J. Am. Chem. Soc. **1996,** *118,* 6105−6109

Rates of Uncatalyzed Peptide Bond Hydrolysis in Neutral Solution and the Transition State Affinities of Proteases

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Received December 5, 1995[⊗]

Abstract: To assess the relative proficiencies of enzymes that catalyze the hydrolysis of internal and C-terminal peptide bonds, the rates of the corresponding nonenzymatic reactions were examined at elevated temperatures in sealed quartz tubes, yielding linear Arrhenius plots. The results indicate that in neutral solution at 25 °C, peptide bonds are hydrolyzed with half-times of approximately 500 years for the C-terminal bond of acetylglycylglycine, 600 years for the internal peptide bond of acetylglycylglycine *N*-methylamide, and 350 years for the dipeptide glycylglycine. These reactions, insensitive to changing pH or ionic strength, appear to represent uncatalyzed attack by water on the peptide bond. Comparison of rate constants indicates very strong binding of the altered substrate in the transition states for the corresponding enzyme reactions, K_{tx} attaining a value of less than 10^{-17} M in carboxypeptidase B. The half-life of the N-terminal peptide bond in glycylglycine *N*-methylamide, whose hydrolysis might have provided a reference for assessing the catalytic proficiency of an aminopeptidase, could not be determined because this compound undergoes relatively rapid intramolecular displacement to form diketopiperazine ($t_{1/2} \sim 35$ days at pH 7 and 37 °C). The speed of this latter process suggests an evolutionary rationale for posttranslational N-acetylation of proteins in higher organisms, as a protection against rapid degradation.

Enzymes catalyzing the hydrolysis of peptide bonds have been characterized extensively with respect to their catalytic efficiencies (k_{cat}/K_m), structures, and sensitivities to reversible and irreversible inhibitors. To appreciate the abilities of peptide hydrolases to enhance the rates of hydrolysis of peptide bonds, it would be desirable to know the rate constants (k_{non}) of nonenzymatic reactions corresponding to those catalyzed by carboxypeptidases, endopeptidases, aminopeptidases, and dipeptidases, under comparable conditions. The rate constant of the uncatalyzed reaction, currently established for only a few enzymes, also provides a basis for estimating “catalytic proficiency”, defined as $(k_{cat}/K_m)/k_{non.}$[1] Expressed in units of M^{-1}, catalytic proficiency furnishes a direct indication of the minimal affinity of an enzyme for the altered substrate in the transition state, and its potential susceptibility to inhibition by stable compounds resembling highly activated reaction intermediates, i.e., transition state analogue inhibitors.

Peptide hydrolysis is known to be catalyzed by acids, bases, and metal complexes. Glycylglycine, for example, is hydrolyzed with a half-time of approximately 2 days in 1 M NaOH and 150 days in 1 M HCl, at 25 °C,[2] and unactivated peptide bonds are cleaved rapidly in the presence of complexes of palladium[3] and copper.[4] The uncatalyzed hydrolysis of peptide bonds had received relatively little attention, however, until Kahne and Still, using a resin-bound peptide with ^{14}C-labeled glycine at its C-terminus, were able to monitor the release of small quantities of glycine with an extrapolated half-time of several years, throughout the pH range near neutrality.[5]

The present work was undertaken to establish whether there might be significant differences between bonds whose hydrolysis is catalyzed by carboxypeptidases, endopeptidases, aminopeptidases, and dipeptidases, in terms of their inherent rates of hydrolysis in the absence of a catalyst. This would allow estimation of the catalytic proficiencies of these enzymes with each other and with those of other C−N hydrolases whose catalytic proficiencies have already been determined.[1] This paper describes the cleavage of glycine-containing peptides of various types, observed by analyzing the contents of sealed tubes that had been exposed to elevated temperatures for timed intervals. Using the integrated intensities of carbon-bound proton resonances observed by high-field NMR, it proved possible to account for all species arising during the cleavage of simple peptides based on glycine and to determine first order rate constants for peptide cleavage. Although susceptible to decarboxylation at higher temperatures, the product glycine itself did not undergo more than 1% decomposition under the most extreme conditions used in these experiments. Rate constants obtained at elevated temperatures were then extrapolated to room temperature.

First order rate constants for hydrolysis of bonds joining two glycine residues were determined for (1) glycylglycine, representing the substrate of a generic dipeptidase, (2) *N*-acetylglycylglycine, representing the substrate of a generic carboxypeptidase, and (3) *N*-acetylglycylglycine *N′*-methylamide, and the results were extrapolated to 25 °C. The rate of hydrolysis of the peptide bond in glycylglycine *N*-methylamide, which might have represented the substrate of a generic endopeptidase, could not be measured because of its rapid cyclization to form diketopiperazine.

Experimental Section

Acetylglycine, glycylglycine, and glycylglycine ethyl ester hydrochloride were obtained from Sigma Chemical Co. and used without further purification. Glycine *N*-methylamide and glycylglycine *N*-methylamide were prepared by the action of 40% aqueous methylamine

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[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1996.

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S0002-7863(95)04077-7 CCC: $12.00 © 1996 American Chemical Society

Table 1. Proton NMR Chemical Shifts, Relative to TSP, in D_2O Solution Buffered at pD 6.8 with 0.1 M Potassium Phosphate

species	chemical shift
acetyglycylglycyine N-methylamide	
$CH_3CONHC\mathbf{H}_2CONHC\mathbf{H}_2CONHCH_3$	3.92 (doublet)
$CH_3CONHCH_2CONHCH_2CONHC\mathbf{H}_3$	2.82
$C\mathbf{H}_3CONHCH_2CONHCH_2CONHCH_3$	2.05
acetylglycylglycine	
$CH_3CONHC\mathbf{H}_2CONHC\mathbf{H}_2COO^-$	3.90 (doublet)
$C\mathbf{H}_3CONHCH_2CONHCH_2COO^-$	1.98
glycylglycine N-methylamide	
$NH_3^+C\mathbf{H}_2CONHC\mathbf{H}_2CONHCH_3$	3.81 (doublet)
$NH_3^+CH_2CONHCH_2CONHC\mathbf{H}_3$	2.73
glycylglycine	
$NH_3^+C\mathbf{H}_2CONHC\mathbf{H}_2COO^-$	3.69 (doublet)
diketopiperazine	
$HNC\mathbf{H}_2CO$ / $OCC\mathbf{H}_2NH$	4.08
acetylglycine	
$CH_3CONHC\mathbf{H}_2COO^-$	3.67
$C\mathbf{H}_3CONHCH_2COO^-$	1.98
glycine N-methylamide	
$NH_3^+C\mathbf{H}_2CONHCH_3$	3.70
$NH_3^+CH_2CONHC\mathbf{H}_3$	2.73
glycine	
$NH_3^+C\mathbf{H}_2CO^-$	3.56
methylamine	
$NH_3^+C\mathbf{H}_3$	2.60
N-methylacetamide	
$CH_3CONHC\mathbf{H}_3$	2.60
$C\mathbf{H}_3CONHCH_3$	1.95
acetate	
$C\mathbf{H}_3COO^-$	1.89

(0.5 mol) on glycine methyl ester or glycylglycine ethyl ester hydrochloride (0.1 mol) at 50 °C for 30 min. After addition of NaOH (0.12 mol) and rotary evaporation to remove excess methylamine, the product was recrystallized from water. Acetylglycylglycine and acetylglycylglycine *N*-methylamide were prepared by the action of a 5-fold excess of acetic anhydride on glycylglycine or glycylglycine *N*-methylamide in boiling acetic acid for 2 h, followed by removal of acetic acid under vacuum.

Each of these products was found to be free of proton-containing impurities by proton NMR in D_2O, observed using a Bruker AMX-500 spectrometer. Chemical shifts of these compounds, and of the products of their decomposition, are shown in Table 1.

In a typical experiment, 0.5 mL of a solution of peptide (0.01 M) in buffer (0.1 M potassium phosphate or potassium acetate, adjusted to ionic strength 2.0 with KCl) was sealed under vacuum in a quartz tube (4 mm internal diameter, 1 mm thick, obtained from G. M. Associates, Inc., Oakland, CA). The sealed tube was placed in an oil bath in an oven for a measured time interval and then opened. An aliquot (0.2 mL) was evaporated to dryness, D_2O (2 mL) was added, and the sample was evaporated to dryness; this procedure was repeated twice to replace exchangeable protons. The sample was then dissolved in D_2O (0.5 mL), and pyrazine (0.01 M in D_2O, 0.5 mL) was added as an internal standard for measuring integrated intensities of nonexchangeable protons by NMR. This procedure, introducing D_2O after incubation at elevated temperature, was adopted (1) to avoid solvent deuterium effects on reaction rates and (2) to avoid exchange of carbon-bound hydrogen atoms. By observing the integrated intensities of the signals from carbon-bound protons (Table 1), it proved possible to follow the course of each of these reactions by high-field NMR, accounting for each of the species arising during the course of peptide hydrolysis. First order rate constants were obtained by dividing log (A_o/A) by the time elapsed, where A_o = the starting concentration of peptide, and A = (A_o minus the concentration of product formed).

Results

Equilibration between Glycylglycine (GG)[6] and Diketopiperazine (G=G). Earlier work has shown that during its slow hydrolysis to glycine in neutral solution, glycylglycine (GG) enters into relatively rapid equilibrium with diketopiperazine (G=G).[7-9] As ring opening is thermodynamically favored by ionization of GG, with pK_a values of 3.1 and 8.1 at 25 °C,[10] cyclization was not found to occur to a significant extent during earlier observations of acid- and base-catalyzed hydrolysis of GG.[2] In neutral solution, we found that GG and G=G were interconverted very rapidly compared with the rate of hydrolysis of GG, with the apparent rate constants for approach to equilibrium shown in Figure 1. Extrapolated to 25 °C, these data indicate that interconversion of GG and G=G occurs with an overall rate constant ($k_1 + k_{-1}$) of approximately 2×10^{-8} s^{-1}, corresponding to a $t_{1/2} \sim 1$ year at pH 7.

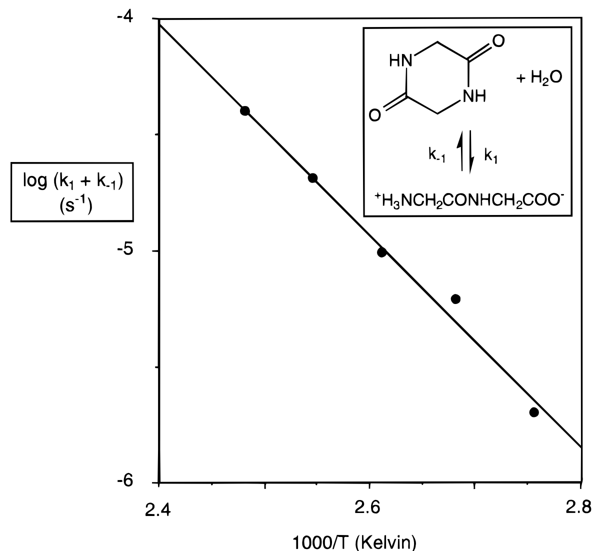

Figure 1. Rate constants ($k_1 + k_{-1}$) for approach to equilibrium between diketopiperazine (G=G) and glycylglycine (GG).

We next examined the position of equilibrium between GG and G=G, starting either from pure GG or pure G=G, in experiments conducted over time intervals equivalent to more than 8 half-lives for their interconversion, with the results shown in Figure 2. The same results were obtained regardless of the position from which equilibrium was approached, and the observed equilibrium constant for conversion of GG to G=G varied with temperature, with values ranging from a value of 0.44 at 160 °C to a value of less than 0.18 at 84 °C.[11] Extrapolation of the line in Figure 2 yields an equilibrium constant of 0.08 for conversion of G=G to GG at 25 °C. In view of the downward curvature of the plot, this should presumably be regarded as an upper limit.

Hydrolysis of Glycylglycine (G-G). Formation of G from GG, after correction for the rapid equilibration of GG with G=G at elevated temperatures to obtain the concentration of GG that was actually present under the reaction conditions, followed satisfactory first order kinetics over the temperature range from 120° to 200°, as indicated by linear semilogarithmic plots of

(6) Abbreviations: AcG = acetylglycine, AcGG = acetyglycylglycine, GG = glycylglycine, G=G = diketopiperazine, GGNHMe = glycylglycine *N*-methylamide, AcGGNHMe = acetyglycylglycine *N*-methylamide.

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(11) A van't Hoff plot of these equilibrium constants, shown in Figure 2, indicates that cyclization of GG to G=G is accompanied by gains in enthalpy and entropy. The downward concavity of this plot also suggests that these compounds differ in heat capacity (see also ref 12).

Table 2. Rates and Equilibria at pH 6.8[a]

reactants	products	$k_{150\,°C}$ (s^{-1})	$k_{25\,°C}$ (s^{-1})	E_{act} (kcal/mol)
AcG-GNHMe hydrolysis	AcG + GNHMe	5.1×10^{-6}	3.6×10^{-11}	+23.5
AcG-G hydrolysis	AcG + G	6.2×10^{-6}	4.4×10^{-11}	+25.0
G-G hydrolysis	G + G	8.9×10^{-6}	6.3×10^{-11}	+23.0
GGNHMe cyclization	G=G + H_2NMe	$>10^{-3}$	8×10^{-8}	+17.2
GG cyclization	G=G + H_2O	$>10^{-3}$ 0.40 (K_{eq})	2×10^{-8} ≥ 0.08 (K_{eq})[b]	not determined

[a] Slopes obtained by linear regression (Figures 1−6) were used to calculate values of E_{act} and are associated with standard errors of ±5%. [b] Maximal value, ignoring downward curvature of Arrhenius plot (see text).

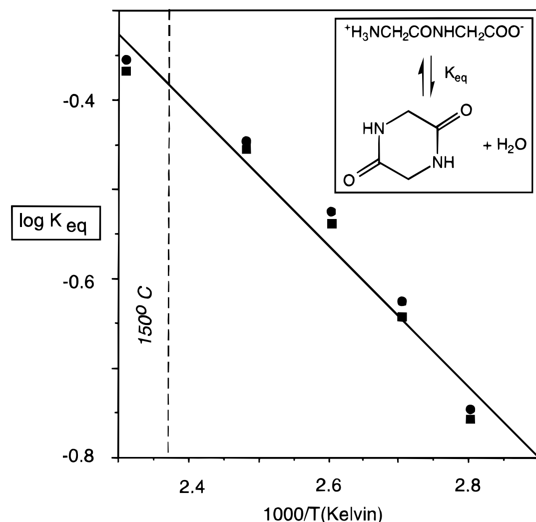

Figure 2. Equilibrium constants for conversion of GG to G=G, starting from GG (squares) or from G=G (circles).

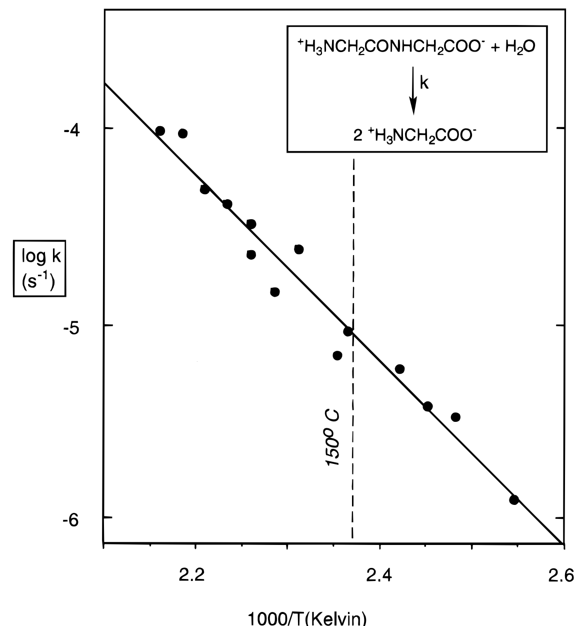

Figure 3. Rate constants for hydrolysis of GG, after correction for the fraction of total glycylglycine that is present as G=G (see text).

A_o/A as a function of time elapsed. To determine the rate constant for hydrolysis of GG to glycine, allowance was made for the "buffering" effect of the relatively rapid equilibrium between GG and G=G described above, by expressing rates of product formation as a function of the reduced concentrations of GG that were actually present at equilibrium under the reaction conditions, as determined in the previous section.[13] In 0.1 M potassium acetate and phosphate buffers in the range from pH 4.2 to pH 7.8, hydrolysis of GG was found to proceed with a rate constant of 8.9 (±0.7) × 10^{-5} s^{-1}. Rates of reaction did not change when buffer concentrations were varied in the range from 0.1 to 0.3 M, nor was the rate of reaction affected by variations of the ionic strength from 0.5 to 2, adjusted by adding KCl to 0.1 M potassium acetate buffer (pH 4.7) or potassium phosphate buffer (pH 6.8). Figure 3 shows apparent rate constants in 0.1 M potassium acetate, pH 5.0, plotted as a logarithmic function of $1/T$, yielding E_{act} = +23 kcal/mol, for the conversion of GG to G + G in the neutral pH range.

Hydrolysis of Acetylglycylglycine (AcGG). Acetylglycylglycine (AcGG), with a peptide bond resembling those cleaved by a carboxypeptidase, was found to decompose mainly by hydrolysis of its peptide bond, to yield AcG + G as the major products, with acetate + GG arising as minor products. At 150 °C, for example, AcGG disappears with a first order rate constant of 1.1 × 10^{-5} s^{-1}, while acetylglycine (AcG) accumulates with an apparent first order rate constant of 5.1 × 10^{-6} s^{-1}, and GG and G=G accumulate together with a combined apparent rate constant of 3 × 10^{-6} s^{-1}. Separate experiments showed that the product AcG is hydrolyzed with a rate constant of 2 × 10^{-6} s^{-1} under these conditions and that the relative rate of this reaction does not change significantly at lower temperatures. After subtracting the rate of disappearance of the product AcG, the rate constant for cleavage of the peptide bond of AcGG was estimated from the rate of appearance of AcG, as approximately 6.0 (±1.3) × 10^{-6} s^{-1} at 150 °C, in 0.1 M potassium acetate and phosphate buffers in the range from pH 4.7 to pH 7.8. Rates of reaction did not vary when the concentrations of acetate or phosphate buffers varied in the range from 0.1 to 0.3 M, nor was the rate of hydrolysis affected by variations in ionic strength produced by addition of KCl, from 0.3 to 2.0. Figure 4 shows rate constants for AcGG hydrolysis, observed over the temperature range between 110 and 170 °C, plotted as a logarithmic function of $1/T$. This plot yields E_{act} = +25 kcal/mol for the conversion of AcGG to AcG + G in the neutral pH range.

Hydrolysis of Acetylglycylglycine *N*-Methylamide (AcG-GNHMe). Acetylglycylglycine *N*-methylamide (AcGGNHMe), with a peptide bond resembling those cleaved by an endopeptidase, undergoes nonenzymatic hydrolysis of its peptide bond to yield AcG and GNHMe as the major products (>90%), with a rate constant of 6.0 (±1.1) × 10^{-7} s^{-1} at 150 °C, in 0.1 M potassium acetate and imidazole-HCl buffers in the range from pH 4.2 to pH 7.8. When buffer concentrations were varied, we detected no evidence of catalysis by acetate or imidazole buffers in the range from 0.1 to 0.3 M. Unlike hydrolysis of GG or AGG, however, hydrolysis of AGGNHMe was found to be subject to significant catalysis by potassium phosphate buffers

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(13) Decarboxylation of glycine, negligible under the conditions of the present experiments, occurs to a significant extent at higher temperatures, as noted earlier by Qian et al. (ref 9). These authors reported rate constants for hydrolysis of GG to G, in unbuffered solution, that are in substantial agreement with the present values at 100 and 120 °C but deviate toward lower values at 160 and 220 °C. The latter discrepancy can probably be ascribed, at least in part, to the lack of correction by Qian et al. for the increasing fraction of total GG that is present as G=G at higher temperatures.

Figure 4. Rate constants for hydrolysis of AcGG to yield AcG and glycine.

Figure 5. Rate constants for hydrolysis of AcGGNHMe to yield AcG and GNHMe.

($k_2 = 5.5\ (\pm 1) \times 10^{-5}\ s^{-1}\ M^{-1}$ at pH 6.8, 150 °C). Figure 5 shows rate constants observed for the uncatalyzed reaction in potassium acetate buffer, pH 5.0, over a temperature range between 100 and 170°, plotted as a logarithmic function of $1/T$. This plot yields $E_{act} = +23.5$ kcal/mol for the conversion of AcGGNHMe to AcG + GNHMe in the neutral pH range.

Cyclization of Glycylglycine *N*-Methylamide (GGNHMe). Hydrolysis of the peptide bond in glycylglycine *N*-methylamide (GGNHMe) might in principle have served as a model for the reaction catalyzed by an aminopeptidase. Instead, this compound undergoes rapid intramolecular cyclization to form diketopiperazine, with displacement of methylamine, as shown in Scheme 2. Aside from methylamine and diketopiperazine, no trace of any other product was observed. This reaction proceeded to completion as a simple first order process, generating linear semilogarithmic plots of A_o/A as a function of time elapsed. At 80 °C, we obtained a rate constant of 6.2 ($\pm$1) $\times$ 10^{-7} s^{-1}, in potassium phosphate and imidazole-HCl buffers at pH 6.8. Figure 6 shows the temperature dependence

Figure 6. Rate constants for cyclization of GGNHMe, with release of G=G and $MeNH_2$.

Scheme 1

Scheme 2

of diketopiperazine formation in 0.1 M potassium phosphate buffer, pH 6.8, over the temperature range between 37 and 130 °C. This plot yields $E_{act} = 17.2$ kcal/mol for cyclization of GGNHMe. At 37 °C, the rate constant for this process was $3.2 \times 10^{-7}\ s^{-1}$, corresponding to a half-time of 25 days.

Discussion

The hydrolyses of AcG-GNHMe, AcG-G, and G-G appear to proceed predominantly by a mechanism involving uncatalyzed attack by water on the peptide bond. Thus, each of these reactions proceeds at a rate that is insensitive to pH in the range between 4.2 and 7.8, and none of these reactions is sensitive to variations in ionic strength in the range between 0.2 and 2, obtained by adding KCl to 0.1 M potassium acetate or potassium phosphate buffers. In earlier work, a similar invariance of rate with pH was reported for release of a C-terminal glycine residue from a resin-bound tripeptide of phenylalanine.[5,14] Also in accord with the present results, the reported rates of acid- and base-catalyzed hydrolysis of GG,[2] extrapolated to pH 7, are too slow by several orders of magnitude to have contributed

(14) The present rate constant for hydrolysis of the peptide bond in AcGG, obtained by extrapolation to 25 °C and pH 7, is $2 \times 10^{-11}\ s^{-1}$, considerably lower than the rate constant ($3 \times 10^{-9}\ s^{-1}$) reported earlier by Kahne and Still [5] for release of C-terminal glycine from a tetrapeptide (-Phe-Phe-Phe-Gly) joined through its N-terminus to a polyacrylamide resin. The nature of the peptide bond undergoing cleavage is similar in these two reactions; and steric hindrance, if any, seems more likely to have occurred in the case of the resin-bound peptide. This suggests the possibility that attack by water on the resin-bound peptide might be assisted by its proximity to the polymer matrix, directly or through some effect on the solvent environment.

Table 3. Catalytic Proficiencies of Some Peptide Hydrolases[a]

enzyme	$t_{1/2\ nonenz}$ (years)	k_{non} (s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)	rate enhancement k_{cat}/k_{non}	catalytic proficiency $(k_{cat}/K_m)/k_{non}$ (M^{-1})
C-terminal peptide bond (exemplified by AcGG) vs carboxypeptidase B, 23 °C[b]	1100	1.8×10^{-11}	238	6×10^{6}	1.3×10^{13}	3.3×10^{17}
internal peptide bond (exemplified by AcGGNHMe) vs angiotensin-converting enzyme, 37 °C[c]	19	1.13×10^{-9}	13.9	6×10^{4}	1.2×10^{10}	5.3×10^{13}
dipeptide bond (exemplified by GG) vs ascites tumor dipeptidase, 40 °C[d]	15	1.5×10^{-9}	1842	7.4×10^{5}	1.2×10^{12}	4.9×10^{14}

[a] Nonenzymatic reaction rates for glycine compounds (k_{non}) are extrapolated from the present data, to the temperature at which the enzyme reaction was investigated. Protease reactions known to proceed through covalent intermediates, such as those catalyzed by chymotrypsin and papain, are not included in this table, because such reactions do not permit straightforward estimation of transition state affinities from comparisons of enzymatic and nonenzymatic reaction rates (ref 1). [b] Carboxypeptidase B + hippuryl-L-arginine, pH 8, 23 °C (ref 18). [c] Angiotensin-converting enzyme + cbz-p-(NO_2)Phe-His-Leu, pH 8.0, 37 °C (ref 19). [d] Ascites tumor dipeptidase + Ala-Gly, pH 8.3, 40 °C (ref 20).

significantly to the rate of hydrolysis of GG at pH 7, obtained by extrapolation in the present work.

Peptide bonds in AcG-GNHMe, AcG-G, and G-G are hydrolyzed at rates that differ by less than a factor of 2 at 150 °C, nor do their energies of activation, obtained by linear regression analysis of the Arrhenius plots, differ by more than the experimental error (~1.2 kcal/mol) estimated from the standard errors of the slopes of each of the individual plots. Substituent effects in this series appear too insignificant to justify any attempt at detailed interpretation. Based on an average value of 23.8 kcal/mol for the energy of activation of all three compounds, AcG-GNHMe, AcG-G and G-G undergo uncatalyzed hydrolysis at 25 °C with half-times of 600, 500, and 350 years, respectively, at pH values near neutrality.

The observed route of decomposition of GGNHMe, via rapid formation of diketopiperazine, agrees with earlier observations of a similar route of degradation of glycylglycine amide at 130 °C[15] and of several peptides that have been shown to be degraded by elimination of diketopiperazines from the N-terminal position at 100 °C.[16] GGNHMe cyclizes too rapidly to permit measurement of the rate of the competing hydrolytic cleavage of the peptide bond in GGNHMe, and G=G and methylamine are the only products observed. In GGNHMe, peptide hydrolysis is evidently much slower than cyclization, so that its rate cannot be measured directly as a model for the reaction catalyzed by an aminopeptidase. From the lack of sensitivity of uncatalyzed peptide hydrolysis to substituent effects, noted above, it seems reasonable to infer that hydrolysis in GGNHMe, if it could be measured, might proceed at a rate similar to those observed in the other cases.

Table 3 compares the present rate constants, for hydrolysis of bonds in glycine peptides, with kinetic constants that have been reported for proteolytic enzymes acting on peptide bonds in similar chemical environments. By these criteria, carboxypeptidase B shows high proficiency as a catalyst, with $(k_{cat}/K_m)/k_{non} \sim 4 \times 10^{17}\ M^{-1}$. The reciprocal of this value corresponds to a maximal dissociation constant of approximately 2.5×10^{-18} M for the enzyme–substrate complex in the transition state. This K_{tx} value is slightly lower than those that have been estimated for adenosine deaminase and cytidine deaminase, which also catalyze the hydrolysis of C–N bonds.[17–20,21] Table 3 shows that an endopeptidase (the angiotensin-converting enzyme) and also a dipeptidase from ascites tumor cells are somewhat less proficient in enhancing the rates of their respective reactions.[22] Nevertheless, enzymes with transition state affinities of this magnitude offer exceptionally promising targets for the design of potent competitive inhibitors.

The present results, summarized in Table 2, indicate that in solvent-exposed positions in proteins, a typical C-terminal peptide bond is hydrolyzed at a rate comparable with that of a typical internal peptide bond. In the absence of inter- or intramolecular catalysis, such a bond would be expected to survive for several centuries in neutral solution at ambient temperatures. In contrast, the peptide bond that joins an unprotected N-terminal dipeptide to the rest of a protein chain undergoes cleavage, to form a diketopiperazine, more than 1000-fold more rapidly. Accordingly, a typical protein would be expected to be completely degraded to diketopiperazines within less time than the time required for hydrolysis of a single internal or C-terminal peptide bond.

With a half-time of approximately 35 days at pH 7 and 37 °C, diketopiperazine formation is rapid enough to pose an apparent threat to the stability of proteins and to suggest a possible rationale for the posttranslational N-acetylation of proteins that has been observed in most proteins in higher organisms.[23] It seems reasonable to speculate that in the relatively long-lived cells of eukaryotes, N-acetylation has evolved as a mechanism for protecting proteins against spontaneous degradation. Short generation times presumably alleviate this problem in prokaryotic organisms, in which mechanisms for N-acetylation appear to be absent.

Acknowledgment. Financial support for this work was provided by Research Grant No. GM-18325 from the NIH.

JA954077C

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(21) The present rate constants for hydrolysis of C–N bonds in GG, AcGG, and AcGGNHMe in neutral solution are surprisingly similar to rate constants reported earlier for hydrolysis of the exocyclic C–N bonds in cytidine and adenosine.[20] Bond distances (*Interatomic Distances*; Chemical Society: London, 1958) suggest that exocyclic C–N bonds of cytidine and adenosine, like peptide bonds, may be markedly stabilized by resonance. Thus, exocyclic CN bonds in derivatives of cytosine (1.31 Å) and adenine (1.30 Å) are slightly shorter than a value considered typical of the C–N bond in peptides (1.325 Å) (Pauling, L. *The Nature of the Chemical Bond*; Cornell: Ithaca, NY, 1960; pp 281–282).

(22) To reduce ambiguities of interpretation and render enzymatic and nonenzymatic reactions more closely comparable for purposes of estimating catalytic proficiency (see ref 1), Table 3 is limited to proteases that do *not* appear act through double displacement mechanisms.

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