Decarboxylation of Peptide-Bound Aminomalonic Acid[1]

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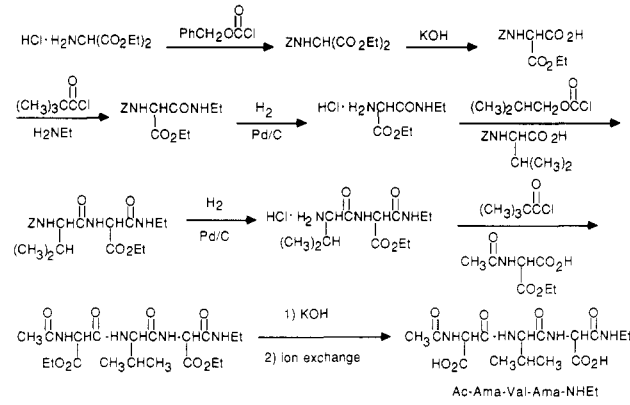
The kinetics of decarboxylation of the monopeptide of aminomalonic acid (Ama), N^{α}-acetyl-Ama *N*-ethylamide, to the corresponding monopeptide of glycine (Gly), N^{α}-acetyl-Gly *N*-ethylamide, are described. The rate constants for decarboxylation of the anionic and neutral species at 120 °C in aqueous medium are 0.002 22 and 0.26 min^{-1}, respectively, and the pK_a relating these species is 2.33. The Arrhenius activation energy and preexponential factor for decarboxylation at the anionic state of the Ama monopeptide are 33.1 kcal/mol and 8×10^{13} s^{-1}, respectively. These values predict a half-life for protein-bound Ama with respect to decarboxylation to protein-bound Gly of 54 years under physiological conditions. They also predict that under the conditions of alkaline protein hydrolysis used in amino acid analysis of malonic acid bearing amino acids, some of the protein-bound Ama will decarboxylate to protein-bound Gly prior to hydrolysis. With the simple peptides N^{α}-Ac-Ama-NHEt and N^{α}-Ac-Ama-Val-Ama-NHEt, 4% and 22%, respectively, of the Ama residues decarboxylated to Gly residues during alkaline hydrolysis.

Introduction

Aminomalonic acid (Ama), the congener of γ-carboxyglutamic acid (Gla)[2] and β-carboxyaspartic acid (Asa),[3] has been found as a constituent of the alkaline hydrolysates of proteins from *Escherichia coli* and atherosclerotic plaque.[4] Gla residues are formed from vitamin-K-mediated posttranslational carboxylation of glutamic acid (Glu) residues[5] and are found especially in blood coagulation[2,6] and bone proteins.[7] The malonic acid unit of Gla imparts the property of calcium binding to the protein. Although an Ama residue bears only a single free carboxylic acid functional group, that group plus the carbonyl of the peptide backbone constitute a malonic acid unit. The potential of an Ama residue for calcium binding and observation of Ama in alkaline hydrolysates of atherosclerotic plaque implicate Ama formation in the pathological calcification of arterial proteins in atherosclerosis. Because the levels of Ama detected in alkaline hydrolysates of atherosclerotic plaque are modestly low, in the range of 1–20 Ama/1000 glycine (Gly), we were concerned that possibly some Ama was lost during the hydrolysis because of decarboxylation to form Gly. In an earlier work, Thanassi studied the decarboxylation of free aminomalonic acid at 45 °C as a function of pH.[8] A rate was found for the decarboxylation of the neutral species, $^{+}H_3NCH(CO_2H)CO_2^{-}$, that was more than 2.5 times the rate for the decarboxylation of the fully protonated form, $^{+}H_3NCH(CO_2H)_2$. He argued that this finding was not consistent with the six-membered cyclic mechanism proposed by Westheimer for the decarboxylation of β-ketocarboxylic acids.[9] Thanassi proposed an anionic mechanism where the developing negative charge at the α carbon is stabilized by the positively charged amino group (Figure 1, structure 1). Decarboxylation was reported not to occur when Ama was in the anionic state $^{+}H_3NCH(CO_2^{-})_2$ under these conditions. Subsequently, Fitzpatrick, Jr., and Hopgood estimated an upper limit for the rate constant for decarboxylation of $^{+}H_3NCH(CO_2^{-})_2$ more than 100 times smaller than the rate constant for decarboxylation of the zwitterion.[10] At physiological pH, peptide-bound aminomalonic acid exists as the anion (Figure 1, structure 2). An anionic mechanism for the decarboxylation of **2** would not be expected to be as efficient as for decarboxylation of **1** due to the decrease in stabilization of the transition state afforded by an amide nitrogen as compared to a protonated amino group. However, the possibility of such a mechanism raised questions regarding the stability of peptide-bound aminomalonic acid at physiological conditions and under the conditions of base hydrolysis.

Scheme I



Other studies of the decarboxylation of these malonic acid bearing amino acids include a comparison of the rates of decarboxylation of Ama, Asa, and Gla in 1 M hydro-

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(2) Stenflo, J.; Fernland, P.; Egan, W.; Roepstorff, P. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *74*, 2730. Nelsestuen, G. L.; Zythovicz, T. H.; Howard, J. B. *J. Biol. Chem.* **1974**, *249*, 6347. Magnusson, S.; Sottrup-Jensen, L.; Petersen, T. E.; Morris, H. R.; Dell, A. *FEBS Lett.* **1974**, *44*, 189.

(3) Christy, M. R.; Barkley, R. M.; Koch, T. H. *J. Am. Chem. Soc.* **1981**, *103*, 3935. Koch, T. H.; Christy, M. R.; Barkley, R. M.; Sluski, R.; Bohemier, D.; Van Buskirk, J. J.; Kirsch, W. M. *Methods Enzymol.* **1984**, *107*, 563.

(4) Van Buskirk, J. J.; Kirsch, W. M.; Kleyer, D. L.; Barkley, R. M.; Koch, T. H. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 722.

(5) *Vitamin K Metabolism and Vitamin K Dependent Proteins*; Suttie, J. W., Ed.; University Park Press: Baltimore, 1980.

(6) Nelsestuen, G. L.; Suttie, J. W. *Biochemistry* **1972**, *11*, 4961.

(7) Hauschka, P. V.; Lian, J. B.; Gallop, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3925. Price, P. A.; Otsuka, A. S.; Poser, J. W.; Kristaponis, J.; Raman, N. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1447.

(8) Thanassi, J. W. *Biochemistry* **1970**, *9*, 525.

(9) Westheimer, F. H.; Jones, W. A. *J. Am. Chem. Soc.* **1941**, *63*, 3283.

(10) Fitzpatrick, J. H., Jr.; Hopgood, D. *Inorg. Chem.* **1974**, *13*, 568.

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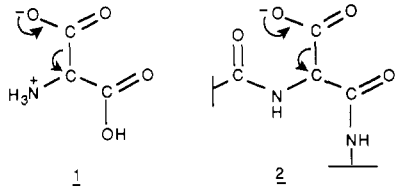
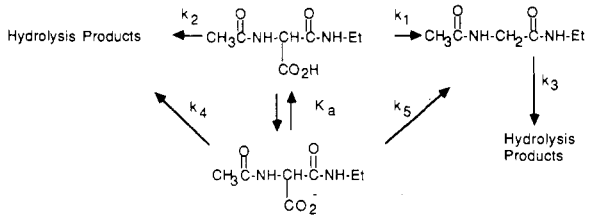


Figure 1.

Scheme II



chloric acid at 100 °C by Hauschka and co-workers.[11] They reported the half-lives to be 1.2, 1.7, and 8.6 min, respectively. Christy and Koch demonstrated that both Asa and peptide-bound Asa decarboxylated at very low pH and eliminated the amino functional group in competition with decarboxylation at higher pH's.[12] Because of these previous observations, the reactivity of peptide-bound aminomalonic acid was investigated as a function of both pH and temperature with use of N^{α}-acetyl-Ama *N*-ethylamide and N^{α}-acetyl-Ama-Val-Ama *N*-ethylamide as model peptides, and the results are reported here.

Results and Discussion

N^{α}-Acetyl-Ama *N*-ethylamide was prepared from commercially available diethyl N^{α}-acetylaminomalonate by saponification of one of the ester functional groups followed by formation of the mixed anhydride with pivaloyl chloride, reaction of the anhydride with ethylamine, and saponification of the second ester group. The tripeptide *N*-Ac-Ama-Val-Ama-NHEt was similarly prepared using mixed anhydride coupling reactions as shown in Scheme I. Peptidelike derivatives of Ama have been synthesized earlier as possible artificial sweeteners by using dicyclohexylcarbodimide and/or formation of the Ama monoacid chloride for creating the amide functionalities.[13,14]

N^{α}-Ac-Ama-NHEt in buffered aqueous medium at elevated temperature decarboxylated to form N^{α}-Ac-Gly-NHEt, identified by comparison with a synthetic sample. Both the destruction of starting material and the formation of the glycine peptide were quantitatively measured by reverse-phase HPLC. The rate of hydrolysis of N^{α}-Ac-Gly-NHEt was determined by following the rate of its disappearance under the same reaction conditions. Hydrolysis products from either starting material or the glycine peptide could not be quantitatively assessed because these materials were transparent to the HPLC detector. The decarboxylation and hydrolysis reactions proceeded according to Scheme II on the basis of the following observations.

Plots of ln ([Ama peptide]$_t$/[Ama peptide]$_0$) vs time at 120 °C were linear and are shown in Figure 2. The destruction of N^{α}-Ac-Ama-NHEt followed pseudo-first-order

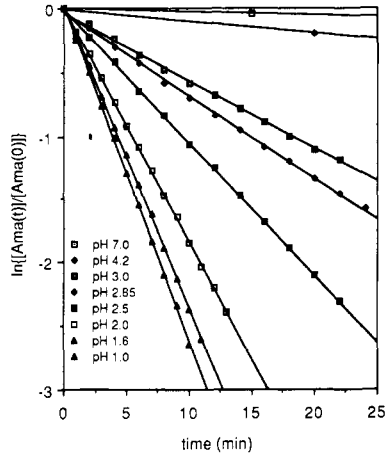


Figure 2. Pseudo-first-order plots for destruction of N^{α}-acetyl-Ama *N*-ethylamide (Ama peptide) versus time as a function of pH. The reactions were monitored at least to 3 half-lives; only 2 out of 10 data points are shown at pH 4.2 and pH 7.0.

Table I. Rate Constants k_{obsd} and k_3 as a Function of pH at 120 ± 0.1 °C

pH[a]	k_{obsd},[b] min^{-1}	half-life (k_{obsd}), min	k_3,[b] min^{-1}
1.00	0.260 ± 0.0002 (2)[b]	2.67	0.042 ± 0.0004 (2)
1.60	0.238 ± 0.001 (2)	2.91	0.0046
2.00	0.20 ± 0.01 (5)	3.52	0.0017 ± 0.0001 (3)
2.50	0.105 ± 0.001 (3)	6.59	3.7×10^{-4}
2.85	0.0656 ± 0.0004 (2)	10.6	2.2×10^{-4}
3.00	0.052 ± 0.002 (3)	13.3	1.4×10^{-4}
4.20	0.0088 ± 0.0008 (2)	79	
7.00	0.002 55 ± 0.000 07 (7)	272	8.4×10^{-5}

[a] The error in pH measurement was ±0.05, and pH was measured at ambient temperature. No correction was made for the reaction temperature of 120 °C. The pH was checked by short-range pH paper during the reaction and at the end of every run and no detectable change in pH was observed. [b] The numbers in parentheses represent the number of determinations, and the errors given are the standard deviations from the mean. All measurements were carried to at least 3 half-lives.

kinetics with the observed rate constant k_{obsd} given by the following expression:

$$k_{obsd} = \frac{(k_1 + k_2)[H^+] + (k_4 + k_5)K_a}{[H^+] + K_a} \quad (1)$$

The formation and destruction of the glycine peptide followed consecutive first-order kinetics, and the concentration of the glycine peptide was given by the expression:

$$[\text{Gly peptide}] = \left\{\frac{k_1[H^+] + k_5K_a}{[H^+] + K_a}\right\} \times [\text{Ama peptide}]_0[\exp(-k_{obsd}t) - \exp(-k_3t)]/(k_3 - k_{obsd}) \quad (2)$$

In the equations [Gly peptide] is the total concentration of N^{α}-Ac-Gly-NHEt at time t and [Ama peptide]$_0$ and [Ama peptide]$_t$ are the initial concentration and the concentration at time t, respectively, of N^{α}-Ac-Ama-NHEt. The values for k_{obsd} obtained at 120.0 ± 0.2 °C are given in Table I and plotted in Figure 3. Correlation coefficients for the first-order kinetic analysis were 1.00 for all individual runs, which were followed to 3 half-lives. The rate constant for the hydrolysis of N^{α}-Ac-Gly-NHEt, k_3, was determined by following the disappearance of synthetic N^{α}-Ac-Gly-NHEt to 3 half-lives for the reactions at pH's of 2.00 and lower. Above pH 2 the reaction was followed to only 1 half-life, and the values obtained probably have

(11) Hauschka, P. V.; Henson, E. B.; Gallop, P. M. *Anal. Biochem.* **1980**, *108*, 57.
(12) Christy, R. M.; Koch, T. H. *J. Am. Chem. Soc.* **1982**, *104*, 1771.
(13) Fujino, M.; Wakimasu, M.; Mano, M.; Tanaka, K.; Nakajima, N.; Aoki, H. *Chem. Pharm. Bull.* **1976**, *24*, 2112. Gansser, C. *Bull. Soc. Chim. Fr.* **1966**, 1713.
(14) Frankel, M.; Harnik, M.; Levin, Y.; Knobler, Y. *J. Am. Chem. Soc.* **1953**, *75*, 78.

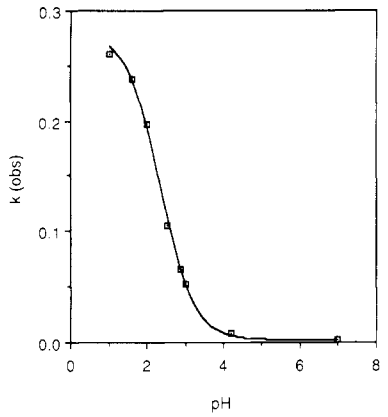


Figure 3. Plot of the observed rate constant for decarboxylation, k_{obsd} (min^{-1}), at 120 °C versus pH. The points represent the observed rate constants and the line results from interpolation of the calculated rate constants from eq 1 and the data in Table II.

Table II. Rate and Equilibrium Constants at 120 ± 0.2 °C As Defined in Scheme II[a]

equilibrium constant:
$K_a = 0.0047 \pm 0.0006$ M and $pK_a = 2.33 \pm 0.06$
combined rates of decarboxylation and hydrolysis:
$(k_1 + k_2) = 0.28 \pm 0.01$ min^{-1}
$(k_4 + k_5) = 0.002\,55 \pm 0.000\,07$ min^{-1}
rates of decarboxylation:
$k_1 = 0.26 \pm 0.001$ min^{-1}
$k_5 = 0.002\,22 \pm 0.000\,05$ min^{-1}

[a] The errors represent the standard deviations from the least-squares analysis.

a correspondingly higher error. The buffer used was 0.1 N ammonium phosphate for most of the determinations; several runs were made using 0.5 and 1.0 N ammonium phosphate buffer with no change in k_{obsd}. At pH 4.2 the buffer was 0.1 N ammonium acetate.

At pH 7, an attempt was made initially to fit the data by using eq 2 and the assumption $k_5 = 0$. However, unreasonable values for k_1 and/or K_a were obtained. That is, the rate of decarboxylation at pH 7 could not be accounted for by only a K_a and k_1 process, and a direct anionic pathway, k_5, must be considered. Using estimates for k_1 and K_a from the data at lower pH values, we found that the terms involving $[H^+]$ in eq 1 could be neglected at pH 7. The value for $k_{obsd} = 0.002\,55$ min^{-1} is then equal to $(k_4 + k_5)$, and the anionic process for decarboxylation is the only significant process at pH 7.

For the data at lower pH values, a nonlinear least-squares computer program and the value for $(k_4 + k_5)$ obtained at pH 7 were used to determine the values for $(k_1 + k_2)$ and K_a listed in Table II. The self consistency of the parameters K_a, $(k_1 + k_2)$, and $(k_4 + k_5)$ was checked by using these values in eq 1 to calculate k_{obsd} as a function of pH. Agreement between the calculated and observed values of k_{obsd} is shown in Figure 3 with the calculated values represented as a curve through the experimental points. The curve was established by a simple interpolation of calculated values.

Equation 2 was then used to determine values for k_1 and k_5. At pH 7, the terms involving $[H^+]$ were again neglected, and the data were fit by using the simplified equation given in eq 3. A value for k_5 of $0.002\,22 \pm 0.000\,05$ min^{-1} was

$$[\text{Gly peptide}] = \frac{k_5[\text{Ama peptide}]_0[\exp(-k_{obsd}t) - \exp(-k_3t)]}{k_3 - k_{obsd}} \quad (3)$$

found. The value obtained for k_5 was then used with eq 2 to determine the value for k_1 at all pH's except pH 1, and the value given in Table II is the average of these calculated values. At pH 1, eq 2 gives a lower value for k_1 than it does at higher pH, probably because at this pH some of the N^{α}-Ac-Ama-NHEt is protonated, resulting in a much higher rate of hydrolysis. Decarboxylation at pH below 1 was not investigated because of the complexities associated with three available carbonyls for protonation.

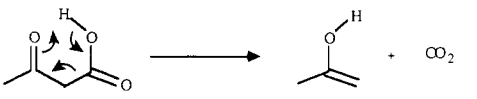


Figure 4.

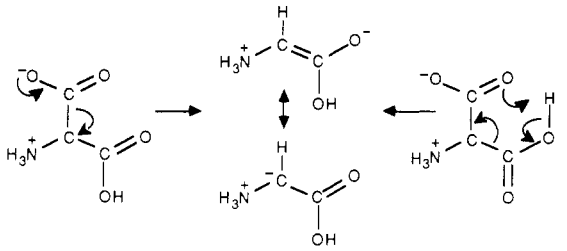


Figure 5.

The mechanism for decarboxylation of β-keto carboxylic acids as proposed by Westheimer proceeds through a cyclic six-membered transition state (Figure 4).[9] The resulting enol tautomerizes to the ketone. In agreement with this mechanism, Westheimer found that the neutral β-keto carboxylic acid, 2,2-dimethyl-3-oxobutanoic acid, decarboxylated approximately 100 times faster than the corresponding anion. Exploring a number of β-keto carboxylic acids, Straub and Bender observed a similar ordering of the rate constants for decarboxylation of neutral and anionic species.[15] They also observed that electron-withdrawing substituents increased the rate constants for decarboxylation of both species, however, significantly more for decarboxylation of the anionic species. The studies of decarboxylation of N^{α}-Ac-Ama-NHEt presented here also show a rate constant for decarboxylation of the neutral form, k_1, that is slightly greater than 100 times the rate constant for decarboxylation of the anionic species, k_5. In contrast, results obtained by Thanassi on the rates of decarboxylation of free aminomalonic acid and α-phenylaminomalonic acid[16] showed a decrease in the rate of decarboxylation at lower values of pH. This was explained in terms of a lower rate constant for decarboxylation of the fully protonated form, $H_3N^+CH(CO_2H)_2$, than for the neutral species, $H_3N^+CH(CO_2H)CO_2^-$. Thanassi[8] noted that the rate of decarboxylation of malonic acid, $CH_2(CO_2H)_2$, is greater than the rate of decarboxylation of sodium hydrogen malonate,[17] consistent with a cyclic transition state for the decarboxylation of both the neutral and anionic species. The reversal of observed rates in free aminomalonic acid and α-phenylaminomalonic acid as a function of pH and the much higher rate constant for decarboxylation of neutral Ama than for decarboxylation of anionic malonic acid led Thanassi to the conclusion that the Westheimer mechanism does not apply to the decarboxylation of neutral aminomalonic acid and α-phenylaminomalonic acid.[8,15] An alternative explanation that was apparent to us is that the higher rate constant for decarboxylation of zwitterionic Ama than for decarboxylation of cationic Ama or anionic malonic acid might result from

(15) Straub, T. S.; Bender, M. L. *J. Am. Chem. Soc.* **1972**, *94*, 8881.
(16) Thanassi, J. W. *Biochemistry* **1973**, *12*, 5109.
(17) Fairclough, R. A. *J. Chem. Soc.* **1938**, 1186.

a decrease in the separation of opposite charges in the transition state for decarboxylation of the zwitterion. The decrease in charge separation would be true for both the Westheimer and Thanassi mechanisms because both yield initially the enol form of the glycine zwitterion as shown in Figure 5. Consequently, the higher rate constant for decarboxylation of the Ama zwitterion than for decarboxylation of the Ama cation may not demand a change of mechanism. Choosing between the two mechanisms involves weighing the relative costs of a proton transfer and the gain from an aromatic transition state.

Neutral, peptide-bound Ama appears to decarboxylate more slowly than neutral, zwitterionic Ama; however, direct comparison of data reported here and by Thanassi is difficult because the measurements were performed at significantly different temperatures. The data obtained by Hauschka[11] were used to calculate a k_{obsd} equal to 3.60 min^{-1} for decarboxylation of free aminomalonic acid in 1 N hydrochloric acid at 120 °C. This k_{obsd}, which is a composite of the rate constants for decarboxylation of neutral and cationic Ama, is more than 10 times the k_{obsd} reported here for the decarboxylation of peptide-bound aminomalonic acid at pH 1 for which k_1 is the predominant contributor. Independent of the mechanism for decarboxylation of the two species, the difference in rate constant probably reflects the difference in electron-withdrawing ability of the ammonium group of Ama and the *N*-acetyl group of peptide-bound Ama. The electron-withdrawing ability of the *N*-acetyl substituent should increase the rate constant for decarboxylation of peptide bound Ama relative to the rate constant for decarboxylation of malonic acid but to a lesser extent than the electron-withdrawing ability of the ammonium substituent should increase the rate constant for decarboxylation of Ama. Evidence for the *N*-acetyl substituent serving as an electron-withdrawing substituent is the low second pK_a observed for peptide bound Ama, 2.33, relative to the corresponding pK_a of malonic acid, 2.85.[18] The rate constants for decarboxylation of malonic acid at 80 and 90 °C reported by Hall[19] give an estimated rate constant for decarboxylation of malonic acid at 120 °C of 1.12×10^{-2} min^{-1}, which is more than 20 times smaller than the rate constant, k_1, for decarboxylation of neutral Ama peptide. The ordering of rate constants for decarboxylation at the respective neutral states, $k_{Ama} > k_{Ama\ peptide} > k_{malonic\ acid}$, is consistent with the observed substituent effects on the rate constants for decarboxylation of β-keto esters.[15]

In comparison of the rate constants for decarboxylation of the free and peptide bound natural malonic acid bearing amino acids, Ama, Asa, and Gla, the general trend of higher stability for peptide-bound amino acids has been observed. Hauschka observed this effect in comparison of the rate constants for decarboxylation of protein-bound γ-carboxyglutamic acid and free γ-carboxyglutamic acid (Gla),[11] and Christy and Koch, for decarboxylation of β-carboxyaspartic acid (Asa) and peptide-bound β-carboxyaspartic acid.[12] The half-life for decarboxylation of free Gla in 1 N hydrochloric acid at 100 °C was found to be 8.6 min while the half-life for decarboxylation of protein bound Gla was 48 min. The half-life for decarboxylation of free Asa in 1 N hydrochloric acid at 70 °C was found to be 31 min and for peptide-bound Asa, 13.7 h. The rate constant for decarboxylation of the peptide-bound Asa may have been unusually low because of the special structure of the peptide, a hydantoin ring. The inherent stability of the malonic acid bearing amino acids when peptide or protein bound may result from special intramolecular hydrogen bonding, which interferes with achievement of the Westheimer transition state. Certainly in the case of protein-bound Ama, also having the amino functional group as an amide rather than as an ammonium ion is important for stability.

Table III. Rate Constants for the Decarboxylation of the Ama Peptide at pH 7 as a Function of Temperature

temp, °C	k_{obsd}, min^{-1}	k_3, min^{-1}	k_5, min^{-1}
100	0.000 262 ± 0.000 005 (2)[a]	0.000 043	0.000 217 ± 0.000 003
110	0.000 80 ± 0.000 02 (2)	0.000 046	0.000 666 ± 0.000 007
120	0.002 55 ± 0.000 07 (2)	0.000 084	0.002 22 ± 0.000 05
130	0.006 75 ± 0.000 07 (2)	0.0001 (estimate)	0.005 97 ± 0.000 07

[a] The numbers in parentheses represent the number of determinations, and the errors given are the standard deviations from the mean.

The rate of decarboxylation at pH 7 was also studied as a function of temperature and the results given in Table III. The values for k_{obsd} and k_3 were used in eq 3 to obtain the values for k_5 (Table III) where the error given is the standard deviation of the average value. The rate constant data followed Arrhenius behavior and gave an activation energy of 33.1 kcal/mol and a preexponential factor of 8×10^{13} s^{-1} for the k_5 process. Energies of activation for the decarboxylation process in 1 N hydrochloric acid for free aminomalonic acid, β-carboxyaspartic acid, and γ-carboxyglutamic acid are 26.9, 26.8, and 26.0 kcal/mol, respectively.[10]

By use of the parameters obtained from the Arrhenius plot, a value for the rate constant for decarboxylation at physiological temperature (37 °C) is estimated to be 2.43×10^{-8} min^{-1} with a half-life of 54.4 years. The half-life for decarboxylation at 110 °C is only 17.3 h and at 120 °C, 5.2 h. The elevated temperatures used in base hydrolysis of biological samples may then result in the loss of Ama due to decarboxylation. For small peptides, the rate of hydrolysis in 2 N potassium hydroxide undoubtedly greatly exceeds the rate of decarboxylation of peptide-bound Ama, and free Ama does not decarboxylate under these conditions. However, for more complex proteins the rate of decarboxylation may become competitive with the rate of hydrolysis. Possibly, the binding of potassium by the Ama residue in an Ama protein during base hydrolysis protects the protein-bound Ama from decarboxylation. The addition of 2 N potassium chloride to the peptide at pH 7.0 and 120 °C, however, lowered the rate constant, k_{obsd}, from 0.002 55 min^{-1} only to 0.001 82 min^{-1}, corresponding to a half-life of 6.3 h.

Possible decarboxylation of peptide-bound Ama residues during the alkaline hydrolysis was tested with the peptides N^{α}-Ac-Ama-NHEt and N^{α}-Ac-Ama-Val-Ama-NHEt. These peptides were each hydrolyzed in 2 M potassium hydroxide at 110 °C for 24 h, and 4% and 22%, respectively, of the Ama residues were converted to Gly residues. The difference in the amount of decarboxylation in the two peptides suggests that internal peptide bonds hydrolyze more slowly than the terminal peptide bonds and, consequently, that decarboxylation of Ama residues bearing only internal type peptide bonds as in proteins might be substantial.

In conclusion, peptide-bound Ama decarboxylates relatively rapidly in the neutral state and slowly in the anionic state. Because peptide-bound Ama decarboxylates in the

(18) Daniele, P. G.; de Robertis, A.; Sammartano, S.; Rigano, C. *Thermochimica Acta* **1984**, *72*, 305.
(19) Hall, G. A., Jr. *J. Am. Chem. Soc.* **1949**, *71*, 2691.

anionic form, some if not substantial amounts of protein-bound Ama will decarboxylate to Gly during protein alkaline hydrolysis. Work is continuing in this laboratory on the calcium binding properties and further reactivity of peptide-bound aminomalonic acid and on the in vivo mechanism of formation of peptide-bound Ama from glycine residues.

Experimental Section

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. HPLC analyses were performed with a Tracor Model 950 pump equipped with a Tracor Model 970A variable wavelength UV–vis detector or with a Hewlett-Packard Model 1090 HPLC equipped with a diode array detector. An Alltech Econosphere C-18 column (4.6 × 250mm, 5 μm) was used unless otherwise indicated. Samples were eluted with 0.1 N aqueous ammonium dihydrogen phosphate, pH 4.5, at a flow of 1.4 mL/min. NMR spectra were obtained with Bruker WM250 or Chemagnetics 200-MHz instruments. Chemical shifts are reported in parts per million on the δ scale with tetramethylsilane as the internal standard. Infrared spectra were obtained with a Mattson Polaris FT spectrometer. Mass spectra were obtained with a VG Instruments 7070 EQ-HF mass spectrometer. Microanalyses were performed by Atlantic Microlab, Atlanta, GA. Chemical reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise specified. *o*-Phthalaldehyde derivatizing solution, 0.8 mg/mL in borate buffer, was obtained from Pierce Chemical (Rockford, IL). Diethyl [*N*-(carbobenzyloxy)amino]malonate[20] and the monopotassium salt of aminomalonic acid[10] were prepared by following literature procedures. Buffers were made from 0.1 N solutions of phosphoric acid, ammonium dihydrogen phosphate, ammonium hydrogen phosphate, and ammonium acetate in HPLC grade water to give the desired pH. In some instances, buffers were made from higher molarity solutions and no change in k_{obsd} was detected.

Kinetic Experiments. Weighed samples of N^{α}-acetylaminomalonic acid *N*-ethylamide or N^{α}-acetylglycine *N*-ethylamide were dissolved in buffer to give final concentrations of (3–6) × 10^{-3} M. Aliquots of approximately 50-μL volumes were sealed in 1.5 × 90 mm capillaries (Kimax). Each capillary was fused to a second capillary and completely submerged in an oil bath at the desired temperature. Individual capillaries were removed at specific time intervals during the reaction and immediately placed in ice water until analysis by HPLC. Control experiments demonstrated that no further reaction occurred after icing at any of the pH values studied. HPLC analyses were performed using 20-μL aliquots from each sample. The Tracor HPLC was employed with the detector set at 210 nm. Repeated injections showed an error in response of less than 1%.

Values for k_{obsd} were obtained by linear least-squares fitting of plots of the natural logarithm of the Ama peptide response vs time. Correlation coefficient for the fitting of the data to first-order kinetics was 1.00 for all individual runs. A nonlinear least-squares computer program utilizing the Marquot algorithm was used to determine individual rate constants and equilibrium constants as described above.

N^{α}-Acetylglycine *N*-Ethylamide. N^{α}-Acetylglycine (1.028 g, 8.77 × 10^{-3} mol) was added to 80 mL of dry chloroform and cooled to 0 °C. Methylmorpholine (0.96 mL, 1.0 equiv) and isobutyl chloroformate (1.14 mL, 1.0 equiv) were added, and the solution was stirred for 5 min. Ethylamine (0.71 mL of a 70% solution, 1.0 equiv) was added, and the reaction mixture was stirred 1 h at 0 °C. The solvent was rotary evaporated, and the product was isolated by dry column silica gel flash chromatography,[21] beginning the elution with a 20-mL fraction of 1:1 methylene chloride–ethyl acetate and increasing the ethyl acetate concentration 10% by volume with each successive fraction. At 100% ethyl acetate, a 5% absolute ethanol gradient was started. The product eluted in the 75% ethyl acetate–25% ethanol through 60% ethyl acetate–40% ethanol fractions. The yield of N^{α}-acetylglycine *N*-ethylamide was 0.84 g (66%). The product was recrystallized from 9:1 hexanes–ethanol (v/v) to give material with mp 145 °C (lit.[22] mp 144 °C). The R_f on silica gel TLC was 0.66 (100% ethanol), 0.47 (1:1 ethyl acetate–absolute ethanol, v/v), 0.40 (85:15 *n*-butanol–acetic acid, v/v), and the HPLC retention time was 8.2 min. The glycine peptide was characterized from the following spectral data: IR (Nujol) 3274, 3092, 1638, 1562 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 8.07 (broad t, *J* = 6 Hz, 1 H), 7.83 (broad t, *J* = 6 Hz, 1 H), 3.62 (d, *J* = 6 Hz, 2 H), 3.08 (m, $J(CH_3)$ = 7 Hz, $J(NH)$ = 6 Hz, 2 H), 1.85 (s, 3 H), 1.01 (t, *J* = 7 Hz, 3 H).

N^{α}-Acetyl-α-carbethoxyglycine. Monosaponification of diethyl N^{α}-acetylaminomalonate was achieved with 2 equiv of potassium hydroxide by using a procedure adapted from Sofia and Katzenellenbogan.[23] Diethyl N^{α}-acetylaminomalonate (1.103 g, 5.08 mmol) was dissolved in 150 mL of 50% ethanol and cooled to 0 °C. Potassium hydroxide (5.8 mL of 1.75 M aqueous solution, 2 equiv) was added, and the reaction mixture was stirred for 20 min at 0 °C. The solution was acidified to pH 4.0 with 1 N hydrochloric acid, and ethanol was removed by rotory evaporation. The aqueous solution was further acidified to pH 1, and water was removed by rotory evaporation followed by high-vacuum rotory evaporation to remove residual water. The residue was dissolved in absolute ethanol (50 mL) and filtered through a sintered-glass funnel. The ethanol was removed by rotory evaporation, and the product was purified by dry column flash chromatography,[21] beginning with a 20-mL fraction of 1:1 methylene chloride–ethyl acetate (v/v) and increasing the ethyl acetate concentration by 5% with each successive fraction. At 100% ethyl acetate, 1 mL of absolute ethanol was added, and the ethanol concentration was increased by 5% with each successive fraction. The product eluted in the fractions starting from 10% methylene chloride–90% ethyl acetate through 50% ethyl acetate–50% ethanol. N^{α}-Acetyl-α-carbethoxyglycine (0.632 g) was isolated in 66% yield and showed the following properties: mp 126–128 °C; FAB mass spectrum (positive ion, glycerol matrix) *m/z* (relative intensity) 188 (50), 144 (100); R_f silica gel 0.39 (100% ethanol); IR (Nujol) 3324, 3200–2300, 1748, 1598, 1532, 1260, 1237 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 13.40 (broad, 1 H), 8.64 (d, *J* = 7.5 Hz, 1 H), 4.95 (d, *J* = 7.5 Hz, 1 H), 4.15 (q, *J* = 7 Hz, 2 H), 1.91 (s, 3 H), 1.20 (t, *J* = 7 Hz, 3 H). Anal. Calcd: C, 44.45; H, 5.86; N, 7.40. Found: C, 44.55; H, 5.87; N, 7.33.

N^{α}-Acetyl-α-carbethoxyglycine *N*-Ethylamide. N^{α}-Acetyl-α-carbethoxyglycine (0.508 g, 2.68 × 10^{-3} mol) was dissolved in 30 mL of dry tetrahydrofuran, and the solution was cooled in a salt ice bath to −10 °C. Methylmorpholine (0.29 mL, 2.68 × 10^{-3} mol) and pivaloyl chloride (0.33 mL, 2.68 × 10^{-3} mol) were added, and the reaction mixture was stirred for 30 min at −10 °C. Ethylamine (0.33 mL of a 70% aqueous solution, 4.02 × 10^{-3} mol) was added, and the reaction mixture was stirred an additional 30 min at −10 °C. The ice bath was removed, and the reaction mixture was stirred overnight at ambient temperature. Evaporation of the solvent was followed by dry column flash chromatography, beginning with 1:1 chloroform–ethyl acetate (v/v) and increasing the ethyl acetate concentration by 5% by volume with each fraction. At 100% ethyl acetate, 5% absolute ethanol was added, and the ethanol concentration was increased by 5% with each additional fraction. The product eluted in the 10% chloroform–90% ethyl acetate fraction through the 80% ethyl acetate–20% ethanol fraction. Evaporation of the solvent gave 0.405 g of product (70%), which was recrystallized from 9:1 hexanes–ethanol to give material with the following properties: mp 145–147 °C; R_f on silica gel 0.74 (1:1 ethyl acetate–ethanol, v/v) and 0.63 (100% ethanol); IR (Nujol) 3292, 3080, 1752, 1642, 1550 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6) δ 8.51 (d, *J* = 8 Hz, 1 H), 8.41 (t, *J* = 5 Hz, 1 H), 4.99 (d, *J* = 8 Hz, 1 H), 4.11 (q, *J* = 7 Hz, 2 H), 3.10 (m, 2 H), 1.90 (s, 3 H), 1.17 (t, *J* = 7 Hz, 3 H), 1.02 (t, *J* = 7 Hz, 3 H). Anal. Calcd: C, 49.99; H, 7.46; N, 12.95. Found: C, 50.09; H, 7.49; N, 13.00.

N^{α}-Acetyl-α-carboxyglycine *N*-Ethylamide. N^{α}-Acetyl-α-carbethoxyglycine *N*-ethylamide (0.214 g, 9.9 × 10^{-4} mol) was dissolved in 10 mL of distilled deionized water. Potassium hydroxide (1.04 mL of 1 N aqueous solution, 1.05 equiv) was added, and the reaction mixture was stirred at ambient temperature for

(20) Cox, D. A.; Johnson, A.; Mauger, A. B. *J. Chem. Soc.* **1964**, 5024.
(21) Harwood, L. M. *Aldrichimica Acta* **1985**, *18*, 25.
(22) Avignon, M.; Huong, P. V.; Lascombe, J.; Marraud, M.; Neel, J. *Biopolymers* **1969**, *8*, 69.
(23) Sofia, M.; Katzenellenbogan, J. *J. Org. Chem.* **1985**, *50*, 2331.

1 h. The solution was concentrated to ~5 mL by vacuum rotary evaporation and then placed on a 2-mL column of wet Dowex 50W-X12 (H^+ form) ion exchange resin, which had been repeatedly washed with distilled deionized water. The column was eluted at a flow of 1 mL/min, and additional water was added as needed. Fractions (1 mL) were collected, and the pH of each fraction was determined. The fractions were spotted on silica gel TLC and then placed in an ice bath. The product eluted in the first 5 fractions at pH 2.2; R_f on silica gel 0.10 (1:1 ethanol:ethyl acetate, v/v). After evaporation and drying in a desiccator, 0.114 g of product was isolated (61% yield) with the following properties: mp 136–137 °C; HPLC t_R 4.2 min; IR (Nujol) 3362, 3290, 3098, 3300–2400, 1714, 1658, 1614, 1500 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6) δ 12.99 (broad, 1 H), 8.35 (d, J = 8.1 Hz, 1 H), 8.34 (unresolved, 1 H), 4.92 (d, J = 8.1 Hz, 1 H), 3.09 (m, J(NH) = 5.7 Hz, $J(CH_3)$ = 7.2 Hz, 2 H), 1.89 (s, 3 H), 1.02 (t, J = 7.2 Hz, 3 H). Anal. Calcd: C, 44.68; H, 6.43; N, 14.89. Found: C, 44.68; H, 6.44; N, 14.84.

N^α-(Carbobenzyloxy)-α-carbethoxyglycine. Diethyl N^α-(carbobenzyloxy)aminomalonate (2.95 g, 9.56 mmol) was dissolved in 300 mL of 50% ethanol and cooled to 0 °C. Potassium hydroxide (10.9 mL of 1.75 N KOH, 1.91 × 10^{-2} mol) was added, and the reaction mixture was stirred at 0 °C for 20 min. The solution was acidified to pH 7 with 1 N HCl, and the ethanol was removed by rotory evaporation. The aqueous solution was kept at 0 °C and further acidified to pH 1.5 with 1 N HCl, which resulted in the precipitation of product. The solution was extracted with 4 × 50 mL of ethyl acetate, and the ethyl acetate layers were combined and dried over anhydrous magnesium sulfate. The magnesium sulfate was removed by filtration and the ethyl acetate by rotory evaporation to give an oil. The product was purified by dry column flash chromatography, collecting 20-mL fractions and eluting with dichloromethane and increasing the ethyl acetate concentration by 5% with each successive 20-mL fraction. The product eluted in the 80% CH_2Cl_2–20% EtOAc fraction through the 55% CH_2Cl_2–45% EtOAc fraction. Rotory evaporation of the solvent left an oil, which quickly solidified to give 2.12 g (79% yield) of product, which had the following properties: mp 68 °C (lit.[13] mp 63–65 °C); silica gel TLC R_f 0.16 (1:1 CH_2Cl_2–EtOAc), 0.55 (1:1 EtOAc–EtOH); IR (Nujol) 3332, 3140, 3300–2800, 3030, 1768, 1712, 1688, 1528, 1253, 1233 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 13.50 (broad, 1 H), 8.14 (d, J = 8 Hz, 1 H), 7.36 (s, 5 H), 5.08 (s, 2 H), 4.83 (d, J = 8 Hz, 1 H), 4.16 (q, J = 7 Hz, 2 H), 1.19 (t, J = 7 Hz, 3 H); FAB mass spectrum (positive ion, glycerol matrix) m/z (relative intensity) 282 (100), 238 (79).

N^α-(Carbobenzyloxy)-α-carbethoxyglycine N-Ethylamide. N^α-(Carbobenzyloxy)-α-carbethoxyglycine (1.60 g, 5.68 mmol) was dissolved in 20 mL of dry THF, and the solution was cooled to −10 °C in an ice salt bath. Methylmorpholine (0.62 mL, 5.68 mmol) was added followed by pivaloyl chloride (0.695 mL, 5.68 mmol). The reaction mixture was stirred at −10 °C for 10 min. Ethylamine (6.9 mL of a 70% solution, 8.52 mmol) was added, and the reaction mixture was stirred at −10 °C for 30 min. The salt ice bath was removed, and the reaction mixture was stirred an additional 2 h. The solvent was rotory evaporated, and the product was isolated by dry column flash chromatography using 20-mL fractions of dichloromethane and increasing the ethyl acetate concentration by 5% with each successive fraction. The product eluted in the 80% CH_2Cl_2–20% EtOAc fraction through the 50% CH_2Cl_2–50% EtOAc fraction. Rotary evaporation of the solvent gave 1.67 g of product (96% yield). The product was recrystallized from 9:1 hexanes–ethanol, and the recrystallized material had the following properties: mp 113 °C; silica gel TLC R_f 0.64 (1:1 CH_2Cl_2–EtOH), 0.41 (1:1 EtOAc–hexanes), 0.79 (1:1 EtOAc–EtOH); IR ($CHCl_3$) 3428, 3022, 2985, 1755, 1725, 1695, 1505 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 8.36 (t, J = 5.4 Hz, 1 H), 7.82 (d, J = 8 Hz, 1 H), 7.36 (s, 5 H), 5.06 (s, 2 H), 4.81 (d, J = 8 Hz, 1 H), 4.12 (q, J = 7 Hz, 2 H), 3.10 (m, 2 H), 1.17 (t, J = 7 Hz, 3 H), 1.02 (t, J = 7 Hz, 3 H). Anal. Calcd: C, 58.43; H, 6.54; N, 9.09. Found: C, 58.53; H, 6.57; N, 9.04.

α-Carbethoxyglycine N-Ethylamide Hydrochloride. N^α-(Carbobenzyloxy)-α-carbethoxyglycine N-ethylamide (1.345 g, 4.36 mmol) was dissolved in 30 mL of absolute ethanol. Hydrochloric acid (5.2 mL, 1 N HCl, 1.2 equiv) was added, and the solution was added to 0.25 g of prehydrogenated 10% Pd/C catalyst in 10 mL of absolute ethanol at 0 °C. The mixture was stirred under hydrogen at atmospheric pressure for 2 h. The catalyst was removed by filtration through Celite, and the Celite washed with 30 mL of additional ethanol. The ethanol was removed by rotory evaporation, the solid was redissolved in 30 mL of absolute ethanol, and the ethanol was evaporated a second time to give 0.898 g (98% yield) of product. The product had the following properties: mp 160.5–161 °C; IR (Nujol) 3238, 3062, 3300–2600, 2637, 2000, 1755, 1680, 1570, 1500 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 9.07 (t, J = 5.4 Hz, 1 H), 8.98 (s, 3 H), 4.75 (s, 1 H), 4.22 (q, J = 7 Hz, 2 H), 3.16 (m, 2 H), 1.21 (t, J = 7 Hz, 3 H), 1.06 (t, J = 7 Hz, 3 H).

N^α-(Carbobenzyloxy)-L-valyl-α-carbethoxyglycine N-Ethylamide. N-(Carbobenzyloxy)-L-valine (0.738 g, 2.90 mmol) was dissolved in 10 mL of dry chloroform, and the solution was cooled to 0 °C. Methyl morpholine (0.32 mL, 2.9 mmol) was added followed by isobutyl chloroformate (0.38 mL, 2.9 mmol), and the reaction mixture was stirred at 0 °C for 15 min. A precooled solution of carbethoxyglycine N-ethylamide hydrochloride (0.619 g, 2.9 mmol) with methylmorpholine (0.32 mL, 2.9 mmol) in 10 mL of dry chloroform was added, and the solution was stirred for 30 min at 0 °C. The reaction mixture was allowed to warm to ambient temperature and stirred an additional 2 h. Additional chloroform was added (50 mL) followed by extraction successively with 15 mL of 1 N sodium bicarbonate, 15 mL of 1 N acetic acid and 15 mL of water. After drying (anhydrous potassium carbonate), filtration, and rotary evaporation of the solvent, 1.13 g of a white solid was obtained (94% yield). The product was recrystallized from ethyl acetate with slow cooling to 0 °C. The recrystallized material had the following properties: mp 185–187 °C; silica gel TLC R_f 0.75 (absolute ethanol), 0.57 (1:5:5 acetic acid–EtOAc–hexanes); IR (Nujol) 3288, 1757, 1740, 1695, 1645, 1540 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 8.52 and 8.28 (d, J = 8 Hz, 1 H total for two diastereomers), 8.39 (broad t, J = 5.3 Hz, 1 H), 7.45 (d, J = 9 Hz, 1 H), 7.35 (s, 5 H), 5.03 (m, 3 H), 4.10 (q, J = 7 Hz, 2 H), 4.08 (m, 1 H), 3.12 (m, 2 H), 1.98 (m, 1 H), 1.16 (t, J = 7 Hz, 3 H), 1.04 and 1.02 (t, J = 7 Hz, 3 H total for two diastereomers), 0.86 (m, 6 H); FAB mass spectrum (positive ion, 3:1 dithiothreitol–dithioerythritol matrix) m/z (relative intensity) 408 (100), 364 (20). Anal. Calcd: C, 58.95; H, 7.17; N, 10.31. Found: C, 58.92; H, 7.18; N, 10.26.

L-Valyl-α-carbethoxyglycine N-Ethylamide Hydrochloride. N^α-(Carbobenzyloxy)-L-valyl-α-carbethoxyglycine N-ethylamide (0.698 g) was dissolved in 50 mL of absolute ethanol with gentle heating. The solution was allowed to cool, and 1 equiv of 1 N HCl (1.7 mL) was added. The solution was then added to prehydrogenated 10% Pd/C catalyst (0.15 g) in 10 mL of ethanol at ambient temperature. The reaction mixture was stirred vigorously under hydrogen at atmospheric pressure for 2 h and filtered through Celite, and the Celite was rinsed with an additional 40 mL of ethanol. Rotory evaporation of the solvent left a light yellow oil, which was refrigerated. After cooling, the oil had solidified and was redissolved in absolute ethanol. Rotory evaporation left a white solid, which amounted to a quantitative yield of product with the following properties: IR (Nujol) 3300, 3100–2300, 2070, 1740, 1670, 1650, 1555, 1530 cm^{-1}; ^1H NMR of major diastereomer (200 MHz, DMSO-d_6) δ 9.12 (d, J = 7.6 Hz, 1 H), 8.67 (broad t, J = 5.5 Hz, 1 H), 8.37 (broad, 3 H), 5.12 (d, J = 7.6 Hz, 1 H), 4.13 (q, J = 7 Hz, 2 H), 3.83 (m, 1 H), 3.13 (m, 2 H), 2.10 (m, 1 H), 1.18 (t, J = 7 Hz, 3 H), 1.04 (t, J = 7 Hz, 3 H), 0.98 (m, 6 H).

N^α-Acetyl-α-carbethoxyglycyl-L-valyl-α-carbethoxyglycine N-Ethylamide. N-Acetyl-α-carbethoxyglycine (0.200 g, 1.04 × 10^{-3} mol) was dissolved in 10 mL of dry THF. Methylmorpholine (0.114 mL, 1 equiv) was added, and the solution was cooled to −10 °C. Pivaloyl chloride (0.127 mL, 1 equiv) was added, and the reaction mixture was stirred at −10 °C for 30 min. A precooled solution of L-valyl-α-carbethoxyglycine N-ethylamide hydrochloride (0.322 g, 1 equiv) with 1 equiv of methylmorpholine in 10 mL of dry chloroform was added, and the reaction mixture was stirred at −10 °C for 30 min. The ice bath was removed, and the reaction mixture was stirred overnight. The THF was rotory evaporated, the solid was triturated with water, and the product was collected by filtration. The residue was washed with diethyl ether to remove pivalic acid. The isolated product weighed 0.234 g (51% yield) and had the following properties: mp 255–256 °C

with decomposition; IR (Nujol) 3290, 3090, 1760, 1640, 1555 cm^{-1}; ^1H NMR for major diastereomer (200 MHz, DMSO-d_6) δ 8.71 (d, J = 8 Hz, 1 H), 8.57 (d, J = 8 Hz, 1 H), 8.42 (unresolved d and t, 2 H), 5.36 (d, J = 8 Hz, 1 H), 5.03 (d, J = 8 Hz, 1 H), 4.45 (m, 1 H), 4.11 and 4.07 (two overlapping q, J = 7 Hz, 4 H), 3.11 (m, 2 H), 2.01 (m, 1 H), 1.89 (s, 3 H), 1.16 (t, J = 7 Hz, 6 H), 1.03 (t, J = 7 Hz, 3 H), 0.88 (m, 6 H). Anal. Calcd: C, 51.34; H, 7.26; N, 12.60. Found: C, 51.43; H, 7.31; N, 12.52.

N^{α}-Acetyl-D,L-Ama-L-valyl-D,L-Ama N-Ethylamide. N^{α}-Acetyl-α-carbethoxyglycyl-L-valyl-α-carbethoxyyglycine N-ethylamide (0.166 g, 3.74×10^{-4} mol) was added to 20 mL of 50% aqueous ethanol. Potassium hydroxide (0.47 mL, 2 N KOH, 2.5 equiv) was added, and the reaction mixture was stirred at ambient temperature until all of the starting material had dissolved, approximately 3 h. The solvent was rotory evaporated, and the residue was triturated with 30 mL of absolute ethanol. The solid material was collected by filtration and washed with additional ethanol. The product was dissolved in 10 mL of distilled deionized water and placed on a 2-mL column of Bio-Rad analytical grade cation exchange resin (AG 50W-X8, H^+ form), which had been repeatedly washed with water. The product was eluted at a flow rate of 1 mL/min, and additional water was added as needed. Fractions were collected in 1-mL increments, placed on ice, and analyzed by HPLC. HPLC analysis shows the presence of four diastereomers, which were individually collected. Reinjection of the separate fractions showed the presence of all four diastereomers, indicating that racemization of the Ama residues had occurred. The pure product eluted in fractions 3-9 from the ion exchange column. The fractions were combined, and the solvent was rotary evaporated. The product was further dried in a vacuum desiccator to give 0.0558 g (38% yield) of product. The product had the following properties: mp decarboxylation at 132 °C, further heating gave mp 206-208 °C with decomposition; IR (Nujol) 3670-2500, 3280, 1750, 1635 (s, broad), 1540 cm^{-1}; ^1H NMR (200 MHz, D_2O) δ 4.31 (m, 1 H), 3.21 (q, J = 7 Hz, 2 H), 2.10 (m, 1 H), 2.08 (s, 3 H), 1.10 (t, J = 7 Hz, 3 H), 0.93 (m, 6 H); FAB mass spectrum (negative ion, 3:1 dithiothreitol-dithioerythritol matrix) m/z (relative intensity) 387 (32), 343 (20), 299 (100). Anal. Calcd for $C_{15}H_{24}N_4O_8 \cdot 0.5H_2O$: C, 45.34; H, 6.34; N, 14.10. Found: C, 45.35; H, 6.38; N, 14.08.

Base Hydrolysis and Amino Acid Analysis.[24] Samples (5-15 mg) were added to 2-4 mL of sterile filtered 2 N KOH in Teflon-lined hydrolysis tubes, and the solutions were degassed with nitrogen for 30 min. Tubes were sealed under nitrogen and placed in an oil bath at 108-110 °C for 24 h. After cooling, samples were transferred to 20-mL beakers. Hydrolysis tubes were rinsed with an additional 2-4 mL of water, and rinsings were combined with the samples. The samples were cooled to 0 °C and acidified with concentrated and then dilute perchloric acid to pH 6.0 while keeping the samples on ice. The samples and precipitate were transferred to centrifuge tubes and centrifuged at 10000 rpm and 4 °C for 20 min. The supernatants were removed and evaporated by high vacuum rotory evaporation. The potassium perchlorate pellets were triturated with an additional 4-5 mL of water, cooled, and centrifuged again. The supernatants were added to the sample and evaporated. The samples were dissolved in an appropriate volume of distilled, deionized water and refrigerated to allow the precipitation of additional potassium perchlorate. The supernatants were filtered through 0.2-μm filters and then diluted with an appropriate volume of 0.4 N borate buffer (pH 10.2) and again filtered prior to HPLC analysis. Aliquots of samples in the borate buffer (typically 20-60 μL) were combined with o-phthalaldehyde derivatizing solution using volumes that gave at least a 5-fold excess of o-phthalaldehyde. Samples were mixed for exactly 60 s, diluted with a measured volume of eluent A, and mixed for 30 s. Aliquots of 20 μL were analyzed by HPLC using a 1090 Hewlett-Packard HPLC system equipped with a diode-array detector monitoring at 338 nm. The column was an Alltech Adsorbosphere OPA-HR 5μm 150 × 4.6 mm cartridge. Eluent A was 98%, 0.05 M NaOAc adjusted with 1 N HCl to pH 5.8, 1% THF, 1% MeOH; eluent B was 99% MeOH, 1% THF. The gradient was 0-5 min, isocratic A; 5-8 min, linear gradient to 10% B; 8-12 min, linear gradient to 20% B; and 12-20 min, isocratic 80% A and 20% B. Retention time for Ama derivative was 3.5 ± 0.1 min and for Gly derivative, 15.4 ± 0.1 min. Calibration curves were determined with use of authentic samples of Ama (monopotassium salt) and glycine.

(24) Jones, B. N.; Pääbo, S.; Stein, S. *J. Liq. Chromatogr.* **1981**, *4*, 565.

Dual Mechanisms of Bimolecular Nucleophilic Substitution of a Heterobenzylic Bromide Related to Thiamin†

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4-Amino-5-(bromomethyl)-1,2-dimethylpyrimidinium ion at 25 °C in 0.9 M KCl made to an ionic strength of 1 M with buffers reacts with hydroxide ion with a rate constant of 725 M^{-1} s^{-1}. Evidence for an intermediate is found in the large common ion rate retarding effect of added bromide ion. An addition-elimination mechanism ($S_N(AE)$) involving the addition of hydroxide ion to give a pseudobase intermediate followed by loss of bromide ion is advanced. Chloride ion reacts by an S_N2 mechanism (5.9×10^{-5} M^{-1} s^{-1}). When 2-thiopyridone acts as a nucleophile by the S_N2 mechanism, its sulfur atom is heterobenzylated with a rate constant of 0.28 M^{-1} s^{-1} (31 °C). Two different bimolecular mechanisms of nucleophilic substitution are thereby observed.

Pyrimidinylmethyl bromide **1**, a "heterobenzylic" bromide, undergoes nucleophilic substitution by a novel combination of bimolecular routes. Cation **1** is an N-methylated analogue of thiamin in which the bromine atom is attached to the methylene side chain in place of the thiazole ring.

In a marked contrast, **1** reacts some 10^6 times faster with

† Dedicated to Professor Edward C. Taylor, Jr., on the occasion of his 65th birthday.

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