Free-Radical Carboxylation of Peptide- and Protein-Bound Glycine To Form Peptide- and Protein-Bound Aminomalonic Acid (Ama)

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A free-radical carboxylation of glycine (Gly) residues in water-soluble peptides and proteins to form aminomalonic acid (Ama) residues is described. Ama residues resulted from the photolysis of a peroxide, preferably *tert*-butyl hydroperoxide, in an aqueous medium containing sodium formate and the Gly peptide or protein. The mechanism is proposed to occur by photochemical cleavage of the peroxide followed by oxy radical abstraction of hydrogen atoms from formate to yield carbon dioxide radical anions. Carbon dioxide radical anions abstract hydrogens from the α-position of Gly residues. Other carbon dioxide radical anions then combine with the resulting glycyl radicals. With excess formate present, destruction of Ac-Gly-NHEt was minimized and formation of Ac-Ama-NHEt was quantitative. Under similar reaction conditions, 2% of the Gly residues of gelatin were carboxylated. The process is discussed in terms of in vivo formation of Ama residues as possible calcium binding sites in dystrophic tissue such as atherosclerotic plaque.

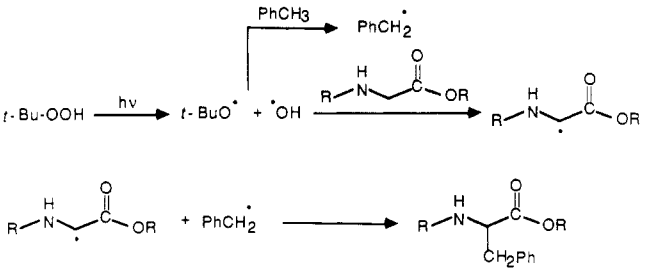
Introduction

Aminomalonic acid (Ama) has been identified as a constituent of alkaline hydrolysates of *E. coli* cytosol proteins, human atherosclerotic plaque, calcified bioprosthetic heart valves, and kidney stones.[1] Ama is a congener of the amino acid γ-carboxyglutamic acid (Gla). Multiple Gla residues in proteins such as prothrombin and osteocalcin are responsible for the calcium binding properties. Because Ama also bears a malonic acid unit capable of serving as a bidentate ligand for calcium, Ama formation in dystrophic tissue such as atherosclerotic plaque might be an explanation for the mineralization of proteins in such tissue. Calcium binding measurements show moderate calcium binding by Ama and Ama peptides relative to calcium binding by Gla and Gla peptides.[2]

Gla residues are formed in protein by enzymic, posttranslational carboxylation of glutamic acid residues with vitamin K as a cofactor.[3] The carboxylation of glutamic acid residues in proteins by a rat liver carboxylase has been modeled with small glutamic acid (Glu) containing peptides. The Gla carboxylase enzyme system was not significantly active in the carboxylation of a small glycine (Gly) containing peptide compared with its activity in carboxylation of a small Glu containing peptide.[4] The carboxylase enzyme is known to be quite sequence specific; consequently, the proper amino acid sequence may not have been provided to the enzyme. Alternatively, an as yet unidentified enzyme may be responsible for the carboxylation of Gly residues. Until a natural Ama-containing protein is isolated and sequenced, selection of a proper peptide for testing in a carboxylase system can be done only at random.

In part because Ama formation appears to be associated with proteins in dystrophic tissue, we have proposed that Ama formation might be modeled by a nonenzymatic, free-radical reaction. Such a chemical process was suggested by the anticipated regioselectivity in the formation of a radical site at the α-position of glycine residues. Many years ago Elad and co-workers demonstrated regioselective hydrogen atom abstraction from the α-position of glycine residues in peptides and proteins by *tert*-butoxy radical and by acetone in its $^3n-\pi^*$ state.[5] The resulting amino acid radicals were subsequently employed in radical combination and radical addition reactions, resulting in the alkylation of glycine residues in peptides and proteins. This is exemplified by the conversion of a glycine residue to a phenylalanine residue as shown in Scheme I.

Scheme I



Regioselectivity was later investigated in polypeptides of molecular weight 2000–5000 Da containing glycine and alanine or glycine and proline.[6] A high degree of selectivity for reaction at glycine residues was observed; 7–25% of the glycine residues were converted to alkylated α-amino acid residues. The preference for reactivity at glycine residues was unexpected in that a secondary rather than a tertiary amino acid radical is involved. Easton and coworkers have recently explored the basis for the glycine regioselectivity by determining relative rates of reaction of amino acid derivatives with *N*-bromosuccinimide.[7] The higher rate of reaction of the glycine residues was attributed to the formation of a more stable, planar radical with fewer nonbonded interactions, as shown in Figure 1.

Because acyloxy radicals readily lose carbon dioxide, a mechanism involving radical addition to carbon dioxide to form an acyloxy radical followed by hydrogen atom

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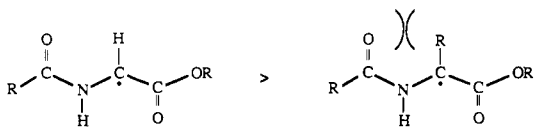


Figure 1. Basis for the greater stability of the glycyl radical.[7]

Table I. Irradiation at 254 nm of the Glycine Monopeptide with Hydrogen Peroxide and Formate

entry	$[Gly]_i$,[a] M	$[H_2O_2]$,[b] M	$[HCO_2^-]_i$,[a] M	% Gly destroyed	% yield of Ama[f]
1	1.40×10^{-2}	3.00×10^{-2}	3.00×10^{-2}	29[c]	0.4
2	8.41×10^{-3} (×10)[e]	8.34×10^{-3}	8.38×10^{-1}	5.0[d]	7.7
3	8.41×10^{-3}	1.67×10^{-2} (×10)[e]	8.38×10^{-1}	6.2[d]	7.3
4	8.41×10^{-3}	4.17×10^{-2} (×10)[e]	8.38×10^{-1}	18[d]	2.5
5	8.41×10^{-3}	8.34×10^{-2} (×10)[e]	8.38×10^{-1}	42[d]	0.5

[a] Initial concentrations. [b] Hydrogen peroxide was added to give the initial concentration indicated. [c] This sample was irradiated with 254-nm light for 24 h. [d] These samples were irradiated with 254-nm light for 0.5 h, at which time all the peroxide had been consumed. Additional aliquots of peroxide were added to give the same concentration, and the sample was irradiated for 0.5 h after each addition. [e] The number in parentheses indicates the number of additions made. [f] Based upon Gly monopeptide destroyed.

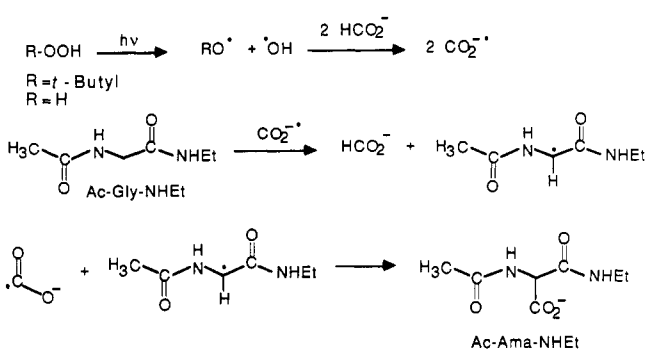
abstraction to form an Ama residue was viewed as less probable. A radical combination reaction between the α-glycyl and carbon dioxide radical anion was then hypothesized, and the results of its in vitro investigation are reported here.

Results and Discussion

Irradiation at 254 nm of phosphate-buffered, aqueous, deoxygenated solutions of hydrogen peroxide or *tert*-butyl hydroperoxide in the presence of sodium formate and N^{α}-acetylglycine *N*-ethylamide (Ac-Gly-NHEt) produced N^{α}-acetylaminomalonate *N*-ethylamide (Ac-Ama-NHEt) as reported in Tables I and II, respectively. The formation of Ama monopeptide and the destruction of Gly monopeptide were monitored by HPLC with detection by UV absorption at 210 nm. The Ama monopeptide was identified by co-injection with an authentic sample under several different eluting conditions. Furthermore, a sample of Ama monopeptide formed from Gly monopeptide was collected as it eluted from the chromatography column, and the kinetics for destruction subsequently determined at pH 7 and 120 °C. The disappearance was first-order and the observed rate constant was identical within experimental error to the rate constant for disappearance of authentic Ama monopeptide.[8] The predominant product under these conditions was Gly monopeptide, formed by decarboxylation of Ama monopeptide.

The yield of Ac-Ama-NHEt from the carboxylation reaction based upon destruction of Ac-Gly-NHEt varied from quantitative to less than 1%, depending upon the conditions and extent of reaction. Quantitative formation of Ama monopeptide was achieved using an excess of both *tert*-butyl hydroperoxide (added in 10 portions) and formate, minimizing destruction of Gly monopeptide. In general, yields were lower when hydrogen peroxide was employed (compare entry 3 of Table I with entry 3 of Table II) and when higher levels of Gly monopeptide destruction occurred. Yields were especially low when formate was not present in excess relative to peroxide (cf entries 7, 8, and 9 of Table II).

Scheme II



Formation of Ama monopeptide is explained by the mechanism shown in Scheme II and discussed as follows. The primary, photolabile, light-absorbing species present is the peroxide. At 254 nm with *tert*-butyl hydroperoxide present at 2.92×10^{-2} M and formate present at 1.25 M, the peroxide absorbs 95% of the incident light. Glycine monopeptide is transparent at this wavelength. The very reactive *tert*-butoxy and hydroxy radicals, formed by bond homolysis of the excited states of *tert*-butyl hydroperoxide and hydrogen peroxide, abstract hydrogen atoms from either the monopeptide or formate at near diffusion controlled rates. When formate is present in large excess, the major radical product initially is the carbon dioxide radical anion. It is shown in Scheme II as the anion because the solutions were buffered to pH 7.4 and the pK_a of $\bullet CO_2H$ is 1.4.[9] The carbon dioxide radical anion then reacts with Gly monopeptide to produce an α-radical. Radical combination of the α-glycyl radical with another carbon dioxide radical anion results in formation of Ama monopeptide.

When the concentration of peroxide is increased, a competing reaction of the carbon dioxide radical anion is the induced decomposition of peroxide to produce additional oxy radical.[10] Under these conditions, nonregio-

$$CO_2^{\bullet -} + HO_2R \rightarrow CO_2 + RO^{\bullet} + OH^-$$

(where R = H or *tert*-butyl)

selective hydrogen atom abstraction from Gly monopeptide and radical combination of the Gly α-radical with radicals other than carbon dioxide radical anion become more likely.

When the concentration of formate was doubled (cf entries 5 and 6 of Table II), much less destruction of the Gly peptide occurred and a higher yield of the Ama monopeptide was obtained. These results suggest that if only the carbon dioxide radical anion is produced, then the only peptide-derived product will be the Ama monopeptide. The formation of the Ama monopeptide under these conditions must only compete with dimerization of the carbon dioxide radical anion to produce oxalate. Dimerization of carbon dioxide radical anion, which occurs with a rate constant of 5×10^8 M^{-1} s^{-1},[11] probably accounts for the large excess of peroxide necessary to form even small amounts of Ama monopeptide.

$$CO_2^{\bullet -} + CO_2^{\bullet -} \rightarrow C_2O_4^{2-}$$

Formation of substantial quantities of other products was not observed by the HPLC analytical method, with

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Table II. Irradiation at 254 nm of the Glycine Monopeptide with *tert*-Butyl Hydroperoxide and Formate

entry	$[Gly]_i$,[a] M	[*t*-BuOOH],[b] M	$[HCO_2^-]_i$,[a] M	% Gly destroyed	% yield of Ama[f]
1	2.52×10^{-2}	1.22	2.25	2.1[c]	56
2	8.41×10^{-3}	1.10×10^{-2} (×10)[e]	8.38×10^{-1}	0.8[d]	100
3	8.41×10^{-3}	1.83×10^{-2} (×10)[e]	8.38×10^{-1}	5.3[d]	20
4	8.41×10^{-3}	2.92×10^{-2} (×8)[e]	8.38×10^{-1}	8.6[d]	21
5	8.41×10^{-3}	2.92×10^{-2} (×12)[e]	8.38×10^{-1}	12[d]	30
6	8.41×10^{-3}	2.92×10^{-2} (×12)[e]	1.68	2.6[d]	51
7	8.41×10^{-3}	3.65×10^{-2} (×10)[e]	8.38×10^{-1}	6.9[d]	33
8	8.41×10^{-3}	5.84×10^{-2} (×10)[e]	8.38×10^{-1}	26[d]	3.7
9	8.41×10^{-3}	8.40×10^{-2} (×10)[e]	8.38×10^{-1}	74[d]	1.4

[a] Initial concentrations. [b] *tert*-Butyl hydroperoxide was added to give the initial concentration indicated. [c] This sample was irradiated with 254-nm light for 24 h. [d] These samples were irradiated with 254-nm light for 0.5 h, at which time all the peroxide had been consumed. Additional aliquots of peroxide were added to give the same concentration, and the sample was irradiated for 0.5 h after each addition. [e] The number in parentheses indicates the number of additions made. [f] Based upon Gly monopeptide destroyed.

the exception of some materials including oxalate eluting at the solvent front. This suggests that the peptide byproducts could be numerous, significantly more polar than Ama monopeptide, and/or do not have a chromophore absorbing at 210 nm. Reasonable possibilities, which were not explored, are the products of radical combination containing functionality subject to facile hydrolysis of either peptide linkage such as the aminol group.

The apparent regioselectivity observed upon hydrogen abstraction by carbon dioxide radical anion likely results from the moderate C–H bond energy of formate, 87 kcal/mol in formic acid,[12] and the substituent stabilization of the resulting α-glycyl radical. The α-glycyl radical is stabilized by electron-donating carbonylamino and electron-withdrawing aminocarbonyl substituents, and consequently, some additional stabilization might result from the captodative (cd) effect.[13] Captodative stabilization appears to be significant at least with electron-donating amino and electron-withdrawing carbonyl substituents.[14] Selectivity for hydrogen atom abstraction from the Gly monopeptide at low extent of reaction likely results from the low relative concentration and low reactivity of the Ama monopeptide at this point. In a control experiment starting with Ama monopeptide in place of Gly monopeptide and the conditions of entry 1 of Table II, no destruction of the Ama monopeptide was observed. Consequently, steric inhibition to hydrogen abstraction from Ama monopeptide, as illustrated for the resulting radical in Figure 1, and the statistical factor of only a single α-hydrogen must lower the rate of hydrogen abstraction from the Ama monopeptide such that this reaction does not compete with other pathways which destroy carbon dioxide radical anion.

Tetraglycine and gelatin also were subjected to reaction with *tert*-butyl hydroperoxide, UV light, and formate as described above. After the last addition of peroxide and dose of irradiation, the water was evaporated and the residue was subjected to base hydrolysis. HPLC analysis of the *o*-phthalaldehyde derivatives[8,15] gave the yields of glycine and aminomalonic acid as reported in Table III. The presence of Ama was verified by co-injection with an authentic sample. The presence of Ama in the alkaline hydrolysates was further established by diluting aliquots with concentrated hydrochloric acid and heating the solution in sealed tubes at 110 °C for 20 min. Subsequently, the solvent was evaporated and the residue was derivatized with *o*-phthalaldehyde; HPLC analysis showed complete loss of the Ama signal, consistent with the known acid lability of Ama.[16]

Table III. Ama Incorporation in Tetraglycine and Gelatin with Irradiation of *tert*-Butyl Hydroperoxide at 254 nm in the Presence of Formate

initial substrate (conc)	$[t\text{-BuOOH}]_i$,[a] M	$[HCO_2^-]_i$,[a] M	% $[Ama]_f$[b]/$[Gly]_f$[b]
tetraglycine (5.67×10^{-3} M)	2.92×10^{-2} (×10)[c]	8.38×10^{-1}	1.2
gelatin (10 mg/mL)	2.92×10^{-2} (×10)[c]	8.38×10^{-1}	1.8

[a] Initial concentrations. [b] Final concentrations. [c] Additions of peroxide were made as indicated in Table I.

Some of the amino acid residues of gelatin and other proteins, particularly tryptophan, absorb at 254 nm and are photoreactive.[17] Consequently, the application of 300-nm light for the carboxylation reaction was explored briefly as reported in Table IV. Formation of Ama monopeptide or Ama residues occurred in approximately the same yield as with 254-nm irradiation; however, the required reaction time was much longer, most likely because of low peroxide optical density at this wavelength.

Elad and co-workers extended the photoalkylation reaction from simple dipeptides to polypeptides and proteins using visible light and triplet photosensitizers such as biacetyl and camphorquinone to cleave the peroxides. Again, longer wavelength light was selected to protect sensitive amino acid residues from direct excitation. We attempted to apply this technique using black lamps with principal emission in the region of 350 nm to effect sen-

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Table IV. Ama Incorporation with 300-nm Irradiation of *tert*-Butyl Hydroperoxide in the Presence of Formate

substrate (conc)	$[t\text{-BuOOH}]_i$,[a] M	$[HCO_2^-]_i$,[a] M	% $[Ama]_f$[b]/$[Gly]_f$[b]	% substrate destroyed	% yield of Ama[e]
Gly monopeptide[c] (8.41×10^{-3} M)	2.92×10^{-1}	8.38×10^{-1}	1.5	6.6	21
Gly monopeptide[c] (8.41×10^{-3} M)	3.65×10^{-1}	8.38×10^{-1}	1.1	3.2	33
diketopiperazine[c] (9.34×10^{-3} M)	3.48×10^{-1}	7.98×10^{-1}	3.4	45	8
gelatin[d] (4 mg/mL)	1.83×10^{-1}	8.38×10^{-1}	0.1	–	–

[a] Initial concentrations. [b] Final concentrations. [c] Irradiation time was 18 h. [d] Irradiation time was 40 h. [e] Based upon substrate destroyed.

sitized decomposition of peroxides in the presence of formate and the glycine monopeptide. The sensitizer and peroxide combinations that were used included biacetyl with di-*tert*-butyl peroxide, biacetyl with *tert*-butyl hydroperoxide, and benzophenone with *tert*-butyl hydroperoxide. These experiments did not produce Ama monopeptide most likely because of reactivity between carbon dioxide radical anion and the photosensitizer. The carbon dioxide radical anion is a strong reducing agent for which redox activity with biacetyl has been demonstrated.[10]

Elad and co-workers also successfully used the photoalkylation reaction to modify glycine residues in insoluble polypeptides and proteins such as $[Ala\text{-}Gly\text{-}Ala]_n$, collagen, lysozyme, and ribonuclease.[6] The reaction of collagen with $n\text{–}\pi^*$ excited acetone in the presence of *p*-fluorotoluene yielded 4 *p*-fluorophenylalanine residues/1000 amino acid residues. This level of alkylation can be compared with the level of carboxylation described above. Glycine residues comprise approximately one-third of the total amino acid residues in collagen. The same reaction with gelatin, which also contains about 33% Gly residues, produced 9 *p*-fluorophenylalanine residues/1000 amino acid residues.[6] This corresponds to a 2.7% yield of modified Gly residues, which is slightly greater than the 1.8% yield for the carboxylation of gelatin reported in Table III. Since a reasonable yield of Ama residues in gelatin was obtained, the free-radical carboxylation of Gly residues in insoluble proteins was explored.

The insoluble proteins selected were bovine elastin, type I collagen, and glutaraldehyde cross-linked bovine pericardium. Bovine pericardium consists predominantly of type I collagen. Elastin and collagen are arterial structural proteins, and cross-linked bovine pericardium is used in the manufacture of artificial heart valves. These proteins were selected because of the possibility that they are carboxylated in vivo to produce Ama residues that serve as calcium binding sites and contribute to the pathological mineralization observed in calcified atherosclerotic lesions and bioprosthetic heart valves. The reactions employed direct excitation of aqueous *tert*-butyl hydroperoxide with 254- or 300-nm light in the presence of excess sodium formate and suspended protein. The insoluble reacted protein was collected by filtration, washed, and subjected to alkaline hydrolysis. HPLC analysis of the *o*-phthalaldehyde amino acid derivatives showed no Ama formation. Filtration of an elastin sample treated with 254-nm light led to the recovery of only about 50% of the starting protein. The possibility that the modification of glycine residues produced a soluble material was checked with a second sample of treated elastin. The water was evaporated from the entire sample, and the residue was subjected to alkaline hydrolysis. HPLC analysis showed a ratio of Ama residues to Gly residues of 0.1%. Ama residues may have been formed in the protein resulting in fragmentation of a soluble peptide or, under the reaction conditions, fragmentation of the elastin occurred with or without Ama formation, and Ama was subsequently incorporated in the soluble fragments. Suspension of elastin in the reaction mixture for the same period of time without irradiation resulted in only 10% loss of insoluble material. Consequently, the photochemistry must have assisted the fragmentation. The simple Ama peptide, Ac-Ama-NHEt, undergoes hydrolysis of an amide linkage by a factor of only two to four faster than the simple Gly peptide, Ac-Gly-NHEt.[8] Possibly, an Ama residue in a macromolecule assists the fragmentation in an as yet unknown manner or some other chemistry results in the fragmentation.

The results presented here suggest that Ama residues may be formed in soluble peptides and proteins under free-radical conditions when carbon dioxide radical anion is present. The reaction does not appear to be very efficient under heterogeneous conditions, most likely because of competitive reactions of carbon dioxide radical anion such as dimerization and oxidation. Certainly under in vivo conditions, oxidation by molecular oxygen to form superoxide would be a serious competitive reaction. Of course, the development of calcified lesions in arterial tissue and in bioprosthetic heart valves requires years in the human body; hence, even an inefficient reaction might be responsible.

An important question is whether an in vivo source of the carbon dioxide radical anion exists. Recently, carbon dioxide radical anion has been identified by spin trapping as an in vivo product of abnormal metabolism. The radical was spin trapped during liver metabolism of carbon tetrachloride. The amount of spin-trapped radical was correlated with hepatocellular damage although the role of carbon dioxide radical anion in initiating cell death was not determined.[18] Interestingly, the greatest amount of hepatic injury following exposure to carbon tetrachloride occurred in the centrilobular region where the concentration of oxygen is lowest.[19] A low oxygen concentration might permit carbon dioxide radical anion attack at neighboring glycine residues before oxidation to carbon dioxide. Carbon dioxide radical anion has also been identified as a metabolite of rat liver mitochondria in the presence of the hypotensive drug hydralazine (1-hydrazinophthalazine).[20]

A possible consequence of carbon dioxide radical anion production in the liver may be the carboxylation of soluble lipoproteins. Some modifications of low density lipoproteins (LDL) result in increased macrophage uptake of LDL in vitro, resulting in generation of foam cells which are a constituent of atherosclerotic plaque.[21] This was initially demonstrated by acetylation of the lysines of LDL to give a more negatively charged LDL, the positively

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charged ammonium groups being transformed into uncharged acetamide groups. Acetylation of LDL is unlikely in vivo, however, and extensive effort has been directed toward identifying the in vivo modification that results in increased macrophage uptake of cholesterol. In vitro modifications of LDL and albumin that result in formation of carboxylate groups, as in the reaction of lysine ϵ-amino groups with maleic anhydride, also result in an increased macrophage response.[22] Conversion of glycine residues to Ama residues in LDL would also result in an increased negative charge. Macrophage-derived foam cells in atherosclerotic plaque have been suggested as one of the sites of calcification in advanced atherosclerosis.[23]

We have attempted to produce Ama in LDL using UV light, *tert*-butyl hydroperoxide, and sodium formate, as was successful in the carboxylation of Gly residues in gelatin. However, amino acid analysis of the reacted LDL did not show Ama in the alkaline hydrolysate. This may not exclude the possibility that Ama could be formed in protein in the liver before formation of the LDL matrix. Carboxylation of protein and lipids in rat liver nuclei was established in early work by G. Scholes using ionizing radiation in aqueous bicarbonate solutions.[24] The reactive species was the carbon dioxide radical anion formed by the reaction of bicarbonate with radiation-produced radicals derived from water. Using ^{14}C-labeled bicarbonate, carboxylation of both lipid and protein fractions was established. The nature of the carboxylated species was not established but the present research suggests that Ama residues may have been formed in the protein fractions.

In summary, we have demonstrated that carbon dioxide radical anion, formed in aqueous, deoxygenated solutions, carboxylates the α-position of glycine residues in Ac-Gly-NHEt, tetraglycine, and gelatin to produce aminomalonic acid residues. We propose that such a reaction might be responsible for in vivo formation of some Ama residues in protein.

Experimental Section

General Methods. HPLC analyses of the Gly and Ama monopeptides were performed with a Tracor Model 950 pump equipped with a Tracor Model 970A variable-wavelength UV–vis detector set at 210 nm, a Hewlett-Packard Model 3390 integrating plotter, and an Alltech Econosphere C-18 column (4.6 × 250 mm, 5 μm). Injections were performed with a 20-μL fixed loop injector, and detector response was calibrated with standard solutions. The detection level of N-Ac-Ama-NHEt relative to N-Ac-Gly-NHEt was 1/1000. HPLC analyses of the *o*-phthalaldehyde derivatives of amino acids[8,15] were performed with a Hewlett-Packard Model 1090 HPLC instrument equipped with a diode array detector set at 338 nm, and data processing work station. The column was an Alltech Adsorbosphere OPR-HR, 5 μm, 150 × 4.6 mm cartridge. The detection level of Ama relative to Gly was similar to that described above. Chemical reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. *N*-Acetyl-D,L-aminomalonate *N*-ethylamide and *N*-acetylglycine *N*-ethylamide were prepared as described earlier.[8] Pure aminomalonic acid was obtained from the monopotassium salt of aminomalonic acid by ion exchange chromatography.[2]

Irradiation Experiments. *N*-Acetylglycine *N*-ethylamide, peroxide, and sodium formate were added to 2–4 mL of 0.1 N potassium phosphate buffer, pH 7.4, to give the concentrations indicated in Tables I and II. The samples were placed in 1 × 10 cm quartz tubes with stir bars and degassed by bubbling purified nitrogen through the solution for 15–30 min. Serum stoppers were used to seal the tubes, and the tubes were suspended in a Rayonet Photochemical Reactor (Southern New England Ultraviolet Co.). The samples were stirred while irradiating with four low-pressure mercury lamps with principal emission at 254 nm with the cooling fan on. The temperature remained at 25 ± 2 °C during the irradiations. Additional peroxide was added at 0.5-h intervals to give the concentrations indicated in the tables. HPLC analyses were performed using the Tracor HPLC system and 0.1 N aqueous ammonium dihydrogen phosphate eluent, pH 4.5, at a flow rate of 1.4 mL/min. Identification of (*N*-acetylamino)malonate *N*-ethylamide as a product was established by co-injection with an authentic sample with elution at several different flow rates. In addition, the Ama monopeptide was collected after HPLC separation from a sample that gave a final ratio of Ama monopeptide to Gly monopeptide at 4% Ama peptide. The sample was concentrated and the pH was adjusted to 7. Aliquots of 50 μL each were sealed in melting point capillary tubes and the tubes were placed in an oil bath at 120 °C. Samples were removed at 50-min time intervals over a period of 550 min and analyzed by HPLC. The chromatographic data showed first-order kinetics for the disappearance of the suspected Ama monopeptide with formation of a peak corresponding to the glycine peptide. The determined k_{obs} was 0.00257 min^{-1}. The same procedure was used for the substrates listed in Tables III and IV except in the experiments involving gelatin, where the buffered solutions of sodium formate were degassed before the addition of gelatin. The solutions were gently heated to dissolve completely the gelatin, and after cooling, *tert*-butyl hydroperoxide was added in the concentrations indicated. All the samples were irradiated using four lights while stirring the solution, except for the gelatin experiment with 300-nm irradiation where 16 lamp bulbs with maximum emission at 300 nm were used. The temperature for this experiment remained at about 40 °C. Samples were placed in Pyrex test tubes (1 × 10 cm) for irradiations using 300-nm light. The extent of formation of Ama residues was determined by base hydrolysis and amino acid analysis[8] for samples other than the glycine monopeptide. The irradiation times indicated in Table III led to complete destruction of the peroxide.

In the attempts to carboxylate Gly residues in the insoluble protein materials, elastin, type I collagen, and glutaraldehyde cross-linked bovine pericardium, the reaction conditions were similar to those described for the carboxylation of soluble peptides and proteins except that the insoluble proteins were suspended in the aqueous reaction medium. The solutions were oxygen degassed before the substrate was added. The water from one elastin reaction mixture was removed by rotary evaporation, and amino acid analysis of the alkaline hydrolysate gave a ratio of Ama residues/glycine residues of 0.1%. Amino acid analysis of all the other samples, which were filtered before base hydrolysis, showed no Ama formation. The alkaline hydrolyses and amino acid analyses were performed as described earlier.[8]

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Registry No. Ac-Gly-NHEt, 24847-33-4; (±)-Ac-Ama-NHEt, 119183-70-9; H-Gly-OH, 56-40-6; tetraglycine, 637-84-3.

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