Vitamin K-dependent carboxylase: Synthesis of an inhibitor of the glutamyl binding site

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Liver microsomes contain a vitamin K and O_2-dependent carboxylase that converts peptide-bound glutamyl residues to γ-carboxyglutamyl residues. The peptide Boc-*O*-phospho–Ser-*O*-phospho–Ser–Leu-OMe has now been synthesized. This peptide inhibits the carboxylation of endogenous protein precursors by a detergent-solubilized preparation of the carboxylase and is an apparent competitive inhibitor of the carboxylation of Phe–Leu–Glu–Glu–Leu.

Vitamin K *Carboxylase* *Phosphoserine* *Peptide substrates* *γCarboxyglutamic acid*

1. INTRODUCTION

The reduced form of vitamin K is required for the action of a liver microsomal enzyme that carboxylates glutamyl residues in precursor proteins to γ-carboxyglutamyl (Gla) residues in completed proteins [1]. This enzyme will carboxylate low-M_r peptides that are homologous with carboxylated sequences in precursor proteins [2]. The results of structure–activity studies designed to define the substrate specificity of the rat liver vitamin K-dependent carboxylase [3] using analogs of substrate peptides Phe-Leu-Glu-Glu-Leu and Boc-Glu-Glu-Leu-OMe clearly indicate that the enzyme efficiently carboxylates only L-glutamyl residues.

A number of lines of evidence [1,4–6] suggest that this oxygen-dependent carboxylase activity is catalyzed by the same enzyme that converts reduced vitamin K to vitamin K 2,3-epoxide, but the detailed mechanism of these reactions has not been clarified. These activities are inhibited by vitamin K antagonists and a number of non-specific reagents, but specific inhibitors of the glutamyl-binding site have not been reported [1]. Structure activity data [3] suggest that for good binding to the carboxylase, a peptide must contain an anionic group, located two methylene units from the peptide chain, and that the residue must have the L-chirality. This could be achieved by replacing the carboxyl group with a phosphate group. We report here the synthesis of peptides containing phosphoserine residues which, because of the placement of an oxygen, rather than a carbon atom at the γ-position, might be expected to be competitive inhibitors of the carboxylase.

2. EXPERIMENTAL

2.1. *Peptide synthesis*

Melting points were determined on a Fischer-Johns melting point apparatus and are corrected. Proton nuclear magnetic resonance spectra were recorded on a Bruker model Hx90E spectrometer. Chemical shifts are reported as values (ppm) relative to TMS (tetramethylsilane) as an internal standard. Analytical thin-layer chromatography was carried out using thin-layer chromatography plates precoated with silica gel 60F-254. Spots were visualized as in [3]. Solvent systems used were: (1) 6% methanol in chloroform; 95% ethanol; (3) 15% methanol in chloroform; (4) methanol; (5)

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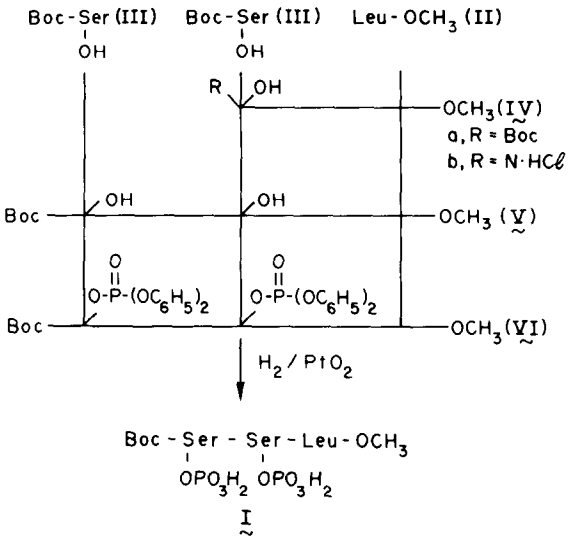

Fig.1. General scheme for the synthesis of peptides containing phosphoserine residues.

chloroform–methanol–acetic acid (70:25:5), and (6) 10% methanol in chloroform. Boc-Ser–Leu-OMe (IV) (m.p. 143–144°C; $R_f(1) = 0.31$ $R_f(2) = 0.76$), Boc-Ser–Ser–Leu-OMe (V) ($R_f(3) = 0.45$) and Boc-Phe–Leu–Ser–Ser–Leu-OMe (m.p. 184°C; $R_f(3) = 0.54$) were synthesized from Leu-OMe (II) and Boc-Ser dicyclohexylamine salt (III) using methods reported for the synthesis of related analogs [3]. Details of these syntheses will be reported separately, and the general synthesis scheme utilized is shown in fig.1.

2.2. *O-Phosporylation of (V) (VI)*

Compound (V) (420.0 mg, 0.001 mol) was dissolved in 20 ml of methylene chloride and triethylamine (0.422 ml, 0.003 mol) was added. After 10 min, diphenyl chlorophosphate (0.622 ml, 0.003 mol) was added and the mixture was stirred at 0°C for 1 h and at room temperature for 2 days. After evaporation of solvent, the residue was purified by silica gel chromatography eluting with 1% methanol in chloroform to give 814.0 mg (92.1%) of (VI) as an oil: $R_f(1) = 0.69$. ^{1}H NMR ($CDCl_3$) 0.85 (*d*, 6H, $J = 5.8$ Hz, Leu 2 $-CH_3$) 1.42 (*s*, 9H, Boc) 1.59–1.68 (*m*, 3H, Leu β-CH_2, γ-CH) 3.68 (*s*, 3H, Leu-OCH_3) 4.29–4.91 (*m* complex, 7H, 3α-CH, 2Ser β-CH_2) 5.63 (*d*, 1H, $J = 6.0$ Hz, NH) 7.04–7.51 (*m*, 22H, 4 Aromatic, 2NH). This material undergoes rapid β-elimination and was converted immediately to the free phosphate derivative I.

2.3. *Boc-O-phospho-Ser–O-phospho-Ser–Leu-OMe (I)*

Compound (VI) (707.2 mg, 0.8 mmol) was dissolved in 40 ml of dry methanol, platinum oxide (PtO_2, 500 mg) was added, and the mixture was hydrogenated for 12 h. After removal of the catalyst by filtration, new catalyst (200 mg) was added and the mixture hydrogenated again for 8 h. The solution was filtered through a celite 545 column and evaporated to dryness. The residue was recrystallized from 1.5 ml methanol and 30 ml ether to give 192.0 mg (41.7%) of crystalline I: m.p. 174–176°C. $R_f(4) = 0.28$. ^{1}H NMR (MeOH-d_4) $\delta = 0.93$ (br, *s*, 6H, Leu 2 $-CH_3$) 1.45 (*s*, OH, Boc) 1.60–1.82 (br, 3H, Leu β-CH_2, γ-CH) 3.70 (*s*, 3H, Leu-OCH_3) 4.08–4.27 (br, 4H, 2Ser β-CH_2) α-CH region (overlapped with MeOH peak). *Anal.* calc. for $C_{18}H_{35}N_3O_{14}P_2$: C, 37.31; H, 6.09; N, 7.25. Found: C, 37.50; H, 5.98; N, 7.14. Using the procedures described for the synthesis of VI and I, Boc–Phe–Leu–Ser–Ser–Leu–OMe was *bis*-phosphorylated (m.p. 139–140°C; $R_f(6) = 0.71$) and hydrogenated to Boc–Phe–Leu–phospho-Ser–phospho-Ser–Leu–OMe (VII): $R_f(5) = 0.05$; ^{1}H NMR is the same as obtained for I except for the added signals for the Phe and Leu residues.

2.4. *Carboxylase assays*

Vitamin K-dependent carboxylase activity was assayed in stored liver microsomes prepared from vitamin K-deficient rats. Incorporation of $^{14}CO_2$ into the peptide substrate Phe–Leu–Glu–Glu–Leu (peptide carboxylase activity) was measured as in [3] and into endogenous microsomal protein (protein carboxylase activity) as in [7]. Peptides being assayed for inhibitor activity were dissolved in a small amount of resuspension buffer [3] containing 5% Triton X-100 before being added to incubation tubes. The substrate Phe–Leu–Glu–Glu–Leu was obtained from Bachem (Torrance, CA). Other reagents used were obtained as indicated in cited procedures.

3. RESULTS

The synthesized peptides Boc-Ser–Ser–Leu-OMe (V), Boc-*O*-phospho-Ser–*O*-phospho-Ser–Leu-OMe (I), and Boc-Phe–Leu–*O*-phospho-Ser–*O*-phospho-Ser–Leu (VII) were assayed as substrates for the vitamin K-dependent carboxylase at 0.5 and

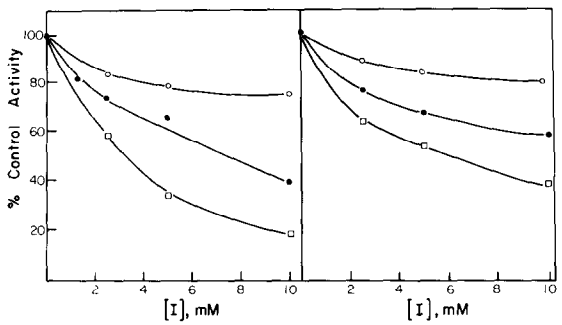

Fig.2. Effect of other peptides on the vitamin K-dependent carboxylation of Boc–Glu–Glu–Leu–OMe (left), or endogenous protein precursors (right): (○—○) Boc-Ser–Ser–Leu-OMe; (●—●) Boc-*O*-phospho-Ser–*O*-phospho-Ser–Leu-OMe; (□—□) Boc-Phe–Leu–O-phospho-Ser–*O*-phospho-Ser–Leu.

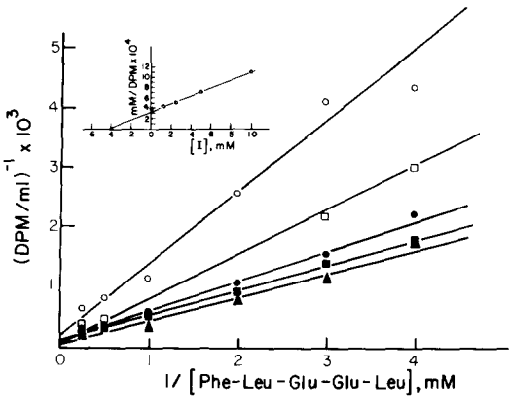

Fig.3. Double reciprocal plot of the effects of Boc-*O*-phospho-Ser–*O*-phospho-Ser–Leu-OMe on the vitamin K-dependent carboxylation of Phe–Leu–Glu–Glu–Leu. Inhibitor concentrations used were: 1.25 mM (■—■); 2.5 mM (●—●); 5.0 mM (□—□); and 10.0 mM (○—○). A replot of the slope is shown in the insert.

2.5 mM. No $^{14}CO_2$ incorporation into these peptides was observed. When these compounds were tested for their ability to inhibit carboxylation of other substrates, the data in fig.2 were obtained. All 3 compounds inhibited the carboxylation of both an added peptide substrate and endogenous protein precursors. In both cases, the non-anionic Ser-containing peptide was a relatively weak inhibitor, while the phosphoserine-containing compounds were more effective. The strongest inhibition was exhibited by the close analog of the pentapeptide substrate, compound VII. Inhibition of the added peptide substrate was more substantial than the endogenous protein.

The structural similarity of the phosphoserine-containing peptides to substrates of the enzyme suggest that these compounds may act as competitive inhibitors at the Glu binding site of the enzyme. This was investigated in more detail utilizing compound I as an inhibitor of the carboxylation of Phe–Leu–Glu–Glu–Leu. The double reciprocal plot shown in fig.3 is consistent with the action of compound I as a competitive inhibitor of this substrate, and the slope replot data in the insert suggest a K_i of ~4 mM. The reversible nature of the inhibition was checked by dialysis of the completely resuspended microsomes plus 5 mM compound I for 30 min or 12 h against the resuspension buffer before the standard assay was carried out. A 30 min dialysis was sufficient to reverse the inhibition of protein carboxylation and decrease the inhibition of peptide carboxylation. A 12-h dialysis decreased the inhibition of peptide carboxylation by compound I to <10%.

4. DISCUSSION

A number of inhibitors of the detergent-solubilized vitamin K-dependent carboxylase have been described in [1]. These have included vitamin K analogs such as Chloro-K [8], 2,3,5,6-tetrachloro-4-pyridinol [9,10], reduced vitamin K esters [11] and a series of substituted forms of vitamin K [12,13]. Some thiol-reactive agents [15,16] and spin trapping reagents [15] and superoxide dismutase [16,17] have also been reported to inhibit this enzyme. Relatively high concentrations of cyanide inhibit the carboxylation reaction [18]. This response is at least partially competitive with respect to CO_2 [19] but other effects may be involved [5].

Specific inhibitors of the Glu binding site have not yet been described. A large number of peptides which contained Asp, homo-Glu, Gln or *D*-Glu residues which were not efficient substrates for the carboxylase were not found to be inhibitors of the reaction [3]. A cyclic hexapeptide which contains a disulfide bridge corresponding to bovine prothrombin precursor sequence 18–23 has been reported to inhibit carboxylation of Phe–Leu–Glu–Glu–Leu [20], but the nature of this inhibition has not been

described. The peptides reported here, which contain phosphoserine rather than glutamyl residues, appear to satisfy the criteria of competitive inhibitors of the Glu binding site and should be useful probes of the mechanism of action of this multisubstrate unique carboxylase.

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