Arg, 0.97; Gly, 1.00; NH_3, 2.01. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-*n*-propyl)tyrosine,4-valine,8-D-arginine]vasopressin (XII). The analogue XII was prepared from intermediate IV (115 mg, 0.076 mmol) in the manner detailed above for IX: yield 11.1 mg (13%); $[\alpha]^{22}_D$ -29.7° (*c* 0.2, 50% AcOH); TLC R_f (C) 0.49, R_f (D) 0.23. Amino acid analysis: Tyr, 1.00; Phe, 1.03; Val, 1.01; Asp, 1.02; Pro, 0.99; Arg, 0.97; Gly, 1.00; NH_3, 1.96. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-methyl)tyrosine,4-valine]arginine-vasopressin (XIII). Treatment of the protected acyl octapeptide V (174 mg, 0.119 mmol) as detailed for IX yielded the analogue XIII (51.5 mg, 35.6%): TLC R_f (A) 0.28, R_f (C) 0.60; $[\alpha]^{23}_D$ -66.3° (*c* 0.4, 1 M AcOH). Amino acid analysis: Tyr, 0.99; Phe, 1.01; Val, 1.02; Asp, 1.01; Pro, 1.00; Arg, 1.01; Gly, 1.00; NH_3, 2.11. Analysis following performic acid oxidation prior to hydrolysis have a Cys(O_3H)-Gly ratio of 1.03:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-ethyl)tyrosine,4-valine]arginine-vasopressin (XIV). The peptide intermediate VI (115 mg, 0.078 mmol) was reduced by sodium in liquid ammonia, reoxidized, deionized, and purified as for IX to give XIV: yield 44 mg (40%); TLC R_f (A) 0.31, R_f (C) 0.62; $[\alpha]_D$ -65.1° (*c* 0.2, 1 M AcOH). Amino acid analysis: Tyr, 1.00; Phe, 1.01; Val, 1.01; Asp, 1.01; Pro, 1.01; Arg, 1.00; Gly, 1.00; NH_3, 1.97. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-isopropyl)tyrosine,4-valine]arginine-vasopressin (XV). Treatment of the protected acyl octapeptidyl amide VII (140 g, 0.094 mmol) as detailed above for IX gave the analogue XV: yield 46 mg (43.3%); TLC R_f (C) 0.63, R_f (D) 0.29; $[\alpha]^{23}_D$ -49.5° (*c* 1.0, 50% AcOH). Amino acid analysis: Tyr, 1.02; Phe, 1.03; Val, 1.01; Asp, 1.02; Pro, 1.03; Arg, 0.97; Gly, 1.00; NH_3, 1.96. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.02:1.00.

[1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),2-(*O*-*n*-propyl)tyrosine,4-valine]arginine-vasopressin (XVI). The analogue XVI was prepared from intermediate VIII (140 mg, 0.094 mmol) in the manner detailed above for IX: yield 28 mg (26.4%); $[\alpha]^{22}_D$ -46.7° (*c* 0.8, 50% AcOH); TLC R_f (C) 0.63; R_f (D) 0.30. Amino acid analysis: Tyr, 1.00; Phe, 1.02; Val, 1.02; Asp, 1.01; Pro, 1.00; Arg, 0.97; Gly, 1.00; NH_3, 1.96. Analysis following performic acid oxidation prior to hydrolysis gave a Cys(O_3H)-Gly ratio of 1.01:1.00.

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Synthesis of Peptide Analogues of Prothrombin Precursor Sequence 5–9. Substrate Specificity of Vitamin K Dependent Carboxylase

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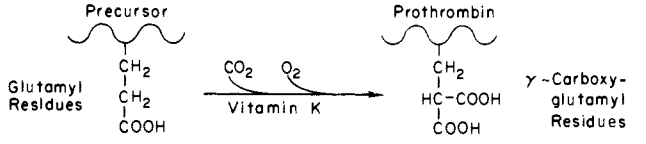
Thirty-five analogues of Phe-Leu-Glu-Glu-Leu, the pentapeptide sequence 5–9 of bovine prothrombin precursor, were synthesized and assayed as potential substrates or inhibitors of rat liver vitamin K dependent carboxylase. Carboxylation of substrate was determined by measuring the incorporation of carbon-14 labeled bicarbonate into product. Changes in substrate carboxylation produced by changing peptide chain length, amino acid chirality, or the distance separating the peptide chain backbone from the carboxyl group were measured. The data suggest that the carboxylase carboxylates L-glutamic acid residues and does not carboxylate L-aspartic acid, L-homoglutamic acid, glutamine, or D-glutamic acid residues; tri- through pentapeptides are better substrates than mono- or bis(amino acid) derivatives, and hydrophobic groups added to the N-terminus can produce better substrates for the enzyme. None of the synthetic substrates is carboxylated as effectively as the endogenous protein substrates for the enzyme. The effect of structure on additional parameters affecting carboxylation is discussed.

Prothrombin (factor II) and the other vitamin K dependent plasma clotting factors (VII, IX, and X) are synthesized in the liver by a reaction involving the vitamin K dependent conversion of specific glutamic acid residues of microsomal precursor proteins to γ-carboxyglutamic acid (Gla)[1] residues of finished proteins.[2-4] In prothrombin, ten glutamyl residues in positions 7, 8, 15, 17, 20, 21, 26, 27, 30, and 33 of the protein chain are carboxylated. The enzyme that catalyzes this reaction, vitamin K dependent carboxylase,[5] requires reduced vitamin

Scheme I



(1) Abbreviations used follow IUPAC-IUB tentative rules as described in *J. Biol. Chem.*, 247, 977 (1972). Additional abbreviations used are: Gla, γ-carboxyglutamic acid; K-H_2, reduced vitamin K; Aad, homoglutamic acid, or α-aminoadipic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; Bzl, benzyl.

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Table I. Physical Properties of Deprotected-Glutamyl Peptides

no.	compd	mp, °C of O-Bzl esters	TLC, R_f (system)[a]	mp, °C	other
7	Boc-Glu-Leu-OMe	65-66	0.11 (A); 0.80 (B)	N[e]	b, c
8	Boc-Glu-Glu-Leu-OMe	90-92	0.08 (C); 0.16 (D)	125-127	b, c
9	Boc-Leu-Glu-Glu-Leu-OMe	158-161	0.88 (A); 0.81 (D)	189-190	b, c
10	Boc-Phe-Leu-Glu-Glu-Leu-OMe	180.5-182	0.82 (A); 0.63 (E)	215-218 dec	b, c
11	Boc-Glu-Asp-Leu-OMe	N[f]	0.82 (A)	119-120	b, c
12	Boc-Asp-Glu-Leu-OMe	N	0.84 (A)	N[f]	b, c
13	Boc-Asp-Asp-Leu-OMe	88-90	0.82 (A)	N	b, c
14	Boc-Glu-D-Glu-Leu-OMe	124-125	0.58 (I); 0.70 (J)	83-85	b, d
15	Boc-D-Glu-D-Glu-Leu-OMe	99-100	0.64 (J); 0.34 (K)	82-83	b, d
16	Boc-D-Glu-Glu-Leu-OMe	87-88	0.58 (I); 0.77 (J)	86	b, d
17	Boc-Glu-Glu-D-Leu-OMe	98-99	0.70 (N)	118	b, d
18	Boc-D-Leu-Glu-Glu-D-Leu-OMe	143-144	0.07 (F); 0.45 (K)	124-126	b, d
19	Boc-D-Leu-Glu-Glu-Leu-OMe	133-135	0.21 (G); 0.45 (K)	101-102	b, d
20	Boc-Phe-Leu-Glu-D-Glu-Leu-OMe	186-187	0.90 (H); 0.69 (J)	184-186	b, c
21	Boc-Phe-Leu-D-Glu-D-Glu-Leu-OMe	164-166	0.50 (K); 0.77 (I)	182-183	b, c
22	Boc-Phe-Leu-D-Glu-Glu-Leu-OMe	186-187	0.44 (I); 0.69 (J)	181-183	b, c
23	Boc-Aad-Aad-Leu-OMe	132-133	0.24 (G); 0.74 (H)	>200	b, d, h
24	Boc-Ala-Glu-Glu-Leu-OMe	132-133	0.68 (H)	128-131	b, d, i
25	Boc-Gly-Glu-Glu-Leu-OMe	127	0.66 (H)	119-120	b, d
26	Boc-Phe-Ala-Glu-Glu-Leu-OMe	183-185	0.73 (H)	196-197	b, d
27	Boc-Phe-Gly-Glu-Glu-Leu-OMe	137	0.70 (H); 0.66 (J)	185-186	b, d
28	propionyl-Phe-Leu-Glu-Glu-Leu-OH	g	0.82 (A); 0.78 (L)	N	b, c
29	Bz-Phe-Leu-Glu-Glu-Leu-OH	g	0.86 (A); 0.82 (L)	220 dec	b, c
30	H-Phe-Glu-Leu-Glu-Leu-OH	g	0.57 (J); 0.48 (M)	N	c
31	Boc-Gln-Gln-Leu-OMe		0.69 (E)	212-214	b, c
32	H-Leu-Glu-Glu-Leu-OH	g	0.40 (E); 0.57 (L)	N	b, c
33	H-Phe-Ala-Glu-Glu-Leu-OH	g	0.46 (E); 0.69 (J)	N	b, c
34	H-Phe-Gly-Glu-Glu-Leu-OH	g	0.36 (E); 0.50 (J)	N	b, c
35	isovaleryl-Met-Glu-Leu-OMe	N	0.37 (G); 0.86 (H)	200	b, d
36	Boc-Glu-Ala-Glu-OMe	108-109	0.79 (H); 0.62 (K)	114-117	b, d
37	Boc-Pro-Leu-Glu-Ala-Glu-OMe	180-181	0.78 (H); 0.49 (K)	193-194	b, d
38	Ac-Pro-Leu-Glu-Ala-Glu-OMe	N	0.69 (H); 0.41 (K)	189-191	b, d
39	Boc-Leu-Glu-Ala-Glu-OMe	138-139	0.81 (H); 0.68 (K)	162-163	b, d
40	Ac-Phe-Ala-Glu-Glu-Leu-OMe	N	0.68 (H)	228-229 dec	b, c
41	H-Phe-Glu-Ala-Leu-Glu-Ser-Leu-OH	g	0.33 (E); 0.61 (L)	N	b, c

[a] TLC solvent systems utilized are designated in parentheses following R_f value. The following solvent systems were used: (A) acetone/methanol/water, 2:2:1; (B) methanol/chloroform, 5:95; (C) Skelly B/ethyl acetate/acetic acid, 65:30;5; (D) benzene/ethyl acetate, 1:1; (E) butanol/acetic acid/water, 4:1:1; (F) 2% MeOH/$CHCl_3$; (G) 4% MeOH/$CHCl_3$; (H) 6% MeOH/$CHCl_3$; (I) 15% MeOH/$CHCl_3$; (J) chloroform/methanol/acetic acid, 70:25:5; (K) 1-butanol/acetic acid/water, 4:1:5; (L) 1-butanol/pyridine/acetic acid/water, 15:10:3:12; (M) 1-butanol/acetic acid/water/ethanol, 1:1:1:1; (N) 1-propanol/37% ammonium hydroxide, 3:7. [b] Satisfactory NMR spectrum obtained for compound. [c] Satisfactory amino acid analysis obtained for compound. [d] Satisfactory elemental analysis obtained for compound. [e] No melting point obtained. [f] Compound isolated as an amorphous solid. [g] Prepared by solid-phase synthesis as described in ref 7. [h] H: calcd, 7.83; found, 7.26. [i] C: calcd, 47.76; found, 47.31.

($K\text{-}H_2$),[1] oxygen, and carbon dioxide to carboxylate endogenous protein substrates (Scheme I). Extensive studies provide evidence that cofactors for other biochemical carboxylations, e.g., biotin, ATP, or acetylcoenzyme A (AcCoA), are not utilized by the vitamin K dependent carboxylase. Thus, vitamin K dependent carboxylation of specific glutamyl residues appears to be a new type of carboxylation reaction with an unknown mechanism.

One aspect of interest in characterizing this enzyme system has been a determination of the specificity of the carboxylase toward various glutamyl-containing low-molecular-weight peptides as potential substrates or inhibitors.[6] We have shown that a pentapeptide, H-Phe-Leu-Glu-Glu-Val-OH (1), which is homologous with residues 5 through 9 of the bovine prothrombin precursor would serve as a substrate for the carboxylase,[7] and other substrates have been identified subsequently.[8,9] These peptides have proven to be very useful for assaying vitamin K dependent carboxylase activity, particularly during enzyme purification. However, rate studies show that the synthetic peptides, e.g., H-Phe-Leu-Glu-Glu-Leu-OH (2), are carboxylated more slowly than an endogenous prothrombin precursor,[8] and sequencing of the product established that only one glutamyl residue is carboxylated.[10,11] No explanation for these differences is known. We report here the results of experiments to define further the specificity of rat liver vitamin K dependent carboxylase for various substrates.

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Chart I. Pathway for Synthesis of C- and N-Protected Tripeptides and Pentapeptides

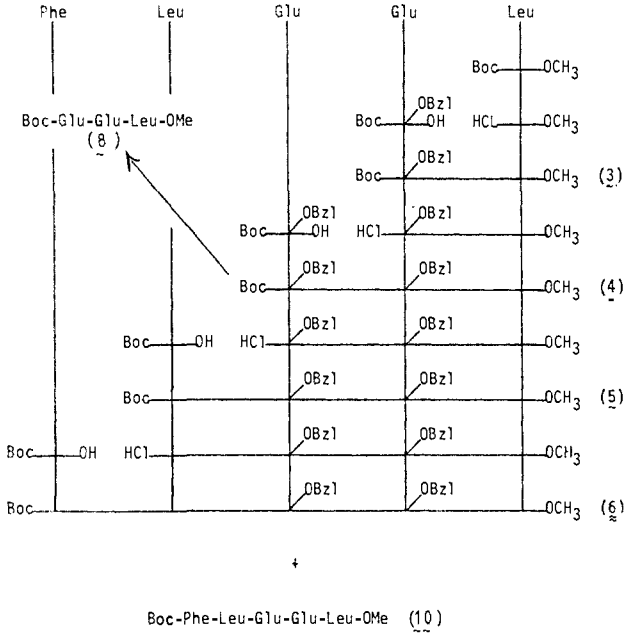

Table II. Effect of Side-Chain Structure on Extent of Carboxylation

no.	structure	rel act.,[a] %
8	Boc-Glu-Glu-Leu-OMe*	107
11	Boc-Glu-Asp-Leu-OMe*	11
12	Boc-Asp-Glu-Leu-OMe*	11
13	Boc-Asp-Asp-Leu-OMe*	<1
23	Boc-Aad-Aad-Leu-OMe	<1
31	Boc-Gln-Gln-Leu-OMe*	<1
35	isovaleryl-Met-Glu-Leu-OMe	6
36	Boc-Glu-Ala-Glu-OMe	4

[a] All peptides were incubated at a concentration of 1 mM with vitamin K-H_2 as the source of the vitamin. The activity is expressed as the percent of the incorporation into 1 mM pentapeptide 2 utilizing the same microsomal preparation. Values are means of duplicate incubations differing by less than 10%. Those peptides marked with an asterisk were tested at 0.25 mM as well as at 1 mM; the relative activities determined at 0.25 mM vs. pentapeptide 2 as standard were comparable with relative activities obtained at 1 mM.

Results

Synthesis of Peptides. Most peptides utilized in this study were synthesized in solution by the methods outlined in Chart I. Synthetic details are provided for the preparation of peptides **3–6**, **8**, and **9** under Experimental Section. The δ-benzyl-L-α-aminoadipate was synthesized by the method of Kubota et al.[18] and converted to the *N*-(*tert*-butyloxycarbonyl) derivative **49**. The remaining peptides listed in Table I were prepared in a similar manner. All compounds were shown to be homogeneous by thin-layer chromatography (TLC) and were characterized by nuclear magnetic resonance (NMR) spectrometry, amino acid analysis, or microanalysis. Melting points of benzyl ester precursors are given in column 3 of Table I. These peptides were shown to be homogeneous by TLC and were characterized by NMR and conversion to the debenzylated products. Peptides **28–34** and **41** were prepared by a solid-phase synthesis procedure as previously described.[7]

Biological Results. The peptides listed in Table I were incubated with preparations of the microsomal vitamin K dependent carboxylase obtained from rats kept on a diet deficient in vitamin K as previously described.[8] The extent of incorporation of ^{14}C-labeled bicarbonate was determined by precipitation of the incubation mixture with trichloroacetic acid and measuring $^{14}CO_2$ incorporation into the soluble peptides in the presence of added vitamin K. Control experiments were performed in which vitamin K was omitted. The extent of carboxylation of peptides **7–41**,

Table III. Effect of D-Amino Acid Residues on Extent of Carboxylation

no.	structure	rel act.,[a] %
8	Boc-Glu-Glu-Leu-OMe*	107
14	Boc-Glu-D-Glu-Leu-OMe	<1
15	Boc-D Boc-D-Glu-D-Glu-Leu-OMe	<1
16	Boc-D-Glu-Glu-Leu-OMe	<1
17	Boc-Glu-Glu-D-Leu-OMe	19
19	Boc-Leu-Glu-Glu-Leu-OMe	77
18	Boc-D-Leu-Glu-Glu-D-Leu-OMe	19
19	Boc-D-Leu-Glu-Glu-Leu-OMe	55
10	Boc-Phe-Leu-Glu-Glu-Leu-OMe	24
20	Boc-Phe-Leu-Glu-D-Glu-Leu-OMe	<1
21	Boc-Phe-Leu-D-Glu-D-Glu-Leu-OMe*	<1
22	Boc-Phe-Leu-D-Glu-Glu-Leu-OMe*	<1

[a] Relative activity is defined in footnote *a* of Table II.

Table IV. Effect of Chain Length and Sequence on Extent of Carboxylation

no.	structure	rel act.,[a] %
2	H-Phe-Leu-Glu-Glu-Leu-OH	100
42	Boc-Glu-OBzl[b]	5
43	Boc-Glu-NHBzl[b]	<1
7	Boc-Glu-Leu-OMe*	<1
8	Boc-Glu-Glu-Leu-OMe*	107
32	H-Leu-Glu-Glu-Leu-OH*	8
9	Boc-Leu-Glu-Glu-Leu-OMe*	77
10	Boc-Phe-Leu-Glu-Glu-Leu-OMe*	24
28	C_2H_5CO-Phe-Leu-Glu-Glu-Leu-OH	149
29	C_6H_5CO-Phe-Leu-Glu-Glu-Leu-OH	27
30	H-Phe-Glu-Leu-Glu-Leu-OH	<1
37	Boc-Pro-Leu-Glu-Ala-Glu-OMe	19
38	Ac-Pro-Leu-Glu-Ala-Glu-OMe	25
39	Boc-Leu-Glu-Ala-Glu-OMe	46
40	H-Phe-Glu-Ala-Leu-Glu-Ser-Leu-OH	6

[a] Relative activity is defined in footnote *a* of Table II. [b] Value taken from data reported in ref 12.

Table V. Effect on Carboxylation of the Amino Acid Preceding the Glu-Glu Sequence

no.	structure	rel act.,[a] %
9	Boc-Leu-Glu-Glu-Leu-OMe*	77
24	Boc-Ala-Glu-Glu-Leu-OMe	19
25	Boc-Gly-Glu-Glu-Leu-OMe[c]	6
2	H-Phe-Leu-Glu-Glu-Leu-OH	100
33	H-Phe-Ala-Glu-Glu-Leu-OH	72
34	H-Phe-Gly-Glu-Glu-Leu-OH	4
10	Boc-Phe-Leu-Glu-Glu-Leu-OMe	24
27	Boc-Phe-Gly-Glu-Glu-Leu-OMe	25
40	Ac-Phe-Ala-Glu-Glu-Leu-OMe	N[b]
45	propionyl-Phe-Leu-Glu-Glu-Leu-OMe	N
46	propionyl-Phe-Ala-Glu-Glu-Leu-OMe	N
47	Ac-Phe-Gly-Glu-Glu-Leu-OMe	N
48	propionyl-Phe-Gly-Glu-Glu-Leu-OMe	N

[a] Relative activity is defined in footnote *a* of Table II. [b] N = compound not assayed due to insolubility in media. [c] These compounds were tested at a concentration of 4.5 to 5.0 mM and the data normalized to the 1 mM reference concentration.

given in Table II–V, refers only to carboxylation that is dependent on vitamin K, and each value represents the percentage carboxylation measured relative to the standard pentapeptide **2**, which was assayed at the same time.

Table II shows the results obtained when the glutamic acid residues in a substrate are modified. During this work, tripeptide **8** was found to be a substrate for the carboxylase and is carboxylated under standard conditions, as well as pentapeptide **2**. Analogues of tripeptide **8** were prepared in which the distance between the peptide backbone and the side-chain carboxyl groups were lengthened (homoglutamyl or α-aminoadipic acid analogue **23**) or shortened (aspartyl analogue **13**). Neither analogue was carboxylated and neither inhibited carboxylation of pentapeptide substrate **2** at 4:1 and 10:1 ratios, respectively. Replacement of the carboxylate group by a carboxamide group, as in the glutamine analogue **31**, also led to a totally inactive compound. Peptides **11** and **12** were carboxylated about one-tenth as effectively as pentapeptide **2**. The site of carboxylation has not been established but is assumed to be at the glutamic acid position in each peptide.

The specificity of the carboxylase for L-amino acid residues was examined in several peptide substrates (Table III). Peptides containing one or two D-glutamyl residues were not carboxylated. It is interesting that only one D-glutamyl group appears sufficient to suppress carboxylation. When the amino acids adjacent to the Glu-Glu unit were replaced with the corresponding D-amino acids, e.g., peptides **17–19**, the extent of carboxylation was reduced but not eliminated.

The effect of peptide chain length on carboxylation also was examined (Table IV). The α-benzyl ester derivative, Boc-Glu-OBzl (**42**), is carboxylated at high substrate concentrations, but several closely related derivatives of glutamic acid, such as Boc-Glu-NHBzl (**43**), are not carboxylated,[12] and neither is dipeptide **7**. However, substantial carboxylation was observed in the protected tripeptide **8**. Further extension of substrate chain length did not produce substantially better substrates.

Protection of the peptide chain termini, particularly at the N terminus, gave improved substrates. Thus, protected tetrapeptide **9** is a much better substrate than the unprotected derivative **32**. Similarly, the *N*-propionyl pentapeptide **28** is carboxylated about 50% more than pentapeptide **2**. However, the *N*-benzoyl analogue **29** and the Boc derivative **10** are both poorer substrates than **2** and **28**, respectively, which may indicate some steric constraint about this position in the peptide chain. The *N*-propionyl pentapeptide methyl ester, propionyl-Phe-Leu-Glu-Glu-Leu-OMe (**43**), which might be expected to be an even better substrate than the corresponding acid **28**, was not sufficiently soluble in the assay media to be tested as a substrate.

Five γ-carboxyglutamyl residues in prothrombin are preceded by leucine or phenylalanine, and the effect of this amino acid on substrate specificity was examined (Table V). The data show that replacement of leucine by alanine or glycine in both the protected tetrapeptide series (**9**, **24**, and **25**) and the unprotected pentapeptide series (**2**, **33**, and **34**) gave significantly poorer substrates. These results indicate that the isobutyl group of leucine in this position of the peptide substrate increases the extent of carboxylation. However, it is not possible to determine whether this is caused by increased affinity of the substrate for the enzyme, by altered substrate conformations, or by changes in the distributional properties of these analogues. Several additional analogues shown in Table V (**45–48**) were synthesized to distinguish between these possibilities but could not be evaluated because of insolubility in the assay media.

Peptides containing two glutamic acids in nonadjacent positions were tested. Peptide **41** (homologous with residues **29–35** of the bovine prothrombin precursor) had low but significant activity. Peptides **37–39** correspond to analogues of the amino acid sequence 15–19 in chicken osteocalcin.[13] These data plus the results obtained with the protected amino acid **42**, Boc-Glu-OBzl, establish that adjacent glutamic acid residues are not an absolute requirement for carboxylation to occur.

Inhibition Studies. All compounds listed in Table I were tested as potential inhibitors of the carboxylation of pentapeptide. The protocol for these experiments has been described.[6] Most compounds were tested at concentrations four times the substrate concentration, and in some cases up to ten times the substrate concentration was used. Under these conditions, no inhibition of carboxylation was detected.

Discussion

Most studies of the vitamin K dependent carboxylase have followed the incorporation of $H^{14}CO_3^-$ into the endogenous microsomal precursors of the vitamin K dependent clotting factors or have assessed the conversion of these proteins to biologically active prothrombin. Graves and Grabau[14] have isolated prothrombin precursors from cultured cell homogenates and demonstrated the presence of at least three major prothrombin precursor species. The levels of these proteins can be altered by administration of vitamin K, but a detailed understanding of the precursor–product relationship between the various species is still lacking. It is not known which species is the substrate for the carboxylase in the in vivo system.

These uncertainties prompted a search for a simpler substrate. We have demonstrated that the pentapeptide Phe-Leu-Glu-Glu-Val (**1**), which is analogous to residues 5–9 of the presumed bovine precursor, is a substrate for the carboxylase, and Houser et al.[9] subsequently demonstrated that similar peptides with a carboxyl-terminal Leu or Ile residue also are substrates and are carboxylated to the same extent as pentapeptide **1**. These results suggested that modification of this position of the peptide does not significantly alter the extent of carboxylation of adjacent glutamyl residues. Relatively high concentrations of pentapeptides are required for maximum activity, and the peptide substrate most studied, Phe-Leu-Glu-Glu-Leu (**2**), has an apparent K_m of about 4 mM.[8] Pentapeptide **2** is carboxylated at a slower initial rate than the endogenous precursor proteins, and the presence of peptide substrates studied previously has not effect on carboxylation of endogenous substrates.[8]

These results indicated that the peptide substrate **2** is not carboxylated as efficiently as endogenous protein substrates. This could be caused by any of several factors. The peptide may not have the proper distributional properties to reach the enzyme effectively or it may lack the proper affinity or conformation for either binding or subsequent carboxylation steps.

To distinguish between these possibilities we initiated studies of the substrate specificity of rat liver vitamin K

(12) Finnan, J. L.; Goodman, H. L.; Suttie, J. A. In ref 4, pp 480–483.

(13) Carr, S.; Biemann, K.; Hauska, P., 1980, personal communication. The correct 15–19 sequence is -Pro-Ile-Gla-Ala-Gln-.

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dependent carboxylase. Our results are consistent with the following conclusions: (1) the carboxylase is specific for L-glutamyl residues (L-aspartyl, L-homoglutamyl (Aad), glutamine, and D-glutamyl units are not carboxylated), (2) tri- through pentapeptides are better substrates than mono- or bis(amino acid) derivatives, and (3) adding hydrophobic substituents to the N terminus of the peptide produces a better substrate, provided the substituent is not too bulky. The propionyl pentapeptide derivative 28 is the best substrate we have tested.

The peptide sequence around the carboxylated residue may be an important factor in determining the extent of carboxylation. The best evidence for this is the effect of decreasing steric bulk on the amino acid preceding the Glu-Glu unit. In two series of good substrates, we find that replacing the isobutyl group (Leu) by a proton (Gly) diminishes carboxylation 13- and 30-fold (Table V). These data suggest that the Leu^6 position in the prothrombin precursor is a point of recognition or binding by the carboxylase. The *tert*-butyloxycarbonyl group of Boc-Glu-Glu-Leu-OMe also provides a hydrophobic group preceding the Glu-Glu unit, and 5 of 10 Glu residues carboxylated in prothrombin are preceded by a Leu or Phe.

Finnan and Suttie showed that H-Phe-Gla-Glu-Leu-OH is formed from pentapeptide 2 and only a small amount of the dicarboxylated peptide (Gla-Gla) was formed.[10] It is not known why pentapeptide 2 is carboxylated only at one glutamic acid position while endogenous proteins are carboxylated more than once. The fact that the -Asp-Glu- peptide 12 is carboxylated establishes that carboxylation in low-molecular-weight peptides is not suppressed completely by an acidic residue preceding Glu. A possible explanation for the predominant formation of H-Phe-Leu-Gla-Glu-Leu-OH is that this product is a poor substrate for the carboxylase relative to pentapeptide 2 because of its decreased lipophilicity. Experiments to test this hypothesis are in progress.

In summary, low-molecular-weight peptides corresponding to prothrombin precursor sequences 5–9 and 29–35 are carboxylated by vitamin K dependent carboxylase but, apparently, not as efficiently as are the endogenous protein substrates. Thus, while the carboxylase selects certain structural features of the substrate such as a -Leu-Glu- sequence or a structural equivalent of this structural unit, it appears that additional structural features contributing to efficient carboxylation are required and remain to be identified. It is possible these will be found in peptides with longer primary sequences. Alternatively, the tertiary structure of the endogenous protein or its subcellular location may be the critical factors regulating efficient carboxylation by the enzyme.

Experimental Section

Melting points were determined on a Fischer-Johns melting point apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were recorded on Varian Model EM-390 and Bruker Model HX90E pulse Fourier transform NMR interfaced with a Nicolet 1080 computer disk unit and Bruker WH 270 spectrometers. Chemical shifts were reported as δ units relative to Me_4Si as an internal standard. Amino acid analyses were determined on a Durrum D500 amino acid analyzer following hydrolysis in degassed 6 N HCl at 100 °C for 24 h.[15] Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN. Analytical thin-layer chromatography was carried out using precoated TLC plates (silica gel 60F-254, catalog no. 5765, E. Merck) and polygram plastic sheets (Silg/UV_{254}, Brinkman). Spots were visualized by ultraviolet light, ninhydrin, and chlorox-tolidine reagent. TLC solvents used are given in the legend to Table I. L-Aminoadipic acid was purchased from Vega-Fox, Inc., Tucson, AZ.

General Procedure A. Removal of the *tert*-Butoxycarbonyl Group. The Boc amino acid or peptide ester (1 mmol) was dissolved in 4 N HCl in dioxane (about 5 mL) and the solution stirred at room temperature for 45–60 min. After excess reagent was removed under reduced pressure, the residue was reevaporated twice from anhydrous ether and then dried over potassium hydroxide in a dessicator in vacuo (oil pump) for at least 3 h. It was not characterized unless stated otherwise.

General Procedure B. Coupling Reactions via DCC-HOBt. Amino acid ester or peptide ester hydrochloride (1 mmol) was dissolved in methylene chloride and cooled in an ice bath with stirring. *N*-Methylmorpholine (1 mmol) was added. After 5–10 min, the Boc amino acid (1 mmol) and HOBt (1.1 mmol) were added, followed by a solution of DCC (1 mmol) in methylene chloride. The reaction was carried out at 0 °C for 5 h, at 4 °C overnight (12–14 h, unless otherwise specified), and at room temperature for 3–5 h. The reaction mixture was filtered to remove DCU, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate, washed successively with water, saturated $NaHCO_3$ (3 times), water, 1 N citric acid (3 times), and water, and then dried onver anhydrous sodium sulfate. It was then recrystallized or purified by chromatography.

General Procedure C. Catalytic Hydrogenolysis. The Boc amino acid ester or peptide ester was dissolved in a suitable amount of *tert*-butyl alcohol, and 10% palladium on charcoal as added. The system was purged with nitrogen for 3 min and then a moderately rapid stream of hydrogen was passed through the reaction mixture for 5.5–8 h. The mixture was passed through a column of Celite 545 and the eluant evaporated to dryness. The residue was triturated with ether and then purified by recrystallization or chromatography.

***N*-(*tert*-Butoxycarbonyl)-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (3).** *N*-(*tert*-butoxycarbonyl)-γ-benzyl-L-glutamic acid (1.68 g, 5 mmol), leucine methyl ester hydrochloride (0.91 g, 5 mmol), *N*-methylmorpholine (0.55 mL, 5 mmol), HOBt (0.84 g, 5.5 mmol), and DCC (1.03 g, 5 mmol) were reacted in 25 mL of methylene chloride according to procedure B. The crude product (2.20 g) was recrystallized from a mixture of 8 mL of ethyl acetate and 60 mL of Skellysolve A to yield 2.14 g (92.2%) of pure 3: mp 69 °C; ^1H NMR ($CDCl_3$) δ 0.92 (d, 6 H, J = 5.6 Hz, Leu 2 δ-CH_3), 1.44 (s, 9 H, Boc), 1.64 (m, 3 H, Leu β-CH_2, γ-CH), 2.12 (m, 2 H, Glu β-CH_2), 2.43 (m, 2 H, Glu γ-CH_2), 3.72 (s, 3 H, Leu-OCH_3), 4.47–4.71 (m, 2 H, Leu α-CH, Glu α-CH), 5.14 (s, 2 H, benzyl CH_2), 5.27 (m, 1 H, Glu NH), 6.57 (m, 1 H, Leu NH), 7.35 (s, 5 H, benzyl aromatic). Amino acid composition: Leu, 1.10; Glu, 1.0.

***N*-(*tert*-Butoxycarbonyl)-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (4).** L-γ-*O*-Benzyl-L-glutamyl-L-leucine methyl ester hydrochloride, prepared from dipeptide 3 (0.30 g, 6.5 mmol) via procedure A, was reacted with *N*-(*tert*-butoxycarbonyl)-L-γ-*O*-benzylglutamic acid (0.219 g, 6.5 mmol), *N*-methylmorpholine (0.715 mL, 6.5 mmol), HOBt (7.15 mmol), and DCC (0.134 g, 6.5 mmol) following procedure B. The crude product was recrystallized from 4 mL of ethyl acetate and 24 mL of Skellysolve B to give pure 4: yield 354.0 mg (79.6%); mp 87–88 °C; ^1H NMR ($CDCl_3$) δ 0.91 (d, 6 H, J = 4.6 Hz, Leu 2 δ-CH_3), 1.25–1.69 [m, 12 H, Leu γ-CH_2, α-CH, includes δ 1.42 (s, 9 H, Boc)], 1.88–2.23 (br m, 4 H, Glu β-CH_2), 2.44–2.55 (m, t, 4 H, 2 Glu γ-CH_2), 3.70 (s, 3 H, Leu-OCH_3), 4.0–4.26 (m, 1 H Leu α-CH), 4.40–4.69 (m, 2 H, 2 Glu α-CH), 5.12–5.25 [m, 5 H, Glu NH, includes δ 5.12 (s, 4 H, 2 benzyl CH_2)], 6.76 (d, 1 H, J = 8.0 Hz, Leu NH), 7.02 (d, 1 H, J = 7.4 Hz, Glu^2 NH), 7.34 (s, 10 H, 2 benzyl aromatic). Anal. ($C_{36}H_{39}N_3O_{10}$) C, H, N.

***N*-(*tert*-Butoxycarbonyl)-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (8).** Protected tripeptide 4 (3.0 g, 4.38 mmol) and 10% palladium on carbon (0.995 g) were suspended in *tert*-butyl alcohol as described in general procedure C for removal of the *O*-benzyl groups. The solution was filtered and evaporated to dryness to give tripeptide 8 (2.03 g, 90%): mp 125–127 °C; NMR

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(methanol-d_4) δ 0.91 (br, 6 H, Leu δ-CH_3), 1.27–14.7 [m, 12 H, Leu β-CH_2, γ-CH, includes δ 1.45 (s, 9 H)], 1.63–1.69 (br, 2 H, 2 Glu β-CH_2), 1.97–2.14 (br q, 2 H, 2 Glu β'-CH_2), 2.41 (t, 4 H, J = 7.0 Hz, 2 Glu γ-CH_2), 3.68 (s, 3 H, Leu-OCH_3), 3.92–4.44 (m, 3 H, 3 Glu α-CH), 7.66 (d, 1 H, J = 3.0 Hz, NH). Anal. ($C_{22}H_{27}N_3O_{10}$) C, H, N.

***N*-(*tert*-Butoxycarbonyl)-L-leucyl-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (5).** Tripeptide **4** (8.2 g, 12 mmol) was converted to the amine hydrochloride in 85 mL of 4 N hydrochloric acid in dioxane via procedure A. This was dissolved in methylene chloride (120 mL) and converted to tetrapeptide **5** by reaction with *N*-methylmorpholine (0.55 mL, 5 mmol), *N*-(*tert*-butoxycarbonyl)-L-leucine hemihydrate (1.246 g, 5 mmol), HOBt (0.842 g, 5.5 mmol), and DCC (1.032 g, 5 mmol) following general procedure B. The crude product was crystallized from a mixture of ethyl acetate (20 mL) in hexane (100 mL) to give pure tetrapeptide **5** (3.58 g, 90%): mp 164–166 °C; ^{1}H NMR ($CDCl_3$) δ 0.92 (d, 12 H, J = 4.6 Hz, 2 Leu 2 δ-CH_3), 1.39 (s, 9 H, Boc), 1.54–1.74 (m, 6 H, 2 Leu β-CH_2, γ-CH), 1.97–2.27 (m, 4 H, 2 Glu β-CH_2), 2.44–2.61 (m, 4 H, 2 Glu γ-CH_2) 3.69 (s, 3 H, Leu-OCH_2), 4.00 (q, 1 H, J = 8.0 Hz, Leu^2 α-CH), 4.26–4.64 (m, 3 H, Leu^1, Glu^1, Glu^2 α-CH), 4.78 (d, 1 H, J = 0.4 Hz, Leu^1 NH), 5.11 (s, 4 H, 2 benzyl CH_2), 6.93 (d, 2 H, J = 8.0 Hz, 2 Glu NH), 7.33 (s, 5 H, benzyl aromatic), 7.34 (s, 5 H, benzyl aromatic). Anal. ($C_{42}H_{60}N_4O_{11}$) C, H, N.

***N*-(*tert*-Butoxycarbonyl)-L-phenylalanyl-L-leucyl-L-γ-*O*-benzylglutamyl-L-γ-*O*-benzylglutamyl-L-leucine Methyl Ester (6).** Tetrapeptide **5** (3.188 g, 4 mmol) was deprotected in 35 mL of 4 N HCl in dioxane following general procedure A. The hydrochloride salt was dissolved in 25 mL of methylene chloride and converted to pentapeptide **6** using *N*-(*tert*-butoxycarbonyl)-L-phenylalanine (1.05 g, 4 mmol), *N*-methylmorpholine (0.44 mL, 4 mmol), HOBt (0.67 g, 4.4 mmol), and DCC (0.82 g, 4 mmol) via general procedure B. The crude product was purified by chromatography over silica gel eluting with 2% methanol in chloroform to give pure pentapeptide **6** (1.3 g, 35%): mp 206–209 °C; ^{1}H NMR ($CDCl_3$) δ 0.90 (br s, 12 H, 2 Leu 2 δ-CH_3), 1.35 (s, 9 H, Boc), 1.50–1.70 (, 6 H, 2 Leu β-CH_2, γ-CH), 1.95–2.27 (m, 4 H, 2 Glu β-CH_2), 2.45–2.56 (m, 4 H, 2 Glu γ-CH_2), 2.99 (d, 2 H, J = 6.0 Hz, Phe β-CH_2), 2.69 (s, 3 H, Leu-OCH_3), 4.02–4.56 (m, 5 H, Phe, 2 Leu, 2 Glu α-CH), 4.87 (d, 1 H, J = 5.2 Hz, Phe NH), 5.09 (s, 4 H, 2 benzyl CH_2), 6.41 (d, 1 H, J = 5.0 Hz, NH), 6.88–6.96 (m, 2 H, 2 Glu NH), 7.29 (s, 5 H, Phe aromatic), 7.31 (s, 10 H, 2 benzyl aromatic), 7.64 (d, 1 H, J = 5.8 Hz, Phe NH). An additional 2.29 g (60%; mp 205–208 °C), contaminated with traces of DCU, could be recovered from other fractions and was suitable for preparation of other pentapeptide analogues. Anal. ($C_{51}H_{69}N_5O_{12}$) C, H, N.

***N*-(*tert*-Butoxycarbonyl)-L-phenylalanyl-L-leucyl-L-glutamyl-L-glutamyl-L-leucine Methyl Ester (10).** Pentapeptide **6** (250 mg, 0.265 mmol) was deprotected in 40 mL of *tert*-butyl alcohol with 10% palladium on carbon (50 mg) following general procedure C. Pentapeptide **9** was obtained in 92% yield (187 mg): mp 218–220 °C; ^{1}H NMR (Me_2SO-d_6; 270 Hz) 0.82–0.89 [m (dd), 12 H, 2 Leu 2 δ-CH_3], 1.30 (s, 9 H, boc), 1.49–1.96 (m, 10 H, 2 Glu β-CH_2, 2 Leu β-CH_2, γ-CH), 2.22–2.30 (br, 4 H, 2 Glu γ-CH_2), 2.75 (t, 1 H, J = 12.5 Hz, Phe β-CH_2), 3.00 (d, 1 H, J = 12.5 Hz, Phe β'-CH_2), 3.61 (s, 3 H, Leu-OCH_3), 4.26–4.42 (br m, 5 H, 5 α-CH), 6.97 (d, 1 H, J = 8.0 Hz, NH), 7.24 (s, 5 H, Phe aromatic), 8.05 (d, 1 H, J = 7.0 Hz, NH), 8.12 (d, 1 H, J = 7.0 Hz, NH), 8.25 (d, 1 H, J = 7.5 Hz, NH), 8.38 (d, 1 H, J = 7.0 Hz, NH). Amino acid composition: Phe, 1.00; Leu, 2.00; Glu, 2.00.

***N*-(*tert*-Butoxycarbonyl)-δ-benzyl-L-α-aminoadipic Acid (Boc-δ-Bzl-Aad; 49).** δ-Benzyl-L-α-aminoadipic acid (0.50 g, 2 mmol), Boc-ON (0.54 g, 2.2 mmol), and triethylamine (0.42 mL, 3 mmol) were added to a mixture of dioxane–water (10 mL each) as described for the synthesis of a homologue.[18] Upon workup, the Boc-Aad was obtained as a noncrystalline oil, which was used without purification in the synthesis of peptide **23**. An analytical sample was obtained by dissolving the oil in ether (10 mL) and adding dicyclohexylamine (DCHA) (0.4 mL). The solution was cooled to 4 °C for 72 h and filtered, and the DCHA salt was dried in vacuo to give 0.70 g (66%), mp 118–119 °C. Anal. ($C_{30}H_{48}N_2O_6$) C, H, N.

Biological Assay. Male 250–300 g Holtzman strain rats were placed on a vitamin K deficient diet for 7 days[16] in coprophogy preventing cages[17] and fasted 18–24 h before decapitation. The livers from six rats were excised, minced, and homogenized in two parts (w/v) ice-cold SI buffer (0.25 M sucrose, 0.025 M imidazole, pH 7.2). The homogenate was centrifuged at 10000*g* for 10 min. The supernatant was centrifuged at 105000*g* for 60 min, and the pellets were surface-washed twice with SIK buffer (SI buffer containing 0.5 M KCl). The drained pellets were stored in liquid nitrogen until ready for use. Carboxylase activity in the stored pellets was stable over at least several months. Thawed pellets were resuspended with seven to eight strokes of a loose-fitting Dounce homogenizer (Kontes, type A pestle) in sufficient buffer to give a final volume equal to that of the original postmitochondrial supernatant. One of two resuspension buffers was used, either buffer A (SIK buffer containing 1.5% Triton) or buffer B (0.25 M sucrose, 0.1 M potassium phosphate, pH 7.25, 0.5 M KCl, 2 mM phenylmethylsulfonyl fluoride, and 1.5% Triton X-100). The resuspended pellets were centrifuged at 165000*g* for 30 min to remove a small amount of insoluble material. Incubation tubes were prepared by adding 400 μL of the microsomal preparation and 20 μL of 0.5 mCi/mL $NaH^{14}CO_3$ to 100 μL of resuspension buffer containing the substrate(s) under study. The reaction was initiated by adding 10 μL of vitamin K-H_2 in ethanol (5 mg/mL) to tubes which had been briefly preincubated in a 17 °C bath. Corked tubes were vortexed briefly and incubated at 17 °C with gentle shaking. Incubations were carried out for 10–20 min, depending upon the individual experiment. At the end of the predetermined period, duplicate 200-μL aliquots of the incubating mix were squirted into 1 mL of cold 10% trichloroacetic acid for determining the incorporation of $^{14}CO_2$ into Cl_3AcOH-soluble material ("peptide assays"). Nonspecific incorporation (background) was determined by using ethanol in lieu of vitamin K-H_2. Differences in absolute disintegrations per minute incorporated with different microsomal preparations, resuspension buffers, etc. were standardized by comparing relative activities (Phe-Leu-Glu-Glu-Leu = 100%). Such comparisons were consistent from one experiment to another.

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