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VITAMIN K_1 DEPENDENT CARBOXYLASE: β-CARBOXYLATION OF t-BUTYLOXYCARBONYLASPARTIC ACID α-BENZYL ESTER

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Summary: Rat liver microsomes solubilized by Triton X-100 catalyze the vitamin K_1 dependent incorporation of carbon-14 from [^{14}C] $NaHCO_3$ into t-butyloxycarbonylaspartic acid α-benzyl ester. High voltage electrophoresis of the *alkaline* hydrolysate of the products of this reaction demonstrates the presence of a labelled species (A) whose electrophoretic mobility is identical to that of β-carboxyaspartic acid. High voltage electrophoresis of the *acid-treated* products reveals the disappearance of A and the appearance of a labelled species whose electrophoretic mobility is identical to that of aspartic acid. These experiments provide unequivocal evidence for the vitamin K_1 dependent β-carboxylation of an aspartic acid side chain, and they constitute the first report of such an enzymatic activity in microsomes.

The vitamin K_1 dependent carboxylase from rat liver catalyzes the carboxylation of specific glutamic acid residues in precursors of the blood clotting factors VII, IX, X and prothrombin, to form the corresponding γ-carboxyglutamic acid (Gla)[1] derivatives (1). The enzyme will also catalyze the carboxylation of Glu in a variety of synthetic peptides which contain either a single Glu residue or a pair of them (2-4). The pentapeptide Phe-Leu-Glu-Glu-Leu-OH, for example, is carboxylated to Phe-Leu-Gla-Glu-Leu-OH, and Boc-Glu-OBzl is converted to Boc-Gla-OBzl (5). The rates of carboxylation are increased 3-fold and 1.3-fold respectively by 1 mM pyridoxal phosphate (6). In contrast, the closely related Asp derivatives Boc-Asp-Asp-Leu-OMe (7) and Boc-Asp-OBzl (8) are reported by Suttie's group not to be carboxylated by the rat liver preparation. It should be noted, however, that the conditions used for these experiments were almost certainly not optimal since (i) the substrate concentrations employed were in the range 1-2 mM, whereas the K_m for Boc-Glu-OBzl at pH 7.2 is reported to be ~ 6 mM (9), and (ii) pyridoxal phosphate was not included in the experiments involving the Asp derivatives. For these reasons, and because there would appear to be no reasonable chemical basis for the stringent substrate specificity for the carboxylase implied by Suttie's results (10), we have re-examined the specificity of the rat liver vitamin K_1 dependent carboxylase towards Boc-Asp-OBzl. The results are reported in this Communication.

Experimental Section

All chemicals were Analytical Reagent grade, and solutions were prepared in distilled, deionized water. Vitamin K_1 (Sigma Chemical Co.) was reduced with sodium dithionite (11) immediately prior to use. [^{14}C] $NaHCO_3$ (~ 50 mCi/mmol) was obtained from New England Nuclear. Substrates Boc-Asp-OBzl, Boc-Asp(OBzl) and Boc-Glu-OBzl were obtained from Vega Biochemicals. Pyridoxal phosphate was obtained from Sigma Chemical Co.. Gla was synthesized by Dr. P.W. Riddles as described by Fernlund *et al.* (12). Asa was kindly supplied by Professor Alan Sargeson (13).

The microsomal carboxylase was prepared from the livers of male Wistar rats which had been fed a vitamin K_1 deficient diet (14) for seven days. Microsomes were prepared by homogenizing livers in five volumes

[1] Abbreviations: Gla, γ-carboxyglutamic acid; Asa, β-carboxyaspartic acid; Boc-Asp-OBzl, t-butyloxycarbonylaspartic acid α-benzyl ester; Boc-Asp(OBzl), t-butyloxycarbonylaspartic acid β-benzyl ester; Boc-Glu-OBzl, t-butyloxycarbonylglutamic acid α-benzyl ester.

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TABLE I

Substrate Specificity of the Vitamin K_1 Dependent Carboxylase

Substrate	Substrate Concentration (mM)	[Vitamin K_1H_2] (μg/ml)	Radioactivity in TCA-Supernatant[a] (dpm)
None	–	140	360
Boc-Asp-OBzl	22	0	360
Boc-Asp-OBzl	22	140	463
Boc-Asp-OBzl	16	140	418
Boc-Asp(OBzl)	22	140	280
Boc-Glu-OBzl	14	140	4,990

[a]Radioactivity was measured after sampling 0.1 ml of each assay and treatment as described in the text. Each number is the result of duplicate determinations which differed by less than 5 percent.

of ice-cold 0.025 M imidazolium chloride buffer, pH 7.2 (0.5 M in KCl), then centrifuging the homogenate at 10,000 X *g* for 10 min. The supernatant was centrifuged at 100,000 X *g* for 60 min. The pellet was resuspended in the minimum volume of buffer [0.025 M imidazolium chloride, pH 7.2 (0.5 M in KCl) made 10% (v/v) in glycerol, 0.5% (v/v) in Triton X-100, 10 mM in $MnCl_2$ and 1 mM in dithiothreitol] and dialyzed for 6 h against the same buffer at 4°C. Carboxylase assays were performed at 18°C in capped 5-ml vials which were stirred magnetically during the equilibration. Substrates [Boc-Asp-OBzl (30 mM), Boc-Asp(OBzl) (20 mM) and Boc-Glu-OBzl (20 mM)] and pyridoxal phosphate (14 mM) were prepared in 0.025 M imidazolium chloride buffer, pH 7.2 (0.5 M in KCl) made 1 mM in dithiothreitol and 1% (v/v) in Triton X-100. The final pH of all solutions was adjusted to 7.2 with 0.1 M NaOH. Vitamin K_1H_2 (10 mg/ml) was prepared in redistilled ethanol. Carboxylase assays contained the following final concentrations of substrates and other reagents in a total volume of 2.68 ml or 5.36 ml: Boc-Asp-OBzl, 22 mM; Boc-Asp(OBzl), 14 mM; Boc-Glu-OBzl, 14 mM; pyridoxal phosphate, 1.04 mM; vitamin K_1H_2, 0.14 mg/ml; [^{14}C]$NaHCO_3$, 1.49 mM. After a 60-min equilibration at 18°C, 0.1-ml aliquots of each assay were added to 0.5-ml aliquots of ice-cold 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation for 2 min at 10,000 X *g*, and 0.5 ml of the supernatant was vacuum dried in a scintillation vial. Radioactivity was determined in a Beckman model LS-250 scintillation counter after adding 10 ml of scintillation fluid (4 g of Omnifluor, 700 ml of Triton X-100 and 1000 ml of Mallinckrodt ScintillAR toluene) to each sample. Counting efficiency was determined using [^{14}C] toluene as an internal standard.

Reaction products were partially purified by chromatography of the remainder of the large-scale assays (5.26 ml after sampling) on a column (70 X 2.5 cm) of Sephadex G-10 equilibrated with 50 mM NH_4HCO_3 at 4°C. Fractions (2 ml/15 min) were monitored for radioactivity and absorbance at 257 nm, λ_{max} for Boc-Asp-OBzl. Pooled fractions were lyophilized and subjected to alkaline hydrolysis (2 M KOH, 22 h, 110°C) or acid treatment (6 M HCl, 22 h, 110°C). Under these conditions, Boc-Asp-OBzl yielded 90% and 97% respectively of the theoretical yield of Asp, determined by amino acid analysis on a Technicon model TSM automated amino acid analyser. Authentic Asa was quantitatively decarboxylated to Asp under the conditions of the acid treatment. Alkaline hydrolysates were desalted on a column of Dowex 50W-X8 (NH_4^+ form), and the desalted alkaline hydrolysates and the acid-treated products were lyophilized. High voltage electrophoresis of these materials was performed in 0.05 M pyridine (pH 3.1 with formic acid) for 1 h at 2,500 V, using Whatman 3 MM paper. Sample strips were eluted with 3 ml of water, dried and re-electrophoresed under the same conditions as the first run. Authentic Asa, Gla and Asp were added to samples prior to electrophoresis. The second electrophoretogram was sectioned at 1-cm intervals to provide samples for radioactivity measurements. Amino acids were visualized by spraying the edges of the residuals with ninhydrin reagent [0.4% (w/v) ninhydrin in ethanol-water-2,4,6-collidine, 190:10:3].

Results and Discussion

Table I gives the results of typical carboxylase assays using Boc-Asp-OBzl, Boc-Asp(OBzl) and Boc-Glu-OBzl as substrates. In the absence of any added substrate, there is an endogenous activity which is not dependent on added vitamin K_1 and which results in "background" radioactivity (360 dpm) in the TCA-soluble fraction. Incorporation of label into this fraction was stimulated by the simultaneous presence of Boc-Asp-OBzl and vitamin K_1H_2. No stimulation was observed when vitamin K_1H_2 was omitted or when Boc-Asp(OBzl) was used as substrate. Indeed, the latter compound inhibited the endogenous activity. Comparison of the radioactivity incorporated when Boc-Asp-OBzl is the substrate with that when Boc-Glu-OBzl is the substrate suggests that the aspartic acid derivative is *ca* fifty times as poor a substrate as is the glutamic acid analogue, at the maximum concentrations used here.

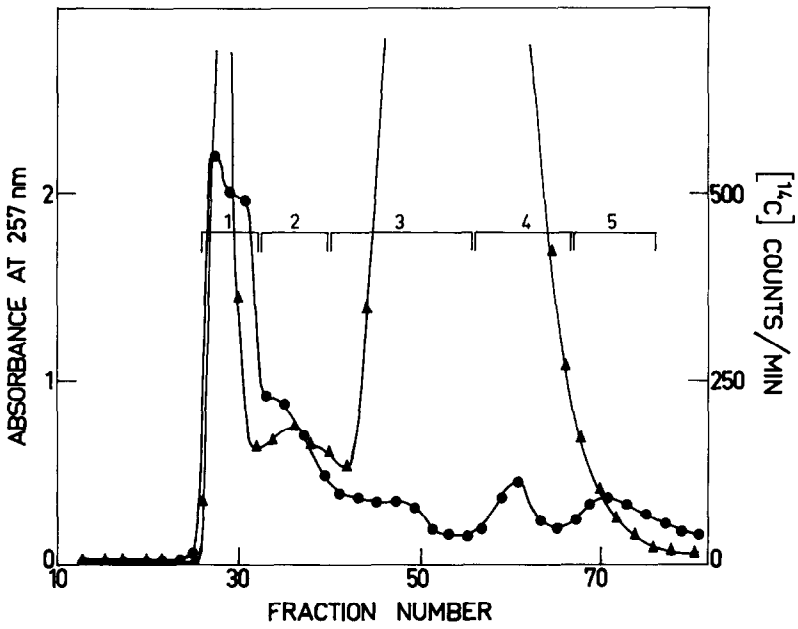

Figure 1. Sephadex G-10 chromatography of the products obtained when Boc-Asp-OBzl was substrate. Column dimensions: 70 X 2.5 cm; column equilibrated with 50 mM NH_4HCO_3; flow rate: 8 ml/h; A_{257} (●); carbon-14, cpm/0.1 ml (▲); pooled fractions (⌐¬).

Partial purification of the reaction products from the above experiments was achieved by chomatography on Sephadex G-10. The elution profile for the chromatography of the Boc-Asp-OBzl experiment is shown in Fig. 1. Fractions were pooled as shown. Similar results were obtained when Boc-Glu-OBzl was substrate, except that the peak of radioactivity associated with fraction 2 was much larger, suggesting that the products of the Boc-Glu-OBzl and Boc-Asp-OBzl carboxylation eluted from Sephadex G-10 in this region of the elution profile. The bulk of the unincorporated [^{14}C]$NaHCO_3$ eluted in fractions 3 and 4. Alkaline hydrolysis of fraction 1 from *both* experiments yielded a labelled compound which co-migrated with authentic Gla on high voltage electrophoresis. Acid treatment of fraction 1 resulted in the disappearance of this compound, and the exclusive appearance of labelled Glu, thus establishing the identity of the labelled compound in the alkaline hydrolysates as Gla. This Gla is reasonably attributed to carboxylation of endogenous protein substrates, the products of which elute in the void volume from Sephadex G-10.

Alkaline hydrolysis of fraction 2 from the Boc-Asp-OBzl experiment yielded two labelled compounds, one of which co-migrated with authentic Asa. Acid treatment of fraction 2 caused the disappearance of this compound, and the appearance of labelled Asp. Analysis of fraction 2 from the Boc-Glu-OBzl experiment demonstrated the presence after alkaline hydrolysis of only one labelled species and it co-migrated with Gla. Acid treatment caused the disappearance of this fraction and the exclusive appearance of labelled Glu. Analysis of fractions 3 and 4 from Sephadex G-10 demonstrated the absence of labelled products in these fractions. The second labelled species which appears in the alkaline hydrolysate of fraction 2 from the Boc-Asp-OBzl experiment but not in that of the Boc-Glu-OBzl assay has not been identified. The possibility that it arises from an impurity in the Asp substrate has not been completely eliminated.

It was noted in preliminary experiments, albeit done under less optimal conditions, that no detectable incorporation of label into Boc-Asp-OBzl occurred if pyridoxal phosphate were omitted from the assay. We plan to investigate further the presently undefined role of pyridoxal phosphate in the carboxylation of Boc-Asp-OBzl and Boc-Glu-OBzl. However, the present results demonstrate unequivocally that a rat liver vitamin K_1 dependent carboxylase catalyzes the carboxylation of the aspartic acid side chain of a simple substrate. In 1979, we anticipated the existence of Asa (10), and it was first unequivocally identified as a component of the ribosomal proteins of *E.coli* in 1981 (15). So far there have been no reports of Asa in any other system. The results of this study reinforce our projection that Asa will be widely distributed as a component of vertebrate mineralized tissues although it may be present in much lower amount than Gla. Therefore, we are currently undertaking a search for Asa in the protein from the dentine of human teeth and also from hermatypic corals where we have recently identified Gla (B. Zerner, unpublished results).

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