Evidence for the vitamin K-dependent γ -carboxylation of the first glutamic acid residue in peptide substrates containing a diglutamyl sequence

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The peptide substrate commonly used in vitamin K-dependent carboxylation, Phe-Leu-Glu-Glu-Val, has been shown, by the use of high-voltage paper electrophoresis, to be degraded from the *N*-terminus by a microsomal leucine aminopeptidase. The replacement of phenylalanine with a *N*-t-butoxycarbonyl group resulted in a tetrapeptide substrate with a blocked *N*-terminus resistant to enzymic degradation. Vitamin K-dependent carboxylation of this non-degradable substrate gave a unique carboxylated product, which was separated from microsomal protein and unchanged substrate by using DEAE-Sephadex A25 as a final step. The carboxylated product was subsequently decarboxylated in ^2HCl and analysed by using g.l.c. coupled to a mass spectrometer. This showed that only the first glutamic acid residue in the peptide substrate was carboxylated.

The post-translational carboxylation of specific glutamic acid residues to form γ -carboxyglutamic acid (Gla) residues requires vitamin K as a cofactor (Stenflo, 1974; Shah & Suttie, 1974). Thus γ -carboxyglutamic-acid-containing proteins are dependent on vitamin K for their synthesis. Such proteins include seven plasma proteins (prothrombin, Factors VII, IX and X, plus proteins C, S and Z), osteocalcin (Hauschka *et al.*, 1975; Price *et al.*, 1976), ovocalcin (Krampitz *et al.*, 1980), atherocalcin (Levy *et al.*, 1979), some renal-calculi matrices (Lian *et al.*, 1977), cartilage (Lian *et al.*, 1979), cementum (Glimcher *et al.*, 1979) and antlers (Hauschka, 1977). Vitamin K carboxylase activity has also been demonstrated in the placenta (Friedman *et al.*, 1979), pancreas (Traverso *et al.*, 1979), lung (Bell, 1979), kidney and spleen (Buchthal & Bell, 1979).

Vitamin K-dependent carboxylase activity was measured by using microsomes (microsomal frac-

tions) prepared from vitamin K-deficient rat livers and requires reduced vitamin K, O_2 and CO_2 . The extent of carboxylation is calculated from the incorporation of ^{14}C into the substrate (endogenous prothrombin precursor or added synthetic peptide substrate; Suttie *et al.*, 1976) after the addition of $\text{NaH}^{14}\text{CO}_3$ to the assay mixture.

Analysis of the synthetic substrate after incubation has revealed multiple radioactive products (Suttie *et al.*, 1976; Rikong-Adie *et al.*, 1979), the presence of γ -carboxyglutamic acid in these products being inferred either from the loss of half the radioactivity during heating in 2M-HCl or by alkaline hydrolysis.

Petersen *et al.* (1979) found that certain alkaline hydrolysates could give false positive ninhydrin peaks emerging from the amino acid analyser column in the same position as γ -carboxyglutamic acid.

This work presents evidence for microsomal proteolytic degradation of Phe-Leu-Glu-Glu-Val and development of a non-degradable substrate. This substrate was then used in the unambiguous quantification of carboxylation and determination of the site of carboxylation where there is a diglutamyl sequence.

Abbreviations used: h.v.p.e., high-voltage paper electrophoresis; Boc, *N*-t-butoxycarbonyl; g.l.c.–m.s., g.l.c. coupled to a mass spectrometer; Gla, γ -carboxyglutamic acid.

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Experimental

Materials

Aquasol was from NEN Chemicals (Southampton); leucine *p*-nitroanilide, NADH, vitamin K and the vitamins used for the rat diet were from Sigma (London) Chemical Co. Ltd. (Poole, Dorset, U.K.); casein (fat/vitamin-low) from Fisons Scientific Apparatus (Loughborough, Leics., U.K.); ninhydrin from Pierce and Warriner (U.K.) Ltd. (Chester, Cheshire); 1,10-*o*-phenanthroline from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.); $\text{NaH}^{14}\text{CO}_3$ from Amersham International p.l.c. (Amersham, Bucks., U.K.); $^2\text{H}_2\text{O}$ (>99.75 atom% ^2H) was obtained from E. Merck (Darmstadt, Germany) and ^2HCl (approx. 38% in $^2\text{H}_2\text{O}$, >99.5 atom% ^2H) was from Fluka (Buchs, Switzerland). All other chemicals used were supplied by BDH Chemicals Ltd. (Atherstone, Warwickshire, U.K.), and the chromatography gel was purchased from Pharmacia (G.B.) Ltd. (Hounslow, Middx., U.K.).

Preparation of microsomes

Male Sprague-Dawley rats (200–250 g) were housed in individual cages with wire-mesh bottoms to reduce coprophagy and fed on a vitamin K-deficient diet adapted from that described by Mameesh & Johnson (1959). Drinking water containing 0.1% neomycin sulphate was provided to deplete gut flora and thus endogenous vitamin K biosynthesis. After 7–10 days on this diet, when plasma prothrombin levels were less than 20% of normal, the livers were removed and a microsomal fraction prepared. The liver (kept at 4°C throughout) was chopped and homogenized in 3 ml of buffer A/g of liver (buffer A was 10 mM-potassium phosphate, pH 7.5, containing 0.25 M-sucrose and 80 mM-KCl). The homogenate was spun at 10000 *g* for 10 min in a Beckman L265B ultracentrifuge and the resulting post-mitochondrial supernatant was spun at 105000 *g* for 60 min. The microsomal pellet obtained was surface-washed with buffer A and either frozen in liquid N_2 and stored at –70°C until required or resuspended in buffer A containing 0.5% Triton X-100 (2 ml of buffer A/g of liver) with a loose-fitting Dounce-type homogenizer (Sadowski *et al.*, 1976).

Vitamin K-dependent carboxylase assay

^{14}C incorporation into peptide substrates was measured. The standard assay incubation mixture comprised 0.4 ml of resuspended microsomes, 2 mM-peptide substrate, 5–10 μCi of $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity approx. 60 mCi/mmol), 50–100 μg of vitamin K quinol or 1 mM-NADH, plus 50–100 μg of vitamin K quinone; the final volume was approx. 0.5 ml.

For separation of peptide substrate (carboxylated and uncarboxylated) from microsomal constituents by h.v.p.e., $\text{NaH}^{14}\text{CO}_3$ was added at ten times the standard concentration and twice as much peptide was used in the assay to ensure easy detection of the radioactive products.

The mixtures were incubated at 27°C for 30 min, after which time the reactions were terminated with the addition of 2 ml of 10% (w/v) trichloroacetic acid to a 0.2 ml portion at 4°C. Alternatively, acetone was added (66%, v/v, final concn.) when the supernatants were used for h.v.p.e. The incubation mixes were left on ice for 30 min and the protein precipitate spun down in a bench-top centrifuge. The trichloroacetic acid supernatants were gassed with CO_2 for 45 min to remove unfixed label and samples were counted for radioactivity in an LKB-Wallac liquid-scintillation counter. The acetone supernatants were evaporated to a minimal volume before spotting on to chromatography paper for h.v.p.e.

Leucine aminopeptidase assay

The amount of leucine aminopeptidase activity present in microsomal extracts was measured by using the method of Pfeiderer (1970). The nitro-aniline released was measured by change in absorption at 405 nm. One unit of leucine aminopeptidase activity is defined as that which hydrolyses 1 μmol of leucine *p*-nitroanilide/min at 37°C and at pH 7.2.

Synthesis of peptide substrates

The pentapeptide Phe-Leu-Glu-Glu-Val and the *N*-protected tetrapeptide Boc-Leu-Glu-Glu-Val (all amino acids in the L-form) were synthesized by standard methods. The composition of the peptides was confirmed by amino acid analysis (molar ratios Phe_{1.0}Leu_{1.0}Glu_{1.98}Val_{0.98} and Leu_{1.02}Glu_{2.0}Val_{0.98} respectively).

The peptides moved as single spots on t.l.c. as detected by exposing the plates to Cl_2 and spraying with either 1% starch/KI or 0.5% ninhydrin in butanol, followed by heating. The solvent system used was butanol/acetic acid/water (2:1:1, by vol.). The peptides also moved as single spots on h.v.p.e. at pH 6.5 and gave the expected high-resolution p.m.r. spectra.

H.p.v.e.

A Shandon flat-bed h.v.p.e. instrument was operated at 3 kV for 2 h. Samples were applied to Whatman 3MM paper (57 cm × 23 cm), a buffer system composed of pyridine/acetic acid/water (100:3:897, by vol.), pH 6.5, being used. After electrophoresis, the paper was dried at room temperature and the peptide detected by spraying with a ninhydrin/cadmium reagent. This was made freshly before use by mixing 15 ml of reagent A with

85 ml of reagent B (reagent A was 5.8 g of cadmium acetate + 250 ml of acetic acid + 500 ml of water; reagent B was 1% ninhydrin in acetone). Again the paper was dried, and any ninhydrin/cadmium-positive areas that appeared were circled after 1 h, their colour and intensity being noted. The distribution of radioactivity was measured by cutting a series of 1 cm sections perpendicular to the direction of electrophoresis, which were then placed individually into scintillation vials and 1 ml of 9% (v/v) formic acid was added. These were left for 5–10 min before 15–20 ml of scintillation fluid (Aquasol) was added and the samples counted for radioactivity. This method of measuring radioactivity could be used whether or not the paper had previously been stained with ninhydrin/cadmium reagent.

Column chromatography

The standard carboxylase assay, using Boc-Leu-Glu-Glu-Val as substrate, was scaled up to approx. 20 ml. A small portion was removed after incubation, placed in trichloroacetic acid and the apparent ^{14}C incorporation into the supernatant was measured in the usual way. The remaining incubation was stopped with acetone (66%), centrifuged and the supernatant reduced in volume by gassing with N_2 . The acetone extract was made 0.3 M with respect to NH_4HCO_3 . A 1-litre gradient of 0.3–0.8 M- NH_4HCO_3 was run at 40 ml/h and one hundred 10 ml fractions were collected. The radioactivity in the fractions was measured, and those containing a radioactive peak were pooled and freeze-dried. A portion of this dried extract was then hydrolysed in 6 M-HCl (18 h, 110°C) and an amino acid analysis was performed with an LKB 4400 analyser (LKB Instruments Ltd., Croydon, Surrey, U.K.) fitted to a 2220 recording integrator.

Analysis of the radioactive peptide by g.l.c.-m.s.

A portion of the freeze-dried radioactive material from the ion-exchange column, and 300 nmol of Boc-Leu-Glu-Glu-Val as control, were treated in separate tubes containing $^2\text{H}_2\text{O}$ acidified with ^2HCl (both reagents ≥ 99.5 atom% ^2H) as previously described (Rose *et al.*, 1980). After this treatment, portions of the control were withdrawn and examined in duplicate by h.v.p.e. One 'run' was exposed to trifluoroacetic acid vapour before staining with the cadmium/ninhydrin reagent; the other run was stained directly. After freeze-drying, the samples were trifluoroacetylated and permethylated as previously described (Rose *et al.*, 1980).

G.l.c.-m.s. analysis was performed on a Carlo Erba 4161 gas chromatograph coupled to a Kratos MS50S mass spectrometer (Kratos Ltd., Manchester M31 2LD, U.K.). Samples in solution in chloroform were introduced, via the on-column

injector, on to a glass capillary column [6.5 m $\times$ 0.31 mm (internal diameter) coated with SE 30]. Helium was used as carrier gas. The column temperature was raised from 180°C to 250°C at 4°C/min. The mass spectrometer was operated in the low-resolution mode; other conditions: source temperature, 200°C; accelerating voltage, 8 kV; electron-beam energy, 58 eV. Data were acquired and processed by a DS 55 data system (Kratos Ltd.).

Results

A sample of the acetone-soluble fraction of a carboxylase assay mixture, containing approx. 50 nmol of pentapeptide, was spotted on to the paper next to a synthetic pentapeptide sample, the latter acting as a marker. After electrophoresis and spraying with the ninhydrin/cadmium reagent, one spot was visible from the synthetic peptide and three spots from the microsomal extract. This suggested that the synthetic peptide added to the carboxylase assay mixture was being degraded by a component of the microsomal system, since a blank incubation (no pentapeptide added) gave no spots at all.

In order to confirm that the three spots were hydrolysis products of Phe-Leu-Glu-Glu-Val, samples of dipeptide (Glu-Val), tripeptide (Glu-Glu-Val) and tetrapeptide (Leu-Glu-Glu-Val) were used as markers in addition to the pentapeptide (Phe-Leu-Glu-Glu-Val). It was found that di- and pentapeptide had a mobility corresponding to spot 1 (nearest the origin), tetrapeptide ran in an area corresponding to spot 2 and tripeptide ran in the spot-3 position. This result is consistent with the pentapeptide being cleaved from the *N*-terminus by the microsomal preparation. A different pattern would have been obtained had cleavage occurred from the *C*-terminus.

Further confirmation of this degradation was obtained from a time-course experiment. The microsomes were incubated in a standard carboxylase assay mixture, samples were removed at intervals, and the reaction was terminated with acetone. Samples were electrophoresed and, as predicted, the pentapeptide spot became weaker with increased incubation time in the carboxylase assay mixture, whereas spots 2 and 3 became more intense with time.

The distribution of radioactivity varied considerably, depending on the length of incubation time in the carboxylase assay and between different preparations of microsomes. The general pattern of events was that the radioactivity tended to be associated with the slower-migrating spots up to about 15 min incubation and then with spots 2 and 3 from 30 min onwards (see Fig. 1).

In a second experiment, the supernatants (trichloroacetic acid and acetone) were analysed at time

Fig. 1. Electrophoretograms from microsomal incubation showing radioactivity distribution at different times. All incubations included Phe-Leu-Glu-Glu-Val as peptide substrate. $\square$, Ninhydrin-positive areas; $\bullet$, radioactivity.

intervals, without hydrolysis, and free amino acids released by degradation were detected. The results (see Table 1) indicated that the peptide substrate appeared to be degraded from the *N*-terminus, since free phenylalanine was released first, followed by increasing amounts of free leucine and glutamic acid. Free valine could not be determined accurately as it eluted from the analyser column close to the tetrapeptide, but was seen to be present in small quantities.

The presence of leucine aminopeptidase activity was established in the microsomal extract. A microsomal suspension (10 μ l) was found to have 0.03 unit of leucine aminopeptidase activity and the peptidase was found to be inactivated by 66% acetone. 1,10-*o*-Phenanthroline at 1 mM inhibited the rate of leucine nitroanilide hydrolysis by 75% in the rat microsomal preparation. The carboxylase activity of the microsomes was inhibited by 85% at this concentration of 1,10-*o*-phenanthroline, thus it was impossible to test the effect of inhibiting the

Table 1. Degradation of Phe-Leu-Glu-Glu-Val during microsomal incubation

The sample analysed contained 100 nmol of pentapeptide. The carboxylation assay was terminated with trichloroacetic acid and the supernatant analysed without hydrolysis.

Incubation time (min)	Free amino acid released (nmol)		
	Phenylalanine	Leucine	Glutamic acid
0	—	—	—
15	27.9	9.2	11.6
30	46.6	21.4	30.6
60	67.7	37.0	66.8

leucine aminopeptidase activity on the carboxylation of the pentapeptide.

In order to prevent the digestion of the peptide substrate by leucine aminopeptidase, the *N*-protected tetrapeptide (Boc-Leu-Glu-Glu-Val) was used instead of the pentapeptide. After incubation of this tetrapeptide with the microsomal preparation, the supernatant was subjected to h.v.p.e. and found to give one spot, indicating that no degradation had occurred, the radioactive area being slightly ahead of the ninhydrin/cadmium spot (Fig. 2), indicating that the glutamic acid residues had been γ -carboxylated. In order to identify the peptide on the paper with the ninhydrin/cadmium stain, the Boc group had to be removed by using trifluoroacetic acid vapour. The electrophoresis paper was then divided into strips and counted as before to determine the distribution of radioactivity. Approx. 90% of the radioactivity applied to the paper was recovered after electrophoresis, all in the one spot. The ninhydrin-positive area was shown to be a tetrapeptide, since after elution and hydrolysis with 6M-HCl, the eluate contained leucine, glutamic acid and valine in the molar proportions 1:2.2:1.

In order to separate the carboxylated peptide from the uncarboxylated peptide on a larger scale, ion-exchange chromatography on DEAE-Sephadex A25 was used. The fractions corresponding to the single radioactive peak, eluting at 0.59 M-NH₄HCO₃, were pooled and freeze-dried. A portion of the dried extract was hydrolysed in 6M-HCl and found to contain leucine, glutamic acid and valine in the molar proportions 0.7:2:1. A total of 280 nmol of radioactive peptide was recovered in this way (80 μ mol of peptide was added to the carboxylase assay originally).

The freeze-dried radioactive peptide, and some Boc-Leu-Glu-Glu-Val as control, were then heated in acidified ³H₂O to transform, by γ -exchange and decarboxylation, any γ -carboxyglutamic acid residues to residues of [γ -³H₂]glutamic acid; residues of normal glutamic acid are not affected by this

Fig. 2. Electrophoretogram from a microsomal incubation containing Boc-Leu-Glu-Glu-Val
□, Ninhydrin-positive areas; ●, radioactivity.

procedure (Rose *et al.*, 1980). Electrophoresis of the control sample showed that the acid treatment removed the Boc group quantitatively. After N^{α} -trifluoroacetylation and NO -permethylation, the volatile derivative of the tetrapeptide samples, now containing [γ - $^2H_2$]glutamic acid wherever there had been γ -carboxyglutamic acid, were analysed by g.l.c.-m.s. The mass spectra obtained are shown in Figs. 3(a) and 3(b) for control and radioactive sample respectively.

Proceeding from low to higher mass, the spectrum of the control (Fig. 3a) exhibits a prominent ion at m/z 130. This iminium ion, $+NH(CH_3):CHCH_2CH_2CO_2CH_3$, provides composition rather than sequence data and informs us of the presence of a glutamic acid residue in a position other than N -terminal (Rose, 1979). The N -terminal leucine residue, m/z 224, is followed by a mass interval of $381 - 224 = 157$. This corresponds to glutamic acid, so the sequence Leu-Glu is thereby established. A further interval of 157 ($=538 - 381$) establishes the sequence Leu-Glu-Glu. The rest of the spectrum does not concern us here. The spectrum of the radioactive sample (Fig. 3b) differs from that of the control in having doublets for the glutamic acid-containing ions (m/z 130, 132; 353, 355; 381, 383; 510, 512; 538, 540). The N -terminal leucine residue is followed by a doublet at m/z 381, 383, which corresponds to Leu-Glu and Leu- $[\gamma$ - $^2H_2$]Glu respectively, in a ratio of approx. 1:2. The first glutamic acid residue of the tetrapeptide

therefore existed as 60–70% γ -carboxyglutamic acid before decarboxylation. A similar ratio is preserved at m/z 538, 540, showing that the second glutamic acid residue was not carboxylated to any significant extent. The doublet at m/z 130, 132 may in principle derive from both glutamic acid residues. We see that the contribution from the second glutamic acid residue, though small, is sufficient to dilute the doublet with the non- ^2H -labelled form when compared with the doublets at m/z 381, 383; 510, 512 and 538, 540. The mass spectrum leaves no doubt that the radioactive tetrapeptide sample consisted of a mixture of the sequence Boc-Leu-Glu-Glu-Val and Boc-Leu-Glu-Glu-Val in a ratio of approx. 2:1. The contamination of the carboxylated product with uncarboxylated peptide substrate is not surprising in view of the small conversion yields obtained. There would be sufficient uncarboxylated starting material to explain the results found here. Of the substrate which was carboxylated at all, Fig. 3(b) demonstrates that essentially all of it carried the carboxy group on the first of the glutamic acid residues.

Some divalent-metal ions have been shown to stimulate vitamin K-dependent carboxylation. $MgCl_2$ at 2–10 mM stimulates carboxylation by 172–296%. It is possible that this stimulation was due to carboxylation of the second glutamic acid residue in the peptide substrate. The high negative charge generated with the formation of the first γ -carboxyglutamic acid residue could perhaps be neutralized by the Mg^{2+} , enabling further carboxylation to occur. However, although Mg^{2+} stimulated incorporation of radioactivity into the Boc-Leu-Glu-Glu-Val substrate by 2.6-fold, the g.l.c.-m.s. results were similar to those obtained when no metal was present. Again the ratio glutamic acid/ $[\gamma$ - ^2H]glutamic acid in the Leu-Glu sequence was preserved in the Leu-Glu-Glu fragment, indicating that the first glutamic acid of the pair had been carboxylated. There was no significant peak at m/z 542, demonstrating that no di- γ -carboxyglutamic acid had been formed.

Conclusions and discussion

These experiments demonstrate two important points: firstly, that the incorporation of radioactivity into the peptide Boc-Leu-Glu-Glu-Val can be correlated with the carboxylation of the first glutamic acid residue, since approx. 90% of the incorporated radioactivity was recovered in this peptide after electrophoresis; secondly, that further radioactive species noted by other workers (Rikong-Adie *et al.*, 1979; Suttie *et al.*, 1976) are probably due to the degradation of the peptide substrates by leucine aminopeptidase activity.

The possibility of the formation of Boc-Leu-Glu-Glu-Val is remote, since, firstly, no detectable

Fig. 3. Mass spectrum of (a) *Leu-Glu-Glu-Val* as its N^α -trifluoroacetyl- NO -permethyl derivative, and (b) mass spectrum of the radioactive peak from the ion-exchange column

(a) The spectrum shows m/z 120–570 and was arbitrarily normalized on m/z 28 (N_2). For convenience and clarity, the conventional three-letter amino-acid code is used here to designate permethylated residues. The Boc group originally present was removed during treatment with acidified $^2\text{H}_2\text{O}$ (see the text). (b) Treatment was similar to the control (a).

radioactive spot was seen running ahead of the main product on h.v.p.e., and secondly, the NH_4HCO_3 gradient was extended to 0.8M, which was greatly in excess of that required to elute the singly carboxylated product. At most, the doubly carboxylated product could only account for 10% of the radioactivity.

These experiments show that the *N*-protection prevents the degradation of the peptide, and hence the g.l.c.-m.s. procedure provides an accurate assessment of the relative degree of carboxylation of the glutamic acid residues of the substrate.

The fact that only a small proportion of the substrate is carboxylated by the enzyme suggests that non-carboxylated peptide is the preferred substrate for the enzyme, particularly since it is present in such excess.

It was thought that the stimulation of carboxylation seen at relatively high concentrations of Mg^{2+} might have been the result of the interaction of the metal ions with the peptide, so neutralizing the negative charge and allowing the carboxylation of the second glutamic acid residue. However, this clearly was not the case, and the effect of the metal must be directed towards the carboxylase.

The fact that only the first glutamic acid in the pair is carboxylated in the substrate, whereas the homologous sequence in prothrombin contains a pair of γ -carboxyglutamic acid residues, indicates that perhaps the peptide cannot comply with the stereospecificity required for the carboxylation of both glutamic acid residues. It is noteworthy in this respect that prothrombin isolated from the plasma of warfarin-treated cattle has only one glutamic acid residue of each pair carboxylated (Esnouf & Prowse, 1977). From that study and the results described here, it would seem that the carboxylation of adjacent glutamic acid residues is relatively more difficult than that of the individual residues, which is perhaps due to the generation of a high density of negative charge. However, there may be some additional mechanism(s) to overcome this, perhaps by neutralization of the charge.

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