

PARTIAL PURIFICATION OF BOVINE LIVER VITAMIN K-DEPENDENT CARBOXYLASE BY IMMUNOSPECIFIC ADSORPTION ONTO ANTIFACTOR X

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1. Introduction

Administration of vitamin K-antagonists induces the appearance of abnormal clotting factors in the blood (man, cow) or in the liver (rat). These abnormal clotting factors (descarboxy factors) contain glutamic acid (Glu) residues instead of the γ -carboxyglutamic acid (Gla) residues, which occur in normal clotting factors [1]. In human and bovine plasma ~90% of the abnormal proteins consists of descarboxyprothrombin. Attempts to identify the rat liver abnormal proteins have been done in an in vitro vitamin K-dependent carboxylating enzyme system (carboxylase) and it was shown that only 25% of the carboxylated endogenous substrates bound to antiprothrombin antibodies [2].

Here, we describe the preparation of vitamin K-dependent carboxylase from the livers of warfarin-treated cows and the results of our investigations concerning the distribution of the various clotting factor precursors in this enzyme system.

2. Materials and methods

2.1. Preparation of coagulation factors and antibodies

Bovine prothrombin was prepared and purified as in [3], factor IX as in [4] and factor X according to [5]. The various coagulation factors were detected with the aid of one-stage coagulation assays [6–8]. Antibodies against these proteins were raised in goats and extracted from the various sera by immunospecific adsorption to the respective Sepharose-bound antigens. The purified antibodies were eluted from the solid phase with 3 M NaCNS and gave single precipitation

lines against normal reference plasma in an Ouchterlony diffusion test [9]. These antibodies were coupled to CNBr-activated Sepharose (Pharmacia). Antiprothrombin–Sepharose slurry (1 ml) was able to neutralize the prothrombin content of 8 ml plasma. For antifactor IX–Sepharose and antifactor X–Sepharose these figures were 5 ml and 9 ml, respectively. The various antibody preparations did not bind detectable amounts of any of the other coagulation factors.

2.2. Preparation of carboxylase

One year old cows were anticoagulated by the oral administration of warfarin (Sigma, 10 mg/kg daily) during 1 week. Cows were slaughtered and microsomes were prepared from the livers of normal and anticoagulated animals as in [10]. The crude microsomes were suspended in a buffer containing 0.05 M KCl, 0.02 M Tris–HCl (pH 7.5) and 0.1% Triton X-100. After centrifugation at $100\,000 \times g$ for 1 h the pellet was solubilized by adding a buffer containing 1 M KCl, 0.02 M Tris–HCl (pH 7.5), 1 mM DTT, 0.5 mM EDTA and 0.5% Triton X-100. The final protein concentration was 15 mg/ml and insoluble material was removed by centrifugation ($140\,000 \times g$ for 1.5 h). The supernatant of this run was designated as soluble carboxylase.

2.3. Measurement of carboxylase activity

Unless indicated otherwise the vitamin K-dependent incorporation of $^{14}\text{CO}_2$ was measured by incubating soluble carboxylase (1 mg protein) and $20\ \mu\text{Ci NaH}^{14}\text{CO}_3$ (Radiochemical Centre, Amersham) in reaction mixtures (0.25 ml) containing 0.15 M NaCl, 0.02 M Tris–HCl (pH 7.5), 1 mM DTT, 0.2 mM EDTA

and 0.2% Triton X-100. The reaction was started by adding 15 μ g chemically reduced vitamin K₁ [10] and the mixtures were incubated for 1 h at 25°C in sealed tubes. The reaction was stopped with 2 ml trichloroacetic acid (10%) and the precipitates were washed and counted. The acid-soluble substrate Phe-Leu-Glu-Glu-Leu (FLEEL) was prepared according to [11] and when it was present in the reaction mixtures the trichloroacetic acid supernatants were degassed at elevated temperatures before counting.

2.4. Gel electrophoresis and protein determination

SDS-polyacrylamide gel electrophoresis was performed according to [12]. Gels containing labeled products were sliced with a Gilson gel slicer and counted. Non-labeled gels were stained with Coomassie blue. Protein concentrations were determined according to [13].

3. Results and discussion

In soluble rat carboxylase the incorporation of ¹⁴CO₂ in endogenous substrates is increased at least 20-fold when the rats are treated with warfarin 18 h before preparing the liver homogenates. We observed that in soluble carboxylase obtained from warfarin-treated cows the carboxylation of endogenous substrate was 16-fold higher (80 000 dpm/g liver) than in carboxylase from normal cows (5000 dpm/g liver). These results suggested, that also in the cow liver some endogenous substrate (presumably clotting factor precursors) is accumulated during warfarin treatment and we therefore used carboxylase from anticoagulated cows for our studies concerning the nature of the bovine endogenous substrate.

Because of the presence of abundant proteolytic activity in bovine liver homogenates a direct determination of clotting factor precursors (e.g., with snake venoms) was not possible. In order to establish the nature of the endogenous substrate we therefore analyzed the ¹⁴C-labeled products that were formed during the carboxylation reaction. The analysis was performed by adsorption to immobilized antibodies and, as is shown in table 1, the main part (69%) of the reaction product consists of factor X-like material, whereas antiprotease and antifactor IX bound 21 and 8% of the incorporated label, respectively. In a second experiment the factor X-like material was eluted from the antibodies and characterized further

Table 1

Sephacrose-linked adsorbant	¹⁴ CO ₂ incorporated into non-adsorbed proteins		¹⁴ CO ₂ incorporated into adsorbed proteins	
	dpm/g liver	%	dpm/g liver	%
(a) Antiprotease	62 638	78.7	16 812	21.2
(b) Antifactor X	23 452	29.5	55 090	69.2
(c) Antifactor IX	73 819	92.8	6181	7.8
(d) a + b + c	9407	11.8	70 314	88.3
(e) Albumin	76 321	95.9	204	0.3

Immunospecific adsorption of carboxylated reaction products. Vitamin K-dependent incorporation of ¹⁴CO₂ was performed in standard reaction mixtures (1 ml). After incubation for 1 h at 25°C, Sepharose-bound antibodies (0.4 ml slurry) were added to the various reaction mixtures and the tubes were rotated end-over-end overnight at 4°C. The Sepharose-beads were washed alternately 3 times with 1 M NaCl in 0.1 M acetate buffer (pH 4.0) and with 1 M NaCl in 0.1 M borate buffer (pH 8.0). Subsequently they were eluted with 6 M urea in 2% SDS, dialyzed against 0.1 M NaCl and counted. Non-adsorbed reaction mixtures (100%) contained 79 588 dpm/g liver. Doubling the amount of Sepharose-bound antibodies did not affect the amount of adsorbed proteins

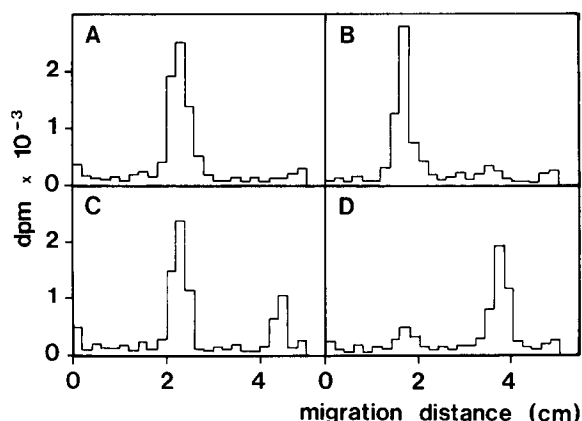


Fig.1. SDS-polyacrylamide gel electrophoresis of carboxylated endogenous substrates. Reaction mixtures (0.5 ml) were incubated for 1 h at 25°C mixed with 0.5 ml antifactor X-Sepharose and rotated end-over-end at 4°C overnight. The Sepharose was washed as described in the legend to table 1 and the labeled antigens were eluted with 3 M NaCNS and dialyzed against 0.15 M NaCl. The samples were incubated at 37°C for 1 h with snake venoms as indicated and SDS was added to 1% final conc. (A) Non-reduced sample; (B) reduced sample; (C) non-reduced sample after treatment with RVV_X; (D) sample treated with RVV_X before reduction.

with the aid of SDS–polyacrylamide gel electrophoresis before and after reduction of the samples (fig.1). The non-reduced samples (A) showed a single band with M_r 55 000, which is in agreement with that of factor X [5,14]. The app. M_r of the reduced protein (B) was found to be 65 000, which is higher than in the non-reduced gels and which shows that the factor X-like material consists of one single polypeptide chain. It turned out that *E. carinatus* venom did not change the observed relative molecular masses (not shown). On the other hand the purified factor X-activating enzyme from Russell's viper venom (RVV_X) induced a small amount of low M_r material (15 000) in the non-reduced gel (C), whereas in the reduced sample (D) nearly all high M_r material had disappeared and was recovered at a position indicating an M_r of 25 000. We concluded that the factor X-like material is a factor X-precursor consisting of one polypeptide chain.

After cleavage of this chain by RVV_X, the molecule is held together by a disulfide bridge, which can be broken by reducing agents. Because the reaction mixture in which the carboxylation occurs is also slightly reducing, a small amount of low M_r material is observed in the non-reduced sample. The increase of the app. M_r in the reduced gels seems to be almost entirely due to changes in the light-chain material. A similar observation was made for the light chain of factor X [14,15]. As plasma factor X contains two peptide chains, in vivo the carboxylation of the precursor probably precedes the conversion of one-chain factor X into two-chain factor X.

Taking into consideration that in bovine plasma circulating prothrombin and factor X are 1.4 and 0.28 μ M, that their half-life times are 80 h and 30 h, respectively, and that factor X contains 2 more Gla residues than does prothrombin, it may be calculated that in vivo at least 1.5 more carboxylation reactions occur in prothrombin precursors than in factor X precursors. The experiments described above demonstrate that this situation is reversed in the in vitro carboxylating enzyme system. The relative abundance of factor X precursors in the carboxylase complex may be caused by a relatively high affinity of carboxylase for this substrate. During the preparation of the liver microsomes unbound factor X precursors might then displace other clotting factor precursors, present in the enzyme complex. A second possibility is that the prothrombin precursors are more susceptible to proteolytic degradation which may occur during the preparation

of the microsomes. The available data provide no basis for distinguishing between these possibilities.

Since we knew that the endogenous substrate for the in vitro carboxylation reaction mainly consisted of precursors of factor X (69%) and prothrombin (21%), we started experiments in which we extracted the crude soluble carboxylase preparation with Sepharose-bound antibodies against factor X and prothrombin, respectively. It turned out that treatment with antiprothrombin removed 21% of the carboxylase activity, whereas antifactor X was able to bind as much as 62% of the total amount of carboxylase. A substantial amount of carboxylase activity was recovered on the Sepharose beads and because most of the microsomal proteins were removed by the washing procedure, the bound enzyme complex had been purified considerably. Solid phase carboxylase was assayed in the absence of Triton X-100 since detergents strongly inhibit its activity, even at low concentration.

A summary of the purification scheme is given in table 2. When we eluted the solid phase carboxylase with 2% SDS in 6 M urea it turned out that our most purified preparation still contained 8 different proteins, when analyzed on polyacrylamide gels. At this moment we do not know which of these proteins belong to the carboxylase complex.

An advantage of the insolubilized enzyme is that it is stable even at elevated temperatures. At 37°C the carboxylation reaction occurred 3 times faster than at 25°C and, in the presence of an excess of exogenous

Table 2

Stage	Protein (mg)	Spec. act. (dpm/mg)	Recovery (%)	Purification (-fold)
Crude microsomes	2400	1420	100	1
Washed microsomes	1800	2085	110	1.5
Soluble carboxylase	1100	3006	97	2.1
Immobilized carboxylase	5	137 276	20	97

Purification of bovine vitamin K-dependent carboxylase. The protein content of immobilized carboxylase was determined after elution of the solid phase with 6 M urea in 2% SDS. The carboxylation of endogenous substrate was measured in the various carboxylase preparations after 1 h incubation at 25°C. When the carboxylase activity was measured with FLEEL the final purification was 115-fold

substrate, the reaction rate was constant for ≥ 2 hours.

Thus far the only way to re-solubilize carboxylase was by allowing the carboxylation reaction to proceed. Preliminary experiments, in which the solid-phase carboxylase was incubated in the presence of vitamin KH_2 , FLEEL and NaHCO_3 showed that $\sim 30\%$ of the enzyme activity could be regained in solution. Preparations thus obtained were unstable, however, and the improvement of the elution procedure is a subject of current studies.

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