In vitro prothrombin synthesis from a purified precursor protein

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IN VITRO PROTHROMBIN SYNTHESIS FROM A PURIFIED PRECURSOR PROTEIN

III. PREPARATION OF AN ACID-SOLUBLE SUBSTRATE FOR VITAMIN K-DEPENDENT CARBOXYLASE BY LIMITED PROTEOLYSIS OF BOVINE DESCARBOXYPROTHROMBIN

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Bovine descarboxyprothrombin and descarboxyfragment-1 can be used as substrates for rat and bovine vitamin K-dependent carboxylase. In both enzyme systems, however, these substrates have a high K_m (0.3–0.4 mM). A better substrate (K_m = 0.001–0.003 mM) was prepared from bovine descarboxyprothrombin by limited proteolysis with subtilisin Carlsberg. This substrate is called Fragment-Su and is composed of the amino acids 13–29 of descarboxyprothrombin.

Introduction

During the last few yeast we have developed a cow liver cell-free system in which purified descarboxyprothrombin is converted into prothrombin [1,2]. The enzyme system which is able to increase the prothrombin concentration in a standard reaction mixture [2] was designated as prothrombin synthase. In our attempts to purify prothrombin synthase [1] we obtained a preparation with a molecular weight of 60 000, in which prothrombin was generated at the lower limit of detection. Whereas in the crude microsomal extract, a parallel incorporation of $^{14}CO_2$ into endogenous proteins was demonstrated, a substantial amount of the carboxylating activity (carboxylase) was lost during the purification procedure. For the work reported here we preferred therefore to use solubilized microsomes as the enzyme preparation in which the carboxylation of various substrates was measured. The main difference between this system and the more frequently used rat carboxylase [3] is that bovine carboxylase may be prepared from non-anticoagulated animals, whereas rat carboxylase has to be prepared from warfarin-treated (or vitamin K-deficient) animals.

In this paper we describe the preparation of a low molecular weight fragment from bovine descarboxyprothrombin that may be used as a trichloroacetic acid-soluble substrate for both rat and bovine carboxylase.

Materials and Methods

Chemicals and buffers

Buffer A: 0.15 M NaCl/0.01 M Tris-HCl, pH 7.5. Vitamin K_1 was obtained from Hoffmann-La Roche (Switzerland) and Triton X-100 was from Sigma (USA). Proteases were obtained from Sigma (USA) and Boehringer (FRG). $NaH^{14}CO_3$ (60 mCi/mmol) was purchased from the Radiochemical Centre Amersham (UK) and Aquasol-2 was from New England Nuclear (USA). Reduced vitamin K (KH_2) was prepared as described in [2]. The synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was obtained from Vega Fox (USA). DEAE-Sephacel and CNBr-activated Sepharose were obtained from Pharmacia

(Sweden) and proteins were coupled to the Sepharose according to the manufacturers prescription. Ultrogel was obtained from LKB (Sweden). All other chemicals were obtained from Merck (FRG).

Preparation of carboxylase

Soluble bovine carboxylase was prepared from non-anticoagulated cows and Sepharose-bound carboxylase was prepared from warfarin-treated cows as described earlier [4]. In order to stabilize the enzyme, 30% ethylene glycol was present in all centrifugation steps. Soluble rat carboxylase was obtained from anticoagulated female rats (200–220 g) of the Lewis strain. The rats were anticoagulated by the intraperitoneal administration of 2 mg sodium warfarin in 0.9% NaCl 18 h before being killed. The animals were killed by decapitation and the livers were removed, washed with 0.25 M sucrose and used for the preparation of soluble carboxylase in a similar way as that described for the bovine system [4].

Measurement of carboxylase activity

Reaction mixtures (0.25 ml) containing 0.1 ml solubilized carboxylase, 0.3 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.2% Triton X-100, 12% ethylene glycol, 20 μCi $NaH^{14}CO_3$, 2 mM dithiothreitol, 10 μM vitamin K_1 hydroquinone and exogenous substrate as indicated were incubated at 25°C in parafilm-sealed tubes. The reaction was stopped at various intervals by diluting the samples with 2 ml ice-cold buffer A and 2 ml 10% trichloroacetic acid. After centrifugation, traces of unbound label were removed from the supernatant by vacuum extraction and the samples were counted in Aquasol-2 in a Packard Tricarb scintillation counter. The trichloroacetic acid-precipitates were dissolved in NaOH and reprecipitated three times before counting. The results are expressed as dpm per gram of liver.

Preparation of purified substrates

Bovine dicoumarol plasma [1] was adsorbed with $BaSO_4$ (200 g/l) and descarboxyprothrombin was purified therefrom by chromatography on QAE-Sephadex, DEAE-Sephadex, hydroxylapatite and Sephadex G-100.

Bovine thrombin was obtained from Hoffman-La Roche and was purified by chromatography on SP-Sephadex and benzamidin-agarose.

Descarboxyfragment-1 was prepared by incubating purified descarboxyprothrombin and thrombin in a molar ratio of 10 : 1 in buffer A at 37°C for 2 h. Descarboxyfragment-1 was resolved from the other components of the digest by chromatography on Sephadex G-100 in 20% acetic acid as described by Stenflo [5] and dialysed against buffer A.

Amino acid analysis, NH_2-terminal sequence and end group determinations

Amino acid analysis was performed after hydrolysis in 6 M HCl in vacuo at 110°C for 20 h using a Beckman 119 CL amino acid analyzer. NH_2-terminal amino acid sequence analysis was performed as described by Edman [6]. In some instances [^{14}C]-phenylisothiocyanate was used. Identification of the phenylthiohydantoins was performed by thin-layer chromatograpy [7]. Radioactive derivatives were identified with a Berthold scanner and quantitated by liquid scintillation counting. COOH-terminal amino acids were determined by digestion with carboxypeptidase Y (Pierce) and amino acid analysis [8].

About 80 nmol of protein was dissolved in 0.05 M sodium acetate buffer, pH 5.5, to a protein concentration of about 1.6 mg/ml. Digestion was performed at 25°C using an enzyme/substrate ratio of 1 : 250 (w/w) and sampling over a time period of 5–90 min. The sample was kept in a boiling water bath for 3 min to inactivate the enzyme and diluted with 0.2 M sodium citrate buffer, pH 2.2, for analysis.

Results

When we tried to carboxylate exogenous substrates in a bovine carboxylating enzyme system, it resulted that at plasma concentrations (1.4 μM) purified bovine descarboxyprothrombin did not affect the carboxylation reaction (Table I). Only at more than 70-fold higher concentrations a significant increase of $^{14}CO_2$ incorporation was measured. In order to exclude the possibility that descarboxyprothrombin merely stimulates carboxylase without being carboxylated itself, we modified either the endogenous substrate or the exogenous one in such a way that they could easily be separated from each other after the carboxylation reaction and occurred. The three modification procedures that we used were: (a) preparing descarboxyfragment-1, which, in

TABLE I

THE CARBOXYLATION OF VARIOUS SUBSTRATES BY RAT AND BOVINE CARBOXYLASE

Mixtures containing rat and bovine carboxylase were incubated at 25°C for 0.5 and 1 h, respectively.

	Vitamin K-dependent incorporation of $^{14}CO_2$			
	By rat carboxylase		By bovine carboxylase	
	In trichloro-acetic acid precipitate	In trichloro-acetic acid supernatant	In trichloro-acetic acid precipitate	In trichloro-acetic acid supernatant
None	10824	58	2967	63
Descarboxyprothrombin (1.4 μM)	10537	62	3005	57
Descarboxyprothrombin (0.1 mM)	19522	85	14829	91
Descarboxyfragment-1 (1.4 μM)	11218	123	2783	212
Descarboxyfragment-1 (0.1 mM)	9971	8854	2012	7882

contrast to the endogenous substrate, is soluble in trichloroacetic acid. (b) Cross-linking the descarboxyprothrombin to CNBr-activated Sepharose and (c) preparing Sepharose-bound carboxylase; in this partly purified enzyme system all endogenous substrate is linked to the solid phase and can thus be separated from the soluble exogenous descarboxyprothrombin.

When using descarboxyfragment-1 we observed that this substrate enhanced the carboxylation reaction in a similar way as descarboxyprothrombin did. Since the additional amount of incorporated label was present in the trichloroacetic acid-soluble fraction (Table I) it is obvious that descarboxyfragment-1 is carboxylated by the rat as well as by the bovine enzyme system. It resulted that rat carboxylase was also able to carboxylate the solid-phase descarboxyprothrombin (Sp-DP) in a vitamin K-dependent way (Table II). In the bovine system, however, it appeared that the cross-linking procedure had changed the descarboxyprothrombin in such a way that the Sp-DP did not stimulate the carboxylation reaction any more and neither was it carboxylated itself. On the other hand, when the enzyme and its endogenous substrate were attached to the solid phase, soluble descarboxyprothrombin could be added to the enzyme system and it could be demonstrated that also in this case the exogenous substrate was carboxylated (Table II).

The carboxylation of descarboxyprothrombin and descarboxyfragment-1 occurred with a very low efficiency, however, (see below) and we tried to prepare better substrates by degrading descarboxyprothrombin with Sepharose-bound proteases. Sixteen proteases were used and the effect of their fragmentation products on the $^{14}CO_2$ incorporation was measured in rat as well as in bovine carboxylase (Table III). By keeping the digested prothrombin at a concentration as low as 2 μM we could rapidly screen the result of the limited proteolysis, because only strongly improved preparations possessed a demonstrable activity in the carboxylation reaction. From these experiments it became clear that four proteases (subtilisin Carlsberg, subtilisin BPN′, pronase P and proteinase K) were able to cleave a highly active fragment from descarboxyprothrombin. In all these cases the carboxylated reaction products were found in the trichloroacetic acid supernatant, and when assayed on Ultrogel columns, all four substrates had a molecular weight which was lower than 5 000. We decided to purify and characterize the substrate that is obtained by the digestion of descarboxyprothrombin with Sepharose-bound subtilisin Carlsberg. This fragment was designated as Fragment-Su and it was purified from the digest by chromatography on DEAE-Sephacel, AcA 54 and Ultrogel A 202 columns (Fig. 1).

The final purification was performed by HPLC-chromatography (Fig. 2). The carboxylatable peptide

TABLE II

CARBOXYLATION OF BOVINE DESCARBOXYPROTHROMBIN BY RAT AND BOVINE CARBOXYLASE

The concentration of exogenous substrate (Sp-DP) was 0.2 ml slurry per ml reaction mixture. The incorporation of $^{14}CO_2$ into Sp-DP was measured after 1 h at 25°C by filtering the reaction mixtures over glass-fiber filters, followed by extensive washing with buffer A. The filters were counted in Aquasol and the results are expressed as dpm per gram liver. The concentration of solid-phase carboxylase (Sp-carboxylase) was 0.2 ml slurry per ml reaction mixture. The incorporation of $^{14}CO_2$ into the soluble substrate was measured after 4 h at 25°C by filtering the reaction mixtures over glass-fiber filters and counting the filtrate.

Sepharose-bound substrate	$^{14}CO_2$ Incorporation by soluble rat carboxylase into Sepharose-bound protein	
A	+Vitamin KH2	−Vitamin KH_2
None	30	28
Descarboxyprothrombin	4 922	88
Prothrombin	62	54
Soluble substrate	**$^{14}CO_2$ Incorporation by Sepharose-bound bovine carboxylase into soluble protein**	
B	+Vitamin KH_2	−Vitamin KH_2
None	138	99
Descarboxyprothrombin	12 720	104
Prothrombin	321	188

TABLE III

CARBOXYLATION OF FRAGMENTED BOVINE DESCARBOXYPROTHROMBIN BY RAT AND BOVINE CARBOXYLASE

The various proteases were coupled to CNBr-activated Sepharose and 1 ml slurry was incubated with 2.5 mg descarboxyprothrombin for 2 h at room temperature, while rotating end over end. The reaction conditions during digestion were: 0.1 M NaCl, 0.02 M Na acetate buffer, pH 6.0 in the case of pepsin and 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.2 for all other proteases. During the carboxylation reaction the concentration of fragmented substrate (if added) was 200 μg/ml. The carboxylation was measured after 60 min at 25°C and blank values (without vitamin K) were subtracted.

Protease used for fragmentation of descarboxyprothrombin	Rat carboxylase		Bovine carboxylase	
	$^{14}CO_2$ Incorporation in:		$^{14}CO_2$ Incorporation in:	
	Trichloroacetic acid precipitate	Trichloroacetic acid supernatant	Trichloroacetic acid precipitate	Trichloroacetic acid supernatant
None	11 595	60	2 840	75
Trypsin	14 410	200	2 685	660
Chymotrypsin	8 510	260	2 715	1 635
Pepsin	11 615	90	2 810	160
Papain	12 560	170	3 725	735
Subtilisin Carlsberg	9 420	1 610	1 605	6 790
Subtilisin BPN'	9 865	1 040	1 970	7 325
Protease II	12 025	220	2 715	1 190
Protease IV	12 505	180	2 985	1 420
Pronase P	9 285	465	1 720	4 210
Pronase AS	12 035	170	2 430	920
Clostripain	10 890	80	3 230	400
Proteinase K	9 850	1 675	1 900	9 105
Thermolysin	11 700	10	2 345	1 370
No substrate added	11 440	115	2 905	85

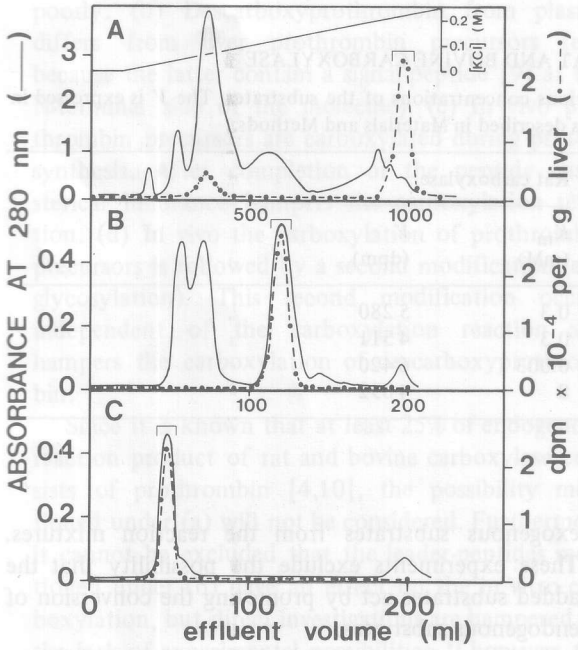

Fig. 1. Fractionation of digested descarboxyprothrombin. Descarboxyprothromin (7 μM) was digested with Sepharose-bound subtilisin (10 ml slurry) in a reaction mixture of 600 ml, containing 10 mM NaCl/1 mM EDTA/10 mM Tris-HCl, pH 7.2. The digest was adsorbed onto a DEAE-Sephacel column (1.5 X 30 cm) in the same buffer and eluted with a linear gradient (2 X 500 ml) from 10 to 250 mM NaCl (panel A). The active fractions were pooled as indicated, concentrated and applied to an AcA 54 column (1.5 X 100 cm) in 1 mM EDTA, 0.5 M KCl and 10 mM Tris-HCl, pH 7.8 (panel B). The active fractions were pooled, concentrated and applied to an Ultrogel A 202 column (1.5 X 100 cm) in distilled water (panel C). After these fractionations the recovery of carboxylatable material was 37%.

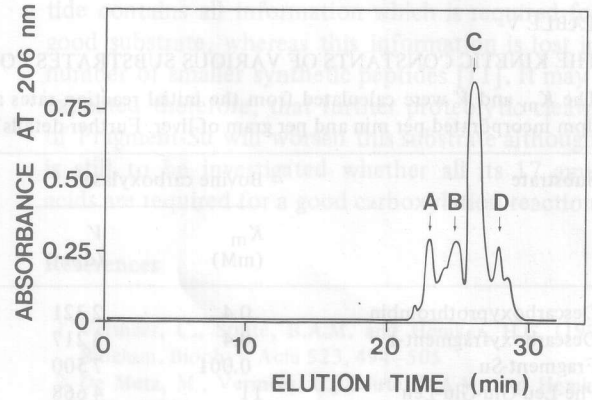

Fig. 2. Purification of Fragment-Su by HPLC-chromatography. The protein solution was adsorbed to an ultrasphere-ODS (5 μM) column (25 X 1 cm, Beckman) and a gradient was applied from 0–30% CH_3CN in 0.1% H_3PO_4. The peaks were eluted with a flow rate of 4 ml per min and assayed as described in the text. The recovery of purified Fragment-Su was 0.26 mol per mol descarboxyprothrombin.

was recovered in peak C. The amino acid composition of the purified peptide is summarized in Table IV and is in agreement with the composition that is expected to be found from the amino acid residues 13–29 of descarboxyprothrombin. Quantitative amino acid analysis revealed that Asn was the N-terminal residue with a yield of 0.86 mol per mol peptide. The sequence of the amino acid residues at the N-terminal side was found to be: Asn-Leu-Glu-Arg and at the C-terminal position we found Phe in 0.93 mol per mol of peptide. This confirmed that Fragment-Su is identical to the amino acid sequence 13–29 in descarboxyprothrombin.

In order to demonstrate that the incorporated label in the carboxylated Fragment-Su was present in gla-residues, we prepared a carboxylating reaction mixture (1 ml) containing 10 μg of the peptide frag-

TABLE IV

AMINO ACID COMPOSITION OF FRAGMENT-Su

The figures in the first column are given as mol amino acid per mol of peptide. The second column represents the composition expected from the sequence 13–29.

Amino acid	Composition observed	Composition expected
Asp	1.1	1
Thr	0	0
Ser	0.8	1
Glu	6.2	6
Pro	1.3	1
Gly	0.2	0
Ala	1.0	1
Val	0.1	0
Met	0	0
Ile	0	0
Leu	2.0	2
Tyr	0.1	0
Phe	1.0	1
His	0	0
Lys	0	0
Arg	2.0	2

TABLE V

THE KINETIC CONSTANTS OF VARIOUS SUBSTRATES FOR RAT AND BOVINE CARBOXYLASE

The K_m and V were calculated from the initial reaction rates at various concentrations of the substrates. The V is expressed in dpm incorporated per min and per gram of liver. Further details are as described in Materials and Methods.

Substrate	Bovine carboxylase		Rat carboxylase	
	K_m (mM)	V (dpm)	K_m (mM)	V (dpm)
Descarboxyprothrombin	0.4	2 321	0.3	5 280
Descarboxyfragment-1	0.4	3 217	0.3	4 511
Fragment-Su	0.001	7 500	0.003	6 420
Phe-Leu-Glu-Glu-Leu	11	4 668	8	4 092

ment. After 1 h at 25°C the proteins were precipitated with trichloroacetic acid and non-bound $^{14}CO_2$ was removed (see Materials and Methods). The trichloroacetic acid supernatant contained carboxylated Fragment-Su (98 512 dpm). After acid hydrolysis in 6 M HCl in vacuo at 110°C for 20 h about 50% of the label (51 326 dpm) had disappeared, showing that the gla-residues had been decarboxylated into glu. Finally, we compared the kinetic constants of descarboxyprothrombin, descarboxy-fragment-1, Fragment-Su and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (Table V). It turned out that the K_m of Fragment-Su was 0.001 mM, whereas the K_m of the other three substrates ranged from 0.4 to 11 mM. Therefore, it has to be concluded that Fragment-Su is a better substrate than any of the other exogenous substrates. Comparison of the maximal carboxylation rates (V) showed that these were all of the same order of magnitude. In contrast to the K_m, however, the V is not independent of the quality of the enzyme system used (e.g. the purity of the system, the relative amount of active enzyme molecules etc.). Therefore the V is a less suitable constant to compare the various substrates.

Discussion

When bovine descarboxyprothrombin and descarboxyfragment-1 were added to a rat liver vitamin K-dependent carboxylating cell-free system, it resulted that both substrates were carboxylated. This could be demonstrated by separating the ^{14}C-labeled exogenous substrates from the reaction mixtures. These experiments exclude the possibility that the added substrates act by promoting the conversion of endogenous substrates.

In bovine liver carboxylase the presence of descarboxyprothrombin and descarboxyfragment-1 induced an increase of the $^{14}CO_2$ incorporation which was similar to that in the rat system. Because descarboxyfragment-1 is not precipitated with trichloroacetic acid it is separated from the endogenous substrates in the reaction mixtures and it could thus be shown that also in this case the exogenous substrate was carboxylated. Insolubilized substrates, however, had lost their ability to stimulate the carboxylation reaction. On the other hand, when we used Sepharose-bound carboxylase and soluble descarboxyprothrombin, it could be demonstrated that bovine carboxylase, as well as rat carboxylase, is able to carboxylate this substrate.

Both in the rat and in the bovine system we observed high K_m values for descarboxyprothrombin and for descarboxyfragment-1. This means that high concentrations (0.5–1 mM) of these substrates have to be added to carboxylase in order to be able to measure their carboxylation. It is not probable that the endogenous substrates are present in carboxylase in these quantities and therefore the endogenous substrates must be far better substrates than the exogenous ones. Possible reasons for this phenomenon might be: (a) The main part of the endogenous substrates does not consist of prothrombin precursors and prothrombin precursors are carboxylated rather

poorly. (b) Descarboxyprothrombin from plasma differs from liver prothrombin precursors (e.g. because the latter contain a signal-peptide [9] at the N-terminal side of the molecule). (c) In vivo prothrombin precursors are carboxylated during protein synthesis. After completion of the peptide chain sterical hindrance hampers the carboxylation reaction. (d) In vivo the carboxylation of prothrombin precursors is followed by a second modification (e.g. glycosylation). This second modification occurs independent of the carboxylation reaction and hampers the carboxylation of descarboxyprothrombin.

Since it is known that at least 25% of endogenous reaction product of rat and bovine carboxylase consists of prothrombin [4,10], the possibility mentioned under (a) will not be considered. Furthermore it cannot be excluded that the leader-peptides mentioned under (b) have an effect on the in vitro carboxylation, but direct investigations are hampered by the lack of experimental possibilities. If however, the structure of the completed descarboxyprothrombin hinders the carboxylation reaction ((c) and/or (d)), it should be possible to remove the main part of the descarboxyprothrombin molecule in order to obtain a substrate that would be more accessible to carboxylase. It resulted that the removal of fragment-2 and thrombin (resulting in descarboxyfragment-1) did not change the kinetic characteristics of the endogenous substrate. Only proteolytic degradation of the latter to a peptide containing the amino acids 13–29 resulted in a substantial improvement of the substrate. It is intriguing that the structure of this peptide contains all information which is required for a good substrate, whereas this information is lost in a number of smaller synthetic peptides [11]. It may be expected therefore, that further proteolytic cleavage of Fragment-Su will worsen this substrate although it is still to be investigated whether all its 17 amino acids are required for a good carboxylation reaction.

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