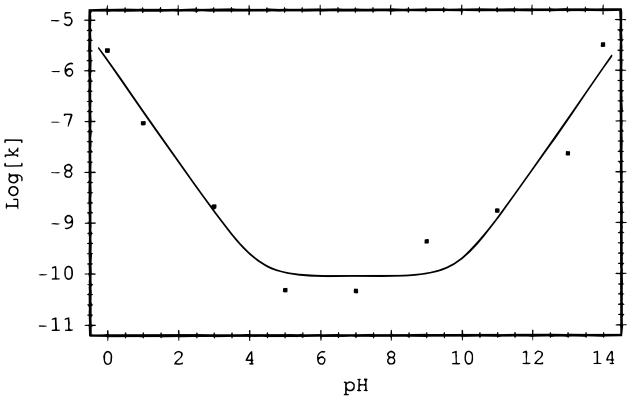

Figure 5. pH-rate profile for the hydrolysis of the (phenylacetyl)-glycyl bond (k_1) in PAGV. Points indicate the rate constants for the production of glycyl-D-valine.

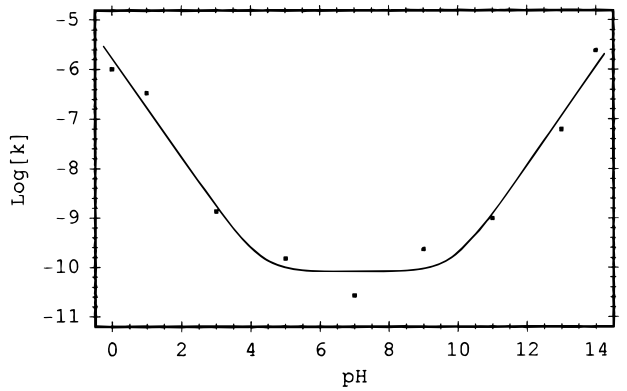

Figure 6. pH-rate profile for the hydrolysis of the glycyl-D-valine bond (k_3) in PAGV. Points indicate the rate constants for the production of D-valine.

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Table 4. Fit of the Data in Table 1 to the equation $k = k_{H_2O} + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-]$ for the Hydrolysis of the (Phenylacetyl)glycyl Bond (k_1) and Glycyl-D-valyl Bond (k_3) in PAGV at 37 °C[a]

bond hydrolyzed	k_{H_2O} (s^{-1})	$k_{H_3O^+}$ (M^{-1} s^{-1})	k_{OH^-} (M^{-1} s^{-1})
(phenylacetyl)glycyl (k_1)	$(9.05 \pm 6.36) \times 10^{-11}$	$(1.60 \pm 1.04) \times 10^{-6}$	$(1.11 \pm 0.73) \times 10^{-6}$
glycyl-D-valine (k_3)	$(8.23 \pm 4.33) \times 10^{-11}$	$(1.67 \pm 0.80) \times 10^{-6}$	$(1.16 \pm 0.56) \times 10^{-6}$

[a] The error limits indicated are asymptotic standard errors.

distorted and *N*-pyramidalized amides;[9,10] and a large plateau, not centered about pH 7, is observed for the hydrolysis of the electronically activated amide *p*-nitrotrifluoroacetanilide.[11] Clearly, no simple relationship exists between reactivity of acyl derivative and the relative magnitudes of k_{H_2O}, $k_{H_3O^+}$, and k_{OH^-}.

Experimental Section

General Methods. HPLC was performed on a Waters 510 dual-pump instrument equipped with a Waters 470 fluorescence detector and a 4.6 × 250 mm Alltima C-18 column (Alltech Associates). Data were analyzed using the software Milennium 2.0 (Waters). ^{1}H NMR spectra were obtained at 400 MHz on a JEOL GSX spectrometer. The data obtained were analyzed using Mathematica 3.0 (Wolfram Research, Inc.).

PAGV was synthesized from phenylacetyl chloride and glycyl-D-valine by a slight modification (NaOH was used in place of $NaHCO_3$) of a published procedure for benzoylglycine.[12] Even after repeated recrystallization from water, the PAGV contained a 0.02% contaminant of glycyl-D-valine. NDA was obtained from Bioanalytical Systems, Inc., and glycyl-D-valine was from BACHEM Bioscience, Inc. All other chemicals were reagent grade and were used without further purification.

Peptide Incubations at pH 3, 5, 7, 9, and 11. The concentrations of PAGV and buffer were chosen such that solutions were no more that 75% saturated with peptide (typically, solutions were well under 50% saturated). Peptide concentrations employed were the following: at pH 3, 1.9 mM; at pH 5, 38 mM; at pH 7, 48 mM; at pH 9, 24 mM; and at pH 11, 4.8 mM. Buffers and concentrations were the following: at pH 3, sodium malonate, 100, 150, and 200 mM; at pH 5, sodium acetate, 100, 150, and 200 mM; at pH 7, imidazole·HCl, 150, 200, and 250 mM; at pH 9, sodium tetraborate, 100, 150, and 200 mM; and at pH 11, triethylamine·HCl, 100, 150, and 200 mM. Each PAGV incubation was prepared as follows. The ionic strength of each of the above buffer solutions was adjusted to 0.25−0.35 with sodium chloride. The resultant solutions were then passed through Chelex 100, the PAGV was added to achieve the concentrations listed above, and the final pH was established. A quantity of 1.2 mL of each solution was then added to a 1.5-mL polyethylene microcentrifuge tube, which had been thoroughly and repeatedly washed with methanol and dried at 140 °C prior to use. The samples were incubated at 37 °C, and depending on the pH, aliquots were removed over a period of between 5 (pH 11) to 28 (pH 7) days for NDA/CN analysis.

NDA Assays. A 50-μL aliquot of a hydrolysis incubation was added to 200 μL of sodium tetraborate buffer (200 mM, pH 9.24) containing 0.25 μM alanine as the internal standard. Methanolic NDA (200 μL, 500 μM) and aqueous sodium cyanide (50 μL, 20 mM) were then added, and the mixture was incubated in the dark at room temperature for 40 min. Ten microliters of the NDA/CN derivatization reaction was injected onto the HPLC, and the CDI derivatives were eluted with a gradient of 40.5 to 70.5% aqueous CH_3CN (containing 0.025% TFA) at a flow rate of 1 mL/min over 30 min: $\lambda_{ex} = 246$ nm and $\lambda_{em} = 490$ nm. Typically, CBI-glycyl-D-valine eluted at approximately 19 min, CBI-alanine at 20 min, and CBI-D-valine at 28 min.

Peptide Incubations at pH 0, 1, 13, and 14. PAGV concentrations and solvents utilized were the following: at pH 0, 4.8 mM PAGV in 1 M DCl; at pH 1. 4.8 mM PAGV in 0.1 M DCl; at pH 13, 9.5 mM PAGV in 0.1 M NaOD; and at pH 14, 9.5 mM PAGV in 1 M NaOD.

^{1}H NMR Assays. One-milliliter samples were incubated at 37 °C directly in 5 mm NMR tubes, and depending on the pH, spectra were recorded over a period of between 8 (pH 14) and 60 (pH 1) days. The relative concentrations of PAGV, glycyl-D-valine, and D-valine were determined at pH 0 and 1 by integration of the respective valyl methyl resonances, and at pH 13 and 14 by integration of the valyl β-methine resonances.

To calculate the rate constants reported for pH 0, 1, and 14, exact analytic solutions were derived from the reaction scheme in Figure 1. The kinetic equations for the system are

$$\frac{d[PAGV]}{dt} = -k_1[PAGV] - k_3[PAGV] \quad (1)$$

$$\frac{d[\text{glycyl-D-valine}]}{dt} = k_1[PAGV] - k_2[\text{glycyl-D-valine}] \quad (2)$$

$$\frac{d[\text{D-valine}]}{dt} = k_2[\text{glycyl-D-valine}] + k_3[PAGV] \quad (3)$$

Moreover, at $t = 0$, $[PAGV] = [PAGV]_0$, and [glycyl-D-valine] = [D-valine] = 0. At all t, [PAGV] + [glycyl-D-valine] + [D-valine] = $[PAGV]_0$. Solving the above equations yields

$$[PAGV]_t = \frac{1}{e^{(k_1+k_3)t}}[PAGV]_0 \quad (4)$$

$$[\text{glycyl-D-valine}]_t = \frac{(e^{(k_1+k_3)t} - e^{k_2 t})k_1}{e^{(k_1+k_2+k_3)t}(k_1 - k_2 + k_3)}[PAGV]_0 \quad (5)$$

$$[\text{D-valine}]_t = \frac{e^{(k_1+k_2+k_3)t}k_1 - e^{(k_1+k_3)t}k_1 + e^{k_2 t}k_2 - e^{(k_1+k_2+k_3)t}k_2 + e^{(k_1+k_2+k_3)t}k_3 - e^{k_2 t}k_3}{e^{(k_1+k_2+k_3)t}(k_1 - k_2 + k_3)}[PAGV]_0 \quad (6)$$

The ^{1}H NMR data were then fit into equations 4−6 to yield values for k_1, k_2, and k_3 (pH 0 and 14) or k_1 and k_3 (pH 1).

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