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Vitamin K Dependent Carboxylation: Determination of the Stereochemical Course Using 4-Fluoroglutamyl-Containing Substrate†

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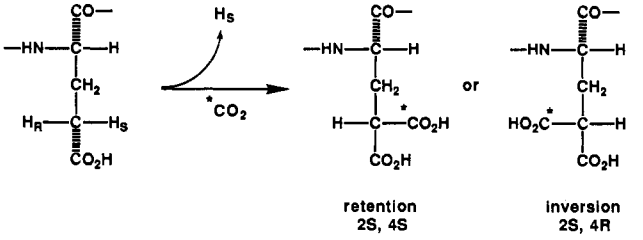
ABSTRACT: The stereochemical course of the vitamin K dependent carboxylation has been elucidated using a (4*S*)-4-fluoroglutamyl-containing pentapeptide as a substrate. The absolute configuration of the [^{13}C]-4-carboxy-4-fluoroglutamate obtained when the carboxylation was carried out with ^{13}C-labeled sodium bicarbonate, was determined after reduction of the [^{13}C]-4-carboxy-4-fluoroglutamyl residue into 4-fluoro-5,5′-dihydroxyleucine, hydrolysis, lactonization, and peracetylation. The absolute configuration at C-4 was determined to be *S* by locating the ^{13}C label in the lactone ring of the trans isomeric lactone and in the hydroxymethyl group of the cis isomer following HPLC separation of both isomers and analysis by GC/MS/MS techniques. It follows that the vitamin K dependent carboxylation occurs with inversion of configuration.

Vitamin K is the cofactor of the posttranscriptional carboxylation of glutamic residues into 4-carboxyglutamic residues which are present in blood coagulation factors, in some bone proteins, and in mineralized tissues (Suttie, 1980, 1985, 1988).

Early studies with *threo*- and *erythro*-4-fluoroglutamate-containing substrates (Dubois et al., 1983) and isotope effect determinations (Decottignies et al., 1984a,b) established that the carboxylase selectively abstracts the 4-*proS* hydrogen (Scheme I).

Scheme I: Possible Stereochemical Course of Vitamin K Dependent Carboxylation of Glutamyl Residues



In order to elucidate the stereochemistry of the reaction, it was necessary to determine the absolute configuration of the 4-carboxyglutamate obtained when ^{14}C- or ^{13}C-labeled carbon dioxide is used: a 4*R* configuration will correspond to an inversion of configuration and vice versa (Scheme I).

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A priori, the malonic nature of the 4-carboxyglutamate raises a problem: the hydrogen at C-4 can exchange rapidly with solvent, resulting in an epimerization of that center, and the loss of stereochemical information. This has been overcome by use of a substrate where the glutamic residue is replaced by an *erythro*-4-fluoroglutamic residue. This analogue is carboxylated at a reasonable rate (Dubois et al., 1983) and leads to a 4-carboxy-4-fluoroglutamic residue that cannot epimerize at C-4 during the reaction or during the purification of the carboxylated peptide. The problem of purification was crucial, especially because of the low yield of the enzymatic carboxylation of peptidic substrates. During simultaneous experiments, the carboxylation was carried out with the non-fluorinated peptide Phe-Leu-Glu-Glu-Val. In this case it has been necessary to reduce the crude product of the reaction containing the carboxylated peptide in order to avoid epimerization. Purification of the reduced peptide, i.e., containing 5,5′-dihydroxyleucine residues, proved to be troublesome and yielded products that were not pure enough to achieve safely the absolute configuration determination by mass spectrometry (vide infra). Ending the enzymatic reaction with a non-epimerizable residue allowed a thorough purification prior to processing the product to achieve the absolute configuration determination.

Experimental Procedures

Chemicals. Dicyclohexylcarbodiimide and Dowex 1-X4 were from Fluka. Diborane in tetrahydrofuran (1.81 M) was prepared according to Brown (1975). NADH, NAD^+, pyridoxal phosphate, dithiothreitol, Triton X-100, sucrose, and leucine aminopeptidase (type III CP, porcine kidney) were from Sigma Chemical Co. The syntheses of 4-fluoroglutamate and of the peptides have been described previously (Dubois et al., 1983). Vitamin K_1 was from Merck Co. $NaH^{13}CO_3$ (99%) and $NaH^{14}CO_3$ (55 mCi/mmol) were from CEA and were purified before use (Guchhait et al., 1974). Sephadex G10, Sephadex G25 and Sephadex A50 were from Pharmacia. High-performance liquid chromatography purification of diacetylated lactones was achieved using two μ-Porasil P/N27477 columns (27 × 0.33 cm) (Waters Associates) with ethyl acetate, 68.2%/hexane, 30%/propan-2-ol, 1.8% as eluent and monitoring with a Waters Associates R401 refractometer. Radioactivity was measured in an Intertechnique SL 30 scintillation counter and corrected for quenching by the double-channel method. Mass spectrometry measurements were run on a Nermag R30-10 coupled with a capillary column. Negative chemical ionization was run with ammonia.

Carboxylation of Phe-Leu-e-4FGlu-Glu-Val. Microsomes from vitamin K deficient rat livers were prepared as previously described (Decottignies et al., 1979). Buffer I was defined as follows: 0.68 g of KH_2PO_4, 4.47 g of KCl, and 8.55 g of sucrose in 100 mL (pH adjusted to 7.58 with 4 M KOH). Vitamin K was reduced with dithionite before use and dissolved in ethanol (10 mM).

The reaction was run into six 250-mL centrifuge bottles. Each bottle contained pyridoxal phosphate (6 mM) and NADH (10 mM) in buffer I (7.8 mL), vitamin K hydroquinone (10 mM) in ethanol (0.78 mL), Phe-Leu-e-4FGlu-Glu-Val (18 mM) in buffer I (7.8 mL), and 380 mg of microsomal proteins in 10% NAD (1 mM)/DTT (0.1 M)/buffer I 1/1/8; final volume 19.4 mL).

Following preincubation (5 min, 20 °C) the carboxylation was started by adding sodium bicarbonate (3.5 mM $NaH^{13}CO_3$, 0.35 mM $NaH^{14}CO_3$ [60 μCi], 3.12 mL). After 4 h at 20 °C, the reaction was stopped by cooling at 0 °C and adding 30% trichloroacetic acid (7.8 mL). The bottles were flushed with nitrogen and kept for 15 h in a desiccator over solid potassium hydroxide to eliminate CO_2 in excess. After centrifugation (5000*g*, 10 min) each pellet was washed with 10% trichloroacetic acid (10 mL and 5 mL) (total radioactivity recovered, 2.5×10^6 dpm). The solution was purified by halves on a Sephadex G25 column (80 × 2.2 cm, 0.1 N acetic acid). Radioactive fractions (255–675 mL) were pooled, lyophilized (2.5×10^6 dpm, 100%/crude product), and purified by halves on a Sephadex G10 column (75 × 2 cm, 0.1 N acetic acid). Radioactive fractions (200–500 mL) were pooled, lyophilized (1.7×10^6 dpm, 68%/crude product), and purified on a Sephadex DEAE A50 column (80 × 5 cm, acetate form). After washing with water (1 L), elution was achieved with acetic acid (2 N). Radioactive fractions (3–4.6 L) were pooled and lyophilized (1.52×10^6 dpm, 60%/crude product).

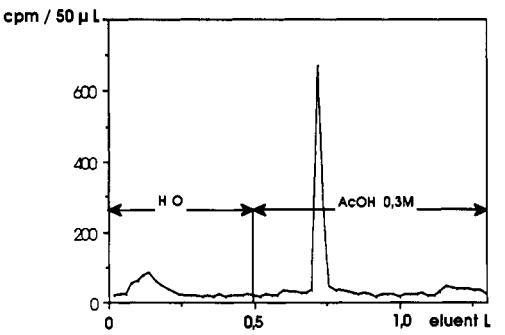


FIGURE 1: Dihydroxyleucine purification on Dowex 1-X4 column. The crude product from leucine aminopeptidase digestion was purified on a 22 × 2.7 cm Dowex 1-X4 column (OH^- form). After washing with water (0.3 L), elution was achieved with 0.3 M acetic acid and monitored by radioactivity determination on aliquots.

Reduction. The lyophilized material was thoroughly dried by evaporating toluene and suspended in dry tetrahydrofuran (10 mL) under argon. Diborane in tetrahydrofuran (1.81 M) was added in two parts (15 and 5 mL after 1 h). After 4 h, diborane in excess was destroyed by addition of methanol (0 °C). Solvents were evaporated under vacuum, and boron derivatives were thoroughly eliminated by evaporating methanol (2 × 20 mL).

Hydrolysis. The crude product was heated in 6 N hydrochloric acid (110 °C, 45 min) and dissolved in Tris-HCl buffer (0.1 M, 60 mL) after elimination of the acid under vacuum. The pH was adjusted to 8.5 (1 M Tris). Magnesium sulfate (0.1 M, 0.465 mL) and leucine aminopeptidase (1.2 mL, 1200 units) were added, and the mixture was incubated for 15 h at 40 °C. After being poured on top of a Dowex 1-X4 column (22 × 2.7 cm, hydroxide form) and washed with water (300 mL), the product was eluted with acetic acid (0.3 N) (Figure 1). Fractions eluted between 390 and 500 mL were pooled and lyophilized (383×10^3 dpm).

Synthesis of Diacetylated Lactones. The hydrolysis product was acetylated (7 h, room temperature) with acetic anhydride (1.32 mL) in methanol (40 mL). Following elimination of solvents the crude product was poured on top of Dowex 50W-X2 column (16 × 1.8 cm, H^+ form), eluted with water, and lyophilized (302×10^3 dpm, 15%/crude product). Lactonization was achieved (15 h, room temperature) in a methylene chloride/dimethylformamide mixture (4/1, 40 mL) with dicyclohexylcarbodiimide (2 M in methylene chloride, 0.8 mL). After evaporation of solvents the residue was dissolved in ethyl acetate. The organic layer was washed with icy water and dried with sodium sulfate (116×10^3 dpm). The aqueous phase (184×10^3 dpm) was evaporated and lactonized again (15 h, room temperature) in a methylene chloride/dimethylformamide mixture (4/1, 8 mL) with dicyclohexyl-

carbodiimide (2 M in methylene chloride, 0.4 mL). Treatment as above yielded a second organic extract (31 × 10^3 dpm).

Both organic extracts were pooled (5.9%/crude product) and acetylated (15 h, room temperature) in an acetic anhydride/pyridine mixture (1/1, 4 mL). After elimination of solvents, the crude product was dissolved in ethyl acetate (10 mL). Washing with brine (3 × 2 mL), drying on sodium sulfate, and evaporating solvent yielded 106.8 × 10^3 dpm (4.2%/crude product).

High-Performance Liquid Chromatography (HPLC).[1] Reference lactones were injected and their retention times determined (2 mL/min; references cis isomer 14.6 min and trans isomer 17.3 min). The radioactive product was injected in ethyl acetate, and 30-s fractions were collected (Figure 2). Fractions 12.5–15 min (cis isomer) and 15–17.5 min (trans isomer) were pooled and purified a second time (1.5 mL/min; references cis isomer 21.5 min and trans isomer 25 min). Fractions 19–23 min (7380 dpm, 0.29%/crude product, 147 ng) and 23–28 min (6220 dpm, 0.25%/crude product, 124 ng) were pooled and used for mass spectrum analysis.

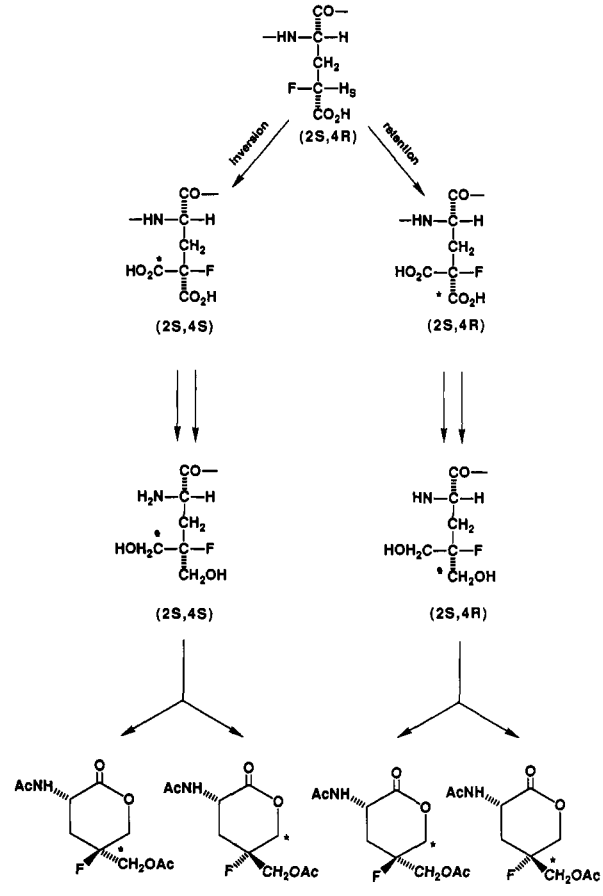
Mass Spectra Analysis. Spectrometric measurements were carried out with a triple-quadrupole mass spectrometer NERMAG R-30-10 (France). The gas chromatography was equipped with a SE52 column (25 m × 0.32 mm, chrompack). Helium (99.99% purity) was used as carrier gas (0.14 bar, corresponding to a 4 mL/min flow at 50 °C). Splitless injections (1-μL maximum volume) were used while the injector temperature was maintained at 220 °C. The oven temperature program for each injection was as follows: 70 °C isothermic 1 min; 40 deg/min up to 180 °C; 180 °C isothermic. The column was inserted directly in the ion source and the interface was maintained at 260 °C.

A modified high-pressure source was used to improve the negative ion chemical ionization (NICI) yield of isomeric deprotonated molecules $(M - H)^-$. Source operating conditions were electron energy 100 eV; emission current 70 mA; and repeller 0 V. The NICI mass spectra were recorded with a source pressure of 0.5 × 10^{-1} torr (in ammonia) with a source temperature of 150 °C. The CAD spectra of selected deprotonated molecules (or fragment anions) were obtained using argon as the collision gas in the collision cell (multiple-collision conditions) at 20 eV as collision energy (E_{lab}). The scan rate was 0.6 s for each full mass and CAD spectra recorded using a PDP 11-73 (Sidar Data System). For quantification of the $^{13}C/^{12}C$ labeling ratios (from labeled molecules) and cis/trans ratios, data acquisition and measurements of peak heights were achieved using selected transition ion monitoring software 240 → 154 (standard sample) and 205 → 154 and 205 → 155 (for labeled lactones). The ratio $(m/z\ 154)/(m/z\ 155)$ from m/z 205 was monitored for extract and for reference lactone (50 ng).

Carboxylation of Phe-Leu-Glu-Glu-Val. The peptide was carboxylated under the same conditions (vide supra) for 30 min, and the reaction was stopped by addition of powdered dry ice. The frozen crude product was lyophilized (the flask being kept at −20 °C) and reduced with diborane in dry tetrahydrofuran as previously described. The proteins were precipitated with 10% trichloroacetic acid, and the supernatant was purified on Sephadex G25 (eluent 10% acetic acid) and Sephadex G10 columns (eluent water). Hydrolysis (hydrochloric acid (6 mol/L), 110 °C, 24 h) and peracetylation

[1] Abbreviations: CAD, collision activated dissociation; HPLC, high-performance liquid chromatography; MS/MS technique, mass spectrometry/mass spectrometry technique; NICI, negative ion chemical ionization.

Scheme II: Strategy for the Determination of the Stereochemistry of the Vitamin K Dependent Carboxylation Using 4-Fluoroglutamyl-Containing Substrates



(acetic anhydride/pyridine 1/1) yielded a crude product that was purified by HPLC as above, and the fractions corresponding to the *cis*- and *trans*-lactones were collected by comparison with reference diacetylated lactones.

Results and Discussion

Strategy. The strategy for the determination of the configuration of the labeled 4-carboxy-4-fluoroglutamic residue is outlined in Scheme II. Following carboxylation of the *erythro*-4-fluoroglutamyl-containing substrate with $^{13}CO_2$, the [4-^{13}C]-4-carboxy-4-fluoroglutamic residue was reduced to a 4-fluoro-5,5′-dihydroxyleucyl residue. After hydrolysis, the resulting fluorodihydroxyleucine was lactonized and peracetylated. The cis and trans isomers were separated, and the label was localized in both isomers. If the label is located at the hydroxymethyl group of the cis isomer and in the lactone ring of the trans isomer, it follows that the absolute configuration of the [4-^{13}C]-4-carboxy-4-fluoroglutamyl residue is 4*S*, which corresponds to an inversion of configuration during carboxylation and vice versa.

Configuration Determination. Preparation and Isolation of Labeled Lactones. Pentapeptide Phe-Leu-e-4FGlu-Glu-Val was carboxylated at 4 mM, to avoid inhibition of the reaction by the substrate, for 4 h (Dubois, 1987), with 99% [^{13}C]sodium bicarbonate diluted with 10% [^{14}C]sodium bicarbonate as tracer. The proteins were precipitated with trichloroacetic acid, and the carboxylated peptide was isolated and purified using gel filtration and ion-exchange chromatography. Reduction was achieved with an excess of diborane in dry tetrahydrofuran for 4 h according to the optimal conditions (Kaminsky, 1981). These conditions ensure a good compromise between the reduction of the malonic moiety which yields the 4-fluorodi-

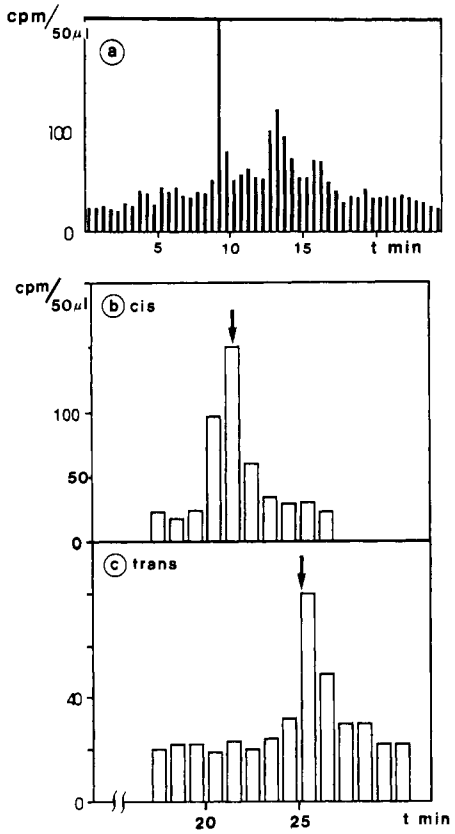
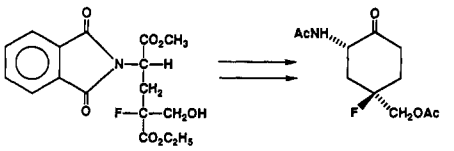


FIGURE 2: Purification of cis and trans isomeric fluorolactones. The separation (part a) and the purification (parts b and c) were achieved using two coupled μ-Porasil columns (0.33 × 25 cm, eluent ethyl acetate/hexane/propan-2-ol 68.2/30/1.8). Separation (part a): The crude product was dissolved in ethyl acetate (500 μL), injected (250 μL), and eluted at 2 mL/min (reference *cis*- and *trans*-lactones were eluted at 14.6 and 17.3 min). Fractions 26–30 (cis) and 31–35 (trans) were pooled and purified. Purification (part b and c): Both cis and trans fractions were dissolved in ethyl acetate (200 μL) and injected. Elution was achieved at 1.5 mL/min (reference *cis*- and *trans*-lactones were eluted at 21.5 and 25 min as indicated by vertical arrows). Fractions 20–23 (cis) and 24–28 (trans) were analyzed by mass spectrometry.

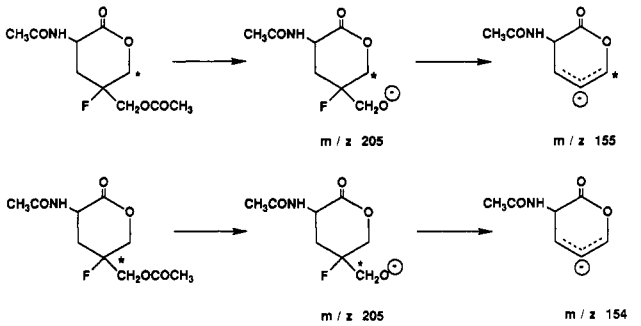
hydroxyleucine and the reduction of the peptidic bonds which lowers the yields of hydrolysis and the recovery of 4-fluorodihydroxyleucine. With Dubois et al. having noticed that 4-fluorodihydroxyleucine was unstable when treated at low pH and high temperature for a long period (Dubois, 1987; Dubois et al., 1991), an enzymatic hydrolysis of the carboxylated peptide was attempted. Leucine aminopeptidase seemed to be well adapted for that hydrolysis, apart from the optimal pH 9 required for activity. We checked that no detectable racemization at the α-carbon of *erythro*-4-fluoroglutamic acid residue, prejudicial to the stereochemistry determination, occurred during incubation for 24 h at pH 9. The yield of enzymatic hydrolysis was significantly improved when the reduced peptide was submitted to a very short acidic hydrolysis before treatment with leucine aminopeptidase.[2] Fluorodihydroxyleucine was isolated by ion exchange chromatography (Figure 1). In contrast with dihydroxyleucine that lactonized almost spontaneously under acidic conditions (Bory et al., 1979), 4-fluorodihydroxyleucine proved to be reluctant to lactonization. The amino group was selectively acetylated with acetic anhydride in methanol, and the lactonization was achieved with dicyclohexylcarbodiimide. The hydroxyl group was then acetylated with acetic anhydride in pyridine, and the separation of the cis and trans isomers was carried out using HPLC (Figure 2). The cis isomer (147 ng) that was eluted before the trans isomer (124 ng) had previously been identified by chemical correlation with (2*S*,4*S*,2*R*,4*R*)-*N*-phthalyl-4-fluoro-4-(hydroxymethyl)glutamic acid, α-methyl, δ-ethyl diester (analyzed by X-ray crystallography) (Dugave et al., 1991) (Scheme III).

[2] During the reduction of the carboxyglutamic residues with diborane in tetrahydrofuran, some of the carbonyl groups of the peptide bonds are reduced to methylene groups (Kaminsky, 1981), and the resulting modified peptides may not be substrates of the leucine aminopeptidase. A short preliminary acidic hydrolysis may yield smaller peptides that do not contain the reduced peptidic bonds and can thus be cleaved by leucine aminopeptidase, improving thus the recovery of the fluorodihydroxyleucine up to 30%.

Scheme III: Structure of (2*S*4*S*,2*R*4*R*)-*N*-Phthalyl-4-fluoro-4-(hydroxymethyl)glutamic acid, α-Methyl, δ-Ethyl Diester (the 2*S*,4*S* Isomer Has Been Represented) and Correlation with the *cis*-Lactone Isomer



Scheme IV: Location of Label by MS/MS Spectrometry Analysis of ^{13}C-Labeled Lactones



Location of the Label in the Lactones. Preliminary studies revealed that with such low amounts of lactones, which were still contaminated with impurities, in spite of a very thorough purification, normal mass spectrometry techniques were not adapted, and gas chromatography coupled with MS/MS mass spectrometry was required (see Appendix). Molecular ions were produced in high yield under negative ion chemical ionization (NICI), and their analysis was performed in a triple-quadrupole spectrometer.

Preliminary studies was unlabeled lactones (see appendix) showed that *m/z* 154 ion, corresponding formally to the loss of the hydroxymethyl chain and of hydrogen fluoride, could be used to locate the label. This ion was generated predominantly by decomposition of *m/z* 204 ion. Upon selection of the corresponding *m/z* 205 ion from ^{13}C-labeled lactones as precursor by the first quadrupole and monitoring *m/z* 154 and *m/z* 155 ions by multiple ion detection in the third quadrupole of the mass spectrometer, it was possible to locate the label in the side chain (formation of *m/z* 154) or in the lactone ring (formation of *m/z* 155) (Scheme IV).

The collision activated dissociation (CAD) spectra of *m/z* 205 ions derived from both ^{13}C-labeled isomeric lactones and the corresponding spectrum of the *m/z* 204 ion derived from unlabeled lactones were recorded under the same conditions (Figure 3). It appeared that in spite of the different purification steps, including the gas chromatography column introduction of the samples into the mass spectrometer, both isomeric lactones were contaminated by impurities that gave rise to extra signals in the CAD spectra of *m/z* 205 ions.

The CAD spectra of the *m/z* 205 ion of the *cis*-lactone isomer (Figure 3a), exhibited a peak at *m/z* 154 and no de-

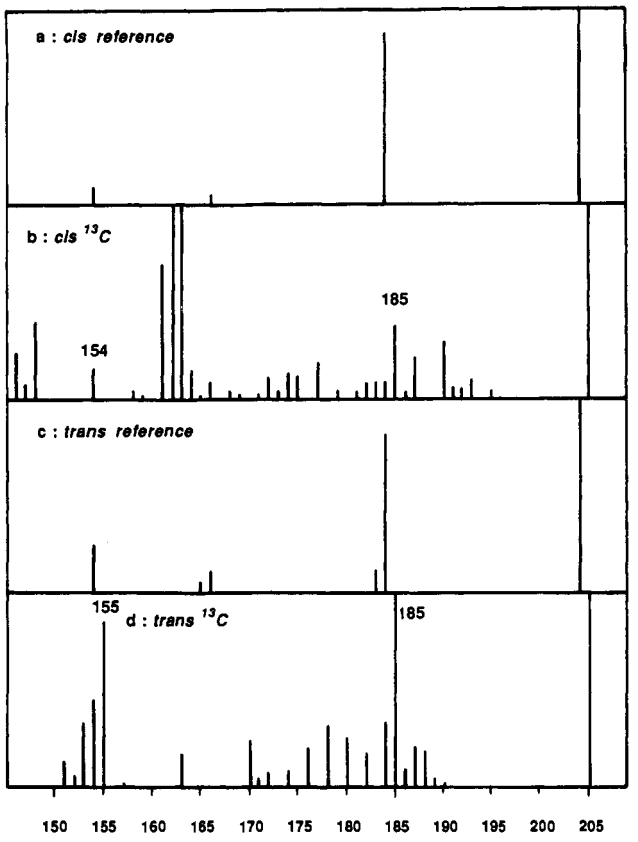


FIGURE 3: GC/MS/MS analysis of ^{13}C-labeled lactones. CAD of m/z 204 ion (reference samples a, cis isomer, and c, trans isomer) and m/z 205 ion (^{13}C-labeled samples b, cis isomer, and d, trans isomer).

tectable signal at m/z 155 whereas ions were detected at m/z 154 and m/z 155 in the corresonding spectrum of the trans isomer.

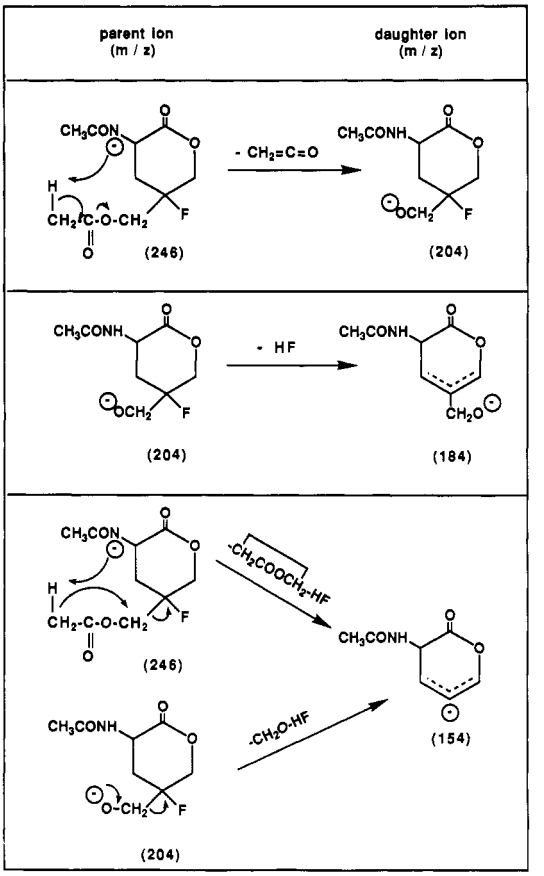
Considering the accuracy observed with such low amounts of product (very low ionic currents), the m/z 154/m/z 185 signal intensity ratio of the cis-labeled lactone and the m/z 155/m/z 185 signals ratio of the trans-labeled lactone (corresponding to the loss of CH_2O + HF) were of the same order of magnitude as the corresponding ratios (m/z 154/m/z 184) of the cis and trans reference lactones.

The presence of the m/z 154 alone in the spectrum of the cis isomer proved that no epimerization at C-4 occurred during the generation and isolation of the lactones. Both isomeric lactones having the same origin, the trans isomer must also be optically pure and should have given rise to a m/z 155 ion alone. The observed m/z 154 signal was thus likely due to contaminants that have not been removed during the purification process.

This established that the ^{13}C label was located in the acetoxymethyl side chain of the cis isomer and in the lactone ring of the trans isomer. It followed that the absolute configuration of the [4-^{13}C]-4-carboxy-4-fluoroglutamyl residue produced by carboxylation of the [2*R*,4*R*]-4-fluoroglutamyl-containing substrate was 4*S*.

As mentioned in the introduction, the determination of the configuration at C-4 was attempted earlier in the case of the carboxylation of Phe-Leu-Glu-Glu-Val with ^{13}C-labeled bicarbonate (Fourès, 1984). In that case, post-carboxylation epimerization at C-4 was possible, excluding preliminary purification of the carboxylated peptide. It has thus been necessary to freeze the crude reaction product with dry ice, in order to slow down epimerization at C-4, and to lyophilize at low temperature before reduction of the 4-carboxyglutamic

Scheme V: Mechanism of Decomposition of Ions Generated by NICI of Acetylated Lactones



residues to 5,5′-dihydroxyleucine with diborane. The crude product was hydrolyzed with 6N hydrochloric acid and acetylated. HPLC separation, monitored by detection of radioactive lactones added as tracer, afforded two fractions corresponding to the cis and trans isomeric lactones. However, in spite of the HPLC separation, both fractions were contaminated with impurities.

The location of the label was attempted by mass spectrometry using MS/MS technique, under electron impact ionization. Preliminary experiments with reference lactones revealed that the ion at m/z 156 which was produced by decomposition of the molecular ion at m/z 229 and corresponded to the loss of the acetoxymethyl side chain was suitable for the location of the ^{13}C (Scheme V): upon selection of the molecular ion at m/z 230 derived from ^{13}C-labeled lactones, an ion at m/z 157 would correspond to a label located in the lactone ring and an ion at m/z 156 would correspond to a location of the label in the acetoxymethyl side chain.

The HPLC fraction corresponding to the trans isomer could be analyzed by mass spectrometry (electron impact), and the decomposition of the molecular ion at m/z 230 yielded an ion at m/z 157, suggesting that the ^{13}C label was located in the lactone ring. The fraction containing the cis isomer was not pure enough for MS analysis and could not be analyzed to confirm the result observed with the trans isomer. Attempts to reproduce this experiment in order to improve the purification failed, presumably at the step of the reduction of the crude incubation mixture.

The conclusion drawn from the analysis of the trans isomeric lactone derived from Phe-Leu-Glu-Glu-Val was that the absolute configuration of [4-^{13}C]-4-carboxyglutamyl residue was 4*R* and was thus consistent with the conclusion derived from the experiments with the fluoroglutamate-containing peptide.[3]

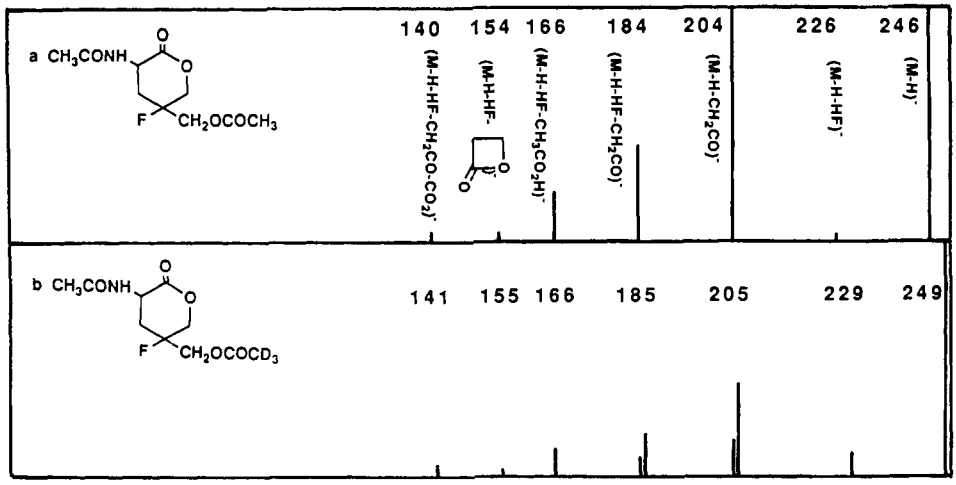


FIGURE 4: CAD spectra of $(M - H)^-$ ion of reference diacetylated lactone (a) and of deuterium labeled diacetylated lactone (b).

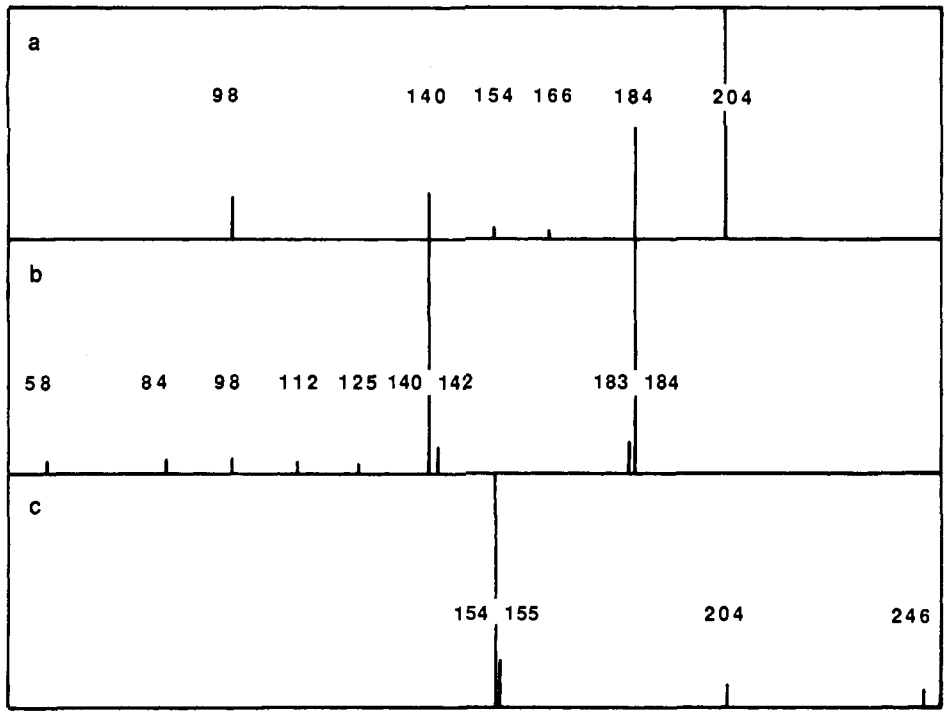


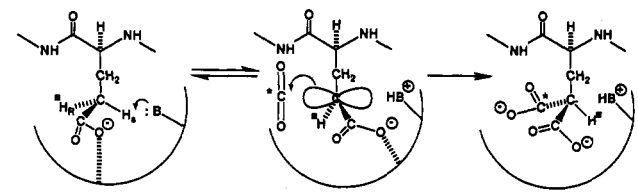
FIGURE 5: CAD spectra of m/z 204 ion (a) and of m/z 184 ion (b). Constant daughter spectrum of m/z 154 ion (c).

CONCLUSION

Thus, a 4*S* configuration of the [4-^{13}C]-4-carboxy-4-fluoroglutamyl residue, established unambigously that the vitamin K dependent carboxylation occurred with *inversion* of configuration. The partial result observed with the substrate that did not contain fluorine was consistent with that conclusion (Scheme VI).

The stereochemistry of an enzymatic reaction is, in most cases, dependent on the binding at the active site. This is a characteristic of the reaction but, in the absence of other indications (kinetics, isotope effects, etc.) it is impossible to draw safe mechanistic conclusions on the basis of a stereochemical study alone. For instance, several authors concluded to a concerted mechanism for biotin-dependent enzymes on the basis of a retention of configuration (Rose, 1970; Mildvan & Scrutton, 1967; Cheung et al., 1975) whereas later works (Kuo & Rose, 1982; Attwood et al., 1986; O'Keefe & Knowles, 1986a,b) based on isotope effect determination revealed that these reactions were not concerted.

Scheme VI: Stereochemistry of the Vitamin K Dependent Carboxylation

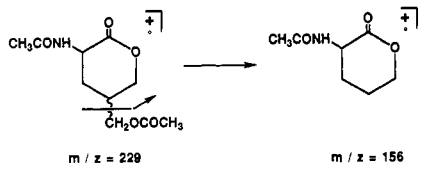


In the case of the vitamin K dependent carboxylation, mechanistic studies concerning the hydrogen exchange with solvent (Decottignies et al., 1984a,b; Anton & Friedman, 1983; McTigue & Suttie, 1983) or the β-elimination during the reaction of a 3-fluoroglutamyl residue (Vidal-Cros et al., 1990) established that the hydrogen abstraction was faster than the overall reaction. This strongly suppport a nonconcerted mechanism involving the reversible formation of a carbanion followed by reaction with CO_2.

The proton abstraction is stereospecific (Dubois et al., 1984; Decottignies et al., 1984a,b), but the actual structure of the

[3] Taking into account the inversion in the priority of the substituents due to the presence of fluorine atom.

Scheme VII: Mechanism of Decomposition of Molecular Ions of Lactones without Fluorine Atoms



carbanion, sp_2 as represented in Scheme VI or rapidly interconverting sp_3 species, is unknown. Anyway, the stereochemistry of the reaction is likely determined by the selection of one of the stereoheterotropic faces of the carbanion at the active site of the carboxylase.

Appendix: Collision Activated Decomposition (CAD) Analysis of the Ions Generated from Isomeric Fluorolactones

Preliminary experiments using different ionization techniques revealed that NICI with ammonia was very efficient, yielding high amounts of the $M - H^-$ ion along with several interesting fragments. The decomposition of the $M - H^-$ m/z 246 ion was studied by MS/MS spectrometry using CAD with argon as collision gas. m/z ions at 226, 204, 184, 154, and 140 were detected (Figure 4). The decomposition $(M - H)^-$ ion derived from the lactone having a deuterated acetoxymethyl group established that, with the exception of the m/z 226 ion which obviously corresponded to the loss of HF $(M - H - HF)^-$, all the fragments corresponded to the loss of a part of the acetoxymethyl group (Figure 4).

The comparison of the decomposition pattern of ions m/z 246, 204, and 184 revealed that ion m/z 154 originated from ion m/z 246 (loss of CH_2COOCH_2 and HF or vice versa) (Figure 5a) and from ion m/z 204 (loss of CH_2O + HF) (Figure 5b) but was not originating from ion m/z 184 as confirmed by the constant daughter ion spectrum of m/z ion 154 (Figure 5c). The pattern for the generation of different ions is outlined in Scheme VII.

The comparison of the ion current revealed that the m/z 204 → m/z 154 was more sensitive than the m/z 246 → m/z 154 by a factor of 2. The former decomposition was thus selected for the location of the ^{13}C label in the acetoxymethyl side chain or in the lactone ring.[4]

[4] The result observed with the cis isomer (a signal at m/z 154 and no detectable signal at m/z 155) proved that the m/z 205 ions, selected in the mass spectrometer for the analysis, corresponded almost exclusively to molecules having incorporated a ^{13}C atom during carboxylation with $^{13}CO_2$, without interference of lactone molecules derived from naturally ^{13}C-labeled glutamyl residues (the natural abundance of ^{13}C being close to 1%, the probability to have one ^{13}C atom present in the diacetylated lactone is roughly 10%) that after carboxylation with $^{12}CO_2$ (which could not be eliminated from the reaction buffers) would yield a m/z 155 signal.

Registry No. Phe-Leu-e-4FGlu-Glu-Val, 86879-14-3; Phe-Leu-Glu-Glu-Val, 61037-79-4; vitamin K, 12001-79-5; 4FGlu, 32563-24-9; γ-Glutamyl(vitamin K dependent) carboxylase, 81181-72-8.

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