Vitamin K-dependent carboxylation

Mechanistic studies with 3-fluoroglutamate-containing substrates

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The tripeptides t-butyloxycarbonyl-Xaa-Glu-[³H]Val, where Xaa is either (2*R*,3*S*)- or (2*R*,3*R*)-3-fluoroglutamate (respectively the *erythro* and the *threo* isomer), were synthesized and their behaviour during vitamin K-dependent carboxylation was studied. Neither peptide was carboxylated. The *erythro* compound gave rise to an HF-elimination product representing 1% of the starting material. This HF elimination did not occur during incubation of the *threo* compound. The formation of the dehydropeptide, probably by elimination of an F⁻ anion from an intermediate carbanion, favours the ionic pathway for vitamin K-dependent carboxylation.

INTRODUCTION

The vitamin K-dependent carboxylation of glutamate residues (Suttie, 1984) is a two-step reaction: the C-4-H-4 bond is cleaved first and the intermediate thus generated reacts with a CO₂ moiety, leading to 4-carboxyglutamate.

The hydrogen-exchange studies by McTigue & Suttie (1983), Anton & Friedman (1983) and Ducrocq *et al.* (1986) established (i) that the reaction is not concerted (the hydrogen exchange occurs in the absence of carboxylation), (ii) that the hydrogen exchange is vitamin K-dependent, (iii) that the hydrogen exchange is faster than the CO₂ fixation and (iv) that the stereospecificity of the hydrogen exchange is the same as the stereospecificity observed during carboxylation.

So far, two mechanisms have been considered for the hydrogen-abstraction step: either a radical-mediated mechanism (pathway a) or a carbanion-mediated mechanism (pathway b) (Scheme 1). The exchange experiments, although they do not strictly rule out the formation of a radical, were interpreted as favouring the carbanion-mediated mechanism. On the other hand, the hypothesis of a radical intermediate is supported by early work by Girardot *et al.* (1976), who described the inhibition of the carboxylation by spin-trapping reagents. However, no adduct has been unambiguously characterized by e.s.r.

We decided to test the carbanion hypothesis by using a β-elimination strategy similar to that developed by Abeles and Stubbe, who investigated the non-concerted nature of propionyl-CoA carboxylase mechanism by using β-fluoropropionyl-CoA (Stubbe & Abeles, 1977; Stubbe *et al.*, 1980). During carboxylation, formation of a carbanion β to the fluorine atom could induce a β-elimination and yield a dehydroglutamyl moiety (Scheme 2). Alternatively, as suggested by our study on model

Scheme 1. Ion-mediated and radical-mediated pathways for vitamin K-dependent carboxylation

Abbreviations used: Boc, t-butyloxycarbonyl; Glu(Δ^{2,3}) and Glu(Δ^{3,4}) (in peptide sequences), 2,3-dehydroglutamic acid and 3,4-dehydroglutamic acid respectively; Glu(3F) (in peptide sequences), 3-fluoroglutamic acid [Glu(3*F*) and Glu(3*F*) represent the *erythro* and *threo* isomers respectively]; Gla (in peptide sequences), 4-carboxyglutamic acid.

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Scheme 2. Expected behaviour of a 3-fluoroglutamate-containing substrate in the carbanion hypothesis and in the radical hypothesis

fluorine-containing radicals, a radical-mediated mechanism would yield the carboxylation product rather than the elimination of a fluorine radical (Vidal-Cros *et al.*, 1989a).

In the present paper we report the synthesis of Boc-Xaa-Glu-Val tripeptides, where Xaa is a 3-substituted glutamic acid, and their behaviour upon interaction with the vitamin K-dependent carboxylating system.

EXPERIMENTAL

Chemicals

Hydroxybenzotriazole, dicyclohexylcarbodi-imide and *N*-hydroxysuccinimide were from Fluka Co. Di-*t*-butyl pyrocarbonate, NADH, NAD⁺, pyridoxal 5'-phosphate, dithiothreitol, Triton X-100, sucrose, leucine aminopeptidase and acylase I were from Sigma Chemical Co., and all amino acids and derivatives were from Bachem.

All other chemicals were of the highest purity available.

The (2*R*,3*R*)-(*threo*)- and (2*R*,3*S*)-(*erythro*)-3-fluoroglutamic acids were synthesized as described by Vidal-Cros *et al.* (1989b), who separated the diastereoisomers chromatographically, after preparing them by reductive amination of racemic 3-fluoro-2-oxoglutarate with glutamate dehydrogenase, thus ensuring the *R*-configuration at C-2. The fluoro-oxoglutarate was made by treating ethyl fluoroacetate with diethyl oxalate to obtain diethyl fluoro-oxaloacetate, and then with ethyl bromoacetate, before decarboxylating and de-esterifying with mild acid treatment.

L-[³H]Valine (25 mCi/mmol) and NaH¹⁴CO₃ (56 mCi/mmol) were from Centre d'Études Atomiques (Saclay, France). The latter was purified before use (Guchhait *et al.*, 1974).

N.m.r.

N.m.r. spectra were recorded on a Bruker WM 250 instrument.

Peptide synthesis

Boc-L-*threo*-3-fluoroglutamyl-L-glutamyl-L-valine,

Boc-L-*erythro*-3-fluoroglutamyl-L-glutamyl-L-valine, Boc-L-glutamyl-L-glutamyl-L-valine, Boc-D-glutamyl-L-glutamyl-L-valine, Boc-L-*threo*-3-hydroxyglutamyl-L-glutamyl-L-valine and Boc-L-*erythro*-3-hydroxyglutamyl-L-glutamyl-L-valine were synthesized by the liquid-phase methodology with *t*-butyloxycarbonyl group for amino-group protection and benzyl ester for carboxy-group protection. Coupling was performed through the *N*-hydroxysuccinimide ester. After purification by column chromatography on silica gel, the protected tripeptides were hydrogenolysed with 10% Pd on charcoal.

Since 3-fluoroglutamic acid is degraded during hydrolysis with 1 M-HCl (Vidal-Cros *et al.*, 1985a), no amino acid analysis could be performed. The purity of peptides was checked by h.p.l.c. and 200 MHz ¹H-n.m.r. spectroscopy.

Boc-L-*threo*-3-fluoroglutamyl-L-glutamyl-L-[³H]valine and Boc-L-*erythro*-3-fluoroglutamyl-L-glutamyl-L-[³H]valine were prepared analogously starting from L-[³H]valine. Radiochemical purity of the two peptides was checked by h.p.l.c. Each peptide was contaminated by less than 0.2% of Boc-Glu(Δ^{2,3})-Glu-Val, arising from spontaneous HF elimination.

Boc-Glu(Δ^{3,4})-Glu-Val was obtained by incorporating a 4-phenylselenoglutarate residue protected as the Boc γ-methyl ester (Bory *et al.*, 1986) into a tripeptide followed by saponification with 1 M-KOH [4 equivalents; in 50% (v/v) ethanol], H₂O₂ oxidation and elimination (8.8 equivalents of H₂O₂, in tetrahydrofuran at 0 °C). ¹H n.m.r. ([²H]chloroform): the ethylenic protons appear as the AB part of an ABX system at δ_A 6.09 p.p.m. and δ_B 6.95 p.p.m. (2H, *J*_{AB} = 15.8 Hz, *J*_{BX} = 5.7 Hz, *J*_{AX} < 1 Hz).

Boc-Glu(Δ^{2,3})-Glu-Val was obtained by isomerization of the 3,4-dehydropeptide in pyridine/methanol or by HF elimination on the fluorinated peptides by NaOH at 40 °C. N.m.r. ([²H]chloroform): δ (p.p.m.) 0.97 (3H, doublet, *J* = 7 Hz), 0.98 (3H, doublet, *J* = 7 Hz), 1.45 (9H, singlet), 1.98 (2H, multiplet), 2.18 (1H, multiplet), 2.43 (2H, multiplet), 3.18 (2H, doublet, *J* = 7 Hz), 4.25 (1H, multiplet), 4.55 (1H, multiplet), 6.28 (1H, triplet, *J* = 7 Hz), 8.15 (2H, multiplet) and 8.37 (1H, multiplet).

Standard assay of carboxylase

Liver microsomal fractions were prepared from warfarin-treated rats (13.4 mM, 10 mg/kg) as previously described (Rikong-Adie *et al.*, 1980; Decottignies-Le Maréchal *et al.*, 1980). The incubation mixture (final pH 7.58) contained 50 mM-KH₂PO₄, 0.6 M-KCl, 0.25 M-sucrose, 1.33 mM-pyridoxal 5'-phosphate, 2.22 mM-NADH, 3.9 μ M-NAD⁺, 0.39 mM-dithiothreitol, 0.22 mM-vitamin K₁ hydroquinone, 1.5 % (v/v) Triton X-100, 7–9 mg of microsomal protein/ml and 1–3 mM-peptide. After preincubation (5 min at 20 °C), the reaction was initiated by addition of NaH¹⁴CO₃ (0.32 mM) and stopped after 30 min by addition of trichloroacetic acid [10 % (w/v) final concentration]. After centrifugation, a sample of the supernatant was evaporated to dryness and radioactivity was measured in an Intertechnique SL 30 scintillation counter in Aquasol-2. Blank experiments without vitamin K or without substrate were run in the same conditions.

Protein was determined by the method of Lowry *et al.* (1951), with BSA as standard.

Isomerization of Boc-Glu($\Delta^{3,4}$)-Glu-Val into Boc-Glu($\Delta^{2,3}$)-Glu-Val

Boc-Glu($\Delta^{3,4}$)-Glu-Val (1 mM) was incubated at 22 °C in the incubation mixture described above but containing heat-denatured microsomal fraction and non-labelled NaHCO₃. The reaction was monitored over a 5 h period on samples previously precipitated with ethanol (50 %, v/v), and centrifuged. H.p.l.c. analysis was performed on a μ Bondapak C₁₈ column, eluted with 0.25 M-triethylammonium phosphate, pH 3, containing 22 % (v/v) acetonitrile at a rate of 1 ml/min and monitored at 210 nm.

Incubation of the Boc-Glu(3F)-Glu-[³H]Val tripeptides

The assay was run on Boc-Glu(3F)-Glu-[³H]Val (3 mM; 0.28 mCi/mmol) in a 2 ml total volume in the standard conditions, except that non-radioactive NaHCO₃ (0.32 mM) was included. After incubation at 20 °C for 2 h, proteins were precipitated by ethanol (2 ml) and centrifuged at 1000 g for 15 min. The whole supernatant was then incubated at 20 °C for 3 h and concentrated to dryness. A sample of the crude mixture was analysed by h.p.l.c. on a Merck Lichrospher 100 RP-8e (5 μ m particle size) column eluted at 1 ml/min with 0.25 M-triethylammonium phosphate, pH 3, containing 18 % (v/v) acetonitrile. Fractions (1 ml) were collected and radioactivity was determined. A blank experiment where vitamin K was omitted was run in the same conditions.

Purification of the product derived from incubation of erythro-Boc-Glu(3F)-Glu-[³H]Val

The crude product was purified by h.p.l.c. on a Merck Lichrosorb RP-18 (1 μ m particle size) column eluted at 3 ml/min with 0.25 M-triethylammonium phosphate, pH 3, containing 18 % (v/v) acetonitrile. Fractions (3 ml) were collected. The dehydropeptide-like fractions, detected by u.v. light at 210 nm, were pooled, concentrated under reduced pressure and freeze-dried. The salts were removed by purification of the recovered material on a C₁₈ Sep-Pak cartridge. After being washed with 70 ml of water, the product was eluted with 3 ml of methanol. The

radiochemical purity of the product was checked again by h.p.l.c.

Identification of the product derived from incubation of erythro-Boc-Glu(3F)-Glu-[³H]Val

The above purified product, diluted with non-labelled Boc-Glu($\Delta^{2,3}$)-Glu-Val (0.4 μ mol), was hydrogenated with 10 % Pd on charcoal in 0.75 ml of methanol. After vigorous stirring for 3 h, the catalyst was filtered off and washed with methanol, and the product was analysed by h.p.l.c. on a Merck Lichrospher 100 RP-8e (5 μ m particle size) column eluted at 1 ml/min with 0.25 M-triethylammonium phosphate, pH 3, containing 12.8 % (v/v) acetonitrile. Fractions (either 1 ml or 0.2 ml) were collected and radioactivity was determined on 0.2 ml samples.

Tritium release assay

Buffers and solutions were prepared with freshly boiled water in order to lower the bicarbonate concentration down to 0.5–1 mM. An incubation was run on 6.75 μ Ci of Boc-(2S)-[2,3-³H]Glu-Glu-Val (1 mM; specific radioactivity 13.5 mCi/mmol) as described for a standard assay. After incubation at 20 °C for 5 h, the proteins were precipitated by addition of trichloroacetic acid [10 % (w/v) final concentration] and centrifuged. The supernatants were freeze-dried and radioactivity was determined on both the recovered water and the freeze-dried material (water, 6172 d.p.m.; freeze-dried material, 42.46 $\times 10^6$ d.p.m.). A blank experiment without vitamin K was run in the same conditions (water, 1959 d.p.m.; freeze-dried material, 32.52 $\times 10^6$ d.p.m.). This corresponded to an exchange of 0.0085 %.

RESULTS

Carboxylation of fluoropeptides

Since the carboxylation of Phe-Leu-Glu-Glu-Val or Boc-Glu-Glu-Val occurs almost exclusively at the first glutamate residue (Decottignies-Le Maréchal *et al.*, 1980), the 3-substituted glutamates were introduced at that position. To facilitate the detection and the purification of the products formed upon reaction of the fluorinated substrates, radioactive peptides labelled with [³H]valine were prepared. The tripeptides were synthesized by the liquid-phase method, with t-butyl-oxycarbonyl group for amino-group protection and benzyl ester for carboxy-group protection, and tested for carboxylation. Results are given in Table 1.

As already observed, the tripeptide Boc-Glu-Glu-Val, although being one of the best of the synthetic peptides commonly used to study vitamin K-dependent carboxylation (Rich *et al.*, 1980), remained a poor substrate for the enzyme (0.1 % reaction at a peptide concentration of 1 mM with 30 min incubation time). However, the ¹⁴CO₂ incorporations with this substrate were significant with respect to the very low value of the blanks (see Table 1). On the other hand the ¹⁴CO₂ incorporation into both fluorinated peptides was not significantly different from the blank and they could not thus be considered as being substrates of the carboxylase.

The lack of carboxylation of the fluorinated peptides prompted us to look for an HF-elimination product. Previous studies in this laboratory had shown that Boc-Glu($\Delta^{3,4}$)-OMe isomerized to Boc-Glu($\Delta^{2,3}$)-OMe (Bory

Table 1. Vitamin K-dependent carboxylation of 3-fluoroglutamate-containing peptides Boc-Xaa-Glu-Val

After preincubation at 20 °C for 5 min, the reaction (final volume 0.225 ml) was initiated by addition of $\text{NaH}^{14}\text{CO}_3$. Then 30 min later the reaction was stopped with trichloroacetic acid, the mixture was centrifuged and the radioactivity was determined on a 0.1 ml sample of the supernatant.

Peptide	Radioactivity incorporated (d.p.m.)*	Carboxylation (% with respect to Boc-Xaa-Glu-Val)	IC ₅₀ †
Boc-Glu-Glu-Val (1 mM)	11 001	0.11	
Boc-Glu(3,F)-Glu-Val‡ (3 mM)	35	0.000115	3 mM
Boc-Glu(3 _f)-Glu-Val‡ (3 mM)	57	0.000187	3 mM

* The blank value without vitamin K (45 d.p.m.) was subtracted.

† [Boc-Glu-Glu-Val] = 1 mM.

‡ Fluorinated analogues bear some resemblance to their hydroxylated analogues (Barnett, 1972; Briley *et al.*, 1975). The 3-hydroxyglutamic acids were prepared, resolved and incorporated into tripeptides. The *threo* and *erythro* isomers (5 mM) were not significantly carboxylated (respectively 56 and 69 d.p.m. incorporation).

et al., 1986) with a half-life of 4 min in the carboxylation conditions (i.e. phosphate buffer, pH 7.58). Similar h.p.l.c. studies, performed on Boc-Glu($\Delta^{3,4}$)-Glu-Val, the expected elimination product, revealed that this peptide also underwent isomerization into Boc-Glu($\Delta^{2,3}$)-Glu-Val. Albeit slower than the isomerization of Boc-Glu($\Delta^{3,4}$)-OMe, this isomerization was relatively fast ($t_{1/2}$ 73 min) with respect to the incubation time (2 h). In order to avoid performing the purification and identification procedures on mixtures of 2,3- and 3,4-dehydropeptides, the 3,4-dehydropeptide was allowed to isomerize as thoroughly as possible before treatment. We checked (detection with u.v. light) that the fluoropeptides did not eliminate HF under these isomerization con-

ditions. As shown in Fig. 1, incubation of *erythro*-Boc-Glu(3F)-Glu-[^3H]Val followed by isomerization resulted in the formation of a radioactive product that was detectable by u.v. absorption at 210 nm (elution between 15 and 17 min) and co-migrated with standard Boc-Glu($\Delta^{2,3}$)-Glu-Val. (The radioactive material that emerged between 18 and 19 min did not absorb at 210 nm, and so was not a peptide.) The enzymic origin of this product was verified by two blanks run either without vitamin K or with heat-denatured liver microsomal fraction. In contrast, the *threo*-3-fluoroglutamate-containing tripeptide did not give rise to this product (Table 2).

This product represented about 1% of the starting

Fig. 1. H.p.l.c. of the *erythro*-Boc-Glu(3F)-Glu-[^3H]Val carboxylation product

erythro-Boc-Glu(3F)-Glu-[^3H]Val (3 mM) was incubated at 20 °C for 2 h in a carboxylation assay containing non-labelled NaHCO_3 (0.3 mM). After precipitation of the proteins by ethanol (50%, v/v) and centrifugation, the supernatant was incubated at 20 °C for 3 h. A blank experiment without vitamin K hydroquinone was run under the same conditions. Samples of these incubation mixtures were analysed by h.p.l.c. on a Merck Lichrospher 100 RP-8e (5 μm particle size) column. Eluting buffer was 0.25 mM-triethylammonium phosphate, pH 3, containing 18% (v/v) acetonitrile, at a rate of 1 ml/min. Monitoring of the eluate was at 210 nm (—). Fractions (1 ml) were collected and the radioactivity was determined (.....). The arrows indicate the retention times of the standard peptides: ∇ , Boc-Glu($\Delta^{2,3}$)-Glu-Val; $\nmid$, *erythro*-Boc-Glu(3F)-Glu-Val or Boc-Glu($\Delta^{3,4}$)-Glu-Val.

Table 2. Incubation of Boc-Glu(3F)-Glu-[³H]Val (1.7 μ Ci): extent of elimination

Analysis of h.p.l.c. profiles was achieved either by measuring the radioactivity of 1 ml collected fractions (*a*) or by integration of the areas under the peaks detected at 210 nm (*b*). The results, expressed as percentages of the ratio of the dehydropeptide formed to the remaining fluorinated peptide, are the averages for three independent determinations.

Peptide (3 mM)	Extent of elimination (%)			
	Vitamin K omitted		Vitamin K present	
	(<i>a</i>)	(<i>b</i>)	(<i>a</i>)	(<i>b</i>)
Boc-Glu(3,F)-Glu-[³ H]Val	1.00*	0.20	1.96	1.00
Boc-Glu(3,F)-Glu-[³ H]Val	0.59	0.22	0.58	0.16

* This value probably reflects the presence of an impurity, labelled but not absorbing at 210 nm, that may be the one referred to later in the text.

material, a value that was larger than the carboxylation yield of Boc-Glu-Glu-Val, which is in the 0.3–0.4 % range under those conditions. This was consistent with the conclusion derived from exchange experiments (see above) that the slower step of the reaction is the addition of CO₂ to the intermediate.

To reinforce the identification of the product as the dehydropeptide, we ran a hydrogenation catalysed by Pd on charcoal. Preliminary experiments on the dehydropeptide, obtained either by isomerization of synthetic

3,4-dehydropeptide or by HF elimination from the fluoropeptide, indicated that the reaction was fast, clean and led to both Boc-L-Glu-L-Glu-L-Val and Boc-D-Glu-L-Glu-L-Val with a diastereoisomeric excess of 43 % favouring the latter. The radioactive fractions eluted between 15 and 17 min (elution time of the dehydropeptide) were hydrogenated. Analysis of the product thus obtained revealed two peaks of radioactivity, eluted respectively as Boc-L-Glu-L-Glu-L-Val and Boc-D-Glu-L-Glu-L-Val (Fig. 2). The amounts of radioactive materials

Fig. 2. H.p.l.c. analysis of the hydrogenation product of Boc-Glu($\Delta^{2,3}$)-Glu-[³H]Val resulting from the carboxylation of *erythro*-Boc-Glu(3F)-Glu-[³H]Val

The carboxylation product of Boc-Glu(3F)-Glu-[³H]Val, purified on a Merck Lichrosorb RP-18 (7 μ m particle size) column and on a C₁₈ Sep-Pak cartridge, was diluted with 0.4 μ mol of synthetic Boc-Glu($\Delta^{2,3}$)-Glu-Val and hydrogenated on Pd on charcoal for 3 h in methanol. The crude product was analysed by h.p.l.c. on a Merck Lichrospher 100 RP-8e (5 μ m particle size) column. Eluting buffer was 0.25 mM-triethylammonium phosphate, pH 3, containing 12.8 % (v/v) acetonitrile, at a rate of 1 ml/min. Monitoring of the eluate was at 210 nm (—). Fractions (1 ml or 0.2 ml) were collected and the radioactivity was determined (· · · · ·, blank; — · — · —, assay). The arrows indicate the retention times of the standard peptides: ↓, Boc-L-Glu-L-Glu-L-Val; ↓, Boc-D-Glu-L-Glu-L-Val.

were almost identical in both peaks, whereas the u.v. monitoring indicated an excess of the DLL-isomer over the LLL-isomer, as observed previously. This suggested that the peak corresponding to the LLL-isomer was contaminated by a radioactive impurity. A peak eluted at the retention time of the DLL-isomer was present in the blank in even higher amount than in the assay (presumably because the fractions containing eventually the dehydro compound were collected blindly to avoid loss of product) whereas nothing was eluted at the retention time of the DLL-isomer. This established (i) that no dehydropeptide was formed in the blank, and (ii) that, even though no unambiguous conclusion can be drawn from the peak eluted at the retention time of the LLL-isomer, the existence of a radioactive peak at the retention time of the DLL-isomer in the assay proves that dehydropeptide was present after incubation with the carboxylase.

DISCUSSION

Mechanism of dehydropeptide formation

Two elementary mechanisms could account for the formation of a dehydropeptide from the *erythro*-3-fluoroglutamate-containing tripeptide: F[•] elimination on a radical intermediate (eqn. 1) or F⁻ elimination on a carbanion intermediate (eqn. 2):

The energy required for the homolytic breaking of the C–F bond is very high (Patai, 1973). Previous studies of α -fluoro-containing radicals suggested that the first possibility (eqn. 1) was very unlikely (Barton *et al.*, 1966; Herdewijn *et al.*, 1987). We also checked (Vidal-Cros *et al.*, 1989a) that no elimination was occurring during the homolytic decarboxylation of the 3-fluoroglutamates according to Barton *et al.* (1983). Thus the fastest reaction undergone by an α -fluoro radical is the trapping of an H[•] radical rather than F[•] radical elimination or dimerization. If a radical intermediate

were involved in the carboxylation, it would probably exhibit the same reactivity and react with another radical (H[•] or CO₂^{•-}) rather than eliminate the F[•] radical. F⁻ anion elimination from a carbanion intermediate (eqn. 2), already used in many studies such as mechanism-based enzyme inhibition (Walsh, 1982; Rando, 1984), seemed to be the most attractive of those simple mechanisms that could account for the dehydropeptide formation. [Stubbe and colleagues (Harris *et al.*, 1987) suggested the existence of a radical cation and the HF elimination to account for the inactivation of ribonucleotide reductase by 2'-deoxy-2'-fluororibonucleotide. However, in this case the radical cation was stabilized by an adjacent donor hydroxy group.]

The dehydropeptide could also be formed by the decarboxylation of Boc-Glu(3F)-Glu-Val (Scheme 3). The fact that no ¹⁴CO₂ was incorporated in the carboxylation assay excluded, *a priori*, a non-stereospecific decarboxylation (¹⁴CO₂ would have been present in the dehydropeptide). It would have required an efficient (concerted?) decarboxylation–fluoride elimination at the active site. This would have implied that the extent of carboxylation of Boc-Glu(3F)-Glu-Val (1% in that hypothesis) exceeded that of Boc-Glu-Glu-Val (0.3–0.4%). This makes that possibility very unlikely, since the CO₂ incorporation is the slow step of the overall reaction (Ducrocq *et al.*, 1986). (Furthermore, malonic protons being very acidic and the presence of fluorine reinforcing this acidity, proton abstraction should be favoured over decarboxylation and should yield a ¹⁴C-labelled product in the carboxylation experiments.) The 2,3-dehydropeptide could have been produced directly by elimination on a carbanion non-specifically generated at the 2-position. However, the stability of the fluorinated peptide under the incubation conditions and the strict dependence of the elimination on the presence of vitamin K make this possibility improbable also. (We checked that no exchange of the 2-hydrogen of Boc-L-[2,3-³H]Glu-Glu-Val with solvent could be detected during the carboxylation. If the same situation holds for the fluorinated peptide, this makes the formation of the 2,3-dehydropeptide by abstraction of the 2-hydrogen very unlikely.)

Provided that the presence of fluorine does not induce a driving force sufficient to modify the reaction pathway when going from Boc-Glu-Glu-Val to Boc-Glu(3F)-Glu-

Scheme 3. Putative formation of dehydropeptide by carboxylation–decarboxylation

This possibility is very improbable (see the text).

Val, our result supports the involvement of a carbanionic intermediate during the enzymic carboxylation.

The difference between the Boc-Glu(3F)-Glu-Val, which yielded a dehydropeptide, and Boc-Glu(3,F)-Glu-Val, which did not eliminate, can be interpreted on the basis of the stereochemical features necessary for the elimination reaction, i.e. the relative orientations of F-3 and H-4 are not fulfilled by the *threo* isomer whereas the proper conformation is achieved with the *erythro* isomer. We have already observed such a reactivity difference between the *threo*- and *erythro*-3-fluoroglutamate isomers during reaction with glutamate decarboxylase (Vidal-Cros *et al.*, 1985b): the *threo* isomer simply decarboxylates whereas the *erythro* isomer decarboxylates and eliminates an F⁻ anion.

CONCLUSION

The *erythro*-Boc-Glu(3F)-Glu-Val yielded an HF-elimination product upon carboxylation. The most attractive mechanism that can rationalize this result is an F⁻ elimination on a carbanion intermediate (Scheme 1, pathway b).

However, the present study does not rule out a radical-mediated mechanism yielding first a radical species that would be reduced into a carbanion in a second step (Scheme 1, pathway c). This pathway has to be tested.

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