Side-Chain Interactions between Arginine and γ-Carboxyglutamic Acid. Studies on Decarboxylation of γ-Carboxyglutamic Acid in the Presence of Guanidine

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γ-Carboxyglutamic acid (Gla) occurs in several proteins associated with the plasma coagulation system. The sequence Gla-Arg and/or Arg-Gla invariably occurs in these vitamin K dependent proteins. The postulated salt bridge formed between the oppositely charged side chains of Gla and Arg residues has been studied by thermal decarboxylation. Guanidine has been used as a model for the side chain of arginine. It is proposed that the one-to-one peptide–guanidine complex decarboxylates and the one-to-two peptide–guanidine complex is "protected" from decarboxylation. The apparent equilibrium constants from curve fitting were K_1 = 0.02 mM and K_2 = 0.5 mM. The rate of decarboxylation of Z-Gla-Arg-OH ($t_{1/2}$ = 3.2 h) is significantly slower than either Gla ($t_{1/2}$ = 0.2 h) or the peptide–guanidine (1:1) complex ($t_{1/2}$ = 0.3 h). It is suggested that the Gla–Arg interaction could account for the different rates of thermal decarboxylation of Gla residues in protein samples as observed by previous workers.

Previous ^{1}H NMR studies[2] on peptides containing adjacent γ-carboxyglutamyl (Gla) and arginyl residues suggested a salt-bridge interaction between the malonyl and guanidinyl side chains of these residues. A similar intramolecular salt-bridge interaction was noted by Lancelot et al.[3] from ^{1}H NMR studies on *N*-acetyl-L-arginyl-L-glutamic acid α-*N*-ethyl amide. Recent X-ray crystallographic structures of L-arginyl-L-glutamic acid and L-arginyl-L-aspartic acid revealed the guanidinyl and carboxylate were involved in an intramolecular type B salt bridge rather than in intermolecular interaction suggested by ^{1}H NMR solution studies.[4] The present paper describes further studies on the Gla–guanidine salt bridge.

Price et al.[5] have reviewed the thermal decarboxylation procedure that has been used to convert Gla to Glu in dry protein samples. When Gla-containing proteins are lyophilized from buffers such as ammonium acetate or ammonium bicarbonate, the resulting ammonium salts of the γ-carboxyl groups are thermally unstable and yield Glu at a decarboxylation rate nearly the same as protonated Gla in solution ($t_{1/2}$ = 0.2 h at 110 °C, Gla lyophilized from ammonium acetate; $t_{1/2}$ = 0.4 h at 100 °C, Gla in aqueous 6 N HCl).[7] Any interaction that displaces the ammonium ions as a proton source, such as binding of sodium, calcium, or magnesium ions, will "protect" the γ-carboxyl groups from decarboxylation. The degree of protection is a function of the number of metal ions bound, and this method has been used to estimate the number of Gla residues involved in the human prothrombin–calcium ion interaction.[6]

We have employed a modification of this published procedure to study the thermal decarboxylation of *N*-(benzyloxycarbonyl)-γ-carboxyglutamic acid α-methyl ester[8] (Z-Gla-OMe) to the corresponding glutamyl product (Z-Glu-OMe) as a function of guanidine concentration (Figure 1). It is evident from these data that only after the first equivalent of guanidine is added does one observe significant "protection" of the Z-Gla-OMe from decarboxylation. Analysis of the guanidine–malonyl side-chain interaction using a curve-fitting program similar to the one described by Reuben[9] yields the following model, which accounts for both the previously obtained ^{1}H NMR data and the decarboxylation "protection" experiments.

As shown in Figure 2, the one guanidine to one Gla complex, PG, contains a guanidine molecule asymmetrically associated to only one of the two carboxylates of the malonyl side chain of Gla. Thus the other carboxylate is still available for decarboxylation ($t_{1/2}$ = 0.5 h). In the two guanidine to one Gla species, PG_2, each carboxylate is hydrogen bonded to a guanidine. Therefore neither carboxylate is available for decarboxylation and the Gla is "protected" ($t_{1/2} \gg$ 1 day).

We have also examined the decarboxylation of the dipeptide Z-Gla-Arg-OMe. If the ^{1}H NMR results[2] that suggest a PG-type interaction of the malonyl and guanidinyl side chains were valid, then one would expect to isolate only Z-Glu-Arg-OMe from the thermal decomposition. This was found to be the case. The rate of decarboxylation of Z-Gla-Arg-OMe ($t_{1/2}$ = 3.2 h) was significantly slower than that of Z-Gla-OMe ($t_{1/2}$ = 0.2 h). Thus Z-Gla-Arg-OMe is not protected from decarboxylation, but the rate is significantly slower. Whether the side-chain interactions of Gla and Arg are inter- or intramolecular cannot be determined by these data, although previous work[2,3] would suggest that an intramolecular interaction occurs in solution.

The demonstration that salt-bridge-type interactions between the malonyl side chain of Gla and guanidine cations can retard or prevent the thermal decarboxylation of Gla residues may be of importance in the study of larger polypeptides containing Gla and Arg residues. Furie et al.[10] reported the effects on the natural-abundance ^{13}C

(1) (a) Undergraduate Honors Student, 1983–1984. (b) This work was supported by Grant HL-20161 from the National Heart, Lung, and Blood Institute, U.S. Public Health Service.
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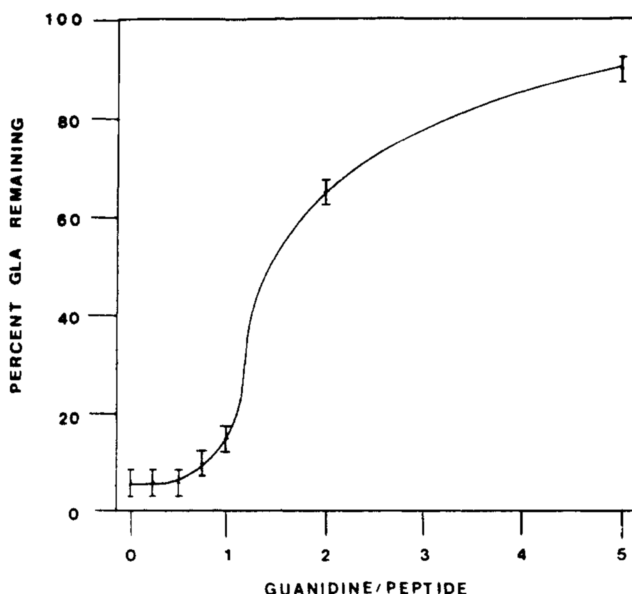

Figure 1. Protection of Z-Gla-OMe from thermal decarboxylation by guanidine. Percent Gla remaining (not decarboxylated) after 1 h at 110 °C is plotted as a function of the guanidine-to-peptide ratio.

NMR spectrum of the binding of Gd(III) ions to the prothrombin 12–44 fragment. Their results indicated that the addition of Gd(III) caused marked line broadening of the resonances associated with the C_γ, C_β, and C_α atoms of the Gla residues and the C_ϵ atom of at least one Arg residue. In retrospect these data are consistent with the asymmetric Gla–Arg salt-bridge interactions of the type we have observed which could stabilize the asymmetric Gla carboxylate–metal ion interactions.

Furthermore, the observation that Gla residues can be "protected" against thermal decarboxylation by malonyl–guanidinyl salt bridges suggests that the "protection" of Gla residues noted in previous protein decarboxylation experiments[6] may not have been entirely due to an added metal ion. The variation of the decarboxylation rate of various Gla residues in prothrombin observed by Bajaj[6] and Tuhy[11] and indeed the number of Gla residues "protected" by various concentrations of added Ca^{2+} ion may well be influenced by Gla–Arg salt-bridge interactions.

Experimental Section

The Gla/Glu analysis was obtained by an isocratic separation with a cation-exchange HPLC column (Aminex a-9, Bio-Rad Laboratories) with a mobile phase of 0.1 M sodium citrate buffer (pH 3.5). The amino acids were detected by postcolumn derivatization with *o*-phthalaldehyde and β-mercaptoethanol (OPA/BME) followed by fluorescence detection. Representative retention times are 4 min for Gla and 11 min for Glu.

The preparation of Z-Gla-OMe has been previously described.[8]

All other chemicals are commercially available and were not purified prior to use.

Decarboxylation of Z-Gla-OMe in the Presence of Guanidine. To a solution of Z-Gla-OMe (2.5 mM, 1.0 mL, 2.5 μmol) in ammonium bicarbonate buffer (0.1 M, pH 7.8) was added an ammonium bicarbonated buffered solution of guanidine (125 mM, 20.0 μL, 2.5 μmol). The sample was lyophilized (<200 μm) overnight. The sample was sealed under vacuum (<200 μm) and then heated in vacuo at 110 °C for 1 h. To the resulting powder was added aqueous sodium hydroxide (2 N, 2 mL). The solution was degassed, sealed under vacuum, and heated at 110 °C for 24 h. Acid (3 N HCl) was added to the solution to lower the pH to 2.0 ± 0.2, and the samples were analyzed as outlined above. A series of guanidine-containing samples were investigated (0.0, 0.5, 1.0, 1.5, 2.0, 5.0, 20.0 molar equiv of guanidine). In order to minimize systematic errors, a complete series of samples were prepared and treated identically. The results represent the data from three complete sets of experiments.

Appendix Calculations

To calculate the different concentrations of species, the following terms and equations were used: $[P_f]$ = free peptide concentration, $[P_t]$ = total peptide concentration, $[G_f]$ = free guanidine concentration, and $[G_t]$ = total guanidine concentration.

$$K_1 = [P_f][G_f]/[PG]$$

$$[PG] = [P_f][G_f]/K_1$$

$$K_2 = [G_f][PG]/[PG_2]$$

$$[PG_2] = [G_f][PG]/K_2 = [G_f][G_f][P_f]/(K_1K_2)$$

The peptide and guanidine concentrations are

$$[P_f] = [P_f][P_t]/[P_t]$$

$$[P_t] = [P_f] + [PG] + [PG_2]$$

$$[G_f] = [G_f][G_t]/[G_t]$$

$$[G_t] = [G_f] + [PG] + [PG_2]$$

Therefore

$$[P_f] = [P_f][P_t]/([P_f] + [PG] + [PG_2])$$

$$[G_f] = [G_f][G_t]/([G_f] + [PG] + [PG_2])$$

$$[P_f] = [P_f][P_t]/([P_f] + [P_f][G_f]/K_1 + [P_f][G_f][G_f]/(K_1K_2))$$

$$[P_f] = [P_t]/(1 + [G_f]/K_1 + [G_f][G_f]/(K_1K_2))$$

$$[G_f] = [G_f][G_t]/([G_f] + [P_f][G_f]/K_1 + [P_f][G_f][G_f]/(K_1K_2))$$

$$[G_f] = [G_t]/(1 + [P_f]/K_1 + 2[P_f][G_f]/(K_1K_2))$$

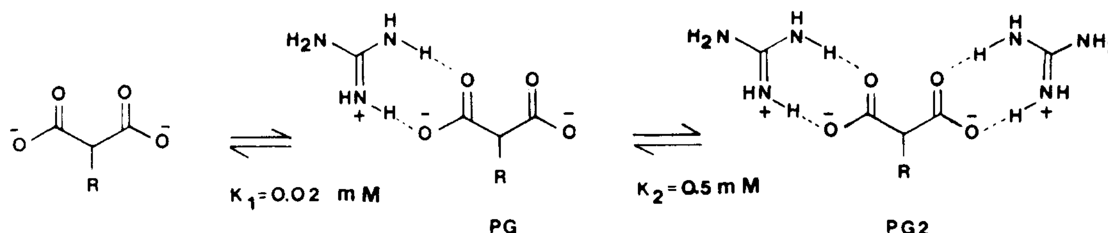

Figure 2. Equilibria involved in the proposed model of guanidine binding to γ-carboxyglutamic acid (Gla). The PG species is identified as being thermally unstable, decarboxylating at a rate nearly equal to that of free Gla. Two guanidine molecules bound to one Gla (PG2), however, confer protection from decarboxylation. Thermodynamic binding constants were obtained by optimized curve fitting.

Substituting the last P_f equation into the last G_f equation,

$$[G_f] = [G_t]/(1 + (1/K_1 + 2[G_f]/(K_1K_2))([P_t]/(1 + [G_f]/K_1 + [G_f][G_f]/(K_1K_2))))$$

This simplifies to

$$f = 0 = [G_f]^3 + (K_2 + 2[P_t] - [G_t])[G_f]^2 + (K_1 + [P_t] - [G_t])K_2[G_f] - K_1K_2[G_t]$$

and

$$f_1 = d(f)/d[G_f]$$

Then Newton's method[9] is used to calculate the $[G_f]$ by

$$[G_f] = [G_f] - f/f_1$$

and this is repeated until f is equal to zero. Then $[P_f]$, [PG], and $[PG_2]$ are calculated. With the concentration of the species calculated, a theoretical point (cp) is calculated by

$$ep(1) = \text{experimental point when } [G_t] = 0$$

$$ep(n) = \text{experimental point when } [G_t] = 20$$

ep = experimental point at the $[P_t]$ and $[G_t]$ used in the calculation for the concentration of species.

$$cp = ep(1)([P_f] + [PG])/[P_t] + ep(n)[PG_2]/[P_t]$$

The quality of the fit is determined by

$$ss = \sum_1^n (cp - ep)^2$$

The fit is optimized by varying K_1 and K_2. All data sets gave the same qualitative K's.

Registry No. Z-Gla-OMe, 96095-90-8; Z-Gla-Arg-OMe, 96095-90-8; guanidine, 113-00-8; L-arginine, 74-79-3; γ-carboxyglutamic acid, 53861-57-7.

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Notes

Reaction of Some 2,3-Steroidal Epoxides with Tri-*n*-butyl(carbethoxymethylidene)phosphorane. Formation of A-Nor Derivatives

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Cyclic epoxides have previously been shown to react with tri-*n*-butyl(carbethoxymethylidene)phosphorane (1) to yield α,β-unsaturated esters in which a ring contraction occurred because of carbon migration; cyclopropyl derivates are formed in minor amounts and in the case of cyclopentene oxide a cyclopropyl derivative is the main reaction product.[1]

In this note we report the reaction of 1[2] with the 2,3-steroidal epoxides 2–6[3] in refluxing toluene (Chart I).

2β,3-Epoxy-5α-cholestane (2) reacts with 1 (epoxide/phosphorane 1:2 molar ratio; reaction time 24 h) to give compound 7 and 8 with satisfactory yields. If the reaction is carried out by using a 1:1 molar ratio, the reaction mixture contains compounds 7 and 8 and aldehyde 9.

Chart I

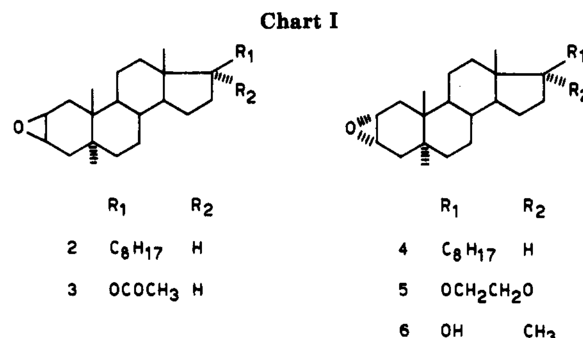
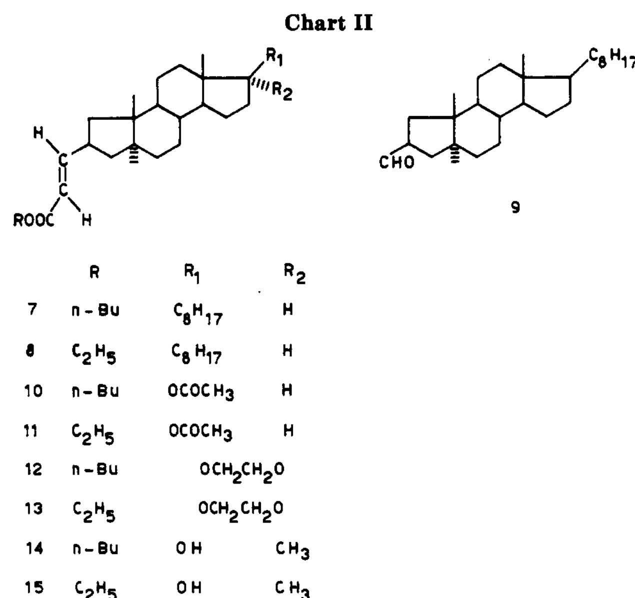


Chart II



Products 7, 8, and 9 are also obtained from the reaction of 2α,3-epoxy-5α-cholestane (4); however, this reaction is slower than that of epoxide 2.

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(2) Phosphorane 1 was prepared from the corresponding phosphonium salt and a commercial solution of *n*-butyllithium in hexane (1.6 M, Fluka). The phosphonium salt was prepared according to Speziale, A. J.; Bissing, D. E. *J. Am. Chem. Soc.* 1963, *85*, 3878.

(3) Epoxides examined were prepared by the following methods. (a) Epoxide 2: Corey, E. J. *J. Am. Chem. Soc.* 1953, *75*, 4832. (b) Epoxide 3 was prepared by acetylation (acetic anhydride and pyridine at room temperature) of 2β,3-epoxy-5α-androstan-17-ol obtained from the corresponding bromohydrin. (c) Epoxide 4 was obtained from the reaction of 5α-cholest-2-ene with *m*-cloroperbenzoic acid in $CHCl_3$ at room temperature. (d) Epoxides 5 and 6: Wolf, G. C.; Blickenstaff, R. T. *J. Org. Chem.* 1976, *41*, 1254.

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