Vitamin K-dependent carboxylation: inhibition by a peptide containing 4-methylene glutamic acid

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The peptide Boc-4-methylene Glu-Glu-Val has been synthesized and shown to be a strong inhibitor of the vitamin K-dependent carboxylation catalyzed by a detergent solubilized rat liver microsome preparation. The inhibition is apparently competitive with respect to the substrate peptide and non-competitive with respect to HCO_3^-

Vitamin K *Carboxylation* *4-Methylene glutamic acid* *γ-Carboxyglutamic acid*

1. INTRODUCTION

Vitamin K is an essential coenzyme in a post-translational reaction which carboxylates selected glutamyl residues in precursor proteins to convert them to the γ-carboxyglutamyl residues of completed proteins [1–3]. A detergent solubilized microsomal preparation from rat liver is able to carboxylate small synthetic peptides containing L-glutamic acid and reproducing part of the prothrombin precursor [4–6].

Modifications of the glutamyl residues are important for studies related to the elucidation of the carboxylation mechanism, and may afford specific inhibitors directed towards the glutamyl binding site of the carboxylase. Authors in [7] have reported the synthesis of a peptide containing residues of *O*-phosphoserine which competitively inhibits the carboxylation of Phe-Leu-Glu-Glu-Leu with an affinity constant close to that of the reference peptide (K_i = 4 mM). Recently, it has been reported [8] that a pentapeptide containing (2*S*,4*S*)-4-methylglutamic acid competitively inhibited the carboxylase with a K_i of 65 μM (IC_{50} = 80 μM), while the (2*S*,4*R*)isomer-containing peptide was a much weaker inhibitor (IC_{50} = 3.6 mM). In contrast, γ-fluoroglutamic acid-containing peptides have been prepared and shown to be substrates and not inhibitors for the vitamin K-dependent carboxylation reaction [9].

We report here the results obtained with a peptide containing a 4-methylene glutamic acid residue, which was expected to react eventually with a nucleophilic site of the carboxylase.

2. EXPERIMENTAL

2.1. *Peptide synthesis*

Proton NMR spectra were recorded on a Bruker WM 250-FT spectrometer. Chemical shifts are reported as ppm relative to tetramethylsilane.

4-Methylene DL-glutamic acid was prepared by a modification of the method of [10]. Resolution was achieved in a quantitative yield on the racemic *N*-acetyl derivative using hog kidney acylase I [11]. 4-Methylene L-glutamic acid I ($[\alpha]_D^{20}$ = 15.0°, *c* = 1, 5 N HCl [12]) was separated from the *N*-acetyl D-isomer by ion exchange chromatography on AGl-X4 resin (AcO^- form) and esterified with HCl/MeOH to give the dimethyl ester derivative II. Treatment of II with di(*t*-butyl) dicarbonate (Fluka) in the presence of dimethylamino-4-pyridine in acetonitrile–water (99:1) gave the Boc-4-methylene L-glutamic acid dimethyl ester III

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which was regioselectively hydrolyzed with α-chymotrypsin to Boc-4-methylene L-glutamic acid γ-methyl ester IV [13]. ^{1}H-NMR ($CDCl_3$):δ = 1.42 (s, 9H, Boc), 2.70 (m, 2H, CH_2), 3.75 (s, 3H, OMe), 5.68–6.20 (2s, 2H, CH_2=). Boc-glutamic acid γ-methyl ester was obtained from the corresponding dimethyl ester by the same method. IV was used for the synthesis of the protected peptide Boc-4-methylene Glu-Glu-Val trimethyl ester using the liquid phase methodology as described in fig.1. ^{1}H-NMR ($CDCl_3$):δ = 1.42 (s, 9H, Boc), 2.70 (m, 2H, β-CH_2), 3.65, 3.71, 3.76 (3s, 9H, OMe), 5.70, 6.25 (2s, 2H, CH_2=). Final deprotection was effected by mild alkaline hydrolysis. Boc-4-methylene Glu-Glu-Val V was purified on a DEAE-Sephadex A-25 column with a linear gradient of acetic acid (0.5–4 N). $[\alpha]_D^{20}$ = −32.2° (c = 0.6, H_2O). ^{1}H-NMR (D_2O):δ = 1.0 (d, 6H, 2 Me), 1.45 (s, 9H, Boc), 2.2–2.7 (3m, 6H, CH_2), 4.0–4.5 (3m, 3H, Hα), 5.8, 6.32 (2s, 2H, CH_2=). The peptide was shown to be pure by thin-layer chromatography on silica gel 60 F-254 (solvent: butanol/acetic acid, 4:1, saturated with water), high-pressure liquid chromatography and high-voltage paper electrophoresis at pH 4.0, 70 V·cm^{-1} (electrophoretic migration relative to Glu = 2.1). Analysis of the *N*-trifluoroacetyl *O*-isopropyl ester derivative of the peptide acidic hydrolysate by gas chromatography on a chiral phase (Chrompack XE-60-S-valine-S-phenylethylamide fused silica capillary column) [14] indicated an optical purity higher than 98% for the constitutive amino acids.

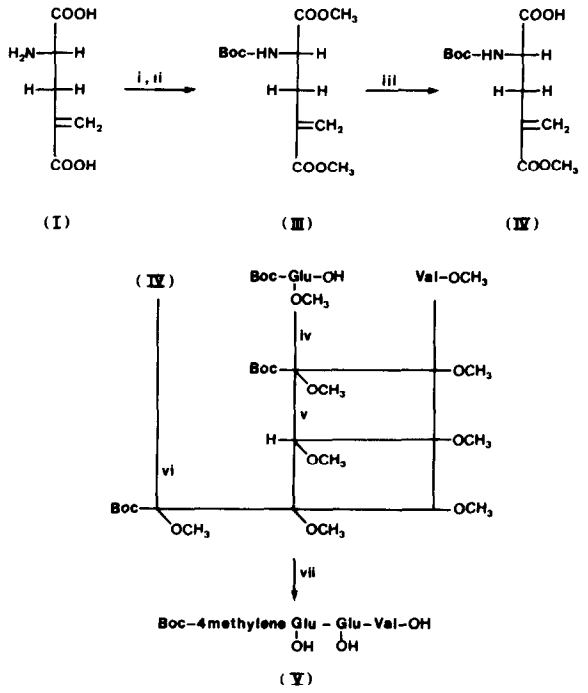

Fig.1. Scheme for the synthesis of the peptide containing 4-methylene glutamic acid. (i) HCl-MeOH, (ii) $(Boc)_2O$-DMAP, (iii) α-chymotrypsin, (iv) DCCI, (v) HCl-AcOH, (vi) DCCI, (vii) 2 N LiOH/MeCN-H_2O (1:1).

2.2. *Carboxylation assays*

Liver microsomes were prepared from vitamin K-deficient rats as in [15]. The microsomal pellets were solubilized in SIKM buffer (0.25 M sucrose, 50 mM imidazole, 0.6 M KCl, 15 mM $MnCl_2$) pH 7.5, containing 1.5% Triton X-100, 1 mM NAD^+ and 1 mM DTT. 0.1 ml of the solubilized preparation (10–15 mg proteins/ml), 50 μl peptide solution in SIKM, 2.5 μl of phylloquinone solution (12 mg/ml) in 5% Triton X-100 and 2.5 μl of 1 mM DTT were incubated with agitation for 5 min, at 20°C. The reaction was initiated with 25 μl of $NaH^{14}CO_3$ (6 μCi, 53 mCi/ mmol), carried out for 30 min at 20°C, and terminated by addition of 25 μl of 60% trichloroacetic acid. After centrifugation, 150 μl of the supernatants were transferred to counting vials and dried in vacuo over NaOH pellets during 2 h. Water (2 ml) and Instagel (14 ml) were added and the radioactivity counted in a Packard liquid scintillation spectrometer. Microsomal proteins were determined according to [16] with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

The peptide which was synthesized contained 4-methylene glutamic acid in place of the first glutamyl residue of the Boc-Glu-Glu-Val substrate because it has been demonstrated, with several similar substrates, that only this residue was carboxylated by the vitamin K-dependent carboxylase [15,17–19]. Incidentally, peptides containing 4-methylene glutamic acid residues have been obtained as chemical modification products of γ-carboxyglutamic acid-containing peptides [20,21].

When Boc-4-methylene Glu-Glu-Val was tested as a substrate for the carboxylase at 0.3 mM concentration, no $^{14}CO_2$ incorporation was observed (table 1). When incubated in the presence of the usual substrate (Boc-Glu-Glu-Val) at the same con-

Table 1

Effect of Boc-4-methylene Glu-Glu-Val on the carboxylation reaction

Substrates	Carboxylase activity	
	dpm/mg protein*	%
Boc-4-methylene Glu-Glu-Val (0.3 mM)	120	-
Boc-Glu-Glu-Val (0.15 mM)	10 030	100
Boc-Glu-Glu-Val (0.15 mM) + Boc-4-methylene Glu-Glu-Val (0.15 mM)	2 700	27

*Incubation blanks without vitamin K subtracted (70–100 dpm/mg protein)

centration (0.15 mM), our peptide inhibited its carboxylation by 73%. However, the inhibition was not time dependent, nor was it increased by preincubation with the inhibitory peptide. The inhibition was essentially reversible after dialysis of the resuspended microsomes for 1 h (table 2). All these results indicate that no covalent reaction of the methylene peptide with the carboxylase had occurred.

The double-reciprocal plot shown in fig.2 is consistent with the action of the methylene peptide as a reversible competitive inhibitor with respect to the peptide substrate, and the slope replot indicates a K_i of 50 μM, slightly lower than that measured for the 4-methyl glutamate-containing pentapeptide [8].

Table 2

Reversibility of the inhibition by Boc-4-methylene Glu-Glu-Val

Preincubation (20 min)	Substrate	Carboxylase activity after dialysis[a]	
		dpm/mg protein	%
Without peptide	Boc-Glu-Glu-Val (0.4 mM)	28 400	100
With Boc-4-methylene Glu-Glu-Val (0.2 mM)	Boc-Glu-Glu-Val (0.4 mM)	26 900	95

[a]After preincubation at 20°C, solubilized microsomes were dialyzed for 1 h against the microsome resuspension buffer (see section 2), then used for the measurement of carboxylase activity

One possible hypothesis for the high affinity of such inhibitors was their possible analogy with a 'transition state' intermediate involving the entering CO_2 molecule, which has been demonstrated to be the reactive carboxylic species [22]; in such a case, these compounds would be similarly competitive inhibitors with respect to CO_2. No such data have been reported for the methyl glutamate-containing peptides. In an experiment with various $H^{14}CO_3^-$ concentrations, the methylene peptide behaved as a perfect non-competitive inhibitor

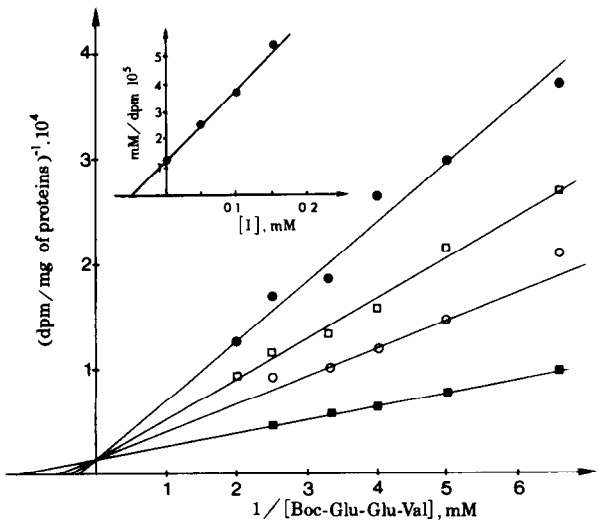

Fig.2. Double-reciprocal plot of the effect of Boc-4-methylene Glu-Glu-Val on the vitamin K-dependent carboxylation of Boc-Glu-Glu-Val at various peptide concentrations. Inhibitor concentration: 0 (■—■), 0.05 mM (○—○), 0.1 mM (□—□), 0.15 mM (●—●). A replot of the slopes is shown in the inset.

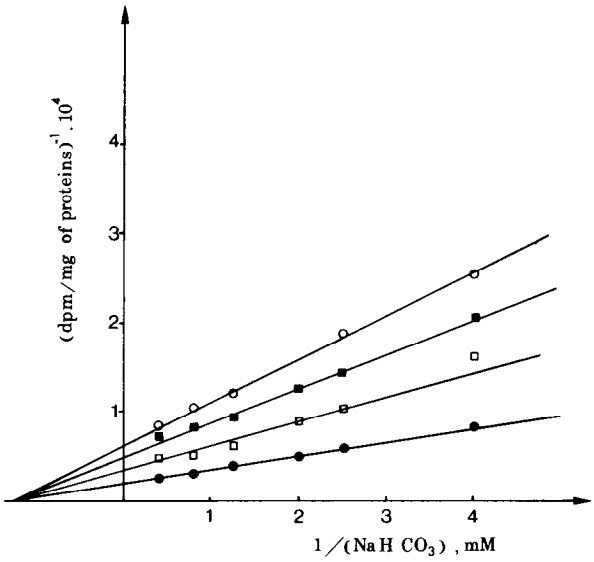

Fig.3. Double-reciprocal plot of the effect of Boc-4-methylene Glu-Glu-Val on the vitamin K-dependent carboxylation of Boc-Glu-Glu-Val (0.3 mM) at various HCO_3^- concentrations. Inhibitor concentrations: 0 (●—●), 0.05 mM (□—□), 0.1 mM (■—■), 0.15 mM (○—○).

(fig.3). We may thus conclude that the binding of the methylene peptide at the substrate site has no effect at all on the binding of CO_2. At this moment, no explanation is available for the high affinity observed for this inhibitor.

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